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Short-term Exercise as a Cognitive Enhancer: an investigation of the underlying mechanisms

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

Submitted October 2011

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II. Summary

Physical exercise is one of the most potent and effective cognitive enhancers we know. Unequivocal evidence in both humans and animals has shown that exercise can induce robust and consistent improvements in performance in different memory tasks. Exercise has also been shown to boost neurogenic and neurotrophic activity in the brain. The mechanisms underlying these effects are still not well understood, and as a result, the therapeutic applicability of exercise has been limited. Although substantial research now points to brain-derived neurotrophic factor (BDNF) as the central mediator of the positive effects of exercise on both cognition and neurogenesis, direct evidence is lacking. Furthermore, it is unclear how exactly exercise causes increases in BDNF, and how these increases translate to improvements in memory. To address these and other gaps in the literature, we used a multidisciplinary approach to investigate the mechanisms of short-term exercise-induced cognitive enhancement in the male Wistar rat, by combining behavioural, cellular, molecular, and imaging techniques.

At the behavioural level, we first confirmed a previous finding in the laboratory that one week of forced moderate exercise improves the performance of rats in a novel object recognition task, but not in a spatial task. We found that choosing a more challenging variant of the spatial task allowed us to detect a significant exercise-induced improvement. In addition, we were able to demonstrate that the effects of exercise are independent of housing conditions, and of treadmill-induced stress. Furthermore, we showed that intracerebroventricular BDNF administration can enhance memory in a manner that mimics exercise. Intravenous BDNF administration, however, enhanced memory only when rats were exercised immediately post-infusion.

At the molecular level, we detected consistent but transient increases in serum BDNF with exercise. Tissue analysis revealed an exercise-induced increase in the regulated release, transcription and expression of BDNF in the dentate gyrus, and an increase in the activation and transcription of its receptor TrkB. Exercise-induced increases were also observed in ERK1 activation and Synapsin-1 expression in the dentate gyrus. Tissue analysis confirmed that a single intracerebroventricular infusion of BDNF induces an increase in BDNF in the dentate gyrus, along with an increase in activated ERK-1 and CaMKII. Intravenous BDNF administration also caused an increase in BDNF in the dentate gyrus, but again only when rats were exercised immediately post-infusion.

At the cellular level, we found that one week of forced moderate exercise induces an increase in cell proliferation, and in the number of immature neurons, but has no effect on the number of mature neurons and apoptosis (all in the dentate gyrus). We also showed that three weeks of environmental enrichment (in the absence of running wheels) are accompanied by an increase in the number of immature neurons in the dentate gyrus, but have no effect on the number of mature neurons or apoptosis.

At the vascular level, we report that blood flow to the hippocampus, but not the cortex or whole brain, increased immediately after an acute bout of exercise. The same result was observed after one week of exercise. Finally, we provide preliminary data to suggest that exercise may be influencing the permeability of the blood-brain barrier in the dentate gyrus, as assessed by the intravenous injection of a fluorescent dye.

Taken together, these results support the hypothesis that short-term exercise-induced cognitive enhancement is mediated by the rapid effects of the neurotrophin BDNF and downstream signalling effectors on synaptic transmission in the dentate gyrus. BDNF could also stimulate the changes in cellular proliferation observed with exercise, but these changes are unlikely to underlie the observed memory improvements. The timing of observed exercise-induced changes, along with our vascular data, raises the possibility that BDNF is originating in part from a peripheral source, and is being transported across the blood-brain barrier into the dentate gyrus by mechanisms that involve acute exercise-induced vascular changes.

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IV. Acknowledgements

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Finally, to Dónal and my amazing family: Joseph, Hoda, Hannah and Naji, I would not have been able to do it without you; this thesis is dedicated to you.

V. List of Abbreviations

7,8-DHF	7, dihydroxyflavone
aCSF	Artificial Cerebrospinal Fluid
ACTH	Adrenocorticotropic hormone
AHN	Adult Hippocamal Neurogenesis
Akt	'Ak': a strain of mice, 't': transforming (also called PKB)
AMPA	α-amino-3hydroxy-5-methyl-4-isoxaloleprorionic acid
ANOVA	Analysis of variance
AP	Action Potential
Arc	Activity-regulated cytoskeletal-associated protein
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BrdU	5'-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
btASL	Bolus-tracking arterial spin labeling
CA 1	Cornu Ammonis 1
CA 3	Cornu Ammonis 3
Ca^{2+}	Calcium
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase

CBF	Cerebral Blood Flow
CBV	Cerebral blood volume
CNS	Central nervous system
Cort	Corticosterone
CREB	c-AMP response element-binding protein
CRH	Corticotropin Releasing Hormone
CSF	Cerebrospinal fluid
CT	Cycle number
CTT	Capillary transit Time
Cyt C	Cytochrome C
DAB	Diaminobenidine
DAG	Diacylglycerol
DCX	Doublecortin
DG	Dentate Gyrus
DNA	Deoxyribonucleic Acid
EC	Entorhinal Cortex
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalography
ELISA	Enzyme-linked immunosorbent assay
ERK	Extacellular signal-related kinase

Ex	Exercise
Ex-NoL	Exercise-no learning
Ex-L	Exercise-learning
Ex-SH	Exercise-standard housed
Ex-En	Exercise-enriched
GAB 1	GRB-associated binder 1
GRB 2	Growth factor-bound 2 adaptor protein
HPA	Hypothalamic pituitary adrenal axis
HRP	Horseradish peroxidase conjugate
i.c.v.	Intracerebroventricular
IGF	Insulin-related Growth Factor
IgG	Immunoglobulin G
Ins(1,4,5)P3	Inositol (1,4,5)-trisphosphate
i.p.	Intraperitoneal
i.v.	Intravenous
KCl	Potassium chloride
KI-67	'Kiel'-67
kDa	Kilodaltons (unit of protein molecular weight)
LPP	Lateral perforant pathway
E-LTP	Early phase long-term potentiation

L-LTP	Late phase long-term potentiation
LTP	Long-term potentiation
LTM	Long term memory
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
mBDNF	Mature BDNF
MEK	Mitogen-activated protein kinase kinase
MPP	Medial perforant pathway
mRNA	Messenger ribonucleic acid
MRI	Magnetic Resonance Imaging
MTT	Mean transit time
MW	Molecular weight
MWM	Morris water maze
NGF	Nerve growth factor
Na-F	Sodium Fluorescein
NMDA	N-methyl-D-aspartate
NOR	Novel object recognition
No-L	No learning
NT-3	Neurotrophin 3
NT-4/5	Neurotrophin 4/5

OCT	Optical cutting temperature
OD	Object displacement
OD	Opitical density
OS	Object Substitution
$p75^{NTR}$	Pan 75 neurotrophin receptor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-Tween®20
PC	Perirhinal Cortex
PCR	Polymerase chain reaction
pERK	Phosphorylated ERK
PI3K	Phosphotidylinositol-3 kinase
PKC	Protein kinase C
PKB	Protein Kinase B (also called Akt)
PLC-γ	Phospholipase C-γ
pPLC-γ	Phosphorylated Phospholipase C-γ
PNS	Peripheral Nervous System
proBDNF	Pro-neurotrophin BDNF
PtdIns(4,5)P2	Phosphotidylinositol-4,5-bisphosphate
Ras	'Rat sarcoma' also called p21

rCBV	Relative cerebral blood flow
RNA	Ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Sed	Sedentary
Sed-L	Sedentary-Learning
Sed-NoL	Sedentary-No Learning
Sed-SH	Sedentary-standard housed
Sed-En	Sedentary-enriched
SEM	Standard error of the mean
SGZ	Subgranular zone
Shc	Scr homologous and collagen-like adaptor protein
SOS	Son of sevenless
STM	Short term memory
SVZ	Subventricular zone
SGZ	Subgranular zone
TBS	Tris buffered saline
TBS-T	Tris buffered saline-Tween®20
tERK	Total ERK

TMB	3,3',5,5'-Tetramethyl-benzidine
tPA	Tissue plasminogen Activator
Trk	Tropomyosin receptor kinase
Trk A	Tropomyosin receptor tyrosine kinase A
Trk B	Tropomyosin receptor tyrosine kinase B
Trk C	Tropomyosin receptor tyrosine kinase C
TrkB-IgG	Tropomyosin receptor tyrosine kinase B immunoglobulin G
VEGF	Vascular Endothelial Growth Factor
Zif268	Zinc Finger Transcription Factor (aslo called EGR1)

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General Introduction

1.1 Rationale

Our ability to recognise familiar objects, remember their locations, and use this information to interact with objects and navigate through space, is such an integral part of our everyday life that we tend to take it for granted. This ability relies on several mental processes, including perception, attention, learning and memory, which collectively fall under the umbrella-term cognition. The focus of this work is on learning and memory; cognitive processes that are especially vulnerable to ageing, trauma, and certain diseases. In this work, we will use the term cognitive enhancement to refer to the process of improving learning and memory, and we will use the term cognitive performance to refer to the performance of animals in tasks that rely on learning and memory.

The field of cognitive enhancement has become a major focus of neuroscience research, the outcomes of which are of crucial importance at both the basic level and the clinical level. Intensive research is now focused on finding drug targets that can be manipulated to improve learning and memory. This endeavour is financially supported by the pharmaceutical industry, and yet, available therapeutic options are sparse and unsatisfactory (Husain and Mehta, 2011, Plath et al., 2011, Roesler and Schroder, 2011). It is easy to overlook the fact that there are simple behavioural changes that when incorporated into one's lifestyle can lead to robust and reliable improvements in cognitive performance. One of the most potent and well-studied of these is physical exercise. Exercise has been shown to effectively prevent, treat, and even reverse cognitive deficits associated with ageing (Lista and Sorrentino, 2009), neurodegenerative and psychiatric disease (Deslandes et al., 2009), and neurological insults and trauma (Lojovich, 2010) in laboratory models, while attempts to use exercise in clinical settings have had an overall positive but more ambiguous outcome (Sofi et al., 2011). This is likely to be due to a combination of factors, including compliance issues, inherent differences between humans and animals, and the vast variety of approaches and exercise paradigms used. However, the greatest obstacle to exploiting exercise therapeutically is that the underlying mechanisms of exercise-induced cognitive enhancement remain largely unknown.

In this work, we use physical exercise as a simple, non-invasive intervention to enhance cognition, and investigate associated cellular, molecular, and vascular changes. This research allows us to gain a better understanding of the neural mechanisms of learning and memory, and has the

gain a better understanding of the neural mechanisms of learning and memory, and has the potential to reveal novel and effective drug targets for cognitive enhancement. Finally, it could aid in the development of multidisciplinary strategies to optimise cognitive enhancement and rehabilitation in a clinical setting.

1.2 Learning and Memory

1.2.1 An Introduction to Learning and Memory

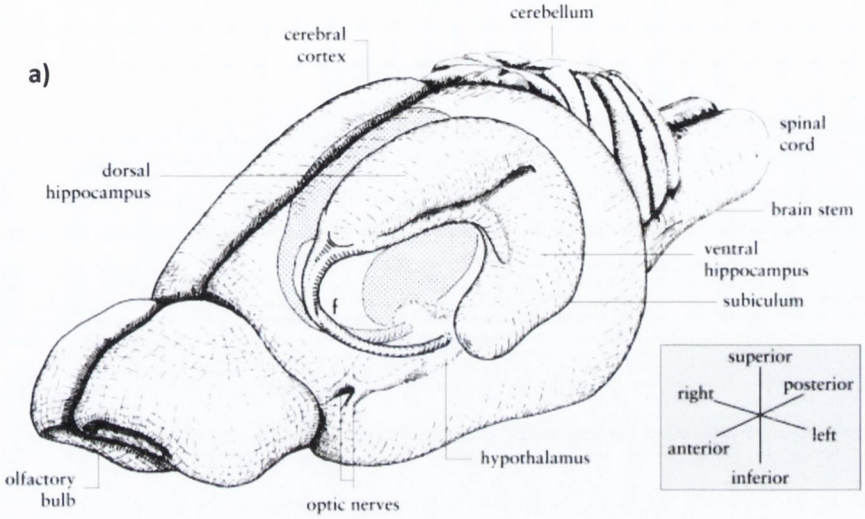
The past fifty years have seen radical advances in our understanding of how memories are formed in the brain. The formation of a new memory involves several processing steps: encoding (or acquisition), storage (or consolidation), and retrieval (or recall) (Kessels et al., 2001). The term 'learning' refers to the mechanisms involved in the first step (acquisition), where a memory trace is initially formed, whereas the mechanisms involved in the storage and recall of information are termed 'memory' (Lynch, 2004), where the memory trace persists and is recalled a time after acquisition. Memory can be categorised as short-term (information is retained over a few seconds), or long-term (information is retained from minutes to years) (Squire, 1986). Long-term memory can be explicit (or declarative) and implicit (procedural, or nondeclarative). Explicit memory refers to the conscious retention of facts, people, places and things, and can be further divided into episodic (contextual, e.g. a place or an object) and semantic (non-contextual, e.g. an abstract fact) memories. Procedural learning refers to skill learning, e.g. learning to play a piano piece (Bird and Burgess, 2008).

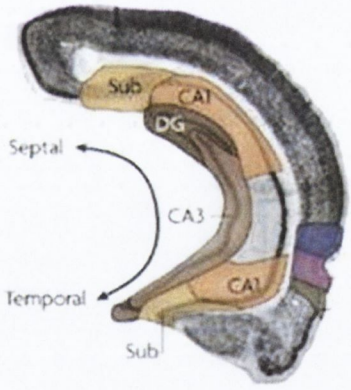
This thesis investigates two types of memory in rodents: spatial memory and object recognition memory. Both of these fall under the long-term, declarative, episodic category, although it is somewhat difficult to apply these categories to non-human animals, since by definition, declarative memories are memories that can be verbally described. However, cognitive tests have been designed to circumvent these challenges (Eichenbaum, 1997), as discussed below. Despite some difficulty in translating rodent research into clinical applications for humans, there are several strong advantages to using rodents in the investigation of learning and memory. First, the absence of a linguistic component in testing for memory enables researchers to assess performance independently of verbal competence (Eichenbaum, 1997). Second, it is widely accepted that the basic cellular and molecular processes underlying learning and memory are similar across species, such that much of the insight we gain from rodent studies can be applied to human research

(Purcell and Carew, 2003). Third, certain interventions (such as inducing selective lesions in particular parts of the brain and observing their effects on cognitive performance) would simply not be possible in humans, and these interventions have revealed a wealth of information about the exact neural structures involved in different stages and types of memory. These studies, combined with lesion mapping studies in humans, have shown that procedural memory requires an intact cerebellum and basal ganglia, while declarative memory requires the medial temporal lobe, particularly the hippocampal formation and associated structures (Young et al., 1997).

1.2.2 The Hippocampal Formation

The hippocampal formation (Fig 1.1) is a subcortical brain structure which encompasses three subregions: the hippocampus proper (which in itself is subdivided into four *cornu ammonis* regions: CA1, CA2, CA3 and CA4), the dentate gyrus (DG), and the subiculum (Afifi and Bergman, 2005). In the rat, only three *cornu ammonis* regions have been identified (CA1, CA2, and CA3) (El Falougy et al., 2008, van Strien et al., 2009). The hippocampal formation as a whole is a trilaminar structure containing three layers of cells: the molecular layer, the pyramidal layer, and the polymorphic layer (or *stratum oriens*). Similarly, the dentate gyrus is composed of a molecular layer (containing the dendrites of granule cells), a granule cell layer (containing the soma of granule cells), and the hilus (containing the axons of granule cells which project to the CA3 region) (van Strien et al., 2009). There is some ambiguity in the literature surrounding the terminology used to refer to the hippocampal formation and its substructures. For the sake of consistency, we will use the term hippocampal formation to refer to the entire hippocampal region (consisting of the hippocampus proper, the dentate gyrus, and the subiculum). The subregion of the hippocampus proper which consists of the Cornu Ammonis (CA) regions will simply be called the hippocampus in this text.





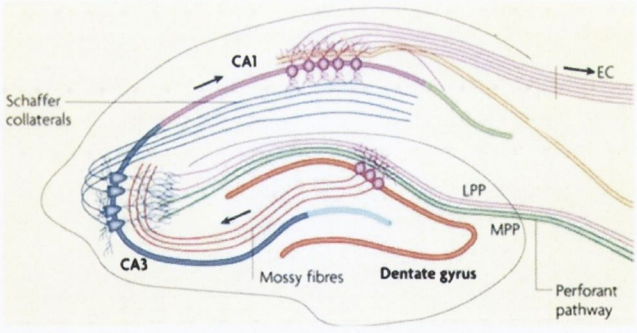


Fig 1.1 Anatomy of the hippocampus

(a) The hippocampal formation is a bilateral structure located between the cortex and the thalamus (van Strien et al., 2009). (b) It consists of the hippocampus proper (CA1 & CA3), the dentate gyrus (DG), and the subiculum (Sub) (van Strien et al., 2009). (c) The lateral and medial perforant pathways (LPP and MPP) carry the main inputs to the hippocampal formation from the perirhinal and parahippocampal cortices via the entorhinal cortex (EC). The main output from the hippocampus is from CA1 to the EC via the subiculum. The mossy fibres connect the granule cells of the dentate gyrus to CA3, while the Schaffer Collaterals connect the CA3 to CA1 (Deng et al., 2010).

The hippocampal formation is part of a network of limbic structures known as Papez's circuit, which includes cortical regions (such as the cingulate cortex and the entorhinal cortex) and subcortical regions (such as the mamillary bodies, and anterior thalamus) (Afifi and Bergman, 2005). The circuit also includes the fibre tracts connecting these structures, such as the fornix. The main inputs to the hippocampal formation are from the parahippocampal cortices via the entorhinal cortex, connected by the lateral and medial perforant pathways. The main output from the hippocampus is from the CA1 neurons of the hippocampus to the entorhinal cortex via the subiculum. Within the hippocampal formation several pathways form connections between subregions: the mossy fiber pathway connects the granule cells of the dentate gyrus to pyramidal cells of the CA3, while the Schaffer Collateral pathway connects the CA3 neurons to the CA1 neurons (Afifi and Bergman, 2005). It is these three pathways, the perforant pathway, the mossy fiber pathway, and the Schaffer Collateral pathway, that have been extensively studied for evidence of experience-dependent plasticity.

The importance of the hippocampal formation in learning and memory has been known for over half a century now, ever since it was noted that bilateral ablation of the hippocampal formation in humans results in a loss of memory and the inability to acquire new memories (anterograde amnesia) (Scoville and Milner, 1957). Long-term memories however, remain intact. There is much debate as to whether the hippocampus is involved in acquisition and/or storage of new memories (Nadel and Hardt, 2011). The dentate gyrus, in particular, has received special attention for its role in memory formation. To date, no studies have shown a direct link between the dentate gyrus and memory formation (Treves et al., 2008), but many of the cellular and molecular changes associated with learning and memory (long-term potentiation, morphological changes, neurogenesis, and neurotrophin-related signalling, introduced in later sections), are well-characterised in the dentate gyrus. As a result, the role of the dentate gyrus in learning and memory was established in a correlational manner, as a result of 1) high rates of neurogenesis and 2) highly plastic properties of connections. It seems like the hippocampus can do its job without the dentate gyrus, as suggested by a study where the DG was lesioned but hippocampal circuitry was not affected (McNaughton and Morris, 1987), since the CA3 receives most of its input directly from the EC. The dentate gyrus has recently been proposed to be crucial for a type of information processing called 'pattern separation' (Aimone et al., 2011). Pattern separation is a general term used to refer to the ability to discriminate between two patterns (including contexts, objects, and locations). The two forms of memory investigated in this study rely heavily on pattern separation, making the dentate gyrus of especial interest in this work.

Parahippocampal structures relevant to this work include the perirhinal (PC) and entorhinal (EC) cortices. The perirhinal cortex (Kealy and Commins, 2011) has been shown to play a role in object familiarity and object recognition memory (Broadbent et al., 2004, Buckley, 2005). The entorhinal cortex (Kerr et al., 2007) is crucial for the processing of spatial information, and represents the main input to the hippocampus.

1.2.3 Spatial Memory

Spatial memory is defined as the ability to encode, store, and retrieve information about the arrangement of objects or routes in space (Kessels et al., 2001). Some researchers add the recognition of objects or routes to this definition, but this is termed spatial perception and is distinct from memory, and the role of the hippocampus in spatial perception is currently under heavy debate (Bird and Burgess, 2008). Spatial memory involves both short-term and long-term memory, and both declarative and procedural processes. There are also several types of spatial memory that can be distinguished according to the type of memory task used to test them (Paul et al., 2009). The main spatial tasks that are currently used are mazes (such as the famous Morris Water Maze) which test the ability to remember routes or paths, and require a more sequential processing of spatial information. Other tests, such as the object displacement task that we use in our laboratory, test object-location memory, which requires both the creation of a spatial map and the association of a specific object with a specific position on the map (Kessels et al., 2001). For simplicity, we will use the broader term spatial memory to refer to object-location memory tested by the object displacement task.

The object displacement (OD) task used in our laboratory exploits the well-established tendency of animals (rats in particular, but including humans) to show a preference for novelty (Ennaceur, 2010). Briefly, the rat is placed in an arena with several distinct objects placed at specific locations and allowed to explore. Twenty-four hours later, one of the objects is displaced to a new location, and the rat is allowed to explore again. Preferential exploration of the displaced object is taken to be an indication that the rat has remembered the positions of the objects from the day before, and is able to identify the displaced object. The performance of the rat in this task depends on its ability to remember the objects and their positions in the arena, and to consequently show a preference for the displaced object. This task is a test for long-term object-location memory (Griffin et al., 2009), and is a subtle assessment of pattern discrimination (Aimone et al., 2011). The simplicity and short duration of this task make it ideal for a quick test of spatial memory immediately after an

intervention, especially if the effects of the intervention (as in the case of exercise) are thought to be transient. Unlike tasks like the water maze, this task does not require the animal to swim (which is an intense form of exercise) and is less stressful: the water maze has been shown to induce significant increases in the stress hormone corticosterone in the blood (Beiko et al., 2004).

1.2.4 Object Recognition Memory

Object recognition memory is defined as the ability to distinguish between a familiar object (one that has been previously presented), and a novel object (one that has not been previously presented) (Squire et al., 2007). The most common way to test an animal's object recognition memory is the novel object recognition task (Squire et al., 2007). Typically in this task, several (two or three) objects are presented for exploration by the rodent in an arena. Twenty-four hours later, familiar object(s) are presented along with a novel object. Again, this task exploits the natural tendency of animals to preferentially explore novel objects, indicating that they remember the familiar object(s). The task used in our laboratory is a variant of this task called the object substitution task (OS) (Griffin et al., 2009). Similarly to the object displacement task, the rat is placed in an arena with several distinct objects placed at specific locations and allowed to explore. Twenty-four hours later, one of the familiar objects is substituted for a novel object, and the rat is allowed to explore again. Preferential exploration of the novel object is taken to be an indication that the rat has remembered the objects from the day before, and is able to identify the novel object. The performance of the rat in this task depends on its ability to remember the objects from the day before.

Although object recognition memory is often described as being a type of memory that is hippocampus-independent, it is likely that the hippocampus plays an indirect role (Buckley, 2005). In a typical novel object recognition task, there is also a spatial component present (such as the arrangement of the objects), even if this component is not crucial to the task itself. Furthermore, object recognition (especially when discriminating two similar objects) relies on pattern separation, a process that has been attributed specifically to granule cells of the dentate gyrus as discussed above (Aimone et al., 2011, Schmidt et al., 2011). It is thought that the hippocampus is involved in these aspects of a novel object recognition task, but not in the actual recognition of the features of the familiar and novel objects, which seems to recruit mostly the perirhinal cortex (Kelly et al., 2003, Buckley, 2005). The perirhinal cortex has been shown to be crucial for both remembering

and recognising the representation of complex objects, i.e. in both object recognition memory and perception (Kealy and Commins, 2011).

1.2.5 Cellular and Molecular Mechanisms of Learning and Memory

1.2.5.1 Synaptic Transmission

Current views on how memories are acquired and stored in the brain require an understanding of the events that allow signals to be transmitted between neurons (Bruel-Jungerman et al., 2007a, Morgado-Bernal, 2011). Neurons communicate with each other across functional connections called synapses. These connections are predominantly between the axon terminal of one neuron (the presynaptic neuron) and the soma or dendrites of another (postsynaptic neuron). Neurotransmitters (we will use glutamate, the main excitatory neurotransmitter of the mature mammalian nervous system, as an example) are sequestered in vesicles and transported to the plasma membrane of the axon terminal. Synaptic vesicles are maintained in two distinct pools, where they are tethered to the cell's cytoskeleton: a reserve pool (where the vesicles remain intact) and the ready-release pool (where the vesicles are brought close to the plasma membrane of the axon terminal, ready to release neurotransmitters). Upon exocytosis, neurotransmitters are released into the synaptic cleft; a gap between pre-and post-synaptic structures, where they bind to receptors on the post-synaptic membrane. Neurotransmitter release involves vesicle docking and fusion, and release of neurotransmitters into the synaptic cleft. This process is mediated by specialised proteins within the vesicle membranes called synaptic vesicle proteins.

The transmission of a signal across a glutamatergic synapse occurs in the following way (Fig 1.2). Depolarisation of the presynaptic neuron causes voltage-gated Ca^{2+} channels in the ER to open and release Ca^{2+} into the cytosol. The Ca^{2+} signal activates synaptic vesicle proteins (such as Synapsin-1 and Synaptophysin) to mediate the release of glutamate into the synaptic cleft. Glutamate diffuses into the cleft and binds to two main receptors on the post-synaptic membrane: AMPA receptors and NMDA receptors, named after their respective ligands (2-amino-3-5-methyl-3-oxo-1,2-oxazol-4-yl propanoic acid and n-methyl-D-aspartate). AMPA-Rs are ligand-gated ionotropic receptors: upon binding to glutamate they open and allow Na+ to pass into the cell. If sufficient depolarisation occurs, the NMDA-Rs are activated. NMDA-Rs are both ligand-gated and voltage-gated: they become activated only if two events occur simultaneously: binding to glutamate, and sufficient depolarisation (which removes an Mg blockade of the ion channel). This allows them to act as

coincidence detectors: when the correct conditions are present they open and allow cation influx (Na^+ and Ca^{2+}) into the cell. Sufficient depolarisation causes voltage gated Ca^{2+} channels in the post-synaptic endoplasmic reticulum (ER) to open, causing an increase in intracellular Ca^{2+} in the post-synaptic cell. As a result, the signal has successfully been transmitted from one neuron to the next. Intracellular signalling cascades transmit the signal from the post-synaptic membrane into the cell. The strength of a synapse depends on how efficiently it is able to transmit a signal between two neurons, and depends on several factors (discussed in the next section).

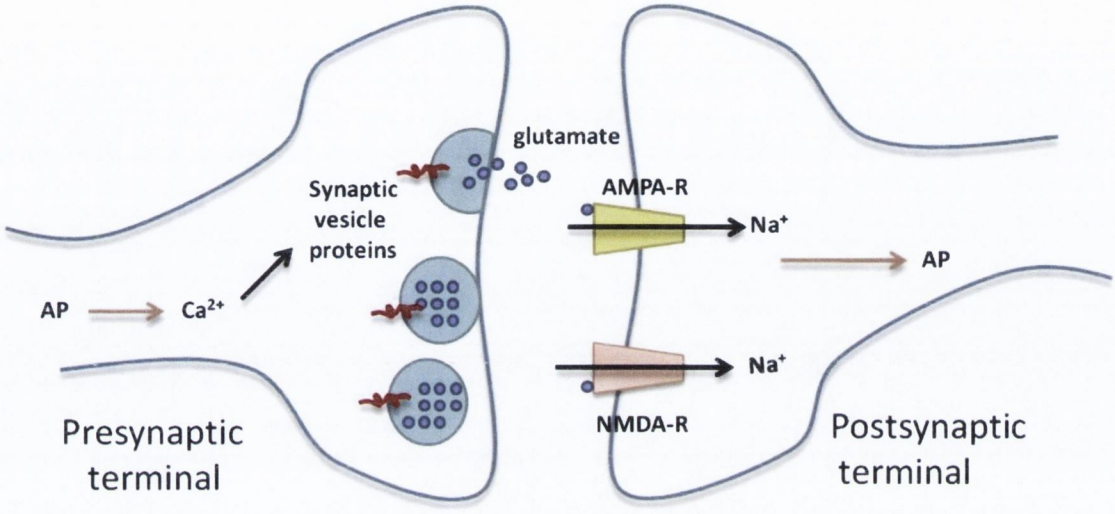


Fig 1.2 Synaptic transmission in glutamatergic neurons

Depolarisation of the presynaptic neuron causes voltage-gated Ca^{2+} channels in the ER to open and release Ca^{2+} into the cytosol. The Ca^{2+} signal activates synaptic vesicle proteins to mediate the release of glutamate into the synaptic cleft, where it binds to two receptors on the post-synaptic membrane: NMDA-Rs and AMPA-Rs. These receptors are ligand-gated voltage channels: when glutamate binds to them they allow an influx of cations into the post-synaptic terminal. If the depolarisation reaches threshold, an action potential is propagated.

1.2.5.2 Long-term Potentiation

The underlying neural mechanisms of the encoding, consolidation, and retrieval of memories are currently a topic of intensive research and debate in neuroscience. Nevertheless, there is a current consensus that the acquisition of a memory trace occurs through changes in the strength of synapses, first predicted by Donald Hebb in 1949 (Cooper, 2005). These changes are activity-dependent, dynamic, and long-lasting. An increase in synaptic strength in response to a stimulus was first recorded by Bliss and Lomo in 1973, and termed long-term potentiation (Bliss and Lomo, 1973).

For long-term potentiation (LTP) of synaptic strength to occur, the synapse must undergo three sequential phases. The first stage is referred to as short-term potentiation (STP), and lasts from milliseconds up to 30 or 45 minutes post-tetanus (Sweatt, 1999). STP relies on activity-dependent changes in the amount of neurotransmitter released by the presynaptic terminals in response to an action potential. In the case of post-tetanic potentiation of the MPP-granule cell synapse, the effect is mainly an increase in the amount of glutamate released. The exact mechanism of how this occurs is still not well understood, but is generally accepted that presynaptic Ca^{2+} levels play an important role (Zucker and Regehr, 2002). Later, we describe a role of BDNF in LTP that involves facilitating glutamate release presynaptically. STP serves as prelude to the two later stages of potentiation: early LTP (E-LTP) and late LTP (L-LTP), both of which are thought to rely mainly on post-synaptic mechanisms.

The second phase of LTP, E-LTP, lasts up to 2 or 3 hours *in vivo*, and depends on post-synaptic mechanisms involving AMPA and NMDA- receptors (Rao and Finkbeiner, 2007). The release of glutamate activates AMPA-Rs and NMDA-Rs in the post-synaptic membrane (as discussed above), which lead to an influx of Ca^{2+} into the cell. This in turn activates several Ca^{2+}-dependent kinases such as the Ca/calmodulin-dependent kinase (CaMKII) and protein kinase C (PKC) (Mayford, 2007). The activation of these kinases by Ca^{2+} is essential to the induction of E-LTP (induction stage), and they are able to auto-phosphorylate, making their continued activation Ca^{2+} - independent. In this way, the E-LTP is maintained even after the Ca^{2+} signal recedes (maintenance stage). In turn, these kinases phosphorylate the post-synaptic AMPA-Rs making them more sensitive to glutamate (Sweatt, 2001), thus causing the same amount of neurotransmitter to induce a larger post-synaptic potential (expression stage).

The third phase of LTP, L-LTP, can last from hours to weeks *in vivo* (Lynch, 2004), and depends on the activation of intracellular signaling pathways which lead to protein synthesis. The same kinases activated during E-LTP activate signaling pathways (such as such as the cAMP pathway and the MAPK pathway) which lead to the phosphorylation of transcription factors and finally, gene expression. A fourth phase of LTP is sometimes dissociated from the third, and it is protein synthesis (Bramham, 2008). Proteins synthesized as a result of the signaling cascades activated during L-LTP include new AMPA-Rs, which are inserted into the post-synaptic membrane and increase the sensitivity of the post-synaptic cell to glutamate.

Consequently, LTP can be facilitated at the synapse by different mechanisms. Presynaptically, any intervention that increases the amount of glutamate exocytosed in an activity-dependent manner at the presynaptic terminal will facilitate LTP (Blundon and Zakharenko, 2008). Glutamate exocytosis can be increased by increasing the amount of glutamate that can be readily released into the synaptic cleft. One mechanism by which this can be achieved is by upregulating and activating synaptic vesicle proteins, which increase the amount of neurotransmitter-filled vesicles that can successfully dock to the membrane and exocytose. Since the activity of some synaptic vesicle proteins is Ca^{2+}-dependent (Schneggenburger and Neher, 2005), increasing presynaptic intracellular Ca^{2+} would also facilitate glutamate release. Postsynaptically, the response to glutamate can be potentiated by increasing the number and sensitivity of AMPA-Rs and NMDA-Rs on the post-synaptic membrane. In addition, increasing the amount of intracellular calcium (or other cations) in the post-synaptic neuron renders the cell more excitable, and thus more likely to depolarise in response to a signal from the presynaptic neuron.

1.2.5.3 Morphological Changes and Synaptogenesis

Although LTP provides is a widely accepted candidate for the neural mechanism underlying the acquisition of new memories, it is thought that simple changes in synaptic strength are not enough for the long-term storage of memories (Bruel-Jungerman et al., 2007a). Widespread plastic changes need to occur across the brain to mediate long-term effects including changes in the number of dendrites and changes in the number, size and density of dendritic spines (tiny structural protrusions from the dendrite). Dendritic spines form synapses with other neurons, and as a consequence, changes in the number, size and density of dendritic spines are directly related to changes in the number, size and density of synapses. Changes in dendritic spines are quite rapid, enlargement of existing spines can happen within minutes (Tanaka et al., 2008); however, the

insertion of AMPA-Rs into these spines takes a few hours (Park et al., 2006), and it will take a few days before these spines form fully functional synapses (Nagerl et al., 2007). The restructuring of existing synapses and even *de novo* synthesis of synapses (synaptogenesis) has been shown to occur with learning (Markham and Greenough, 2004), and is thought to represent a more stable and persistent form of memory storage in the brain.

1.2.5.4 Adult Hippocampal Neurogenesis

Neurogenesis is defined as the process by which mature, functional neurons are generated from precursor stem cells (Ming and Song, 2005). The discovery that neurogenesis continues into adulthood in both humans (Eriksson et al., 1998) and animals (reveiwed in Gross, 2000), and is not confined to development, has revolutionised the field of neuroscience, and over the last decade considerable advances have been made in elucidating its mechanisms and properties. Neurogenesis has since been shown to occur continuously throughout the lifespan of most mammals studied to date, however in a spatially-restricted manner. Two regions in the adult mammalian brain have been identified as neurogenic zones: the subventricular zone (SVZ) of the lateral ventricles, and the subgranular zone (SGZ: a strip of cells between the granule layer and the hilus) of the dentate gyrus in the hippocampal formation. New neurons originating in the lateral ventricles migrate to the olfactory bulb where they become interneurons, while new neurons originating in the dentate gyrus migrate into the granule cell layer to become fully functional granule cells (Gage, 2000).

Adult hippocampal neurogenesis (AHN) has been divided into several stages (Lledo et al., 2006). To identify and characterise the stages of AHN, researchers make use of specific cellular markers for each of the different stages (von Bohlen Und Halbach, 2007). First, progenitor cells in the subgranular zone of the dentate gyrus divide by undergoing mitosis (proliferation stage). These progenitor cells can be divided into several subtypes, depending on the cell-surface markers that they express (Kempermann, 2008). Late pre-mitotic precursors start to express doublecortin (DCX), a microtubule-binding protein involved in cell migration (Couillard-Despres et al., 2006). During and after cell division, cells can be stained for 5-bromo-2'-deoxyuridine (BrdU), currently the most widely used marker for cell proliferation. BrdU is a synthetic thymidine analog: an exogenous nucleoside that can become incorporated into replicating DNA during the S-phase (synthesis phase) of the cell cycle by substituting for endogenous thymidine. As a result, cells that are replicating within a narrow time-window of BrdU administration will stain positively for BrdU upon immunohistochemical analysis (Hayes and Nowakowski, 2000, Taupin, 2007, Landgren and

Curtis, 2011). An alternative marker for cell division is the endogenous nuclear protein KI-67. It is expressed in all phases of the cell cycle except the resting phase and can thus be used to identify mitotic cells (Scholzen and Gerdes, 2000, Kee et al., 2002).

Newly-born cells then differentiate into a neuronal phenotype (differentiation stage). These immature neurons (still expressing DCX) migrate into the granule cell layer (migration stage), and then mature into dentate granule cells (maturation stage) (Abrous et al., 2005). Upon maturation, DCX is down-regulated, and neurons start to express NeuN (Neuronal Nuclei), a neuron-specific protein (Mullen et al., 1992). Then they become functionally integrated into existing neural circuits (Carlen et al., 2002). The first stages (differentiation and migration) happen within the first week of birth, while maturation occurs during the second week. By the end of the second week, these neurons start extending axons towards the CA3, while it takes another week for dendritic spines and synapses to form with cells of the EC. Through this process, the dentate gyrus forms about 10, 000 new neurons per day (Shors, 2009). Of these new cells, more than half (up to 80% in some cases) die within one week of birth (Kempermann et al., 2003) by apoptosis.

Although there is still no direct evidence that AHN contributes to memory formation, a lot of correlative evidence exists (Bruel-Jungerman et al., 2007b). One of the most compelling arguments for the role of newly-born neurons in memory is that they have specific cellular properties that make them especially suited to undergo LTP (Schmidt-Hieber et al., 2004, Ehninger and Kempermann, 2008). Recently, computational models have attributed to new granule cells a specific role in pattern separation (Aimone et al., 2011, Schmidt et al., 2011). This hypothesis proposes that immature neurons are tuned to respond to a broad spectrum of stimuli, due to being highly excitable and receiving more general inputs. Mature neurons, on the other hand, are fine-tuned to respond to specific stimuli, due to higher excitability thresholds and stronger, more specific inputs from the EC. At any one time, the dentate gyrus is composed of both populations of cells, and the combination of both allows for the processing that underlies pattern separation. A higher proportion of new cells allows for higher resolution discrimination. This idea is backed by recent studies have shown that an increase in neurogenesis is both necessary (Tronel et al., 2010) and sufficient (Sahay et al., 2011) to improve pattern separation. Most interventions that improve learning (such as exercise and environmental enrichment) also have been found to increase AHN (Abrous et al., 2005, Ming and Song, 2005). Interestingly, one of the most important regulators of AHN is the neurotrophin brain-derived neurotrophic factor (BDNF) (Choi et al., 2009), which is the focus of the next section.

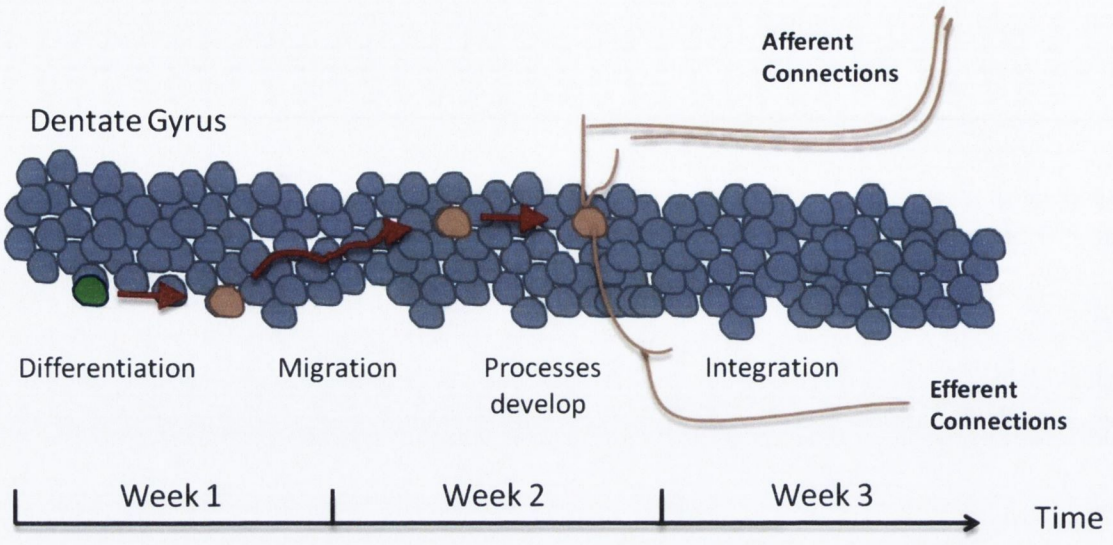


Fig 1.3 Stages of adult hippocampal neurogenesis

Adult hippocampal neurogenesis (AHN) occurs in the dentate gyrus of the hippocampal formation, and encompasses several stages. First, progenitor cells in the subgranular zone (a strip of cells between the granule layer and the hilus) of the dentate gyrus proliferate (proliferation). The new cells then differentiate into a neuronal phenotype (differentiation). These immature neurons then migrate into the granule cell layer (migration), and then mature into dentate granule cells (maturation). They then develop processes and create afferent connections with, and efferent connections with. At this stage, they become functionally integrated into existing neural circuits. Immature neurons exhibit strong plasticity properties around week 3 of development.

1.3 The Role of BDNF in Learning and Memory

1.3.1 An Introduction to Neurotrophins

Neurotrophins are soluble proteins that are produced and secreted from neurons in the central and peripheral nervous system. They are growth factors; first discovered for their role in regulating the proliferation, growth, maturation, and survival of neurons in the developing nervous system. There are four main neurotrophins in the mammalian brain, brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4). These proteins are structurally closely related and bind to a family of tyrosine kinase receptors which include TrkA, TrkB, and TrkC. There is some overlap in receptor binding, but in general, NGF has the highest affinity for TrkA, BDNF has the highest affinity for TrkB, and NT-3 and NT-4 have the highest affinity for TrkC (Lu et al., 2005). Receptor binding activates signalling cascades which lead to the upregulation of specific genes that mediate the neurotrophin's trophic effects on specific subpopulations of neurons (Binder, 2007). There are also several growth factors that do not belong to this family, but have been shown to be induced by exercise, namely, insulin-related growth factor (IGF-1) and vascular endothelial growth factor (VEGF). Upon discovery, neurotrophins received considerable interest from the scientific community, mainly due to their potential as therapeutic agents to counteract the effects of ageing and neurodegenerative orders on the brain. BDNF in particular, has shown the most promise as a therapeutic agent, due to its potent neuroprotective and cognitive enhancing effects (Nagahara et al., 2009), and also due to its high tolerability. However, BDNF has poor pharmacokinetic properties which have derailed attempts to use BDNF in clinical applications.

In this work, we focus mainly on the neurotrophin BDNF, and to a lesser degree on NGF, IGF, and VEGF, since they have been implicated in learning and memory processes in general, and in the effects of exercise in particular.

1.3.2 Brain-derived Neurotrophic Factor (BDNF)

In addition to its developmental role, BDNF has been well-established to play an important functional role in the mature nervous system: it regulates the proliferation, differentiation and survival of neurons, the facilitation and stabilisation of the synapse, synaptogenesis, and activity-dependent forms of neuroplasticity (Carvalho et al., 2008).

BDNF is a 13 kDa protein consisting of 119 amino acid residues, and is produced by the transcription of the *Bdnf* gene. The *Bdnf* gene is highly conserved in species ranging from fish to mammals, highlighting its functional importance (Heinrich and Pagtakhan, 2004, Pruunsild et al., 2007). In the adult brain, BDNF is found and produced in different cell types, most notably neurons (Wetmore et al., 1991, Barakat-Walter, 1996), but also in astrocytes (Moretto et al., 1994). Centrally, the highest levels of BDNF are expressed in the hippocampus, in association with glutamatergic neurons (Yan et al., 1997). In the periphery, circulating BDNF is found mainly stored in platelets (Yamamoto and Gurney, 1990). Significant concentrations of BDNF are also expressed in the heart, liver, spleen, skeletal muscle and lungs (Scarisbrick et al., 1993, Timmusk et al., 1993, Yamamoto et al., 1996). BDNF is also produced and sequestered by endothelial cells, and some have proposed that this could be a source of BDNF in the brain (Bayas et al., 2002).

The transcription of BDNF is temporally-, spatially- and activity-dependent (Greenberg et al., 2009). The *Bdnf* gene is transcribed into mRNA in the soma, and then is either translated into protein on site, or translocated to the dendrites (Tongiorgi and Baj, 2008). The gene has different promoter regions, which seem to control where the mRNA will be translated) and eventually how the protein product will be secreted. Upon translation, BDNF (like the other neurotrophins) is in a pre-pro-from, which is then cleaved in the endoplasmic reticulum (ER) to produce proBDNF. ProBDNF can then be cleaved into its mature form, and whether this cleavage happens intracellularly or extracellularly determines the ultimate mode of secretion. Although evidence has shown that proBDNF is secreted by central neurons and is biologically active (Yang et al., 2009), other studies have reported that 90% of the BDNF found in central neurons is of the mature form (Matsumoto et al., 2008).

Proteins can be secreted in one of two ways: constitutive secretion or regulated secretion (Lessmann and Brigadski, 2009). Constitutive secretion occurs when BDNF is packaged into special synaptic vesicles that automatically exocytose once they reach the plasma membrane. When proBDNF is cleaved intracellularly by furin in the Golgi bodies, it is targeted for constitutive secretion. Regulated secretion is activity-dependent; secretory vesicles remain intact at the presynaptic terminal until a Ca-dependent signal causes them to exocytose. When proBDNF is cleaved extracellularly by plasmin, via the activation of plasminogen by tPa (tissue plasminogen activator), it is targeted for regulated secretion. The amount of BDNF secreted in a regulated manner depends on voltage-gated Ca^{2+} channels and NMDA-R receptors (Hartmann et al., 2001,

Kolarow et al., 2007). BDNF is the only neurotrophin that is preferentially targeted for regulated secretion (Thomas and Davies, 2005), giving it a unique role in activity-dependent processes.

1.3.3 BDNF-TrkB Signaling Cascades

Upon release into the synaptic cleft, BDNF binds with high affinity to TrkB on the post-synaptic membrane. In the adult mammalian brain, the highest levels of TrkB are expressed on granule cells of the dentate gyrus and pyramidal cells of the CA3 (Murer et al., 2001). TrkB levels are also highest in association with glutamatergic synapses, the distribution pattern of which colocalises with BDNF expression. BDNF has a wide range of short-term and long-term effects, most of which are thought to be mediated by its binding to the TrkB Receptor, and the consequent activation of signalling cascades (Cunha et al., 2010).

There are three major TrkB-activated intracellular signalling pathways (Huang and Reichardt, 2003, Minichiello, 2009), initiated by the binding of BDNF to TrkB. This interaction causes the TrkB receptor to dimerise and autophosphorylate. The TrkB is a transmembrane receptor with an extracellular ligand-binding site and several kinase domains: an intracellular kinase domain and a juxtamembrane domain. The phosphorylation of the intracellular domain causes the phosphorylation of the juxtamembrane domain, which in turn acts as a docking site for adaptor proteins such as Shc and phospholipase C (PLC-γ1). Specific phosphorylation sites recruit specific adaptor proteins, and lead to the activation of a specific signalling cascade.

1.3.3.1 The Ras-ERK Pathway

The first is the ERK (extracellular signal-related kinase) pathway (Sweatt, 2001); one of the most efficient pathways used by cells to relay a signal from membrane-bound receptors to the nucleus. ERK belongs to the mitogen-activated kinase (MAPK) superfamily of kinases; enzymes that relay signals into the cell by phosphorylating other proteins. MAPKs have a well-established function in controlling cell proliferation via regulation of the cell cycle. There are two known isoforms of ERK: ERK1 (p44) and ERK2 (p42), both of which are activated by the following pathway. When BDNF binds to TrkB, TrkB autophosphorylation at a specific site (Y515) creates a binding site for the recruitment of certain protein adaptors such as Shc. This activates Ras, which activates Ras-mitogen-activated protein kinase (Ras-MAPK), which in turn activates MEK (the MAPK/ERK Kinase). This leads to the activation of several kinases and transcription factors, notably ERK,

RSK, CREB and CaMKII. The complexity of the protein interactions involved in this cascade is important for the maintenance and amplification of the signal.

Over the past few years, evidence has emerged implicating the ERK pathway as essential to learning and memory (Davis and Laroche, 2006, Peng et al., 2010). ERK activation is a key modulator of synaptic plasticity, necessary for the protein synthesis-dependent stage of LTP. Many different neurotransmitters and signalling systems activate the MAPK pathway and there are about 70 proteins that can directly activate ERK, but it seems all these pathways converge to ultimately upregulate genes involved in promoting neuronal growth and differentiation on one hand (Kaplan and Miller, 2000) and in regulating synaptic plasticity on the other. Gene targets of ERK include transcription factors (such as CREB and Zif268) and plasticity-related proteins such as Arc (Zheng et al., 2009). ERK activation has also been shown to upregulate BDNF (Saarelainen et al., 2001), indicating that the presence of BDNF can activate its own transcription.

There is also some evidence that the two isoforms of ERK (ERK1 and ERK2) may be differentially activated in different types of memory. For example, only ERK1 has been shown to be activated in the dentate gyrus after object recognition learning (Kelly et al., 2003).

1.3.3.2 The PI3K-Akt Pathway

Another pathway activated by BDNF-TrkB binding is the phosphatidylinositol-3 kinase (PI-3K) cascade. The binding of Shc to the TrkB receptor creates a binding site for PI3K, a kinase which changes the composition of phospholipids in the plasma membrane, and results in the translocation of Akt (PKB) to the plasma membrane. Akt plays an important role in neuronal survival (Zhao et al., 2006), and recent evidence has given it a role in synaptogenesis and spinogenesis in the hippocampus (Cuesto et al., 2011). However, little evidence exists of a direct involvement in synaptic transmission, indicating that it is more likely to be involved in the long-term effects of BDNF on learning and memory.

1.3.3.3 The PLCγ-CaMKII Pathway

The third main pathway is the PLC-γ1 cascade. Phosphorylation of TrkB (at Y816) recruits PLC-γ1 which hydrolyses phosphatidylinositol-4,5,-bisphosphate to produce inositoltrisphosphate (IP3) and diacylglycerol (DAG). IP3 leads to the release of Ca^{2+} from internal stores from the endoplasmic reticulum via ligand-gated Ca^{2+} channels, leading to an increase in free intracellular

Ca^{2+} in the cell. DAG activates protein kinase C (PKC), which also causes intracellular Ca^{2+} release and the activation of ERK. The additional Ca^{2+} in turn activates more ERK and more CREB, again upregulating genes that promote cell survival. This additional Ca^{2+} also activates calcium/calmodulin-dependent protein kinase-2 (CaMKII) which plays a central role in plasticity (Wayman et al., 2008). Three features make this kinase crucial to memory formation: 1) It is activated by Calcium/Calmodulin complex, the activity of which is Ca^{2+} dependent, allowing it to become activated by a Ca^{2+}-signal, 2) it is able to phosphorylate many proteins that play a role in synaptic transmission (such as NMDA-Rs and synaptic vesicle proteins) and 3) It is able to autophosphorylate, enabling it to propagate a signal after the original Ca^{2+} increase has worn off. For e.g. CaMKII can bind to AMPA and NMDA receptors in the post-synaptic density and activate them. BDNF rapidly facilitates glutamate exocytosis via this pathway, by mechanisms that are independent of *de novo* protein synthesis (Matsumoto et al., 2001).

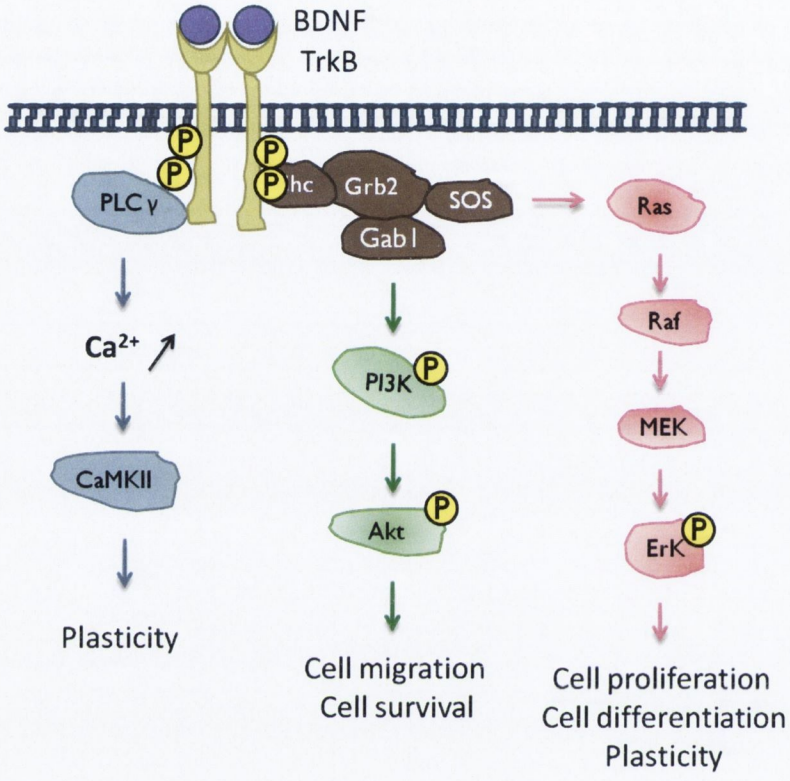


Fig 1.4 Main BDNF-TrkB signalling cascades

BDNF binds to TrkB as a homodimer, causing the dimerisation and autophosphorylation of TrkB at intracellular sites. This causes the recruitment of adaptor proteins (Shc, Grb2, SOS, Gab1), which activate the ERK pathway (via Ras, Raf, and MEK) and the Akt pathway (via PI3K). The ERK pathway primarily regulates cell proliferation and cell differentiation by activating transcription factors for genes, but is also involved in plasticity. The Akt pathway primarily regulates neuronal survival. The third pathway is activated by recruiting PLCγ to a different site on the TrkB receptor, this leads to an increase in intracellular Ca^{2+} and the activation of Ca/Calmodulin-dependent kinase (CaMKII). CaMKII is involved in plasticity, by phosphorylating synaptic vesicle proteins.

1.3.3.4 **BDNF and Learning and Memory**

Of all the neurotrophin family of proteins, there is strongest evidence for a role for BDNF in learning and memory (Hall et al., 2000, Rattiner et al., 2004, Hennigan et al., 2009). The amount of BDNF protein in the hippocampus has been correlated with performance in a spatial memory task (Molteni et al., 2002). Some studies have shown that the exogenous administration of BDNF in vivo induces LTP (Messaoudi et al., 1998, Messaoudi et al., 2002) and memory improvements (Griffin et al., 2009). Recently, the development of novel molecular techniques that allow the selective upregulation of the BDNF gene have also supported a role for BDNF in improving memory and rescuing cognitive decline (Nagahara et al., 2009).

As discussed above, the transcription and release of BDNF are both activity-dependent, making BDNF especially suited to mediate experience-dependent changes in the hippocampus. BDNF-TrkB signalling has been implicated in all the cellular and molecular changes associated with improved memory described in the previous section. BDNF has been shown to influence membrane excitability (Rose et al., 2004) and LTP (Bramham and Messaoudi, 2005), indicating that it can have extremely rapid effects on synaptic transmission. BDNF has also been shown to promote dendritic complexity and density, synapse number and morphology (Hu et al., 2005), and to promote cell proliferation, differentiation, and survival in the dentate gyrus (Young et al., 2007, Choi et al., 2009), indicating that it is also involved in more persistent memory-related changes.

Some of the main data linking BDNF to a key role in learning and memory are provided by studies of behavioural interventions that enhance learning and memory, such as exercise and environmental enrichment. These interventions also cause increases in neurogenesis, and have been shown to robustly and consistently increase BDNF in the hippocampus (Olson et al., 2006).

1.3.4 Nerve Growth Factor (NGF)

Like BDNF, NGF stimulates the proliferation, growth and survival of neurons. Since NGF binds to TrkA with high-affinity, and activates the ERK pathway (described above), it is likely to be involved in stimulating both short-term and long-term effects on synaptic plasticity, morphology, and neurogenesis, similar to BDNF. NGF activity however is largely restricted to a specific population of neurons: the cholinergic neurons of the forebrain which project to the hippocampus

(Chen et al., 1997). These neurons strongly express NGF's receptor, TrkA (Cellerino, 1996). Since these neurons are preferentially targeted in Alzheimer's disease, NGF originally emerged as a potential therapeutic agent to counteract the effects of neurodegeneration, but turned out to cause severe pain as a side-effect (Thoenen and Sendtner, 2002) due to nociceptive properties (Lewin and Mendell, 1993). Unlike BDNF, NGF is present in very low amounts in the hippocampus and its secretion is not activity-dependent, making its involvement in experience-dependent processes in the hippocampus unlikely. However, there have been reports (albeit sparse) of a role for NGF in learning and memory (Gustilo et al., 1999, Conner et al., 2009).

1.4 Behavioural Interventions that Enhance Cognition

1.4.1 Exercise

1.4.1.1 Exercise as a Cognitive Enhancer

It seems intuitive that physical exercise, with its general benefits to health, would also have a positive effect on brain health and function. Thus it comes as no surprise that aerobic fitness has been correlated with improved cognitive function in both children and adults. However, what is less expected is the observation that even short intervals of moderate physical exercise can induce highly robust improvements in learning and memory. Over the last decade, these improvements have been well-documented in both human and animal studies, and over a variety of memory tasks (Vaynman and Gomez-Pinilla, 2005).

The most common tasks currently used to evaluate memory in rodents are spatial tasks. There are many studies that link exercise to improved performance in spatial memory tasks, especially maze-related tasks such as the Morris Water Maze, Y-maze, T-maze and radial arm maze tests (van Praag, 2008). These improvements are usually evident in rats in which a cognitive deficit is present, in the form of ageing or brain injury (Cotman and Berchtold, 2002, Albeck et al., 2006). However, some studies have shown improvements in the water maze in young healthy rats with three weeks (Adlard et al., 2004) and one week (Vaynman et al., 2004) of voluntary running. Exercise has also been shown to improve performance in hippocampus–dependent tasks that do not require movement, e.g. passive avoidance learning and contextual fear-conditioning (Liu et al., 2009). Previous work in our laboratory has shown exercise to enhance object recognition memory, which relies largely on the perirhinal cortex as well as the hippocampus (O'Callaghan et al., 2007).

The cognitive benefits of exercise are affected by the type, intensity, mode (voluntary Vs forced), and duration of exercise paradigms. It has been shown that it is specifically cardiovascular (aerobic) exercise (running, swimming, cycling) that induces cognitive benefits as opposed to strength/resistance training (Liu-Ambrose and Donaldson, 2009). There is also some evidence to suggest that moderate exercise is more beneficial than either mild or highly strenuous exercise (Kashihara et al., 2009). In rodents, running is the most common form of aerobic exercise used. Running can be voluntary (by placing running wheels in the home cage) or forced (animals are placed on treadmills), both of which have been shown to have positive effects on cognition. Most studies use exercise paradigms that last longer than three weeks, however, there is evidence to suggest that the cognitive benefits of exercise can occur with acute bouts of exercise in humans (Ferris et al., 2007, Griffin et al., 2011) and rats (*unpublished data*).

1.4.1.2 Exercise and BDNF

Although significant evidence has accumulated linking exercise to cognitive enhancement, the underlying mechanisms are still debated. Over the past decade, adult hippocampal neurogenesis was suspected to be the main phenomenon underlying exercise-induced cognitive enhancement (van Praag, 2008). Currently, the neurotrophin BDNF has emerged as the primary candidate to mediate the effects of exercise on both cognition and neurogenesis (Bekinschtein et al., 2011). Although the expression of several neurotrophic factors is affected by exercise, only BDNF is elevated consistently and robustly in the brain after short-term regular exercise (Molteni et al., 2002), indeed with as little as one week of exercise, as we and others have shown (Vaynman et al., 2007, Griffin et al., 2009). Since BDNF is a major regulator of neurogenesis, and has been shown to play a central role in learning and memory, it has been proposed that exercised-induced increases in BDNF, and not neurogenesis, are mechanistically responsible for short-term cognitive enhancement, although direct evidence is still lacking.

Many studies have shown that BDNF protein increases consistently in the brain of exercised rodents, whether the exercise is forced (O'Callaghan et al., 2007, Soya et al., 2007, Lou et al., 2008, Griffin et al., 2009) or voluntary (Vaynman et al., 2004, van Praag et al., 2005, Rex et al., 2007). One particular study went on to show that blocking BDNF action with TrkB-IgGs blocks exercise-induced improvements in the MWM (Vaynman et al., 2004). However, these studies are difficult to interpret since BDNF is required for learning and memory, and blocking the TrkB receptor could be producing a generalised impairment in memory.

In addition to affecting BDNF expression in the brain, exercise has been shown to increase BDNF in the blood (both serum and plasma) in both humans and animals (Zoladz and Pilc, 2010). The significance of this peripheral effect is still unknown; it has been suggested that the exercise-induced increase in BDNF originates in the brain, but crosses over the blood-brain barrier into the circulation (Rasmussen et al., 2009). This idea is discussed in greater detail below (Section 1.4.1.6).

1.4.1.3 Exercise and LTP

Few studies have investigated the relationship between short-term forced exercise and LTP in the dentate gyrus. However, there is substantial evidence linking long-term exercise (especially voluntary) to LTP facilitation. One month of voluntary exercise has been shown to facilitate LTP in the hippocampi of young mice (van Praag et al., 1999) and young rats (Farmer et al., 2004). Recently, a study demonstrated that that long-term forced exercise was associated with facilitation in LTP and memory improvements in a mouse model of Alzheimer's disease (Liu et al., 2011). A previous study in our laboratory has shown that one week of forced exercise can facilitate LTP in the dentate gyrus of young rats (O'Callaghan et al., 2007). No studies to date have looked at the effect of a single bout of exercise on LTP.

1.4.1.4 Exercise and Morphological Changes

Several studies have shown that exercise can induce widespread morphological alterations in the hippocampus and entorhinal cortex within a month (Stranahan et al., 2007), and in the dentate gyrus within two weeks (Eadie et al., 2005, Redila and Christie, 2006). These include changes in dendritic length and complexity and spine density.

1.4.1.5 Exercise and Neurogenesis

Physical exercise is the most potent neurogenic intervention we know (Fabel and Kempermann, 2008, van Praag, 2008). Over the past decade, many studies have shown exercise to increase cellular proliferation in the dentate gyrus of rodents with both voluntary (van Praag et al., 1999, Trejo et al., 2001, Fabel et al., 2003, Kitamura et al., 2003, Overstreet et al., 2004, van Praag et al., 2005, Olson et al., 2006, Van der Borght et al., 2007) and forced paradigms (Uda et al., 2006, Leasure and Jones, 2008). Interestingly, exercise does not seem to stimulate neurogenesis in the olfactory bulb (Brown et al., 2003).

The effects of exercise on cell proliferation are almost immediate: in mice, the proliferative rate of cells peaks after 3 days of voluntary running, remains elevated for up to 32 days after which it returns to baseline (Kronenberg et al., 2006). The number of immature neurons however continues to increase beyond that point, indicating that cell survival and/or differentiation are also affected.

1.4.1.6 Exercise, Vascular Endothelial Growth Factor (VEGF), and Vascular Changes

Evidence has accumulated to show that exercise induces an increase in cerebral blood flow (CBF) to the brain, possibly to compensate for increased metabolic demand (Ogoh and Ainslie, 2009a, b). Vascular adaptations to exercise are necessary to sustain regular physical activity. In addition to rapid changes in vasculature (such as vasodilation), exercise induces new vessel formation (angiogenesis) by a mechanism that involves vascular endothelial growth factor (VEGF) (Van der Borght et al., 2009). VEGF has been shown to increase in the blood (Prior et al., 2004) and the brain (Tang et al., 2010) with exercise, possibly in response to hypoxic conditions (the VEGF gene has a promoter region that is hypoxia-sensitive).

An increase in blood flow to the brain enables better oxygenation, and an overall enhancement of neuronal function. In the aged brain, cognitive decline is accompanied by decreased perfusion of brain tissue (Brown and Thore, 2011). Taken together, these phenomena suggest that exercise-induced changes in vasculature could contribute to the improvements in cognitive function associated with exercise. However, direct links between vascular changes and cognition have yet to be established, especially when studying the immediate effects of exercise. Very few studies have looked at the relationship between exercise and blood flow to localised areas of the brain.

1.4.1.7 Exercise and Insulin-related Growth Factor (IGF-1)

Another growth factor that has been shown to be induced by exercise is insulin-like growth factor (IGF-1). Exercise increases the amount of IGF-1 in the serum (Llorens-Martin et al., 2008). The significance of this increase is still unclear, but it has been suggested that IGF-1 could be a mediator of the positive effects of exercise on cognition (Ding et al., 2006a). Circulating IGF-1 is produced by the liver (Butler and LeRoith, 2001), and has been shown to cross the blood-brain barrier using a saturable transport system (Pan and Kastin, 2000). In the brain, IGF-1 acts by binding to its receptor (IGF-1-R), which is a member of the tyrosine kinase receptor family (like TrkA, TrkB, and TrkC) (LeRoith et al., 1993). This leads to the activation of signaling cascades,

similar to those activated by BDNF and NGF, including the PLCγ-CaMKII pathway, the PI3K-Akt pathway, and the Ras-ERK pathway. As a result, IGF-1 could have direct effects on neuroplasticity, neurogenesis, and neuronal survival (Torres Aleman, 2005), similar to those of BDNF. Studies have shown that blocking serum IGF-1 using antibodies represses exercise-induced increases in neurogenesis in the dentate gyrus (Trejo et al., 2001), exercise-induced neuroprotection following injury (Carro et al., 2000). In addition, mice with congenitally low serum levels of IGF-1 were shown to have impairments in spatial memory which were rescued by exogenous IGF-1 administration (Trejo et al., 2008). This would suggest that serum IGF-1 could be an important mediator of the effects of exercise on the brain. However, it is also possible that IGF-1 exerts its effects indirectly, by regulating the transcription of BDNF (Carro et al., 2001, Trejo et al., 2001), since the exogenous administration of IGF-1 into the circulation has been shown to upregulate BDNF in the brain (Carro et al., 2000). There is also some evidence to suggest that IGF-1 might play a role in regulating the activity of VEGF in stimulating angiogenesis (Lopez-Lopez et al., 2004, Ding et al., 2006a).

1.4.1.8 Exercise and the Blood-Brain Barrier

The blood-brain barrier segregates brain cells from the circulation via complex tight junctions (Abbott, 2002, Ballabh et al., 2004). Under normal circumstances, the blood-brain barrier is not permeable to molecules with a molecular weight above 180 Da (Gloor et al., 2001), which would include neurotrophins such as BDNF (13 kDa). Early reports indicated that BDNF can cross the blood-brain barrier bi-directionally via a possible saturable transport system (Poduslo and Curran, 1996, Pan et al., 1998), and these reports are often cited by papers today (Rasmussen et al., 2009, Zoladz and Pilc, 2010). However, since then a series of studies has contradicted these findings (Pardridge et al., 1994, Pardridge et al., 1998, Zhang and Pardridge, 2001, 2006, Nagahara and Tuszynski, 2011), and a consensus is yet to be reached.

Limited evidence suggests that the blood-brain barrier may become more permeable with exercise. This idea seems counterintuitive, since the blood-brain barrier performs the necessary function of protecting the brain from changes in plasma concentration of solutes, especially with events such as meals, exercise or infection. In most of these studies, blood-brain barrier (BBB) permeability was assessed by detecting levels of S-100β, a protein specific to the CNS (Kapural et al., 2002, Sendrowski et al., 2004). The presence of this protein in the serum indicates blood-brain barrier disruption. An early study found an increase in BBB permeability with exercise (Sharma et al.,

1991). Subsequent studies have shown that the BBB may become more permeable after swimming in humans (Dietrich et al., 2003), and with exercise in the heat in rodents (Watson et al., 2005, Watson et al., 2006). More recently, one study reported that BDNF can cross the blood-brain barrier from the brain into the circulation with exercise (Rasmussen et al., 2009).

The question of whether BBB permeability changes with exercise is an important one, for several reasons. Since BDNF is currently thought to be the main mediator of exercise-induced enhancements on brain function and cognition, it is of primary interest to clarify how exactly exercise causes an increase in BDNF. Some have suggested it originates in the brain (Rasmussen et al., 2009), but it is still unclear how physical exercise, a peripheral activity which involves skeletal muscles, heart, and circulation, can stimulate the production of BDNF in the brain.

1.4.2 Environmental Enrichment as a Cognitive Enhancer

Environmental enrichment is defined as an experimental paradigm in which animals are housed in an environment designed to facilitate motor, sensory, cognitive, and social stimulation (Bennett et al., 1969). This traditionally involves housing the rats in larger cages with a larger number of animals, and adding toys, nest boxes, special bedding, tunnels, and running wheels to the cages. The toys and tunnels are changed frequently to ensure continuous novelty. Environmental enrichment paradigms have been shown to enhance the performance of animals in several cognitive tests (Dahlqvist et al., 2004, Brillaud et al., 2005, Leggio et al., 2005, Costa et al., 2007, Cao et al., 2008). These improvements are accompanied by increases in certain neurotrophins (Ickes et al., 2000) , synaptic density (Gelfo et al., 2009), and neurogenesis (Kempermann et al., 2002, Brown et al., 2003, Bruel-Jungerman et al., 2005, Fan et al., 2007).

Many studies have been published on environmental enrichment, but most environmental enrichment protocols include a running wheel in the cage which makes it difficult to separate out the effects of the cognitive enrichment from exercise (van Praag et al., 2000). This also makes the underlying neural mechanisms more difficult to interpret, and deters the attempt to use these mechanisms in the search for drug targets that could therapeutically replicate or enhance the effects of environmental enrichment on cognition (Sztainberg et al., 2010).

The environmental enrichment protocol used in our laboratory removes all confounds of exercise (physical enrichment) and animal number per cage (social enrichment), and focuses entirely on the effect of cognitive enrichment in the form of toys, nest boxes, and special bedding (Birch and

Kelly, 2012). Recent literature provides evidence to suggest that the documented beneficial effects of environmental enrichment on cognition and neurogenesis can be attributed entirely to the exercise component (Kobilo et al., 2011).

1.5 Conclusion and Objectives

Over the last few years the neurotrophin BDNF has emerged as the primary candidate to mediate exercise-induced cognitive enhancement. However, a direct link between exercise and BDNF has not yet been established, and many questions pertaining to the exact mechanisms involved remain unanswered. In this work, a combination of multidisciplinary techniques were used to study the cognitive, cellular, molecular, and vascular effects of one week of moderate forced exercise in the adult Wistar rat. We have two main hypotheses. Our first hypothesis posits that short-term forced moderate exercise confers robust improvements in both spatial memory and object recognition memory, in a manner that is independent of housing conditions and treadmill-induced stress, and that these effects are mediated by rapid effects of BDNF and BDNF-activated signalling cascades on synaptic transmission in the dentate gyrus. Our second hypothesis posits that exercise-induced vascular changes facilitate the delivery of BDNF to the brain from the circulation.

The following is a more detailed break-down of the questions we were interested in answering through this work.

1) Can one week of forced moderate exercise improve performance of rats in both an object recognition task and a spatial task?

2) Can we improve the sensitivity of our cognitive tasks by varying the number of objects and training time?

3) Can one week of forced exercise improve performance of rats in a more challenging variant of the spatial task?

4) Do housing conditions (standard housing Vs enriched housing) affect exercise-induced improvements in cognitive performance?

5) Do housing conditions and forced exercise affect the stress response and anxiety behaviour?

6) How does exercise affect BDNF concentrations in the serum and plasma of rats?

7) Does exercise have an effect on the BDNF-TrkB signalling system in the brain, with a focus on the hippocampus and parahippocampal structures?

8) Does exercise have an effect on downstream signalling effectors such as ERK1 and ERK2, PLC-γ, CaMKII, and synaptic vesicle proteins in the dentate gyrus?

9) What cellular changes in the dentate gyrus (cell proliferation, neuronal phenotypes, and apoptosis)?

10) Can exogenous BDNF administration (intracerebroventricular and intravenous) mimic some the behavioural and molecular effects observed with exercise?

11) Does exercise induce changes in regional cerebral blood flow that can be investigated using arterial spin labelling (an MRI technique)?

12) Does exercise induce changes in blood brain barrier permeability that facilitate the delivery of BDNF from the circulation to the brain?

Chapter 2 General Materials and Methods

2.1 Materials

2.1.1 Animals

Wistar rats (3 month old, males)	BioResources Unit, TCD
Irradiated laboratory animal diet	Harlan™, Madison, WI, USA

2.1.2 ELISA kits

Human BDNF Duoset® ELISA kit	R&D Systems Europe, Oxon, UK
Rat β-NGF Duoset® ELISA kit	R&D Systems Europe, Oxon, UK
Human Total TrkB Duoset® ELISA kit	R&D Systems Europe, Oxon, UK
Human p-TrkB Duoset® ELISA kit	R&D Systems Europe, Oxon, UK
Corticosterone HS EIA ELISA Kit	Immunodiagnostic Systems, UK
Substrate Solution	R&D Systems Europe, Oxon, UK

2.1.3 General laboratory chemicals

Acrylamide electrophoresis reagent	Sigma, Wicklow, Ireland
Ammonium persulphate	Sigma, Wicklow, Ireland
Aprotinin	Sigma, Wicklow, Ireland
Bio-Rad dye reagent concentrate	Bio-Rad, California, USA

Bovine serum albumin (BSA)	Sigma, Wicklow, Ireland
Bromophenol blue sodium salt	Sigma, Wicklow, Ireland
5-bromo-2'-deoxyuridine	Sigma, Wicklow, Ireland
Calcium chloride ($CaCl_2$)	Lennox, Dublin, Ireland
Dimethyl sulphoxide (DMSO)	Sigma, Wicklow, Ireland
Di-Sodium hydrogen orthophosphate ($Na_{2\ hrs}PO_4$)	Sigma, Wicklow, Ireland
DL-Dithiothreitol (DTT)	Sigma, Wicklow, Ireland
Ethanol	Sigma, Wicklow, Ireland
Glucose	Lennox, Dublin, Ireland
Glycerol	Sigma, Wicklow, Ireland
Glycine	Sigma, Wicklow, Ireland
Hydrochloric acid (HCl)	Lennox, Dublin, Ireland
Hydrogen peroxide (H_2O_2)	Sigma, Wicklow, Ireland
Leupeptin	Sigma, Wicklow, Ireland
Magnesium sulphate ($MgSO_4$)	Sigma, Wicklow, Ireland
Magnesium Chloride ($MgCl_2$)	Sigma, Wicklow, Ireland
β-Mercaptoethanol	Sigma, Wicklow, Ireland
Methanol (MeOH)	Sigma, Wicklow, Ireland
N, N' – Methylenebisacrylamide	Sigma, Wicklow, Ireland
Nitrocellulose membrane	Amersham Bioscience, Stockholm, Sweden

Normal Goat Serum	Vector
Normal Rabbit Serum	Chemicon
NP-40	Sigma, Wicklow, Ireland
OCT™ compound	Tissue Tek®, Zoeterwoude, The Netherlands
Potassium chloride (KCl)	Sigma, Wicklow, Ireland
Potassium dihydrogen orthophosphate (KH_2PO_4)	Sigma, Wicklow, Ireland
Potassim hydroxide (KOH)	Sigma, Wicklow, Ireland
Potassium phosphate (KH_2PO_4)	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 (NaCl)	Sigma, Wicklow, Ireland
Sodium dodecylsulphate (SDS)	Sigma, Wicklow, Ireland
Sodium hydrogen carbonate ($NaHCO_3$)	Sigma, Wicklow, Ireland
Sodium hydroxide (NaOH)	Lennox, Dublin, Ireland
Sodium orthovanadate (Na_3VO_4)	Sigma, Wicklow, Ireland
Sodium phosphate, monobasic (NaH_2PO_4)	Sigma, Wicklow, Ireland
Sodium phosphate, dibasic ($Na_{2\ hrs}PO_4$)	Sigma, Wicklow, Ireland
3,3',5,5'-Tetramethylbenzidine (TMB)	Sigma, Wicklow, Ireland
N,N,N',N'-Tetramethylethylene-diamine (TEMED)	Sigma, Wicklow, Ireland

Tris-base	Sigma, Wicklow, Ireland
Tris-HCl	Sigma, Wicklow, Ireland
Tween®-20	Sigma, Wicklow, Ireland

2.1.4 General laboratory products and plastics

Biosphere filter pipette tips	Sarstedt, Nümbrecht, Germany
Cork discs	R.A. Lamb Ltd., Sussex, UK
Falcon tubes (15ml, 50ml)	Sarstedt, Nümbrecht, Germany
Microtest 96-well flat bottomed plates	Sarstedt, Nümbrecht, Germany
Microtubes (0.5ml, 1.5ml)	Sarstedt, Nümbrecht, Germany
96 Microwell Nunc ELISA plates	Nunc, Roskilde, Denmark
Needles 26G, 21G	BD Microlance, Oxford, UK
Optical adhesive covers	Applied Biosystems, Warrington, UK
96-well optical reaction plates	Applied Biosystems, Warrington, UK
Parafilm	Lennox, Dublin, Ireland
PCR tubes	Sarstedt, Nümbrecht, Germany
Pipette tips	Sarstedt, Nümbrecht, Germany
Plastic transfer pipettes	Sarstedt, Nümbrecht, Germany
Scalpels (disposable)	Swann-Morton, Sheffield, UK
Standard grade No.1 filter paper	Whatman Ltd., UK

Standard grade No.3 filter paper	Whatman Ltd., UK
Sterile syringes (1ml, 2ml, 10ml, 50ml)	BD Plastpak, Oxford, UK
Syringe-drive filter unit	Millipore, Chemicon, Cork, Ireland

2.1.5 Molecular reagents

Absolute ethanol	Sigma, Wicklow, Ireland
BCA Protein Assay Kit	Pierce, Rockford, USA
High capacity cDNA reverse transcription kit	Applied Biosystems, Warrington, UK
Molecular grade water	Sigma, Wicklow, Ireland
RNA*later*™	Ambion, Warrington, UK
RNase-free microtubes	Ambion, Warrington, UK
RNaseZap® wipes	Ambion, Warrington, UK
Nucleospin® RNA II isolation kit	Macherney-Nagel, Limerick, Ireland
Taqman gene expression assays	Applied Biosystems, Warrington, UK
Taqman universal PCR master mix	Applied Biosystems, Warrington, UK

2.1.6 Western Immunoblotting reagents and antibodies

Anti-mouse (goat) IgG peroxidase conjugate	Sigma, Wicklow, Ireland
Anti-rabbit (goat) IgG peroxidase conjugate	Sigma, Wicklow, Ireland

β-Actin mouse monoclonal IgG	Sigma, Wicklow, Ireland
Rabbit anti- phospho-p44/42 MAPK (ERK1/2) IgG	Cell Signaling Technology®, Massachusetts, USA
Rabbit anti- p44/42 MAPK (ERK1/2) IgG	Cell Signaling Technology®, Massachusetts, USA
Hybond-C extra nitrocellulose membrane	Amersham Biosciences, Buckinghamshire, UK
Precision Plus Protein Standards (Dual Colour)	Bio-Rad Laboratories, California, USA
MagicMark™ XP Western Protein Standard	Invitrogen, UK
ReBlot Plus strong antibody stripping solution	Millipore, Cork, Ireland
SuperSignal® chemiluminescence	Pierce, Rockford, USA

2.2 Animals

Three-month old male Wistar rats (BioResources Unit, Trinity College Dublin, Ireland) were used in all experiments. All animals were experimentally naïve, and weighed between 350 and 450 g before starting the experiments. They were group-housed (3 rats per cage) in a standardised environment under a 12 hrs light/12 hrs dark cycle (lights on at 7a.m). Ambient temperature was controlled (20-22°C). Water and food were provided *ad libitum*, and the rats were allowed to acclimatise to the laboratory environment and were handled by the experimenter prior to the onset of the experiment. All experiments were conducted under national law and European Union directives on animal experiments.

2.3 Exercise training

Animals were randomly assigned to either an exercise group or a sedentary group. All animals were familiarised to motorised treadmills (Fig 2.1, Exer 3/6 treadmill, Columbus Instruments) by being placed on the treadmill for 15min on two consecutive days (belt speed 5 m/min). The acute exercise protocol consisted of a single bout of running (belt speed, 1 km/hr) for 60 min. The one week exercise protocol (Griffin et al., 2009) consisted of an acute bout of exercise (60 min) daily for 7 consecutive days (belt speed, 1 km/hr). The treadmill is equipped with wire loops at one end of the belt through which a mild electric shock can be delivered; these act to motivate the rats to run continuously and were activated at low levels (on average an intensity of 2 on a scale of 0–10; this represents a current of 0.7mA with an inter-pulse interval of 2s) throughout all exercise sessions. Rats were observed while exercising to ensure they ran continuously and to monitor for signs of stress. Sedentary rats were placed on stationary treadmills with shock loops activated at the same low levels for the same duration.

2.4 Environmental Enrichment

The environmental enrichment protocol (Birch and Kelly, 2012) involved housing the rats in larger cages, while food, water, and RT were kept standard as described above. Cages were randomly assigned as either 'enriched' or 'standard-housed'. Each enriched cage was given one nest box and additional special bedding. The cages also each contained three different toys which were changed on a weekly basis (Fig 2.2). The protocol lasted three weeks after which the animals were tested in

a cognitive task. Standard house cages did not contain toys, nest boxes, or bedding. None of the cages contained running wheels or tunnels.

2.5 BrdU injection protocol

Animals received daily intra-peritoneal injections of BrdU (50mg/kg) during the 7-day forced exercised period (in Chapters 4 and 5). BrdU was dissolved in 0.9% NaCl at a concentration of 20mg/ml. The solution was filter-sterilized before injection (van Praag et al., 1999). On each day, animals were injected 15 minutes prior to being placed on the treadmill.

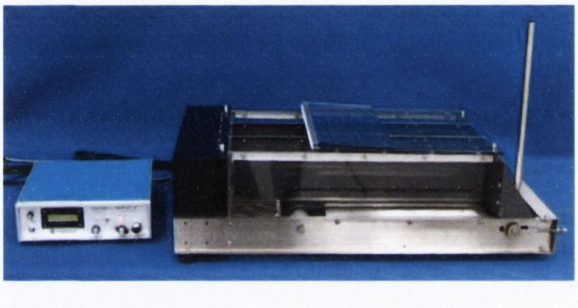


Fig 2.1 Motorised treadmill used for all exercise experiments.
Exer 3/6 treadmill, Columbus Instruments, equipped with electrical stimulus system and 3 individual lanes.

Fig 2.2 Example of toys used in environmental enrichment protocol
Each cage received 4 different toys, all of which were changed weekly, for three weeks.

2.6 Cognitive Tests

2.6.1 Object Displacement Paradigm

All animals were familiarised to the empty open-field setup for two days (10 mins on each day) prior to starting the learning protocol. The setup for the object displacement paradigm (Griffin et al., 2009) consisted of a black circular arena (diameter 0.9m, height 0.48m) placed in a dimly lit room. Spatial cues in the form of cardboard shapes were fixed to the walls of the arena. Objects were constructed from large Lego blocks and positioned at fixed coordinates in the arena (Fig 2.3). The number of objects varied between two and three. The learning protocol consisted of two days: a training day (day 7) and a testing day (day 8). On the training day, animals were placed into the arena at a specific entry point for several 5 min trials with an inter-trial rest period of 5 min. An examiner recorded the time spent exploring each object using stopwatches. In addition, an EthoVision tracking system (Noldus Information Technology, UK) was used to track the movement of the animals in the arena. Objects and arena were thoroughly cleaned between trials to ensure the absence of olfactory cues. Twenty-four hours later, on the testing day, one of the objects was displaced to a new position, and rats were reintroduced into the open field for a single 5 min period. The time spent exploring each object was recorded as before. The time (in seconds) spent exploring each object was expressed as a percentage of the total exploration time. The criteria for exploration were strictly based on active exploration, in which rats had to be touching the object with their noses, paws or whiskers.

2.6.2 Object Substitution Paradigm

The object displacement paradigm (Griffin et al., 2009) is a novel object recognition task, and was conducted in the same open-field setup as described above. Objects were constructed from large lego blocks and positioned at fixed coordinates in the arena (Fig 2.4). The number of objects varied between two and three. Again, animals were familiarised to the open-field two days prior to starting the learning protocol (10 minutes on each day). On the training day, animals were placed into the arena at a specific entry point for several 5 min trials with an inter-trial rest period of 5 min. An examiner recorded the time spent exploring each object using stopwatches. In addition, an EthoVision tracking system (Noldus Information Technology, UK) was used to track the movement of the animals in the arena. Objects and arena were thoroughly cleaned between trials to ensure the absence of olfactory cues. Twenty-four hours later (testing day), one of the objects was

removed from the arena and replaced by a new object, and rats were reintroduced into the open field for a single 5 min period. The time spent exploring each object was recorded as before. The time (in seconds) spent exploring each object was expressed as a percentage of the total exploration time. The criteria for exploration were strictly based on active exploration, in which rats had to be touching the object with their noses, paws or whiskers.

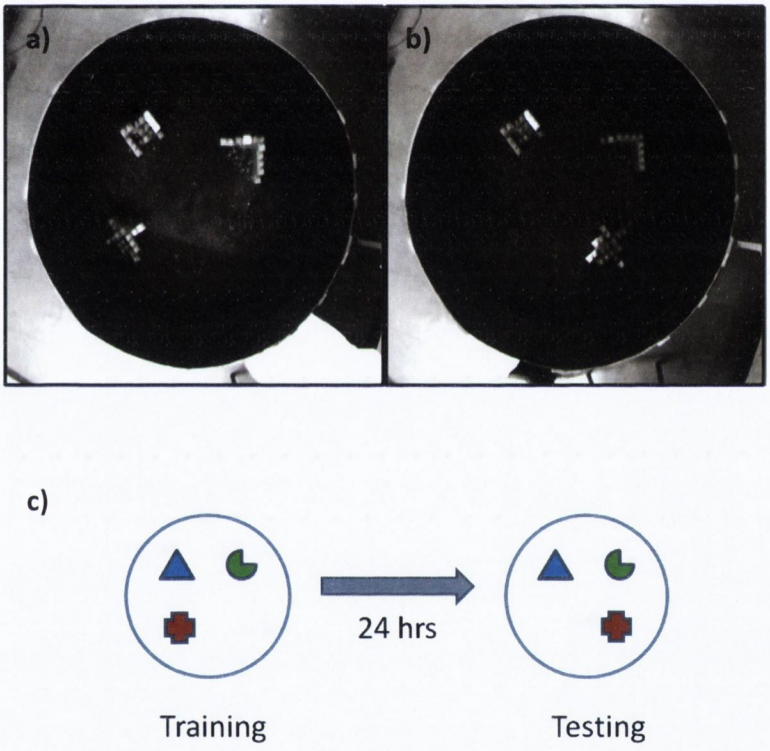
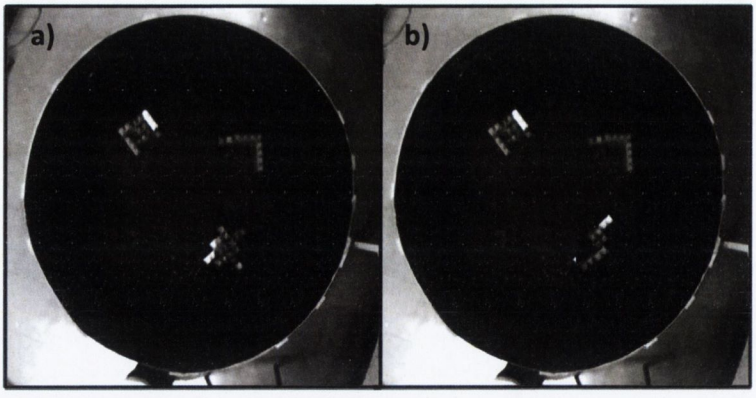


Fig 2.3 Object Displacement Paradigm

An example of an object displacement task with three objects. The pictures (a,b) show the arena and typical objects used. (a) represents the arrangement of objects on training day; (b) represents the arrangement of objects on testing day after one of the objects has been displaced. The schematic (c) summarises the task.



c)

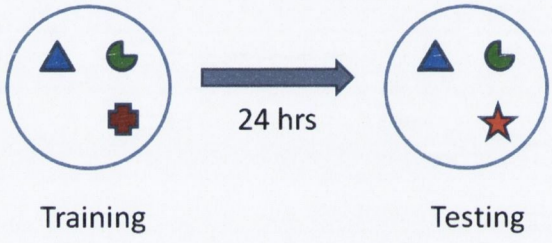


Fig 2.4 Object Substitution Paradigm

An example of an object substitution task with three objects. The pictures (a,b) show the arena and typical objects used. (a) shows the arrangement of objects on training day; (b) shows the arrangement of objects on testing day after one of the objects has been replaced by a novel object. The schematic (c) summarises the task.

2.7 Open-field test for anxiety behaviour

The open-field test is a classical and popular test for measuring anxiety-related behaviour in a variety of animals (Prut and Belzung, 2003). In this test, the animal is typically allowed to explore an unfamiliar, enclosed arena for a fixed duration. Animals tend to spend more time close to the walls of the arena, a behaviour known as thigmotaxis. Thigmotaxis is used as a measure of anxiety, since an animal that is less anxious will spend more time in the central areas of the arena and less time close to the walls (Simon et al., 1994). In Chapter 4, we used a variant of this test to investigate anxiety behaviour in rats. The circular arena used for the cognitive tasks was also used for this test. All cues and objects were removed from the arena. Using the tracking software Ethovision (Noldus Information Technology, UK), the arena was divided into two parts: a central part which included the central 75% of the arena, and a peripheral part which included the peripheral 25% of the area. This method eliminates the problem of assessing the time the animal spends in corners, which occurs with the traditional square open field. On the first day of habituation to the open-field, rats were allowed to explore the unfamiliar arena for 5 minutes, the preferred duration for open-field tests in the literature (Prut and Belzung, 2003). During this time, the time spent in each of the central and peripheral areas was recorded in seconds. Time spent in periphery was calculated as a percentage of total exploration time.

2.8 Blood Analysis

2.8.1 Collection of whole blood

To monitor the levels of BDNF in the serum and plasma during certain experiments, whole blood was collected in one of four ways (Diehl et al., 2001), depending on whether a terminal or non-terminal method was required, and on the volume of blood required. Non-terminal methods include collection from the saphenous vein and tail veins. Terminal methods include cardiac puncture and exsanguination post-decapitation.

2.8.1.1 Saphenous vein blood collection

Whole blood was collected from the lateral saphenous vein of the upper hind leg in the following way. The rat was anesthetized with isofluorane by placement in a gas chamber, maintained under anaesthesia using a face-mask (4% induction, 1.5–2% maintenance in 100% oxygen; Abbot

Laboratories, Ltd, UK), and kept on a warming pad throughout the procedure to avoid hypothermia. The hind leg fur was shaved with an electric razor, and a tourniquet was administered using a rubber band to expose the vein. The shaved area was cleaned and a lubricant gel was applied for extra visibility. The vein was punctured close to the calf with a 25 gauge needle, and the accumulating blood was collected using a hematocrit tube (Microvette CB300Z, Sarstedt, Ireland). For plasma collection, the hematocrit tube was replaced by a heparin-coated hematocrit tube (Microvette CB300Z, Sarstedt, Ireland). This method was used in Chapter 3, however, the method was discontinued in later chapters and substituted for tail vein blood collection as described below (Section 2.8.1.2). The tail vein method was found to be easier and produce a higher volume of blood.

2.8.1.2 Tail vein blood collection

The rat was anesthetized with isofluorane by placement in a gas chamber, maintained under anaesthesia using a face-mask (4% induction, 1.5–2% maintenance in 100% oxygen; Abbot Laboratories, Ltd, UK), and kept on a warming pad throughout the procedure to avoid hypothermia. The tail was washed thoroughly with soap and water to aid visibility of the veins. The tail was then heated slightly using a warming pad, and a tourniquet was applied to the proximal end of the tail. The vein was punctured at about one-third the distance from the tail tip using a 26 gauge needle. Only the lateral veins were sampled, and no more than 2mls was taken per rat (1ml for serum, 1ml for plasma) per sampling. Hematocrit tubes were used as described for saphenous vein collection. This method was preferred to collection from the saphenous vein due to ease of access to the vein and the collection of larger blood volumes.

2.8.1.3 Cardiac puncture

The rat was overdosed with urethane in preparation for transcardial perfusion (Section 2.9.2). Following complete anaesthesia the abdomen was cut open below the diaphragm, and the rib cage was cut rostrally on the lateral edges to expose the diaphragm. The diaphragm was carefully removed and the sternum was cut through to expose the heart. Using a heparinised 26-gauge needle, whole blood was collected via cardiac puncture directly into a syringe containing heparin (heparin sodium, Leo Laboratories Ltd, Dublin 12, Ireland, 25units/ml) for plasma preparation. This method was used only in experiments where the rats were transcardially perfused, since exposure of the heart allowed easy access to the blood, and exsanguination post-decapitation could

not be performed. The advantage of this method is that it allows large quantities of blood to be collected from a single animal (Beeton et al., 2007).

2.8.1.4 Exsanguination

Immediately post-decapitation, trunk blood was collected by exsanguination (Grouzmann et al., 2003) into an eppendorf tube in the absence of anticoagulant for serum preparation. This method was used when cardiac perfusion was not performed and rats were killed immediately post-testing by decapitation.

2.8.2 Preparation of serum and plasma samples for ELISA

Whole blood samples for serum analysis were left at RT for half an hour to clot. The samples were then centrifuged at 1500 g for 20 minutes at RT, after which the supernatant was removed and stored at -80°C. All serum samples were diluted 1:2 in distilled water and assayed according to protocol (Section 2.12). This is the dilution at which previous assays in the lab determined optimal readings for the BDNF ELISA. Whole blood samples for plasma analysis were placed on ice immediately after collection. Samples were centrifuged at 1500g for 10 min at 4°C. The platelet-rich plasma supernatant was recovered and stored at -80°C for future analysis. Methods for blood processing were adapted from protocols in the literature (Elfving et al., 2010) and optimised in the laboratory.

2.9 Preparation of tissue samples

2.9.1 Tissue collection

Animals were stunned and decapitated. After collection of trunk blood, brains were rapidly removed and placed on ice. Alternatively (in Chapter 6), animals were transcardially perfused (Section 2.9.2). The brains were hemisected, and the left hemisphere was taken for sectioning. The right hemisphere was dissected freehand according to Paxinos and Watson (Paxinos and Watson, 2004). The hippocampus (H), dentate gyrus (DG), perirhinal cortex (PC) and entorhinal cortex (EC) were dissected out as follows: to remove the perirhinal cortex, tissue on either side of the rhinal sulcus was pinched off using forceps. To remove the entorhinal cortex, tissue at the posterior edge of the hemisphere was pinched off using forceps. To remove the hippocampus, the brain was

cut above the olfactory bulbs and the cerebellum and brain stem were removed. The brain was then placed upright on its anterior end, and the thalamic tissue was removed to reveal the hippocampus. The hippocampus can then be rolled gently to separate from the rest of the tissue. On the hippocampus, a discrete line indicates the outline of the dentate gyrus, which can then be gently pushed off from the rest of the hippocampus. The entire procedure takes approximately two minutes. Each dissected region was divided into three pieces for processing.

2.9.2 Transcardial perfusion

Animals were overdosed with urethane (1.5mg/kg i.p.). Absence of pedal and eye reflexes was taken to indicate deep anesthesia. Following complete anaesthesia and cessation of breathing, the animal was cut open below the diaphragm, and the rib cage was cut rostrally on the lateral edges to expose the diaphragm. The diaphragm was carefully removed and the sternum was cut through to expose the heart. While the heart was still beating, a small hole was cut into the right atrium, and a gavage (connected to peristaltic pump via tubing) was inserted into the left ventricle and clamped. The animal was transcardially perfused with ice-cold heparinised (heparin sodium, Leo Laboratories Ltd, Dublin 12, Ireland, 25units/L) saline solution (0.89% NaCl) for 10 minutes. Post-perfusion, dissections proceeded as described in Section 2.9.1.

2.9.3 Preparation of tissue slices

Tissue slices were stored according to the method of Haan and Bowen (Haan and Bowen, 1981) . Freshly dissected tissue was sliced bi-directionally to a slice thickness of 350μm with a McIlwain tissue chopper (Campden Instruments Ltd., UK). All samples were rinsed in ice-cold Krebs buffer (composition: NaCl, 136mM; KCl, 2.54mM; KH_2PO_4 1.18mM; $MgSO_4.7H_2O$, 1.18mM; $NaHCO_3$, 16mM; Glucose, 10mM) containing $CaCl_2$ (final concentration: 2mM). The slices were re-suspended after each wash, allowed to settle on ice (approximately 30 s) and rinsed twice more with Krebs $CaCl_2$ buffer. Finally, slices were re-suspended in ice-cold Krebs $CaCl_2$ containing dimethylsuphoxide (DMSO; final concentration: 10%) and stored at -80°C until further analysis.

2.9.4 Preparation of homogenate

Freshly dissected tissue samples from each region were immediately homogenized using a 1ml glass homogeniser in 350 μl of buffer. Buffers were used were as follows: Krebs solution (NaCl,

136mM; KCl, 2.54mM; KH_2PO_4 1.18mM; $MgSO_4.7H_2O$, 1.18mM; $NaHCO_3$, 16mM; Glucose, 10mM) containing $CaCl_2$ (final concentration: 2mM) for use with BDNF and NGF ELISAs, and Lysis Buffer (NP-40, 1% (v/v); Tris (pH8.0), 20mM; NaCl, 137mM; glycerol, 10% (v/v); ethylenediaminetetracetic acid (EDTA), 2mM; activated sodium orthovanadate (Na_3VO_4), 1mM; aprotinin; leupeptin) for use with TrkB/p-TrkB ELISAs and Western Blotting.

2.9.5 Preparation of tissue samples for RNA extraction

Freshly dissected tissue samples were placed in tubes containing 350 µl of RNA*Later*™ (Ambion, Warrington, UK), and kept at -4°C for up to two weeks until used for RNA extraction.

2.9.6 Preparation of whole brains for sectioning

The left hemisphere was propped up on a cork disk in the correct orientation, covered in O.C.T compound (R.A. Lamb LTD, Sussex, UK), and quickly immersed in liquid nitrogen until O.C.T turned white. Hemispheres were covered in aluminium foil and stored at -80°C until sectioning. Alternatively (in Chapters 5 and 6), whole brains were flash-frozen in isopentane (on dry ice) and then wrapped in aluminium foil and stored at -80°C. At a later date, the brains were propped up on a cork disk and covered with OCT on dry ice until OCT turned white. Hemispheres were covered in aluminium foil and stored at -80°C until sectioning.

2.10 PCR (Polymerase Chain Reaction)

2.10.1 RNA extraction

To prepare the tissue samples for PCR, RNA extraction was performed using a total RNA isolation kit (Macherney-Nagel). Samples that had been stored in RNA buffer were removed from the -80°C freezer, placed in 350µl of RA1 buffer and 3.5µl of β–mercaptoethanol and homogenised using a polytron tissue disrupter (Kinetatica). As per the user manual provided with the kit, the sample homogenate was added to Nucleospin® filter units and filtered by centrifugation at 13000rpm for 1min. 350µl of 70% ethanol was added to each sample lysate and mixed by pipetting vigorously, approximately 5 times. Each sample mix was placed in a Nucleospin® RNA II column and centrifuged at 13,000 rpm for 30 seconds, allowing the RNA to bind to the silica membrane. Following centrifugation, the column was placed in a new collecting tube and 350µl of membrane

desalting buffer (supplied) was added to the column. The column was again centrifuged at 13,000 rpm for 1 minute. rDNase (supplied) was diluted 1:10 in DNase Reaction Buffer (supplied) and 95µl of this solution was pipetted directly into the centre of the silica column, to digest DNA. Samples were incubated with the DNase solution for 15 minutes at RT. 200µl of buffer RA2 was then added to the column and centrifuged at 13000 rpm for 30 seconds. The column was placed in a new collecting tube. 50ml of ethanol was added to 25ml of RA3 buffer concentrate and 600µl of this was added to the column and centrifuged at 13000 rpm for 30 seconds. The flow-through was discarded and the column was washed a second time with 250µl of RA3 buffer, and then centrifuged at 13000 rpm for 2 minutes. Finally, the column was placed in a fresh RNase–free microtube and 60µl of RNase–free H_2O (supplied) was added to the column and the RNA was eluted during centrifugation at 13000 rpm for 1 minute. The eluted RNA was stored at -80°C for qualification, quantification and reverse transcription (see below).

2.10.2 RNA quantification

The optical density of the extracted RNA was measured using a spectrophotometer (NanoDrop-1000) 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:

$RNA = OD_{260}$ x dilution factor x 40µg/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 approximately 1.8-2.0 is indicative of pure RNA. RNA concentrations were equalized with RNase-free H_2O so that equal concentrations of RNA could be used as a template for cDNA transcription.

2.10.3 Reverse transcription of RNA

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 10 mins, then at 37°C for 2 hrs. The cDNA was stored frozen at -20°C for later real-time polymerase chain reaction (PCR) analysis.

2.10.4 Polymerase chain reaction (PCR)

Gene expression of targets (see Table 2.1) was assessed using Taqman gene expression assays (Applied Biosystems, UK) 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.

Brain regions were tested for mRNA of different targets using multi-target (multiplex) Q-PCR. cDNA was diluted with RNase-free H_2O (1:4 dilution) and 10µl of this diluted cDNA was pipetted onto a PCR plate. Following this, target primer/probe (1.25µl), β-actin primer/probe (1.25µl) and Taqman master mix (12.5µl) were mixed and added to each well (25µl reaction volume). Electronic pipettes (EDP3 2-20µl, 10-100µl and 20-200µl) were used to ensure pipetting accuracy. RT-PCR measurements were performed using an ABI Prism 7300 instrument (Applied Biosystems). Samples were placed in a real-time PCR thermocycler and measured according to the following consecutive steps: step 1: 95°C for 10 min and step 2: 95°C for 15 s followed by the final transcription step at 60°C for 1 min for each cycle. Step 2 was repeated forty times and the fluorescence was read during the annealing and extension phase (60°C) for the duration of the programme.

The following primers were used:

Gene name	Assay number	NCBI Gene reference*
BDNF	Rn00560868_m1	----------
TrkB B	Rn00820626_m1	NM_012731.1
β-NGF	Rn01533872_m1	XM_227525.3
TrkA A	Rn00572130_m1	NM_021589.1
IGF-1	Rn00710306_m1	NM_178866.4 NM_001082478.1 NM_001082479.1 NM_001082477.2
KI-67	Rn01451466_m1	XM_225460.4

Table 2.1 List of gene assays used for PCR

*Gene reference as listed on the National Centre for Biotechnology Information (NCBI). Entrez-Nucleotide website: http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=search&db=nucleotide

2.10.5 RT-PCR analysis

The ΔΔCT method (Applied Biosystems 7300 RQ software) was used to assess gene expression for all RT-PCR analysis. This method was used to assess relative gene expression by comparing the gene expression of experimental samples to the mean of control samples, rather than quantifying the exact copy number of the target gene. In this manner, the fold-difference (increase or decrease) can be compared between experimental and control samples. The fold change is assessed using the cycle number (CT) difference between samples. Briefly, the threshold for fluorescence is set and the CT is measured against this value. In order to accurately assess the difference between gene expressions, the threshold was set when the PCR reaction was in the exponential phase.

2.11 BDNF release experiment

2.11.1 BDNF release protocol

Slices were thawed rapidly at 37°C and washed three times with ice-cold oxygenated Krebs $CaCl_2$. Slices were then incubated (pre-incubation step) in 250µl of Krebs $CaCl_2$ with O_2 at 37°C (using a water bath) for 3 minutes. Slices were spun down at 500g for 20 seconds, and the supernatant was discarded. This process was repeated (incubation step) and the supernatant was removed for later assessment for BDNF (basal condition). The slices were then stimulated with 250µl of Krebs $CaCl_2$ containing 50mM KCl and incubated with O_2 at 37°C (using a water bath) for 3 minutes. Slices were spun down at 500g for 20 seconds, and the supernatant was removed for later assessment of BDNF (stimulated condition). Slices were then homogenised in ice-cold Krebs $CaCl_2$ and spun down at 4°C for 10 minutes. The supernatant was removed for BDNF assessment (cell condition).

2.12 ELISA (Enzyme-Linked Immunosorbent Assay)

2.12.1 Total protein quantification using the Bradford Protein Assay

Samples were spun at 1400 rpm for 15 min at 4°C and supernatants were removed. Protein content of tissue samples in Krebs Buffer was quantified using the Bradford Protein Assay (Bradford, 1976). Samples were analysed in triplicate on a 96-well plate (Microtest plate, Sarstedt, Ireland). A standard curve was prepared from a stock solution of 1000µg/ml bovine serum albumin (BSA) diluted in deionised water. The standard curve consisted of seven 1:2 serial dilutions, with the eighth standard containing only deionised water. For each sample, 5µl of sample was pipetted into each well. All wells (standards and samples) received 195µl of diluted Bio-Rad. Absorbances were assessed at 630nm using a 96-well plate reader. Protein concentrations were calculated using regression analysis, and values were expressed as mg protein/ml. Tissue samples were equalized to the lowest value for BDNF quantification.

2.12.2 Protein quantification using the BCA protein assay

The Bradford protein assay was not compatible with tissue samples stored in Lysis buffer due to the presence of detergent in the buffer, thus protein concentrations in Lysis buffer were quantified using the BCA protein assay method (Pierce®, Thermo Scientific). All standards and samples were

analysed in triplicate (10µl/well) on a 96-well plate (Microtest plate; Sarstedt, Ireland). A standard curve was prepared by appropriately diluting a 1ml ampule of 2.0mg/ml albumin standard (BSA) with Lysis buffer, as per the manufacturer's guidelines. The standard curve ranged from 2000µg/ml down to 25µg/ml, with a blank of deionised water included as the lowest standard. BCA working reagent was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B (50:1, reagent A:B) and 200µl of this working reagent was added to the standards and samples. Each plate was mixed with gentle agitation for 30 seconds, covered and incubated at 37°C for 30 minutes. Plates were cooled to RT and the absorbance of the standards and samples was measured at 562nm using a 96-well plate reader (Synergy HT Multi-Mode Microplate Reader, BioTek®). The regression equation of the curve was used to calculate the concentration of protein in each sample and values were expressed as mg protein/ml. All samples were equalised to the lowest value by adding Lysis buffer to ensure that each sample contained similar concentrations of protein before any further neurochemical analysis.

2.12.3 BDNF quantification

BDNF concentrations in serum, plasma, and tissue samples were assessed by an R&D sandwich ELISA (DuoSet Quantikine IC Human BDNF ELISA, R&D Systems, UK). Serum samples were diluted 1:2 in double distilled water before quantification. A 96-well plate (NUNC-immuno™ MaxiSorp™ plate; Denmark) was coated with capture antibody (2µg.ml-1 mouse anti-human BDNF diluted in phosphate buffered saline (PBS; 50µl/well)) and incubated overnight at RT. The plate was washed with wash buffer (PBS-T; 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 hr at RT. After washing, standards (recombinant human BDNF diluted in reagent diluent, top standard concentration: 1500 pg/ml) and samples were added in duplicate (50µl/well) and incubated for 2 hrs at RT. The plate was washed again and incubated with detection antibody (25ng/ml biotinylated mouse anti-human BDNF in reagent diluent; 50µl/well) for 2 hrs at RT. Following this, the plate was washed and reacted with Streptavidin-HRP complex (1:200 dilution in reagent diluent; 50µl/well) for 20 min at RT. After washing, either substrate solution (1:1 mixture of colour reagent A and colour reagent B) or 3,3',5,5'- Tetramethylbenzidine (TMB) one solution was added (50µl/well) and incubated in the dark at RT for a minimum of 20 min or until sufficient colour development had occurred. The reaction was stopped using 1M H_2SO_4 (50µl/well). The optical density (OD) was measured at 450nm using a 96-well plate reader (Synergy HT Multi-Mode Microplate Reader, BioTek®). A standard curve was constructed and the

regression equation of the curve was used to calculate the concentration of BDNF in each sample. Values were expressed as pg BDNF/mg protein for tissue homogenate or as pg BDNF/ml serum for serum samples.

2.12.4 β-NGF quantification

β-NGF concentration in tissue was assessed by an R&D sandwich ELISA (Rat β-NGF Duoset® ELISA kit) according to manufacturer's instructions. Briefly, a 96-well plate (NUNC-immuno™ MaxiSorp™ plate; Denmark) was coated with capture antibody (0.4μg/ml goat anti-rat β-NGF diluted in phosphate buffered saline (PBS; 50μl/well)) and incubated overnight at RT. The plate was washed with wash buffer (PBS-T; 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 hr at RT. After washing, standards (recombinant rat β-NGF diluted in reagent diluent, top standard concentration: 2000pg/ml) and samples were added in duplicate (50μl/well) and incubated for 2 hrs at RT. The plate was washed again and incubated with detection antibody (100ng/ml biotinylated goat anti-rat β-NGF in reagent diluent; 50μl/well) for 2 hrs at RT. Following this, the plate was washed and reacted with Streptavidin-HRP complex (1:200 dilution in reagent diluent; 50μl/well) for 20min at RT. After washing, either Substrate solution (1:1 mixture of colour reagent A and colour reagent B) or TMB solution was added (50μl/well) and incubated in the dark at RT for a minimum of 20 min or until sufficient colour development had occurred. The reaction was stopped using 1M H_2SO_4 (50μl/well). The OD was read at 450nm wavelength using a 96-well plate reader (Synergy HT Multi-Mode Microplate Reader, BioTek®). The regression equation of the standard curve was used to calculate the β-NGF concentrations of the tissue homogenate and was expressed as pg NGF/mg protein.

2.12.5 TrkB and p-TrkB quantification

TrkB concentration in tissue was assessed by an R&D sandwich ELISA (DuoSet IC Human Total TrkB) according to manufacturer's instructions. Briefly, a 96-well plate (NUNC-immuno™ MaxiSorp™ plate; Denmark) was coated with capture antibody (8 μg/ml mouse anti-human TrkB antibody diluted in phosphate buffered saline (50μl/well)) and incubated overnight at RT. The plate was washed with wash buffer (PBS-T; 0.05% Tween®20 in PBS) using an automated plate washer (Columbus Plus, Tecan, Austria) and blocked with reagent diluent (1% BSA in PBS; 150μl/well) for 1h at RT. After washing, standards were diluted in the recommended IC diluent (1% NP-40,

20mM Tris (pH 8.0), 137mM NaCl, 10% glycerol, 2mM EDTA, 1mM activated sodium orthovanadate) to a concentration of 6000 pg/ml (top standard). The standard curve consisted of eight 1:2 serial dilutions, with the ninth standard containing only the IC diluent. Standards and samples (equalised in Lysis Buffer) were added in duplicate (50µl/well) and incubated for 2 hrs at RT, or overnight at 4°C. The plate was washed again and incubated with detection antibody (50ng/ml biotinylated goat anti-human TrkB antibody in IC diluent; 50µl/well) for 2 hrs at RT. Following this, the plate was washed and reacted with Streptavidin-HRP complex (1:200 dilution in IC diluent; 50µl/well) for 20 min at RT. After washing, either Substrate solution (1:1 mixture of colour reagent A and colour reagent B) or TMB one solution was added (50µl/well) and incubated in the dark at RT for 20 minutes. The reaction was stopped using 1M H_2SO_4 (50µl/well). The OD was read at 450nm wavelength using a 96-well plate reader (Synergy HT Multi-Mode Microplate Reader, BioTek®). The regression equation of the standard curve was used to calculate the TrkB concentrations of the tissue homogenate and was expressed as pg TrkB/mg protein.

Phosphorylated TrkB was detected using a Human Phospho-TrkB ELISA (R&D Systems). The capture antibody used was mouse anti-human TrkB antibody diluted to a concentration of 2µg/ml in PBS. The standard used was recombinant human phosphorylated TrkB at a top concentration of 300pg/ml. The detection antibody used was a mouse anti-phospho-tyrosine antibody conjugated to HRP. No Streptavidin-HRP step was required.

2.12.6 Corticosterone Quantification

Corticosterone concentrations in serum were assessed using a Corticosterone HS EIA ELISA Kit (Immunodiagnostic Systems, UK) according to manufacturer's instructions. Serum samples were diluted 1:10 before quantification. 100 µl of calibrator (lyophilized horse serum containing corticosterone and preservative, provided in kit), controls (control 1: lyophilised horse serum containing preservative, control 2: lyophilised horse serum containing rat serum and preservative, provided in kit), and sample were diluted in 400 µl of buffer (phosphate buffered saline containing preservative, provided in kit), vortexed, incubated at 80°C for 30 mins, and allowed to cool. 100µl of calibrator, controls, and sample diluted in buffer were added in duplicate to a 96-well plate (Microplate coated with polyclonal sheep anti-corticosterone antibody, 12 x 8-well strips in a foil pouch with desiccant provided in kit). 100µl of an enzyme conjugate (aqueous solution containing corticosterone labeled with horseradish peroxidase, protein, enzyme stabilisers and preservative) were added to each well the plate was and incubated for 4hr at RT. The plate was washed in with

wash buffer (PBS-T; 0.05% Tween®20 in PBS) using an automated plate washer (Columbus Plus, Tecan, Austria) and incubated with 3,3',5,5'- Tetramethylbenzidine (TMB) solution (100µl/well) in the dark at RT for a minimum of 20 min or until sufficient colour development had occurred. The reaction was stopped using 1M HCL (100µl/well). The optical density (OD) was measured at 450nm using a 96-well plate reader (Synergy HT Multi-Mode Microplate Reader, BioTek®). A standard curve was constructed and the regression equation of the curve was used to calculate the concentration of corticosterone in each sample. Values were multiplied by 10 to get the concentration of corticosterone in each serum sample in ng/ml.

ELISA	Capture Antibody (concentration)	Standard (top concentration)	Detection Antibody (concentration)
BDNF	Mouse anti-human BDNF (2µg/ml)	Recombinant human BDNF (1500pg/ml)	Biotinylated mouse anti-human BDNF (25ng/ml)
NGF	Goat anti-rat β-NGF (0.4µg/ml)	Recombinant rat β-NGF (2000pg/ml)	Biotinylated goat anti-rat β-NGF (100ng/ml)
TrkB	Mouse anti-human TrkB antibody	Recombinant human TrkB (6000pg/ml)	Biotinylated goat anti-human (50ng/ml)
p-TrkB	Mouse anti-human TrkB antibody (2µg/ml)	Recombinant human phosphorylated TrkB (300pg/ml)	Anti-phospho-tyrosine-HRP (1:2000)

Table 2.2 ELISA antibodies and concentrations

2.13 Western Immunoblotting

2.13.1 Preparation of samples for gel electrophoresis

Aliquots of equalised tissue sample in Lysis buffer were thawed on ice. 100 µl of sample was added to 100 µl of sample buffer (Tris-HCl pH 6.8, 0.5M; sodium dodecyl sulphate (SDS), 10% (w/v); glycerol, 10% (v/v); 2-β-mercaptoethanol, 5% (w/v); bromophenol blue, 0.05% (w/v)). Samples were then boiled in a heating block for 5 min.

2.13.2 Gel electrophoresis

10% Acrylamide gels were made up and allowed to set between two glass plates and inserted into an electrophoresis unit (BioRad Mini-Protean 3, BioRad Laboratories, Herfortshire, England). Electrode Running buffer (composition: Tris base, 25mM; glycine, 200mM; SDS, 17mM) was added to the inner and outer reservoirs. 10µl of prepared sample or 5µl of prestained molecular markers were loaded onto the wells. The gel was run at 30mA for 30 mins.

2.13.3 Western Immunoblotting

The gel was washed in transfer buffer (25mM Tris Base, 192 mM Glycine, 20% MeOH, 0.05% SDS, 80% dH_2O). One sheet of nitrocellulose paper (Amersham) and two sheets of filter paper (Whatman No.3) were pre-cut to the size of the gel and soaked in transfer buffer for 5 mins. A layered sandwich was prepared by stacking the nitrocellulose paper on top of the filter paper followed by the gel and the second piece of filter paper. Air bubbles were removed by rolling a pencil over the sandwich, and the sandwich was placed on the anode of a semi-dry blotter (Apollo Instruments, Alpha Technologies, Dublin, Ireland) pre-moistened with transfer buffer. The transfer was carried out at 225mA for 75 mins. Blots were blocked in blocking buffer (5% BSA in TBS-T) for nonspecific binding for 2 hrs at RT, and probed with the primary antibody (diluted in 2% BSA in TBS-T). This was washed off and a secondary antibody (diluted in 2% BSA in TBS-T) was added. Immunoreactive bands were detected using SuperSignal West Dura chemiluminescence reagents (Pierce). The membranes were then developed using a gel reader (Fujifilm Las-3000 Imaging System) and analysed using the Fujifilm Multigauge Software.

Primary Antibody	Dilution	Secondary Antibody	Dilution
p-Erk (Cell Signaling Technology)	1:1000	Goat anti-Rabbit (Sigma)	1:1000
ERK (Cell Signaling Technology)	1:1000	Goat anti-Rabbit (Sigma)	1:2000
p-PLCγ (Cell Signaling Technology)	1:1000	Goat anti-Rabbit (Sigma)	1:250
PLCγ (Cell Signaling Technology)	1:1000	Goat anti-Rabbit (Sigma)	1:1000
p-CaMKII (Cell Signaling Technology)	1:1000	Goat anti-Rabbit (Sigma)	1:500
CaMKII (Cell Signaling Technology)	1:1000	Goat anti-Rabbit (Sigma)	1:1000
Synapsin (Cell Signaling Technology)	1:1000	Goat anti-Rabbit (Sigma)	1:1000
p-Synapsin (Cell Signaling Technology)	1:1000	Goat anti-Rabbit (Sigma)	1:250
Synaptophysin (Millipore)	1:1000	Goat anti-mouse (Sigma)	1:1000
β-actin (Sigma)	1:1000	Goat anti-mouse (Sigma)	1:2000

Table 2.3 List of Antibodies used for Western Immunoblotting

2.14 Immunohistochemical Staining

2.14.1 Preparation of hippocampal sections for immunostaining

On the day of sectioning, the hemisphere was allowed to sit at -20°C for 20-30 minutes. Cutting was done using a cryostat (Leica CM1900), 50μm saggittal sections were cut until the hippocampus started to appear (~150 x 50μm). Once the hippocampus became clearly visible, three 10μm saggittal slices were transferred onto a subbed slide and stained with 0.1% toluidine blue for 1 min and viewed by light microscopy (Nikon Labophot, Nikon Instech co., Ltd, Kanagawa, Japan) to confirm the correct position. Then 10μm sections were transferred onto subbed slides for light microscopy, such that 6 slides (3 sections per slide) were taken for each animal. 20μm sections (3 slides, 3 sections per slide) were transferred onto subbed slides for confocal microscopy. Slides were coded and relabeled to ensure objectivity during analysis. All sections were stored at -20 until stained.

2.14.2 BrdU immunostaining and analysis

Quantification of proliferating cells in the dentate gyrus was performed using a 3,3'-diaminobenzidine (DAB)-linked staining. Frozen sections were allowed to thaw to RT for 10 mins. Water-repellent circles were drawn around mounted tissue sections with a Pap pen (Invitrogen). Slides were fixed in pre-cooled 100% methanol (Sigma-Aldrich) for 10 minutes, and then washed in PBS for 3x3 minutes. Slides were incubated in 2N HCL (Fluka, 75μl per section) for 30 mins at 37°C to denature the DNA and allow the antibody to bind to the incorporated BrdU. Slides were washed in 0.1M Borate buffer (0.1M sodium borate in deionised water, pH 8.5) for 2x5 min to neutralise the sections and then washed in PBS for 3x3 mins. Slides were incubated in 0.3% H_2O_2 (Sigma, 75μl per section) for 20 mins at RT to block endogenous peroxidase. Slides were incubated in blocking buffer consisting of a 1:5 dilution of Normal Rabbit Serum (Vector Laboratories, UK) in 1% BSA in PBS (75μl per section) for 60 mins at RT to block non-specific binding sites. Primary rat anti-BrdU (AbCam) was diluted in blocking buffer to a concentration of 1:75. Slides were incubated in primary rat BrdU (75μl per section) overnight at 4°C.

The next day, the secondary antibody (Rabbit anti-rat, AbCam) was diluted to a concentration of 1:500 in blocking buffer. Slides were washed in PBS for 3x3 mins, and then incubated in secondary

antibody for 30 mins at RT. Slides were washed again in PBS for 3x3 mins, and then incubated in Vectastain ABC solution (Vector Laboratories, UK) for 30 minutes at 4°C to amplify the signal.

DAB chromagen (Dako, Denmark), a colour reagent, was activated by adding 1µl of 30% H_2O_2 to 1 ml of DAB. 50µl of colour reagent was added to each section and colour development was observed under the microscope. The colour reaction was stopped by immersing the slides in PBS as soon as brown cells started to appear. Slides were counterstained using filtered hematoxylin (Sigma) for 4x3 mins, and washed with running tap water in between. Slides were washed in distilled H_2O for 3 mins. Slides were dehydrated by washing with increasing concentrations of ethanol (70%, 90%, and 100%) and then immersed in xylene for a second. Slides were mounted with DPX mountant (Sigma) and a coverslip and left to dry before counting.

Slides were examined using a Nikon light microscope. A total of three sections per animal, with six views in each section, were counted. Brown cells were identified as all cells since hematoxylin stains all cells. Blue cells were identified as BrdU positive cells. BrdU positive cell quantities were expressed as a percentage of total cell count.

2.14.3 BrdU/NeuN staining

Frozen sections (20µm) were allowed to thaw to RT for 10 mins. Water-repellent circles were drawn around mounted tissue sections with a Pap pen (Invitrogen). Slides were fixated in 4% formaldehyde in PBS and then washed in PBS for 3 mins. Slides were incubated with 0.5 M NaOH in a humidified chamber for 30 mins at RT to denature the DNA. Slides were washed in PBS for 3x 3 mins. A blocking solution was prepared containing a 1:4 ratio of Normal Goat Serum (NGS) and 0.2% Triton-X in PBS. Slides were incubated in the blocking solution for 60 mins to block non-specific binding sites. A primary antibody solution was prepared by diluting the BrdU primary antibody (Chicken polyclonal to BrdU, 1:600) and the NeuN primary antibody (Anti-NeuN, Millipore, 1:100) in blocking solution. Slides were incubated in primary antibody solution overnight at 4°C in a humidified chamber. The next day the secondary antibody solution was prepared by diluting the BrdU secondary antibody (goat anti-chicken Alexa-488, 1:1000) and the NeuN secondary antibody (goat anti-mouse Alexa-633, 1:2000) in blocking solution. Slides were washed in PBS for 6x3 mins, and then incubated in secondary antibody solution for 30 mins at RT. Slides were washed again in PBS for 12x3 mins, and then counterstained by incubating in Hoechst (1:1000 dilution in PBS) for 10 minutes at RT to visualise all nuclei. After washing again in PBS

for 3x3 mins, slides were mounted using Vectashield® and a coverslip and sealed with nail varnish. Slides were stored at 4°C in the dark and coded and relabelled to ensure objectivity during analysis. Slides were observed using a LSM 700 Confocal Laser Scanning Microscope and LSM software (Carl Zeiss, Germany) the next day. Green fluorescence indicated BrdU labelling and red fluorescence indicated NeuN labelling. DAPI-stained cells appeared blue. All slides were scanned the day after staining to maintain consistent conditions. A total of three sections per animal, with six views in each section, were counted. The number of BrdU/NeuN positive cells was expressed as a percentage of total BrdU+ve cells.

2.14.4 BrdU/Doublecortin staining

The same protocol was followed for BrdU/DCX Staining as for BrdU/NeuN staining. The DCX antibody was a rabbit polyclonal antibody (H-280, sc-28939, Santa Cruz) diluted to a concentration of 1:100 in blocking solution. The secondary antibody was goat anti-rabbit Alexa-633 diluted to a concentration of 1:2000 in blocking solution.

2.14.5 TUNEL staining

The amount of apoptotic tissue was assessed using the DeadEnd™ Fluorometric TUNEL System (Promega, Ireland) according to manufacturer's instructions. Briefly, slides with adhering tissue sections (20μm) were fixed by immersing in 4% formaldehyde in PBS for 15 mins. Slides were washed twice in PBS for 5 mins each time, and then permeabilised by incubating in a Proteinase K solution (20μg/ml) for 10 mins at RT. Slides were washed in PBS for 5 mins, and then fixed again in 4% formaldehyde in PBS. Slides were then equilibrated using the provided Equilibration Buffer for 10 mins at RT. A TdT reaction mix was applied to the sections and incubated in the dark for 60 minutes at 37°C in a humidified chamber. The reaction was then stopped by immersing in 2x SSC for 15 mins, and then washed three times in PBS (5 mins per wash). The slides were counterstained with DAPI (1:1000 dilution in PBS) to visualise all nuclei and then mounted using Vectashield®. Slides were stored at 4°C and coded and relabelled to ensure objectivity during analysis. All slides were scanned using a confocal microscope (LSM 700 Confocal Laser Scanning Microscope and LSM software; Carl Zeiss, Germany) the day after staining to maintain consistent conditions. Green fluorescence indicated apoptotic tissue and DAPI-stained cells appeared blue.

Primary Antibody	Dilution	Secondary Antibody	Dilution
Chicken polyclonal to BrdU (Millipore)	1:600	Goat anti-chicken Alexa-488 (Invitrogen)	1:1000
Anti-NeuN Antibody (Millipore)	1:100	Goat anti-mouse Alexa-488 (Invitrogen)	1:2000
Rabbit polyclonal anti-doublecortin antibody (Millipore)	1:100	Goat anti-rabbit (Invitrogen)	1:2000

Table 2.4 List of antibodies used for immunohistochemical staining

2.15 BDNF ICV Infusions

2.15.1 Canula Implantation

Animals were single-housed and weighed prior to surgery. Animals were anesthetised with ketamine (100 g/kg; Bayer Healthcare) and xylazine (100 mg/kg: Rompun1, Bayer Healthcare) and supplemented throughout the surgical procedure as necessary.). Alternatively, an isofluorane system (4% induction in a gas chamber, 1.5–2% maintenance using a face mask, in 100% oxygen; Abbot Laboratories, Ltd, UK) was used when it became available (preferred method). Absence of pedal reflexes was taken to be an indicator of deep anaesthesia. If no reflexes were observed, the rat's head was shaved and placed in a stereotaxic frame with ear-bars and a mouthpiece. All surgical equipment was sterilised using 100% alcohol. The scalp was swabbed with iodine using cotton buds and an incision was made using a scalpel. Skin was fixed out of the way using crocodile clamps. The underlying membranes were scraped away using a scalpel and the skull was dried using cotton buds to visualise the bregma. Skull surface was confirmed to be level (parallel to horizontal side-bars of stereotactic frame), clean, and dry before proceeding with the craniotomy. Bregma (Fig 2.5a) was identified and the coordinates for the left lateral ventricle were calculated relative to bregma (coordinates, bregma, 0.9 mm; midline, 1.3 mm, Fig 2.5a) according to Paxinos and Watson (Paxinos and Watson, 2004). Using a surgical drill, a 1mm hole was drilled for the guide canula. Drill was held normal to the surface of the skull and care was taken to perforate only the skull and not the dura mater. Three additional 1mm holes were drilled part-way into the skull in

locations anterior, posterior, and lateral to the canula hole for the screws (Fig 2.5a). After all holes were drilled, screws were screwed into the screw holes using a screw driver. Care was taken to perforate the skull too deeply, but to screw in the screws only as much as was required to get a firm hold (about three turns). The dura mater in the canula hole was carefully pierced using a bent sterile needle. A guide canula (Plastics 1, Bilaney Consultants Ltd., Kent, UK, Fig 2.5c) was fixed to the moveable arm of the stereotaxic frame and placed directly above bregma. The guide canula was custom-built to reach a depth of 2.6mm from the surface of the skull. The guide canula was lowered carefully into the hole and fixed in place by cementing it to the screws using dental cement (Dentalon plus, Hereaus Kulzer Ltd., Belgium) applied with a small metal spatula. Special care was taken to mould the cement in such a way that there were no sharp edges to irritate the scalp. Once the cement had dried, the canula-holder was detached from the guide canula and a dummy canula (Plastics 1, Bilaney Consultants Ltd., Kent, UK, Fig 2.5c) was used to seal the guide canula. The incision was closed using surgical staples and iodine and an antiseptic cream were applied to the wound. The entire procedure takes about 30 minutes. Animals were given Carprofen (Rimadyl, 5mg/kg, s.c.) immediately after closing the incision. Post-operative care included single-housing the animals individually in cages with a separator to prevent the implant from catching on the lower parts of the cage's metal grid. Paper towels were used instead of bedding for the first 24 hours to avoid getting bedding in the wound. Animals were allowed to recover from surgery for two weeks during which their weight and behaviour were closely monitored for signs of distress or pain. After the wound had healed (1 or 2 days), the dummy canula was unscrewed daily to avoid jamming and to habituate animals to the infusion procedure.

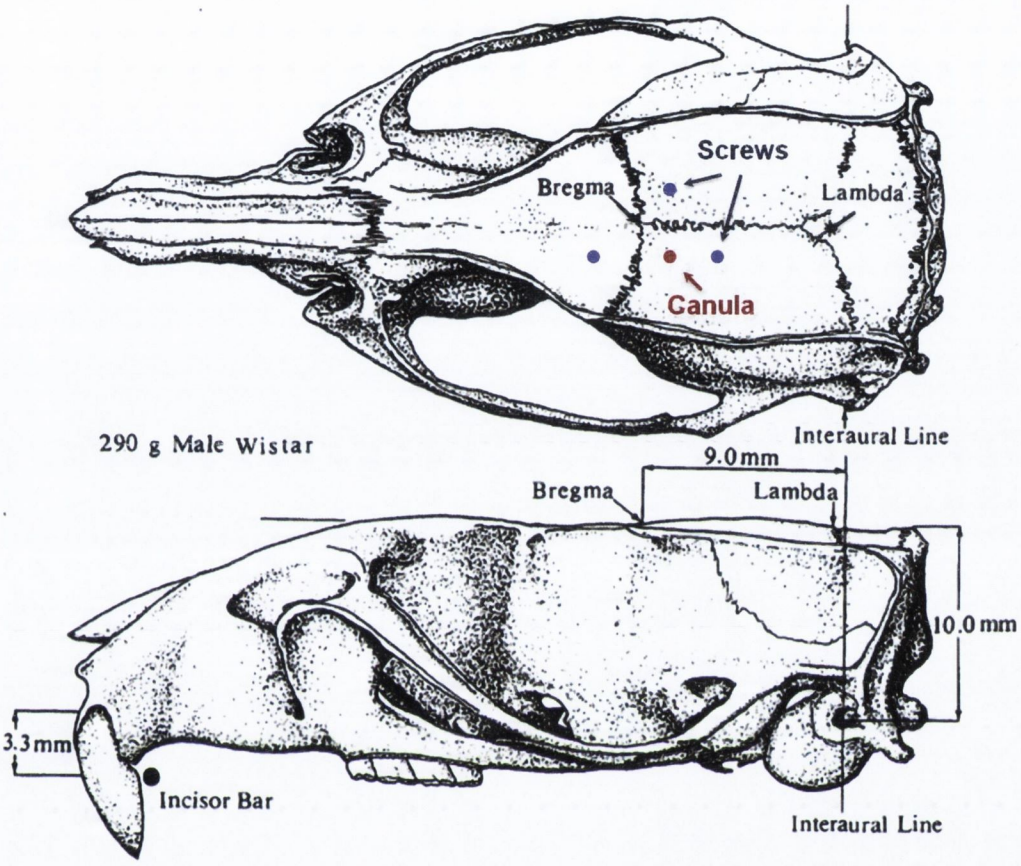


Fig 2.5 Position of guide canula and screws

This schematic shows dorsal (top) and lateral views of a male Wistar rat skull. It was modified from Paxinos and Watson (Paxinos and Watson, 2004) to show the canula and screw positions used for canula implantation. Bregma was identified and the coordinates for the left lateral ventricle were calculated relative to bregma (coordinates, bregma, 0.9 mm; midline, 1.3 mm). At this position, a 1mm hole was drilled for the guide canula (shown in red). Three additional 1mm holes were drilled part-way into the skull in locations anterior, posterior, and lateral to the canula hole for the screws (shown in purple).

2.15.2 ICV Infusion

After recovery, for infusions into the left ventricle, the dummy canula was replaced with an internal canula (Plastics 1, Bilaney Consultants Ltd., Kent, UK, Fig 2.5c) with a 1mm protrusion. Thus the depth of the infusion was 3.6mm in total from the surface of the skull. One bolus infusion of recombinant Human BDNF (10ng), was administered into the left ventricle (i.c.v., Fig 2.5b). The dosage was calculated based on the exercise-induced increase in hippocampal BDNF protein (48.22pg/mg protein) as observed in previous experiments in the laboratory (Griffin et al., 2009). The calculated dose (7.72ng) was rounded up to allow for loss during infusion. Control animals received an infusion of cytochrome C (10ng, i.c.v.) to control for the effect of infusing a large molecule (BDNF) directly into the brain. All infusions were diluted in aCSF (NaCl, 150mM; KCl, 3mM, $CaCl_2$, 0.19mM, $MgCl_2$, 0.8mM, Na_2HP0_4, 0.8mM, NaH_2PO_4, 0.2mM. The solutions were infused using a 10µl Hamilton syringe (Microliter Syringes, Hamilton) connected via sterile rubber tubing to an internal canula, inserted into the guide canula of each rat. Each rat was given 0.5µl of solution, over a two minute time period. 30 mins post-infusion, all animals were trained in a cognitive task (section 2.6). Animals were tested 24 hours later. Exploration of objects was recorded and expressed as a percentage of the total exploration time.

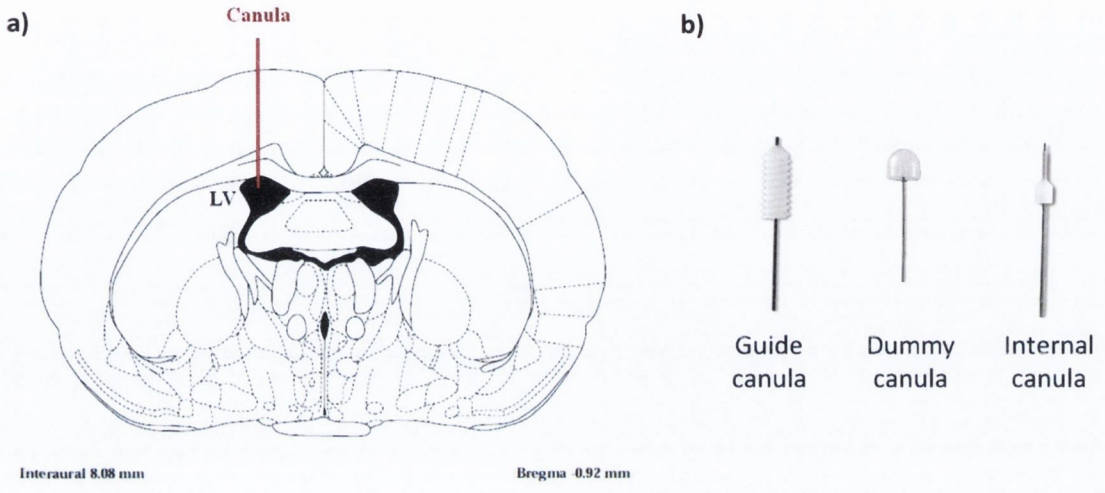


Fig 2.6 Coronal view of infusion site and canulae

(a) shows the infusion site in a coronal section at 0.9mm posterior to bregma (adapted from Paxinos and Watson, 2004). (b) shows the three types of canula used: the guide canula was implanted into the skull, the dummy canula was used to seal the guide canula before infusions, the internal canula was connected to a syringe and inserted into the guide canula for infusions.

2.16 BDNF IV Infusions

Tail veins were accessed as described in section 2.81.2 (tail vein blood collection). One bolus of human recombinant BDNF (volume 500µl; 10ng/ml) dissolved in 0.89% saline was injected into the lateral tail vein of rats. Rats were allowed to recover for 1 hour, after which they were trained in a cognitive task. Animals were tested 24 hours later. Exploration of objects was recorded and expressed as a percentage of the total exploration time.

2.17 MRI Analysis

2.17.1 Bolus tracking Arterial Spin Labeling (btASL)

Bolus tracking Arterial Spin Labelling (BtASL) analysis was performed according to the method of Kelly (Kelly et al., 2009). This technique uses magnetically labelled arterial blood as an

endogenous contrast agent (bolus) to quantify the perfusion of specific brain regions. It constitutes a noncompartmental approach to ASL perfusion quantification based on a Fokker-Planck equation for changes of the distribution of labelled blood in the brain over time.

2.17.2 Image acquisition

Rats were anesthetized with isofluorane (4% induction, 1.5–2% maintenance; Abbot Laboratories, Ltd, UK) in 100% oxygen and secured in a custom-built cradle with ear bars and a bite-bar to support the head. Respiration rate and temperature were monitored using purpose-built MRI-compatible monitoring equipment (SA Instruments, Stony Brook, NY). Temperature was maintained at 37.0–38.0°C using a feedback-controlled water-circulating heating system. All imaging experiments were conducted on a seven Tesla BioSpec animal scanner (Bruker BioSpin, Ettlingen, Germany) equipped with a 20cm actively-shielded gradient system and actively decoupled transmit (12 cm Helmholtz) and receive (3cm surface) coils. Gradient-echo pilot scans were used to ensure the animal was correctly positioned in the magnet, with the hippocampal region of interest (typically centred at 3.10mm posterior to bregma) in the isocenter of the magnet to minimize imaging artefacts. Coronal images were obtained with a 5 sec preparation interval followed by a fast low angle shot (FLASH) sequence to image the flow of 'tagged' blood with the following acquisition parameters: FOV=3.00 x 3.00 cm, image matrix = 128 x 64, 1 x 2mm slice, TR = 6.94 ms, TE = 2.63 mm with 22 x repetition images per slice to enable the calculation of blood flow in that slice. The selection of the slice was selected to provide the best view of the hippocampal region (Fig 6.10).

2.17.3 BtASL analysis

Regions of interest (ROIs) were drawn in ImageJ (Rasband W.S., Bethesda, MD, USA.) Regions of interest (left and right hippocampus, left and right cortex, and whole brain) were manually selected from a high-resolution scan of the previously selected slice (Fig 6.10).

Difference images were generated by subtracting the control image from the labelled image to give an image with signal intensities proportional to the concentration of excited spins (Fig 6.11). The ROIs were superimposed on the difference images and mean intensity measures were plotted against time to produce intensity time-curves. These curves were fitted to a noncompartmental model of cerebral perfusion (Kelly et al., 2009) and two parameters were calculated: the mean

transit time (MTT) and capillary transit time (CTT) of the 'tagged blood'. The mean transit time (MTT) refers to the average time taken for labelled arterial water to traverse the vasculature (Meier and Zierler, 1954). The capillary transit time (CTT) refers to the time taken for labelled arterial water to become distributed at the region of interest (Kelly et al., 2010). The MTT and CTT were calculated from the first and second statistical moments of the signal-time curves, respectively. Changes in MTT and CTT are inversely proportional to cerebral blood flow. Relative cerebral blood volume (rCBV) was calculated by dividing the absorbance by the mean cerebral blood flow (as measured from the middle cerebral arteries).

2.18 Na-F Infusions

Sodium fluorescein (Na-F, MW 376 Da, Sigma Aldrich, UK) was dissolved in 0.89% saline, at a concentration of 0.02g/ml (Kaya and Ahishali, 2011). The solution was filtered and 1ml was injected into the tail vein of each rat while under isofluorane anesthesia (4% induction, 1.5–2% maintenance in 100% Oxygen; Abbot Laboratories, Ltd, UK). The tail vein was accessed as described for blood sampling (section 2.8.1.2). 10 minutes later, the animals were injected with urethane (1.5mg/kg i.p.) to obtain surgical anesthesia. Animals were then transcardially perfused. Whole brains were hemisected and flash-frozen in isopentane (on dry ice) and then wrapped in aluminium foil and stored at -80°C. At a later date, the brains were propped up on a cork disk and covered with OCT on dry ice until OCT turned white. On the day of sectioning, the hemisphere was allowed to sit at -20°C for 20-30 minutes. Cutting was done using a cryostat (Leica CM1900), 50μm saggittal sections were cut until the hippocampus started to appear (~150x50μm). Once the hippocampus became clearly visible, three 15μm saggittal slices were transferred onto a subbed slide and stained with 0.1% toluidine blue for 1 min and viewed by light microscopy (Nikon Labophot, Nikon Instech co., Ltd, Kanagawa, Japan) to confirm the correct position. 15μm sections were transferred onto subbed slides for fluorescent microscopy, such that 6 slides (3 sections per slide) were taken for each animal. Slides were coded and relabelled to ensure objectivity during analysis. Slides were mounted using using Vectashield® containing DAPI and a coverslip and sealed with nail varnish. Slides were immediately viewed under the fluorescent microscope. 6 views of the dentate gyrus were taken for each animal, and the mean intensity fluorescence was averaged among the 6 views. Corresponding DAPI-stained images were acquired to indicate the position of the views along the dentate gyrus.

2.19 Statistical analysis

All data were analysed in Prism (Prism GraphPad Software, La Jolla California USA) or SPSS (SPSS, Inc., Chicago IL) in the following way. For two groups and one independent variable, a Student's *t*-Test was used to assess whether the means are significantly different. For more than two groups, a one-way ANOVA with *Post-hoc* Tukey comparisons was used to analyse data with one independent variable (factor) and one dependent variable (factor), and a two-way ANOVA with Bonferroni *Post-hoc* comparisons was used to analyse data with two independent variables (factors) and one dependent variable (factor). A multivariate two-way ANOVA was used to analyse data with three independent variables (factor) and one dependent variable (factor).Additionally, post-hoc two-way ANOVAs were performed for each level of the third independent variable. A value of $p < 0.05$ (*) was considered to be significant, and $p < 0.001$ (**) was considered to be highly significant. All data are presented as mean ± S.E.M.

Chapter 3 One week of forced exercise improves object recognition learning in the adult Male Wistar rat

3.1 Introduction

Exercise is known to improve learning in both humans and animals (Radak et al., 2001, Cotman and Berchtold, 2002), and these cognitive effects have been correlated with increases in BDNF and neurogenesis in learning-related brain regions such as the dentate gyrus (van Praag et al., 1999, Vaynman and Gomez-Pinilla, 2005). However, most of the literature on this topic involves long-term (more than three weeks) of exercise, whereas improvements in cognitive performance have been observed in as little as one week of daily exercise (Vaynman et al., 2004, Griffin et al., 2009). The exact mechanisms underlying acute improvements in memory following short-term exercise have not been determined, but are thought to be mediated by rapid effects of BDNF on synaptic transmission.

In this first experiment, we aimed to test the effect of a one-week exercise program on the cognitive performance of rats, and then test blood and brain tissue for certain changes. Rats were subjected to a one-week exercise forced paradigm, and learning was assessed in both a spatial task (object displacement) and an object recognition task (object substitution). Serum levels of BDNF were monitored at regular intervals. Tissue samples from the dentate gyrus (DG), perirhinal cortex (PC), and entorhinal cortex (EC) were analysed for the presence of BDNF mRNA and protein, TrkB mRNA and IGF-1 mRNA. Finally, we tested for Ki-67 mRNA in the dentate gyrus, as a cellular marker for neurogenesis.

3.2 Methods and Study Design

Four-month old male Wistar rats were used in these experiments (n=28), and were randomly assigned to either an exercise group (n=15) or a sedentary group (n=13). Two experiments (Experiments 1 and 2) were performed on the same animals, separated by a month-long wash-out period. Each experiment consisted of an exercise protocol (days 1-7), and a learning protocol (days 7 and 8). The animals were habituated to the treadmills for two days (15 min per day) before starting the exercise protocol, and they were habituated to the open field two days (15 min per day) before starting the learning protocol (Fig 3.1). The cognitive tests used were a 3-object object

displacement task (OD) with 3x5 minutes of training (Experiment 1, Fig 3.2a), and a 3-object (OS) task with 3x5 minutes of training (Experiment 2, Fig 3.2b).

Animals were randomly assigned to 4 different experimental groups: Ex-L (exercised and learning, n=8), Ex-NoL (exercised and not learning, n=7), Sed-L (sedentary and learning, n=7), Sed-NoL (sedentary and not learning, n=6), depending on whether or not they 1) received exercise training and 2) performed the learning paradigm. Sed-NoL and Ex-NoL (non-learning groups) did not perform a cognitive task, but were placed in an empty open field for the same duration as Sed-L and Ex-L (learning groups).

Blood samples were collected at several time-points for serum analysis. 24 hours before the first bout of exercise for Experiment 1 (Baseline 1), blood samples were collected from the saphenous vein of 6 animals representing all four groups (Section 2.8.1.1). 24 hours after the last bout of exercise (Experiment 1), after the rats were tested on the object displacement task, blood samples were collected from the saphenous vein of 6 animals from each group. After the month-long washout period, 24 hours before the first bout of exercise for Experiment 2 (Baseline 2) blood samples were collected from the saphenous vein of 6 animals from each group. 24 hours after the last bout of exercise, after the rats were tested in the novel object recognition task, blood samples were collected from trunk blood of all animals upon decapitation. The brains were dissected on ice, and samples of dentate gyrus, hippocampus, perirhinal and entorhinal cortex were collected for analysis.

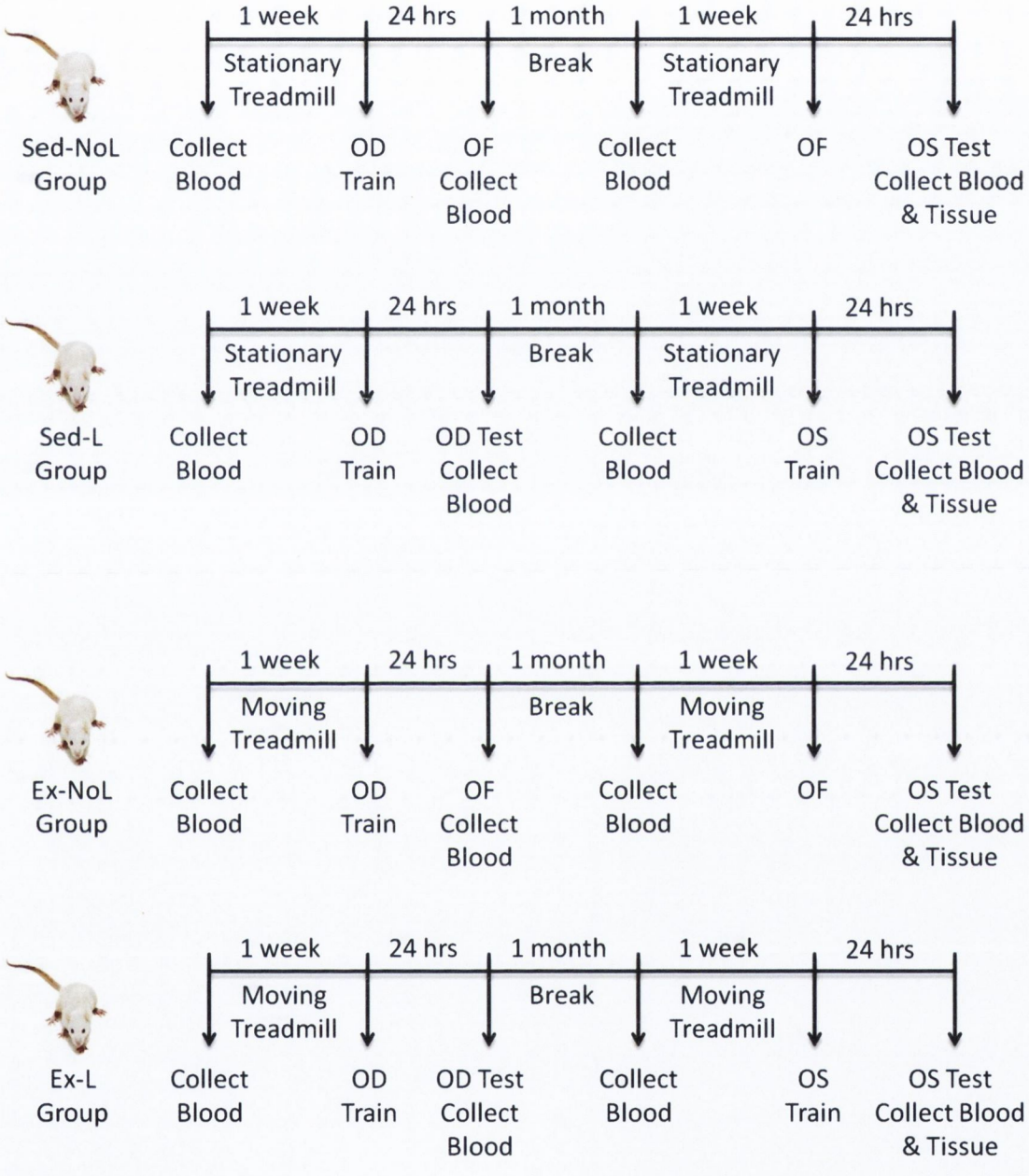
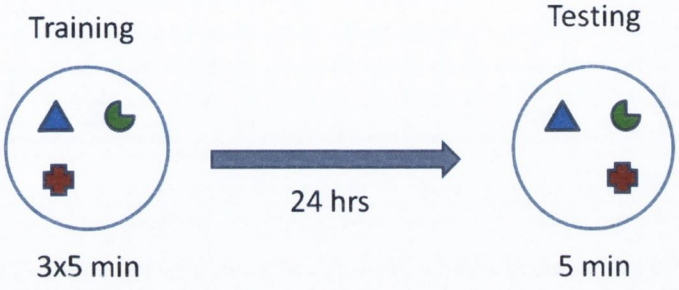


Fig 3.1 Study design for experiments in Chapter 3

Animals were divided into four groups: Sed-NoL (sedentary non-learning, n=7), Sed-L (sedentary learning, n=8), Ex-NoL (exercise non-learning, n=7), Ex-L (exercise learning, n=8). Exercised groups (Ex-NoL and Ex-L) were exercised on the rodent treadmills for one hour daily for one week at 1km/hour. Sedentary groups (Sed-NoL and Sed-L) spent the same amount of time on stationary treadmills. After the last bout of exercise, learning groups (Sed-L and Ex-L) were trained in an

object displacement (OD) task and tested 24 hours later. After the last bout of exercise, non-learning groups (Sed-NoL and Ex-NoL) were placed in an empty open field (OF) for the same time as the learning groups on both the training and testing days. After a month-long wash-out period during which no experiments were performed, the exercise and learning protocols outlined above were repeated, with an object substitution task (OS) instead of the OD task. Blood samples were collected before and after each experiment. After testing in the OS task, rats were decapitated and brain and blood samples were collected for analysis.

a) Object Displacement Task



b) Object Substitution Task

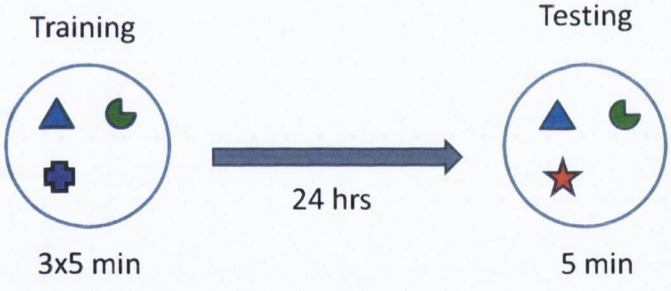


Fig 3.2 Cognitive tasks used in Chapter 3

This diagram summarises the cognitive tasks used in this chapter. Animals are allowed to explore for 3x5 minute trials (Training), and are tested 24 hours later for 5 minutes (Testing). For the object displacement task (a), the third object is displaced to a new position on the testing day. For the object substitution task (b), the third object is removed and replaced by a fourth (novel) object on the testing day.

3.3 Results

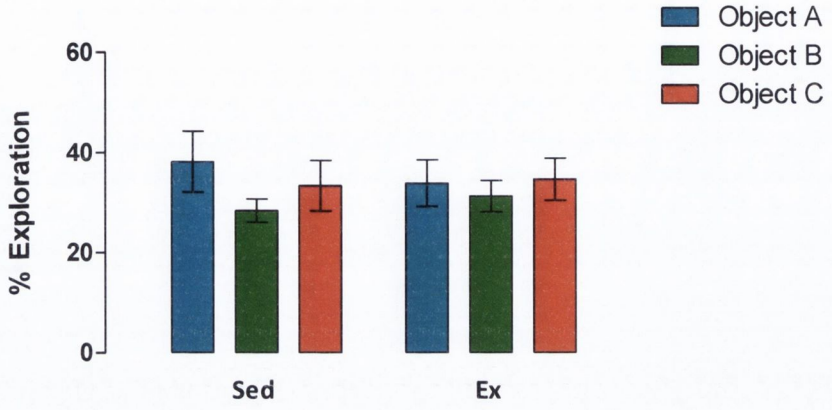
3.3.1 Both exercised rats and sedentary controls preferentially explore the displaced object in a 3-object OD task with 3x5 minutes of training

Spatial memory was tested using a 3-object object displacement task with 3x5 minute trials of exploration on the training day. Twenty-four hours later (testing day), one of the objects (C) was displaced to a new location, and animals were allowed to explore for 5 minutes. Time spent exploring each of the objects was recorded and reported as a percentage of the total exploration time, mean ± SEM.

During the training period, both exercised (Ex) and sedentary (Sed) rats spent a similar amount of time exploring each of the three objects (A, B and C), indicating that animals had no preference for any specific object (Fig 3.3a, Sed: Object A: 38.21 $\pm$ 6.09 %, Object B: 28.4 $\pm$ 2.34 %, Object C: 33.39 $\pm$ 5.05 %, Ex: Object A: 33.92 $\pm$ 4.66 %, Object B: 31.35 $\pm$ 3.15 %, Object C: 34.73 $\pm$ 4.2 %). During the testing period, both groups preferentially explored the displaced object C (Fig 3.3b, Sed: Object A: 25.44 $\pm$ 2.3 %, Object B: 21.68 $\pm$ 4.65 %, Object C: 52.88 $\pm$ 4.9 %, Ex: Object A: 29.56 $\pm$ 2.4 %, Object B: 28.76 $\pm$ 2.96 %, Object C: 41.67 $\pm$ 2.01).

A two-way ANOVA was performed with 'exercise' as the first independent variable (factor) and 'object' as the second. There was an overall effect of object ($P<0.0001$, $F_{(1,14)}=26.74$) indicating a general preference for the displaced object. *Post-hoc* analysis was performed using the Bonferroni multiple comparisons post-hoc test. *Post-hoc* analysis revealed that both the Sed group ($p<0.001$) and the Ex group ($p=0.05$) spent significantly more time exploring the displaced object C compared to stationary objects A and B. This indicates that both groups remembered the stationary objects (A and B) and could identify the displaced object C. A significant interaction ($p=0.019$) indicates that the independent variables of 'exercise' and 'object' had a significant effect on each other.

a) Training



b) Testing

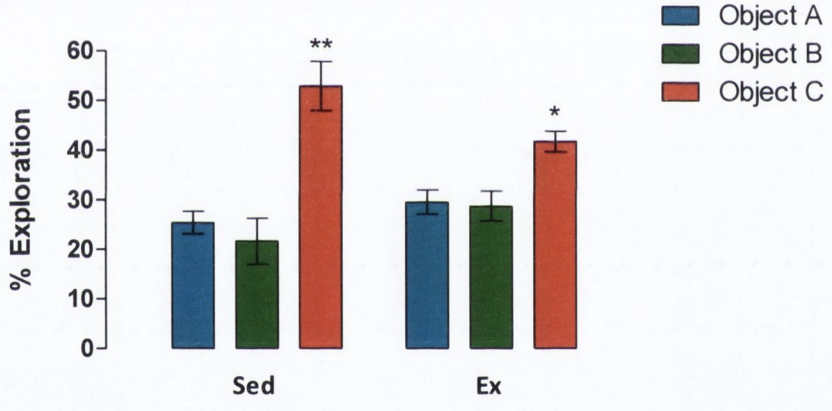


Fig 3.3 Both exercised rats and sedentary controls preferentially explore the displaced object in an object displacement task.

Data are presented as exploration time for each object (A, B or C, where C is the displaced object) as a percentage of the total exploration time. 'Sed' and 'Ex' refer to sedentary (control) group (Sed-L, n=8) and exercised group (Ex-L, n=8) respectively. On the training day (a), both groups spent a similar amount of time exploring each of the three objects (A, B and C), indicating that they had no preference for any one object. On the testing day (b), both groups preferentially explored the displaced object (C), indicating that they could remember the location of the objects and identify the displaced object (C). All data are shown as means $\pm$ SEM. The asterisk denotes data sets of significant difference as assessed by a two-way ANOVA with *post-hoc* Bonferroni comparisons. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

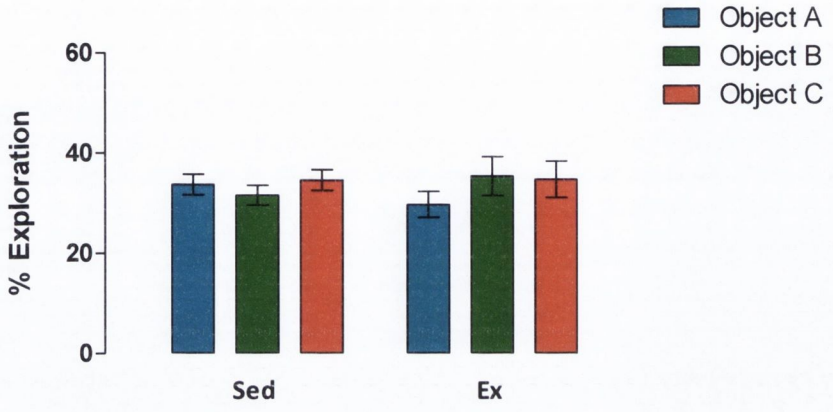
3.3.2 Exercised rats, but not sedentary controls, preferentially explore the substituted object in 3-object (OS) task with 15 minutes of training

Object recognition memory was tested using a 3-object object substitution task with 3x5 minute trials of exploration on the training day. Twenty-four hours later (testing day), one of the objects (C) was replaced by a novel object (D), and animals were allowed to explore for 5 minutes. Time spent exploring each of the objects was recorded and reported as a percentage of the total exploration time, mean ± SEM.

During the training period, both exercised (Ex, n=8) and sedentary (Sed, n=8) rats spent a similar amount of time exploring each of the three objects (A, B and C), indicating that animals had no preference for any specific object (Fig 3.4a, Sed: Object A: 33.76 $\pm$ 2.05 %, Object B: 31.6 $\pm$ 1.98 %, Object C: 34.64 $\pm$ 2.07 %, Ex: Object A: 29.78 $\pm$ 2.56 %, Object B: 35.43 $\pm$ 3.88 %, Object C: 34.79 $\pm$ 3.6 %). During the testing period, the Ex group preferentially explored the novel object D while the Sed group did not. (Fig 3.4b, Sed: Object A: 37.66 $\pm$ 3.26 %, Object B: 35.43 $\pm$ 3.88 %, Object C: 34.79 $\pm$ 3.6 %, Ex: Object A: 30.83 $\pm$ 3.9 %, Object B: 19.61 $\pm$ 4.9 %, Object C: 57.05 $\pm$ 7.9 %).

A two-way ANOVA was performed with 'exercise' as the first independent variable and 'object' as the second. *Post-hoc* analysis was performed using the Bonferroni multiple comparisons *post-hoc* test. There was an overall effect of object ($P=0.0044$, $F_{(1,14)} = 6.245$) indicating a preference for the novel object. *Post-hoc* analysis revealed that the Ex group ($p<0.001$) spent significantly more time exploring the novel object C compared to familiar objects A and B. This indicates that both groups remembered the familiar objects (A and B) and could identify the novel object C. A significant interaction ($p=0.002$) indicates that the independent variables of 'exercise' and 'object' had a significant effect on each other.

a) Training



b) Testing

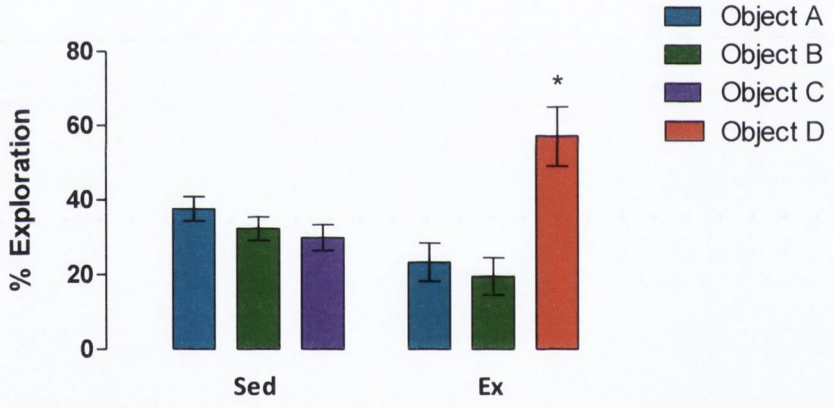


Fig 3.4 Exercised rats, but not sedentary controls, preferentially explore the novel object in an object substitution task

Data are presented as exploration time for each object (A, B ,C, or D, where D is the novel object) as a percentage of the total exploration time. 'Sed' and 'Ex' refer to sedentary (control) group (Sed, n=8) and exercised group (Ex, n=8) respectively. On the training day (a), both groups spent a similar amount of time exploring each of the three objects (A, B and C), indicating that they had no preference for any one object. On the testing day (b), the exercised group preferentially explored the novel object (D), indicating that rats could remember the familiar objects (A and B) and identify the displaced object (D). All data are shown $\pm$ SEM. The asterisk denotes data sets of significant difference as assessed by a two-way ANOVA with *post-hoc* Bonferroni comparisons. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

3.3.3 Exercise does not affect total exploration of objects

Total exploration time was summed (in seconds) on the training day for each group. A *t*-Test revealed no significant difference between groups (p=0.099, Fig 3.5, Sed Total Exploration: 107.9 ± 27.17 s, Ex Total Exploration: 171.4 ± 22.86 s).

3.3.4 Exercise induces an acute and transient increase in the concentration of BDNF protein in the serum.

Analysis of serum samples from the first experiment using a BDNF ELISA showed that exercise significantly increased the concentration of BDNF in the serum of the exercised group compared with that of the sedentary group ($F_{(2,30)}$=3.922, p=0.0307, one-way ANOVA, Fig 3.6, Baseline BDNF: 318.1 ± 81.73 pg/ml, Sed BDNF: 279.2 ± 52.97 pg/ml, Ex BDNF: 528 ± 77.34 pg/ml). A *Post-hoc* Tukey test showed that this increase was significant when compared to Sed BDNF, but not when compared to baseline.

Analysis of serum samples from the second experiment using a BDNF ELISA showed that exercise significantly increased the concentration of BDNF in the serum of the exercised group compared with that of the sedentary group ($F_{(2,30)}$=6.14, p=0.0054, one-way ANOVA, Fig 3.6, Baseline BDNF: 286.6 ± 72.56 pg/ml, Sed BDNF: 303.3 ± 66.35 pg/ml, Ex BDNF: 620.4 ± 92.06 pg/ml). A *Post-hoc* Tukey test showed that this increase was significant when compared to Sed BDNF, but not when compared to baseline.

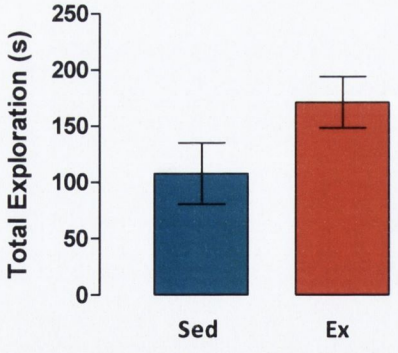


Fig 3.5 Exercise does not affect total exploration of objects

Data are presented as total exploration of objects in seconds. All data are shown ± SEM.

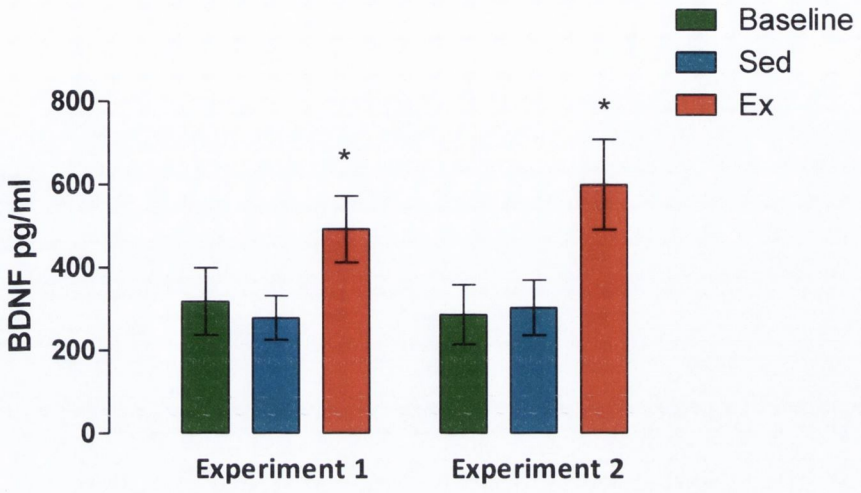


Fig 3.6 Exercise induces an acute and transient increase in the concentration of BDNF protein in the serum.

BDNF concentrations are expressed in pg per μl of serum for each group, as assessed by ELISA. The graphs depict the results for samples taken before (Baseline, n=6) and after (Sed=sedentary, n=6, Ex=exercised, n=6) the first experiment, and for samples taken before and after the second experiment. All data are shown ± SEM. The asterisk denotes data sets of significant difference as assessed by a one-way ANOVA with *post-hoc* Tukey comparisons. (*p<0.05,**p<0.01, ***p<0.001)

3.3.5 Exercise induces an increase in BDNF mRNA expression in the dentate gyrus, but not in the perirhinal cortex and entorhinal cortex.

In the dentate gyrus, BDNF mRNA expression was significantly increased in exercised rats compared to sedentary rats (p=0.0013, Student's *t*-Test, Fig 3.7a, Sed: 1 ± 0.11, Ex fold change: 1.82 ± 0.2) as evaluated by PCR. The Ex-L group showed a significant increase in BDNF mRNA compared to the other groups ($F_{(2,30)}$=3.888, p=0.0115, one way ANOVA, Fig 3.7b, Sed-NoL: 1 ± 0.22, Sed-L BDNF fold change: 0.89 ± 0.1, Ex-NoL BDNF fold change: 1.2 ± 0.22, Ex-L BDNF fold change: 1.98 ± 0.19). A *Post-hoc* Tukey test showed that Ex-L BDNF mRNA was significantly elevated compared to Sed-NoL and Sed-L but not Ex-NoL. In the perirhinal cortex, no differences were observed for BDNF mRNA between sedentary and exercised groups (Fig 3.7a, Sed BDNF: 1 ± 0.16, Ex BDNF fold change: 0.85 ± 1.7). In the entorhinal cortex, no differences

were observed for BDNF mRNA between sedentary and exercised groups (Fig 3.7b, Sed BDNF: 1 ± 0.46, Ex BDNF fold change: 1.1 ± 0.2).

3.3.6 Exercise induces an increase in TrkB mRNA expression in the dentate gyrus and perirhinal cortex, but not in the entorhinal cortex

In the dentate gyrus, TrkB mRNA expression was significantly increased in exercised rats compared to sedentary rats ($p=0.0327$, Student's *t*-Test, Fig 3.8a, Sed TrkB: 1 ± 0.11, Ex TrkB fold change: 2.37 ± 0.52). In the perirhinal cortex, TrkB mRNA expression was significantly increased in exercised rats compared to sedentary rats ($p=0.0406$, Student's *t*-Test, Fig 3.8b, Sed TrkB: 1 ± 0.12, Ex TrkB fold change: 1.5 ± 0.22). In the entorhinal cortex, no significant differences were observed for TrkB mRNA expression between sedentary and exercised groups (Fig 3.8c, Sed TrkB: 1 ± 0.17, Ex TrkB fold change: 1 ± 0.23).

3.3.7 Exercise induces an increase in IGF-1 mRNA expression in the dentate gyrus and entorhinal cortex, but not in the perirhinal cortex

In the dentate gyrus, IGF-1 mRNA expression was significantly increased in exercised rats compared to sedentary rats ($p=0.035$, Student's *t*-Test, Fig 3.9a, Sed IGF-1: 1 ± 0.31, Ex IGF-1 fold change: 2.4 ± 0.52). In the perirhinal cortex, IGF-1 mRNA expression did not differ between exercised and sedentary rats (Fig 3.9b, Sed IGF-1: 1 ± 0.17, Ex IGF-1 fold change: 1 ± 0.23). In the entorhinal cortex, IGF-1 mRNA expression was significantly increased in exercised rats compared to sedentary rats ($p=0.027$, Student's *t*-Test, Fig 3.9c, Sed IGF-1: 1 ± 0.14, Ex IGF-1 fold change: 1.52 ± 1.16).

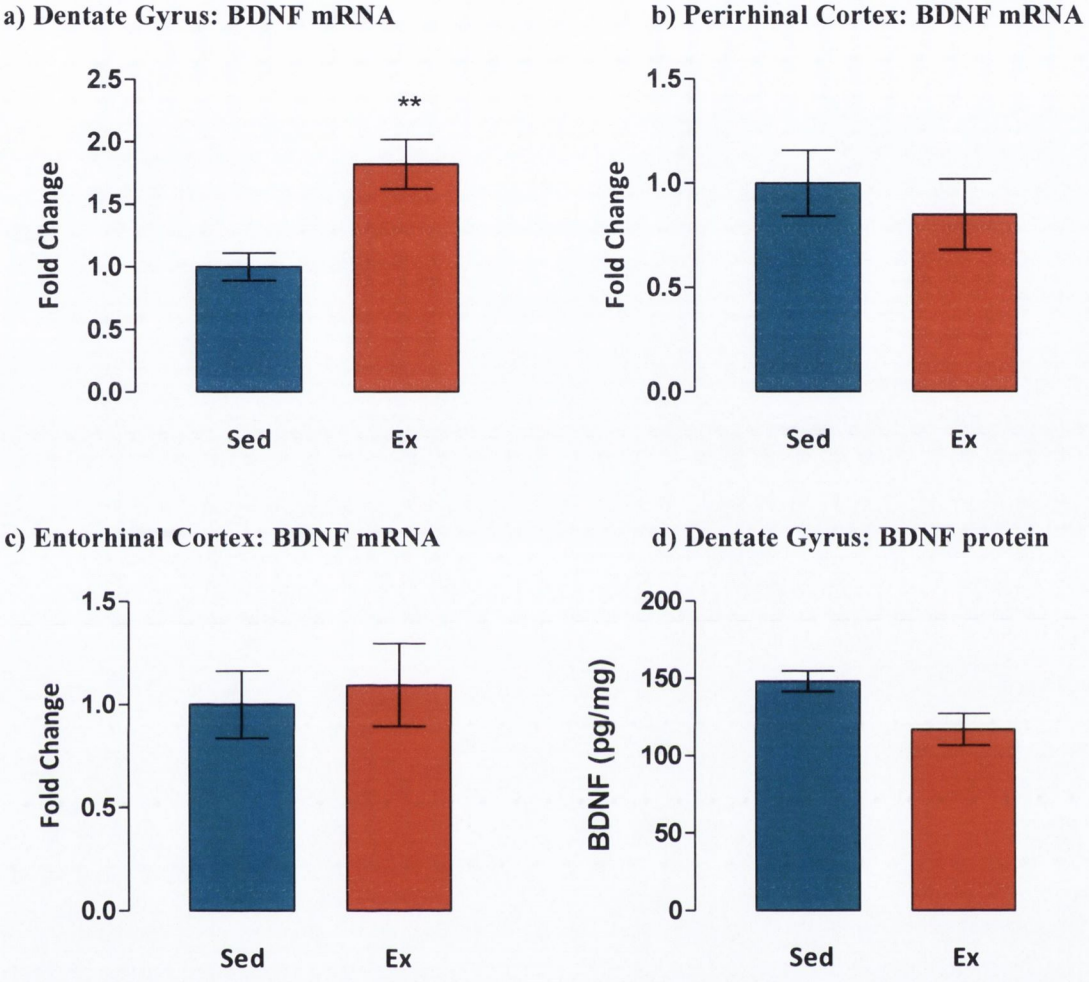


Fig 3.7 Exercise induces an increase in BDNF mRNA expression in the dentate gyrus, but not in the perirhinal and entorhinal cortex.

This graph shows the fold changes in BDNF mRNA in (a) dentate gyrus, (b) perirhinal cortex, and (c) entorhinal cortex as assessed by PCR. BDNF mRNA in the dentate gyrus is elevated in the exercised group (Ex, n=6) relative to sedentary controls (Sed, n=6). All mRNA data are expressed as fold change ± SEM. No changes in BDNF protein were observed across groups or brain regions when normalised to total protein, as assessed by an ELISA. All protein data are expressed in pg/mg protein ± SEM._The asterisk denotes data sets of significant difference as assessed by a one-way ANOVA. . (* $p<0.05$)

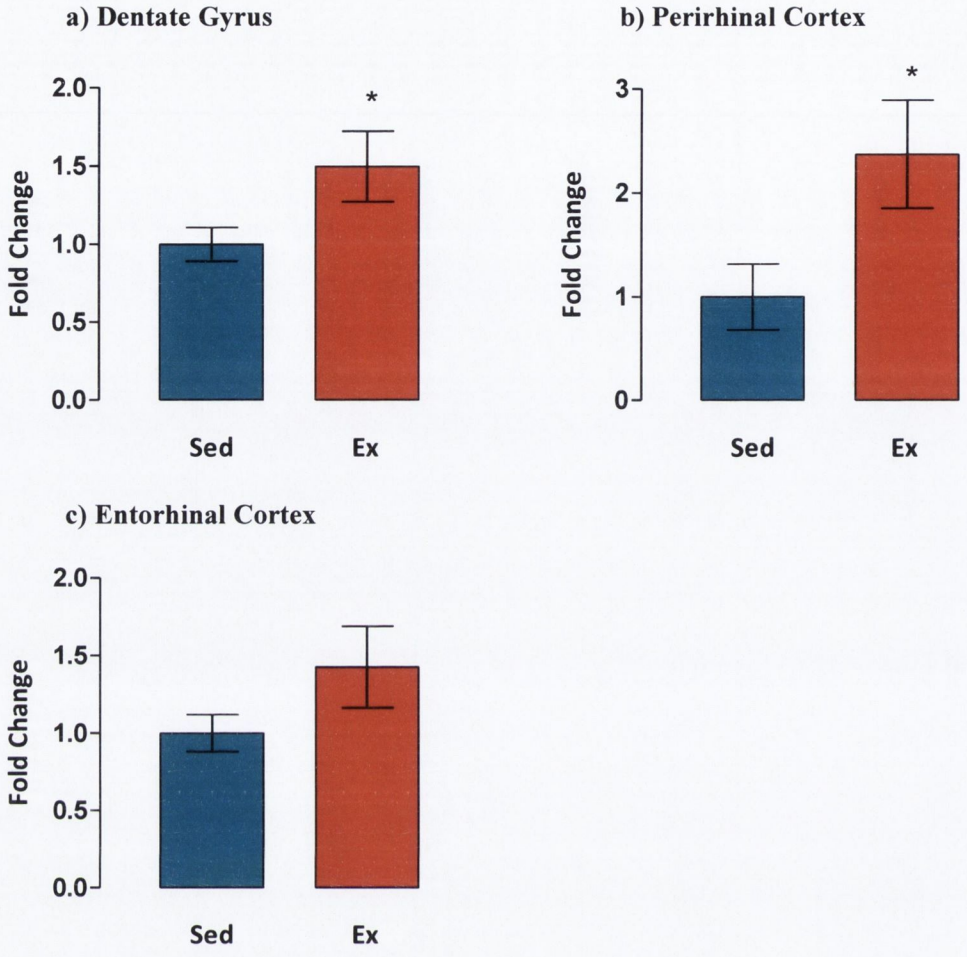


Fig 3.8 Exercise induces an increase in TrkB mRNA in the dentate gyrus and perirhinal cortex, but not in the entorhinal cortex.

This graph shows the fold change in TrkB mRNA in (a) dentate gyrus, (b) perirhinal cortex, and (c) entorhinal cortex as assessed by PCR. TrkB mRNA is elevated in both the dentate gyrus and perirhinal cortex in the exercised group (Ex, n=6) relative to sedentary controls (Sed, n=6). No significant change is observed in the entorhinal cortex. All data are expressed as fold change $\pm$ SEM. The asterisk denotes data sets of significant difference as assessed by a Student's *t*-Test (*$p<0.05$).

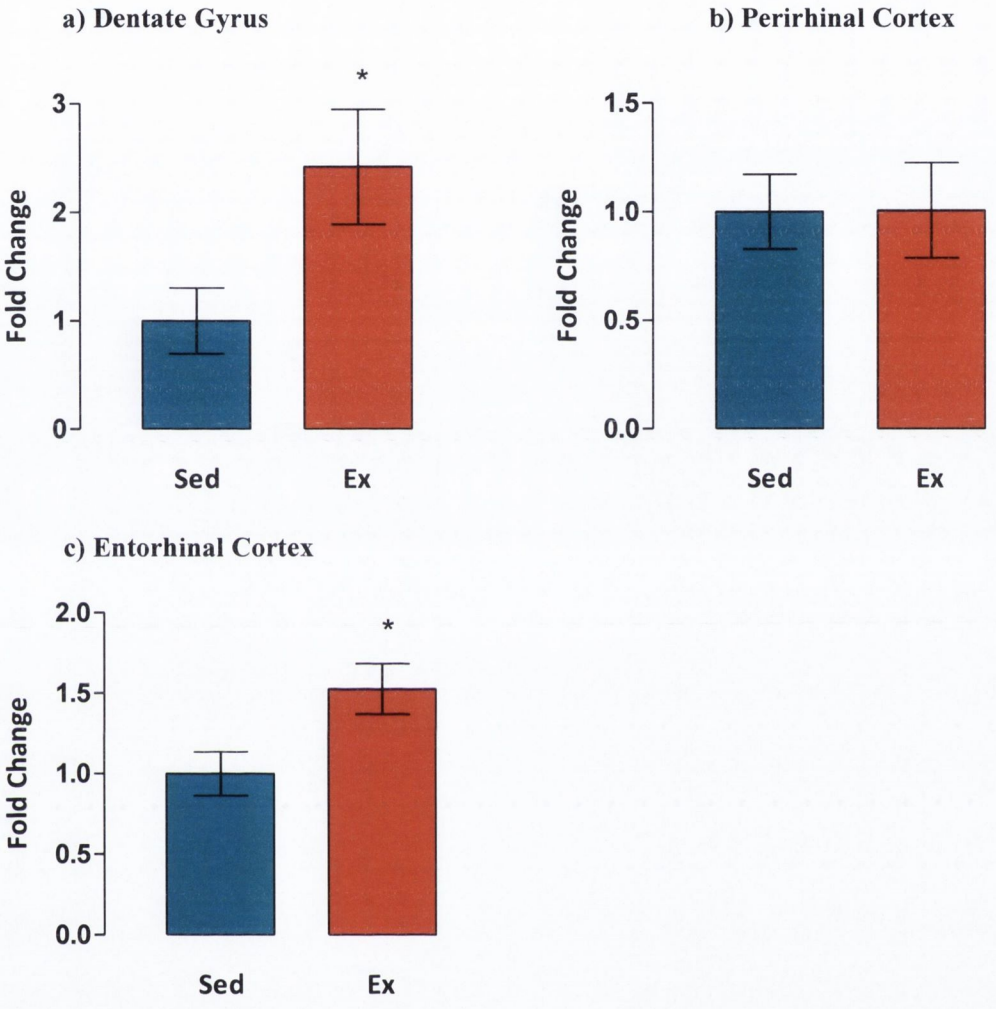


Fig 3.9 Exercise induces an increase in IGF-1 in the dentate gyrus and entorhinal cortex but not in the perirhinal cortex.

This graph shows the fold change in IGF-1 mRNA in (a) dentate gyrus, (b) perirhinal cortex, and (c) entorhinal cortex as assessed by PCR. IGF-1 mRNA is elevated in dentate gyrus (a) and entorhinal cortex (c) of Ex rats (n=6) compared to Sed rats (n=6). No changes were observed in the perirhinal cortex. All data are expressed as fold change± SEM. The asterisk denotes data sets of significant difference as assessed by a Student's *t*-Test.

3.3.8 Exercise has no effect on KI-67 mRNA expression in the dentate gyrus

In the dentate gyrus, KI-67 mRNA expression was not significantly increased in exercised rats compared to sedentary controls (p=0.087, Student's *t*-Test, Fig 3.10, Sed IGF-1: 1 $\pm$ 0.2, Ex IGF-1 fold change: 1.8 $\pm$ 0.38).

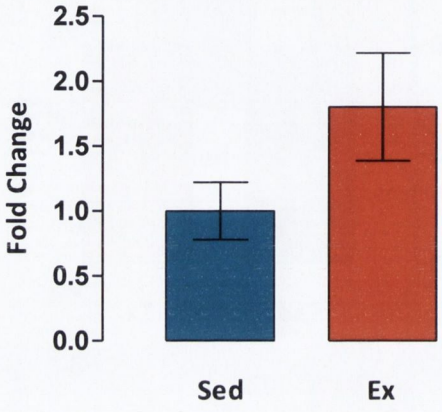


Fig 3.10 Exercise does not affect KI-67 mRNA expression in the dentate gyrus

This graph shows the fold change in KI-67 mRNA in exercised rats (Ex, n=6) compared to sedentary controls (Sed, n=6) in the dentate gyrus (DG) as assessed by PCR. The difference is not significant (p=0.088) as evaluated by a Student's *t*-test. All data are expressed as fold change$\pm$ SEM.

3.4 Discussion

3.4.1 Behavioural Analysis

The data generated in this study show that a 7-day exercise program enhanced the rats' performance in the object substitution task, but not in the object displacement task. These two tasks are thought to enlist two separate forms of learning. Object displacement requires the rat to remember the original position of the object using spatial memory, a hippocampus-dependent type of learning. Both groups performed very well in this task regardless of whether or not they had exercised. This raises the possibility that the level of difficulty of the task was low, and a ceiling effect occurred whereby interventions such as exercise cannot improve performance in the task. The object substitution task on the other hand (Kelly et al., 2003), requires a different type of memory: object recognition memory. This task is thought to depend on both the hippocampus and the perirhinal cortex (Broadbent et al., 2004, Buckley, 2005). The rat has to remember the familiar objects and identify the novel object, and indicate successful discrimination of this difference by preferentially exploring the novel object. Sedentary rats explored all objects equally on the testing day, indicating that they could not remember the familiar objects and identify the novel object. Exercised rats however preferentially explored the novel object, indicating that they could remember the familiar objects. This corroborates previous studies where exercise was shown to enhance object recognition learning in a similar task to ours, but not spatial learning in the Morris Water maze (O'Callaghan et al., 2007).

In previous studies in our laboratory, sedentary rats did preferentially explore the novel object in an object substitution task (O'Callaghan et al., 2007). In our study, the sedentary rats did not preferentially explore the novel object. The reason for this discrepancy could lie in the level of difficulty of the tasks. In previous studies, the object substitution task was performed with only two objects, where one of these two familiar objects was substituted for a third on testing day. In this study, the task was performed with three objects, with one of them substituted for a fourth on testing day. This suggests that increasing the number of objects in such a task would increase the difficulty of the task by increasing the cognitive load.

In the literature, most studies that have reported exercise-induced enhancements in spatial learning show that it reverses an existing cognitive deficit due to ageing (Radak et al., 2001, Albeck et al., 2006) or an experimental insult (Christie et al., 2005, Gobbo and O'Mara, 2005). Very few studies have reported exercise-induced improvements in spatial memory in healthy young adult rats (Vaynman 2005: 1-week voluntary, MWM, Van der Borght 2007: 2 week forced, Y-maze). Taken together with our results, this suggests that the difficulty of a task needs to be taken into account when assessing the effect of exercise on cognitive performance. It may be the case that rats are simply inherently better at tasks that require spatial learning than object recognition, when similar tasks are presented. Factors such as the number of objects, amount of training exploratory time, time delay between training and testing, and external spatial cues, could contribute to defining the level of difficulty of a cognitive task within this experimental setup. The way to test this hypothesis would be to design an experiment in which the spatial task is made more challenging, perhaps by increasing the number of objects, decreasing the amount of training exploratory time, increasing the delay between training and testing, or removing some of the external spatial cues.

3.4.2 BDNF Analysis

Our 7-day exercise program resulted in acute increases in BDNF protein in the serum. This increase was observed after both experiments. In addition, differences within the four groups were observed. The group that had exercised and performed the learning task (Ex-L) showed the highest amounts of serum BDNF in both experiments. This could represent a composite effect of both learning and exercise, which would indicate that that BDNF is initially being upregulated in the brain, and then crossing the blood-brain barrier to enter the circulation. When comparing serum BDNF levels of experiment 1 to those of experiment 2, we notice that they returned to baseline after the month-long break. This indicates that exercise exerts immediate, acute and transient effects on peripheral BDNF. However, a closer look at the exercised levels from the two experiments shows a slight increase. A similar effect has been reported before in the hippocampus (Berchtold et al., 2005), and is thought to prove that exercise forms a molecular memory for BDNF that remains long after the exercise training has ceased. Similarly in our study, the experience of exercise could have primed the animal's system for a future BDNF response.

Tissue analysis revealed an increase in BDNF mRNA in the dentate gyrus. Interestingly, individual groups showed differences in BDNF mRNA; the group that had exercised and learned (Ex-L) had significantly higher amounts than the three remaining groups (Ex-NoL, Sed-L, and Sed-NoL). The

transcription of BDNF, as discussed above, is activity-dependent, and learning has been shown to increase BDNF in the brain (Chen et al., 2010). Taken together with the results of the serum BDNF analysis, these results indicate that both peripheral and central BDNF increases are exercise-linked and learning-linked. The similarities observed between the central and peripheral BDNF responses also suggest that they have a common source which until recently has been thought to be the hippocampus (Rasmussen et al., 2009). Another possibility could be that the BDNF is being released into the periphery as a direct result of exercise, and is somehow crossing the blood-brain barrier into the dentate gyrus. Since BDNF has been shown to upregulate its own transcription via the ERK pathway (Saarelainen et al., 2001), a peripheral increase in BDNF could be translated into a central one, provided BDNF crosses the blood-brain barrier.

Analysis of BDNF protein in the dentate gyrus did not show an increase, a finding that contradicts previous results (Griffin et al., 2009). However, it is important to take the time scale of BDNF gene expression, transcription, and translation to protein into consideration (Adlard et al., 2004). It is possible that we would have detected more BDNF protein at a later time-point, after the mRNA we did detect has been translated to protein.

3.4.3 TrkB Analysis

TrkB receptor mRNA was found to be significantly increased in both the dentate gyrus and the perirhinal cortex. Upregulation of the TrkB receptor has been reported to occur with exercise ((Liu et al., 2008) and to improve learning (Koponen et al., 2004), and could be one of the mechanisms by which exercise facilitates BDNF-TrkB signalling, and as a result, enhances cognition. The upregulation and targeting of TrkB (like BDNF) in hippocampal neurons has been shown to be activity-dependent: neurons that are frequently depolarised were shown to have higher amounts of TrkB mRNA (Tongiorgi et al., 1997, Nagappan and Lu, 2005). Also, BDNF has been shown to induce the transcription and accumulation of TrkB receptors in hippocampal neurons (Righi et al., 2000). This indicates that the effect of exercise on TrkB could be an indirect result of the increase in BDNF, and the subsequent synaptic facilitation.

Since we performed the spatial and recognition tasks in succession on the same animals, and later performed the neurochemical analysis, it is impossible to discern which effects correspond to each particular task. One hypothesis would be that the increases in TrkB mRNA observed in the

perirhinal cortex occur only when the animal performs the object recognition task and not the spatial variant. This hypothesis is investigated in the next chapters.

3.4.4 IGF-1 Analysis

A significant increase in IGF-1 was observed in the dentate, and entorhinal cortex, but not in the perirhinal cortex. The recent literature suggests that IGF-1 plays an important role in modulating the effects of exercise on spatial learning and adult hippocampal neurogenesis (AHN). Exercise has been shown to increase the amount of IGF-1 in the serum (Llorens-Martin et al., 2008), and IGF-1 has been shown to cross the blood-brain barrier using a saturable transport system (Pan and Kastin, 2000). It has also been suggested that the reuptake of IGF-1 from the blood into the brain is necessary for its increased expression in the CNS (Ding et al., 2006a). In the study presented here, we observed in an increase in IGF-1 mRNA in the CNS in two brain regions, which suggests a central source for IGF-1. The mechanism and significance of exercise-induced upregulation of the IGF-1 gene in the brain is unclear. Further studies are required to elucidate the interface of BDNF and IGF-1 at the cellular level, and the observed pattern of increased expression.

3.4.5 Cell Proliferation Analysis

Finally, we observed no significant changes in KI-67 mRNA in the dentate gyrus. KI-67 is a nuclear protein with a short half-life, expressed in dividing cells during mitosis. This makes it a reliable marker for cellular proliferation, which occurs during the first stages of neurogenesis (Kee et al., 2002). Exercise has been shown to consistently increase neurogenesis in the dentate gyrus (van Praag et al., 1999, Lista and Sorrentino, 2009) in previous studies (most of these studies used BrdU, the classical thymidine analog used for the detection of neurogenesis). In our case, the exercise regimen was much shorter (7-days) which could account for the fact that we did not detect major differences in KI-67. Furthermore, it would be interesting see whether one week of forced moderate exercise is enough to cause an increase in cellular proliferation in the dentate gyrus as detected by BrdU immunohistochemistry. This question is investigated in Chapters 4 and 5. Neurogenesis in the dentate gyrus has been repeatedly linked to exercise-induced improvements in learning (Ernst et al., 2006, Leuner et al., 2006), although the exact functional relevance of the new hippocampal neurons remains debated (Ehninger and Kempermann, 2008). Cellular changes induced by our 7-day exercise protocol are investigated and discussed in greater detail in the next chapter.

3.5 Summary

We have shown that one week of moderate forced treadmill running improves cognitive performance of adult male Wistar rats in an object recognition task, but not in a spatial variant of the task. This improvement is accompanied by an acute and transient increase in BDNF protein in the serum, and by the selective upregulation of BDNF, TrkB and IGF-1 genes in the dentate gyrus, perirhinal cortex and entorhinal cortex. These data are consistent with other reports in the literature that short-term exercise can enhance learning in the rat via mechanisms involving BDNF expression and signalling.

Chapter 4 Exercise improves object recognition memory and induces BDNF expression and cell proliferation in environmentally enriched rats

4.1 Introduction

Environmental enrichment (Pham et al., 1999b, Brillaud et al., 2005) and exercise are two behavioural interventions that have been shown consistently to improve cognition in both spatial (Schrijver et al., 2004) and object recognition tasks (Leal-Galicia et al., 2008). Typically, these two interventions are combined by including a running wheel in the cages of enriched animals, in addition to the toys, nest boxes, tunnels, and special bedding that are traditionally used to create an enriched environment (Bennett et al., 1969). Studies in our lab (Birch et al, *in preparation*) indicate that environmental enrichment without running wheels ('cognitive' enrichment), can still confer cognitive benefits in healthy young adult rats, and protect against age-related cognitive decline.

The beneficial effects of exercise on cognition and neurogenesis have been challenged by several suggestions. The first suggestion stems from the observation that laboratory animals are physically and cognitively deprived (Martin et al., 2010) compared to wild animals, since housing conditions in the laboratory facility provide very little stimulation. Thus, it can be argued that exposing the animals to a new environment (the treadmill) and a new activity (the running) in itself could be stimulating the animals cognitively, such that it is not the physical activity itself but the cognitive stimulation that is causing the observed cognitive enhancement. To tackle this question, we environmentally enriched rats for a short period of time, and tested whether exercise conferred an additional advantage at the behavioural level, and whether it induced changes at the cellular, and molecular levels compared to animals that had only been enriched, exercised, or neither.

In addition, this experiment allows us to answer some questions regarding the physical and cognitive components of environmental enrichment. Typically, environmental enrichment paradigms include running wheels (physical enrichment), and larger numbers of animals per cage (social enrichment), in addition to toys, nest-boxes, and special bedding (cognitive enrichment). It is important to separate out which effects are related to 'physical' and 'social' enrichment, and which are specific to 'cognitive' enrichment (van Praag, 2008, Bekinschtein et al., 2011). The recent literature suggests that the beneficial effects of environmental enrichment on cognition and

neurogenesis can be attributed exclusively to the 'physical enrichment' component (Kobilo et al., 2011).

The final question is whether stress could be playing a role in the observed exercise-induced cognitive enhancement. Running (forced running in particular) is a stressful activity, especially when electric shockers are used to motivate the animals to run. The literature on the effects of stress and cognition is vast and at times contradictory, but in general it is thought that mild, acute stress has a positive effect on learning and hippocampal plasticity (de Kloet et al., 1999) and that high intensity and particularly chronic stress has detrimental effects (Kim and Haller, 2007). It could be suggested that the electric shocks are providing a mild source of stress that could be stimulating cognitive enhancement. In addition, it could also be argued that forced exercise in itself is mildly stressful on the rats, and that it is the mild stressor, and not the exercise that is inducing the cognitive enhancement and the neurochemical changes.

In this chapter, two experiments were designed to answer these questions. The first experiment aims to separate out and then recombine the effects of short-term physical and cognitive enrichment in young adult rats. In addition, it tests whether the cognitive and neurochemical effects of exercise are diminished in environmentally enriched rats, in comparison to standard-housed laboratory rats. To avoid confusion, we will refer to our environmental enrichment paradigm as 'environmental enrichment' (or simply enrichment) and not 'cognitive enrichment', even though it contains only the cognitive component.

The second experiment investigates the role of stress in our exercise and enrichment paradigms, particularly whether the electric shockers used to motivate the rats to run on the treadmill have an effect on anxiety behaviour, exploratory behaviour, cognitive performance, neurochemistry, and levels of the stress hormone corticosterone in the blood (de Kloet et al., 2005).

4.2 Methods and Study Design

Three-month old Wistar rats were randomly assigned to one of four groups: Sed-SH (sedentary standard-housed, n=9), Sed-En (sedentary enriched, n=9), Ex-SH (exercised standard-housed, n=9), and Ex-En (exercised enriched, n=12). Animals from enriched groups (Sed-En, Ex-En) were housed in an enriched environment for three weeks, while standard-housed groups (Sed-SH, Ex-SH) were housed in a standard environment. After a three-day habituation period to the treadmill, Animals from exercised groups (Ex-En, Ex-SH) were exercised for one hour daily at 1 km/hr for one week on a rodent treadmill, while sedentary animals (Sed-SH, Sed-En) were placed for the same amount of time on a stationary treadmill. The week of exercise overlapped with the third week of enrichment, during which all animals were given daily BrdU injections (50mg/kg, i.p.). The protocols for environmental enrichment and exercise are detailed in Chapter 2. On the last day of exercise, enrichment, or the combination (Fig 4.1), all animals were tested for general locomotor activity and exploratory behaviour in an open field.

On the last day of treatment, all rats were trained in a 3-object OS test with 2x5 minutes of training, and tested 24 hours later (Fig 4.2). Immediately after testing, animals were euthanised by stunning and decapitation. Trunk blood was collected for serum analysis, and the brain was hemisected on ice. The right hemisphere was flash frozen in liquid nitrogen for sectioning. The left hemisphere was dissected further, and tissue samples from the dentate gyrus, perirhinal cortex, and hippocampus were collected for neurochemical analysis.

To rule out the effect of the electric shocks on behaviour and neurochemistry, we conducted a small parallel experiment in which rats (n=5) were placed on the shocker-part of the treadmill for 1 hour a day for seven consecutive days (with the rest of the treadmill blocked off), and were shocked at a rate of one low-intensity shock every 5 minutes to roughly mimic the amount of shocking typically experienced by an exercising animal. The same behavioural tests (open-field, object substitution) were conducted on ES animals. Trunk blood was collected for serum analysis, and the brain was hemisected. The right hemisphere was flash frozen in liquid nitrogen for sectioning. The left hemisphere was dissected further, and tissue samples from the dentate gyrus, perirhinal cortex, and hippocampus were collected for neurochemical analysis.

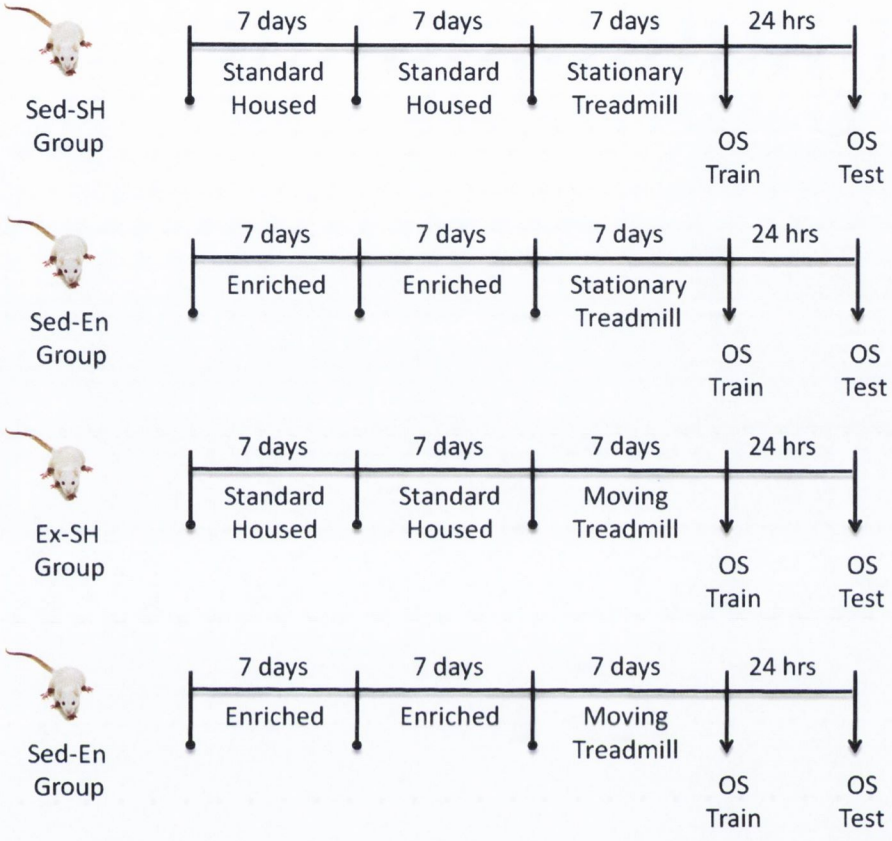


Fig 4.1 Study design for experiments in Chapter 4

Animals were divided into four groups: Sed-SH (sedentary standard-housed, n=9), Sed-En (sedentary enriched, n=9), Ex-SH (exercised standard-housed, n=9), and Ex-En (exercised enriched, n=12). Animals from enriched groups (Sed-En, Ex-En) were housed in an enriched environment for three weeks, while standard-housed groups (Sed-SH, Ex-SH) were housed in a standard environment. Animals from exercised groups (Ex-En, Ex-SH) were exercised for one hour daily at 1 km/hr for one week on a rodent treadmill, while sedentary animals (Sed-SH, Sed-En) were placed for the same amount of time on a stationary treadmill. The week of exercise overlapped with the third week of enrichment, during which all animals were given daily BrdU injections (50mg/kg, i.p.). All animals were trained in an object substitution task (OS) at the end of the three weeks (object substitution), and tested 24 hours later. After testing, animals were decapitated and tissue and blood samples were collected for analysis.

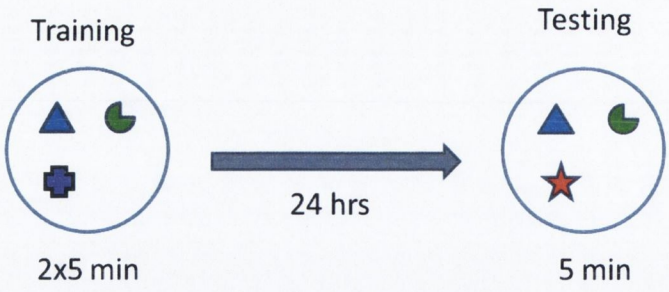


Fig 4.2 Cognitive task used in Chapter 4

This diagram summarises the cognitive task used in this chapter. Animals are allowed to explore for 2x5 minute trials on the training day (Training), and are tested 24 hours later for 5 minutes (Testing) after one of the objects has been replaced by a novel object.

4.3 Results

4.3.1 Exercise, enrichment, and the combination have no effect on open field behaviour

All four groups (Sed-SH, Sed-En, Ex-SH, Ex-En) performed similarly in the open field test, with no significant changes observed in % time spent in periphery (Fig 4.3, Sed-SH: 63.66 $\pm$ 4.62 %, Sed-En: 52.24 $\pm$ 2.93 %, Ex-SH: 57.36 $\pm$ 6.73 %, Ex-En: 68.24 $\pm$ 4.12 %) as assessed by a two-way ANOVA.

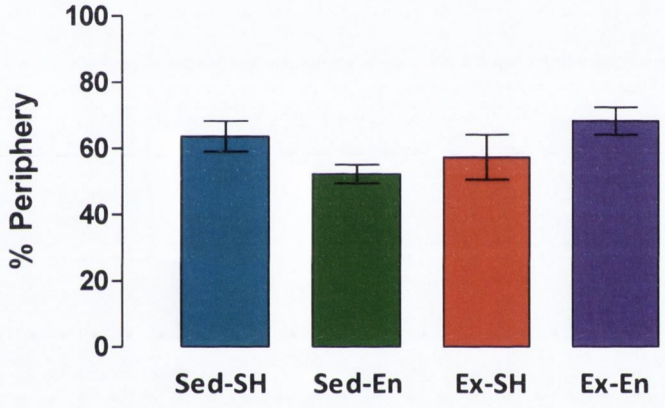


Fig 4.3 Exercise, enrichment, and combination have no effect on anxiety behaviour in an open-field test.

All animals (n=9-12 per group) exhibited similar behaviour in an open-field anxiety-test in which the time spent in the periphery is taken as a percentage of the total time spent in the open field. All data are presented as % time spent in the periphery $\pm$ SEM. No differences were present as evaluated by a two-way ANOVA.

4.3.2 Exercise, enrichment, and the combination have no effect on total exploration time

When total exploration time was summed (in seconds) on the training day for each group, analysis revealed no significant difference between groups (Fig 4.4, Sed-SH: 257 ± 19.33 s, Sed-En: 215.8 ± 29.06 s, Ex-SH: 210.5 ± 22.02 s, Ex-En: 217.3 ± 17.91 s) as assessed by a two-way ANOVA.

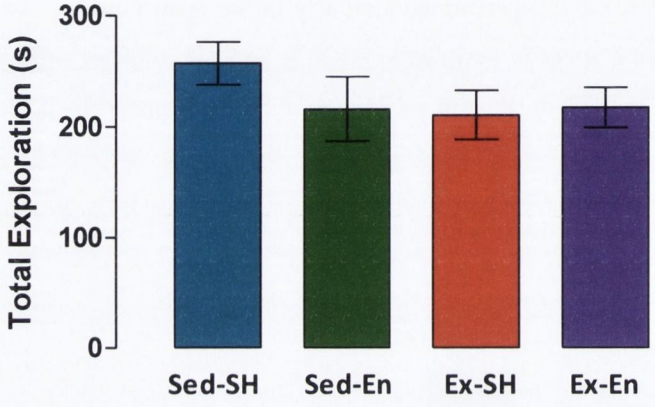


Fig 4.4 Exercise, enrichment, and combination have no effect on total exploratory behaviour. All animals (n=9-12 per group) explored similarly on training day. Data are presented as total exploration of objects in seconds. All data are shown ± SEMs. No differences were present as evaluated by a two-way ANOVA.

4.3.3 Exercised rats preferentially explore the novel object in a 3-object OS task with 2x5 minutes of training

Object recognition learning was tested with a 3-object object substitution task (3rd object is substituted by a 4rth novel object) with 2x5 minutes of training (Fig 4.2).

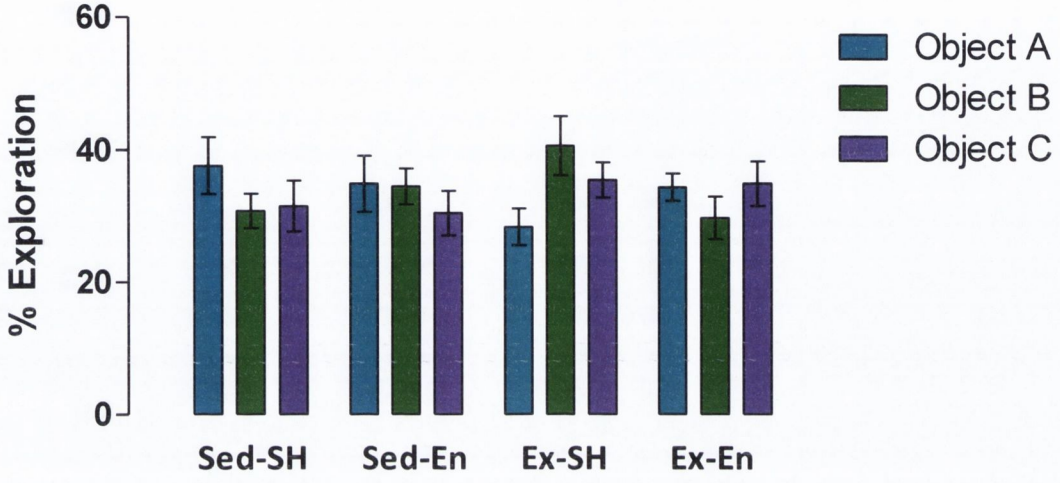
During the training period, time spent exploring each of the objects was recorded and reported as a percentage of the total exploration time, mean ± SEM. (Fig 4.5a, Sed-SH: Object A: 34.36 ± 2.94 %, Object B: 32.12 ± 2.17 %, Object C: 33.52 ± 3.36 %; Sed-En: Object A: 34.10 ± 3.03 %, Object B: 34.50 ± 1.8 %, Object C: 31.40 ± 2.34 %; Ex-SH: Object A: 33.07 ± 3.89 %, Object B: 31.64 ± 2.2 %, Object C: 35.29 ± 3.79 %; Ex-En: Object A: 33.20 ± 1.63 %, Object B: 33.53 ± 2.86 %, Object C: 33.27 ± 1.7 %).

During the testing period, time spent exploring each of the objects was recorded and reported as a percentage of the total exploration time, mean ± SEM. (Fig 4.5b, Sed-SH: Object A: 37.11 ± 4.01 %, Object B: 31.35 ± 3.95 %, Object D: 31.54 ± 3.2 %; Sed-En: Object A: 34.40 ± 3.03 %, = Object B: 28.75 ± 2.56 %, =Object D: 40.46 ± 10.66 %,=; Ex-SH: Object A: 27.89 ± 3.16 %, Object B: 29.72 ± 2.45 %, Object D: 42.39 ± 3.76 %; Ex-En: Object A: 22.86 ± 1.6 %, Object B: 25.21 + 2.1 %, Object D: 51.92 ± 2.86 %).

A multivariate two-way ANOVA (MANOVA) was performed in SPSS with three independent variables: exercise (2 levels: sedentary, exercise), enrichment (2 levels: standard-housed, enriched), and object (3 levels: A,B,C or A,B,D), and one dependent variable: exploration time. On training day, none of the independent variables had an effect on exploration time. On testing day, there was a main effect of exercise ($F_{(3,105)} = 9.448$, $p=0.004$) on the exploration of object D, indicating that exercised groups could identify the novel object and preferentially explored it. There was no effect of enrichment and no interaction. *Post-hoc* analysis was performed by conducting a two-way ANOVA with two independent variables: exercise (2 levels: sedentary, exercise), enrichment (2 levels: standard-housed, enriched), and one dependent variable: exploration time of each object. During the training period, there were no main effects for either of the independent variables and no interaction for any of the groups or any of the objects. This indicates that all groups (Sed-SH, Sed-En, Ex-SH, Ex-En) spent a similar amount of time exploring each of the three objects (A, B and C) on training day, and that animals had no preference for any specific object. During the testing period, there were no main effects for either of the independent variables and no interaction

for any of the groups for objects A and B. However, for object D, there was a main effect of exercise and a main effect of enrichment (two-way ANOVA, effect of enrichment: $F_{(3,37)}=7.64$, $p=0.009$, effect of exercise: $F_{(3,37)}=11.7$, $p=0.002$). There was no interaction.

(a)



(b)

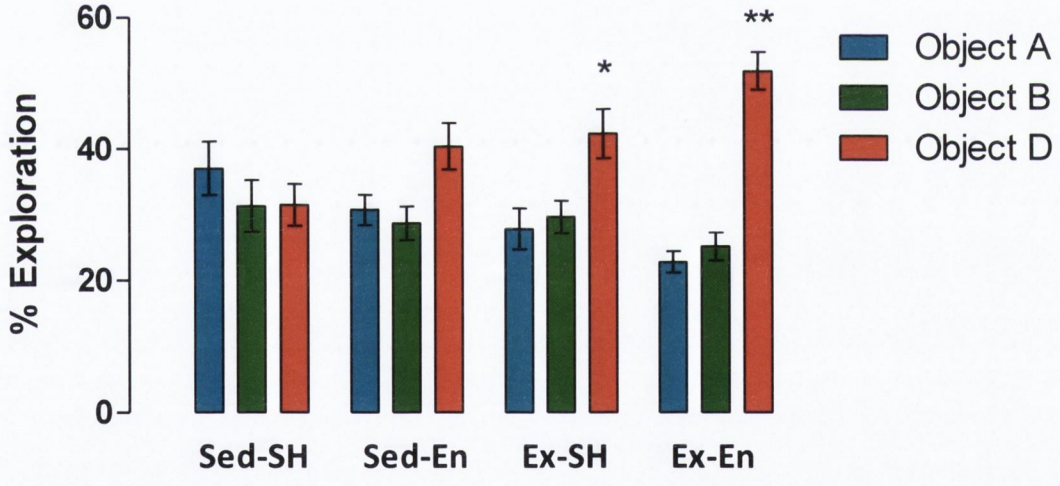


Fig 4.5 Exercised rats preferentially explore novel object in an OS task

Data are presented as exploration time for each object (A, B or C, where object B is replaced by D on the testing day) as a percentage of the total exploration time. On the training day (a), all groups (n=9-12 per group) explored all three objects to a similar degree. On the testing day (b), all three treatment groups (Sed-En, Ex-SH, and Ex-En) preferentially explored the substituted object, but this was not significant for the Sed-En group. All data are shown as % exploration $\pm$ SEM. The asterisk denotes data sets of significant difference as assessed by a multivariate two-way ANOVA (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

4.3.4 Exercise, enrichment, and combination have no effect on BDNF protein the serum, dentate gyrus, perirhinal cortex, or hippocampus

Analysis of serum samples using a BDNF ELISA showed no significant difference between groups when assessed using a two-way ANOVA (Fig 4.6, Sed-SH: 151.7 ± 28.74 pg/ml, Sed-En: 253.2 ± 46.79 pg/ml, Ex-SH: 147.3 ± 23.68 pg/ml, Ex-En: 165.2 ± 22.01 pg/ml). There was no interaction, indicating that the independent variables 'enrichment' and 'exercise' had no effect on each other.

Analysis of brain samples using a BDNF ELISA showed no significant difference between groups in the dentate gyrus (Fig 4.7a, Sed-SH: 143.2 ± 58.41 pg/mg, Sed-En: 152.2 ± 45 pg/mg, Ex-SH: 102.6 ± 29.21 pg/mg, Ex-En: 166.2 ± 40.79 pg/mg), perirhinal cortex (Fig 4.7b, Sed-SH: 374.3 ± 40.34 pg/mg, Sed-En: 362.2 ± 67.27 pg/mg, Ex-SH: 320.9 ± 47.80 pg/mg, Ex-En: 343.4 ± 53.65), and hippocampus (Fig 4.7c, Sed-SH: 556.1 ± 51.51 pg/mg, Sed-En: 719 ± 41 pg/mg, Ex-SH: 619.3 ± 83.68 pg/mg, Ex-En: 458.3 ± 46.27 pg/mg) when normalised to total protein. Statistical analysis was performed using a two-way ANOVA.

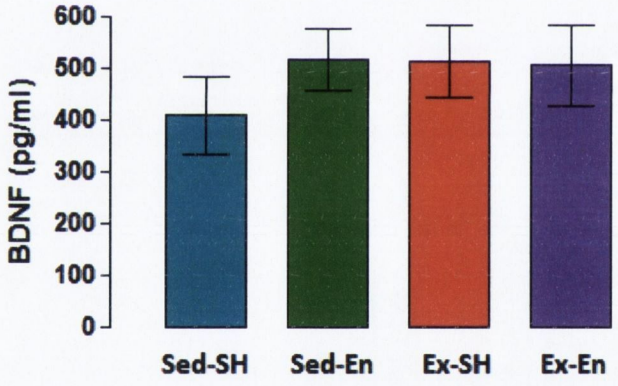
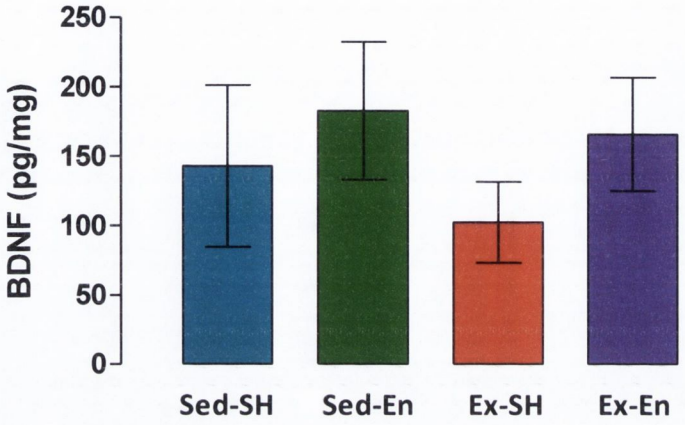
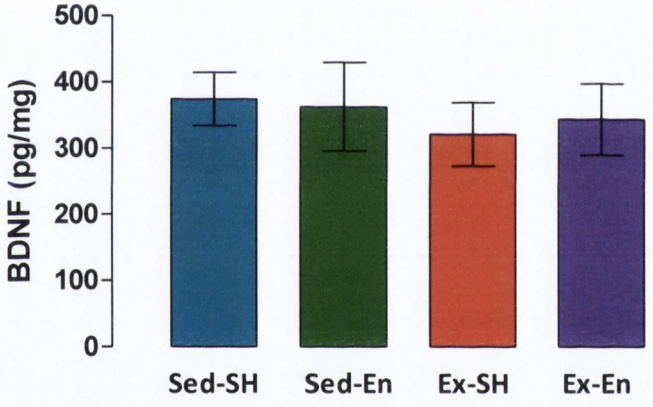


Fig 4.6 Exercise, enrichment, and combination have no effect on BDNF in the serum.
Concentrations of BDNF protein in serum are presented as assessed by an ELISA for each of the four groups (Sed-SH: sedentary standard-housed, Sed-En: sedentary-enriched, Ex-SH: exercised standard-housed, Ex-En: exercised-enriched, n=9-12 per group). Concentrations are in pg BDNF per ml serum. All data are shown ± SEM.

a) Dentate Gyrus



b) Perirhinal Cortex



c) Hippocampus

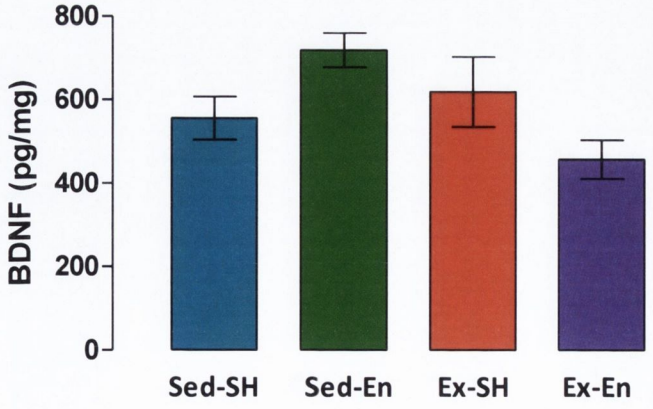


Fig 4.7 Exercise, enrichment, and combination have no effect on the concentration of BDNF protein in the dentate gyrus, perirhinal cortex and hippocampus.

This graph shows the concentration of BDNF in (a) dentate gyrus, (b) perirhinal cortex, and (c) entorhinal cortex as assessed by ELISA (n=9-12 per group). No changes in BDNF protein were observed across groups or brain regions when normalised to total protein. All data are expressed in pg/mg protein $\pm$ SEM. Statistical significance was assessed by a two-way ANOVA.

4.3.5 Exercise, but not enrichment, induces a significant decrease in NGF-β protein in the dentate gyrus

In the dentate gyrus, NGF-β protein concentrations were assessed by an ELISA (Fig 4.8, Sed-SH: 303.5 ± 16.47 pg/mg, Sed-En: 355.8 ± 21.32 pg/mg, Ex-SH: 268.8 ± 27.58 pg/mg, Ex-En: 291.1 ± 19.3 pg/mg) when normalised to total protein. A two-way ANOVA was conducted to examine the effect of exercise (first factor) and enrichment (second factor) on NGF-β protein in the dentate gyrus. There was a main effect of exercise ($F_{(3,30)}$=5.339, p=0.0269) but not of enrichment, indicating that NGF-β protein was significantly decreased in the exercised groups compared to the other groups. There was no interaction, indicating that the independent variables 'enrichment' and 'exercise' had no effect on each other.

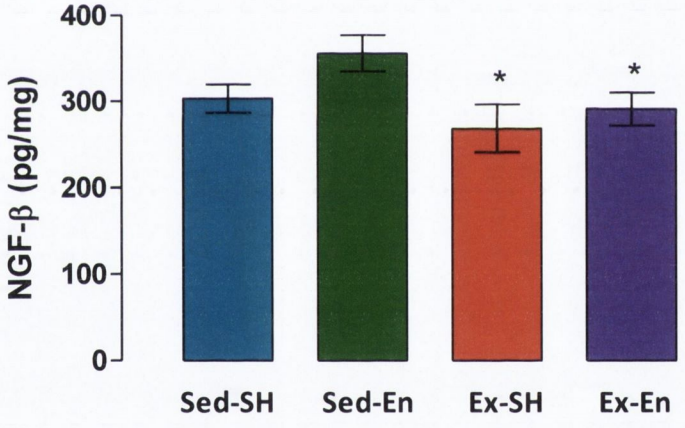


Fig 4.8 Exercise, but not enrichment, induces a decrease in β-NGF protein in the dentate gyrus

This graph shows the concentration of β-NGF protein in the dentate gyrus as assessed by ELISA (n=9-12 per group). All data are expressed in pg/mg protein ± SEM. Statistical significance was assessed by a two-way ANOVA.

4.3.6 Exercise induces an increase in BDNF mRNA expression in the dentate gyrus

In the dentate gyrus, BDNF mRNA expression was assessed by PCR (Fig 4.9, Sed-SH: 1 ± 0.42, Sed-En fold change: 0.94 ± 0.28, Ex-SH fold change: 1.55 ± 0.53, Ex-En fold change: 1.5 ± 0.27).

A two-way ANOVA was conducted to examine the effect of exercise (first factor) and enrichment (second factor) on BDNF mRNA expression in the dentate gyrus. There was no significant interaction between the effects of exercise and enrichment on BDNF mRNA expression. There was a main effect of exercise ($F_{(3,30)} = 15.31$, $p=0.0006$) but not of enrichment.

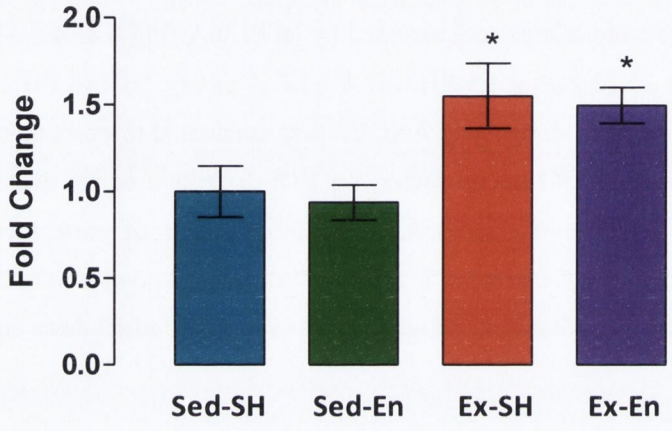


Fig 4.9 Exercise, but not environmental enrichment, induces an increase in BDNF mRNA expression in the dentate gyrus

This graph shows the fold change in BDNF mRNA in the dentate gyrus (DG) as assessed by PCR. BDNF mRNA expression in the dentate gyrus is elevated in the exercised groups (Ex-SH, Ex-En) relative to the non-exercised groups (Sed-SH, Sed-En). All data are expressed as fold change $\pm$ SEM. The asterisk denotes data sets of significant difference as assessed by a two-way ANOVA. (*$p<0.05$)

4.3.7 Exercise, enrichment, and the combination have no effect on TrkB and TrkA mRNA expression in the dentate gyrus

Tissue analysis by PCR revealed no differences in TrkB mRNA in the dentate gyrus in the Ex-SH group (two-way ANOVA, Fig 4.10a, Sed-SH: 1 $\pm$ 0.05, Sed-En fold change: 1.24 $\pm$ 0.06, Ex-SH fold change: 1.37 $\pm$ 0.09, Ex-En fold change: 1.16 $\pm$ 0.07)

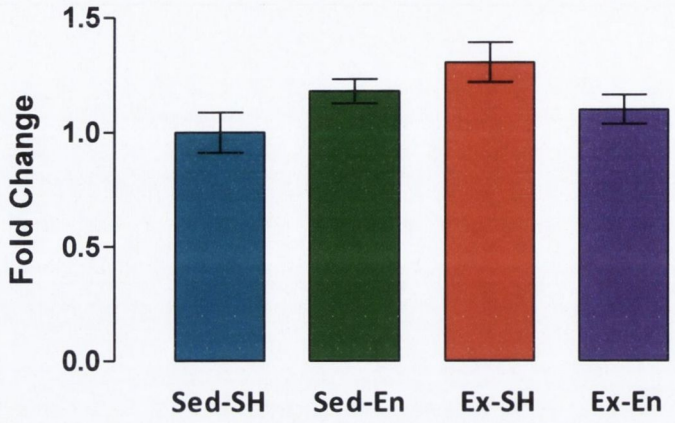
Tissue analysis by PCR revealed no differences in TrkA mRNA in the dentate gyrus in the Ex-SH group (two-way ANOVA, Fig 4.10c, Sed-SH: 1 $\pm$ 0.5, Sed-En fold change: 1.94 $\pm$ 0.91, Ex-SH fold change: 2.25 $\pm$ 1.29, Ex-En fold change: 1.6 $\pm$ 0.36).

4.3.8 Exercise, enrichment, and combination have no effect on NGF and KI-67 mRNA expression in the dentate gyrus

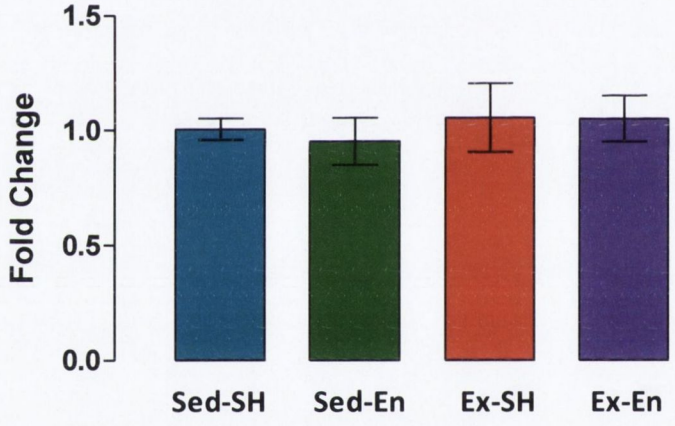
Tissue analysis by PCR revealed no significant differences in NGF mRNA levels in the dentate gyrus (Fig 4.10b, Sed-SH: 1 $\pm$ 0.14, Sed-En fold change: 0.95 $\pm$ 0.23, Ex-SH fold change: 1.06 $\pm$ 0.45, Ex-En fold change: 1.05 $\pm$ 0.35).

Finally, we also assessed KI-67 mRNA (Fig 4.10d, Sed-SH: 1 $\pm$ 0.36, Sed-En fold change: 1.05 $\pm$ 0.4, Ex-SH fold change: 1 $\pm$ 0.16, Ex-En fold change: 1 $\pm$ 0.24) and found no significant changes (consistent with earlier results).

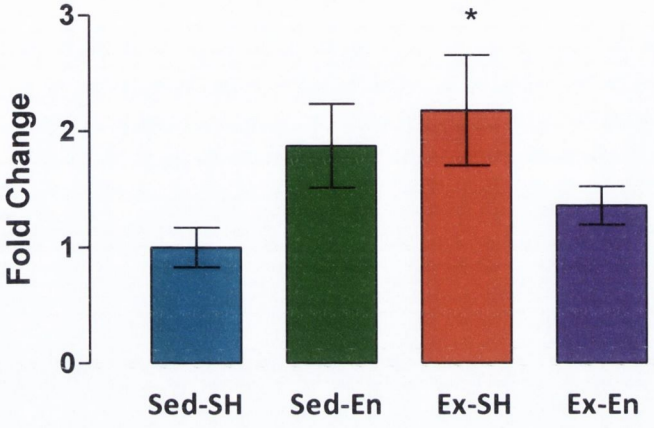
(a) TrkB mRNA



b) NGF mRNA



c) TrkA mRNA



d) KI-67 mRNA

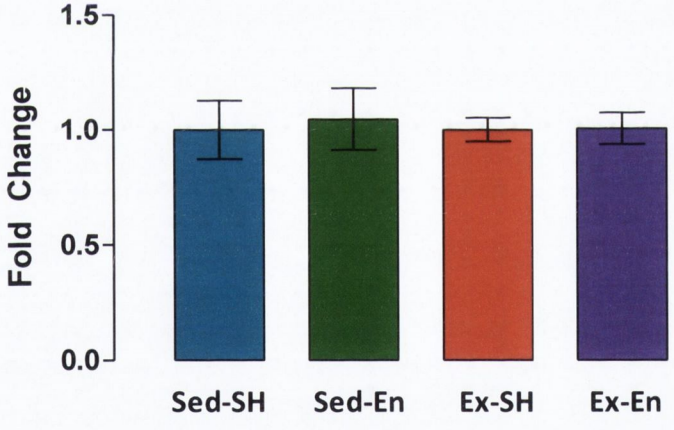


Fig 4.10 Exercise induces does not affect TrkB, TrkA, NGF, and KI-67 mRNA in the dentate gyrus.

This graphs shows the fold change in mRNA for TrkB (a), NGF (b), TrkA (c) and KI-67 (d) in the dentate gyrus of the four groups (Sed-SH, Sed-En, Ex-SH, Ex-En, n=9-12 per group) as assessed by PCR. No changes were observed when assessed by a two-way ANOVA. All data are expressed as fold change $\pm$ SEM.

4.3.9 Exercise induces an increase in BrdU+ve cells in the dentate gyrus

In the dentate gyrus, the average number of BrdU positive cells was calculated as a percentage of the total number of cells counted. (Fig 4.11, Sed-SH: 0.72 $\pm$ 0.16 %,, Sed-En: 1.34 $\pm$ 0.2 %, Ex-SH: 2.29 $\pm$ 0.26 %, Ex-En: 2.68 $\pm$ 0.4 %) A two-way ANOVA was conducted to examine the effect of exercise (first factor) and enrichment (second factor) on cell proliferation in the dentate gyrus. There was no significant interaction between the effects of exercise and enrichment on cell proliferation. There was a main effect of exercise ($F_{(3,23)} = 15.48$, $p=0.0008$) but not of enrichment.

4.3.10 Exercise, enrichment, and combination do not affect number of newly born mature neurons in the dentate gyrus

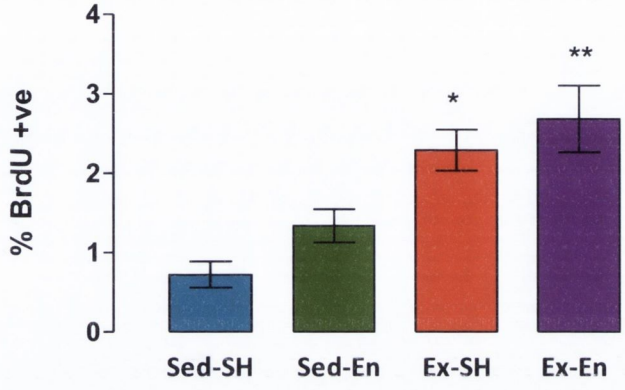
Mature neurons were identified as cells that were positive for both NeuN and BrdU, taken as a percentage of the total number of BrdU+ve cells. No differences were observed in the number of BrdU+ve/NeuN+ve cells across groups when assessed by a two-way ANOVA (Fig 4.12, Sed-SH: 46.08 $\pm$ 1.968 %, Sed-En: 54.8 $\pm$ 2.865 %, Ex-SH: 54.82 $\pm$ 3.753 %, Ex-En: 56.96 $\pm$ 4.198 %).

4.3.11 Exercise, enrichment, and combination induce an increase in DCX +ve cells in the dentate gyrus

Since BrdU and DCX were not colocalised in the cells, the average number of DCX positive cells was taken as the average number of DCX+ve cells summed over 6 views from 3 slides (Fig 4.13, Sed-SH: 7.43 $\pm$ 1 cells, Sed-En: 17.5 $\pm$ 1.19 cells, Ex-SH: 13.4 $\pm$ 0.93 cells, Ex-En: 20.280 $\pm$ 2.48 cells).

A two-way ANOVA was conducted to examine the effect of exercise (first factor) and enrichment (second factor) on number of DCX+ve cells in the dentate gyrus. There was no significant interaction between the effects of exercise and enrichment on cell proliferation. There was a main effect of exercise ($F_{(3,23)} = 1.414$, $p=0.012$) and of enrichment ($F_{(3,23)} = 28.47$, $p=0.0001$).

(a)



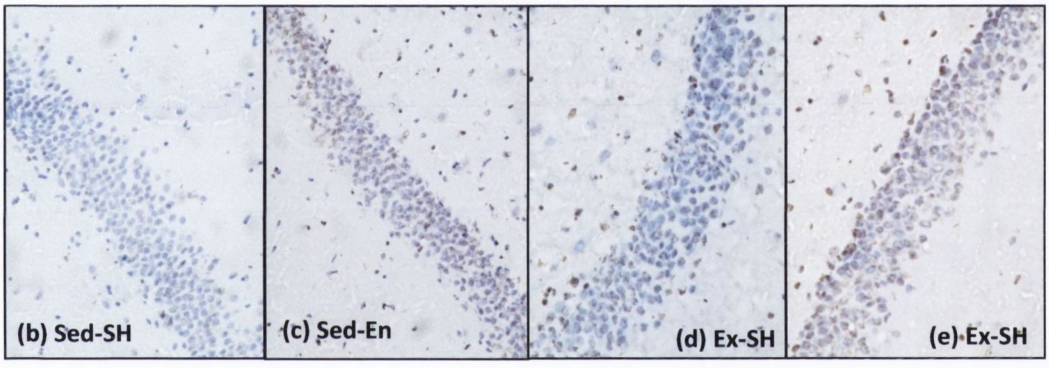


Fig 4.11 Exercise, but not environmental enrichment, increases the number of proliferating cells in the dentate gyrus.

A two-way ANOVA revealed a significant increase in the number of BrdU+ve cells in the dentate gyri of the exercised groups compared to controls (Sed-SH). Data are expressed as a percentage of total number of cells ± SEM, with n=6 in each group (a). Shown are representative pictures the staining of the dentate gyrus for each of the four groups, where hematoxylin-stained cells appear blue and BrdU+ve cells appear brown (3b-3e).

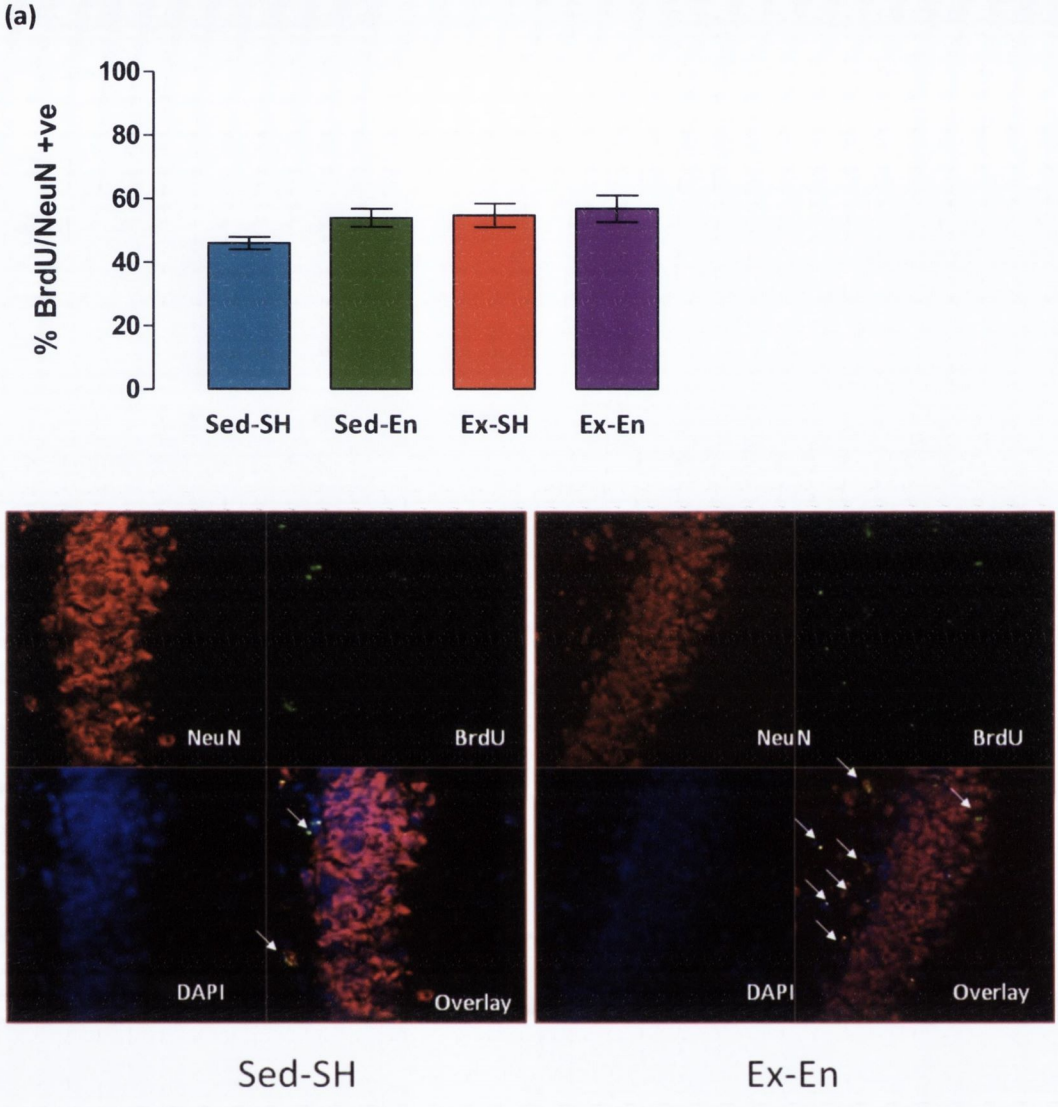
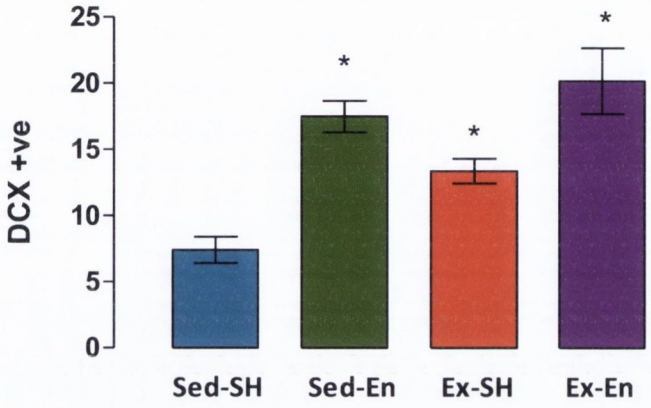


Fig 4.12 Exercise, cognitive enrichment, and the combination, do not affect the number of NeuN+ve/BrdU+ve cells in the dentate gyrus.

A two-way ANOVA revealed no change in the increase in the number of BrdU+ve cells as a percentage of NeuN +ve cells. Data are expressed as a percentage of total number of cells $\pm$ SEM, with n=6 in each group (a). Shown are representative pictures the staining of the dentate gyrus for each of the four groups, where DAPI-stained cells appear blue, NeuN-stained cells appear red, and BrdU+ve cells appear yellow/green.

(a)


DCX +ve
25
20
15
10
5
0
*
*
*
Sed-SH
Sed-En
Ex-SH
Ex-En

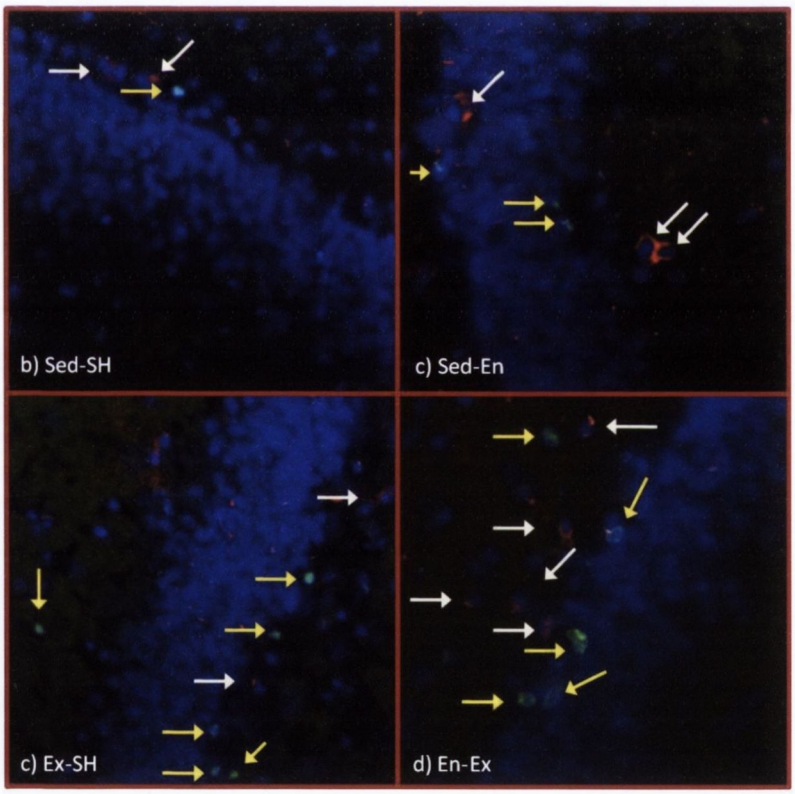

b) Sed-SH
c) Sed-En
c) Ex-SH
d) En-Ex

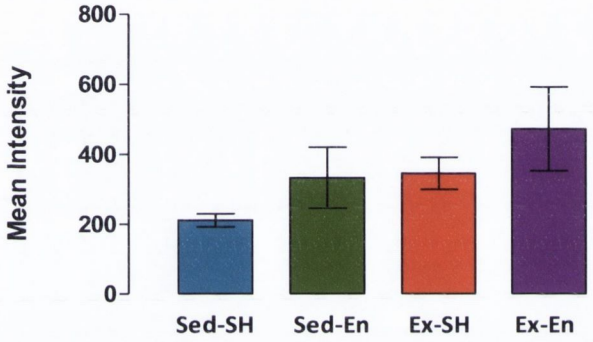
Fig 4.13 Enrichment, exercise, and the combination increase the number of immature neurons in the dentate gyrus of adult rats.

A two-way ANOVA revealed a significant increase in the number of DCX +ve cells in the dentate gyrus of the three treatment groups (n=6 per group) compared to controls (Sed-SH, n=6). Data are expressed as average number of DCX +ve cells per 6 views $\pm$ SEM. Shown are representative pictures the staining of the dentate gyrus for each of the four groups, where DAPI-stained cells appear blue, DCX-stained cells appear outlined in red (highlighted by white arrows), and BrdU+ve cells appear yellow/green (highlighted by yellow arrows).

4.3.12 Exercise, enrichment, and combination have no effect on the number of apoptotic cells in the dentate gyrus

TUNEL staining revealed no significant differences among the groups. Data are presented as mean intensity ± SEM (Fig 4.14, Sed-SH: 211.9 ± 19.1, Sed-En: 333.6 ± 87.2, Ex-SH: 346.4.5 ± 45.81 s, Ex-En: 473.2 ± 119), and were assessed by a two-way ANOVA.

(a)



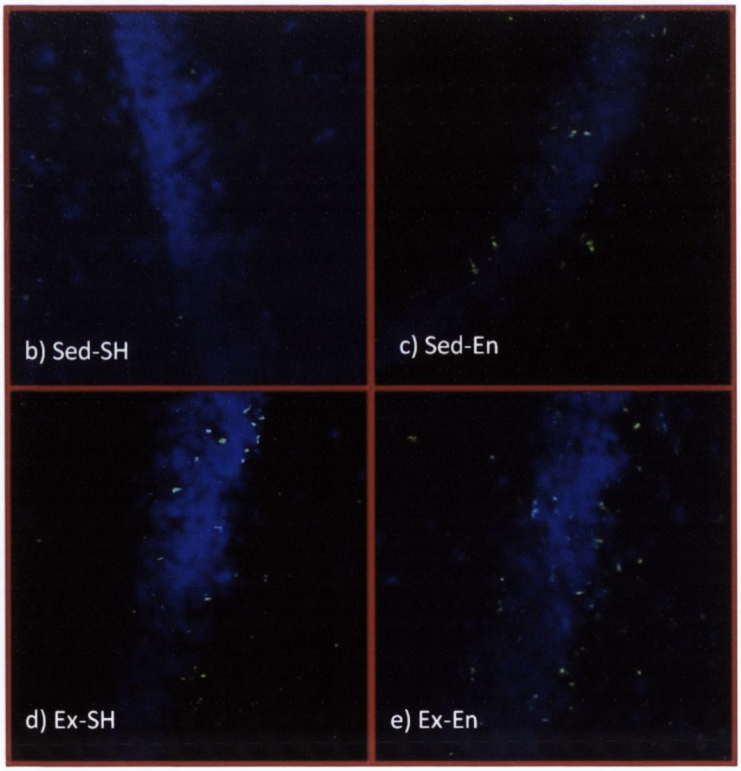


Fig 4.14 Exercise and cognitive enrichment do not affect the amount of apoptotic cells in the dentate gyrus.

A two-way ANOVA revealed no differences in fluorescent intensity with TUNEL staining in the dentate gyrus of rats from all four groups. Data are presented as mean fluorescent intensity ± SEM (a). Shown are representative pictures of the staining for the four groups (4.14b-e). (Sed-SH: sedentary standard-housed, Sed-En: sedentary-enriched, Ex-SH: exercised standard-housed, Ex-En: exercised-enriched, n=6 per group).

4.3.13 ES (Electric shock) rats do not preferentially explore the novel object in a 3-object OS task with 2x5 minutes of training

ES rats (n=5) were tested in a three-object OS task (third object is substituted for a fourth), in which they were given 2x5 minute trials of exploration on the training day (Fig 4.2). During the training period, ES rats spent a similar amount of time exploring each of the objects (A, B and C), indicating that animals had no preference for any specific object (Fig 4.15, Object A: 31.95 $\pm$ 3.7 %, Object B: 38.20 $\pm$ 4.64 %, Object C: 29.84 $\pm$ 2 %). During the testing period, control rats spent a similar amount of time exploring the familiar and novel objects (A, B and D), indicating that they could not remember the familiar objects and identify object D as the novel object (Fig 4.15, Object A: 34.73 $\pm$ 4.64 %, Object B: 37.12 $\pm$ 5.26 %, Object D: 28.15 $\pm$ 6.09 %). Statistical significance was assessed using a two-way ANOVA with Bonferroni *Post-hoc* comparisons.

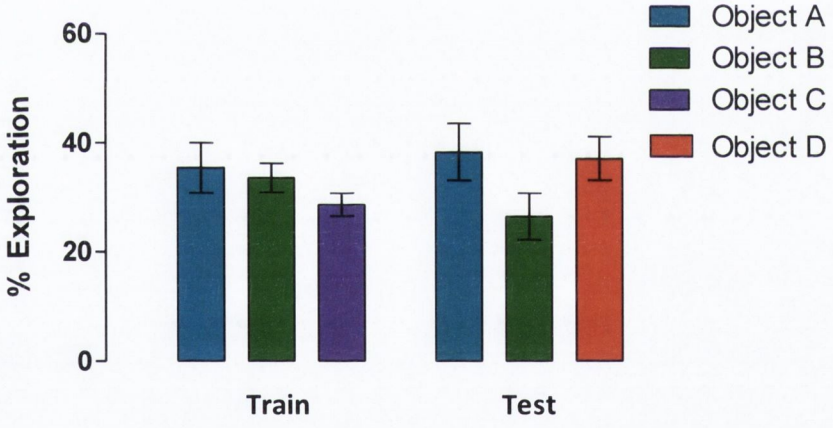


Fig 4.15 ES group does not preferentially explore substituted object in an OS task.
Data are presented as exploration time for each object (A, B ,C, or D, where D is the novel object) as a percentage of the total exploration time (n=5 per group). On the training day (a), ES animals spent a similar amount of time exploring each of the three objects (A, B and C), indicating that they had no preference for any one object. On the testing day (b), ES animals spent a similar amount of time exploring each of the three objects (A, B and D), indicating that they could not remember the familiar objects (A and B) and identify the displaced object (D). All data are shown as % Exploration $\pm$ SEM.

4.3.14 Enrichment, but not exercise, induces a decrease in serum corticosterone

A corticosterone ELISA was performed on serum collected from all animals (Fig 4.16, Sed-SH: 27.73 ± 2.4 ng/ml, Sed-En: 19.98 ± 1.9 ng/ml, Ex-SH: 24.74 ± 2.07 ng/ml, Ex-En: 22.59 ± 1.3 ng/ml, ES: 22.2 ± 0.94 ng/ml). A two-way ANOVA was conducted to examine the effect of exercise (first factor) and enrichment (second factor) on corticosterone concentration in the serum. There was no significant interaction between the effects of exercise and enrichment on serum corticosterone. There was a main effect of enrichment ($F_{(3,23)} = 6.62$, $p=0.015$) but not of exercise. The ES group was compared to the other groups using a one-way ANOVA, to reveal no significant differences.

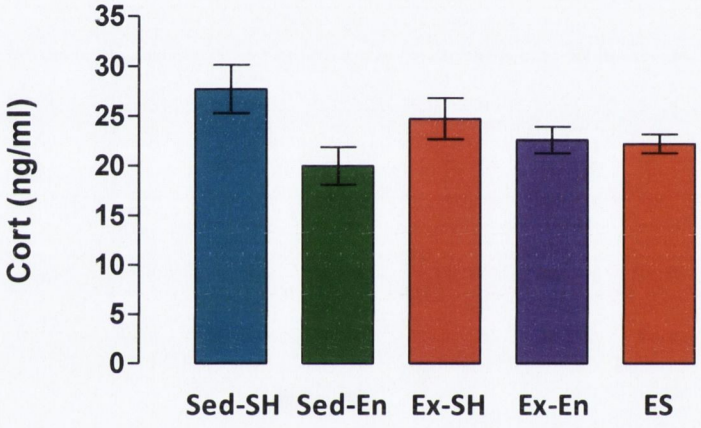


Fig 4.16 Enrichment, but not exercise, induces a decrease in serum corticosterone

The graph shows the amount of corticosterone in the serum of each treatment group (Sed-SH: sedentary standard-housed, Sed-En: sedentary-enriched, Ex-SH: exercised standard-housed, Ex-En: exercised-enriched, ES: electric shock group). Concentrations are presented in ng corticosterone per ml serum as assessed by a corticosterone ELISA kit. All Data are presented s mean concentration ± SEM.

4.3.15 ES (electric shock) treatment has no effect on BDNF protein concentration in the dentate gyrus

In the dentate gyrus, BDNF protein concentration of the ES group did not differ significantly from that of the other four groups when normalised to total protein, as assessed by an ELISA (Fig, 4.17, Sed-SH: 143.2 ± 58.41 pg/mg, Sed-En: 152.2 ± 45 pg/mg, Ex-SH: 102.6 ± 29.21 pg/mg, Ex-En: 166.2 ± 40.79 pg/mg, ES: 96.72 ± 43.44 pg/mg). All data are shown in pg/mg ± SEM and were analysed with a two-way ANOVA.

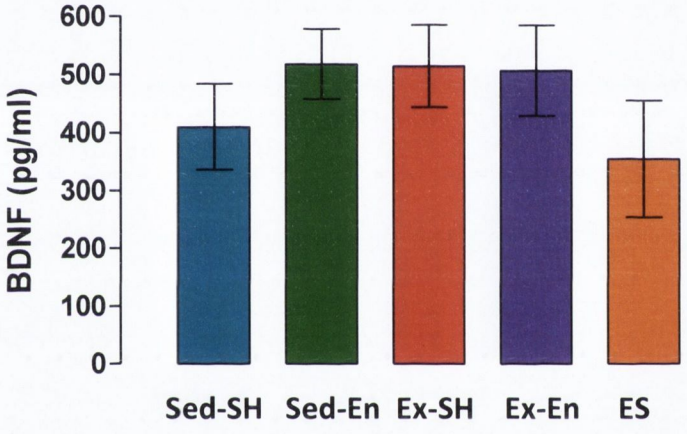


Fig 4.17 ES treatment does not affect the concentration of BDNF protein in the dentate gyrus. No differences in BDNF concentration were observed in the ES group compared to the other four groups when normalised to total protein, as assessed by an ELISA. All protein data are expressed in pg/mg protein ± SEM. Statistical significance was assessed by a two-way ANOVA. (Sed-SH: sedentary standard-housed, n=6, Sed-En: sedentary-enriched, n=6, Ex-SH: exercised standard-housed, n=6, Ex-En: exercised-enriched, n=6, ES: electric-shock, n=5).

4.4 Discussion

4.4.1 Behavioural Analysis

To test the effect of exercise or enrichment interventions on learning, we used a novel object recognition task (3 objects, 2x5 min of training). The time spent exploring the objects on training day was similar for all groups, indicating that neither intervention alone, nor a combination of both, has an effect on general locomotor activity or motivation to explore. On the testing day, the sedentary standard-housed controls (Sed-SH) explored all three objects similarly, indicating that they could not remember the familiar objects and identify the novel object. The three intervention groups (Sed-En, Ex-SH, Ex-En) preferentially explored the novel object, indicating that they could remember the familiar objects and identify the novel object. For the Sed-En (sedentary enriched), this effect was not as clear as for the exercised groups. These results demonstrate that one week of forced moderate exercise induces a clear improvement in object recognition memory compared to standard-housed controls, in agreement with the results of Chapter 3. Three weeks of environmental enrichment induce a less clear effect, but the data suggests that some improved learning has occurred. Therefore, exercise-induced cognitive improvements are not only evident in standard-housed, cognitively deprived animals, but also in animals that have been enriched continuously for three weeks. In addition, these results suggest that improvements in hippocampal-dependent learning reported with typical environmental enrichment paradigms could be a result of the combined effect of exercise and cognitive enrichment. One way to obtain a more conclusive answer to this question would be to test the animals in a more challenging task. The effect of task difficulty on cognitive performance is investigated in detail in the next chapter.

4.4.2 Neurotrophin and neurotrophin receptor analysis

Tissue analysis revealed increases in BDNF mRNA in the dentate gyri of the two exercised groups (Ex-SH and Ex-En), but not the sedentary-enriched group (Sed-En). Exercise-induced cognitive enhancement has been consistently correlated with increases in the neurotrophin BDNF in the hippocampus (Vaynman et al., 2003, Farmer et al., 2004, Gomez-Pinilla et al., 2008). However, for environmental enrichment there have been contradictory reports in regard to the role of neurotrophins (Neeper et al., 1996, Pham et al., 1999a, Ickes et al., 2000). This is likely to stem from the different experimental paradigms used in different laboratories for environmental

enrichment. Studies that have reported increases in BDNF in the hippocampus in response to environmental enrichment typically include running wheels in their enriched cages. Our data are consistent with previous findings in our laboratory (unpublished results), that suggest that BDNF mediates the improvements in object recognition memory caused by exercise, but not by environmental enrichment. This has also been suggested by a recent study in mice (Kobilo et al., 2011).

Ongoing studies in our laboratory have reported increases in NGF-β in the dentate gyrus with 6 weeks of cognitive enrichment, but not with exercise. We observed a decrease in NGF-β protein in the dentate gyrus in the exercised groups compared to the other two groups. The significance of this finding is unclear. NGF has been proposed as a possible mediator of the effects of environmental enrichment by increasing neurogenesis (Birch et al, in preparation); however, certain characteristics of NGF make its involvement in experience-dependent plasticity unlikely. For instance, unlike BDNF and TrkB, NGF and TrkA are expressed at very low levels in the hippocampus (Cellerino, 1996, Narisawa-Saito and Nawa, 1996), and the secretion of NGF from cells occurs constitutively, and not in an activity-dependent manner. As a result, further studies are needed to establish a convincing link between NGF and learning and memory.

We found no changes in TrkB and TrkA mRNA expression in the dentate gyrus of the Ex-SH group. The TrkB Data are inconsistent with our findings in Chapter 3, where we suggested that exercise-induced upregulation of the TrkB gene could be a possible mechanism by which BDNF-TrkB signalling is strengthened. This could be caused directly by an unknown mechanism, or indirectly, by increasing BDNF. An increase in BDNF causes increased neuronal excitability, and as a consequence, increased activity which has been shown to activate both BDNF (Tabuchi et al., 2002) and TrkB (Kingsbury et al., 2003) transcription. However, further studies are required to determine the reliability and underlying mechanisms of this effect.

4.4.3 Neurogenesis and Apoptosis

Both exercise and environmental enrichment have been shown to increase neurogenesis in the dentate gyrus of adult rats (van Praag et al., 2000, Kempermann et al., 2002, Brown et al., 2003, Olson et al., 2006), but again, most enrichment paradigms that report this include running wheels. The first study to separate out the effect of exercise from environmental enrichment was in 1999 (van Praag et al., 1999), where it was shown that environmental enrichment on its own did not

stimulate cellular proliferation in the dentate gyrus. Exercise, however, did. This finding has since been replicated (Steiner et al., 2008), and it is currently thought that exercise and enrichment affect different stages in neurogenesis (Olson et al., 2006). Exercise is thought to increase both cellular proliferation and the differentiation of these cells into neurons, while enrichment seems to be more important in the survival of neurons long-term (Kronenberg et al., 2003). This suggests that environmental enrichment on its own does not increase cellular proliferation in the dentate gyrus, but does increase the number of new neurons present in the dentate gyrus in the long-term (Steiner et al., 2008). This view fits in with the results of this chapter, where we observed a significant increase in proliferating cells in the dentate gyrus in the exercised groups, but not in the enriched group. Previous studies in our laboratory have found significant increases in cell proliferation with six weeks of cognitive enrichment, but not with three weeks. This could be due to an increased survival rate in new cells with enrichment.

An investigation of the phenotype of the newly generated cells revealed that about half of these were mature neurons (positive for both BrdU and NeuN). NeuN is a cell surface marker that stains for mature neurons (Mullen et al., 1992). This finding was at first surprising, since we only started injecting the animals with BrdU one week before tissue collection. The literature indicates that it takes at least two weeks for new cells to develop into mature neurons and start expressing NeuN. However, most studies with adult hippocampal neurogenesis have been done in mice, and recent studies in have reported that in rats cells can express NeuN within one week of being born (Snyder et al., 2009). However that does not explain why we found no changes between groups, despite the observed differences in the number of BrdU positive cells. It should be noted that the measure we used to assess the number of newly proliferated mature cells was the ratio of NeuN+ve to BrdU+ve cells. As demonstrated in the representative images of our NeuN/BrdU staining, an increase was observed in both total NeuN and total BrdU cells with treatment. This could explain why the ratio remained unchanged.

We observed an increase in the number of doublecortin-positive cells with exercise, enrichment and the combination. Doublecortin is a cell surface marker expressed by immature neurons in the first few days of their life (Couillard-Despres et al., 2006). 90% of new cells express the immature neuronal marker DCX within a few days of birth. Interestingly, most of the doublecortin-positive cells were not colocalised with BrdU. One possibility is that the doublecortin-positive cells are cells that were born before we started BrdU injections. In the case of cognitive enrichment, since the protocol is three weeks long, it is likely that the effects on cell proliferation preceded the BrdU

injections. It is possible that in both exercise and cognitive enrichment, the interventions caused the differentiation of cells into immature neurons to speed up, leading to an increase in stained cells. There is evidence that BDNF influences the differentiation of progenitor cells into neurons in the hippocampus (Babu et al., 2009), and this could be a mechanism by which exercise induces an increase in doublecortin positive cells.

Another possibility is that the DCX-labeled neurons are pre-mitotic stem cells that have not yet proliferated. Kempermann and colleagues have shown that Type-2b and Type-3 cells express doublecortin (Kempermann, 2008, Lafenetre et al., 2011). An increase in the number of DCX+ve (but BrdU-ve) cells in the dentate gyrus with environmental enrichment and exercise could indicate that more cells have started to commit to the neuronal lineage than in controls. This could attribute to exercise and enrichment a role in pre-mitotic processes that initiate the transition of cells from Type-1 and Type-2a into Type 2b and Type 3 cells.

4.4.4 Effects on Stress and Anxiety

Environmental enrichment and exercise are both thought to modulate the stress response and produce anxiolytic effects that have been suggested to play a part in the cognitive improvements that accompany these interventions (Moncek et al., 2004, Greenwood and Fleshner, 2008). This is based on the observation that stress has been shown to disrupt learning and LTP *in vivo* (Cazakoff and Howland, 2009, Howland and Cazakoff, 2010), thus an intervention that relieves stress could improve memory in part by diminishing the negative effects of stress on memory processing. The physiological changes that occur in the mammalian body in response to an acute stressor are now well-understood. Briefly, the stress response involves the activation of the hypothalamic-pituitary-adrenal (HPA) axis, where the hypothalamus secretes corticotropin-releasing hormone (CRH), which stimulates the anterior pituitary gland to release ACTH (adrenocorticotropic hormone) (Rivier and Vale, 1983), which in turn stimulates the adrenal glands to release glucocorticoids. The main glucocorticoid in humans is known as the stress hormone cortisol, and in rodents it is corticosterone. In our study, we assessed differences in stress levels between treatment groups by testing the serum of the rats for corticosterone using an ELISA (de Kloet et al., 2005, McEwen, 2007).

The results of the corticosterone ELISA revealed a significant decrease in corticosterone levels with enrichment. This has been shown in other studies (Belz et al., 2003), although some reports

have been contradictory. There does however seem to be a consensus in the literature that environmental enrichment produces a generalised anxiolytic effect and attenuates stress responses (Benaroya-Milshtein et al., 2004, Fox et al., 2006), but only in the long-term (months). The data presented here suggests that the stress response may be attenuated after as little as three weeks of treatment.

In addition, we briefly investigated the effect of our interventions on anxiety behaviour in the open-field test. The open-field test is considered a classical test for anxiety (Prut and Belzung, 2003). All groups spent a similar amount of time in the periphery as a percentage of total time spent in the open field. It is possible that we are only just starting to see an anxiolytic effect in the enriched rats that with further enrichment, would become more pronounced.

Stress could also play a role in our exercise protocol. The advantage of using treadmills as opposed to voluntary wheel-running is that they allow precise control of the duration and intensity of running for all animals. However, treadmills do present a higher level of stress for the rat (Leasure and Jones, 2008) since it is being forced to run and will be shocked if it does not run (albeit a mild shock). High levels of the stress hormone corticosterone have been shown to transiently down-regulate BDNF in the hippocampus (Schaaf et al., 2000). This could explain why significant increases in BDNF mRNA and protein in the dentate gyrus are not observed with high-intensity running (Soya et al., 2007). The results of the corticosterone analysis revealed no significant changes in the serum of exercised rats compared to standard-housed controls, indicating that the stress induced by the treadmill is likely to be minimal. In addition, the ES rats showed no changes in corticosterone in the serum, and no changes in behaviour, suggesting that the electric shocks have a negligible effect on both behaviour and corticosterone levels in the blood.

4.5 Conclusion

We have shown that short-term environmental enrichment and exercise both induce improvements in cognitive performance in a challenging object recognition task. These enhancements in cognition are likely to be mediated by separate mechanisms. The improvements can be induced in enriched rats as well as standard-housed rats, and are likely to be mediated by the short-term effects of the neurotrophin BDNF on synaptic plasticity. In addition, we have shown that the exercise-induced cognitive enhancement is unlikely to be affected by any stress-effect induced by the treadmill. The cognitive enhancement produced by environmental enrichment was not associated with any

significant changes in neurotrophin levels in the hippocampus and related structures; however, it could be mediated by changes in the number of immature neurons in the dentate gyrus. This suggests that short-term environmental enrichment could have an effect on the differentiation and/or survival of neurons, if not on cellular proliferation per se.

Chapter 5 One week of exercise improves performance in a challenging spatial task by activating BDNF-stimulated cell signaling pathways

5.1 Introduction

The results of Chapter 3 showed that one week of forced exercise improves the performance of rats in the object substitution task but not in the object displacement task. This is in agreement with previous experiments in our laboratory (O'Callaghan et al., 2007) which showed that an identical exercise protocol did not improve spatial learning in the Morris Water Maze. However, the literature suggests that if there is an existing deficit in spatial learning due to ageing or an insult, exercise can rescue this impairment (Christie et al., 2005, Gobbo and O'Mara, 2005, Albeck et al., 2006). This led us to hypothesise that the reason why we do not observe an improvement in the object displacement task with exercise is because the spatial task is too easy. This hypothesis would agree with previous studies that have showed that it is easier to observe exercise-induced differences in spatial learning when the tasks are made more challenging (van Praag et al., 1999, 2000).

In the first experiment described in this chapter, we aimed to test this hypothesis by varying the level of difficulty of the two cognitive tasks, and evaluating the ability of healthy controls to perform in the different variants. As a result, we were able to determine a variant of the object displacement task in which control rats performed poorly.

In the second experiment, we aimed to test whether we could tease apart a difference in performance between sedentary and exercised rats by choosing a more difficult variant of the spatial task. In parallel, we sought to investigate possible mechanisms underlying exercise-induced improvements in cognitive performance. Since exercise-induced increases in neuronal proliferation have been reported along with improvements in learning in several laboratories (reviewed in van Praag, 2009), we tested whether our one-week forced exercise paradigm was sufficient to induce cell proliferation in the dentate gyrus of adult rats, in addition to our usual BDNF analyses.

In the third experiment, we confirmed the effect of one-week of forced exercise on the challenging spatial task, and analysed parallel BDNF-stimulated changes in the dentate gyrus, including activation of the TrkB receptor and stimulation of downstream signalling pathways. Since the

signalling pathways under investigation are also activated by learning (Minichiello, 2009) we added an additional sedentary non-learning group to control for any learning-induced effects.

In previous chapters, we established that one week of forced moderate exercise is accompanied by an increase in BDNF in the blood and in the brain, and we have suggested that this increase in BDNF (and the subsequent stimulation of downstream signaling pathways) is directly responsible to the improvements that we observe in spatial and object recognition learning. In the fourth experiment of this chapter, we aimed to test whether the administration of exogenous BDNF directly into the lateral ventricle of the brain (intracerebroventricularly: i.c.v.) is sufficient to mimic the exercise-induced cognitive improvements observed in previous chapters. The dosage of BDNF administered was calculated from the increases observed in the brain with one week of exercise (Griffin et al., 2009). In addition, we were interested in the question of persistence: how long do the BDNF-induced effects on cognition last? Finally, the fifth experiment investigates whether a single BDNF i.c.v. infusion of the administered dosage causes an appropriate increase in BDNF in the dentate gyrus, and whether this increase is accompanied by BDNF-stimulated signaling events that facilitate synaptic transmission and plasticity.

5.2 Methods and Experimental Design

5.2.1 Experiment 1: The effect of varying training time and number of objects on cognitive performance

Three-month-old adult Wistar rats (n=12) were divided into two groups (n=6 per group). Each group performed a series of cognitive tasks, each task separated from the next by a 3-day wash-out period (total time = 3 weeks). The cognitive tasks consisted of either the object displacement task or the object recognition task, conducted with ascending levels of difficulty. The difficulty of the task was increased by either 1) increasing the number of objects (from two to three) or 2) decreasing the number of exploration trials on the training day (three, two or one 5 min trial; see Fig 5.1 for study design).

5.2.2 Experiment 2: The effect of one week of exercise on performance in a challenging spatial task

Three-month-old Wistar rats (n=12) were randomly assigned to sedentary or exercise groups (n=6 per group), and subjected to the one week forced exercise protocol as previously described in section 2.3, All rats were injected with 50mg/kg BrdU (i.p.) daily during the 7-day exercise protocol. After the last bout of exercise, both groups were tested in a three-object object displacement task, with 1x5 min of exploration on the training day (see Fig 5.5 for study design). Whole blood was collected after testing and spun down for serum analysis (Section 2.8.2), and the dentate gyrus was dissected from the right hemisphere for neurochemical analysis (Section 2.9.1). The left hemisphere was flash-frozen and sectioned (Section 2.9.6) for staining with a BrdU antibody to test for cellular proliferation in the dentate gyrus (Section 2.14.2).

5.2.3 Experiment 3: The effect of one week of exercise on BDNF-stimulated signaling events

Three-month-old Wistar rats (n=18) were randomly assigned to one of three groups (n=6 per group): exercise (Ex), sedentary (Sed), and a sedentary non-learning group (Non-L). Exercised and sedentary groups performed the one-week forced exercise protocol as described in section 2.3. After the last bout of exercise, both groups were tested in a three-object object displacement task, with 1x5 min of exploration on the training day. The sedentary non-learning group was placed on the stationary treadmill, similarly to the sedentary group, but did not perform the cognitive task. Instead, these rats were placed in the empty open field (objects and spatial cues removed) for 5 minutes on both the training and testing day (see Fig 5.11 for study design). Immediately after testing, the dentate gyrus was removed from both hemispheres. Dentate gyrus tissue from the right hemisphere was cross-chopped for the BDNF release experiment. Dentate gyrus tissue from the left hemisphere was homogenised for ELISAs and Western Blots.

5.2.4 Experiment 4: The effect of a single BDNF infusion (i.c.v.) on spatial memory

Three-month-old Wistar rats (n=12) were cannulated as described in General Methods (2.14). The rats were allowed two weeks to recover from surgery during which their behaviour and weight were carefully monitored. After a two-day habituation period to the open field, rats were randomly assigned to a BDNF group (BDNF, n=6) or a control group (Cyt C, n=5). BDNF rats were infused

with a single bolus of human recombinant BDNF (10ng in CSF, i.c.v.). Control rats received a similar infusion of cytochrome c (10ng in CSF, i.c.v.).

30 minutes post-infusion, all animals were trained in a 3-object object displacement task with 1x5 minutes of training (Section 2.6). Animals were tested 24 hours later. One week later, the BDNF group was infused with Cytochrome c (10ng in CSF, i.c.v.) and then trained and tested in a new object displacement task (3 objects, 1x5 minutes of training) to test for persistence of the effect of BDNF.

5.2.5 Experiment 5: The effect of a single BDNF infusion (i.c.v.) on object recognition memory and serum BDNF

Young rats (n=12) were cannulated as described in General Methods (Section 2.15.1). The rats were allowed two weeks to recover from surgery during which their behaviour and weight was carefully monitored. After a two-day habituation period to the open field, rats were randomly assigned to a BDNF group (n=6) or a control (Cyt C) group (n=5). Blood was collected from the tail vein of each animal for plasma and serum analysis (Section 2.8.1.2). Twenty-four hours after blood collection, rats (n=6) were infused with a single bolus of human recombinant BDNF (10ng in CSF, i.c.v.). Control rats (n=6) received a similar injection of cytochrome c (10ng in CSF, i.c.v.).

30 minutes post-infusion, all animals were trained in a 3-object object displacement task with 1x5 minutes of training (Section 2.6). 1.5 hours post-infusion, blood was collected again for serum and plasma analysis. Animals were tested 24 hours later.

After a wash-out period of three days, the groups were switched (BDNF group received Cyt C injection, Cyt C group received BDNF injection) and infused again as described above. 30 minutes post-infusion, all animals were trained in a 4-object object recognition task with 2x5 minutes of training (Section 2.6). Animals were tested 24 hours later (see Fig 5.23 for study design).

5.2.6 Experiment 6: The effect of a single BDNF infusion (i.c.v.) on BDNF-stimulated signaling events in the dentate gyrus

After a wash-out period of a week, 12 cannulated animals were randomly assigned to a BDNF group (n=6) or a control (Cyt C) group (n=6).). BDNF rats were infused with a single bolus of human recombinant BDNF (10ng in CSF, i.c.v.). Control rats received a similar injection of

cytochrome c (10ng in CSF, i.c.v.). 30 minutes post-perfusion, rats were overdosed with urethane (0.3g/ml, i.p.) and blood was collected for serum and plasma analysis via cardiac puncture. After blood collection, the animals were perfused with heparinised 0.89% ice-cold saline for 10 minutes (Section 2.9.2) and brain tissue (dentate gyrus and hippocampus) was collected and flash-frozen for further analysis (see Fig 5.27 for study design).

When thawed, tissue samples were divided into two pieces: one was homogenised in Krebs buffer (for BDNF analysis by ELISA) and the other was homogenised in Lysis buffer (for TrkB ELISAs and Western Blots). Tissue was assessed for BDNF, p-TrkB and TrkB, p-ERK and ERK, p-PLCγ and PLCγ, p-Synapsin and Synapsin, and p-CaMKII and CaMKII (See Table 2.3).

5.3 Results

5.3.1 Experiment 1: The effect of varying training time and number of objects on cognitive performance

5.3.1.1 Control rats preferentially explore the novel object in a 2-object OS task with 10 minutes of training, but not with 5 minutes of training

Control rats (n=6) were tested in a 2-object object substitution task (second object is substituted for a third), in which they were given 2x5 minute trials of exploration on the training day (Fig 5.2a). During the training period, control rats preferentially explored Object B, indicating that animals had a preference for that object ($p=0.024$, Student's t-Test, Fig 5.2a, Object A: 46.21 $\pm$ 2 %, Object B: 53.79 $\pm$ 2 %). During the testing period, control rats preferentially explored the novel object C ($p=0.039$, Student's *t*-Test, Fig 5.2a, Object A: 38.82 $\pm$ 6.7 %, Object B: 61.18 $\pm$ 6.7 %).

In a separate experiment, control rats (n=6) were tested in a 2-object object substitution task (second object is substituted for a third), in which they were given 1x5 minute trial of exploration on the training day (Fig 5.2b). During the training period, control rats spent a similar amount of time exploring each of the objects (A and B), indicating that animals had no preference for any specific object (Fig 5.2b, Object A: 54.49 $\pm$ 5.17 %, Object B: 45.51 $\pm$ 5.17 %). During the testing period, control rats spent a similar amount of time exploring the familiar and novel object (A and C respectively), indicating that they could not identify object C as the novel object (Fig 5.2b, Object A: 47.99 $\pm$ 2.8 %, Object C: 52 $\pm$ 2.8 %).

5.3.1.2 Control rats do not preferentially explore the novel object in a 3-object OS task with 15 or 10 minutes of training

Control rats (n=6) were tested in a three-object OS task (third object is substituted for a fourth), in which they were given 3x5 minute trials of exploration on the training day (Fig 5.3a). During the training period, control rats spent a similar amount of time exploring each of the objects (A, B and C), indicating that animals had no preference for any specific object (Fig 5.3a, Object A: 33.76 $\pm$ 2.05 %, Object B: 31.60 $\pm$ 1.99 %, Object C: 34.64 $\pm$ 2.07). During the testing period, control rats spent a similar amount of time exploring the familiar and novel objects (A, B and D), indicating that they could not remember the familiar objects and identify object D as the novel object (Fig 5.3a, Object A: 37.66 $\pm$ 3.26 %, Object B: 32.38 $\pm$ 3.15 %, Object C: 29.96 $\pm$ 3.49 %).

In a separate experiment, control rats (n=6) were tested in a 3-object object substitution task (third object is substituted for a fourth), in which they were given 2x5 minute trials of exploration on the training day (Fig 5.3b). During the training period, control rats spent a similar amount of time exploring each of the objects (A, B and C), indicating that animals had no preference for any specific object (Fig 5.3b, Object A: 38.23 $\pm$ 4.84 %, Object B: 30.79 $\pm$ 2.6 %, Object C: 31.55 $\pm$ 3.86 %,). During the testing period, control rats spent a similar amount of time exploring the familiar and novel objects (A,B and D), indicating that they could not remember the familiar objects and identify object D as the novel object (Fig 5.3b, Object A: 37.11 $\pm$ 4.08 %, Object B: 31.35 $\pm$ 3.95 %, Object D: 31.54 $\pm$ 3.2).

5.3.1.3 Control rats preferentially explore the displaced object in a 3-object OD task with 15 or 10 minutes of training, but not with 5 minutes of training.

Control rats (n=6) were tested in a three-object object displacement (OD) task (third object is displaced), in which they were given 3x5 minute trials of exploration on the training day (Fig 5.4a). During the training period, control rats spent a similar amount of time exploring each of the objects (A, B and C), indicating that animals had no preference for any specific object (Fig 5.4a, Object A: 38.21 $\pm$ 6 %, Object B: 28.40 $\pm$ 2.3 %, Object C: 33.39 $\pm$ 5 %). During the testing period, control rats preferentially explored the displaced object C, indicating that they could remember the location of the objects and identify the displaced object ($F_{(2,18)}=17.02$, $p<0.0001$, one-way ANOVA, Fig 5.4a, Object A: 25.44 $\pm$ 2.3 %, Object B: 28.40 $\pm$ 2.3 %).

Control rats (n=6) were tested in a three-object object displacement (OD) task (third object is displaced), in which they were given 2x5 minute trials of exploration on the training day (Fig 5.4b). During the training period, control rats spent a similar amount of time exploring each of the objects (A, B and C), indicating that animals had no preference for any specific object (Fig 5.4b, Object A: 32.46 $\pm$ 1.4 %, Object B: 35.67 $\pm$ 0.96 %, Object C: 31.87 $\pm$ 0.89 %). During the testing period, control rats preferentially explored the displaced object C, indicating that they could remember the location of the objects and identify the displaced object ($F_{(2,18)}$=9.44, p=0.0022, one-way ANOVA, Fig 5.4b, Object A: 29.41 $\pm$ 2.82 %, Object B: 28.86 $\pm$ 2.5 %, Object C: 41.73 $\pm$ 1.5 %).

Control rats (n=6) were tested in a three-object object displacement (OD) task (third object is displaced), in which they were given 1x5 minute trial of exploration on the training day (Fig 5.4c). During the training period, control rats spent a similar amount of time exploring each of the objects (A, B and C), indicating that animals had no preference for any specific object (Fig 5.4c, Object A: 30.09 $\pm$ 3.64 %, Object B: 35.33 $\pm$ 3.97 %, Object C: 34.57 $\pm$ 3.09 %). During the testing period, spent a similar amount of time exploring each of the objects (A, B and C), indicating that they were unable to remember the location of the objects and identify the displaced object (Fig 5.4c, Object A: 31.83 $\pm$ 3.15 %, Object B: 39.16 $\pm$ 6.2 %, Object C: 29.01 $\pm$ 4.16 %).

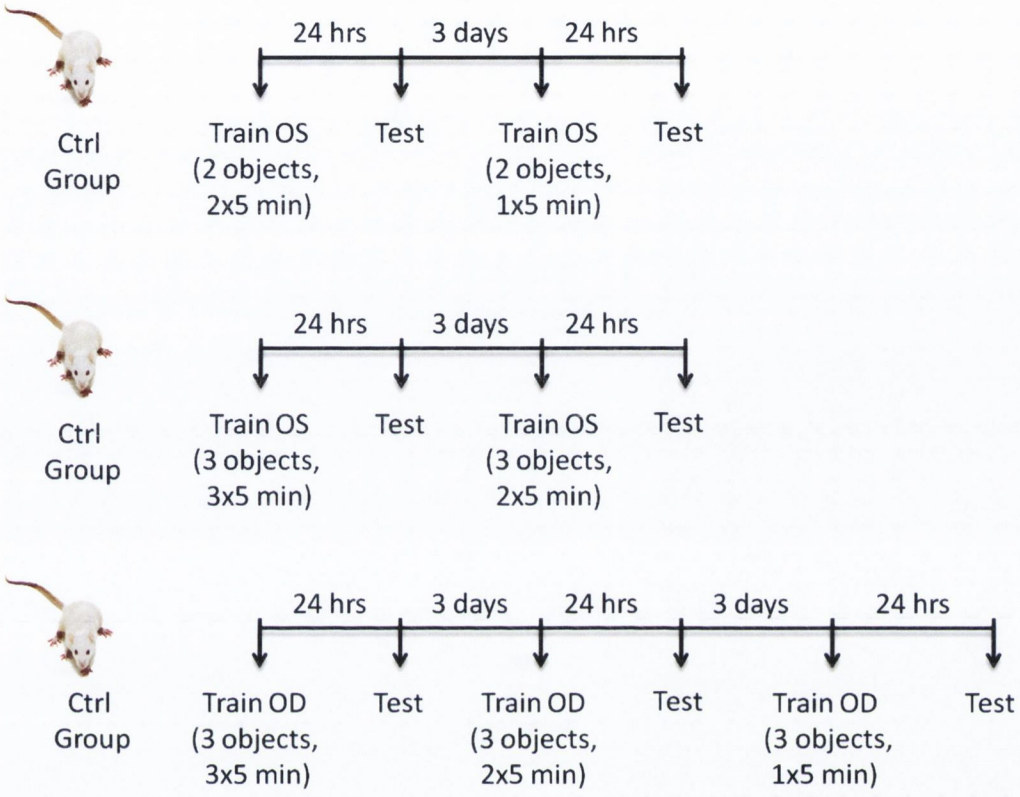
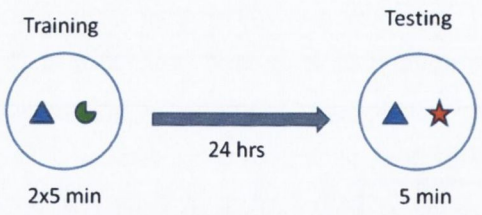
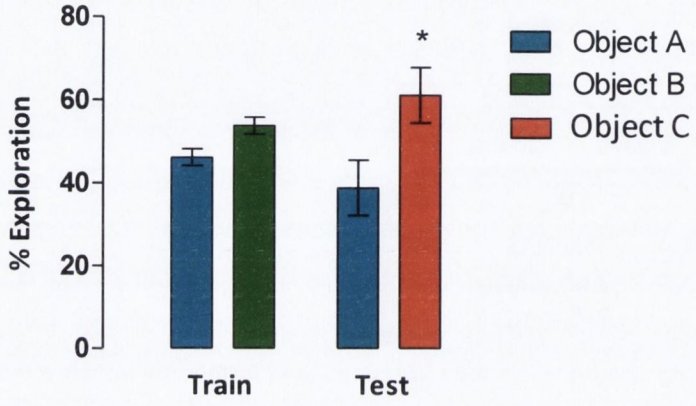


Fig 5.1 Study design of Experiment 1: The effect of varying training time and number of objects on cognitive performance

Control rats (n=6) were trained and tested in a series of object substitution and object displacement tasks. The level of difficulty of the tasks was changed by changing the training time (time spent exploring the objects on training day) and the number of objects (two or three). The rats were given a three-day wash-out period in between each task, and the level of difficulty was increased in each task (by increasing the number of objects or decreasing the training time).

a)


Training
Testing
24 hrs
2x5 min
5 min


80
60
40
20
0
% Exploration
*
Object A
Object B
Object C
Train
Test

b)


Training
Testing
24 hrs
5 min
5 min

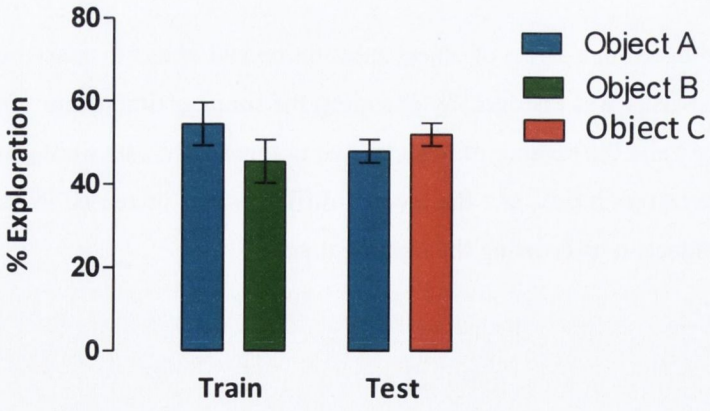
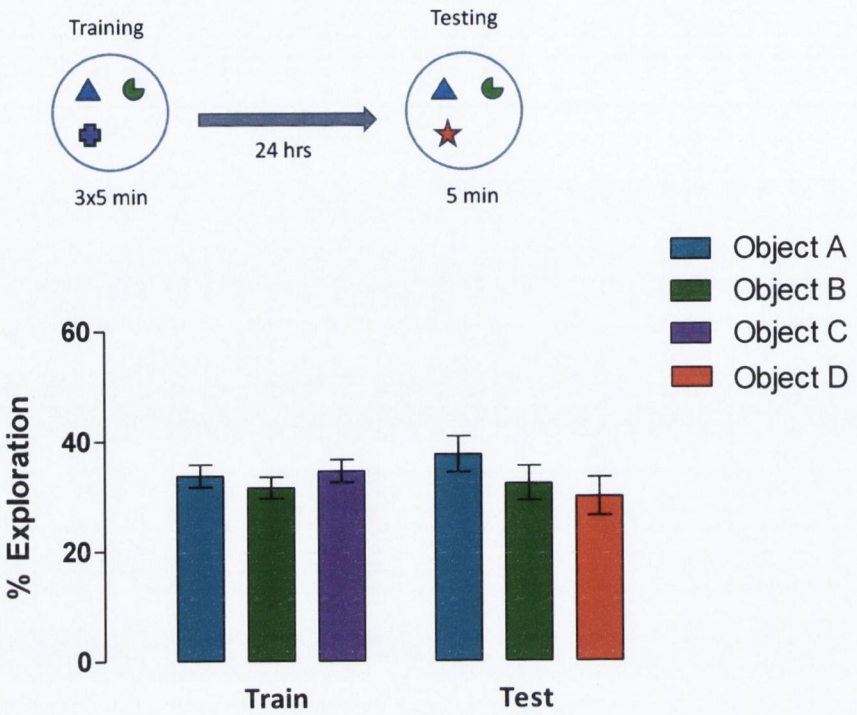

80
60
40
20
0
% Exploration
Object A
Object B
Object C
Train
Test

Fig 5.2 Control rats preferentially explore the novel object in a 2-object OS task with 10 minutes of training, but not with 5 minutes of training.

Data are presented as exploration time for each object (A, B or C, where C is the novel object) as a percentage of the total exploration time. Schematics above each graph describe the cognitive task. The graphs show the results for training day (Train) and testing day (Test). With 2x5 minute trials of exploration (a), control rats spent a similar amount of time exploring each of the three objects on training day. On testing day, control rats preferentially explored the novel object (C), indicating that they could remember the familiar objects and identify the novel object. With 1x5 minute trial of exploration (b), rats spent a similar amount of time exploring each of the three objects on training day. On testing day, control rats spent a similar amount of time exploring each of the three objects, indicating that they could not remember the familiar objects and identify the novel object (C). All data are shown as means $\pm$ SEM. The asterisk denotes data sets of significant difference as assessed by a Student's t-Test. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

a)


Training
Testing
24 hrs
3x5 min
5 min
Object A
Object B
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% Exploration
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40
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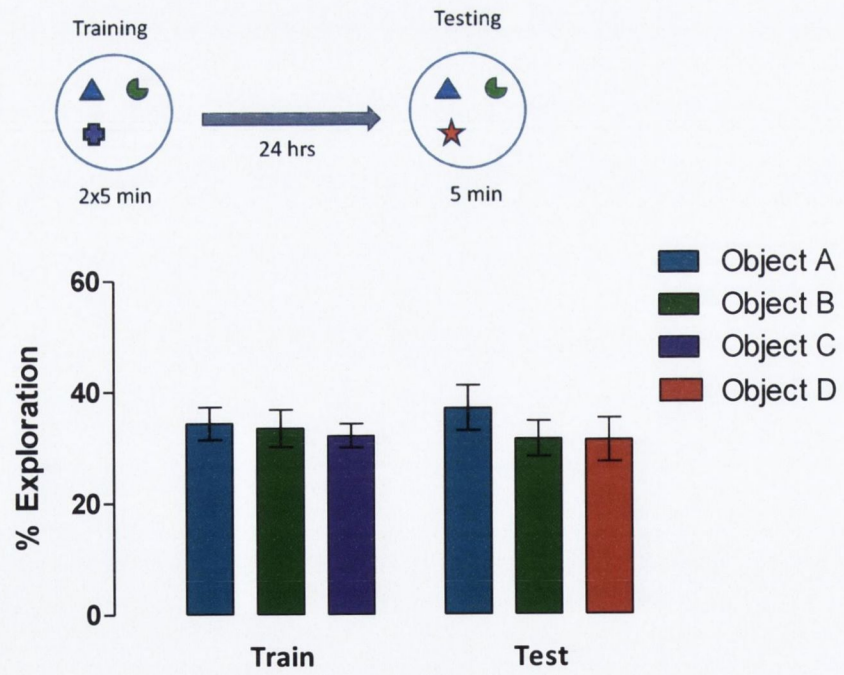
Train
Test

b)


Training
Testing
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2x5 min
5 min
Object A
Object B
Object C
Object D
% Exploration
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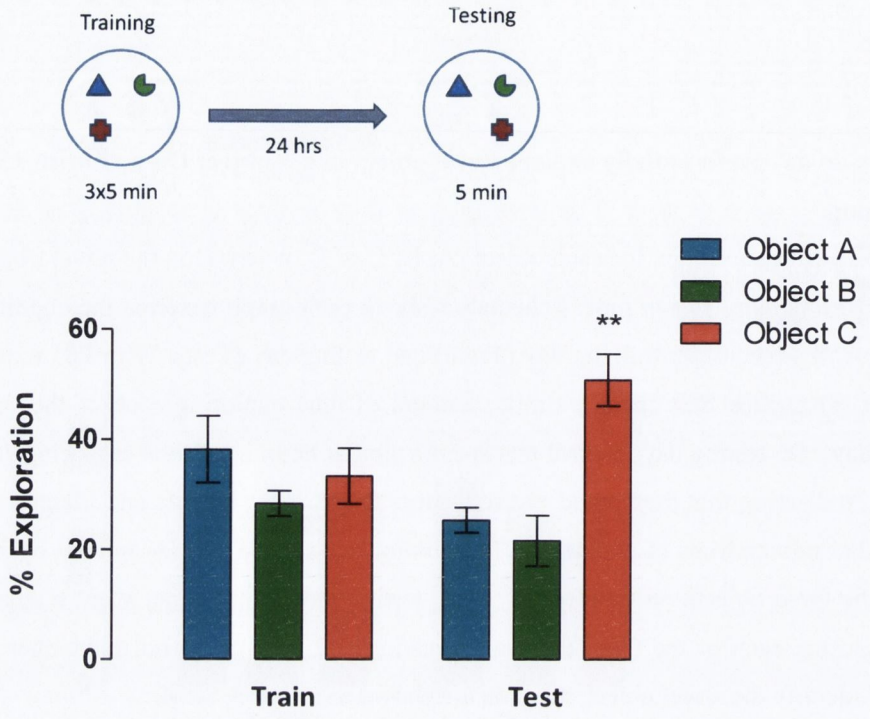
40
20
0
Train
Test

Fig 5.3 Control rats do not preferentially explore novel object in a 3-object OS task with 15 or 10 minutes of training.

Data are presented as exploration time for each object (A, B, C or D, where D is the novel object) as a percentage of the total exploration time. Schematics above each graph describe the cognitive task. The graphs show the results for training day (Train) and testing day (Test). With 3x5 minute trials of exploration (a), control rats spent a similar amount of time exploring each of the three objects on training day. On testing day, control rats spent a similar amount of time exploring each of the three objects, indicating that they could not remember the familiar objects and identify the novel object. With 2x5 minute trials of exploration (b), control rats spent a similar amount of time exploring each of the three objects on training day. On testing day, control rats spent a similar amount of time exploring each of the three objects, indicating that they could not remember the familiar objects and identify the novel object. All data are shown as means $\pm$ SEM.

a)


Training
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24 hrs
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Object A
Object B
Object C
% Exploration
60
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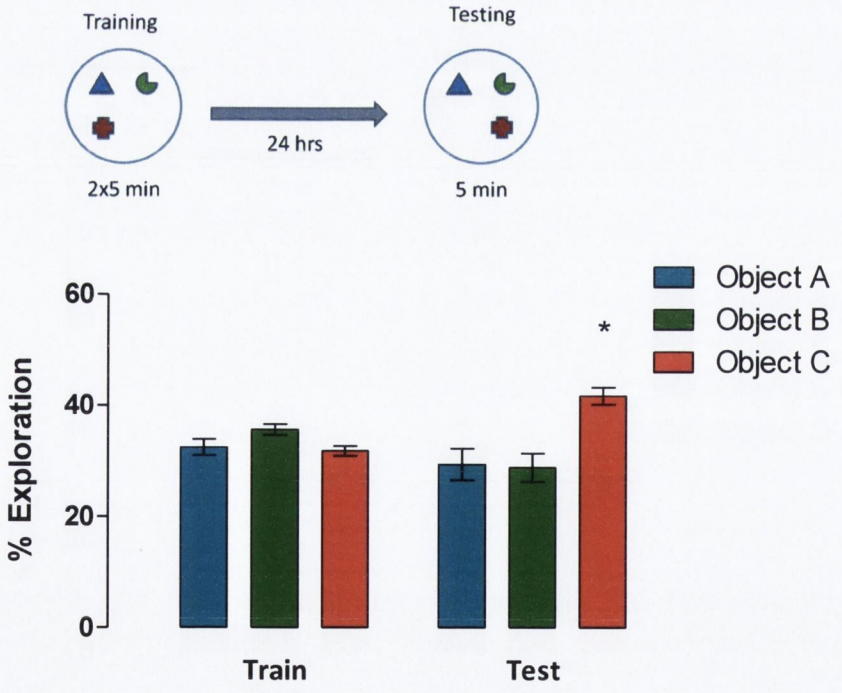
Train
Test

b)


Training
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Object A
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Train
Test

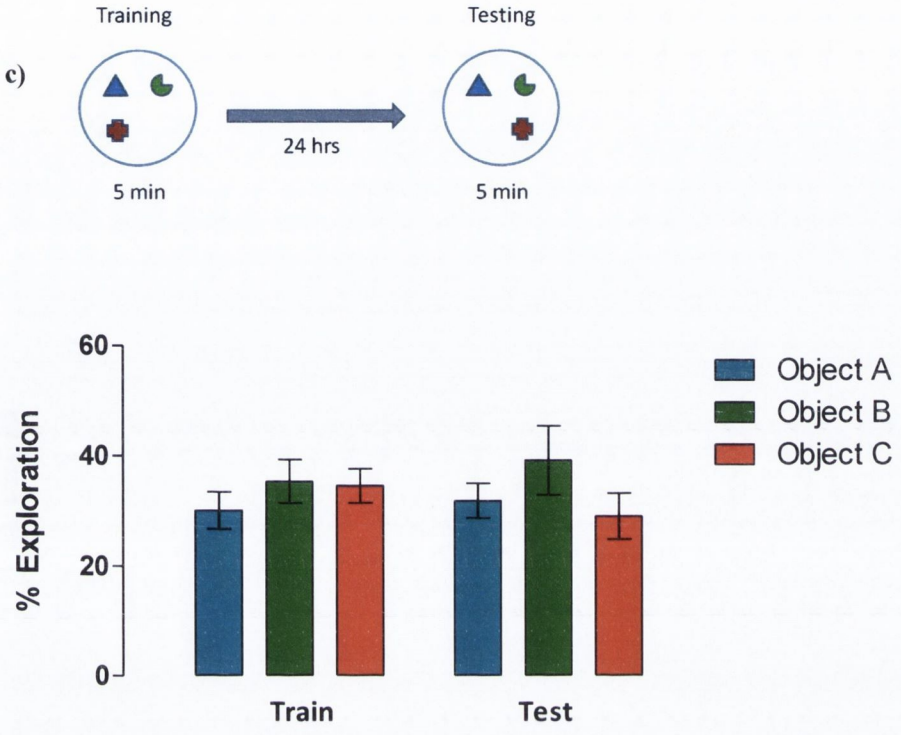


Fig 5.4 Control rats preferentially explore displaced object in a 3-object OD task with 3x5 and 2x5 minutes of training, but not with 1x5 minutes of training.

Data are presented as exploration time for each object (A, B or C, where C is the displaced object) as a percentage of the total exploration time. The schematics describe the cognitive tasks. The graphs (a,b,c) depict the results for training day (Train) and testing day (Test). With 3x5 minutes of training (a), control rats (n=6) preferentially explore the displaced object (C) on the testing day. With 2x5 minutes of training (b), control rats (n=6) preferentially explore the displaced object (C) on the testing day. With 1x5 minutes of training (c), control rats (n=6) do not preferentially explore any one object on testing day. All data are shown as mean % exploration time for each object $\pm$ SEM. The asterisk denotes data sets of significant difference as assessed by a two-way ANOVA with *Post-hoc* Bonferroni comparisons. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

5.3.2 Experiment 2: The effect of one week of exercise on performance in a challenging spatial task

5.3.2.1 Exercised rats preferentially explore the displaced object in a 3-object OD task with 5 minutes of training

During the training period, both exercised (Ex) and sedentary (Sed) rats spent a similar amount of time exploring each of the three objects (A, B and C), indicating that animals had no preference for any specific object (Fig 5.5a, Sed: Object A: 38.13 $\pm$ 6.64 %, Object B: 27.53 $\pm$ 6.13 %, Object C: 34.34 $\pm$ 2.9 %, Ex: Object A: 34.03 $\pm$ 1.76 %, Object B: 32.15 $\pm$ 3.5 %, Object C: 33.82 $\pm$ 4 %). During the testing period, the Ex group preferentially explored the displaced object C, while the sedentary group spent a similar amount of time exploring each of the three objects (A, B and C) (Fig 5.5b, Sed: Object A: 31.15 $\pm$ 5.22 %, Object B: 36.65 $\pm$ 3.44 %, Object C: 32.2 $\pm$ 5.8 %, Ex: Object A: 22.68 $\pm$ 3.38 %, Object B: 26.07 $\pm$ 5.87 %, Object C: 51.25 $\pm$ 4.13 %).

A two-way ANOVA was performed with 'exercise' as the first independent variable and 'object' as the second. *Post-hoc* analysis was performed using the Bonferroni multiple comparisons *Post-hoc* test. There was an overall effect of object ($P=0.0008$, $F_{(2,30)} = 9.076$) indicating a preference for the displaced object. *Post-hoc* analysis revealed that the Ex group spent significantly more time exploring the displaced object C compared to stationary objects A and B ($p<0.001$). This indicates that the exercised group could remember the location of the objects and identify the displaced object C. The Sed group spent a similar amount of time exploring each of the three objects indicating that they could not remember the location of the objects and identify the displaced object C. A significant interaction ($p=0.0013$) indicates that the independent variables of 'exercise' and 'object' had a significant effect on each other. Time spent exploring each of the objects was recorded and reported as a percentage of the total exploration time, mean ± SEM.

5.3.2.2 One week of exercise increases the concentration of BDNF in the serum

Analysis of serum samples using a BDNF ELISA showed a significant increase in serum BDNF concentration in the exercised group compared to the sedentary group ($p=0.035$, Student's *t*-Test, Fig 5.7, Sed: 400 $\pm$ 108.7 pg/ml, Ex: 621 $\pm$ 162.4 pg/ml).

5.3.2.3 One week of exercise increases BDNF mRNA expression the dentate gyrus

In the dentate gyrus, BDNF mRNA expression was significantly increased in exercised rats compared with sedentary rats, ($p=0.03$, Student's t-Test, Fig 5.8, Sed BDNF : 1 ± 0.42, Ex BDNF fold change: 1.77 ± 0.2.

5.3.2.4 One week of exercise does not significantly affect the concentration of BDNF in the dentate gyrus, perirhinal cortex, and hippocampus

No significant changes in BDNF protein concentration were observed in the dentate gyrus (Fig 5.9a, Sed: 77.13 ± 4.46 pg/mg, Ex: 108.6 ± 15.97 pg/mg), perirhinal cortex (Fig 5.9b, Sed: 71.78 ± 10.59 pg/mg, Ex: 72.99 ± 10.37 pg/mg), and hippocampus (Fig 5.9c, Sed: 146 ± 15.59 pg/mg, Ex: 140 ± 12.88 pg/mg) when normalised to total protein.

5.3.2.5 One week of exercise increases the number of proliferating cells in the dentate gyrus

The average number of BrdU positive cells (taken as a percentage of the total number of cells counted) was significantly increased in the dentate gyrus of exercised rats compared to sedentary controls ($p=0.0167$, Student's *t*-Test, Fig 5.10, Sed: 1.69 ± 0.74 %, Ex: 4.69 ± 2.11 %).

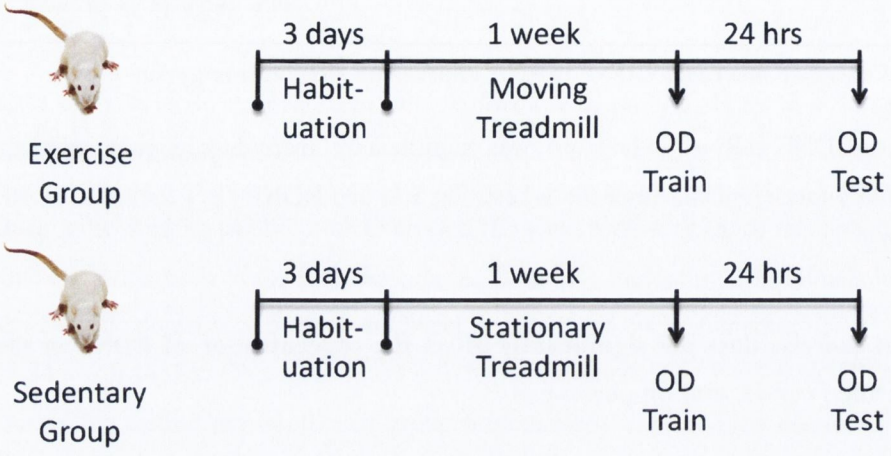
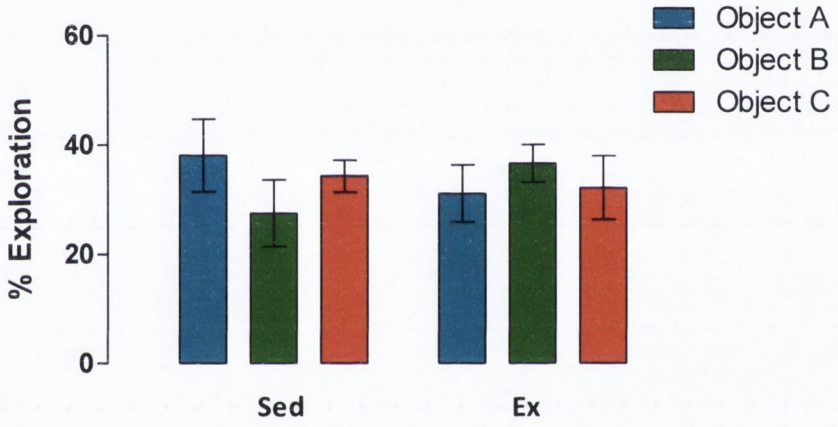


Fig 5.5 Study Design of Experiment 2: The effect of one week of forced exercise on the cognitive performance of rats in a challenging spatial task.

Rats (n=12) were randomly assigned to an exercise (Ex, n=6) group and a sedentary control group (Sed, n=6). After a period of habituation to the treadmill (3 days), the Ex group was exercised at 1 km/hour for one hour daily for seven consecutive days, while the Sed group was placed on a stationary treadmill for the same duration. On the last day of exercise, all rats were trained in a 3-object object displacement task with 5 minutes of training, and tested 24 hours later after displacing one of the objects.

a) Training



b) Testing

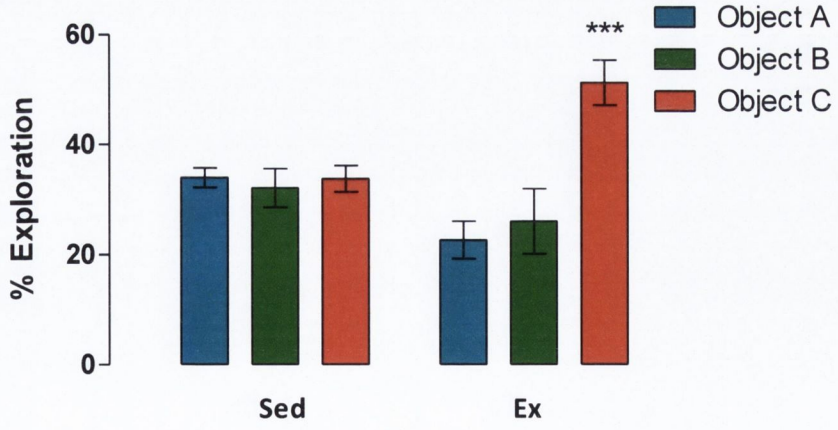


Fig 5.6 Exercised rats preferentially explore the displaced object in a 3-object OD task with 5 min training.

Data are presented as exploration time for each object (A, B or C, where C is the displaced object) as a percentage of the total exploration time. The schematic (top) describes the task. The graphs depict the results for training day (a) and testing day (b). Neither sedentary rats (Sed, n=6) nor exercised rats (Ex, n=6) showed a preference for any one object on the training day (a). On the testing day, the exercised rats preferentially explored the displaced object, while the sedentary rats did not (c). All data are shown as mean % exploration time for each object + SEM. The asterisk denotes data sets of significant difference as assessed by a two-way ANOVA with Bonferroni multiple comparisons as a post-hoc test. (*p<0.05, **p<0.01, ***p<0.001)

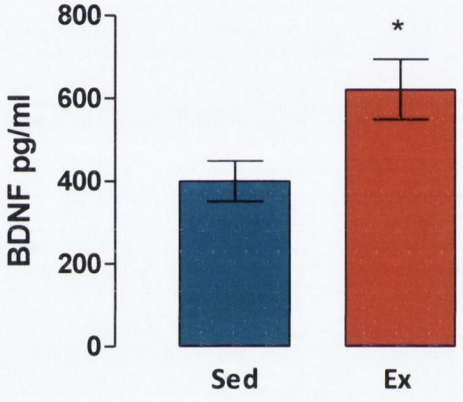


Fig 5.7 One week of exercise induces an increase in BDNF protein in the serum.
Concentrations of BDNF protein in serum are presented as assessed by an ELISA. In the serum, the concentration of BDNF was increased compared to that of sedentary controls (p=0.035, t-Test). Concentrations are in pg BDNF per ml of serum. All data are shown $\pm$ SEM.

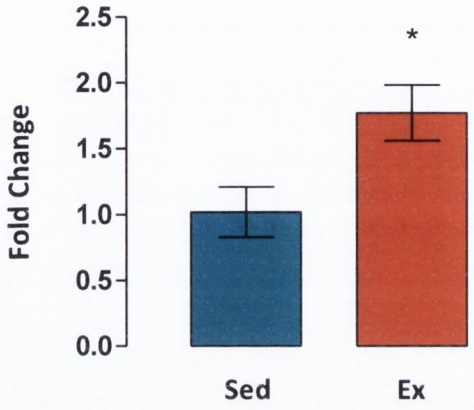
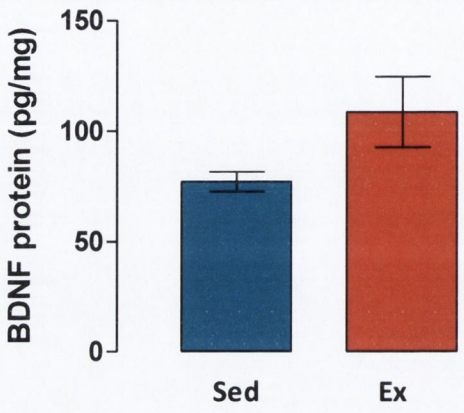


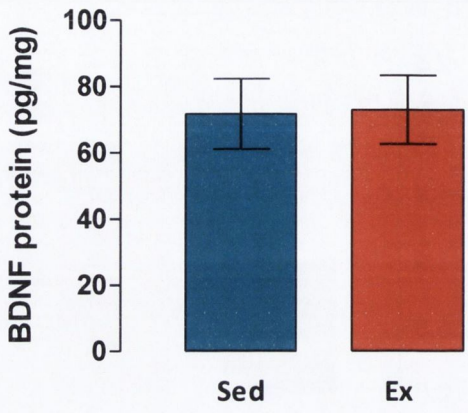
Fig 5.8 One week of exercise induces an increase in BDNF mRNA expression in the dentate gyrus.

Concentrations of BDNF mRNA in the dentate gyrus are presented as a fold change increase above the control group as assessed by a PCR. All data are shown ± SEM. The asterisk denotes data sets of significant difference as assessed by a Student's *t*-Test. (* p=0.03)

a) Dentate Gyrus



b) Perirhinal Cortex



c) Hippocampus

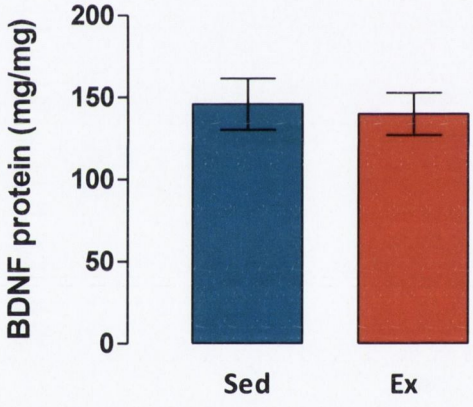


Fig 5.9 One week of exercise has no effect on BDNF protein in the dentate gyrus, perirhinal cortex, and hippocampus

Concentrations of BDNF protein normalised to total protein are presented as assessed by an ELISA. In the dentate gyrus (a), perirhinal cortex (b) and hippocampus (c) no difference was observed between Ex and Sed groups All data are shown in pg/mg $\pm$ SEM.

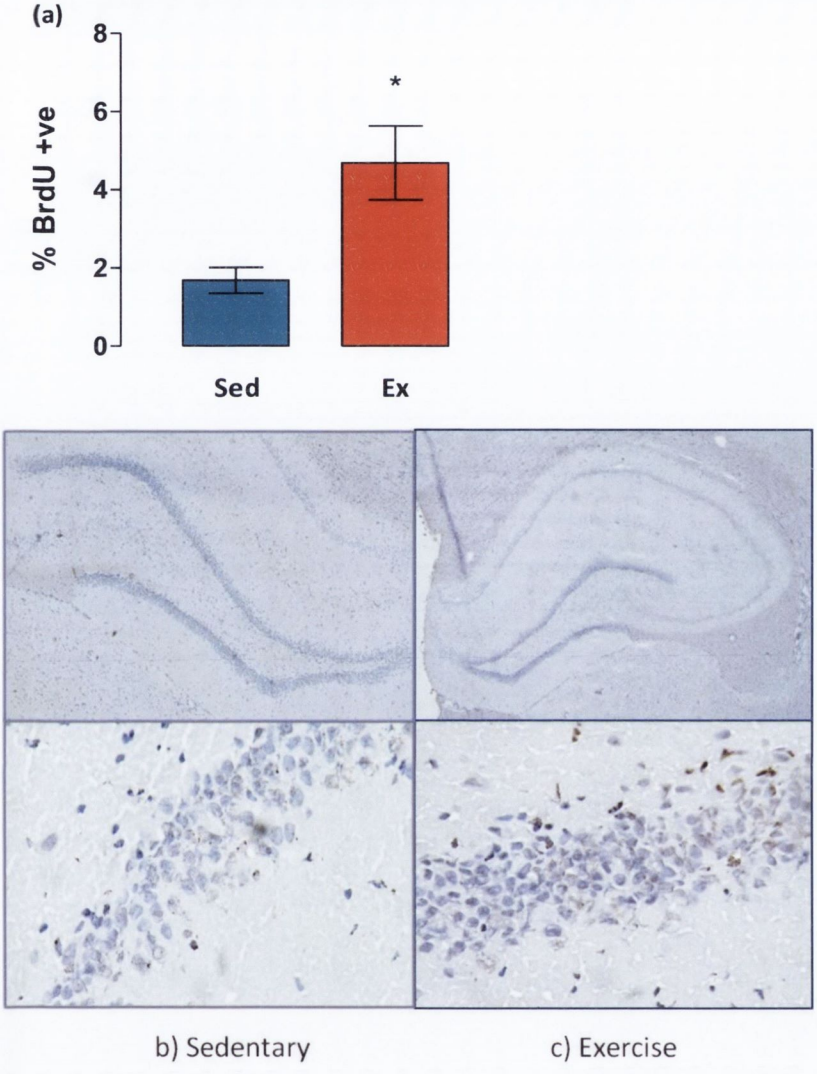


Fig 5.10 One week of exercise induces an increase in BrdU positive cells in the dentate gyrus. The graph (a) presents the data as average number of BrdU positive cells taken as a percentage of the total number of cells counted in the dentate gyri of sedentary (Sed) and exercised (Ex) rats. All Data are mean % BrdU +ve cells ± SEM. The asterisk denotes a significant difference between the groups as evaluated by a Student's t-Test. The representative pictures (b,c) show the hippocampus from a sedentary rat (b, top) and an exercised rat (c, top), and the dentate gyrus from an exercised rat (b, bottom) and a sedentary rat (c, bottom). Blue cells are hematoxylin stained (stains all nuclei), while brown cells are BrdU +ve (DAB chromagen stained).

5.3.3 Experiment 3: The effect of one week of exercise on BDNF-stimulated signaling events

5.3.3.1 Exercised rats preferentially explore the displaced object in a 3-object OD task with 5 minutes of training

During the training period, both exercised (Ex) and sedentary (Sed) rats spent a similar amount of time exploring each of the three objects (A, B and C), indicating that animals had no preference for any specific object (Fig 5.12a, Sed: Object A: 28.26 ± 3.41 %, Object B: 37.16 ± 3 %, Object C: 34.58 ± 5 %, Ex: Object A: 36.13 ± 6.43 %, Object B: 32.61 ± 5.65 %, Object C: 31.27 ± 6.06 %). During the testing period, the Ex group preferentially explored the displaced object C, while the sedentary group spent a similar amount of time exploring each of the three objects (A, B and C) (Fig 5.12b, Sed: Object A: 31.08 ± 3.05 %, Object B: 33.51 ± 5.2 %, Object C: 35.41 ± 2.6 %, Ex: Object A: 27.78 ± 4.1 %, Object B: 26.88 ± 2.18 %, Object C: 45.34 ± 2.96 %).

A two-way ANOVA was performed with 'exercise' as the first independent variable and 'object' as the second. *Post-hoc* analysis was performed using the Bonferroni multiple comparisons *Post-hoc* test. There was an overall effect of object ($P=0.0061$, $F_{(2,30)} = 6.083$) indicating a preference for the displaced object. *Post-hoc* analysis revealed that the Ex group spent significantly more time exploring the displaced object C compared to stationary objects A and B ($p<0.01$). This indicates that the exercised group could remember the location of the objects and identify the displaced object C. The Sed group spent a similar amount of time exploring each of the three objects indicating that they could not remember the location of the objects and identify the displaced object C. The interaction was not significant ($p=0.058$), indicating that the independent variables of 'exercise' and 'object' did not have a significant effect on each other. Time spent exploring each of the objects was recorded and reported as a percentage of the total exploration time, mean ± SEM.

5.3.3.2 Exercise induces an increase in KCl-stimulated release of BDNF from dentate gyrus.

Under constitutive conditions, no difference was observed in the amount of BDNF released from slices of dentate gyrus in any group (Fig 5.14, Non-L: 250.1 ± 58.23 pg/mg, Sed: 295.2 ± 95.9 pg/mg, Ex: 305.4 ± 7.07 pg/mg). Upon stimulation with 50mM KCl, BDNF release was significantly increased from the dentate gyrus of the exercised group compared with the non-learning group ($p=0.0342$. $F_{(2,12)}=4.82$, one-way ANOVA, Fig 5.14, Non-L: 203.2 ± 70.96 pg/mg,

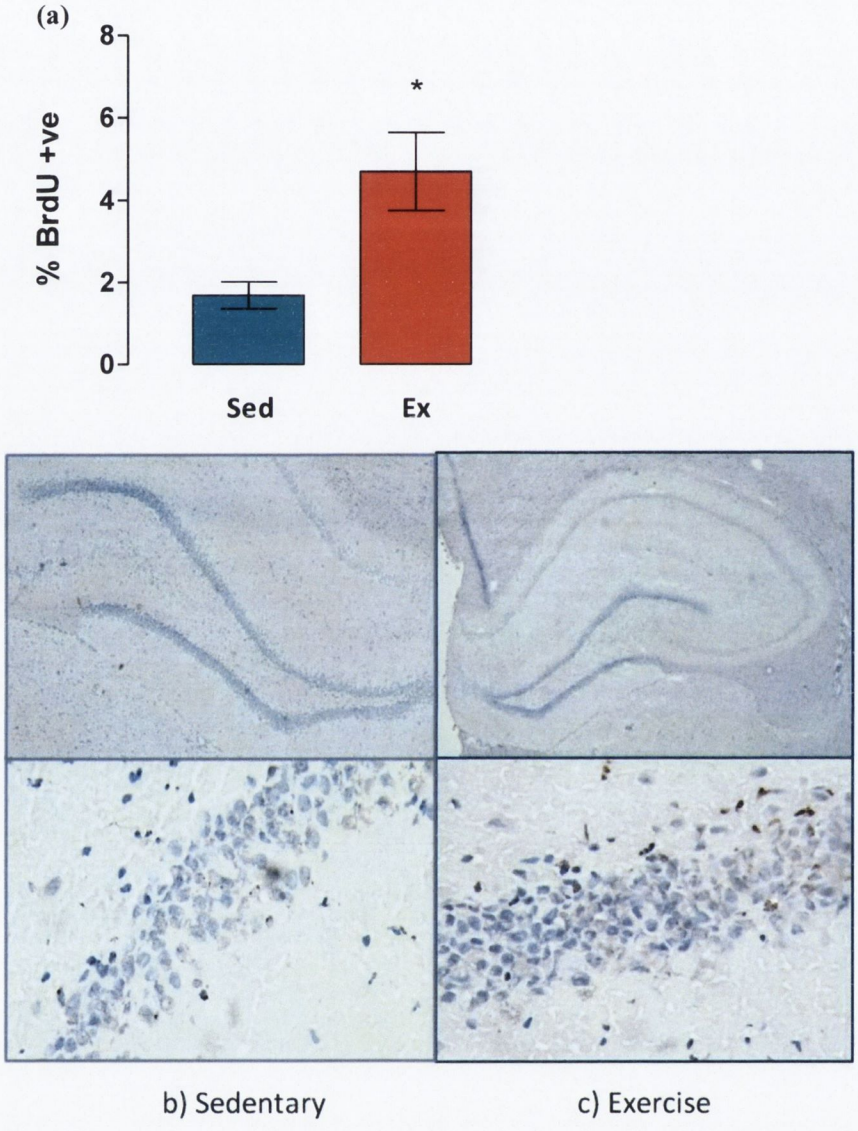


Fig 5.10 One week of exercise induces an increase in BrdU positive cells in the dentate gyrus. The graph (a) presents the data as average number of BrdU positive cells taken as a percentage of the total number of cells counted in the dentate gyri of sedentary (Sed) and exercised (Ex) rats. All Data are mean % BrdU +ve cells ± SEM. The asterisk denotes a significant difference between the groups as evaluated by a Student's t-Test. The representative pictures (b,c) show the hippocampus from a sedentary rat (b, top) and an exercised rat (c, top), and the dentate gyrus from an exercised rat (b, bottom) and a sedentary rat (c, bottom). Blue cells are hematoxylin stained (stains all nuclei), while brown cells are BrdU +ve (DAB chromagen stained).

Sed: 358.2 ± 31.73 pg/mg, Ex: 488.5 ± 75.89 pg/mg). *Post-hoc* analysis showed that the increase in the Ex group was significant only when compared to the Non-L group ($p<0.05$, Tukey). After stimulation, the remaining pellet was homogenised and tested for BDNF remaining in the cells. No significant differences were observed in BDNF released from the cell pellet between the groups (Fig 5.14, Non-L: 161.8 ± 50.77 pg/mg, Sed: 121.8 ± 36.15 pg/mg, Ex: 360.9 ± 129.5 pg/mg).

5.3.3.3 Exercise induces an increase in activated TrkB receptor in the dentate gyrus

Dentate gyrus homogenate was analysed for TrkB expression and activation using ELISA. There was a significant increase in phosphorylated TrkB in the dentate gyrus of exercised rats compared with controls (Fig 5.15a, $p=0.03$, one-way ANOVA, Non-L: 58.93 ± 8.55 pg/mg, Sed: 71.56 ± 13.55 pg/mg, Ex: 104.4 ± 10.86 pg/mg), when normalised to total protein. Post-hoc comparison of individual groups revealed that the increase was significant only in comparison to the non-learning group ($p<0.05$, Tukey). The expression of the TrkB receptor did not vary significantly among the three groups (Fig 5.15b, Non-L: 137.7 ± 34.9 pg/mg, Sed: 183 ± 56.75 pg/mg, Ex: 281 ± 61.43 pg/mg).

5.3.3.4 Exercise induces an increase in TrkB mRNA expression in the dentate gyrus, but not in the perirhinal cortex and hippocampus.

In the dentate gyrus, TrkB mRNA expression was significantly increased in exercised rat ($p= 0.0183$, one-way ANOVA, Fig 5.16a, Non-L : 1 + 0.16, Sed fold change: 1.1 ± 0.08, Ex fold change: 1.36 ± 0.28). In the perirhinal cortex, TrkB mRNA expression was similar in all three groups (Fig 5.16b, Non-L : 1 ± 0.3, Sed fold change: 1.23 ± 0.14, Ex fold change: 1.36 ± 0.28).In the hippocampus, TrkB mRNA expression was similar in all three groups (Fig 5.16c, Non-L : 1 ± 0.16, Sed fold change: 1.1 ± 0.52, Ex fold change: 0.95 ± 0.29).

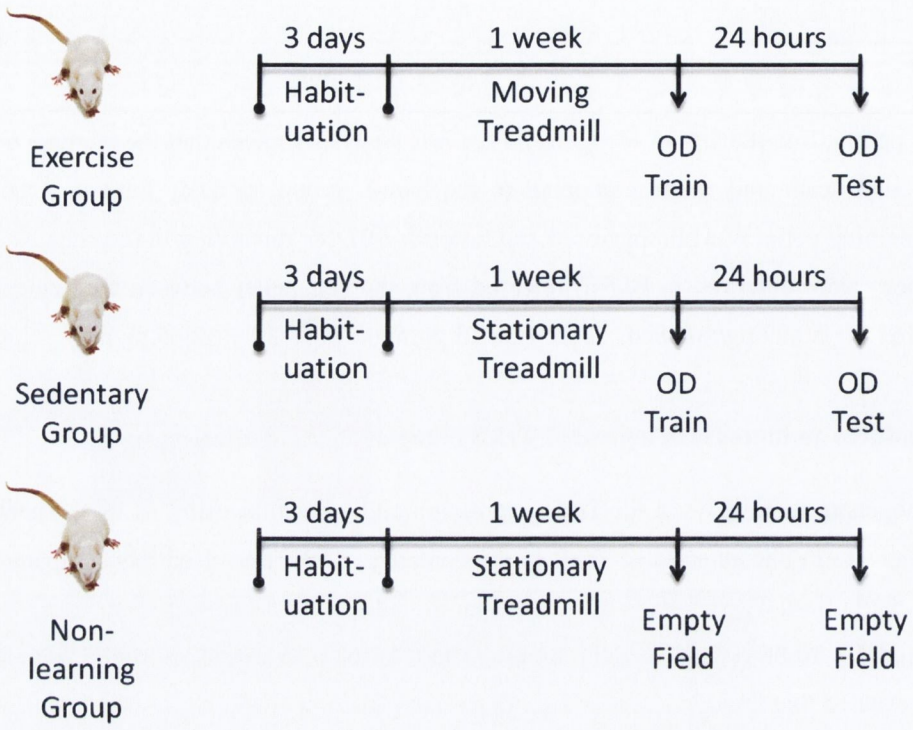


Fig 5.11 Study design of Experiment 3: The effect of exercise on BDNF-stimulated cell signaling mechanisms

Rats (n=6 per group) were randomly assigned to one of three groups: exercise (Ex), sedentary (Sed), and a non-learning group (Non-L). The exercised group (Ex) performed the one-week forced exercise protocol as described in section 2.3, while sedentary groups (Sed and Non-L) were placed on a stationary treadmill for the same duration. After exercise on the 7th day, Sed and Ex groups were tested in an object displacement task. Non-L rats did not perform the cognitive task, but were placed in the empty open field (objects and spatial cues removed) for 5 minutes on both the training and testing day. Immediately after testing (8th day), tissue and blood was collected for analysis.

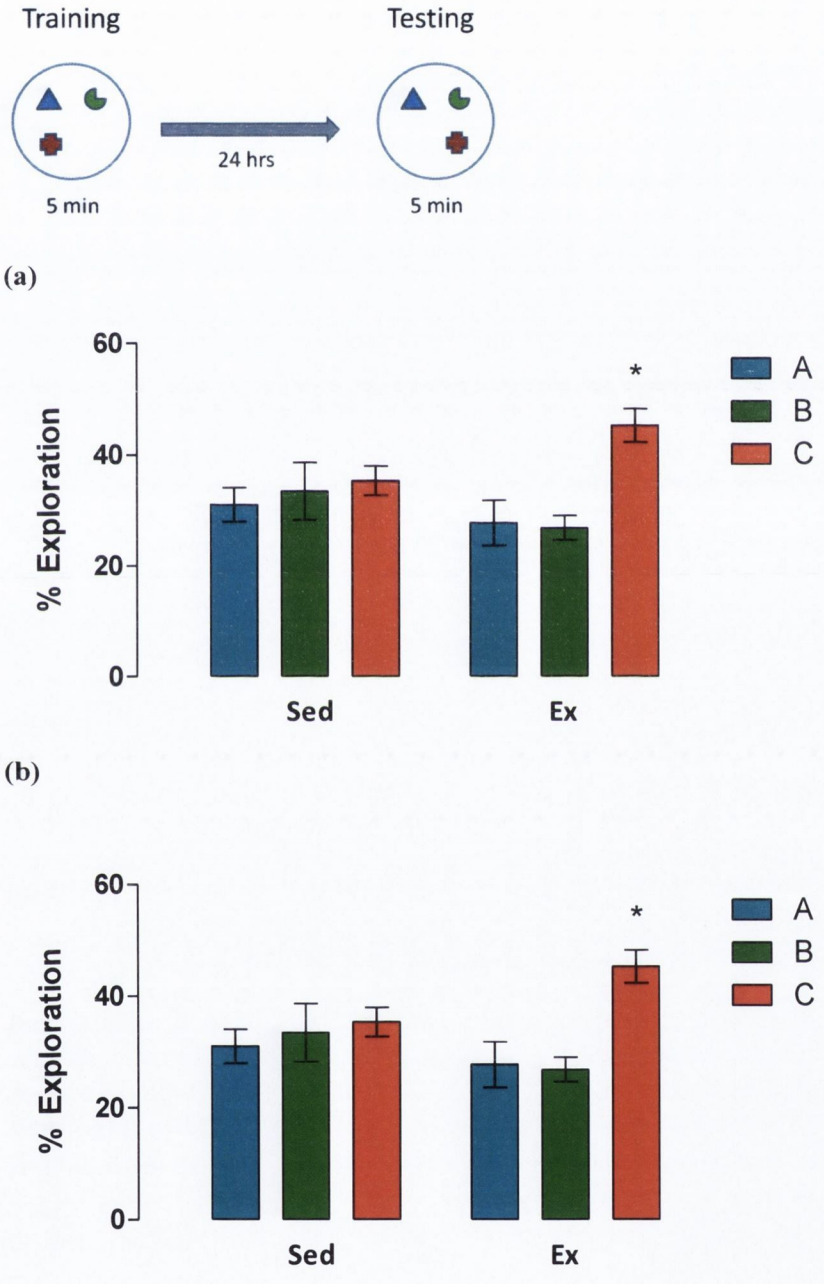


Fig 5.12 Exercised rats preferentially explore the displaced object in a 3-object OD task with 5 min training.

Data are presented as exploration time for each object (A, B or C, where C is the displaced object) as a percentage of the total exploration time. The schematic (top) shows the cognitive task. The graphs depict the results for training day (a) and testing day (b). Neither sedentary rats (Sed, n=6)

nor exercised rats Ex, (n=6) showed a preference for any one object on the training day. On the testing day, the exercised rats preferentially explored the displaced object, while the sedentary rats did not. All data are shown as mean % exploration time for each object ± SEM. The asterisk denotes data sets of significant difference as assessed by a two-way ANOVA with *Post-hoc* Bonferroni comparisons. (*p<0.05, **p<0.01, ***p<0.001)

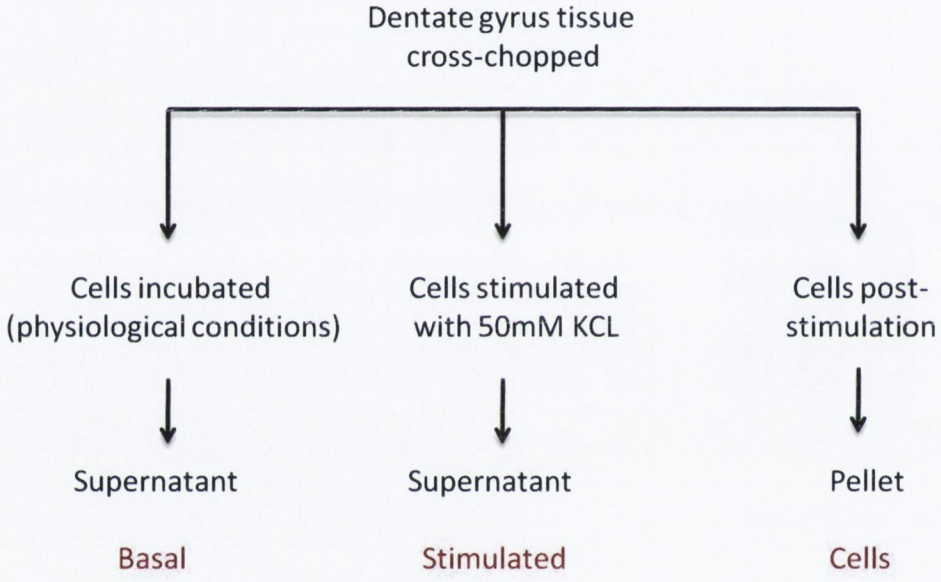


Fig 5.13 Study design for BDNF release experiment

Tissue collected from the dentate gyrus of the hippocampus was cross-chopped, washed and suspended in oxygenated Krebs at physiological conditions (37°C). The supernatant from the physiological condition (Basal) was removed and assessed for BDNF. The cells were then resuspended and stimulated with a high KCL solution, the supernatant (Stimulated) was removed and assessed for BDNF. The remaining pellet was homogenized and assessed for BDNF (Cells).

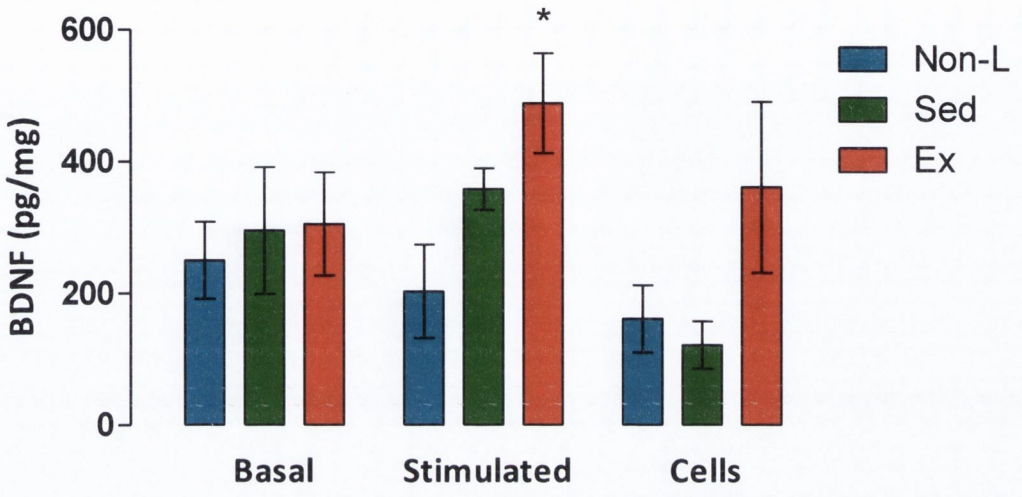


Fig 5.14 Exercise induces an increase in the amount of BDNF released from cells of the dentate gyrus upon stimulation.

For the basal condition, the amount of BDNF was similar for all three groups. For the stimulated condition, the amount of BDNF released by cells from the exercised groups was increased over that of the Non-L controls (one-way ANOVA, p=0.028). The amount of BDNF remaining in the cells seemed higher in the exercised group, but this did not reach statistical significance (one-way ANOVA).

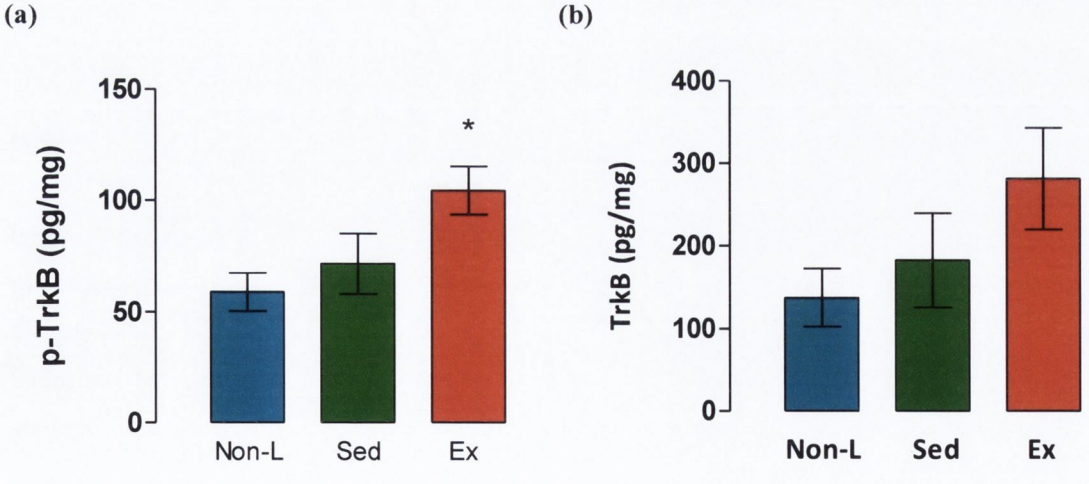


Fig 5.15 Exercise induces an increase in phosphorylation of TrkB in the dentate gyrus.

Concentrations of phosphorylated TrkB (a) and total TrkB (b) protein are presented as assessed by an ELISA. The amount of p-TrkB is significantly increased in the dentate gyrus of exercised rats

compared to Non-L controls (a). The amount of total TrkB is not significantly affected (b). All data are shown in pg/mg protein ± SEM. The asterisk denotes data sets of significant difference as assessed by a one-way ANOVA with *Post-hoc* Tukey comparisons.

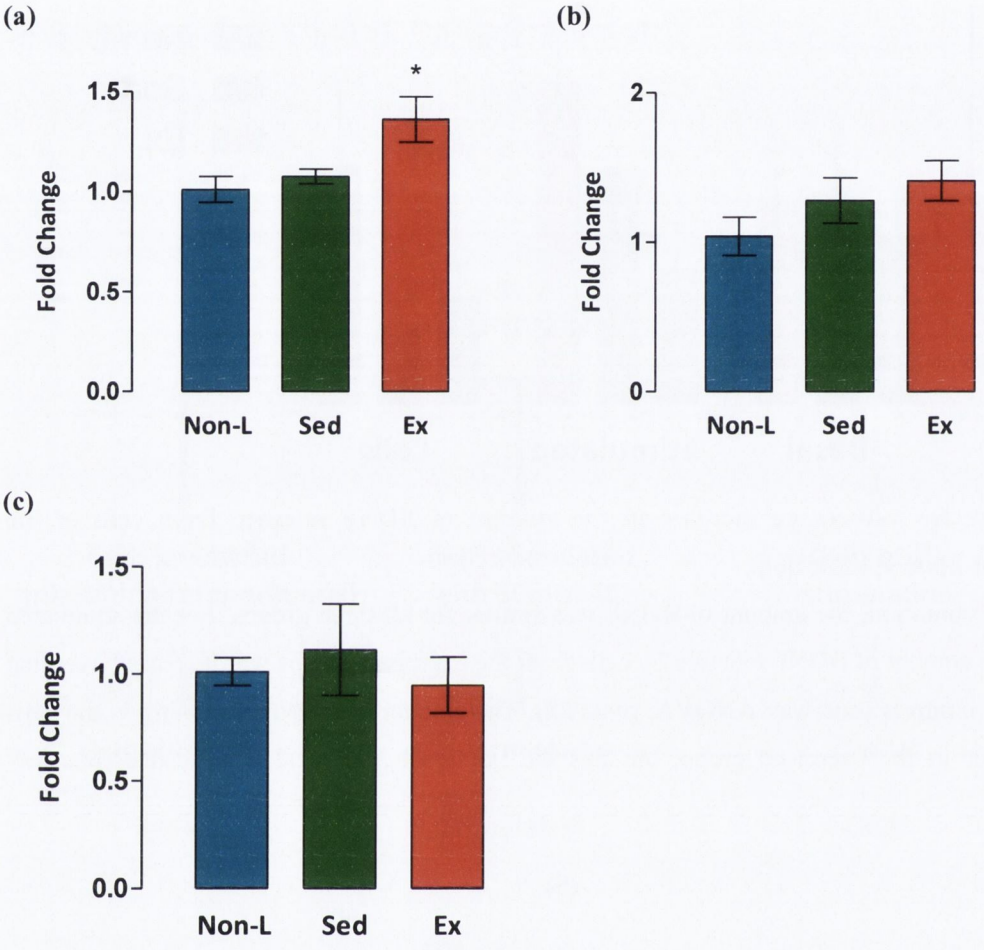


Fig 5.16 Exercise induces an increase in TrkB mRNA expression in the dentate gyrus, but not in the perirhinal cortex and entorhinal cortex.

This graph shows the fold changes in TrkB mRNA in (a) dentate gyrus, (b) perirhinal cortex, and (c) hippocampus as assessed by PCR. TrkB mRNA expression in the dentate gyrus is elevated in the exercised group (Ex) relative to sedentary controls (Sed, Non-L). All mRNA data are expressed as fold change ± SEM. No changes in TrkB mRNA expression were observed in the perirhinal cortex (b) or hippocampus (c) across groups. The asterisk denotes data sets of significant difference as assessed by a one-way ANOVA with *Post-hoc* Tukey comparisons. (* $p<0.05$)

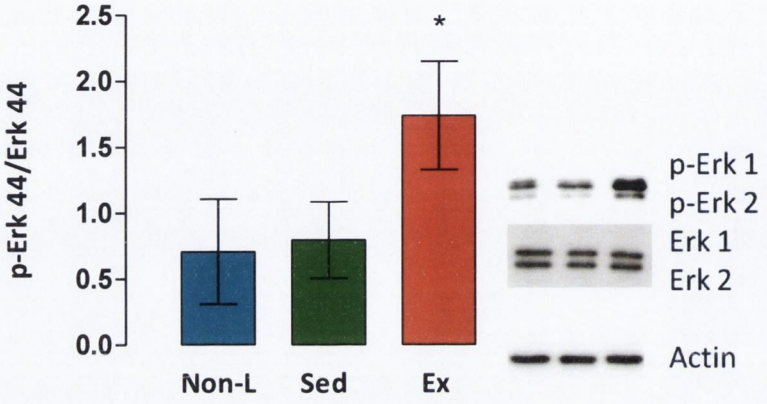
5.3.3.5 Exercise induces an increase in phosphorylation of ERK 1 (ERK 44) in the dentate gyrus.

Western blot analysis revealed a significant increase in phosphorylated ERK 1 (ERK44) in the exercised group compared with controls (Fig 5.17a, $p=0.049$; one-way ANOVA), when normalised to total ERK 1. A post-hoc test revealed that this increase was significant only when compared to the non-learning group ($p<0.05$, Tukey post-Test). No changes were observed in p-ERK2 (Fig 5.17b) when normalised to total ERK2. No changes were observed in total ERK1 (Fig 5.17c) or total ERK2 (Fig 5.17d) when normalised to β-actin.

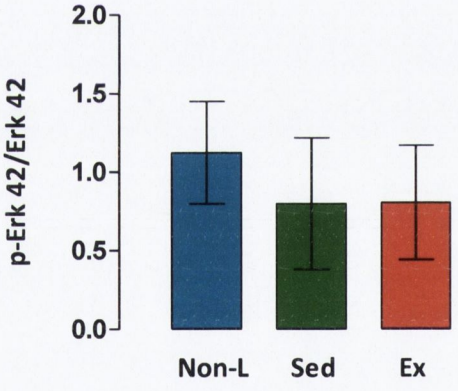
5.3.3.6 Exercise induces an increase in Synapsin-1 in the dentate gyrus

Western blot analysis revealed an increase in Synapsin-1 in dentate gyrus with exercise, when normalised to β-actin (Fig 5.18a, $p=0.042$, one way ANOVA). This increase was only significant when compared to the non-learning group, as revealed by a post-hoc test (Tukey). Phospho-synapsin-1 was also investigated, but showed no changes when normalised to Synapsin-1. No significant changes were observed in Synaptophysin (Fig 5.18b).

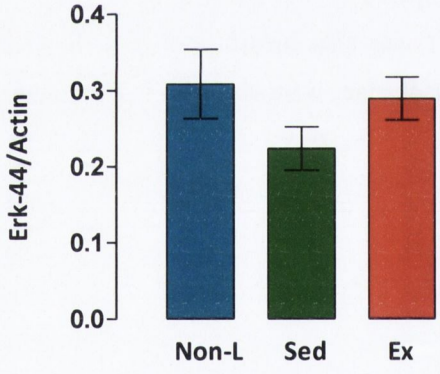
a)


p-Erk 44/Erk 44
2.5
2.0
1.5
1.0
0.5
0.0
*
Non-L
Sed
Ex
p-Erk 1
p-Erk 2
Erk 1
Erk 2
Actin

b)


p-Erk 42/Erk 42
2.0
1.5
1.0
0.5
0.0
Non-L
Sed
Ex

(c)


Erk-44/Actin
0.4
0.3
0.2
0.1
0.0
Non-L
Sed
Ex

d)

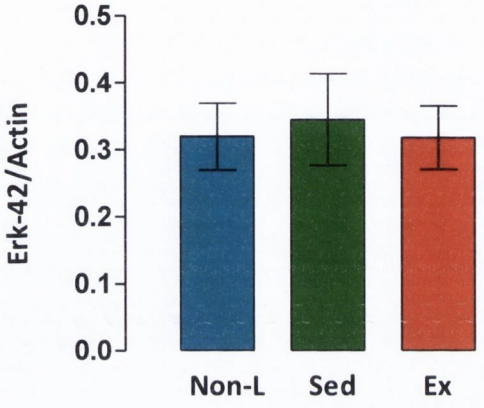
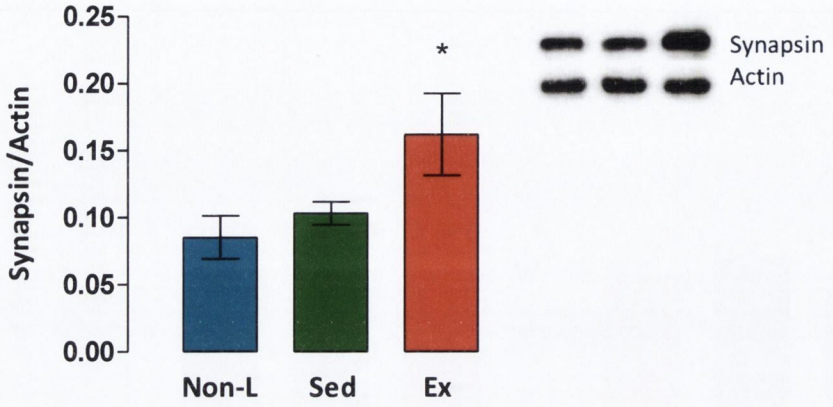


Fig 5.17 Exercise induces an increase in phosphorylated ERK1 (p44-ERK) in the dentate gyrus.

This figure (a) shows that exercise induced a significant increase in the amount of phosphorylated ERK1 (p44-ERK) in the exercised group compared to both controls (one-way ANOVA, $p=0.049$), when normalised to total ERK 1. No changes were observed in p-ERK2 when normalised to total ERK2 (b). No changes were observed in total ERK1 (c) or total ERK2 (d) when normalised to β-actin. All data are shown $\pm$ SEM. Representative bands are shown for the three groups (Non-L, Sed, Ex) and for all five target proteins (p-ERK1, p-ERK2, ERK1, ERK2, and β-actin).

a)



b)

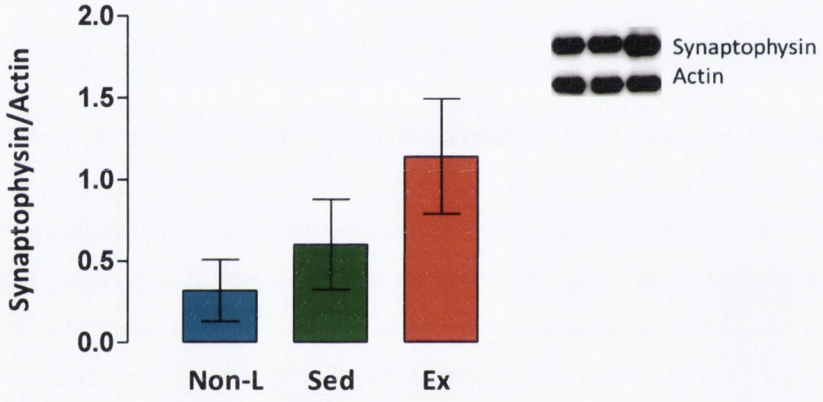


Fig 5.18 Exercise induces an increase in expression of Synapsin-1 in the dentate gyrus.

Graph (a) shows an increase in Synapsin-1 in the dentate gyrus with exercise, when normalised to β-actin (one-way ANOVA, $p=0.042$). This increase was only significant when compared to the non-learning group, as revealed by a post-hoc test (Tukey). No significant changes were observed in Synaptophysin. All data are shown $\pm$ SEM. Representative bands are shown for the three groups (Non-L, Sed, Ex) and for the target proteins (Synapsin-1, Synaptophysin, and β-actin).

5.3.4 Experiment 4: The effect of a single BDNF infusion (i.c.v.) on spatial memory

5.3.4.1 A single BDNF infusion (i.c.v.) does not affect total exploration of objects

Rats from both the BDNF and control groups exhibited normal exploratory behaviour in the open field on the training day. When total exploration time (in seconds) was summed for each group, there was no significant difference between the two groups as assessed by a Student's *t*-test (Fig 5.20, Cyt C: 58.61 ± 7.94 s, BDNF: 44.17 ± 6.97 s).

5.3.4.2 Rats infused with BDNF (i.c.v.) preferentially explore the displaced object in a 3-object object OD task with 1x5 minutes of training.

During the training period, both Cyt C and BDNF rats spent a similar amount of time exploring each of the three objects (A, B and C), indicating that animals had no preference for any specific object (Fig 5.21a, Cyt C: Object A: 35.3 ± 9.85 %, Object B: 22.7 ± 6.13 %, Object C: 41.99 ± 13.14 %, BDNF: Object A: 38.59 ± 7.82 %, Object B: 26.81 ± 8.34 %, Object C: 34.60 ± 9.69 %).

During the testing period, the BDNF group preferentially explored the displaced object C, while the Cyt C group spent a similar amount of time exploring each of the three objects (A, B and C) (Fig 5.21b, Cyt C: Object A: 32.12 ± 6.1 %, Object B: 31.14 ± 6.9 %, Object C: 36.74 ± 5.32 %, BDNF: Object A: 23.53 ± 4.57 %, Object B: 24.53 ± 4.88 %, Object C: 51.94 ± 8.86 %).

A two-way ANOVA was performed with 'BDNF' as the first independent variable and 'object' as the second. *Post-hoc* analysis was performed using the Bonferroni multiple comparisons *Post-hoc* test. There was an overall effect of object ($P=0.02$, $F_{(2,24)} = 2.21$) indicating a preference for the displaced object. *Post-hoc* analysis revealed that the BDNF group spent significantly more time exploring the displaced object C compared to stationary objects A ($p<0.01$, Bonferroni) and B ($p<0.05$, Bonferroni). This indicates that the BDNF group could remember the location of the objects and identify the displaced object C. The Cyt C group spent a similar amount of time exploring each of the three objects indicating that they could not remember the location of the objects and identify the displaced object C. The interaction was not significant. Time spent exploring each of the objects was recorded and reported as a percentage of the total exploration time, mean ± SEM.

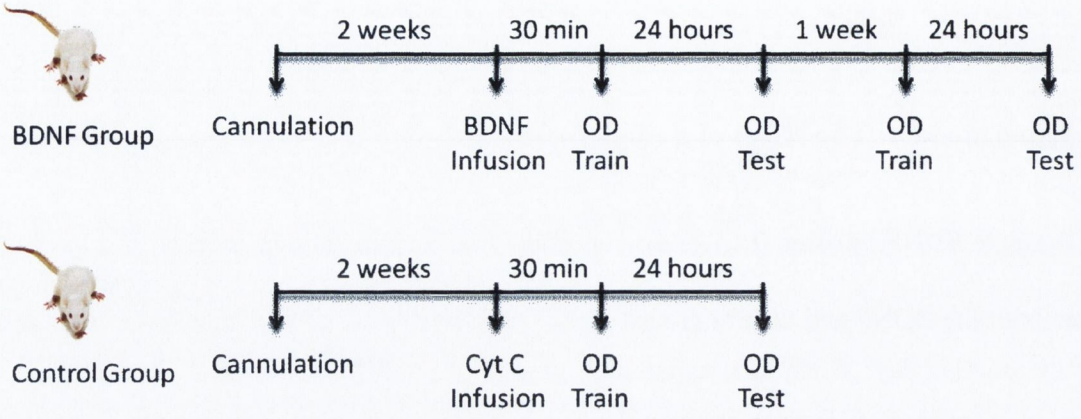


Fig 5.19 Study Design of Experiment 4: The effect of a single BDNF infusion (i.c.v.) on spatial memory.

Cannulated rats were allowed two weeks of recovery and then infused with a single bolus injection of human recombinant BDNF (10ng, i.c.v.). Control rats received a similar injection of Cytochrome c. All rats were trained in an object displacement task 30 minutes post-infusion, and were tested 24 hours later. One week later, the BDNF group was trained and tested in a new object displacement task to evaluate the persistence of the cognitive effect induced by the BDNF injection.

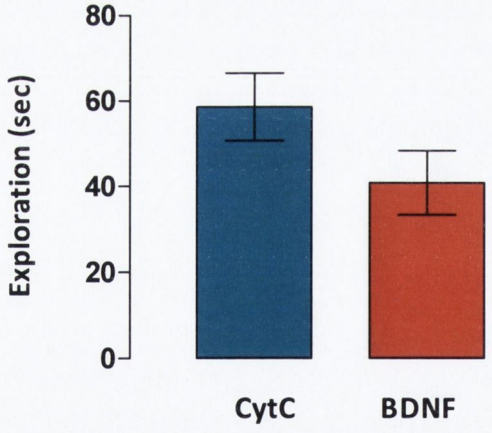
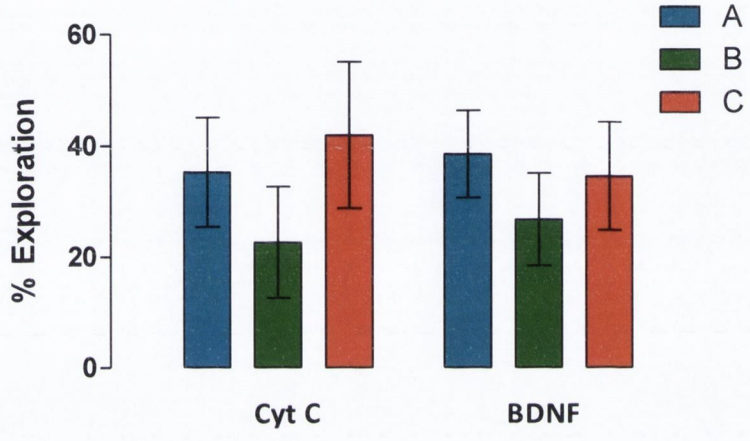


Fig 5.20 A single BDNF Infusion (i.c.v.) has no effect on total exploration.

Rats that received a single intracerebroventricular infusion of BDNF (BDNF group) and controls (Cyt C group) exhibited similar total exploratory behaviour on the training day. Data are presented as total exploration of objects in seconds. All data are shown $\pm$ SEMs. No significant difference was observed (Student's *t*-Test).

(a)



(b)

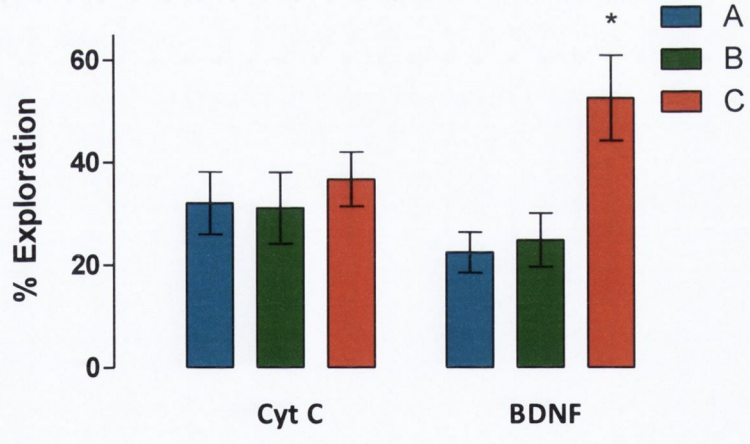


Fig 5.21 Rats infused with BDNF (i.c.v.) preferentially explore the displaced object in a 3-object OD task with 5 minutes of training.

Data are presented as exploration time for each object (A, B or C, where C is the displaced object) as a percentage of the total exploration time. The schematic (top) describes the cognitive task. The graphs depict the results for training day (a) and testing day (b). Rats from either group showed no preference for any one of the objects on training day (a). On the testing day (b), BDNF rats preferentially explored the displaced object (object C), while the control group (Cyt C) did not. All

data are shown as mean % exploration time for each object ± SEM. The asterisk denotes data sets of significant difference as assessed by a two-way ANOVA with *Post-hoc* Bonferroni comparisons. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

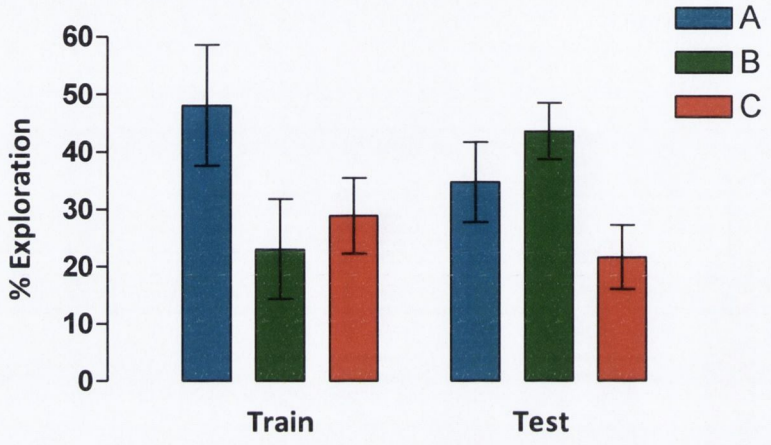


Fig 5.22 Rats infused with BDNF (i.c.v.) do not preferentially explore the displaced object when tested one week post-infusion

One week after receiving the BDNF infusion, the BDNF group was infused with cytochrome c and then trained and tested in a new object displacement task. The rats did not preferentially explore the displaced object. All data are shown as mean % exploration time for each object + SEM. The asterisk denotes data sets of significant difference as assessed by a two-way ANOVA. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

5.3.4.3 Rats infused with BDNF (i.c.v.) do not preferentially explore displaced object one week post-infusion

One week after the BDNF infusion, the BDNF group was given an infusion of Cyt C (10ng, i.c.v.) and then trained and tested again in a new 3-object object displacement task with 1x5 minutes of training. During the training period, rats spent a similar amount of time exploring each of the three objects (A, B and C), indicating that animals had no preference for any specific object (Fig 5.22, Object A: 48.07 $\pm$ 10.51 %, Object B: 23.04 $\pm$ 8.75 %, Object C: 28.89 $\pm$ 6.57 %).

During the testing period, rats spent a similar amount of time exploring each of the three objects (A, B and C) indicating that they could not remember the location of the objects and identify the displaced object (Fig 5.22, Object A: 34.75 $\pm$ 6.98 %, Object B: 43.61 $\pm$ 4.92 %, Object C: 21.64 $\pm$ 5.68 %).

5.3.5 Experiment 5: The effect of a single BDNF infusion (i.c.v.) on object recognition memory and BDNF in serum and plasma

5.3.5.1 Rats infused with BDNF (i.c.v.) preferentially explore the displaced object in a 3-object OD task with 5 minutes of training.

During the training period, BDNF and Cyt C rats spent a similar amount of time exploring each of the three objects (A, B and C), indicating that animals had no preference for any specific object (Fig 5.24a, Cyt C: Object A: 36.29 $\pm$ 4.33 %, Object B: 27.33 $\pm$ 2.69 %, Object C: 36.38 $\pm$ 5.56 %, BDNF: Object A: 29.59 $\pm$ 3.98 %, Object B: 43.87 $\pm$ 5.78 %, Object C: 26.54 $\pm$ 4.05 %). During the testing period, the BDNF group preferentially explored the displaced object C, while the control group spent a similar amount of time exploring each of the three objects (A, B and C) (Fig 5.24b, Cyt C: Object A: 32.63 $\pm$ 4.89 %, Object B: 36.87 $\pm$ 2.50 %, Object C: 30.91 $\pm$ 4.38 %, BDNF: Object A: 25.59 $\pm$ 3.98 %, Object B: 25.28 $\pm$ 2.86 %, Object C: 54.91 $\pm$ 7.89 %).

A two-way ANOVA was performed with 'BDNF' as the first independent variable and 'object' as the second. *Post-hoc* analysis was performed using the Bonferroni multiple comparisons *Post-hoc* test. During testing, there was an overall effect of object ($P=0.016$, $F_{(2,24)} = 8.476$) indicating a preference for the displaced object. *Post-hoc* analysis revealed that the BDNF group spent significantly more time exploring the displaced object C compared to stationary objects A and B

($p<0.001$). This indicates that the BDNF group could remember the location of the objects and identify the displaced object C. The Cyt C group spent a similar amount of time exploring each of the three objects indicating that they could not remember the location of the objects and identify the displaced object C. A significant interaction ($p=0.0016$) indicates that the independent variables 'BDNF' and 'object' had a significant effect on each other. Time spent exploring each of the objects was recorded and reported as a percentage of the total exploration time, mean ± SEM.

5.3.5.2 Rats infused with BDNF (i.c.v.) and control rats preferentially explore the novel object in a 3-object OS task with 2x5 minutes of training.

During the training period, BDNF rats spent significantly less time exploring object B compared to objects A and B, while Cyt C rats spent a similar amount of time exploring each of the three objects (A, B and C) (Fig 5.25a, Cyt C: Object A: 32.98 ± 2.44 %, Object B: 34.19 ± 3.18 %, Object C: 32.83 ± 2.23 %, BDNF: Object A: 33.94 ± 1.25 %, Object B: 25.18 ± 1.55 %, Object C: 40.88 ± 0.64 %). During the testing period, both the Cyt C and BDNF group preferentially explored the novel object D (Fig 5.25b, Cyt C: Object A: 26.15 ± 9.24 %, Object B: 27.21 ± 5.25 %, Object C: 46.65 ± 12.48 %, BDNF: Object A: 26.04 ± 5.33 %, Object B: 15.30 ± 2.54 %, Object C: 58.66 ± 6.27 %).

A two-way ANOVA was performed with 'BDNF' as the first dependent variable and 'object' as the second. *Post-hoc* analysis was performed using the Bonferroni multiple comparisons *Post-hoc* test. During training, there was an overall effect of object ($P=0.0074$, $F_{(2,24)} = 8.65$) indicating an aversion for object B. *Post-hoc* analysis revealed that the BDNF group spent significantly less time exploring object B compared to objects A ($p<0.05$) and C ($p<0.001$). During testing, there was an overall effect of object ($P<0.0001$, $F_{(2,24)} = 3.356$) indicating a preference for the novel object D. *Post-hoc* analysis revealed that the BDNF group spent significantly more time exploring the novel object D compared to familiar objects A ($p<0.001$) and B ($p<0.001$). This indicates that the BDNF group could remember the familiar objects and identify the novel object D. *Post-hoc* analysis revealed that the Cyt C group spent significantly more time exploring the novel object D compared to familiar objects A ($p<0.01$) and B ($p<0.01$). This indicates that the Cyt C group could also remember the familiar objects and identify the novel object D. The interaction was not significant ($p=0.05$) indicating that the independent variables 'BDNF' and 'object' did not have a significant effect on each other. Time spent exploring each of the objects was recorded and reported as a percentage of the total exploration time, mean ± SEM.

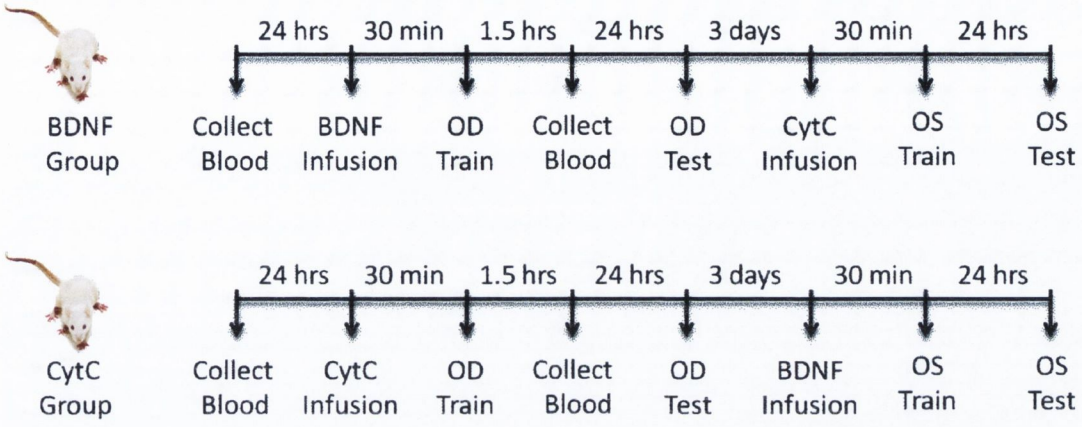


Fig 5.23 Study Design of Experiment 5: The effect of a single BDNF infusion (i.c.v.) on object recognition memory and BDNF in serum and plasma

Baseline blood was collected from all animals 24 hours prior to infusion and processed for serum analysis. Animals received a single bolus BDNF infusion (10ng, i.c.v.) and were trained in an object displacement task 30 minutes later. Control animals received a similar injection of Cytochrome c. Blood was collected again 1.5 hours post training (1.5 hours post-infusion) and processed for serum analysis. All animals were tested 24 hours post-training. After a 3 day wash-out period, groups were switched (BDNF group received a Cyt C injection, Cyt C group received a BDNF injection). 30 minutes post-infusion, animals were trained in an object substitution task and tested 24 hours later.

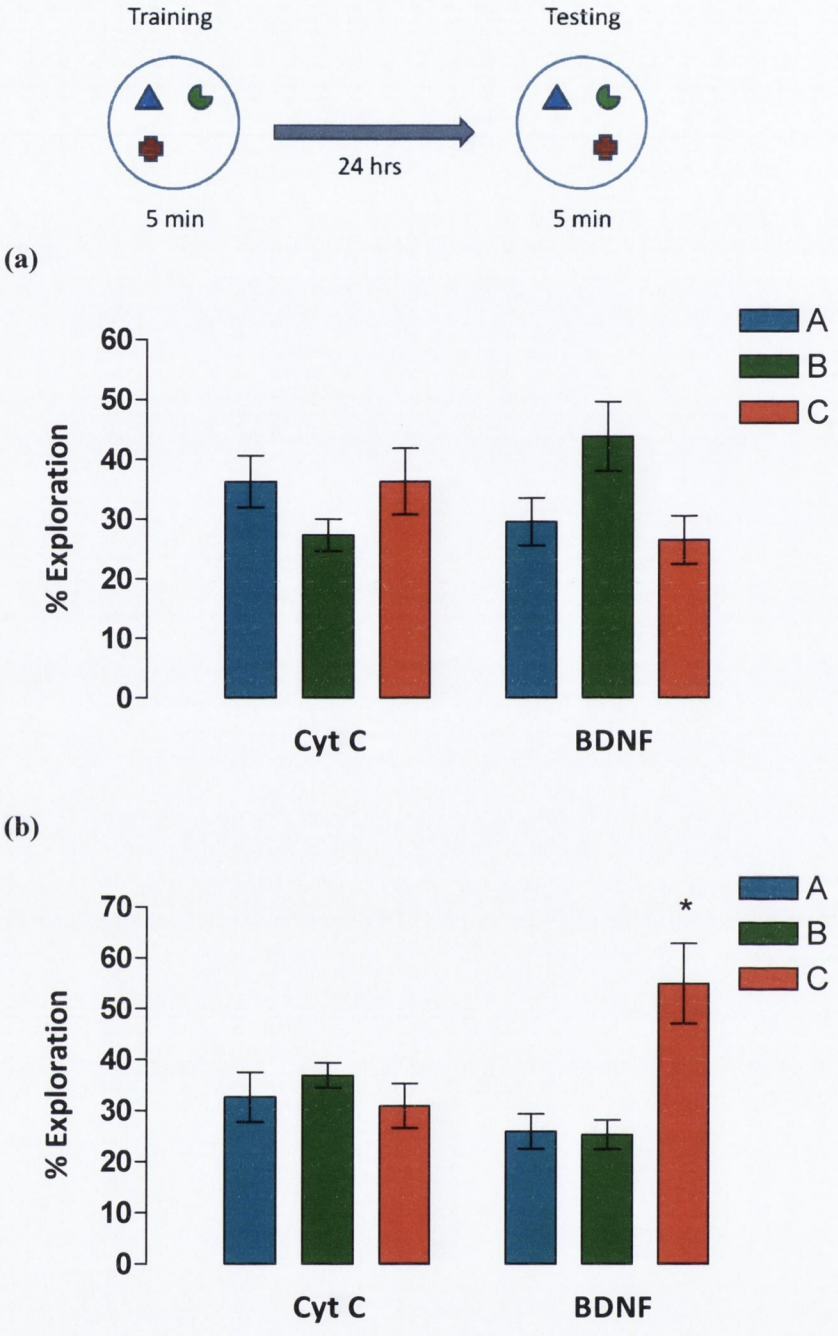
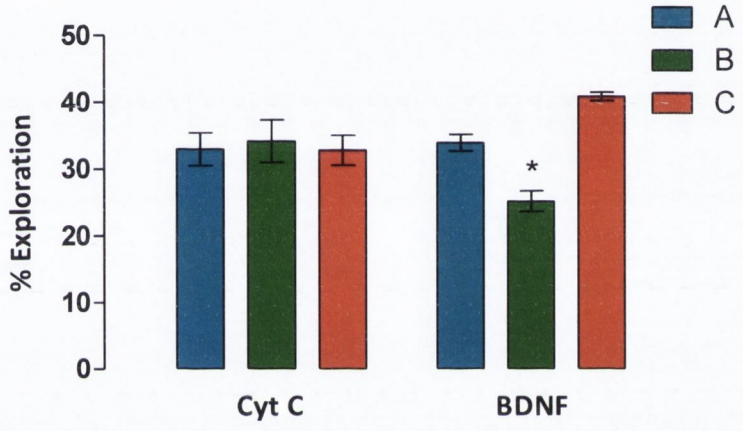


Fig 5.24 Rats infused with BDNF (i.c.v.) preferentially explore the displaced object in a 3-object OD task with 5 minutes of training.

Data are presented as exploration time for each object (A, B or C, where C is the displaced object) as a percentage of the total exploration time. The schematic (top) shows the cognitive task. The graphs depict the results for training day (a) and testing day (b). Neither group displayed a preference for any one of the objects on training day (a). On the testing day (b), BDNF rats

(a)



(b)

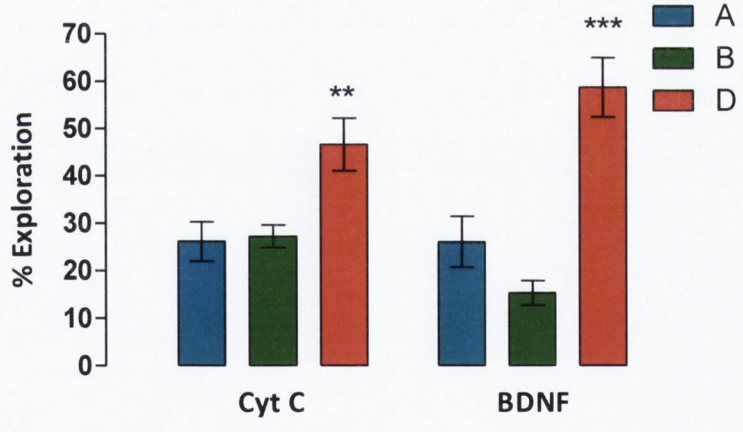


Fig 5.25 Rats infused with BDNF (i.c.v.) preferentially explore the novel object in a 3-object OS task with 2x5 minutes of training.

Data are presented as exploration time for each object (A, B or C, or D where D is the novel object) as a percentage of the total exploration time. The schematic (top) shows the cognitive task. The graphs depict the results for training day (a) and testing day (b). Rats from the control group showed no preference for any one of the objects on training day (a), whereas the BDNF group showed a preference for object B. On the testing day (b), both Cyt C and BDNF rats preferentially

explored the novel object (object D). All data are shown as mean % exploration time for each object $\pm$ SEM. The asterisk denotes data sets of significant difference as assessed by a two-way ANOVA. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

5.3.5.3 A single BDNF infusion (i.c.v.) does not affect the amount of BDNF in serum and plasma collected 1.5 hours post-infusion

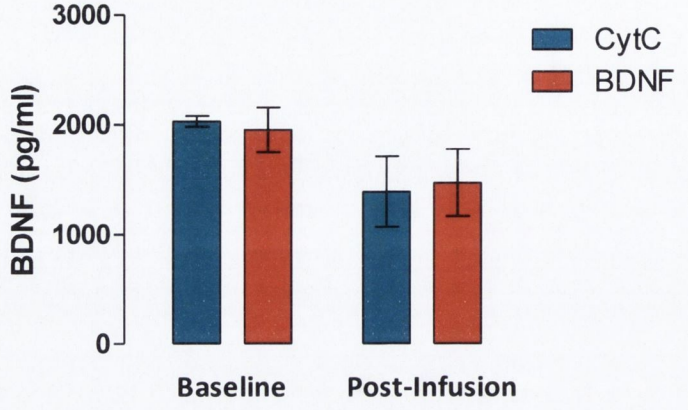
Analysis of blood samples taken 24 hours pre-infusion (baseline) using an ELISA showed no difference in BDNF content in serum (Fig 5.26a, Serum Baseline Cyt C: 2032 $\pm$ 50.4 pg/ml, Serum Baseline BDNF: 1955 $\pm$ 202.8 pg/ml) and plasma (Fig 5.26b, Plasma Baseline Cyt C: 27.24 $\pm$ 3.7 pg/ml, Plasma baseline BDNF: 28.24 $\pm$ 4.8 pg/ml) between groups. Analysis of blood samples collected 1.5 hrs post-infusion showed no difference in BDNF content in the serum (Fig 5.26a, Serum Cyt C: 1392 $\pm$ 320 pg/ml, Serum BDNF: 1474 $\pm$ 305 pg/ml) and plasma (Fig 5.27b, Plasma Cyt C: 27.52 $\pm$ 3.7 pg/ml, Plasma BDNF: 28.24 $\pm$ 4.8 pg/ml) between groups. There was also no difference in BDNF content of serum and plasma in blood samples collected 1.5 hours post-infusion when compared to blood samples taken 24 hours pre-injection (baseline) from either group.

5.3.6 Experiment 6: The effect of a single BDNF infusion (i.c.v.) on BDNF-stimulated signaling events in the dentate gyrus

5.3.6.1 A single BDNF infusion (i.c.v.) induces an increase in BDNF in the contralateral dentate gyrus of the hippocampus 30 mins post-infusion

Analysis of homogenised tissue from the dentate gyrus collected 30 minutes post-infusion using a BDNF ELISA showed an increase in BDNF in the right dentate gyrus of the BDNF group compared to controls when normalised to total protein ($p=0.028$, Student's *t*-Test, Fig 5.29, Cyt C: 71 $\pm$ 9.98 pg/mg, BDNF: 104 $\pm$ 7.57 pg/mg).

(a) Serum



(b) Plasma

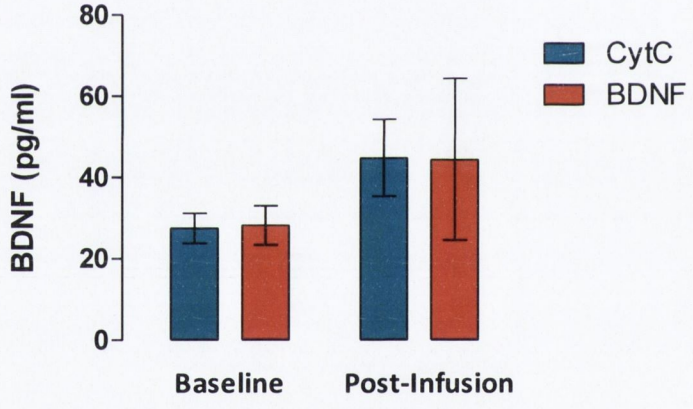


Fig 5.26 BDNF Infusion has no effect on the amount of BDNF present in the serum or in the plasma when measured 1.5 hours post-infusion.

Concentrations of BDNF protein in serum (a) and plasma (b) are presented as assessed by an ELISA. Concentrations are in pg per ml of serum or plasma. The data for the Baseline condition represent blood sampled 24 hrs before infusion. The data for the Post-infusion condition represent blood sampled 1.5 hrs post-infusion. All data are shown $\pm$ SEM. The asterisk denotes data sets of significant difference as assessed by a Student's *t*-Test. (* $p<0.05$)

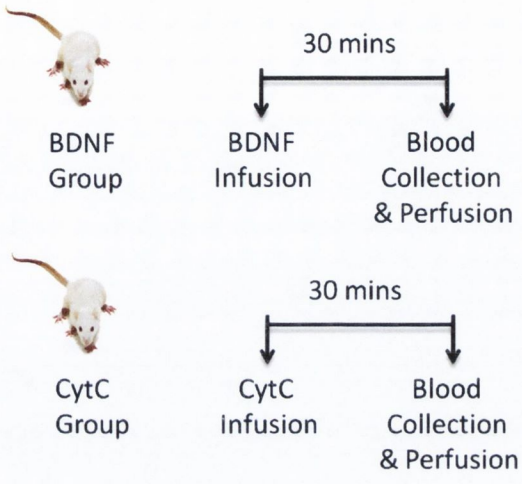


Fig 5.27 Study Design of Experiment 6: The effect of a single BDNF infusion (i.c.v.) on BDNF-stimulated signaling events in the dentate gyrus

Animals were infused with either BDNF or cytochrome c (10ng, i.c.v.). 30 minutes post-infusion, blood was collected (cardiac puncture) after which the animals were perfused and tissue was collected.

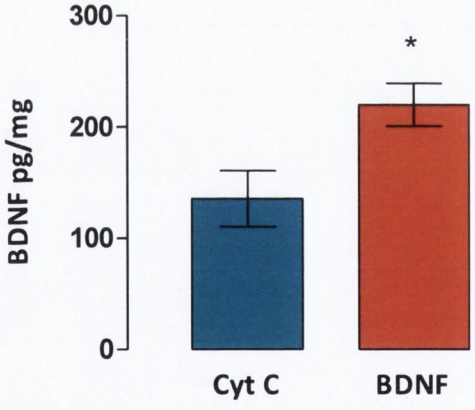


Fig 5.28 A single BDNF Infusion (i.c.v.) induces an increase in the concentration of BDNF in the contralateral dentate gyrus 30 mins post-infusion

Concentrations of BDNF in the right dentate gyrus are presented in pg per mg of total protein as assessed by an ELISA. All data are shown $\pm$ SEM. The asterisk denotes data sets of significant difference as assessed by a Student's *t*-Test.

5.3.7 A single BDNF infusion (i.c.v.) induces an increase in activated ERK1 in the dentate gyrus

Western blot analysis revealed a significant increase in the amount of p-ERK 1 (p44-ERK) in the dentate gyrus of BDNF rats compared to the control group when normalised to total ERK 1 ($p=0.028$, Student's *t*-Test, Fig 5.29a). p-ERK2 was also increased in the dentate gyrus when normalised to ERK2, but this increase was almost statistically significant ($p=0.054$, Student's *t*-Test, Fig 5.29b). Total ERK1 (Fig 5.29c) and total ERK2 (Fig 5.30d) were not significantly affected when normalised to β-actin.

5.3.8 A single BDNF infusion (i.c.v.) does not significantly affect the amount of p-PLCγ in the dentate gyrus.

Western blot analysis showed that the amount of phosphorylated PLCγ was not affected by the infusion of BDNF when normalised over total PLCγ (Fig 5.30a). The amount of total PLCγ was not affected when normalised over β-actin (Fig 5.30c). The increase in p-PLCγ was not significant ($p=0.058$, Student's *t*-Test, Fig 5.30b) when results were normalised over β-actin.

5.3.9 A single BDNF infusion (i.c.v.) induces an increase in p-CaMKII and CaMKII in the dentate gyrus

Western blot analysis revealed an increase in the amount of p-CaMKII in the dentate gyrus of BDNF rats compared to Cyt C controls when normalised to total CaMKII ($p=0.0123$, Student's t-Test, Fig 5.31a). The amount of CaMKII was increased in the dentate gyrus of BDNF rats compared to Cyt C controls when normalised to total β-actin ($p=0.005$, Student's *t*-Test, Fig 5.31b).

5.3.9.1 A single BDNF infusion (i.c.v.) does not significantly affect the amount of Synapsin-1 in the dentate gyrus

Western blot analysis revealed no significant increase in the amount of Synapsin-1 in the dentate gyrus of BDNF rats compared to Cyt C controls when normalised to total β-actin ($p=0.09$, Student's *t*-Test, Fig 5.32).

(a)

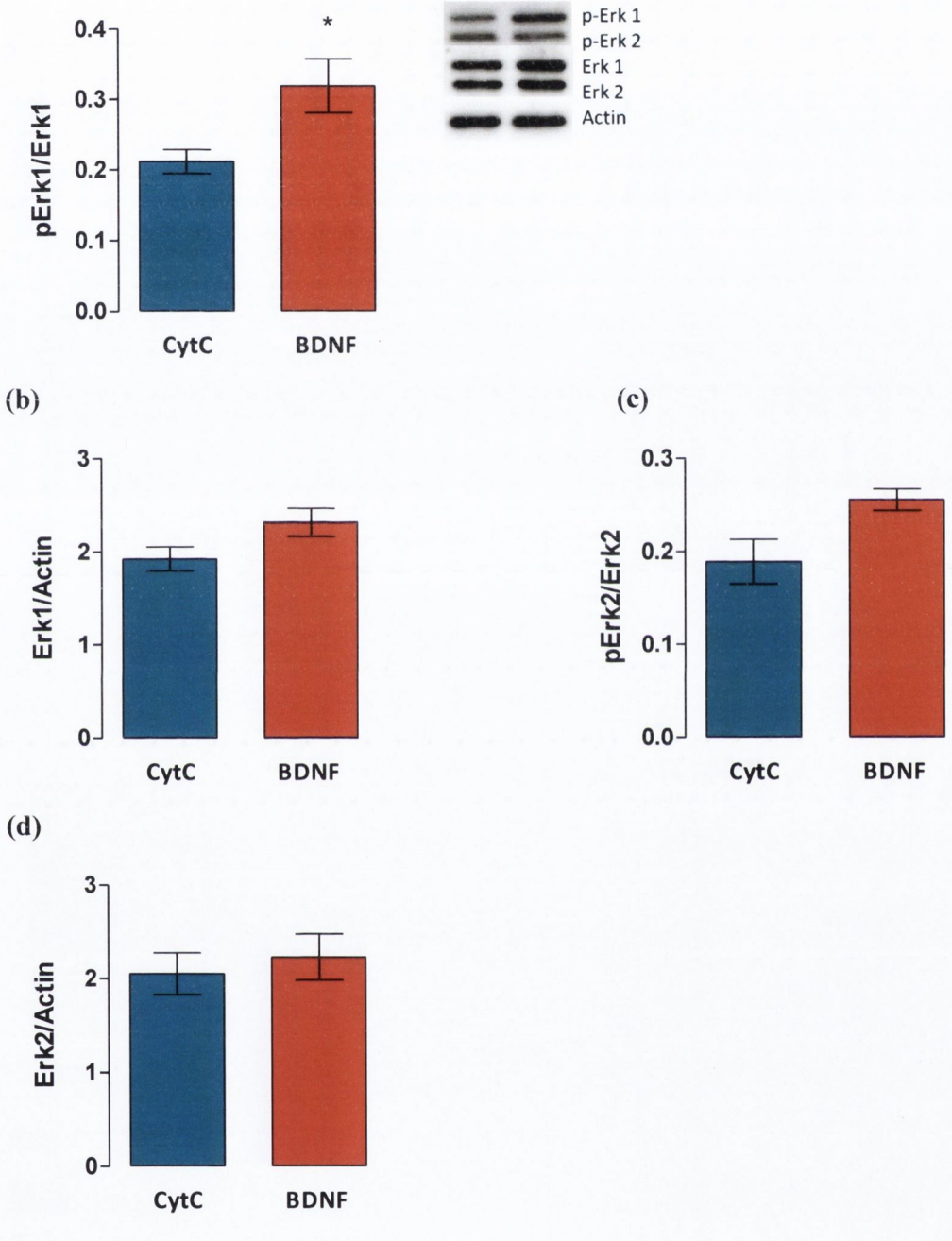


Fig 5.29 BDNF infusion induces an increase in the concentration of p-ERK1 in the dentate gyrus.

(a) Western blot analysis shows an increase in the amount of p-ERK 1 (p44-ERK) in the dentate gyrus of BDNF rats compared to Cyt C controls when normalised to total ERK 1 (p44-ERK). (b) Levels of p-ERK2 were not significantly increased in the dentate gyrus of BDNF rats compared to

Cyt C controls when normalised to total ERK2. (c,d) Levels of total ERK1 and ERK2 were not significantly affected when normalised to β-actin. Representative bands from Western blot analysis probing for p-ERK1, p-ERK2, ERK1, ERK2 and β-actin are shown. Groups are Cyt C controls (left band) and BDNF (right band). All data are presented as mean ± SEM. The asterisk denotes data sets of significant difference as assessed by a Student's t-Test. (* $p<0.05$, individual p-values are reported in the text

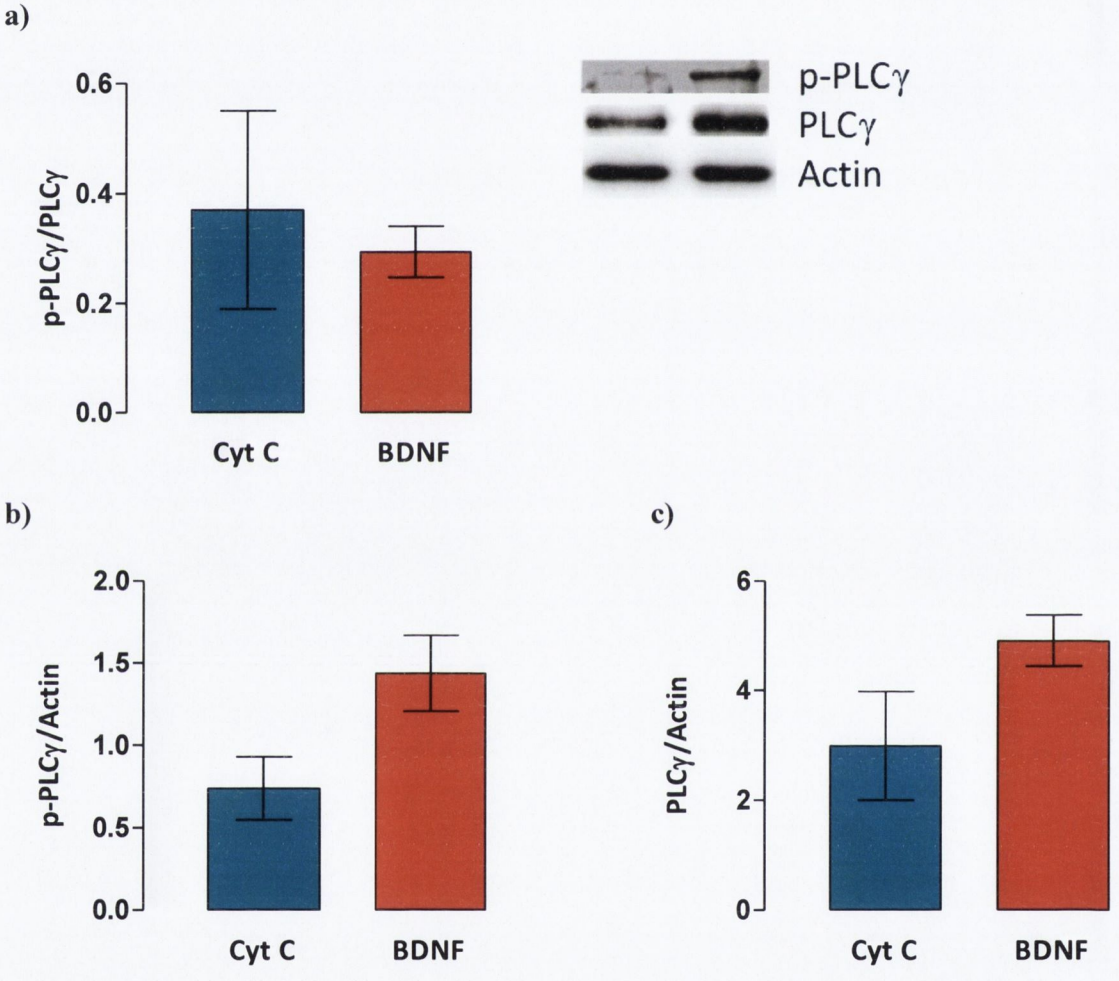


Fig 5.30 BDNF i.c.v. infusion does not have a significant effect on p-PLCγ and PLCγ in the dentate gyrus

(a) Western blot analysis shows that the amount of phosphorylated PLCγ was not affected by the infusion of BDNF when normalised to total PLCγ (Fig 6.16a). (b) No change was observed in p-

PLCγ and PLCγ when normalised over β-actin. Representative bands from Western blot analysis probing for p-PLCγ PLCγ and β-actin. Groups are Cyt C controls (left band) and BDNF (right band). All data are presented as mean ± SEM.

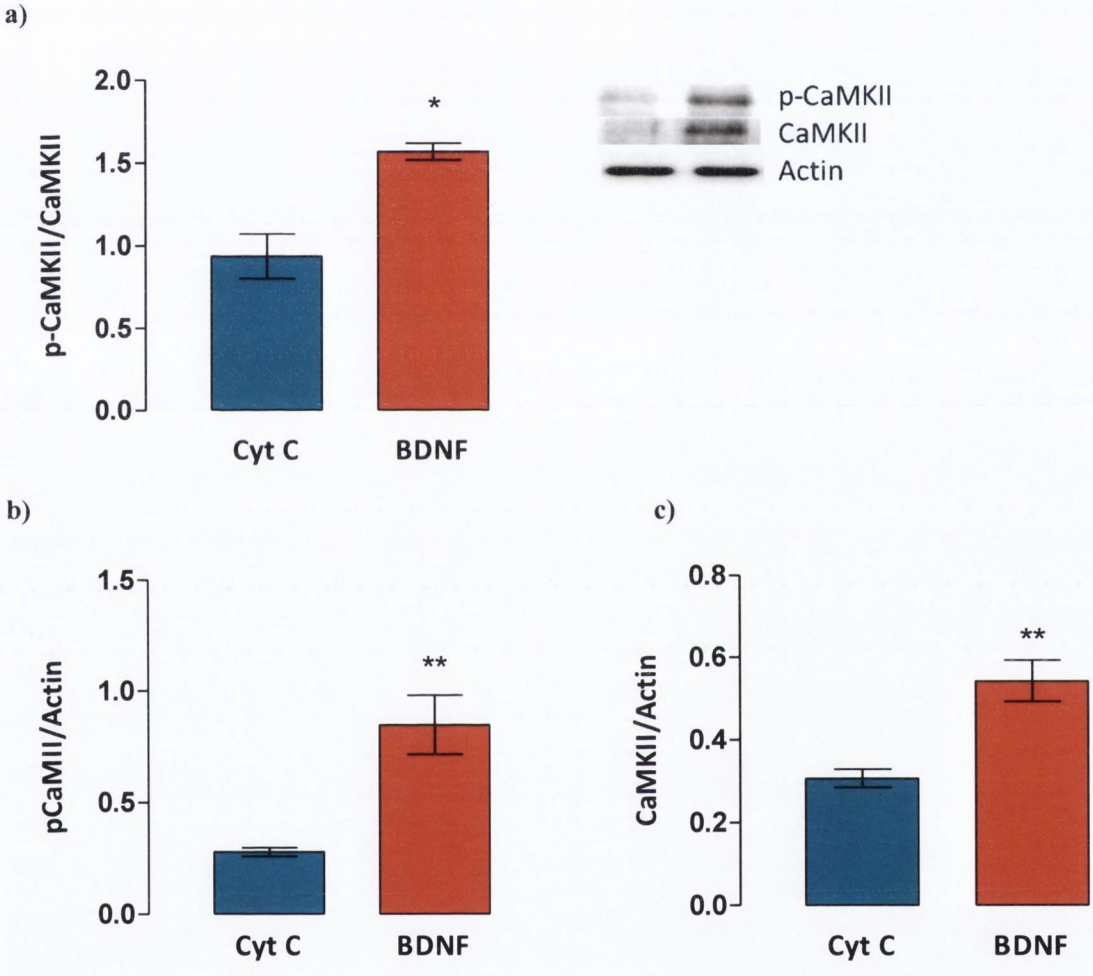


Fig 5.31 BDNF i.c.v. infusion causes an increase in p-CaMKII and CaMKII in the dentate gyrus.

(a,b) Western blot analysis shows an increase in the amount of p-CaMKII in the dentate gyrus of BDNF rats compared to Cyt C controls when normalised to total CaMKII (a) and β-actin (b). (c) The amount of CaMKII was increased in the dentate gyrus of BDNF rats compared to Cyt C controls when normalised to total β-actin. Representative bands from Western blot analysis probing for p-CaMKII, CaMKII and β-actin are shown. Groups are Cyt C controls (left band) and BDNF

(right band). All data are presented as mean ± SEM. The asterisk denotes data sets of significant difference as assessed by a Student's t-Test. (* $p<0.05$, individual p-values are reported in the text).

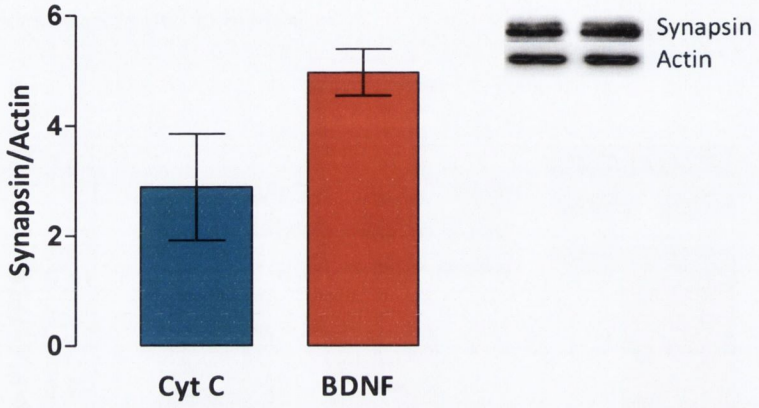


Fig 5.32 BDNF i.c.v. infusion does not significantly affect the amount of Synapsin-1 in the dentate gyrus.

(a) Western blot analysis shows that the amount of Synapsin-1 was not affected by the infusion of BDNF when normalised over total β-actin. Representative bands from Western blot analysis probing for Synapsin-1 and β-actin. Groups are Cyt C controls (left band) and BDNF (right band). All data are presented as mean ± SEM.

5.4 Discussion

5.4.1 Behavioural Analysis

The results of Experiment 1 demonstrate the effect of varying the difficulty of a cognitive task on the performance of the rat. The difficulty of the task was manipulated by changing two variables: the training time (5, 10, or 15 minutes) and number of objects (two or three). The shorter the training time, the harder the task, as the rat is given less time to explore and retain the position and features of the objects. Increasing the number of objects also makes the task harder, as the rat has to retain more information. This series of experiments allowed us to pinpoint which variants of the cognitive tasks would allow us to detect subtle but significant improvements in performance caused by short-term behavioural interventions. If both control and treatment groups preferentially explore the displaced or novel object, the results become more difficult to interpret (even if the amount of preferential exploration varies between the groups). Thus having a testing system where rats either learn or do not learn allows a more straightforward evaluation of the effect of treatment on cognitive performance.

Having identified a spatial task on which the control rats performed poorly (a 3-object OD-task with 5 min training), it was used to test the cognitive performance of rats after a 7-day forced exercise protocol (Experiment 2). The results indicate that one week of exercise can improve performance in an object displacement task as long as the level of difficulty of the task is tailored to tease out fine differences between sedentary and exercised groups (published in Griffin et al 2009). This is in agreement with previous studies that have reported that differences in learning between exercised and sedentary animals become more apparent if the cognitive tasks are made more challenging (van Praag et al., 1999). In Experiment 3, we repeated the challenging spatial task (3-object OD task with 1x5 minutes of training) with a new set of animals, and found that one week of forced moderate exercise improves performance of rats in this task. This shows that the exercise-induced improvement in spatial memory is strong and reproducible.

In a complementary experiment (Experiment 4), a single intracerebroventricular injection of human recombinant BDNF improved performance in the same spatial task (3-object OD-task with 5 min training). Exogenous BDNF administration has been shown to improve learning in an inhibitory avoidance task (Alonso et al., 2005) and a novel object recognition task (Griffin et al., 2009). One study reported some improvements in the MWM with a single BDNF infusion into the right

hippocampus, however, the behavioural results were ambiguous (Cirulli et al., 2004). Dosages administered in the literature are usually much higher than the one used in our laboratory (Cirulli et al., 2004, Alonso et al., 2005). In our experiment, the dosage administered was based on the exercise-induced increase in BDNF observed in the dentate gyrus in previous experiments in the laboratory (Griffin et al., 2009). This is the first time that an exogenous BDNF infusion at physiologically relevant concentrations has been reported to clearly improve memory in a spatial task. Taken together, these experiments provide compelling evidence that an exercise-induced increase in BDNF in the dentate gyrus is sufficient to induce significant improvements in spatial memory.

When trained and tested again one week later, the rats were unable to successfully perform the task, indicating that the beneficial effects of a single BDNF infusion (i.c.v.) are transient. Since the BDNF group explored object B significantly less than the other two objects on training day, this experiment merits replication to ensure adequate training. However, the results of the testing day suggest that a one-week wash-out period is an adequate time window for the effects of a BDNF injection (10ng, i.c.v.) to wear off.

In a separate set of experiments (Experiment 5), we repeated the procedures of Experiment 4 with the same effect, confirming that a single intracerebroventricular injection of human recombinant BDNF improves performance in a spatial task (3-object OD-task with 5 min training) in a robust and consistent manner. Three days later, the groups were switched (rats that had previously received BDNF now received Cyt C, and *vice versa*) and trained and tested in a challenging novel object recognition task (3-object OD-task with 2x5 min trials of training). Both groups successfully identified the novel object in this task. Since this is a task that we have shown repeatedly to be too challenging for control rats, we conclude that the effect of the previous BDNF injection (3 days earlier) may still have persisted in the Cyt C rats, allowing them to successfully perform this task. This result has two main implications. First, the cognitive effects of a single BDNF infusion persist 3 days post-infusion, implying that a three-day week wash-out period is an inadequate time window for the effects of a BDNF injection (10ng, i.c.v.) to wear off.

Second, a single intracerebroventricular infusion of human recombinant BDNF improves performance in a novel object recognition task (3-object OS-task with 2x5 min training). This was shown previously in our lab in a slightly less challenging variant of the task (3-object OS-task with 3x5 min training, Griffin et al., 2009).

5.4.2 BDNF Analysis

Analysis of serum BDNF protein concentrations in Experiment 2 revealed a significant increase with exercise. This is consistent with previous results, reiterating that one week of forced moderate exercise increases BDNF in the serum in a robust and reproducible manner. Similarly, analysis of BDNF mRNA in the dentate gyrus showed an increase in response to one week of exercise, and no significant changes were observed in BDNF protein. These results were also consistent with previous experiments described in this thesis (Chapter 3), but not with previous experiments performed in the laboratory (Griffin et al., 2009, O'Callaghan et al., 2007), where the opposite was shown. Several factors may contribute to this discrepancy: including the time-delay between last bout of exercise and blood collection, and methods used for blood collection and analysis (discussed further in General Discussion).

Since the signalling pathways under investigation are also activated by learning (Minichiello et al., 2009), we added an additional sedentary non-learning group (NoL) in Experiment 3 to control for any learning-induced effects. After testing, tissue from the dentate gyrus was cross-chopped and processed for analysis of BDNF release (2.7.2). Under constitutive conditions, we observed no difference in the amount of BDNF released from slices of dentate gyrus in any group. Upon stimulation with 50mM KCl (stimulated condition) BDNF release was significantly increased in exercised group compared with the non-learning group. The following mechanisms could be involved:

1) Exercise could simply be increasing the amount of BDNF readily available for release. Since a general increase in BDNF in the dentate gyrus has been observed with exercise, this is likely. The mechanisms by which exercise causes an increase in BDNF in the dentate gyrus are still unknown and will be investigated further in the next chapter.

2) Exercise could be increasing the number and activity of synaptic vesicle proteins (such as Synapsin-1 and Synaptophysin). This could also be an indirect effect of increasing BDNF, since there is evidence that BDNF-stimulated signalling events can affect synaptic vesicle protein activity (discussed later).

3) Since homogenised tissue from the dentate gyrus contains both neurons and glia, it is also possible that exercise is increasing the activity-dependent release of BDNF from glia. For example, astrocytes have been shown to take up pro-BDNF from the extracellular space and release it upon

stimulation (Bergami et al., 2008). However, since most of the BDNF (90%) present in the brain is in the mature form, it is unlikely that release of pro-BDNF from astrocytes provides a substantial contribution to the BDNF we detected.

In Experiment 5, blood samples for BDNF analysis were collected at two time-points: 24 hours before BDNF i.c.v. infusion (baseline) and 1.5 hours after BDNF i.c.v. infusion (1.5 hours). No changes in BDNF were detected in serum or plasma across groups or time-points. This finding is consistent with the idea that BDNF, under normal circumstances, does not cross the blood-brain barrier (Pardridge et al., 1994). As a result, a bolus injection of BDNF into the brain would not have any effect on concentration of BDNF in the blood, which is what we observed. In addition, we detected a 100-fold increase in the amount of BDNF in the serum compared to plasma. This is consistent with human studies that report that serum contains about 200 times more BDNF than plasma (Zoladz and Pilc, 2010). This has been attributed to the finding that most the circulating BDNF is sequestered in platelets (Yamamoto and Gurney, 1990, Radka et al., 1996) from which it can be released when clotting is activated by thrombin (Fujimura et al., 2002). In the absence of an anticoagulant (blood collection for serum analysis), blood samples start clotting rapidly: in this process platelets are activated by thrombin and release BDNF into the serum. Serum samples thus contain most of the BDNF that was previously sequestered in the platelets. When an anticoagulant is used (blood collection for plasma analysis), platelets remain intact, thus very little BDNF is released from platelets into the plasma. The function of platelet BDNF is to be released at sites of injury to encourage peripheral nerve regeneration (Fujimura et al., 2002) . However, since platelets do not synthesise BDNF, the source of platelet BDNF is still unknown.

Finally, we found an increase in BDNF protein in the contralateral dentate gyrus of rats that had received a single intracerebroventricular infusion of recombinant BDNF. This result is important because it provides evidence that BDNF is able to diffuse bilaterally from the left ventricle into hippocampal tissue. It also shows that the dosage chosen (10ng) is sufficient to increase the concentration of BDNF in the dentate gyrus by approximately 100pg/mg of tissue, despite substantial losses. This increase mimics that induced by one week of exercise (Griffin et al., 2009). This finding is of particular interest, since it has been suggested that the diffusion of exogenously administered BDNF is restricted, due in part to the large number of TrkB receptors that bind and rapidly internalise BDNF (Yan et al., 1997). This could account for the difference in BDNF detected in the dentate gyrus compared to the amount infused.

5.4.3 Cell Proliferation Analysis

BrdU staining revealed a significant increase in the number of BrdU+ve cells in the dentate gyrus. This is in agreement with results of chapter 4, and a few other studies that have reported cellular proliferation in the dentate gyrus with exercise that is both forced and of short duration (less than three weeks) (Kim et al., 2002, Uda et al., 2006).

Cellular proliferation has been shown in mice to start increasing after as little as three days of voluntary exercise, but to only become significant after 10 days (Van der Borght et al., 2009). Others have described the 10^{th} day of voluntary exercise as being the 'peak of the pro-proliferate effect' (Fabel and Kempermann, 2008), after which the effect of exercise on cellular proliferation seems to taper off. There is some evidence that the stages of neurogenesis occur at a different rate in rats than in mice (Snyder et al., 2009).

As we established in Chapter 4, it is unlikely that this increase in cellular proliferation contributes to the observed improvements in spatial memory. First of all, cellular proliferation itself does not correspond to neurogenesis, but is only the first of the stages as discussed in the introduction. Proliferating cells include precursors that could give rise to different types of cells, including glia, and all of these are stained by BrdU. Second, even if the proliferating cells are identified as immature neurons, within the 7-day time-window during which our exercise paradigm exerts its effects, these cells would not have had time to mature or integrate into existing functional neuronal circuits (Ehninger and Kempermann, 2008). It is more likely that these cellular changes prepare the brain for future learning.

5.4.4 Cell Signaling

We found an increase in the amount of phosphorylated TrkB protein in the dentate gyrus of exercised rats when normalised to total protein. When normalised to total TrkB however, no effect was observed since there was an increase in total TrkB (not significant). The increase in phosphorylation of the receptor in the exercised rats was significant only when compared to the non-learning controls, and not the sedentary-learning group. This suggests that exposure to the spatial task may have led to activation of the TrkB receptor, if not as strongly as with exercise. Activation of the TrkB receptor by BDNF, and the subsequent activation of signalling cascades (such as PLCγ-CaMKII and Ras-ERK) has been shown to occur with learning (Minichiello, 2009). The data suggests that exercise, by increasing BDNF, acts on the same pathways that are activated

by learning, causing amplification of the downstream effects of these pathways, and consequent improvements in memory acquisition and consolidation.

We also found increases in TrkB mRNA expression in the dentate gyrus, but not in the hippocampus, and perirhinal cortex. This is interesting since we found an increase in TrkB mRNA in previous chapters in the perirhinal cortex when the novel object recognition task was performed. Taken together with the results of Chapter 3, the data suggest that different types of memory lead to the upregulation of TrkB in different areas of the brain. Spatial memory is not thought to recruit the perirhinal cortex, and TrkB upregulation after performing a spatial task was specific to the dentate gyrus. Object recognition memory is thought to recruit both the dentate gyrus and the perirhinal cortex, and TrkB upregulation after performing a novel object recognition task occurred in both the dentate gyrus and the perirhinal cortex. Since the upregulation of TrkB is activity-dependent (Tongiorgi et al., 1997, Kingsbury et al., 2003), it is possible that areas of the brain that are recruited and activated for a specific task upregulate TrkB to strengthen the BDNF-TrkB signalling system and prepare for future learning.

We found an increase in the amount of phospho-ERK 1 in the dentate gyrus after one week of exercise. No changes were observed in ERK2 activation, and in total ERK1 or ERK2. Since exercise has been shown to increase BDNF in the hippocampus, it is not surprising that BDNF-related signalling cascades would become activated in response to exercise. This includes the Ras-ERK pathway, which is activated via TrkB phosphorylation, and leads to the ERK phosphorylation. The activation of ERK is known to play an important role in learning and memory (Davis and Laroche, 2006). What is interesting however is that only ERK1 was significantly activated after exercise, and not ERK2. Since ERK1 has been shown to be activated in the dentate gyrus with simple exposure to objects (training) in an object recognition task (Kelly et al., 2003). It is possible that the activation of ERK1 in the dentate gyrus could be learning-induced and not exercise-induced. We addressed this issue by adding a sedentary non-learning group to control for the effects of simple exposure to the objects. The increase in ERK1 was present in only the exercised group, and not in the two sedentary groups, regardless of exposure to the objects. This provides evidence that the activation of ERK1 in the dentate gyrus is exercise-specific, and that exercise activates signalling cascades required for the acquisition and consolidation of memory.

Interestingly, a single infusion of BDNF (i.c.v.) also resulted in significant ERK1 activation in the dentate gyrus (Experiment 6). This would suggest that the infused BDNF is activating signalling

cascades in a manner similar to endogenous BDNF, resulting in the phosphorylation of ERK1. As with exercise, only the ERK1 isoform was significantly activated. Since no learning paradigms were performed in this experiment, we can conclude that the activation of ERK1 is a direct consequence of the BDNF infusion. This shows that an infusion of BDNF into the ventricle can lead to the activation of BDNF-stimulated signalling cascades in the dentate gyrus, presumably by diffusing into the brain parenchyma and activating TrkB receptors on granule cells, resulting in the subsequent activation of ERK1 in a manner that mimics the effect of exercise.

We observed no significant increases in both p-PLCγ and PLCγ in the dentate gyrus of rats that had received a BDNF infusion (i.c.v.) when normalised to β-actin. Since BDNF is known to cause the phosphorylation of PLCγ by binding to TrkB, we expected to find an increase in the activated form of PLCγ. PLCγ recruitment and activation leads to the production of IP3 and DAG, both which can act to release Ca from intracellular stores. This causes the activation of several Ca-dependent signalling pathways that play a role in plasticity.

We observed an increase in both CaMKII and p-CaMKII in the dentate gyrus of rats that had received a BDNF infusion (i.c.v.) when normalised to β-actin. CaMKII has been shown to play a central role in plasticity (Wayman et al., 2008) and is a mechanism by which BDNF rapidly facilitates glutamatergic transmission (Matsumoto et al., 2001). The transcription of CaMKII is activity-dependent (Thiagarajan et al., 2002), which raises the possibility that an increase in synaptic activity due to rapid BDNF-induced effects (for example, changes in membrane excitability, activation of synaptic vesicle proteins) could in itself act as a signal to upregulate CaMKII.

Activation of CaMKII (Lucchesi et al., 2011) occurs when it binds to a Ca/Calmodulin complex, which causes a change in conformation that exposes a catalytic subunit on CaMKII. As a result, CaMKII is able to phosphorylate itself and other proteins (such as NMDA-Rs and Synapsin-1). An increase in the amount of phosphorylated CaMKII in the dentate gyrus indicates activation by Ca/Calmodulin, and subsequent phosphorylation. This is likely to be induced by the activation of PLCγ by BDNF-TrkB binding, and a consequent increase in intracellular calcium in the cell.

5.4.5 Synaptic Vesicle Proteins

We found an increase in the amount of Synapsin-1 in the dentate gyrus of exercised rats compared to sedentary and Non-L controls. Synapsin-1 is the most abundant phosphoprotein present in

synaptic vesicle membranes of the mammalian central nervous system. It can be phosphorylated by several substrates, including CaMKII and ERK (Jovanovic et al., 2000).It has been shown to be essential for the clustering and release of vesicles at the axon terminal (Shupliakov et al., 2011). The exact mechanism by which Synapsin-1 mediates the release of vesicle contents are still not well understood, but is thought that the unphosphorylated form of Synapsin-1 anchors synaptic vesicles to the actin filaments of the cytoskeleton, maintaining them in the reserve pool. Upon phosphorylation, Synapsin-1 dissociates from the actin filaments, allowing the vesicles to become mobilised into the ready-release pool, where they can then dock, fuse, and exocytose. As a result, the phosphorylation of Synapsin-1 is a crucial mechanism by which synaptic transmission can be controlled and facilitated.

Others have shown (Vaynman et al., 2006) that Synapsin-1 is upregulated in the hippocampus of adult rats with as little as three days of voluntary exercise, and it is interesting to see that our forced moderate exercise protocol has a similar effect. In the study by Vaynman and colleagues, injecting the rats with a TrkB-IgG (which blocks the binding of BDNF to TrkB), prevented the exercise-induced upregulation of Synapsin-1. This indicates that BDNF-TrkB signalling is required for the upregulation of Synapsin-1 by exercise.

How exactly BDNF-TrkB signalling leads to the upregulation of Synapsin-1 is still unknown, although we can assume that one of the pathways activates the transcription of the Synapsin gene. However, BDNF-TrkB signalling has been directly linked to the phosphorylation of Synapsin-1, since there is evidence to show that ERK is a substrate of Synapsin-1 (Jovanovic et al., 2000). We were unable to obtain reliable Western Blot data for phospho-Synapsin-1; however, it would be of great interest to confirm that exercise-induced increases in BDNF and ERK-1 (as discussed above) can lead to an increase in phospho-Synapsin-1. This could provide a direct mechanism by which exercise facilitates synaptic transmission.

We found subtle changes in synaptophysin which did not reach significance. Synaptophysin is another synaptic vesicle protein, thought to play a role in vesicle budding and exocytosis (Daly et al., 2000). The study of Vaynman and colleagues (Vaynman et al 2004) reported an increase in synaptophysin in rats after three days of voluntary exercise, an effect that was blocked upon TrkB-IgG administration. This is in agreement with studies reporting that the transcription of synaptophysin is also controlled by BDNF-TrkB signalling (Tartaglia et al., 2001), and could be another way by which BDNF regulates synaptic potentiation via synaptic vesicle proteins.

5.5 Summary

The temporal dynamics of adult hippocampal neurogenesis make it unlikely to mediate the cognitive benefits of one week of exercise. Although we observed increases in cell proliferation with one week of exercise, these new cells are more likely to be preparing the brain for future learning, which could be the function of many of the long-term effects of BDNF on the cytoarchitecture of the brain (Bekinschtein et al., 2008). The observed cognitive improvements, if mediated by the consistent increases we observe in BDNF expression, are more likely to be due to the short-term effects of this neurotrophin on synaptic transmission, which involve activation of the TrkB receptor, the subsequent activation of ERK-1, and the upregulation and activation of synaptic vesicle proteins. These cognitive improvements can be mimicked by a single intracerebroventricular BDNF infusion, which leads to the induction of signalling cascades and the activation of kinases (CaMKII and ERK-1) that are crucial for synaptic potentiation. We propose that the mechanism by which one week of exercise improves learning is by increasing BDNF in the dentate gyrus, and subsequently inducing rapid pre- and post-synaptic changes that facilitate synaptic transmission. The mechanism by which exercise increases BDNF is unknown, and is a topic of investigation in the next chapter.

Chapter 6 Exercise, BDNF and the Blood-Brain Barrier

6.1 Introduction

6.1.1 Exercise and the source of BDNF

In previous chapters we have shown that one week forced moderate exercise can induce improvements in spatial and object recognition memory in rodents, and that these improvements are accompanied by an increased expression of the neurotrophin BDNF in the dentate gyrus. The relationship between the cognitive improvements and BDNF increases are still purely correlational. However, we have also shown that an increased concentration of BDNF in the dentate gyrus, caused by a single intracerebroventricular infusion of exogenous BDNF, is sufficient to trigger a cascade of signalling events similar to those induced by exercise, and most importantly, to mimic the observed exercise-induced enhancements in both spatial and object recognition memory. Put together, these data suggest that short-term exercise-induced memory improvements are directly dependent on BDNF increases in the dentate gyrus. Interestingly, the mechanisms by which exercise induces an increase in BDNF in the brain are still unclear.

Since we have shown consistently that exercise increases the expression of BDNF mRNA in the dentate gyrus, we can assume that increases in BDNF protein can be attributed, at least in part, to an increase in the transcription of the *Bdnf* gene in cells of the dentate gyrus. However, it remains unclear whether these transcriptional changes are the sole source of BDNF, and more importantly, how exactly exercise brings about these transcriptional changes. Since BDNF regulates its own transcription (Saarelainen et al., 2001), it is possible that BDNF from another source could be reaching the dentate gyrus and stimulating localised transcription of more BDNF in a positive feedback loop .

In parallel, we have shown that one week of moderate forced exercise is accompanied by a robust and transient increase in the concentration of BDNF in the blood. This leads us to the question of whether the source of the exercise-induced increase in BDNF is the periphery (blood or other peripheral tissues) or the brain. Since most of the BDNF in the periphery is sequestered in platelets, one hypothesis is that BDNF is released from the platelets during exercise. Possible stimuli could include sheer stress or changes in core temperature, two factors that have been shown to stimulate

BDNF release from platelets, and both of which occur with exercise. For the circulating BDNF to be delivered to specific regions in the brain such as the dentate gyrus, it would have to cross the blood-brain barrier. The main problem with this hypothesis is that BDNF is a large molecule (13 kDa), and should not, under ordinary circumstances, cross the blood-brain barrier. However, the literature reports conflicting evidence on this topic (reviewed in the General Introduction).

To investigate this hypothesis, we designed a series of three experiments (Experiments 1, 2 and 3) where we administered BDNF peripherally to answer the following questions:

1) Can BDNF, if infused into the tail veins of rats (i.v.) cause a cognitive improvement? (Experiment 1)

2) Can BDNF, if infused into the tail veins of rats (i.v.) in the presence of exercise, cause a larger cognitive improvement over exercise alone? (Experiment 2)

3) Can a single infusion of BDNF (i.v.), in the presence of exercise, cause an increase in BDNF in the dentate gyrus or hippocampus? (Experiment 3)

6.1.2 Exercise and blood flow

Exercise-induced changes in cerebral blood flow could play an important role in cognitive enhancement. Exercise has been shown to increase cerebral blood flow (Ogoh and Ainslie, 2009b) and increased blood flow to the hippocampus has been associated with improved spatial memory performance in aged individuals (Heo et al., 2010). To date, no studies have investigated the relationship between acute cardiovascular exercise and immediate localised changes in cerebral blood flow. Exercise-induced changes in cerebral blood flow are especially relevant to our study, because localised increases in cerebral blood flow could provide a mechanism by which circulating BDNF is delivered to specific regions in the brain.

To investigate localised changes in cerebral blood flow with exercise, we used a novel MRI technique developed in Trinity College Institute of Neuroscience (Kelly et al., 2009), known as Bolus tracking arterial spin labelling (btASL). This is a variant of Arterial Spin Labelling which provides a non-invasive, highly sensitive measure of cerebral blood flow.

We investigated the effect of our exercise protocols (acute bout and one week of forced exercise) on blood flow to the hippocampus, cortex, and whole brain, as assessed by btASL (Fig 6.9).

6.1.3 Exercise and the blood-brain barrier

The question of whether BDNF crosses the blood-brain barrier (BBB) with exercise is central to the study of exercise-induced cognitive enhancement. There are several ways to evaluate changes in the permeability of the blood-brain barrier. Most commonly these involve injecting a protein-based dye of a molecular weight larger than 180 Da into the circulation and testing whether it can be detected in the brain. Under normal circumstances, proteins larger than 180 Da do not cross the blood-brain barrier. One of the most commonly used dyes is Evans blue, a 961 Da protein. However, studies have reported that Evans Blue binds to albumin in the circulation to form a complex of almost 70 kDa (Nag, 2003). This complicates the use of Evans Blue in conditions where only subtle changes in blood-brain barrier are suspected, as with exercise.

A better option is sodium fluorescein (Na-F), a fluorescent dye with a molecular weight of 376 Da (Kaya and Ahishali, 2011). Although Na-F is about 36 times smaller than BDNF, its small size allows the detection of subtle increases in blood-brain barrier permeability, and is thus more suited to our purposes. In addition, the fluorescence is easily quantifiable by confocal microscopy.

6.2 Methods and Study Design

6.2.1 BDNF IV Study

6.2.1.1 Experiment 1: The effect of a single BDNF (i.v.) infusion on cognitive performance

To investigate whether BDNF, if infused into the tail-vein of a rat, can cause a cognitive improvement, we conducted the following experiment. After a two-day habituation period to the open field, young rats (n=12) were randomly assigned to a BDNF group (BDNF, n=6) or a control group (Cyt C, n=6). Tail veins were accessed as described previously, and BDNF rats were infused with a single bolus of human recombinant BDNF (10ng in 500µl saline, i.v.). Control rats received a similar infusion of cytochrome c (10ng in 500µl saline, i.v.).

30 minutes post-infusion, all animals were trained in a 3-object object displacement task with 1x5 minutes of training (Section 2.6). Animals were tested 24 hours later (see Fig 6.1 for study design).

6.2.1.2 Experiment 2: The effect of a single BDNF (i.v.) infusion, followed by an acute bout of exercise, on cognitive performance

To investigate whether BDNF, if infused into the tail-vein of a rat in combination with exercise, can cause a cognitive improvement beyond that caused by exercise alone, we conducted the following experiment. After a two-day habituation period to the treadmills and open field, young rats (n=12) were randomly assigned to a BDNF group (BDNF, n=6) or a control group (Cyt C, n=6). Tail veins were accessed as described previously, and BDNF rats were infused with a single bolus of human recombinant BDNF (10ng in 500µl saline, i.v.). Control rats received a similar infusion of Cytochrome C (10ng in 500µl saline, i.v.). Rats were allowed 15 minutes to recover from anaesthesia, after which all rats were exercised for 1 hour at 1km/hr on rodent treadmills.

30 minutes post-exercise, all animals were trained in a 3-object object novel object recognition task with 2x5 minutes of training (Section 2.6). Animals were tested 24 hours later.

6.2.1.3 Experiment 3: The effect of a single BDNF (i.v.) infusion, followed by an acute bout of exercise, on the concentration of BDNF in the dentate gyrus

To investigate whether BDNF, if infused into the tail-vein of a rat in combination with exercise, can cause an increase in BDNF in the dentate gyrus beyond the increase caused by exercise alone, we conducted the following experiment. After a two-day habituation period to the treadmills, young rats (n=12) were randomly assigned to a BDNF group (BDNF, n=6) or a control group (Cyt C, n=6). Tail veins were accessed as described previously, and BDNF rats were infused with a single bolus of human recombinant BDNF (10ng in 500µl saline, i.v.). Control rats received a similar infusion of cytochrome c (10ng in 500µl saline, i.v.). Rats were allowed 15 minutes to recover from anaesthesia, after which all rats were exercised for 1 hour at 1km/hr on rodent treadmills.

30 minutes post-perfusion, rats were overdosed with urethane (0.3g/ml, i.p.) and were perfused with heparinised 0.89% ice-cold saline for 10 minutes (Section 2.9.2). Brain tissue (dentate gyrus and hippocampus) was collected and flash-frozen for further analysis.

Collected tissue was bisected and homogenized in either Krebs buffer (350µl; for BDNF ELISA) or Lysis buffer (350µl; for TrkB ELISAs and Western Blots).

6.2.2 The effect of exercise on cerebral blood flow as assessed by bolus tracking Arterial Spin Labeling (btASL)

Three month-old Wistar rats (n=12) were randomly assigned to sedentary or exercise groups, and subjected to the one week forced exercise protocol as previously described (Section 2.3). 24 hours before the first bout of exercise, all rats were scanned (baseline condition) as described in section 2.17. Immediately after the first bout of exercise on the first day, all rats were scanned (acute condition). Immediately after the last bout of exercise on the 7th day, all rats were scanned (week condition). Immediately after scanning, rats were overdosed with urethane (0.3g/ml, i.p.) and blood samples were collected for serum and plasma analysis via cardiac puncture. Rats were then perfused with heparinised ice-cold saline (heparin: 25 Units/L, saline: 0.89%) for 10 minutes (Section 2.9.2). Brain tissue (dentate gyrus and hippocampus) was collected and flash-frozen for further analysis. Collected tissue was bisected and homogenized in either Krebs buffer (350µl; for BDNF ELISA) or Lysis buffer (350µl; for TrkB ELISAs and Western Blots).

6.2.3 The effect of exercise on blood-brain barrier permeability to Na-F

Two studies were conducted to evaluate exercise-induced changes in blood-brain barrier permeability using intravenous sodium fluorescein (Na-F).

In the first study, all rats from the MRI study (Ex, n=6, Sed, n=6) were infused with 1ml of Na-F (i.v.) immediately after the last scan (1 hour post-exercise) and transcardially perfused 10 minutes later (Section 2.9.2). This allowed us to compare the blood-brain barrier integrity of rats that had been exercised for a week to sedentary controls, and test for any persistent effects of exercise.

In the second study, three month-old Wistar rats (n=12) were randomly assigned to sedentary or exercise groups, and subjected to the acute exercise protocol as previously described in section 2.3. Immediately post-exercise, all rats were infused with 1ml of Na-F (i.v.) and transcardially perfused 10 minutes later (as described in Section 2.9.2). This allowed us to test for any immediate and transient effects of exercise on blood-brain barrier permeability.

6.3 Results

6.3.1 BDNF IV Studies

6.3.1.1 Rats infused with BDNF (i.v.) do not preferentially explore the displaced object in an object displacement task.

Rats received an infusion of either BDNF (10ng, i.v, n=6) or cytochrome c (10ng, i.v., n=6). 30 minutes post-infusion, all rats were trained in a 3-object object displacement task with 1x5 mins of training (Fig 6.1, Fig 6.2). All animals were tested 24 hours later.

During the training period, both BDNF and Cyt C rats spent a similar amount of time exploring each of the three objects (A, B and C), indicating that animals had no preference for any specific object (Fig 6.3a, Cyt C Object A: 30.99 $\pm$ 17.01 %, Cyt C Object B: 31.93 $\pm$ 9.21 %, Cyt C Object C: 37.08 $\pm$ 10.91 %, BDNF Object A: 31.92.61 $\pm$ 10.79 %, BDNF Object B: 37.81 $\pm$ 16.51 %, BDNF Object C: 30.27 $\pm$ 7.82 %).

During the testing period, both BDNF and Cyt C rats spent a similar amount of time exploring each of the three objects (A, B and C) indicating that they could not remember the location of the objects and identify the displaced object (Fig 6.3b, Cyt C Object A: 36.03 $\pm$ 8.47 %, Cyt C Object B: 32.02 $\pm$ 9.68 %, Cyt C Object C: 31.95 $\pm$ 4.57 %, BDNF Object A: 32.07 $\pm$ 12.33 %, BDNF Object B: 30.23 $\pm$ 7.74 %, BDNF Object C: 37.7 $\pm$ 8.19 %).

6.3.1.2 Rats infused with BDNF (i.v.) followed by an hour of exercise preferentially explore the novel object in an object substitution task.

Rats received an infusion of either BDNF (10ng, i.v, n=6) or cytochrome c (10ng, i.v., n=6). 15 minutes post-infusion, all rats were exercised at 1 km/hour on a rodent treadmill. 30 minutes post-exercise, rats were trained in a 4-object novel object recognition task with 2x5 mins of training (Fig 6.4, Fig 6.5). All animals were tested 24 hours later.

During the training period, both BDNF and Cyt C rats spent a similar amount of time exploring each of the three objects (A, B and C) (Fig 6.6a, Cyt C Object A: 38.37 $\pm$ 3.42 %, Cyt C Object B: 37.03 $\pm$ 1.16 %, Cyt C Object C: 35.58 $\pm$ 3.65 %, BDNF Object A: 30.61 $\pm$4.53 %, BDNF Object B: 33.24 $\pm$ 3.39 %, BDNF Object C: 34.66 $\pm$ 3.95 %).

During the testing period, BDNF rats preferentially explored the novel object D, while Cyt C rats spent a similar amount of time exploring all three objects (Fig 6.6b, Cyt C Object A: 29.60 ± 5.35 %, Cyt C Object B: 29.08 ± 4.07 %, Cyt C Object C: 41.32 ± 4.32 %, BDNF Object A: 25.99 ± 5.34 %, BDNF Object B: 26.02 ± 3.26 %, BDNF Object C: 47.99 ± 7.54 %).

A two-way ANOVA was performed with 'BDNF' as the first dependent variable and 'object' as the second. *Post-hoc* analysis was performed using the Bonferroni multiple comparisons *Post-hoc* test. During testing, there was an overall effect of object ($P<0.003$, $F_{(2,30)} = 7.17$) indicating a preference for the novel object D. *Post-hoc* analysis revealed that BDNF rats spent significantly more time exploring the novel object D compared to familiar objects A ($p<0.05$) and B ($p<0.05$). This indicates that BDNF rats could remember the familiar objects and identify the novel object D. The interaction was not significant. Time spent exploring each of the objects was recorded and reported as a percentage of the total exploration time, mean ± SEM.

6.3.1.3 A single BDNF infusion (i.v.) followed by an hour of exercise induces an increase in BDNF in the dentate gyrus

Rats received an infusion of either BDNF (10ng, i.v, n=6) or cytochrome c (10ng, i.v., n=6). 15 minutes post-infusion, all rats were exercised at 1 km/hour on a rodent treadmill. 30 minutes post-exercise, animals were perfused and tissue was collected (Fig 6.7).

Analysis of homogenized tissue from the dentate gyrus using an ELISA showed an increase in BDNF in the right dentate gyrus of the BDNF group compared to controls when normalised to total protein ($p=0.028$, Student's *t*-Test, Fig 6.8a, Cyt C: 71 ± 9.98 pg/mg, BDNF: 104 ± 7.57 pg/mg).

Analysis of homogenized tissue from the hippocampus using an ELISA showed no significant changes in BDNF rats compared to controls when normalised to total protein (Fig 6.8b, Cyt C: 129.6 ± 62.02 pg/mg, BDNF: 189.6 ± 42.02 pg/mg).

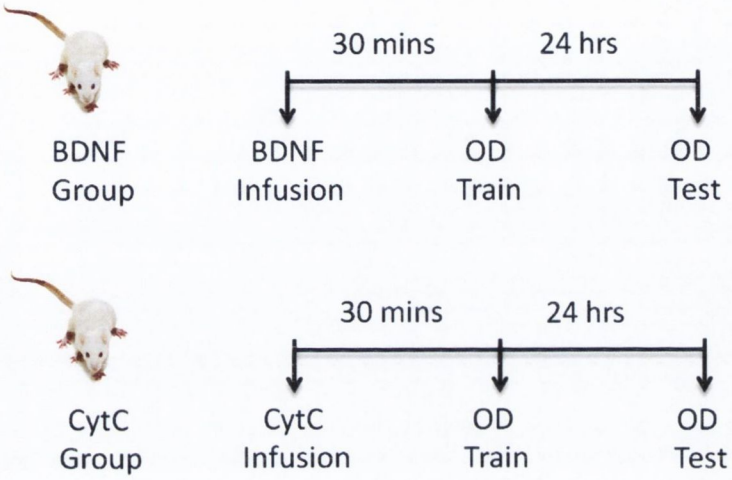


Fig 6.1 Study design of Experiment 1

Rats received a single BDNF infusion (10ng, i.v.) into the tail vein. Control animals received a similar infusion of cytochrome c (10ng, i.v.). All animals were trained in an object displacement test 30 minutes post-infusion, and tested 24 hours later.

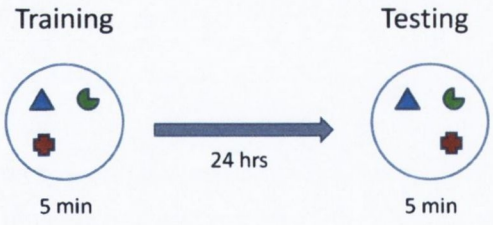
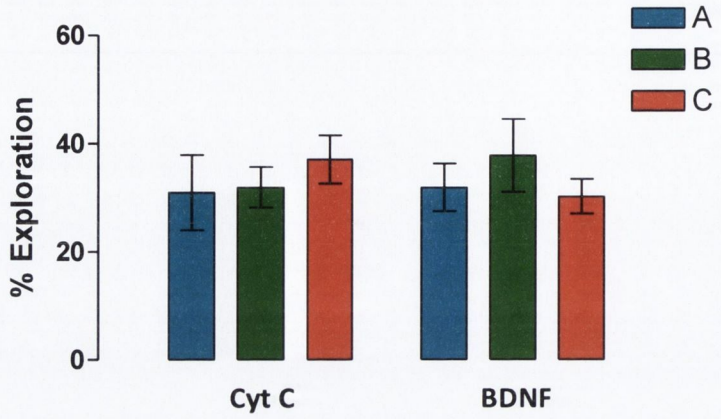


Fig 6.2 Cognitive task of Experiment 1

30 minutes post-infusion, animals were trained in a 3-object object displacement task with 1x5 minutes of training, and were tested 24 hours later with a 5 minute bout of exploration after one of the objects had been displaced.

a) Training Day



b) Testing Day

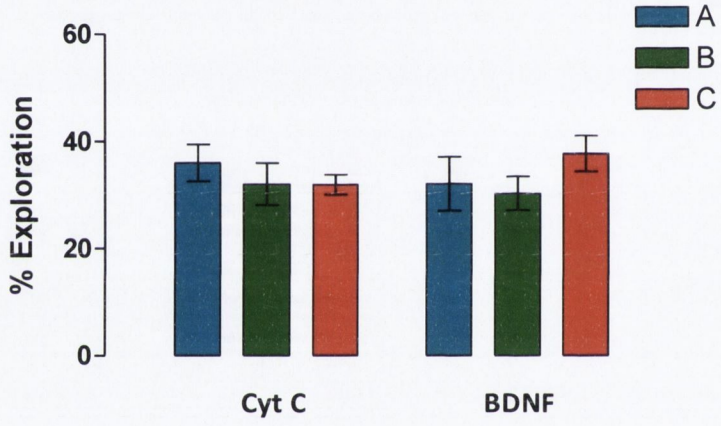


Fig 6.3 Rats infused with BDNF (i.v.) do not preferentially explore the displaced object in a 3-object object displacement test with 1x5 min of training.

Data are presented as exploration time for each object (A, B or C, where C is the displaced object) as a percentage of the total exploration time. The graphs show the results for training day (a) and testing day (b). (a) On the training day, both BDNF (n=6) and Cyt C (control, n=6) group spent a similar amount of time exploring each of the three objects (A, B and C), indicating that they had no preference for any one of the objects. (b) On the testing day, both groups spent a similar amount of time exploring each of the three objects (A, B and C), indicating that they could not remember the location of the objects and identify the displaced object C. All data are shown as mean % exploration time for each object $\pm$ SEM.

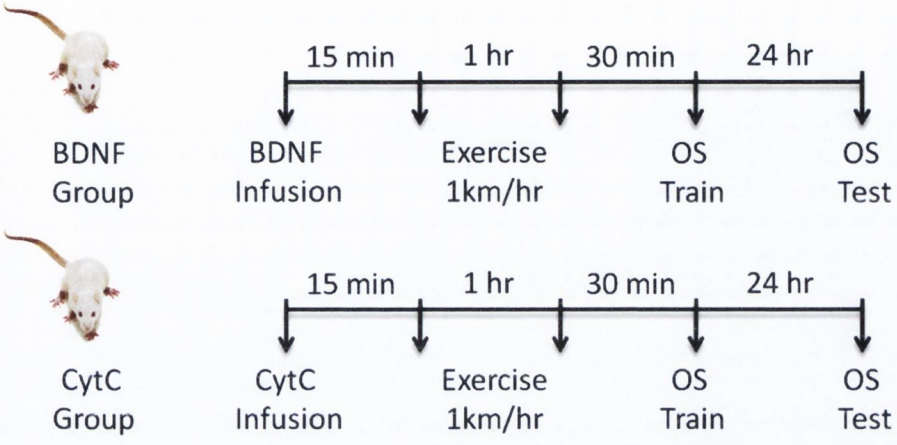


Fig 6.4 Study design of Experiment 2

Rats received a single BDNF infusion (10ng, i.v.) into the tail vein. Control animals received a similar infusion of cytochrome c (10ng, i.v.). All animals were allowed to recover for 15 minutes, and then placed on the treadmills. All animals were exercised for 1 hour (at 1km/hr) and then allowed to rest for 30 minutes. Animals were then trained in an object substitution task, and tested 24 hours later.

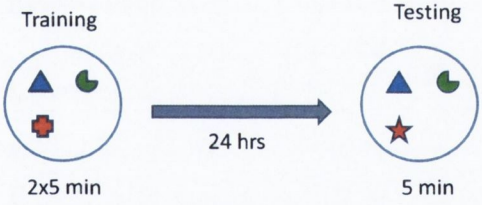
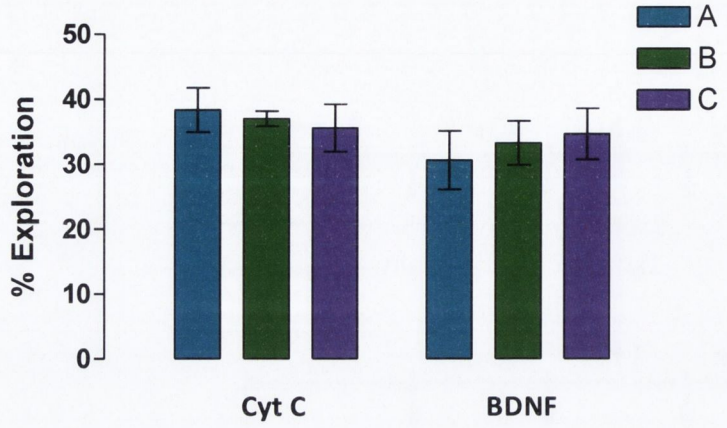


Fig 6.5 Cognitive task of Experiment 2

30 minutes post-exercise, animals were trained in a 4-object object substitution task with 2x5 minutes of training. Animals were tested 24 hours later with a 5 minute bout of exploration after one of the objects had been replaced by a novel object.

a) Training Day



b) Testing Day

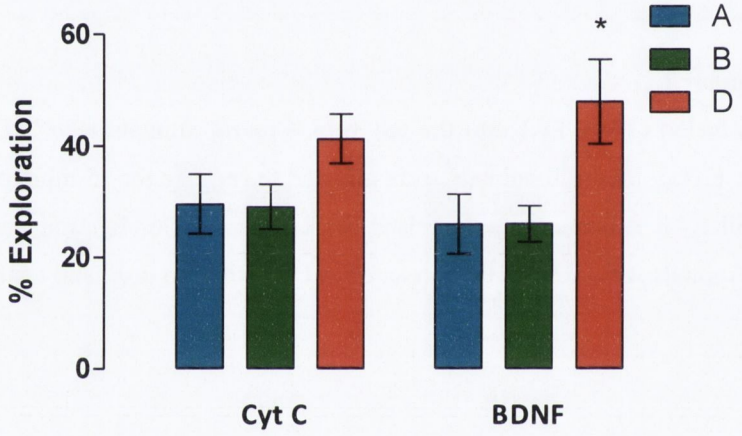


Fig 6.6 Rats infused with BDNF (i.v.) followed by an hour of exercise preferentially explored the substituted object in a 3-object object substitution task with 2x5 minutes training.

Data are presented as exploration time for each object (A, B, C or D) as a percentage of the total exploration time. The graphs show the results for training day (a) and testing day (b). (a) On the training day, both groups spent a similar amount of time exploring each of the three objects (A, B and C), indicating that they had no preference for any one of the objects. (b) On the testing day, the BDNF group preferentially explored the novel object, indicating that it could remember the familiar objects (A, B) and identify the novel object D. The control group explored all the objects similarly (the slight preference is not significant), indicating that it could not remember the familiar objects and identify the novel object D. All data are shown as mean % exploration time for each object $\pm$ SEM.

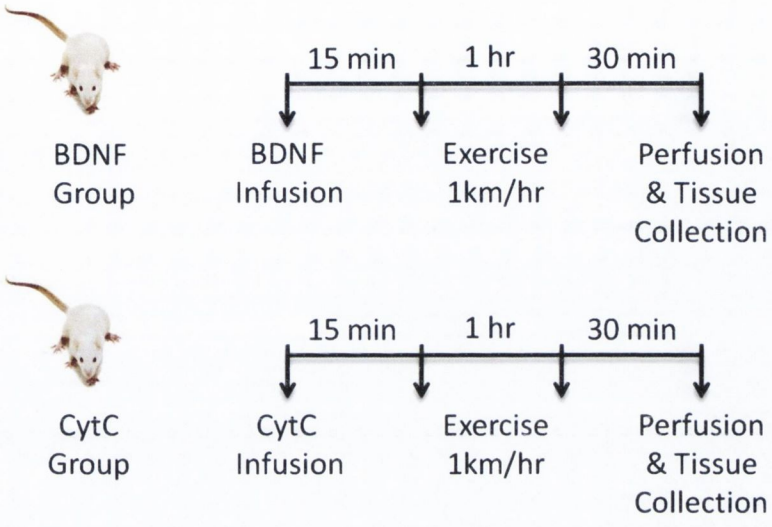
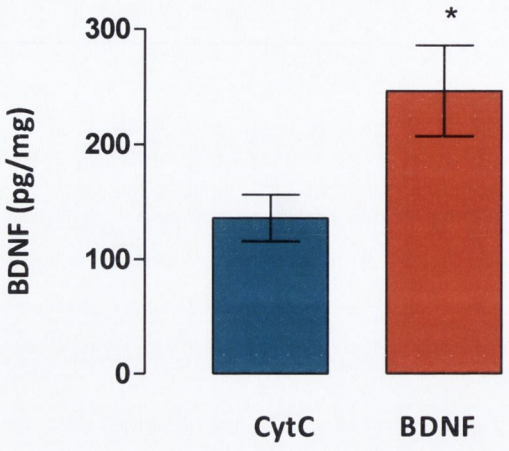


Fig 6.7 Study design of Experiment 3

BDNF Rats (n=6) received a single BDNF infusion (10ng, i.v.) into the tail vein. Control animals (n=6) received a single infusion of cytochrome c (10ng, i.v.) into the tail vein. All animals were allowed to recover for 15 minutes, and were then placed on the treadmills. All animals were exercised for 1 hour (at 1 km/hr) and then allowed to rest for 30 minutes. Animals were then overdosed with urethane (0.3g/L), perfused, and brain tissue was collected for analysis.

a) Dentate Gyrus



b) Hippocampus

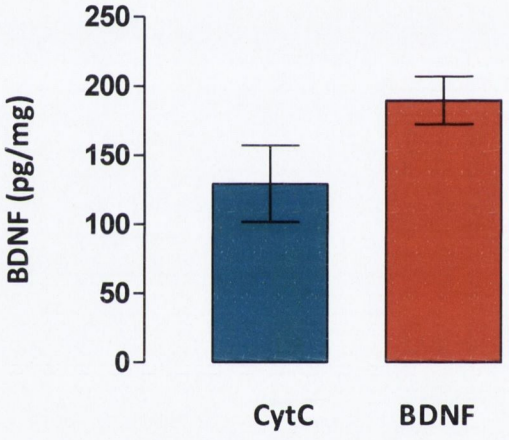


Fig 6.8 A single infusion of BDNF (i.v.) followed by an hour of exercise induced an increase in BDNF in the dentate gyrus.

Concentrations of BDNF in the dentate gyrus (a) and the hippocampus (b) are presented in pg per mg of total protein as assessed by an ELISA. a) In the dentate gyrus, the concentration of BDNF was increased in the BDNF groups compared to controls. B) In the hippocampus, the concentration of BDNF was not significantly increased compared to controls.. All data are shown $\pm$ SEM. The asterisk denotes data sets of significant difference as assessed by a Student's *t*-test.

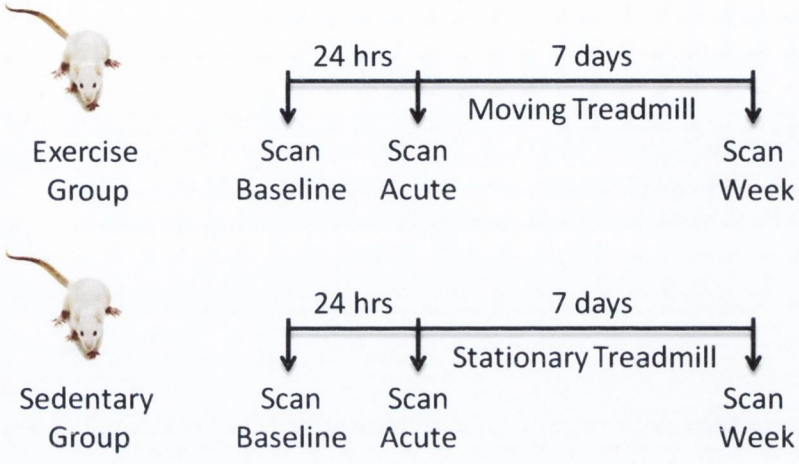


Fig 6.9 Study design of MRI Experiment

Rats (n=12) were randomly assigned to an exercise (Ex, n=6) group and a sedentary control group (Sed, n=6). After a period of habituation to the treadmill (3 days), the Ex group was exercised at 1 km/hour for one hour daily for seven consecutive days, while the Sed group was placed on a stationary treadmill for the same duration. All rats were scanned at three time-points: (1) 24 hours before the first bout of exercise (baseline condition), (2) Immediately after the first bout of exercise on the first day (acute condition) and (3) immediately after the last bout of exercise on the 7^{th} day (week condition). Immediately after scanning on the 7^{th} day, rats were perfused and tissue was collected for analysis.

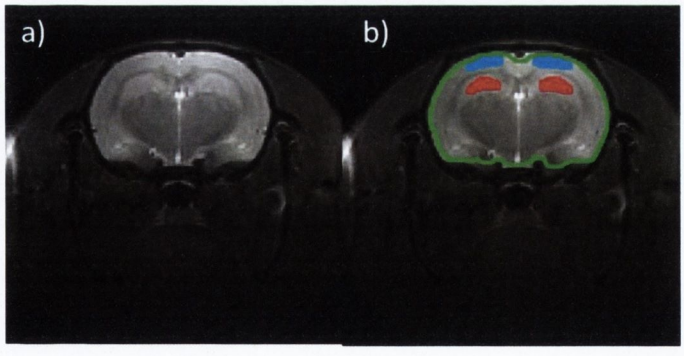


Fig 6.10 Regions of Interest

(a) Representative image of a high resolution anatomical scan of the slice chosen for analysis. The cortex, corpus callosum, hippocampus, and thalamus are clearly visible. (b) Regions of interest are shown as chosen manually for analysis. The left and right cortex are highlighted in blue, the left

and right hippocampus are highlighted in red, and the outline of the whole brain is highlighted in green (this region of interest encompassed all brain regions inside the green outline.)

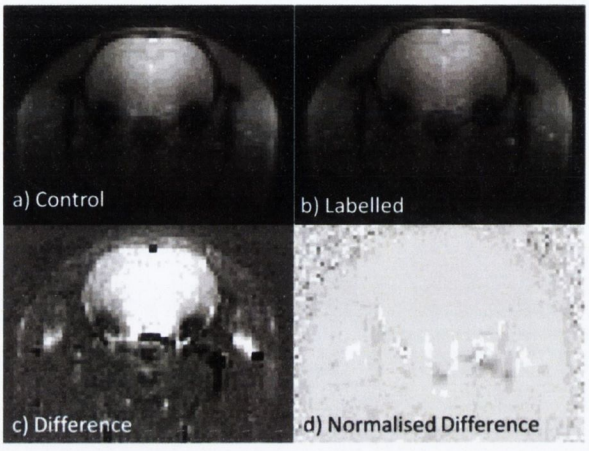


Fig 6.11 Representative labelled and control pictures

Representative images used to get the time-intensity curves for each region of interest. Control images (a) were subtracted from labelled images (b) to obtain the difference image (c) which was then normalised (d).

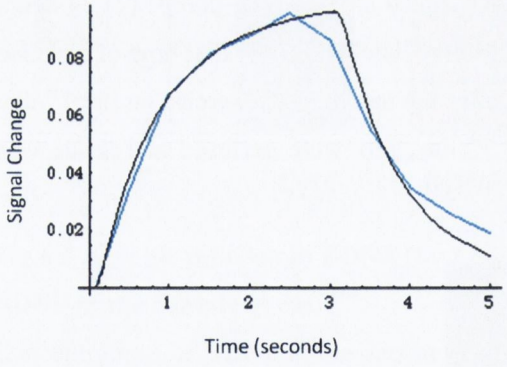


Fig 6.12 Example least square fit of ASL data to model

Representative time-intensity curve for whole brain obtained from normalised difference images (blue curve) fitted to a model (black curve). The parameters MTT and CTT are calculated from this curve.

6.3.2 The effects of exercise on blood flow as assessed by Arterial Spin Labeling

6.3.2.1 Blood flow to the hippocampus increases immediately after an acute bout and one week of exercise compared to sedentary controls.

Relative cerebral blood volume (rCBV) values for the hippocampus were calculated for the baseline condition, acute condition, and week condition (Section 2.17). In the baseline condition, no difference was observed between the sedentary (Sed, n=6) and exercise (Ex, n=6) rats (Fig 6.13a, Sed: rCBV ± SEM: 0.155 ± 0.029, Ex: rCBV ± SEM: 0.166 ± 0.01). In the acute condition, rCBV was increased significantly in the Ex group compared to the Sed group (p=0.0009, Student's *t*-Test, Fig 6.13a, Sed: rCBV ± SEM: 0.133 ± 0.002, Ex: rCBV ± SEM: 0.148 ± 0.002.) In the week condition, rCBV was increased significantly in the Ex group compared to the Sed group (p=0.04, Student's *t*-Test, Fig 6.13a, Sed: rCBV ± SEM: 0.133 ± 0.002, Ex: rCBV ± SEM: 0.151 ± 0.007. When values for Sed and Ex groups were compared across conditions (baseline, acute, week), no significant differences were observed (repeated measures one-way ANOVA.)

The Mean Transit Time (MTT) for the hippocampus was calculated for the baseline condition, acute condition, and week condition (Section 2.17). In the baseline condition, no difference was observed between the Sed and Ex groups (Fig 6.13b, Sed: MTT ± SEM: 1.899 ± 0.05 s, Ex: MTT ± SEM: 1.789 ± 0.13 s). In the acute condition, MTT was decreased significantly in the Ex group compared to the Sed group (p=0.034, Student's *t*-Test, Fig 6.13b, Sed MTT± SEM: 1.841 ± 0.07 s, Ex MTT ± SEM: 1.655 ± 0.275 s). In the week condition, no change was observed in the MTT of the Ex group compared to the Sed group (p=0.136, Student's *t*-Test, Fig 6.13b, Sed MTT ± SEM: 1.868 ± 0.288 s, Ex MTT ± SEM: 1.621 ± 0.288 s). When values for Sed and Ex groups were compared across conditions, no significant differences were observed (repeated measures one-way ANOVA.)

The Capillary Transit Time (CTT) for the hippocampus was calculated for the baseline condition, acute condition, and week condition (Section 2.17). In the baseline condition, no difference was observed between the Sed and Ex groups (Fig 6.13c, Sed: CTT ± SEM: 1.8 ± 0.037 s, Ex: CTT ± SEM: 2.145 ± 0.3 s). In the acute condition, there was no significant change in CTT in the Ex group compared to the Sed group (p=0.056, Student's *t*-Test, Fig 6.13c, Sed CTT± SEM: 1.763 ± 0.103 s, Ex CTT ± SEM: 1.5 ± 0.006 s). In the week condition, no significant change in CTT was observed in the Ex group compared to the Sed group (p=0.085, Student's *t*-Test, Fig 6.13c, Sed

CTT ± SEM: 1.636 ± 0.097 s, Ex CTT ± SEM: 1.419 ± 0.059). When values for Sed and Ex groups were compared across conditions, no significant differences were observed (repeated measures one-way ANOVA.)

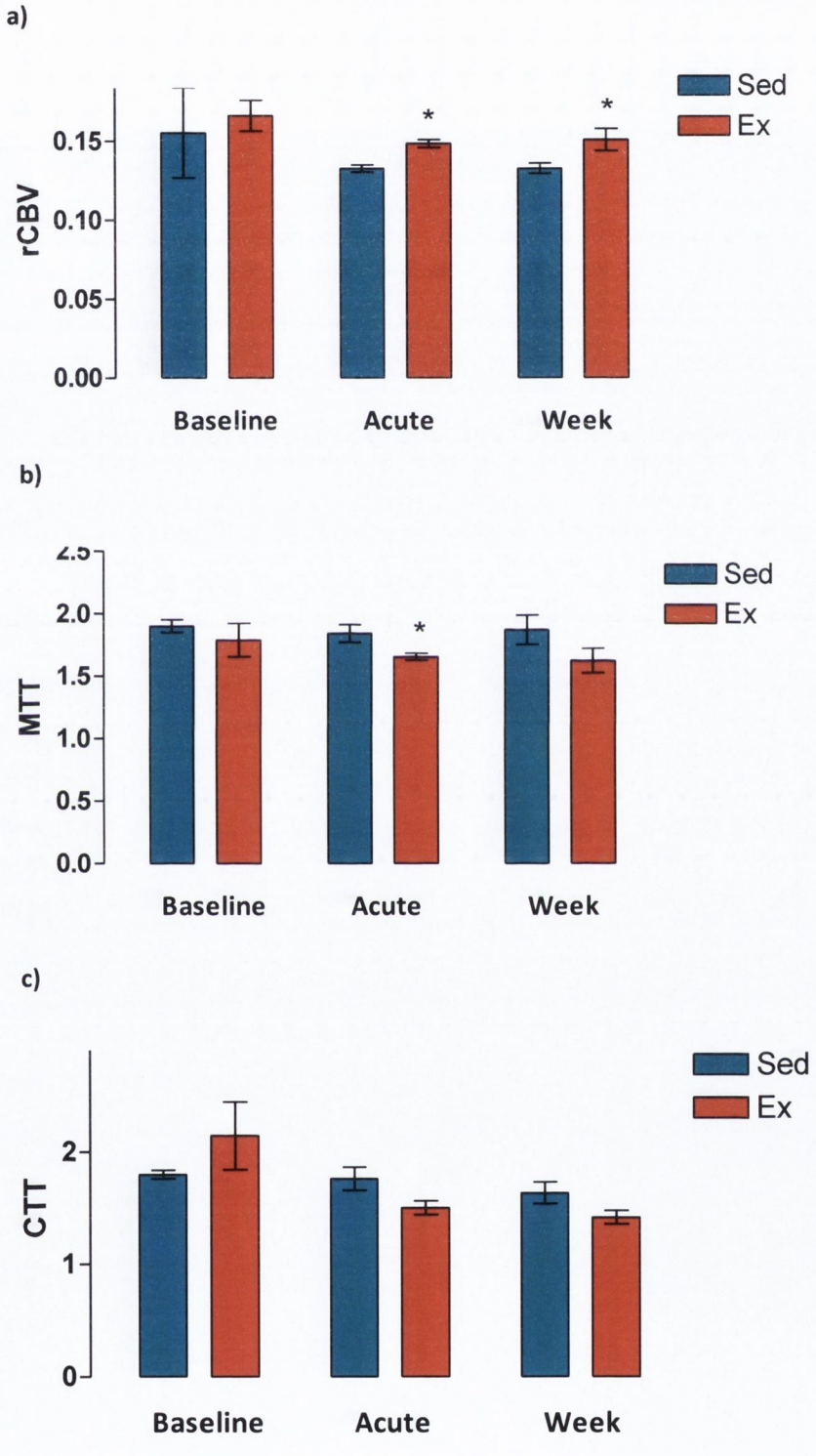


Fig 6.13 Blood flow to the hippocampus increases immediately after an acute bout and one week of exercise compared to sedentary controls.

(a) Relative cerebral blood volume (rCBV) values for the hippocampus are shown for the baseline condition, acute condition, and week condition. In the baseline condition, no difference is observed between the sedentary (Sed, n=6) and exercise (Ex, n=6) groups. In the acute condition, rCBV is increased in the Ex group compared to the Sed group. In the week condition, rCBV is increased in the Ex group compared to the Sed group. Data for the left and right hippocampus were averaged and are shown as mean rCBV in ml/g/min + SEM. The asterisk denotes data sets of significant difference as assessed by a Student's *t*-Test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

(b) Mean Transit Time (MTT) values for the hippocampus are shown for the baseline condition, acute condition, and week condition. In the baseline condition, no difference is observed between the sedentary (Sed, n=6) and exercise (Ex, n=6) groups. In the acute condition, MTT is decreased in the Ex group compared to the Sed group. In the week condition, no significant difference is observed between the Sed and Ex groups. Data for the left and right hippocampus were averaged and are shown as mean MTT in seconds + SEM. The asterisk denotes data sets of significant difference as assessed by a Student's *t*-Test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

(c) Capillary Transit Time (CTT) values for the hippocampus are shown for the baseline condition, acute condition, and week condition. In the baseline condition, no difference is observed between the sedentary (Sed, n=6) and exercise (Ex, n=6) groups. In the acute condition, CTT is increased in the Ex group compared to the Sed group. In the week condition, CTT is increased in the Ex group compared to the Sed group. Data for the left and right hippocampus were averaged and are shown as mean CTT in seconds + SEM. The asterisk denotes data sets of significant difference as assessed by a Student's *t*-Test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

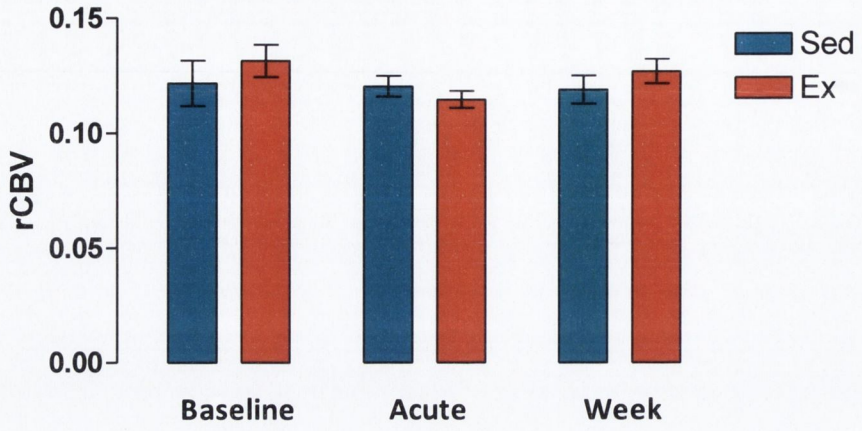
6.3.2.2 Blood flow to the cortex does not change immediately after an acute bout and one week of exercise compared to sedentary controls.

Relative cerebral blood volume (rCBV) values for the cortex were calculated for the baseline condition, acute condition, and week condition (Section 2.17). In the baseline condition, no difference was observed between the sedentary (Sed, n=6) and exercise (Ex, n=6) groups (Fig 6.14a, Sed: rCBV ± SEM: 0.122 ± 0.01, Ex: rCBV ± SEM: 0.138 ± 0.004). In the acute condition, no difference was observed between the sedentary and exercise groups (Fig 6.14a, Sed: rCBV ± SEM: 0.12 ± 0.004, Ex: rCBV ± SEM: 0.114 ± 0.004). In the week condition, no difference was observed between the sedentary and exercise groups (Fig 6.14a, Sed: rCBV ± SEM: 0.119 ± 0.006, Ex: rCBV ± SEM: 0.127 ± 0.005). When values for Sed and Ex groups were compared across conditions, no significant differences were observed (repeated measures one-way ANOVA.)

The Mean Transit Time (MTT) for the cortex was calculated for the baseline condition, acute condition, and week condition (Section 2.17). In the baseline condition, no difference was observed between the Sed and Ex groups (Fig 6.14b, Sed: MTT ± SEM: 2.267 ± 0.063 s, Ex: MTT ± SEM: 2.321 ± 0.975 s). In the acute condition, no difference was observed between the Sed and Ex groups (Fig 7.14b, Sed MTT: ± SEM: 2.129 ± 0.068 s, Ex: MTT ± SEM: 2.051 ± 0.045 s). In the week condition, no change was observed in the MTT of the Ex group compared to the Sed group (Fig 6.14b, Sed MTT ± SEM: 2.166 ± 0.088 s, Ex MTT ± SEM: 2.069 ± 0.088 s). When values for Sed and Ex groups were compared across conditions, no significant differences were observed (repeated measures one-way ANOVA.)

The Capillary Transit Time (CTT) for the cortex was calculated for the baseline condition, acute condition, and week condition (Section 2.17). In the baseline condition, no difference was observed between the Sed and Ex groups (Fig 6.14c., Sed: CTT ± SEM: 1.815 ± 0.084 s, Ex: CTT ± SEM: 1.83 ± 0.13 s). In the acute condition, no difference was observed between the Sed and Ex groups (Fig 7.14c., Sed: CTT ± SEM: 1.585 ± 0.056 s, Ex: CTT ± SEM: 1.565 ± 0.053 s). In the week condition, no difference was observed between the Sed and Ex groups (Fig 6.14c., Sed: CTT ± SEM: 1.738 ± 0.128 s, Ex: CTT ± SEM: 1.508 ± 0.133 s). When values for Sed and Ex groups were compared across conditions, no significant differences were observed (repeated measures one-way ANOVA.)

a)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0.10
0.05
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rCBV
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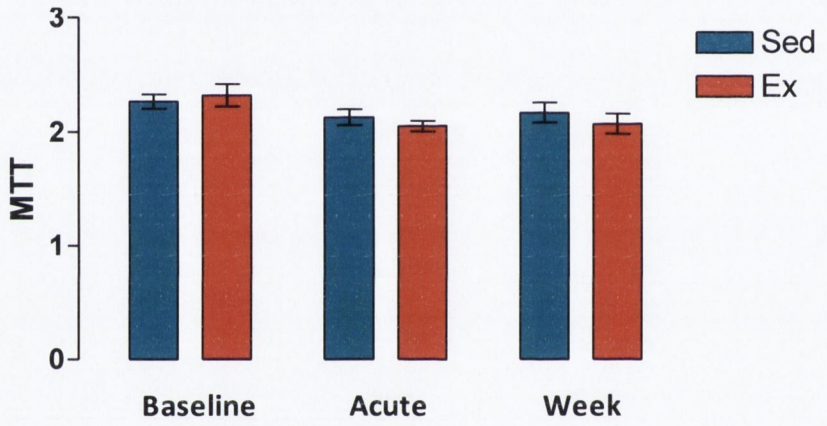
Week

b)


3
2
1
0
MTT
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Week

c)

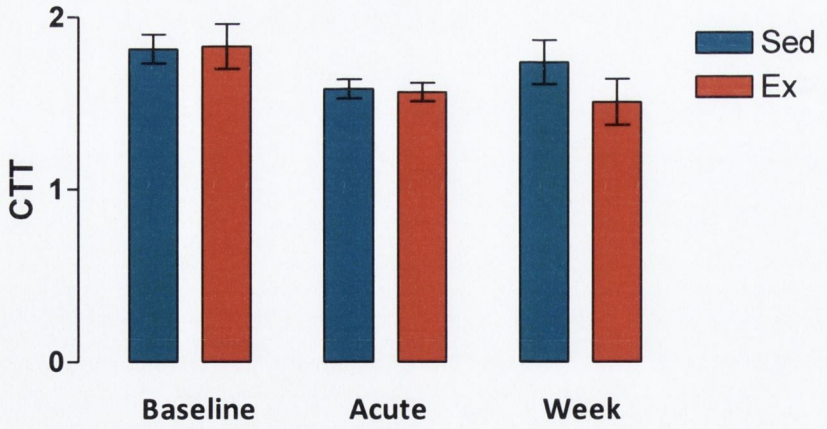
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Fig 6.14 Blood flow to the cortex does not change immediately after an acute bout and one week of exercise compared to sedentary controls.

(a) Relative cerebral blood volume (rCBV) values for the cortex are shown for the baseline condition, acute condition, and week condition. No differences are observed between the sedentary (Sed, n=6) and exercise (Ex, n=6) groups for the baseline condition, acute condition, and week condition. No differences are observed within each group across the three conditions. Data for the left and right cortex were averaged and are shown as mean rCBV $\pm$ SEM.

(b) Mean Transit Time (MTT) values for the cortex are shown for the baseline condition, acute condition, and week condition. No differences are observed between the sedentary (Sed, n=6) and exercise (Ex, n=6) groups for the baseline condition, acute condition, and week condition. No differences are observed within each group across the three conditions. Data for the left and right cortex were averaged and are shown as mean MTT in seconds $\pm$ SEM.

(c) Capillary Transit Time (CTT) values for the cortex are shown for the baseline condition, acute condition, and week condition. No differences are observed between the sedentary (Sed, n=6) and exercise (Ex, n=6) groups for the baseline condition, acute condition, and week condition. No differences are observed within each group across the three conditions. Data for the left and right cortex were averaged and are shown as mean CTT in seconds $\pm$ SEM.

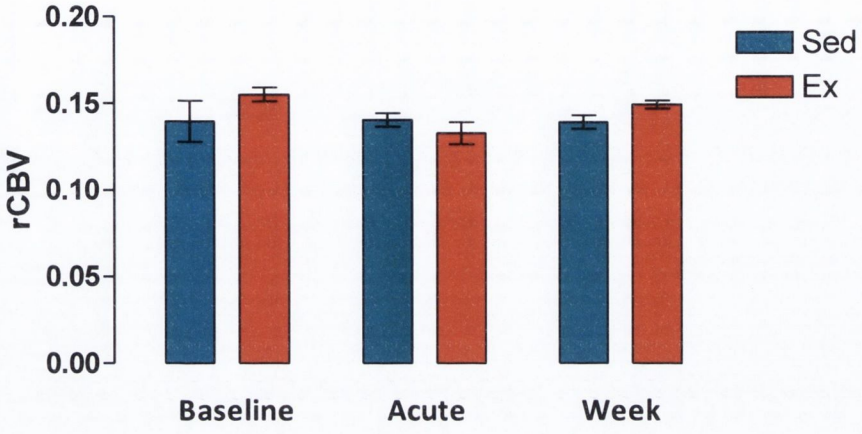
6.3.2.3 Blood flow to the whole brain does not change immediately after an acute bout or one week of exercise compared to sedentary controls.

Relative cerebral blood volume (rCBV) values for the whole brain were calculated for the baseline condition, acute condition, and week condition (2.17). In the baseline condition, no difference was observed between the sedentary (Sed, n=6) and exercise (Ex, n=6) groups (Fig 6.15a, Sed: rCBV ± SEM: 0.14 ± 0.012, Ex: rCBV ± SEM: 0.155 ± 0.004). In the acute condition, no difference was observed between the sedentary and exercise groups (Fig 6.15a, Sed: rCBV ± SEM: 0.14 ± 0.004, Ex: rCBV ± SEM: 0.13 ± 0.006). In the week condition, no difference was observed between the sedentary and exercise groups (Fig 6.15a, Sed: rCBV ± SEM: 0.139 ± 0.004, Ex: rCBV ± SEM: 0.149 ± 0.002). When values for Sed and Ex groups were compared across conditions, no significant differences were observed (repeated measures one-way ANOVA.)

The Mean Transit Time (MTT) for the whole brain was calculated for the baseline condition, acute condition, and week condition (Section 2.17). In the baseline condition, no difference was observed between the Sed and Ex groups (Fig 6.15b, Sed: MTT ± SEM: 1.908 ± 0.047 s, Ex: MTT ± SEM: 1.936 ± 0.072 s). In the acute condition, no difference was observed between the Sed and Ex groups (Fig 6.15b, Sed: MTT ± SEM: 1.769 ± 0.066 s, Ex: MTT ± SEM: 1.829 ± 0.055 s). In the week condition, no change was observed in the MTT of the Ex group compared to the Sed group (Fig 6.15b, Sed MTT ± SEM: 1.816 ± 0.05 s, Ex MTT ± SEM: 1.781 ± 0.054 s). When values for Sed and Ex groups were compared across conditions, no significant differences were observed (repeated measures one-way ANOVA.)

The Capillary Transit Time (CTT) for the whole brain was calculated for the baseline condition, acute condition, and week condition (Section 2.17). In the baseline condition, no difference was observed between the Sed and Ex groups (Fig 6.15c, Sed: CTT ± SEM: 1.708 ± 0.044 s, Ex: CTT ± SEM: 1.793 ± 0.068 s). In the acute condition, no difference was observed between the Sed and Ex groups (Fig 7.15c, Sed: CTT ± SEM: 1.710 ± 0.037 s, Ex: CTT ± SEM: 1.829 ± 0.125 s). In the week condition, no difference was observed between the Sed and Ex groups (Fig 6.15c, Sed: CTT ± SEM: 1.588 ± 0.069 s, Ex: CTT ± SEM: 1.541 ± 0.044 s). When within-group values for Sed and Ex groups were compared across conditions, no significant differences were observed (repeated measures one-way ANOVA.)

a)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rCBV
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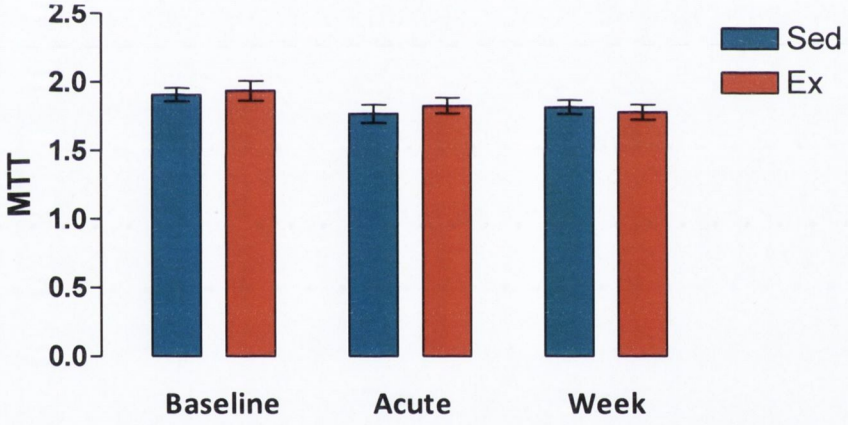
b)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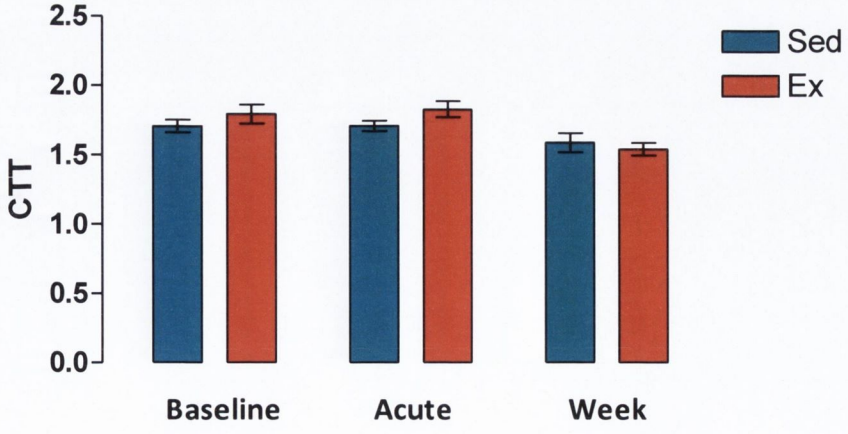
2.5
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Acute
Week

Fig 6.15 Blood flow to the whole brain does not change immediately after an acute bout or one week of exercise compared to sedentary controls.

(a) Relative cerebral blood volume (rCBV) values for the whole brain are shown for the baseline condition, acute condition, and week condition. No differences are observed between the sedentary (Sed, n=6) and exercise (Ex, n=6) groups for the baseline condition, acute condition, and week condition. No differences are observed within each group across the three conditions. Data are shown as mean rCBV ml/g/min $\pm$ SEM.

(b) Mean Transit Time (MTT) values for the whole brain are shown for the baseline condition, acute condition, and week condition. No differences are observed between the sedentary (Sed, n=6) and exercise (Ex, n=6) groups for the baseline condition, acute condition, and week condition. No differences are observed within each group across the three conditions. Data are shown as mean MTT in seconds $\pm$ SEM.

(b) Capillary Transit Time (CTT) values for the whole brain are shown for the baseline condition, acute condition, and week condition. No differences are observed between the sedentary (Sed, n=6) and exercise (Ex, n=6) groups for the baseline condition, acute condition, and week condition. No differences are observed within each group across the three conditions. Data are shown as mean CTT in seconds $\pm$ SEM.

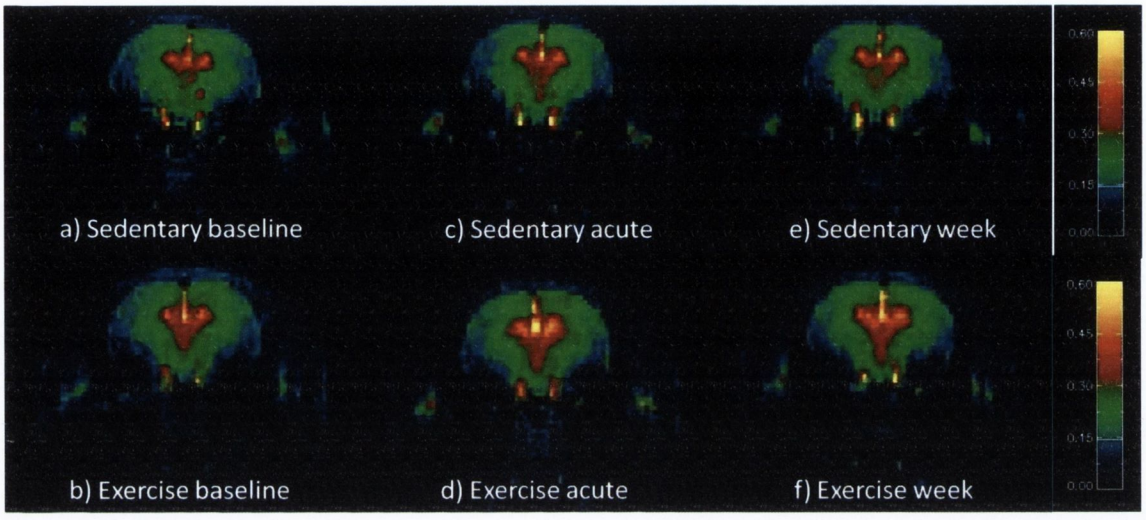

Fig 6.16 Perfusion Maps

ASL Perfusion maps are shown for two representative animals. For all animals, the regions that show the highest perfusion values are the middle cerebral arteries, ventricles, and the hippocampus. For the baseline condition, no significant differences in perfusion are observed between sedentary (a) and exercised (b) rats. For the acute condition, a significant increase in perfusion is observed in the hippocampal area of exercised rats (d) compared to sedentary controls (c). For the week condition, a significant increase in perfusion is observed in the hippocampal area of exercised rats (f) compared to sedentary controls (e).

6.3.2.4 One week of exercise induces an increase in BDNF protein in the dentate gyrus.

Analysis of homogenized tissue from the dentate gyrus of rats from the MRI experiment using an ELISA showed a significant increase in BDNF in the right dentate gyrus of the BDNF group compared to controls when normalised to total protein ($p=0.0075$, Student's *t*-Test, Fig 6.17, Sed: 91 ± 7.44 pg/mg, Ex: 133 ± 9.18 pg/mg).

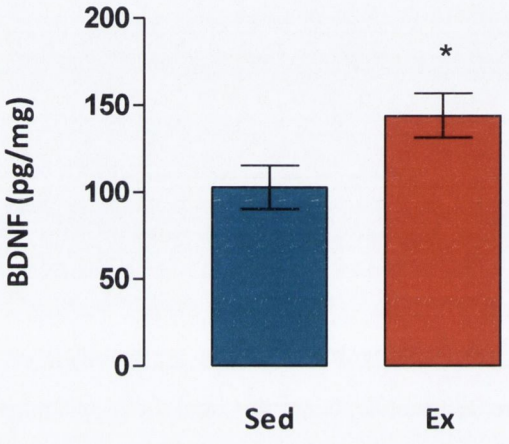

Fig 6.17 One week of exercise induces an increase in BDNF protein in the dentate gyrus.
Analysis of homogenized tissue from the dentate gyrus of rats from the MRI experiment using an ELISA showed a significant increase in BDNF in the right dentate gyrus of the exercised group compared to sedentary controls when normalised to total protein.

6.3.3 Na-F Analysis

We conducted two studies to evaluate exercise-induced changes in blood-brain barrier permeability using intravenous sodium fluorescein (Na-F).

In the first study, we observed no differences in mean fluorescence in the dentate gyrus between sedentary and exercised rats when infused with Na-F after one week of exercise, and one hour after the last bout. The level of detected fluorescence in all brains was negligible (Fig 6.18, Sed: 2.91 $\pm$ 0.07, Ex: 2.86 $\pm$ 0.063).

In the second study, we observed no significant change in mean fluorescence in the dentate gyrus of exercised rats compared to sedentary controls. The level of detected fluorescence in all brains was higher than in the first study and the variability was also much higher ($p=0.07$, Student's *t*-Test, Fig 6.19, Sed: 46.39 $\pm$ 30.01, Ex: 184.7 $\pm$ 92.43).

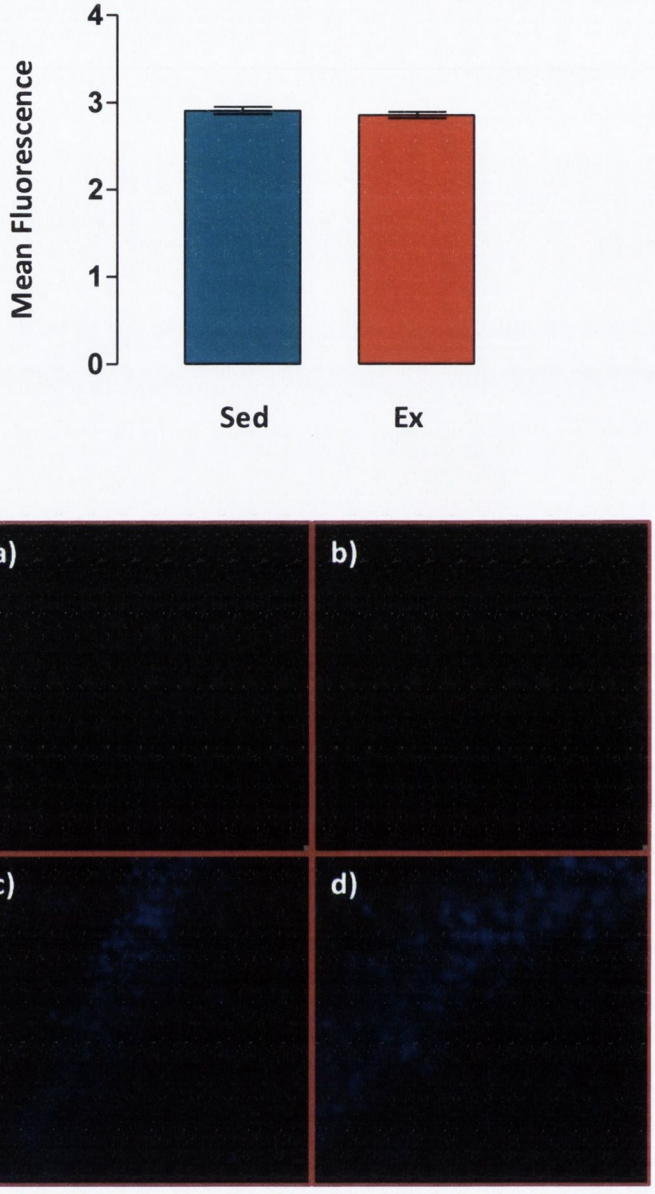

Fig 6.18 Na-F Study: Week Condition

The graph shows the mean fluorescence for the dentate gyrus for sedentary (n=3) and exercised (n=3) rats after one week of exercise, one hour post-exercise. No differences were observed and mean fluorescence was negligible for both groups. Data are shown in mean fluorescence $\pm$ SEM. The images show representative views of Na-F fluorescence for a sedentary (a) and exercised (b) rat, while (c) and (d) show the corresponding DAPI-stained cells for (a) and (b) respectively, to indicate the position of the view along the dentate gyrus .

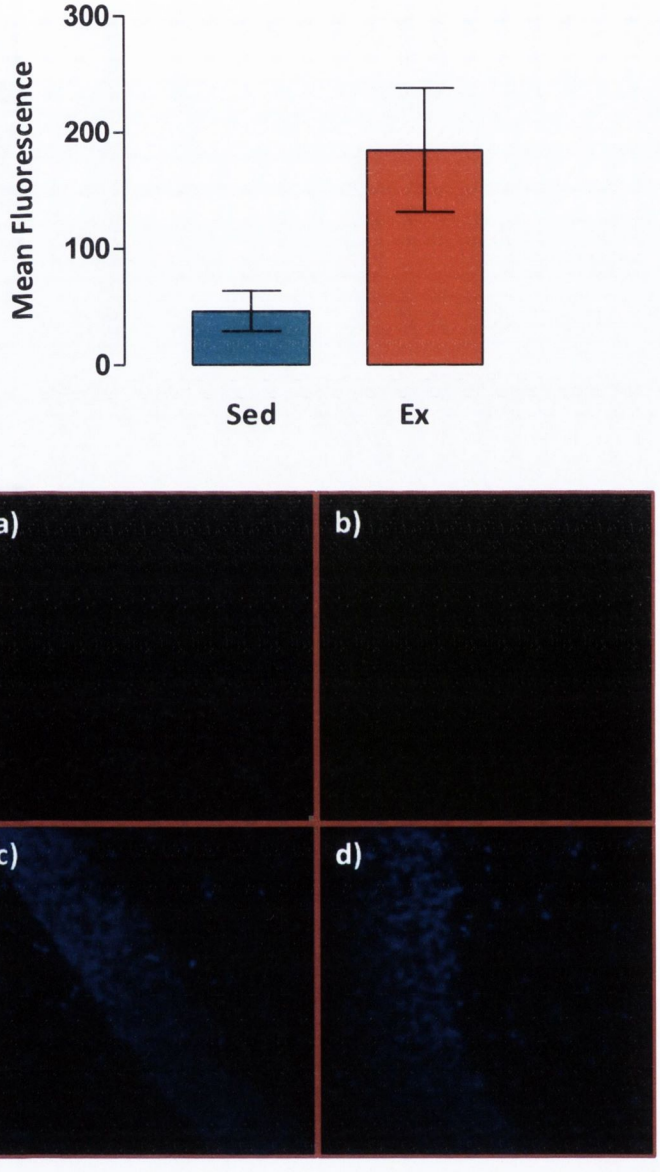

Fig 6.19 Na-F Study: Acute Condition

The graph shows the mean fluorescence for the dentate gyrus for sedentary (n=3) and exercised (n=3) rats immediately post-exercise. No significant difference in fluorescence was observed between groups. Data are shown in mean fluorescence $\pm$ SEM. The images show representative views of a sedentary (a) and exercised (b) rat, while (c) and (d) show the corresponding DAPI-stained cells for (a) and (b) respectively, to indicate the position of the view along the dentate gyrus

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6.4 Discussion

6.4.1 Behavioural Analysis

Infusion of a single bolus of recombinant BDNF (i.v.) did not improve spatial memory in an object displacement task. Since intracerebroventricular infusion of the same dose (10ng) in Chapter 5 did induce a clear improvement in a similar task, it is possible that that recombinant BDNF, when infused into the circulation, is unable to cross the blood-brain barrier. This is consistent with studies (Pardridge et al., 1994, Pardridge et al., 1998, Pardridge, 2006, Zhang and Pardridge, 2006) which showed that BDNF does not cross into the brain from the circulation. There has been a recent report of chronic subcutaneous BDNF infusions causing an increase in BDNF in the brain, (Schmidt and Duman, 2010) along with antidepressant and anxiolytic effects. However it is possible that in this case, BDNF is being sequestered by the platelets or endothelial cells in the periphery, and then transported into the brain by different mechanisms (for example exercise, as discussed below).

Infusion of a single bolus of recombinant BDNF (i.v.) improved novel object recognition memory in an object substitution task, when rats were exercised for 1 hour immediately post-infusion. Rats that received a cytochrome c infusion (10ng, i.v.) prior to exercise did not preferentially explore the novel object. This result suggests that circulating BDNF may be crossing the blood-brain barrier only in the presence of exercise. Since several studies have suggested an exercise-induced increase in blood-brain barrier permeability, it is possible that exercise is regulating that permeability of the blood-brain barrier to BDNF. This is discussed further in the last section.

6.4.2 BDNF Analysis

Rats that received an intravenous BDNF infusion immediately pre-exercise had a significantly higher concentration of BDNF in the dentate gyrus compared to controls that received an intravenous infusion of cytochrome c pre-exercise. These data suggest that an increase in BDNF in the circulation is able to translate into an increase in BDNF in the brain, but only in the presence of exercise. These data also suggest that the increase in BDNF is most pronounced in the dentate gyrus compared to the rest of the hippocampus. One reason for this localised increase could be simple vascularisation, the dentate gyrus is the most highly vascularised structure in the

hippocampus, allowing it to receive the largest blood supply when normalised to tissue volume (Grivas et al., 2003).

In the MRI study, rats were exercised daily for one week and perfused on the last day of exercise. We observed an increase in the concentration of BDNF protein in the dentate gyrus of these rats. This is a particularly interesting finding for two reasons. First, this is the first time animals were perfused after one week of exercise prior to tissue collection. Since the dentate gyrus is highly vascularised, reports of an increase in BDNF in the tissue could be reflecting an increase in the capillaries. Perfusion allows us to remove this confound and confirm that an increase in BDNF is specifically occurring in the tissue and not in the blood. Second, this was the first time the animals were killed on the last day of exercise and not a day later. This experiment has since been replicated in the laboratory with the same result: when tissue samples are collected on the last day of exercise we observe a robust increase in BDNF protein in the dentate gyrus, but not when tissue is collected 24 hours later. Taken together with our results from previous chapters, this result indicates that timing may be crucial in determining whether we find increases in BDNF protein, mRNA, or both in the dentate gyrus. Our results suggest that the increase in BDNF protein precedes that of mRNA, indicating that it may be the protein that is increasing first, and then activating the transcription of more BDNF. This supports the hypothesis that the source of the BDNF increase in the dentate gyrus is outside of the dentate gyrus.

6.4.3 Blood Flow Analysis

We used bolus tracking Arterial Spin Labelling (btASL) analysis to quantify exercise-induced changes in the perfusion of the hippocampus, cortex, and whole brain. We found a highly significant increase in regional cerebral blood volume (rCBV) to the hippocampus after an acute bout of exercise, along with corresponding decreases in mean transit time (MTT: defined as the average time taken for a labelled particle to cross the vasculature) and capillary transit time (CTT: defined as the time taken for a labelled particle to diffuse at a region of interest). We also found a significant increase in regional cerebral blood volume (rCBV) to the hippocampus after a week of exercise, along with corresponding decreases in MTT and CTT. We did not delineate a region of interest specific to the motor cortex, but analysed the cortex as a whole. This could, in part, explain why we did not observe any changes in rCBV to the motor cortex.

Increases in cerebral blood flow in response to cardiovascular exercise have been well established to occur both acutely and persistently (Seifert and Secher, 2011). The mechanisms by which exercise induces acute changes in cerebral blood flow include:

1) Cerebral autoregulation: arteries to the brain respond to decreases in blood pressure by vasodilation, leading to an increase blood flow to the brain.

2) Baroreceptors: activated by drops in arterial CO2 levels to cause vasodilation, leading to an increase in blood flow to the brain

3) Neurovascular coupling: increased neuronal activity during physical activity leads to higher metabolic demand during and straight after exercise. This is especially relevant with regards to increases in blood flow to the motor cortex with exercise, as reported by Smith and colleagues (Smith et al., 2010).

These mechanisms explain why exercise would cause an increase in total cerebral blood flow and blood flow to the motor cortex, but not why it would be restricted to a particular area of the brain: the hippocampus.

Our finding is in agreement with studies reporting hippocampal specificity when investigating exercise-induced increases in vascularisation (Van der Borght et al., 2009) and exercise-induced increases in blood volume (Pereira et al., 2007). In both of these studies, changes were restricted to the dentate gyrus of the hippocampus, and neurogenesis-induced angiogenesis was cited as an explanation (Ward and Lamanna, 2004, Yang et al., 2011). The progression of newborn cells through the stages of neurogenesis is accompanied by parallel changes in the vascular microenvironment. New neurons have a high metabolic demand, and to ensure an adequate supply of oxygen and nutrients they secrete signals that promote the formation of new blood vessels, creating what has been termed a 'vascular niche' (Palmer et al., 2000). Since the dentate gyrus is the only area in the adult brain (outside the olfactory bulb) to undergo neurogenesis, it is possible that the high rates of angiogenesis within these neurogenic niches result in high levels of vascularisation. This hypothesis is supported by the finding that dentate gyrus is the most highly vascularised area of the hippocampus (Grivas et al., 2003). Since improved vascularisation of a particular area corresponds to higher amounts of blood supply, it is possible that the dentate gyrus is more sensitive to increases in CBV than the rest of the brain, simply because of its unique neurogenic and angiogenic properties. This could also explain why the hippocampus is more

vulnerable to blood-borne toxins than the rest of the brain, and why it is the first brain region to deteriorate with ageing.

But why is the effect larger with exercise? In the week condition, increased cell proliferation coupled to angiogenesis could account for the increase in CBV, indicating that a week of exercise could have increased the vascularisation of the dentate gyrus in exercised animals. However, in the acute condition, it is more likely that increases in total cerebral blood flow occurred with exercise but that they are most evident and last longest in the dentate gyrus, due to its unique vascular properties.

6.4.4 Blood-brain barrier Permeability

Acute and persistent exercise-induced changes in blood-brain barrier permeability were investigated using intravenous sodium fluorescein (Na-F).

When Na-F was infused after one week of exercise, one hour after the last bout of exercise, no changes in fluorescence were observed in the dentate gyrus when assessed by fluorescent microscopy. The amount of fluorescence observed was negligible. This is consistent with the idea that Na-F cannot cross the blood-brain barrier under normal circumstances, and shows that if exercise induces any changes in BBB permeability, this effect is transient and disappears within one hour of exercise.

When Na-F was infused after a single bout of exercise, the results were less clear. Both sedentary and exercised rats showed higher levels of fluorescence in the dentate gyrus compared with the first experiment (week condition), despite the fact that all procedures in the two experiments were carried out in an identical manner. In addition, we observed no significant change in mean fluorescence in the exercised rats when compared to sedentary controls. However, the variability of the fluorescence values was very high both within and between groups. Further experiments and a larger sample size are required to clarify whether the observed increase is a reliable effect and not caused by an artifact.

The idea that exercise may be causing an increase in the permeability of the blood brain is not a new one. A review of the literature reveals several possible mechanisms, including exercise-induced hypoxia, since hypoxia has been shown to increase blood-brain barrier permeability (Kaur and Ling, 2008). Hypoxic effects may also be confounding our results in of the Na-F experiment:

since we perfused the brains before tissue collection, it is possible that the blood-brain barrier may have allowed Na-F to pass through as an effect of hypoxia induced by the perfusion. This does not however explain why we found an increase in fluorescence only in the second study, since we perfused the rats in both studies.

If changes in BBB permeability do occur with exercise, it is likely that these changes are region-specific. Studies have shown that the BBB is not homogenous all over the brain, and that it is more vulnerable to leakage in certain locations (Saubamea et al., 2011).

Another possible explanation is that the high rates of angiogenesis in the dentate gyrus are linked to changes in blood-brain barrier permeability. There have been reports of increased BBB permeability with both angiogenesis and VEGF overexpression (Rigau et al., 2007). It appears that newly formed capillaries are leakier than mature capillaries, thus the integrity of the blood-brain barrier is weaker in areas in which high rates of vascular remodelling and angiogenesis are taking place. Exercise-induced increases in VEGF (Fabel et al., 2003) and angiogenesis (Ding et al., 2006b) have been well-established, and a study showing that intravenous infusions of VEGF increased BBB leakage (Zhang et al., 2000) further supports this hypothesis.

This could explain why exercise-induced changes in blood-brain barrier permeability might be confined to the dentate gyrus, ensuring tissue-specific delivery of blood borne growth factors such as BDNF and IGF-1. This could also explain the transient nature of exercise-induced changes: the leaky capillaries are present continuously, but it is only when total cerebral blood flow increases in response to exercise that the pressure in the capillaries is high enough to cause significant leakage. This hypothesis merits further investigation.

6.5 Conclusion

In this Chapter, we present data that support the hypothesis that BDNF can cross the blood-brain barrier from the circulation into the dentate gyrus with exercise. We show that peripheral BDNF administration can enhance memory and induce an increase in BDNF in the dentate gyrus, but only when rats are exercised immediately post-infusion. We also report that blood flow to the hippocampus increased immediately after an acute bout and after one week of exercise, which may constitute a mechanism by which BDNF is delivered to the hippocampus in a spatially restricted manner.

Chapter 7 General Discussion

This work adds several key findings to the rapidly-expanding literature on exercise-induced cognitive enhancement. Our main objective was to assess the beneficial effect of short-term moderate forced exercise on two types of memory, and to investigate the underlying molecular, cellular, and vascular changes as possible mechanisms mediating these improvements.

7.1 Behaviour

The beneficial effects of exercise on cognitive performance have been well established in both humans and animals (Hillman et al., 2008). Most of these studies use long-term (more than three weeks) and voluntary exercise. Here we have shown that one week of forced moderate exercise induces robust and reliable improvements in both spatial memory and object recognition memory in young healthy rats. Both types of memory are heavily dependent on pattern separation, and are thought to recruit primarily the dentate gyrus. The tasks we used to assess these different types of memory were an object displacement task and a novel object recognition task respectively. We have shown that varying the number of objects and the number of training trials can make these tasks more or less challenging, and that this method can be used to establish a highly sensitive testing system for the evaluation of cognitive performance in rats. We have shown that in the case of spatial learning, a more challenging variant of the task is needed to observe an improvement induced by one week of exercise.

We have also provided evidence that the cognitive-enhancing effects of one week of forced moderate exercise are evident in animals housed in both enriched and standard conditions. This indicates that the exercise-induced effects are unlikely to be related to mental deprivation due to housing conditions. In addition, we have shown that treadmill shock-induced stress is unlikely to play a role in the cognitive-enhancing effects of our exercise paradigm. In parallel, we demonstrated that three weeks of environmental enrichment without running wheels may have some beneficial effect on learning in an object recognition task, although it. Finally, we showed that the effects of one week of exercise on both spatial and object recognition memory can be mimicked by a single intracerebroventricular injection of recombinant BDNF.

7.2 Mechanisms

We have shown that exercise is accompanied by a wide range of cellular, molecular, and vascular changes, and that most of these changes are restricted to the hippocampus, and more specifically to the dentate gyrus. Since recent evidence has suggested a crucial role for the dentate gyrus in pattern separation, we hypothesise that one or more of these mechanisms could serve to improve neural function and synaptic transmission in the dentate gyrus and directly cause the improvements we see in memory. Here we review some of the more consistent and interesting exercise-induced changes, and attempt to link them mechanistically.

One of our most consistent observations is a transient exercise-induced increase in BDNF in the serum, which lasts at least 24 hours after the last bout of exercise. Unpublished studies in our laboratory have shown that this increase is apparent as soon as 4 hours after an acute bout of exercise. This finding is quite prominent in the literature (Zoladz and Pilc, 2010) and there is some evidence supporting the hypothesis that the source of circulating BDNF is the hippocampus itself, and that it crosses the blood-brain barrier into the circulation during exercise (Rasmussen et al., 2009). The mechanisms by which exercise could cause an increase in BDNF in the dentate gyrus are unknown. It is also possible that the BDNF originates, at least in part, from sources outside the dentate gyrus. Possible peripheral sources include platelets and endothelial cells. Platelets do not produce BDNF, but only sequester it, and it is possible that BDNF is being released from platelets due to sheer stress or core temperature changes associated with exercise. If this is the case, we would expect to see an increase in BDNF in the plasma but not in the serum. Some studies have shown increases in BDNF in the plasma, but the Data are inconclusive, mainly because of inconsistent blood analysis techniques (Zoladz & Pilc, 2010). Since coagulation causes the release of BDNF from platelets (Fujimura et al., 2002), and increases in temperature speed up this coagulation, it is possible that blood analyses are confounded by the time the samples are left to clot, and the temperature at which they are incubated.

One of our most exciting findings is an increase in blood flow to the hippocampus immediately after acute exercise. We have suggested the possibility that increases in blood flow to the hippocampus could enhance the transport of BDNF to the hippocampus from the circulation, provided it is able to cross the blood-brain barrier. We have also suggested that the permeability of the blood-brain barrier to BDNF could be affected by exercise. The most compelling evidence we have to support this hypothesis is that a single intravenous infusion of BDNF improved memory

only when immediately followed by an acute bout of exercise. This finding has since been replicated in the laboratory. Furthermore, the infusion caused an increase in BDNF in the dentate gyrus of exercised animals compared to animals that received cytochrome c and exercise. Since we have shown that an acute bout of exercise is associated with increased blood flow to the hippocampus, and since the blood-brain barrier may be leakier at the DG than in other regions due to a high rate of angiogenesis (new capillaries are leakier), we conclude that exogenous BDNF may be delivered to the dentate gyrus from the circulation with exercise. This would also lend more weight to the hypothesis that BDNF is being released from a peripheral source (such as platelets or endothelial cells) during exercise. This hypothesis is especially attractive since the mechanisms involved would be simple and physiological, without a need for as yet unknown and possibly non-existent mediators. It would also fit in very well with the rest of the data presented in this work, as the release of BDNF into the circulation, along with changes in blood flow, and changes in blood-brain barrier permeability, could act as an instigator for all other exercise-induced events observed (discussed below).

We did not detect an increase in BDNF protein in the dentate gyrus when tissue was collected 24 hours post-exercise, but we did when tissue was collected one hour post-exercise. We did however observe increases in BDNF mRNA expression in dentate gyrus tissue 24 hours after the last bout of exercise. This is consistent with other studies which have observed increases in BDNF mRNA expression with 3 days of voluntary running (Vaynman et al., 2003, Farmer et al., 2004). If increases in BDNF protein in the dentate gyrus precede the changes in mRNA this could indicate that BDNF from outside the dentate gyrus could be reaching the dentate gyrus and stimulating the transcription of more BDNF. The activation of ERK via the MAPK pathway has been shown to upregulate BDNF (Saarelainen et al., 2001) indicating that the presence of BDNF can activate its own transcription in a positive feedback loop. The source of BDNF could be central (Falkenberg et al., 1993) or peripheral (e.g. platelets and endothelial cells).

Changes in the brain that are likely to underlie improvements in memory induced by one week of exercise must occur within that week. Similarly, changes that are likely to underlie improvements induced by an acute bout of exercise or a single intracerebroventricular injection of BDNF would have to occur within half an hour, since rats are trained within half an hour of the infusion or exercise. This automatically excludes cellular and morphological changes such as neurogenesis, spinogenesis, synaptogenesis, and angiogenesis as possible mechanisms, although all of these have been shown to occur with exercise. Short-term BDNF-induced changes in plasticity can be TrkB-

dependent or independent, and include direct changes in membrane excitability, increases in extracellular calcium and the phosphorylation of synaptic vesicle proteins. We have shown that one week of exercise causes the activation of TrkB and the Ras-MAPK pathway, resulting in the phosphorylation of ERK-1. We also observed increased phosphorylation of ERK-1 and CaMKII within half an hour of an intracerebroventricular BDNF infusion. We conclude that BDNF-stimulated signaling cascades, specifically the Ras-MAPK pathway, and possibly also the PLCγ-CaMKII pathway, are likely to be involved in mediating exercise-induced effects on synaptic transmission in the dentate gyrus, and have an impact on memory. Since these same pathways are activated by learning, loss of function studies which block these mechanisms and observe disruptions in cognitive performance are difficult to interpret. Thus despite the substantial evidence highlighting BDNF as the central mediator of exercise-induced cognitive enhancement, no direct causal links have been established. It is always possible that the beneficial effects of exercise are entirely BDNF-independent. Some of the more interesting hypotheses arguing the BDNF-independence of these effects are outlined below.

7.3 Alternative hypotheses

7.3.1 Changes in core temperature with exercise

Since the membrane fusion dynamics are temperature-dependent, neurotransmitter endo-and exoctyosis at synaptic terminals can be affected by changes in temperature (Smith et al., 2008). Many *in vitro* studies have reported temperature-dependent changes in synaptic transmission (Hardingham and Larkman, 1998, Dinkelacker et al., 2000, Pyott and Rosenmund, 2002, Fernandez-Alfonso and Ryan, 2004, Kushmerick et al., 2006). In particular, there is evidence to suggest that increasing temperature facilitates LTP, mainly by increasing the amount of neurotransmitter released at synaptic terminals.

Since a single bout of forced moderate exercise increases the core temperature of animals by 2-3°C (*unpublished observations*), it is possible that exercise-induced increases in core temperature could facilitate synaptic transmission and enhance learning. However, it must be noted that the effect of temperature changes on synaptic transmission have only been studied *in vitro*, and most were performed to compare between RT and physiological temperatures. One way to test this hypothesis *in vivo,* would be to heat animals up in the absence of exercise (by changing holding RTs) and test cognitive performance and/or LTP induction. This study is currently ongoing in our laboratory.

7.3.2 Increased blood flow to the brain with exercise

We discussed the link between increased blood flow to the hippocampus and exercise-induced cognitive enhancement in the context of enhanced BDNF delivery to the hippocampus during exercise. However, enhanced blood flow to the hippocampus could improve neural function in a BDNF-independent manner, simply by increased oxygen and nutrient supply. However, while meeting metabolic demands would have a general effect on neuronal health, it is unclear if and how this could impact plasticity on the short-term.

7.3.3 Other growth factors

Besides BDNF, there is evidence that other growth factors are induced with exercise: IGF-1 and VEGF-1 are of particular interest. IGF-1 has direct effects on neuroplasticity, neurogenesis, and neuronal survival (Torres Aleman, 2005), similar to those of BDNF. In addition, IGF-1 has been shown to cross the blood-brain barrier from the serum via a saturable transport system (Pan and Kastin, 2000). It has been shown to be necessary for exercise-induced cognitive enhancement (Trejo et al., 2001, Trejo et al., 2008), but it remains to be seen whether it is sufficient and acts in a manner that is independent of BDNF. In addition to its role in promoting vascular changes, VEGF-1 has also been shown to have direct effects on synaptic plasticity (Kim et al., 2008), possibly by acting directly on CaMKII. Since some studies have shown VEGF-1 induction in the brain with exercise (Fabel et al., 2003), it is possible that VEGF-1 could play a role in exercise-induced cognitive enhancement, although this requires further investigation.

7.3.4 Theta rhythm induction with exercise

Hippocampal theta rhythms are strong oscillatory patterns that can be recorded from within the hippocampus using electroencephalography (EEG). These oscillations are produced when populations of hippocampal neurons fire in synchrony at a frequency of 4-8 Hz, and have been observed to occur in states of alertness and exploration in the rat. Theta oscillations have been linked to spatial memory performance (Buzsaki, 2005). Direct evidence and exact mechanisms have yet to be revealed, but in general, it is thought that enhanced synchronous activity in the hippocampus results in improved spatial learning (Jutras and Buffalo, 2010). Since treadmill running has been shown to robustly induce theta activity in healthy young male rats, it has been proposed that theta rhythms could serve as a possible mechanism for short-term exercise-induced

cognitive enhancement (Kuo et al., 2011). The main problem with this hypothesis is one of timing: theta–activity usually stops when running stops (Kuo et al., 2011), while training in a cognitive task usually occurs at least half an hour later. It is also possible that theta rhythms and BDNF are not two mutually exclusive mechanisms: BDNF increases the excitability of neurons, hence BDNF-induction increases the likelihood of neurons firing and hence their synchronicity. Again, the timing of these events is inconsistent with this hypothesis: theta–activity stops when running stops (Kuo et al., 2011) whereas the increase in BDNF has been shown to last up to 24 hours post-running (Griffin et al., 2009). An interesting study that might clarify some of these issues would be to acutely exercise rats or exogenously administer BDNF, and then record theta rhythms from the hippocampus using EEG, in combination with LTP.

7.4 Future Directions

7.4.1 Timing of exercise-induced changes

The timing of exercise-related events is crucial to assessing their involvement in short-term cognitive enhancement. A study in which samples of blood and tissue are taken at different time-points post-exercise and assessed for BDNF protein and mRNA would clarify some of the uncertainty related to the time-scale and sequence of the effects of exercise. This could be conducted with an acute bout of exercise, since it removes any confounds related to multiple bouts of exercise.

7.4.2 Blood-brain barrier permeability with exercise

The question of whether BDNF is crossing the blood-brain barrier with exercise could be tackled by tagging exogenous BDNF and tracing its movement post-administration in rodents. One possible method could be to biotinylate recombinant BDNF, inject it peripherally, and assess its presence in the brain (and specifically the dentate gyrus) using standard immunohistochemical techniques. This study could be performed bidirectionally: the biotinylated BDNF could also be infused intracerebroventricularly, and then assessed in serum and plasma.

In parallel, further studies are required to investigate whether exercise induces changes in the permeability of the blood-brain barrier. The serum of exercised animals could be tested for the CNS-specific protein S100-β. In addition, the permeability of the blood-brain barrier to molecules

of varying molecular weights can be evaluated using dextrans (Hoffmann et al., 2011). If BDNF is shown to cross the blood-brain barrier, but other particles of similar or smaller size are not, this would indicate the existence of a BDNF-specific transport system.

7.4.3 Short-term exercise and angiogenesis

There are several methods to visualize the microvasculature in brain sections (Argandona and Lafuente, 1996). It would be interesting to look at how fast exercise-induced changes in angiogenesis occur, as a possible mechanism for the changes we see in blood flow. Parallel changes in VEGF-1 and its receptor could be assessed.

7.4.4 Peripheral sources of BDNF: platelets and endothelial cells

The source of BDNF during exercise has still not been properly identified. In the periphery, platelets and endothelial cells have been suggested as possible contributors. It would be of great interest to perform well-controlled plasma Vs serum studies immediately post-exercise, and to isolate intact platelets and endothelial cells and evaluate their BDNF content before and after an acute bout of exercise.

7.4.5 Acute bouts of exercise and LTP

Unpublished experiments in the laboratory have shown cognitive improvements with as little as a single acute bout of exercise. We have shown that these improvements are accompanied by increases in blood flow to the hippocampus, and by an increase in BDNF in the serum. To date, no studies have looked at whether an acute bout of exercise facilitates LTP in the MPP-DG pathway, and whether this can be blocked by infusing p-MAPK inhibitors and p-CaMKII inhibitors.

7.4.6 Small molecule mimetics for BDNF

Since BDNF is a large molecule with a short half-life (De Young et al., 1999) and poor pharmacokinetic properties, attempts at delivering BDNF to the brain in human clinical trials have been largely unsuccessful, presumably due to a difficulty in crossing the blood-brain barrier (Nagahara and Tuszynski, 2011). One way to circumvent these difficulties is to develop small molecule mimetics for BDNF. These molecules could potentially be injected peripherally, cross the

blood-brain barrier to activate TrkB receptors in the brain, and initiate downstream signalling cascades in a manner that mimics BDNF.

Until very recently, such endeavours were hindered by the lack of availability of potent and selective TrkB agonists. Within the past year, the identification of a novel TrkB agonist: 7,8 dihydroxyflavone (Jang et al., 2010) has caused a new surge of interest in this approach. The agonist is a flavinoid derivative that has been shown to bind to the TrkB receptor with a high affinity. It has also been shown to dimerise and activate the autophosphorylation of TrkB as efficiently as BDNF itself. Importantly, it was reported to penetrate the blood-brain barrier: i.p. injections of this compound caused activation of TrkB receptors in the hippocampus (Jang et al., 2010).

Furthermore, 7,8-DHF has been reported to mimic some of BDNF's neuroprotective properties: the compound protected cultured hippocampal neurons against apoptosis in a model of neuronal injury (Jang, et al., 2010). In addition, two recent studies have demonstrated the ability of 7,8-DHF to mimic some of the cognitive-enhancing effects of BDNF: i.p. administration in mice enhanced the acquisition of fear memory (Andero et al., 2011) and reversed spatial memory impairments in animal models of stress (Andero et al., 2010) and Alzheimer's disease (Devi and Ohno, 2011).

If the exercise-induced enhancements in memory we have observed throughout this work are directly caused by the binding of BDNF to TrkB and subsequent activation of signalling cascades, we hypothesise that activation of the TrkB receptor via a small molecule agonist would have a similar effect. To test this hypothesis, it would be interesting to investigate whether a single i.p. injection of the selective TrkB agonist (7,8-DHF) could mimic the observed enhancements in spatial memory induced by exercise and BDNF i.c.v. infusions. If the TrkB agonist can be shown to work in vivo, this could have enormous clinical implications.

7.5 Implications of this work

The literature on cognitive enhancement suggests that there are no other behavioural or pharmacological interventions to-date that are as potent and as effective as exercise in 1) increasing BDNF, 2) increasing neurogenesis, 3) improving spatial and object recognition memory and 4) reversing deficits in all of the above.

The finding that just one week of moderate forced treadmill running can have robust beneficial effects in young adult rats highlights just how potent and efficient this simple lifestyle intervention really is. We observed enhancements in two different types of memory, increases in blood flow to the brain, increases in neurotrophin release and activity, and increases in neurogenesis, all with only a week of exercise. Long-term, regular, high-intensity exercise regimes may be intimidating, especially in ageing, sick or sedentary populations, while short-term, low-intensity regimens may be seem more accessible, providing reluctant humans with a compelling incentive to exercise.

We have also eliminated some confounds associated with translating exercise research in rodents to humans. We have shown that it is simply the physical activity component of exercise, and not mental stimulation or stress that induces cognitive benefits and BDNF-related changes. Human beings, unlike laboratory animals, live in a world of constant social and cognitive enrichment, but are still likely to benefit highly from moderate physical exercise.

We have provided evidence that improvements in memory tasks that rely on pattern separation can occur within a time-frame that is too short for newly born granule cells in the dentate gyrus to become functional. This Data are inconsistent with the hypothesis that neurogenesis is necessary for improved pattern separation. We have also presented compelling evidence that an exercise-induced increase in BDNF in the dentate gyrus is sufficient to induce significant improvements in spatial memory. This strengthens the case for a BDNF-related mechanism for exercise-induced cognitive enhancement, and also suggests that the intracerebroventricular injection of physiological doses of BDNF could be developed into a therapeutic application for the treatment of cognitive deficit and decline.

Furthermore, if exercise is opening up the blood-brain barrier, this finding could be of particular clinical significance. The integrity of the blood-brain barrier is one of the biggest challenges to the therapeutic delivery of drugs to the CNS. A transient, safe, and spatially-restricted method of increasing the permeability of the blood-brain barrier is of paramount interest in a clinical setting.

We have also demonstrated that the acute exogenous administration of recombinant BDNF is well-tolerated, can activate BDNF-related signaling events in the dentate gyrus that facilitate synaptic transmission, and is accompanied by improvements in both spatial and object recognition memory. As a result, the successful delivery of BDNF could be extremely promising in a clinical setting, especially if it can be administered via peripheral injections combined with acute bouts of exercise, which would bypass the need for neurosurgery.

In conclusion, this work beings us one step closer to elucidating the mechanisms by which short-term exercise induces cognitive enhancement, and answers several outstanding questions in the field. In addition, it demonstrates the importance of pursuing this line of research, since both exercise and BDNF have exciting therapeutic potential which should be exploited to improve cognition in health and disease.

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X. Selected Publications and Abstracts

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