LEABHARLANN CHOLÁISTE NA TRÍONÓIDE, BAILE ÁTHA CLIATH | TRINITY COLLEGE LIBRARY DUBLIN
Ollscoil Átha Cliath | The University of Dublin

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

Copyright statement

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

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

Liability statement

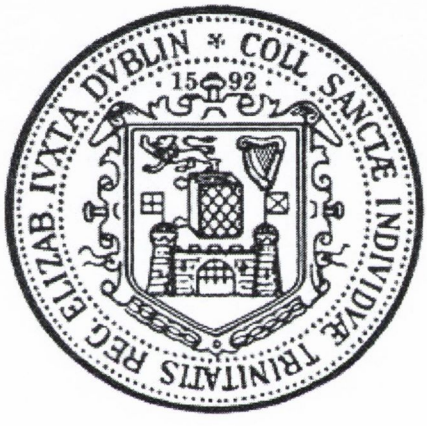
By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses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

Access Agreement

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

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

The Efficacy of Environmental Enrichment as Cognitive Enhancer: An Evaluation of the Underlying Mechanisms



by Amy Marion Birch

Thesis submitted for the degree of Doctor of Philosophy

at University of Dublin, Trinity College

2012

TRINITY COLLEGE
2 5 SEP 2012
LIBRARY DUBLIN

Thesis 9634

Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this orany other university and it is entirely my own work.

I agree to deposit this thesis in the University's open access institutional repository orallow the library to do so on my behalf, subject to Irish Copyright Legislation andTrinity College Library conditions of use and acknowledgement.

I Summary

Environmental manipulations can enhance neuroplasticity in the brain, with enrichment-induced cognitive improvements being linked to increased expression of growth factors, such as neurotrophins, and enhanced hippocampal neurogenesis. Environmental enrichment can ameliorate the memory decline reported in normal ageing and in animal models of Alzheimer's disease, Huntington's disease and depression. Thus, this may be a particularly beneficial therapeutic intervention against age-related cognitive decline. Environmental enrichment is defined as the addition of social, physical and somatosensory stimulation into an animal's environment via larger group housing, extra objects and often, running wheels. Previous studies from our lab report that physical activity alone is a potent memory enhancer but that long-term environmental stimulation can be as effective as exercise at ameliorating age-related memory decline. Therefore, it is crucial to dissociate the different factors of environmental enrichment, and to elucidate the associated neurochemical mechanisms. The aim of this study is to evaluate the efficacy of environmental enrichment, in the absence of exercise, as a cognitive enhancer and assess the underlying mechanisms involved, with particular focus upon the roles of neurotrophins and neurogenesis in mediating this effect.

To assess the minimum period of environmental enrichment necessary to elicit an improvement in hippocampal-dependent memory, male Wistar rats were housed in standard (SH) or environmentally enriched (EE) housing for two, three and six weeks. Following this, rats' recognition memory was tested using the Novel Object Recognition (NOR) task.To measure the effect of environmental enrichment on additional forms of memory, spatial (using the Object Displacement [OD] task) and working memory (using the T maze task) of 6 week EE rats was also tested. 3 week and 6 week EE rats displayed improved recognition memory and 6 week EE rats also displayed improved spatial and working memory. Neurochemical analyses revealed a significant increase in βNGF and cell proliferation (as measured by BrdU+ nuclei) in the dentate gyrus of 6 week EE rats, and further analysis revealed a significant positive correlation between NOR task performance and both βNGF and BrdU+ nuclei, suggesting that they may mediate the enrichment-induced memory improvements. In addition, 6 week EE rats showed a significant increase in synaptophysin and synapsin I in the dentate gyrus, indicating that environmental enrichment can enhance synaptic plasticity.

To further analyse the effect that βNGF might play in enhancing memory function, male Wistar rats were given a single intracerebroventricular infusion or, in some

experiments,infused chronically (42 days) with βNGF. All βNGF-infused rats displayed significantly improved recognition memory, as measured using the 3 object NOR task. Chronic βNGF-infused rats displayed a significant increase in βNGF, TrkA and synapsin I in the hippocampus and a parallel increase in ERK phosphorylation when compared with controls, suggesting a learning-induced activation of the MAPKinase pathway. Chronic βNGF-infused rats also showed an increase in cell proliferation in the dentate gyrus, suggesting that βNGF can enhance hippocampal neurogenesis.

To assess the neuroprotective effect of environmental enrichment, in the absence of exercise, rats were housed in continuous enriched conditions for approximately 20 months and their memory was assessed at young age, prior to their housing, middle age and old age using the two object and three object NOR task, OD task, Morris water maze and T maze task. Rats were also scanned in a 7T Bruker MRI scanner at young, middle age and old age to assess regional changes in grey matter (using Voxel-Based Morphometry) and blood flow (using bolus-tracking Arterial Spin Labelling) with age, and the effect environmental enrichment may have upon these measures. There was an age-related decline in recognition, spatial and working memory which is prevented with environmental enrichment. Neurochemical analysis revealed an age-related reduction in βNGF and VEGF in hippocampus, and cell proliferation in the dentate gyrus, that is prevented by environmental enrichment. Furthermore, environmental enrichment attenuated the age-related increase in apoptosis and the pro-inflammatory markers IL-1β and CD68. Long-term environmental enrichment also induced increases in grey matter intensity in the S1 cortex and partially rescued the age-related reduction in cerebral blood flow.

Taken together, the data in this study provide clear and powerful evidence that environmental enrichment can induce memory improvements in young rats, and can prevent age-related memory loss. We consistently demonstrate that these memory improvements are associated with upregulation of βNGF and hippocampal neurogenesis in both the long-term and short-term. This study also demonstrates that environmental enrichment can ameliorate many features typical of the ageing brain, such as increases in apoptosis and pro-inflammatory markers. Furthermore, we provide novel data on enrichment-induced regional grey matter increases and age-related alterations in blood flow in the rat.

II Acknowledgments

First, I wish to thank my supervisor Dr Áine Kelly for her continued support and guidance. Her seemlingly never ending enthusiasm and encouragement has been a great support for me in the last year. I genuinely don't think I would have be able to do this without her.

I am forever grateful to Prof. Marina Lynch and Dr Áine Kelly for giving me the opportunity to undertake a PhD in Trinity College Institute of Neuroscience and the Department of Physiology. I would also like to thank the technical and administrative staff of TCIN and the Physiology department, without whom I would have fallen at many hurdles before now. They are Kieran Walsh, Alice Jordan, Ann Connick, Gillian Roddie, Ciaran Conneely, Lisa Beattie with special thanks to Lesley Penney for all her help and advice with brain sectioning and animal care and Quentin Comerford for his computing advice.

I wish to thank Health Research Board (HRB), Ireland for providing financial suppport for this study.

I also wish to thank Ranya Bechara for her continued help and support in the lab, she has always been there with a helping hand or a hug! I wish the best of luck to both Jack Prenderville and Suzanne McCreddin as they continue their PhDs in the lab, you have both become really great friends & I'll miss you so much. A special thanks must also go to a previous Kelly lab member Éadaoin Griffin, without whom I definitely would not have had the confidence to complete this PhD. She taught me everything from animal handling to protein assays and is always available for advice and a cup of tea! I extend a massive hug and thanks to Katey McKayed, my fellow partner in crime in the office, for all the laughs over the past two years. Good luck K Mac – I'll be back for your hand-in without a doubt!

Thanks also to my fellow HRBs: Jen, Liz, Donal and Rodrigo who welcomed me so warmly to Ireland and whohave made the last 4 years so much fun. I also mention my office, lab and PhD mates who have made my time at TCIN fly by: Janis, Michael, Aoife, Sabina, Laura, Jen, Raasay, Luke, Deb, Priyanka, Gillian, Donal, Aisling, Jimmy, Ronan, Valentina, Barry, and Colleen.

Finally, I want to say a huge thank you to Ciaran and his family for always providing a bolt-hole in Strabane or Dunfanaghy when I need some peace and quiet and to my family for their never-ending patience, encouragement and love and for always teaching me that I was able to do anything.

III List of Abbreviations

ANOVA	Analysis of variance
Arc	Activity-regulated cytoskeletal-associated protein
BDNF	Brain derived neurotrophic factor
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
btASL	Bolus-tracking arterial spin labelling
CA1	Cornu Ammonis 1
CA2	Cornu Ammonis 2
CA3	Cornu Ammonis 3
CREB	c-AMP response element-binding protein
DAB	Diaminobenidine
DAG	Diacylglycerol
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-related kinase
Gab-1	Growth factor receptor-bound protein 2-associated-binding protein 1
GABA	Gaba-Aminobutyric acid
Grb	Growth factor receptor-bound protein
HRP	Horseradish peroxidase conjugate
ip	Intraperitoneal
IP3	Inositol (1,4,5)-trisphosphate
KCl	Potassium chloride
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid

MW	Molecular weight
MWM	Morris water maze
NGF	Nerve growth factor
NMTS	Non-matching to sample
NOR	Novel object recognition
NT-3	Neurotrophin 3
NT-4/5	Neurotrophin 4/5
OD	Object displacement
OF	Open field
$p75^{NTR}$	p75 neurotrophin receptor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphotidylinositol-3 kinase
PLC-γ	Phospholipase C-γ
RSK	Ribosomal S6 kinase
RT-PCR	Real-time polymerase chain reaction
SDS	Sodium dodecylsulphate
SEM	Standard error of the mean
SOS	son of Sevenless
TBS	Tris buffered saline
TBS-T	Tris buffered saline-Tween
Trk	Tropomyosin-related kinase
Trk A	Tropomyosin-related kinase receptor tyrosine kinase A
Trk B	Tropomyosin-related kinase receptor tyrosine kinase B
Trk C	Tropomyosin-related kinase receptor tyrosine kinase C
VBM	Voxel-based morphometry

IV List of Tables

V List of Figures

Table of Contents

Chapter 1: Introduction

1.1 General Introduction

Understanding the process of neuroplasticity in the brain is fundamental to furthering knowledge about how learning takes place and memories are formed. The groundbreaking proposal that plasticity is experience dependent and that this process can be demonstrated to occur at a cellular level (Bliss and Lomo, 1973) has provided the basis for research into the cellular mechanisms behind learning that are still crucial in neuroscience to this day. This framework also influenced the early research into whether external environmental stimulation could be used as an experimental manipulation to induce behavioural improvements. Since that time this kind of stimulation has been defined as environmental enrichment and has been used experimentally to induce cognitive improvements, modulate the expression of growth factors, such as neurotrophins, and enhance neurogenesis (Rosenzweig and Bennett, 1996, van Praag *et al.*, 2000). Recent data on lifestyle trends in the U.S. show that most people spend twice as much time watching television as participating in cognitive stimulating activities such as reading or socialising and only 6% of their leisure time exercising (U.S. Bureau of Labor Statistics, 2011). Given the impact that a cognitively and physically active lifestyle can have on the health of the mind and body, a lack of stimulation may be causing detrimental effects in the general population impacting on future mental health. Furthermore, both cognitive and social stimulation are crucial for normal development in childhood: additional cognitive stimulation for children from lower socioeconomic backgrounds, at home or in a preschool setting, can significantly improve their academic achievements (Gray and Klaus, 1965, Crosnoe *et al.*, 2010).

Environmental enrichment has also been shown to be protective against the normal decline in memory functioning associated with ageing and against a number of different neurological and psychological pathologies, such as depression, Huntington's disease and Alzheimer's disease in both humans and animal models (Mora *et al.*, 2007, Laviola *et al.*, 2008, Brenes *et al.*, 2009, Nithianantharajah and Hannan, 2011). This shows that environmental enrichment has the ability to play a therapeutic role in a health system that is becoming increasingly burdened by an ageing population and associated age-related cognitive diseases for which, to date, there are no highly effective treatments or cure

(Fratiglioni *et al.*, 2004). In addition to this, if the mechanisms underlying the cognitive enhancement or neuroprotective effects of environmental enrichment are elucidated, these mechanisms have the potential to become molecular targets to enhance the current treatment of these neurological conditions.

Environmental enrichment has been classically defined as "a combination of complex inanimate and social stimulation" (Rosenzweig *et al.*, 1978), with many experiments also including running wheels in their enrichment protocol. This is a very broad definition and has led to a difficulty in understanding the exact component of an enriched environment that can induce cognitive improvements; hence there are many theories suggesting that it is the combination of all of these factors that is the key aspect. Exercise alone however, can induce cognitive improvements via similar neurochemical and neuroplastic changes to those that have been demonstrated in enrichment experiments (O'Callaghan *et al.*, 2007, Griffin *et al.*, 2009). This research is therefore focused on distinguishing whether cognitive stimulation without the inclusion of physical stimulation can bring about similar cognitive improvements via similar mechanisms to those seen in enrichment protocols that include physical exercise. Following this, this research will focus upon long-term environmental enrichment in the absence of exercise and whether it can have a neuroprotective effect on age-related cognitive decline in healthy ageing rats, and what changes this enrichment may have on the brain structure, function and neurochemistry.

1.2 Hippocampus and Associated Structures

The association between the hippocampus and memory function was initially documented after a bilateral temporal lobe resection surgery was carried out by William Schoville on Henry Gustav Molaison, better known as H. M., a patient with severe localised epileptic seizures, in 1953. In this surgery two thirds of his hippocampal formation, parahippocampal gyrus and the amygdala along with his entire entorhinal cortex were destroyed. After the surgery H. M. was left with a dramatic loss in memory function characterised by a specific deficit in his ability to store new short-term memories into long-term memory (Schoville and Milner, 1957). This meant that whilst he was still able to remember information from past events, he could not recognise new people or places (anterograde amnesia). Further case studies have shown that patients with lesioning to the limbic association areas of the temporal lobe have similar memory deficits to those reported by H. M (Zola-Morgan *et al.*, 1986, Victor and Agamanolis, 1990). This gave

researchers new insight into the role of the hippocampal region in cognitive function, and experimental lesioning of this region together with selective lesioning of hippocampal sub-structures has provided further insight into their cognitive roles (for review see Squire, 1992).

The hippocampal formation is a subcortical brain structure that forms part of the limbic system. It can be further classified into 3 subregions: the dentate gyrus, the hippocampus proper consisting of the CA1, CA2 and CA3 regions, and the subiculum. The hippocampal formation appears as two interlocking C-shaped structures, with the dentate gyrus occupying the area between the hippocampus proper and the subiculum (figure 1a). The subiculum directly continues from the hippocampus (CA1 region) into the parahippocampal gyrus (entorhinal cortex). The neuronal population in the hippocampus is made up of two different types: the principal neurons (pyramidal cell) and intrinsic neurons (polymorphic, basket cell). Axons of the pyramidal cell neurons in the hippocampus and subiculum project into the entorhinal cortex, where impulses are passed on to the limbic, sensory and multimodal association cortical areas. The hippocampus and subiculum also project into the fornix, the main output tract of the hippocampal formation. The intrinsic neurons have axons that remain within the hippocampus and are inhibitory (GABAergic) to the pyramidal cell activity. The main input to the hippocampal formation is via the entorhinal cortex, travelling through the subiculum and on to the dentate gyrus then CA1 and CA3 regions unidirectionally. This circuit is completed with signals originating from the CA1 and subiculum relaying back to the entorhinal cortex, making this structure an important gateway for the hippocampus. Associated with the parahippocampus is the perirhinal cortex, a structure that has been described as playing an important role in visual recognition memory. Whilst it seems that full amnesia occurs only with damage to all of the hippocampal formation and associated parahippocampus, many of these structures have been shown to play separate roles in different types of memory and selective damage to specific regions can cause specific memory deficits (for review see Eichenbaum et al., 2007).The dissociation between areas within the hippocampal and parahippocampal regions has been studied extensively, with data showing that dorsal hippocampal lesions can induce impairments in spatial memory in the rodent whilst sparing recognition memory (Duva *et al.*, 1997, Broadbent *et al.*, 2004). In fact, lesioning 30% of the dorsal hippocampus is sufficient to induce deficits in spatial memory task performance in rodents with a corresponding negative correlation between the size of the lesion and the

performance of the rodent (Broadbent et al., 2004). This correlation holds true up to 50% lesioning, after which the size of the lesion does not cause any further deficit. It is argued therefore that spatial memory is heavily reliant on a large amount (up to 50%) of hippocampal tissue, whereas there is no deficit on object recognition tasks with up to 75% hippocampal lesioning indicating that recognition memory utilises a much smaller amount of hippocampal tissue for normal functioning. Recent research into the role of the perirhinal cortex in memory has supported this dissociation; lesioning within this region induces deficits in a variety of recognition memory tasks in the both non-human primates and rodent models whilst spatial memory remains intact (Brown and Aggleton, 2001, Buckley, 2005).

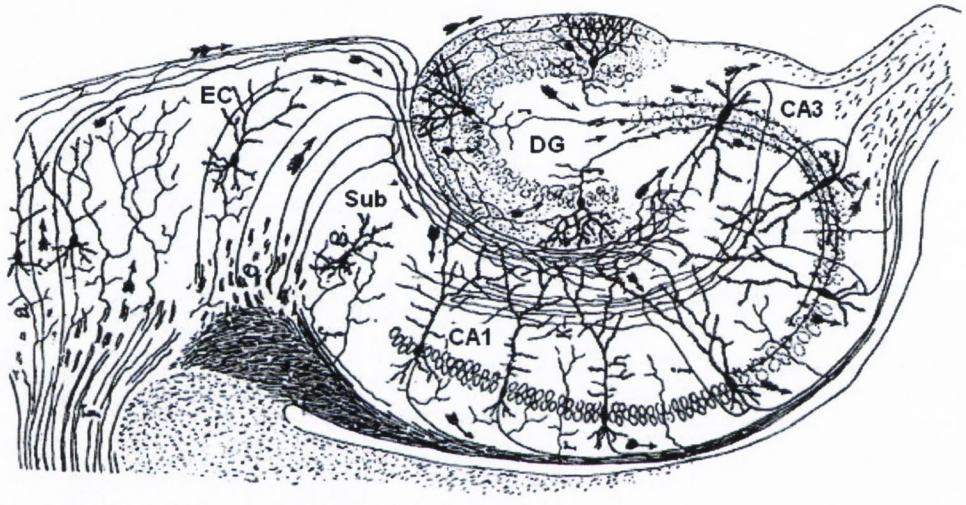


Figure 1a. An illustration of the subregions of the hippocampal formation modified from a drawing by Ramón y Cajal (1911). EC = Entorhinal Cortex; DG = Dentate Gyrus; Sub = Subiculum.

1.2.1 Short-Term and Long-Term Mechanisms of Neuroplasticity

External stimulation via motor and sensory inputs has a direct impact on neuronal circuitry in the brain. It is the fine-tuning of this circuitry that enables normal brain functioning to occur and because of this all of our past experiences play a role in shaping our future behaviours and abilities. This is made possible via a process of experience-dependent plasticity, where neuronal pathways and connections that are heavily stimulated are strengthened and connections that are seldom used are pruned away. First hypothesised by Hebb (Hebb, 1949), this theory has been used to model synaptic transmission in the brain, particularly using long-term potentiation (LTP), where high-frequency stimulation of a neural pathway can elicit long-lasting changes in synaptic efficacy (Bliss and Lomo, 1973).

Consequently, stimulation of these strengthened pathways at a later date would be reactivating the memory of the past experiences. This theory of memory formation is called the synaptic plasticity and memory (SPM) hypothesis and, although not universally accepted, constitutes a leading hypothesis of memory formation. A large amount of research has concentrated on gaining data to connect this hypothesis to actual learning processes (Martin and Morris, 2002), and whilst there is no conclusive evidence to date it is widely accepted that there is synaptic strengthening following certain types of learning.

Repeated high frequency stimulation of a neural pathway at certain intervals (long-lasting, late-phase LTP; L-LTP) induces protein synthesis and this can lead to morphological changes such as increases in post-synaptic spine area and number of spines (Lee *et al.*, 1980) and increases pre-synaptically in synaptic vesicle number which can enhance neurotransmitter release (Meshul and Hopkins, 1990). Blockade of CAMKII or ERK, both important in the LTP signalling cascade, has been shown to block long-term memory formation (Blum *et al.*, 1999, Frankland *et al.*, 2001), whilst an increase in glutamate release has been shown both after LTP and learning in the Morris water maze (Richter-Levin *et al.*, 1998). Changes in neurotransmitter release could be associated with increases in synaptic vesicle availability; increases in synaptic vesicle proteins synaptophysin, synapsin and synatotagmin have been shown 3 hours post LTP-induction (Lynch *et al.*, 1994).

This classical definition of synaptic plasticity only models changes in LTP or long-term depression (LTD) via alterations such as neurotransmitter release or receptor density, however there is also evidence to suggest experience-dependent neurogenesis may play a role in learning processes.

Neurogenesis in the adult mammalian brain is typically confined to two areas: the subventricular zone (SVZ), where neural stem cells and progenitor cells differentiate and subsequently travel to the olfactory bulb, and the subgranular zone (SGZ) of the dentate gyrus, where granule cells are produced from neural stem cells and progenitor cells continuously throughout adult life (Ehninger and Kempermann, 2008). Neural stem cells do exist in other areas of the brain, however in these non-neurogenic regions the stem cells do not differentiate. Transplanting these cells into the dentate gyrus does induce their differentiation and this would suggest that there are environmental factors within the niche of the SGZ (or any other neurogenic region) that play significant roles in the regulation of

this differentiation (Shihabuddin et al., 2000). In the adult SGZ, proliferating radial and nonradial precursor cells generate intermediate progenitor cells which in turn give rise to neuroblasts. These migrate from the SGZ to the granule cell layer and within 7 days begin to project axons through the hilus.At this stage, these dentate granule cells are tonically activated via ambient GABA but are not synaptically integrated into the neural network (Zhao *et al.*, 2006). In the second week, the granule cells begin to look more neuron-like: their dendrites begin to extend towards the molecular layer and axons growing through the hilus. These axons reach the CA3 area within 10-11 days and spine formation begins at around 16 days (Zhao *et al.*, 2006). At this point, the immature neurons exhibit different firing properties than mature neurons and also lack glutamatergic input, although they do receive synaptic GABAergic input by local interneurons (Espósito *et al.*, 2005). By 21 days, these progenitors begin to resemble mature neurons and receive activation via glutamatergic inputs by 28 days (Wang *et al.*, 2003, Ge *et al.*, 2006). GABA is an important regulator of neurogenesis, as GABAergic activation on neural progenitor cells increases expression of NeuroD, a positive regulator of neuronal differentiation, and increases the number of new neurons as measured by 5-bromo-2'-deoxyuridine (BrdU) labelling (Ge *et al.*, 2006).

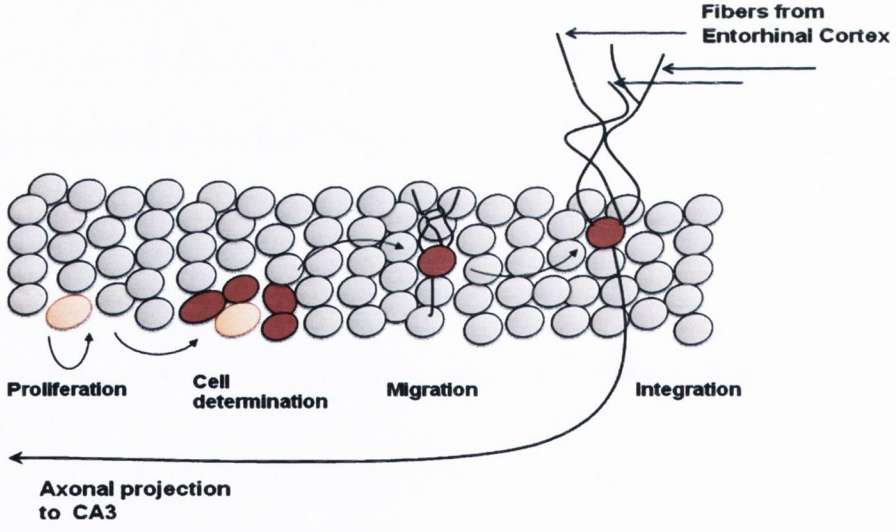


Figure 1b.Adult neurogenesis in the dentate gyrus. Neural stem cells proliferate and differentiate into granule cells which migrate from the sub granular zone into the granule cell layer. By day 7, granule cells begin to project axons through the hilus and by day 14 , dendrites begin to extend towards the molecular cell layer. By day 28, these young progenitor cells begin to resemble mature neurons and can receive activation via glutamatergic inputs.Adapted from Lie *et al (2004).*

Given current knowledge of the importance of the hippocampus in both memory consolidation and retrieval, it has been theorised that neurogenesis in this region may serve a significant role in this process. Existing data show that immature neurons exhibit electrophysiological properties, some of which are similar to adult neurons (van Praag *et al.*, 2002) and some of which are unique to their population. For example, they are more excitable and induce LTP more readily than more mature neurons (Schmidt-Hieber *et al.*, 2004, Ge *et al.*, 2007). This enhanced synaptic plasticity seem to rely upon NR2B-containing NMDA receptors (Ge *et al.*, 2007). This has led some researchers to suggest that an increase in this population of immature neurons may be ideally suited to undergo activity-dependent plasticity and hence facilitate learning and memory processes (Bruel-Jungerman *et al.*, 2007b).

The function of adult neurogenesis in the hippocampus remains a hotly debated issue, the main challenge centring on associating neurogenesis with hippocampal network function and behavioural outputs. Hippocampal-dependent learning paradigms (for example the Morris water maze) have been shown to selectively increase neurogenesis in the hippocampus, whereas a similar task that is not hippocampal-dependent does not (Gould *et al.*, 1999a). Whilst other studies have replicated this result (Kempermann and Gage, 1999), there is also evidence of learning induced apoptosis in the hippocampus (Ambrogini et al., 2004). This indicates that learning can functionally regulate mature neurons and neuronal precursor cells by selectively promoting proliferation, survival or in fact apoptosis of different populations in an activity dependent manner (Dobrossy *et al.*, 2003, Dupret *et al.*, 2007). Nevertheless, these correlations between neurogenesis and learning cannot conclusively prove that neurogenesis is a requirement for hippocampal-dependent learning. To test whether neurogenesis is a vital component in the learning process, different techniques for the ablation of dividing cells in the dentate gyrus have been developed via neurochemical agents, radiological approaches or different genetic manipulations. Evidence from these experiments has been mixed: using methylaxomethanol acetate (MAM) or genetic manipulation to prevent cell division has induced impairments in some hippocampal-dependent conditioning tasks whereas other hippocampal-dependent spatial tasks were unaffected (Shors *et al.*, 2002, Saxe *et al.*, 2006). Improvements in object recognition memory have also been prevented via a MAM-induced loss of neurogenesis (Bruel-Jungerman et al., 2005). The ablation of neurogenesis via focal X-irradiation however, does not affect improvements in spatial learning due to environmental

enrichment (Meshi *et al.*, 2006) but has been shown to impair contextual fear conditioning (Saxe *et al.*, 2006). New genetic techniques have been developed more recently that can be utilised to knockdown neurogenesis for a set period of time in a specific region: Jessberger and colleagues use lentiviral techniques to block neurogenesis in the dentate gyrus of rats and have shown that this impairs both spatial and object recognition learning (Jessberger et al., 2009). These data suggest that there could be some different types of memory processes that may be more sensitive to changes in neurogenesis than others.

In recent years, the focus of research has become heavily biased towards the role that young, immature neurons play in memory formation. Kee and colleagues show that after mice perform a water maze task, there is a significant increase in expression of the immediate-early genes *c-fos* and *Arc* on neurons that were 4-8 weeks old (Kee *et al.*, 2007), suggesting that these immature neurons are involved in spatial processing. Neurons only 2 weeks old that are exposed to an enriched environment are more likely to become activated again at a later date when they are exposed to the same environment than a different one (Tashiro *et al.*, 2007), which would indicate that there is preferential activation of these new neurons when exposed to a new stimulus and this activation is associated with memory formation. Further evidence for the role of young neurons in learning processes comes from their importance in the process of activity dependent synaptic plasticity. One of the unique properties of young neurons is their ability to exhibit LTP even in the presence of GABAergic inhibition, whereas mature neurons do not, and therefore they display a lower threshold for LTP induction (Wang *et al.*, 2000). However, when neurogenesis is abolished via gamma irradiation 3 weeks prior to high frequency stimulation, LTP cannot be evoked (Snyder *et al.*, 2001). These studies show that neurons that are less than 3 weeks old are necessary for LTP induction under physiologically relevant conditions (when GABAergic inhibition remains). Whilst the functional connection between immature neurons and the formation of new memories has not been fully elucidated, it is clear that they play a different role in hippocampal plasticity mechanisms than mature neurons and their preferential activation during hippocampal-dependent memory tasks would suggest they are important for the processing of novel information. However, it is not fully understood how they are functionally integrated into existing neuronal networks and whether this process requires activity-dependent synaptic modulation such as during a learning paradigm (Mongiat and Schinder, 2011).

An alternative hypothesis proposed for the role of adult-born neurons in dentate gyrus is that they are indirectly involved via a process of functional integration into hippocampal circuitry that enables an improved capacity for memory storage in the future (Ehninger and Kempermann, 2008). The suggestion that adult neurogenesis may play a long lasting role in hippocampal function has been proposed via the 'neurogenic reserve' hypothesis (Kempermann, 2008). This theory proposes that sustained cellular activity during adult life can provide a greater capacity and efficiency within the hippocampal neural circuitry and may also protect against age-related neuropathological damage. Activity-dependent regulation of hippocampal neurogenesis is therefore argued not only to recruit young neurons into a functioning memory circuit, but also to stimulate mechanisms that can increase the number of young neurons available for recruitment on a much longer timescale (Kempermann, 2008). Experimentally, activities such as exercise and environmental enrichment have been shown to stimulate such mechanisms and increase neurogenesis via neurochemical changes in the neurogenic niche within the dentate gyrus (van Praag *et al.*, 2000).

Synaptogenesis is an attractive mechanism to associate with memory formation because remodelling of neural networks would seem likely to occur if the theory of experience-dependent plasticity is followed. The induction of LTP and learning has been shown to bring about synaptogenesis or alter the morphology of current synapses to increase strength such as spine branching and boutons with multiple synapses, however a comprehensive analysis of these changes after learning has not been undertaken (for reviews, see Bruel-Jungerman *et al.*, 2007a, Kelsch *et al.*, 2010). Both synapsin and synaptophysin are synaptic vesicle proteins found in abundance in synaptic boutons. The synapsins are known to play an important role in synaptic plasticity and neurotransmission and are regulated by a number of different protein kinases and phosphatises such as cAMP-dependent protein kinase (PKA), CAMKII and MAPK. Synapsin I binds to synaptic vesicles and is thought to be important in the clustering of synaptic vesicles together, which helps to maintain a functioning vesicle pool in the synaptic bouton at active zones and the organisation of the reserve pool of synaptic vesicles. This function is maintained possibly via its tethering to Actin filaments during its dephosphorylated state, where upon phosphorylation these vesicles may be released which could facilitate their movement into the active zone (for reviews, see Cesca *et al.*, 2010, Shupliakov *et al.*, 2011). Additionally, increases in synapsins are found during synaptogenesis, making them a useful protein

marker for evidence of synaptic remodelling (Lohmann *et al.*, 1978). Synaptophysin is one of the most abundant synaptic vesicle proteins and whilst it was the first vesicle protein to be discovered, its role is not yet fully understood. Recent data show that it regulates endocytosis to maintain the availability of synaptic vesicles during and after neuronal stimulation (Kwon and Chapman, 2011), however it has been reported to be unnecessary for neurotransmitter release as synaptophysin homozygous knockout mice exhibit normal neurotransmission (McMahon *et al.*, 1996). More recently, there is evidence that these knockout mice have deficits in exploratory behaviour, recognition and spatial memory (Schmitt *et al.*, 2009), suggesting that it may play a role in cognitive function. Additionally, because of its abundance in the synaptic bouton, it provides a useful measure to determine synapse number and hence synaptogenesis,

The substantial amount of data that is available at present shows there is an important role for adult neurogenesis and synaptogenesis in the hippocampus in both short-term memory processes as well as providing protection in the longer term against age-related memory decline. The specific mechanisms behind these patterns however, are only very recently becoming more evident and many are yet to be elucidated.

1.3 Measuring Cognitive Function in the Rat

Memory is a dynamic system involving classification, encoding, storage and recovery of information. Memory can be divided into two major classes: declarative, which is information that can be consciously stored and retrieved; and procedural or non-declarative, which is information about motor or perceptual skills (Squire, 1986). Procedural memory typically requires extensive acquisition phases whereas declarative memory can be acquired through relatively few exposures to the information that is to be learnt. The two forms of declarative memory that are particularly well studied in humans and animals are recognition memory and spatial memory. Recognition memory can be generally defined as the ability to discriminate the novelty or familiarity of previous experiences. This can be associated with differences in an individual object, a whole environment or the spatial arrangement of objects within an environment. There are several regions that have been implicated in the neural basis of recognition memory: lesions to the perirhinal cortex have been shown to disrupt recognition of novel objects (Ennaceur *et al.*, 1996, Bussey *et al.*, 1999) but not spatial memory (Barker *et al.*, 2007).There is a great degree of controversy in the literature surrounding the contribution of the hippocampus to

the processing of recognition memory, indeed many hippocampal lesion studies report no deficits in novel object recognition memory (Mumby, 2001, Barker and Warburton, 2011)but some do show deficits within specific parameters, particularly with a longer period between acquisition and testing (Clark *et al.*, 2000). Nevertheless, it is evident that intact spatial memory is heavily reliant upon a large portion of hippocampal tissue whereas object recognition memory relies upon a much smaller amount, and it is the perirhinal cortex that is most valuable in processing in novel object recognition memory (Duva *et al.*, 1997, Broadbent *et al.*, 2004, Buckley, 2005). The development of numerous tasks that allowspecific aspects of these different types of memory to be tested have been crucial in aiding our understanding of the roles of different brain regions and have helped to provide insights into the neurochemistry of learning.

One of the first behavioural tasks to be designed was used to test visual recognition memory by using the delayed non-matching to sample task (DNMS). This task was initially developed for monkeys and later adapted for rodents (Prusky et al., 2004). In this task, an animal is trained to displace an object in order to obtain a food reward (sample phase) and then, following a set delay period, the sample object is presented again with a novel object and the animal must displace the novel object in order to obtain the food reward (choice phase). More recently however, use of this task has been overtaken by the novel object recognition task (NOR) which uses an animal's spontaneous preference for novelty as its measure of memory function. This is particularly useful because it takes away the pre-training that is needed to learn the non-matching rule as is the case in the DNMS. This virtually eliminates the variability associated with rule learning across different species and within test groups of animals, and furthermore the results of this task are arguably more sensitive to recognition memory impairments and show consistency in behavioural findings across species (Nemanic *et al.*, 2004)

The NOR task has three different phases: familiarisation, delay and test. In the rodent version of this test, the animals actively explore objects for a set period of time in the familiarisation phase, and then following a delay period one of the familiar objects is replaced by a novel object and animals are tested on the amount of time they spend exploring the novel object. One of the reasons why this task has become so widely used is because of its flexibility; manipulation of the delay phase (varying the amount of time between the familiarisation and test phases) enables researchers to change the memory load on the rodent without introducing any changes to motivation, perception or attention and

thus is a very powerful tool in testing for memory impairments. Further manipulation can alter the memory load of the rodent by changing the number of objects or the time the animals are given to explore the objects in the familiarisation phase. Consequently, it is possible to determine behavioural differences due to experimental manipulation with a very high degree of sensitivity providing the appropriate test design is utilised. This type of task has also proved invaluable in understanding the mechanisms involved in specific aspects of memory, because it is possible to disrupt the memory formation specifically at any one of the encoding (familiarisation), consolidation (delay) or retrieval (test) phases and measure the behavioural outcomes (Winters and Bussey, 2005a, Winters and Bussey, 2005b).

The spatial variant of this task, the object displacement task (OD), is based on the same familiarisation and delay phase, but in this case instead of a novel object being introduced in the test phase, one of the familiar objects is moved to a new position and the spontaneous exploration of the displaced object is used a measure of spatial memory (Poucet, 1989, Larkin *et al.*, 2008).

A more popular test of spatial memory is the Morris water maze (Morris, 1984). This is one of the most frequently used measures of hippocampal-dependent memory in rodents (for review, see Paul et al., 2009). In this task, rodents swim around a circular arena and use distal spatial cues to find a static hidden platform and escape the water. This task is performed with a certain number of trials over a number of days, with the speed at which animals find the platform decreasing as they perform more trials. The latency to find the platform is used as a measure of acquisition of this spatial information. This is not however, an ideal measure of spatial memory as it is open to disruption by other factors such as differential search strategies. Therefore a probe trial is often used in this task, where the platform is removed after a set number of acquisition trials and the time animals spend in the quadrant where the platform was positioned is used a measure of their memory for the spatial location of the platform. Successful completion of this task has been shown to be hippocampal-dependent, and particularly sensitive to dorsal hippocampal lesions (Moser *et al.*, 1993, Broadbent *et al.*, 2004).

One of the main advantages of the water maze is that animals are incentivised to escape from the water onto the platform which is a strong negative reinforcer for learning. This is especially important for testing groups of animals that may be exhibiting depressive or

sickness-behaviour and therefore would not be expected to show the natural exploratory tendencies necessary for successful performance on the NOR and OD tasks. Unfortunately, this is also a significant disadvantage of the water maze because the negative effects of stress from the aversive stimuli of immersion in water may limit this test's ability to measure the spatial memory relevant to natural and real-life situations. In this sense, the OD task has an inherent advantage over this task because it is not stressful and directly taps into natural exploratory behaviour of rodents.

Another more traditional way of measuring spatial memory in rodents is using spontaneous alteration behaviour. This test is based on the principle that animals tend to explore areas that have been explored less recently (Dennis, 1939). Typically this test is performed using a 'T' or 'Y' maze, where the animals are allowed to freely explore each arm of the maze and the number of visits to each arm is recorded. This test can be performed as a free or forced test: the free test allows animals to freely explore and the forced test will block one of the arms of the device to favour alternation behaviour or a positive reinforcer, such as a food reward, can be placed at the end of the arms to reward alternation behaviour (Dember and Fowler, 1959). As with the NOR and OD tasks, increasing the interval between each trial (one trial being equal to exploration of a single arm) increases the memory load on animals. Performance on this task is sensitive to hippocampal lesions but other regions of the brain have also been associated with successful alternation, such as the thalamus, neocortical areas and the basal ganglia (Lalonde, 2002).

In addition to measuring memory in rodents, it is often common to take a more general measure of innate behavioural characteristics such as grooming, rearing, intruder aggression and exploration of a novel environment. All of these tests are based on the theory that they will be affected by the level of anxiety that the animals are experiencing. These tests do not give a measure of the memory abilities of animals, but differences within groups can serve as a confounding factor in interpreting results from the different behavioural tests outlined above. The NOR, OD and T or Y maze tasks all require animals to exhibit natural exploratory behaviours in order that they can effectively perform the task and the swimming strategy of animals in the water maze could affect the time it takes them to find the hidden platform.

The earliest test of anxiety developed for rodents was open field exploration (Hall, 1934). In this test, naive animals are placed in a large circular arena and their exploration of this

arena is recorded. In this situation, rodents typically exhibit thigmotactic behaviours; they tend to avoid the 'exposed' areas in the arena and mainly explore around the edges. Therefore, exploration of the central area in this test is generally used as an index of anxiety (Treit and Fundytus, 1988), although whether a test based on locomotor activity can be a full measure of emotionality is contentious in the literature (Ramos, 2008). Nevertheless, this test is useful to establish whether there are different exploratory strategies between treatment groups. Indeed, studies have shown that environmental enrichment can change locomotor activity and reduce thigmotactic exploration (Zimmermann *et al.*, 2001, Harris *et al.*, 2009). These studies highlight the importance for sufficient habituation of animals with the test environment before the NOR, OD and T/Y maze tests to ensure their tendency to explore overrides the rodents' anxiety.

The most popular test of anxiety at present in the literature is probably the elevated plus maze. As with open field exploration, this test is based on the knowledge that rodents will typically avoid exposed areas. The maze is designed as a '+' shape in which two of the arms are open and exposed and two of the arms are enclosed with high walls. The amount of times the rodents enter either the closed or open arms is then used as a measure of anxiety. Animals treated with anxiolytic drugs show a higher number of entries into the open arms and correspondingly, animals treated with anxiogenic drugs show a reduced number of entries into open arms. Animals that are confined on the open arms of the maze also measure higher levels of plasma corticosterone than when confined to the closed arms (Pellow *et al.*, 1985). This test is most frequently used to measure the effects of different drugs that are hypothesised to have either an anxiolytic or anxiogenic effect (for review, see Rodgers and Dalvi, 1997), nonetheless it is an important measure to assess when studying memory changes because chronic stress can upregulate glucocorticoids, which have been shown to impair memory performance (Bodnoff *et al.*, 1995). Furthermore, environmental enrichment has been shown to reduce anxiolytic behaviours in rodents (Zhu *et al.*, 2006, Galani *et al.*, 2007, Hattori *et al.*, 2007, Brenes *et al.*, 2009).

1.4 Neurotrophins

The neurotrophin family of proteins are known to play a key role in neurodevelopment, but are also vital for many functions in the adult brain. They are closely linked to the maintenance and support of adult neurons and the regulation of activity-dependent synaptic plasticity (Hennigan et al., 2007). There are four neurotrophins that have been described in

mammals: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). NGF and BDNF are the most extensively researched to date.

Neurotrophins are synthesised as proneurotrophin precursor proteins that undergo enzymatic cleavage to yield mature neurotrophins. The actions of neurotrophins are mediated by a receptor signalling system that involves many different ligand-receptor interactions. Signalling via Trk (tropomyosin receptor kinase) receptors is commonly associated with cell survival and the enhancement of synaptic transmission (Tyler *et al.*, 2002, Bramham and Messaoudi, 2005), whilst there is strong evidence to suggest that signalling via $p75^{NTR}$ (p75 neurotrophin receptor, a member of the tumour necrosis factor, TNF, receptor family) plays a role in mediating cell death and functional impairment (Barker, 1998). The different neurotrophins are specific to different Trk receptors: TrkA binds with high affinity to NGF, TrkB to BDNF and NT-4 and TrkC to NT-3. The $p75^{NTR}$binds all neurotrophins with approximately equal low affinity although recent evidence suggests that the proneurotrophins are chemically active proteins and this receptor binds them with high affinity (Lee *et al.*, 2001, Volosin *et al.*, 2008).

The dual and opposing processes that are triggered through neurotrophin-receptor interactions are indicative of the number of different downstream pathways that can be activated. Activation via the Trk receptors results in autophosphorylation of the tyrosine residues on the cytoplasmic domains of these receptors which in turn can trigger intracellular signalling cascades that include the Ras-ERK (extracellular signal-related kinase) protein kinase pathway, PI-3 kinase (phosphotidylinositol-3-kinase)/Akt kinase pathway and PLC-γ1 (figure 1b).

Action via the PLC-γ1 pathway is through the recruitment of PLC-γ1 by phosphorylated residues on the Trk receptor. It is then itself phosphorylated and activated, and hydrolyzes phosphatidylinositides to create diaglycerol (DAG) and inositol 1,4,5 trisphosphate (IP_3). IP_3 induces the release of Ca^{2+} from stores which increases the Ca^{2+} in the cytoplasm and hence activates Ca^{2+}-dependent pathways, such as activation of Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) which plays a crucial role in the maintenance of LTP (Miyamoto, 2006). Activation of Ras has been shown to promote survival and proliferation of neurons through the ERK family of MAP kinases (Grewal et al., 1999). Recruitment of Shc and phosphorylation leads to the recruitment of the Grb-2 and son of Sevenless (SOS)

complex. SOS is a Ras exchange factor and therefore this recruitment induces transient activation of Ras. Downstream, pp90 ribosomal S6 kinases (RSK) are activated and in turn phosphorylate a number of transcription factors, including CREB (cAMP regulated enhancer binding protein). These factors regulate the expression of many genes that are known to be under the control of neurotrophins (Xing et al., 1996). CREB, for example, regulates genes that have been implicated in long-term neurotrophin-dependent neuronal survival (Bonni et al., 1999). Additionally, phosphorylation of MAP kinase isoforms p42 and p44 (pERK-1 and pERK-2) has been shown to stimulate the phosphorylation of synapsin I which reduces its actin bundling and therefore may play a significant role in synaptic plasticity (Jovanovic *et al.*, 1996). Primarily PI3-kinase acts via Akt to induce neuronal survival (Dudek *et al.*, 1997) and can be activated via Trk-mediated phosphorylation of Grb-associated binder-1 (Gab-1) (Holgado-Madruga *et al.*, 1997), however Ras has also been shown to activate PI3-kinase to promote survival in sympathetic neurons (Mazzoni *et al.*, 1999). Conversely, the binding of neurotrophins to $p75^{NTR}$ can induce the activation of the JNK (c-Jun N-terminal kinase) pathway and downstream activation of caspases, which in turn cause DNA fragmentation and neuronal apoptosis (for review, see Roux and Barker, 2002).

Thus, whilst the main function of neurotrophins is generally accepted to be neuronal survival, the complexity of their receptor interactions and signalling cascades suggests that they should be more appropriately classified as mediators of neuronal differentiation and growth. There is also a great deal of data supporting the hypothesis that neurotrophins, particularly BDNF and NGF, are neuromodulators involved in plasticity mechanisms in the brain; a specific role for NGF in the expression of long-term potentiation (LTP) has been shown (Kelly *et al.*, 1998a, Conner *et al.*, 2009) however the vast majority of literature implicates a more central role for BDNF in this form of cellular plasticity (Bramham and Messaoudi, 2005, Hennigan *et al.*, 2007).

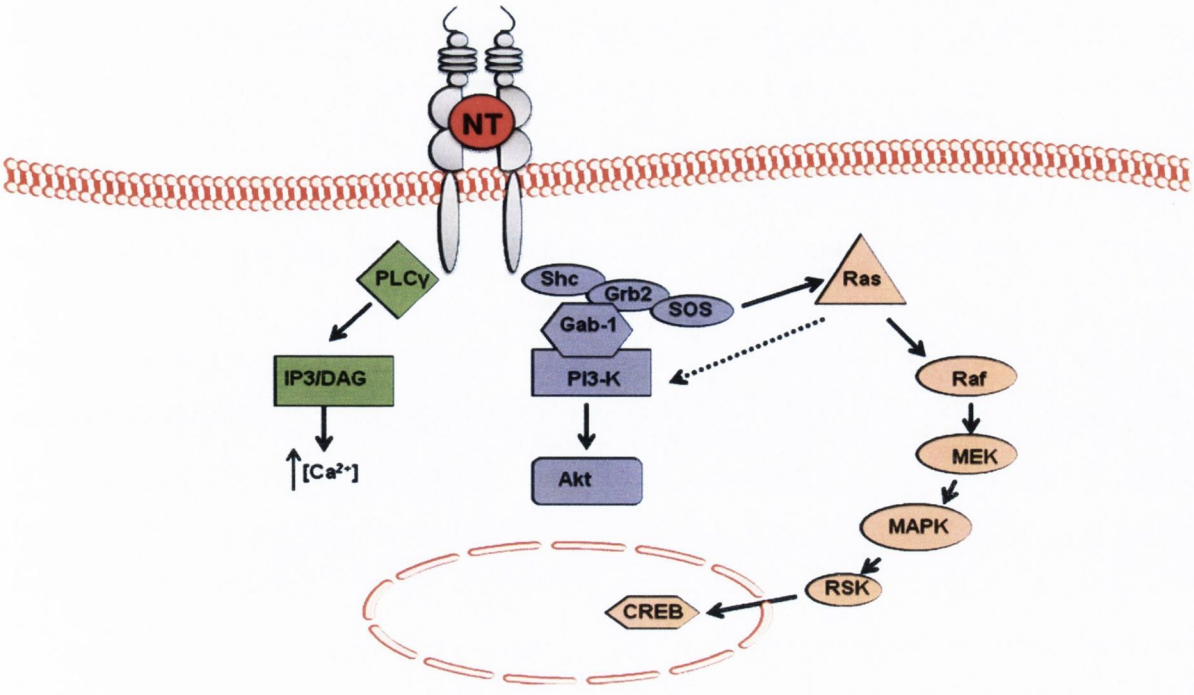


Figure 1c. Basic pro-survival signalling pathways of Trk receptors. Phosphorylation of the Trk receptor activates three major signalling pathways: Ras/MAPK, PI3Kinase/Akt and IP3-dependent Ca^{2+} release. Adapted from Segal *et al*(2003) and Patapoutian and Reichardt (2001).

1.4.1 NGF

NGF was the first neurotrophin to be discovered, described as a factor that could promote the survival and development of maturing neurons in culture (Levi-Montalcini and Hamburger, 1951). NGF is expressed during development and throughout adult life in the central nervous system (CNS). Trk A is the main receptor for NGF although at very high concentrations it can also be activated by NT-3. Signalling via this receptor elicits neurite outgrowth and cell survival (Loeb et al., 1991).

During development, NGF plays a major role in the survival and maturation of many populations of neurons: namely basal forebrain and striatal cholinergic neurons (for review, see Sofroniew et al., 2001). Animals heterozygous for the NGF gene (NGF +/-) show a reduction in the number of basal forebrain cholinergic neurons and also a loss of cholinergic innervation to the hippocampus. In the mature brain, NGF is widely expressed in the hippocampal formation by pyramidal and dentate granule neurons (French et al., 1999), by subpopulations of GABAergic interneurons (Lauterborn et al., 1993) and by a subpopulation of small interneurons in the striatum (Bizon et al., 1999). In the hippocampus, its expression is regulated by neuronal activity, with glutamatergic and

cholinergic neurotransmission increasing expression and GABAergic neurotransmission decreasing expression (Thoenen *et al.*, 1991, Zafra *et al.*, 1991, Lindefors *et al.*, 1992, French *et al.*, 1999). NGF has also been shown to be produced throughout the CNS by microglia and astrocytes (Heese et al., 1997), largely in response to neuronal injury indicating its role in the promotion of survival and regeneration in the brain (Tonchev et al., 2008). Selective inhibition of NGF in an animal model of epilepsy prevented the typically seen post-seizure axonal sprouting and cell body increases, indicating that this neurotrophin may be important for synaptic remodelling after this type of injury (Holtzman and Lowenstein, 1995). An exogenous infusion of NGF within 5 hours of middle cerebral artery occlusion significantly reduced infarct volume and apoptosis levels in a rabbit model of stroke and therefore may be an important therapeutic target for neuroprotection against cerebral ischemia (Yang *et al.*, 2011).

Whilst all neurotrophins have been proposed as mediators of both structural and functional plasticity, NGF has been implicated more specifically as being important in structural plasticity rather than having direct effects on neuronal activity. NGF has been shown to play a role in memory processes however, with blockade of endogenous NGF in the dentate gyrus impairing spatial memory (Conner et al., 2009) and contextual memory consolidation (Woolf et al., 2001). Direct infusion of NGF into the CA1 region of the hippocampus has also been shown to stimulate enhanced retention on an inhibitory avoidance task via MAP kinase activation (Walz et al., 2000) and improve spatial memory in aged rats (Niewiadomska et al., 2006). In NGF(+/-) mice however, only prolonged exposure to NGF can reverse spatial memory acquisition when compared with brief exposure; after 5 weeks of exogenous NGF infusions, NGF(+/-) mice showed a reversal in a spatial memory acquisition deficit, as tested by the Morris water maze, but this reversal was not evident after a short infusion period of 3 days (Chen et al., 1997). Furthermore, continuous infusions of exogenous NGF have also been shown to enhance the survival of new neurons in the granule cell layer of the dentate gyrus and increase activity in hippocampal cholinergic neurons (Frielingsdorf et al., 2007). Interestingly, NGF infusions did not directly increase neurogenesis, suggesting that this neurotrophin exerts its main effects in trophic support as opposed to directly stimulating proliferation.

There is no evidence of reductions in NGF in normal ageing, MCI or Alzheimer's disease patients but an increase in proNGF and a reduction in Trk A receptor expression has been shown (Fahnestock *et al.*, 2001, Mufson *et al.*, 2003). No corresponding decrease in

$p75^{NTR}$ expression has been shown and therefore NGF may more frequently bind to $p75^{NTR}$ and elicit activation of pro-apoptotic pathways. Given the high affinity of $p75^{NTR}$ for proneurotrophins, their increased expression may also facilitate a tipping of the balance of NGF signalling away from survival and towards apoptosis in neurodegenerative diseases such as Alzheimer's disease. A recent study has also found reductions in mature NGF and phospho-TrkA receptors with a corresponding increase in proNGF and $p75^{NTR}$ in the prefrontal cortex and hippocampus of aged rats however (Terry Jr *et al.*, 2011), suggesting that there may be changes in NGF levels with age that are not detectable post-mortem. These studies show that there is a dysfunction in normal NGF production and signalling in the aged brain, and this could be associated with the age-related loss of memory function (Terry Jr *et al.*, 2011).

1.4.2 BDNF

BDNF is the most widely studied of all the neurotrophins; there is hope that it will yield therapeutic value in the future for a number of neurological and neurodegenerative conditions including depression and Alzheimer's disease. It is expressed in the developing and adult hippocampus and neocortex (Hofer *et al.*, 1990, Friedman *et al.*, 1991), by excitatory pyramidal neurons but not GABAergic inhibitory interneurons. Both populations of these neurons express the primary BDNF receptor, Trk B, but its expression is higher in interneurons. Electrical activity can stimulate release of BDNF, with hippocampal seizures inducing its expression (Kornblum et al., 1997). In the hippocampus, neuronal activity can also stimulate expression of BDNF mRNA and subsequent release of BDNF protein (Nanda and Mack, 2000).

Initially, BDNF was described as being slow-acting and regulating the proliferation, survival and growth of populations of neurons in both the developing and adult brain. It was also shown to enhance axonal growth of granule cells from the dentate gyrus *in vitro* (Patel and McNamara, 1995). The last decade of evidence however, has revealed a major role for BDNF in synaptic transmission and neuroplasticity particularly, but not exclusively, in the hippocampus.

BDNF has been shown to play a crucial role in LTP (for review, see Bramham and Messaoudi, 2005). Both homozygous and heterozygous knockout animals (BDNF -/-, BDNF +/-) show reduced LTP, which is rescued by the reintroduction of BDNF. Direct incubation of hippocampal sections with BDNF also induces LTP (Ying et al., 2002), most

likely via stimulation of MAP kinase mediated phosphorylation of synapsin which has been shown to enhance glutamate release (Jovanovic *et al.*, 2000). These data are evidenced by behavioural results: BDNF mRNA expression is selectively increased in hippocampal CA1 pyramidal cells in a contextual fear conditioning task (Hall *et al.*, 2000b). During short-term and long-term memory formation in an inhibitory avoidance task there is also a transient increase in BDNF mRNA. Additionally, short-term memory performance on this task can be improved via exogenous infusions of BDNF and long-term memory performance impaired following infusions of anti-BDNF or protein synthesis inhibitors in a time-dependent manner (Alonso *et al.*, 2002, Bekinschtein *et al.*, 2008). These papers suggest that the action of BDNF is via the MAP kinase signalling pathway, in an ERK-dependent manner. The action of BDNF has also been shown to affect many different hippocampal-dependent memory systems; protein synthesis inhibition in MAP kinase pathway can cause deficits in spatial memory tasks (McGauran *et al.*, 2008) and hippocampal specific deletion of BDNF also induces spatial and object recognition memory impairments (Heldt *et al.*, 2007).

The wealth of data available with respect to the role of BDNF in synaptic plasticity is indicative of its proposed role as one of the major molecular mediators of learning and memory. Its importance in hippocampal neurogenesis is also becoming increasingly recognised following the discovery that Trk B is expressed on stem cells and neural progenitor cells in the dentate gyrus (Donovan *et al.*, 2008, Li *et al.*, 2009). This discovery places a greater amount of weight on the importance of adult hippocampal neurogenesis in neuroplasticity and memory functioning, and the potential roles of BDNF in these processes. Conditional knockout or knockdown of BDNF in mice induces reduced levels of neural progenitor cell survival and dendritic development in the dentate gyrus and BDNF (+/-) mice do not show the enrichment induced increase in neurogenesis that is exhibited with wild-type mice (Rossi *et al.*, 2006, Choi *et al.*, 2009, Taliaz *et al.*, 2010). This would indicate that BDNF is an important factor in the neurogenic niche necessary for efficient neurogenesis in the dentate gyrus. Indeed, a continuous infusion of BDNF into the hippocampus increased neurogenesis in adult rats (Scharfman *et al.*, 2005). These studies, together with the emerging theories regarding the function of young neurons in learning and memory, make it seems highly likely that BDNF has a number of different roles to play in synaptic and neuronal plasticity.

As with NGF, there is mixed evidence regarding the expression of BDNF with age: increases in BDNF in the hippocampus of aged mice have been reported, whereas some studies report decreases in the aged rat and others report no changes (Katoh-Semba *et al.*, 1998, Karege *et al.*, 2002, Silhol *et al.*, 2005). In humans, BDNF plasma levels are found to decrease with age, whereas there is no change in BDNF expression in the hippocampus (Lommatzsch *et al.*, 2005b, Webster *et al.*, 2006). Similar to NGF however, there is a decrease in Trk B receptor expression reported in the hippocampus over the life span in both humans and rats (Silhol *et al.*, 2005, Webster *et al.*, 2006). BDNF may play an important role in synaptic plasticity in ageing, as blocking BDNF attenuates LTP induction in aged but not young rats and an exogenous infusion of BDNF can rescue deficits in LTP in middle-aged rats (Rex *et al.*, 2006, Diógenes *et al.*, 2011).

1.5 Environmental Enrichment

Environmental enrichment within an experimental setting requires the enhancement of the conditions of standard laboratory housing. This can be done in many different ways but typically involves making the animals' environment more complex by adding toys, tunnels, bridges, nesting materials and running wheels. The animals are also often housed in larger cages with a larger number of co-inhabitants in each cage to increase the number of social interactions. The additional objects can enhance the sensory and physical experiences of the animals as well as their experience of novelty in their home environment, since objects will be replaced or moved frequently. In addition to this, the inclusion of a running wheel will increase the physical activity of the animal and previous studies in our lab have shown that this enhances their cognitive function (O'Callaghan *et al.*, 2007, Griffin *et al.*, 2009). The large number of different combinations that can be used within an enriched environment does cause a degree of difficulty in explaining any behavioural or neurochemical changes that may occur; specific factors cannot be easily pinpointed as the main cause of these changes and hence it is commonly interpreted to be an interaction of factors that is an essential part of the enrichment protocol (van Praag *et al.*, 2000, Kumar *et al.*, 2011).

There are two main cognitive theories regarding the way in which environmental enrichment can have an effect on the brain. These are the 'arousal hypothesis' (Walsh and Cummins, 1975) and the 'learning and memory hypothesis' (Rosenzweig and Bennett, 1996). The arousal hypothesis argues that the enhanced 'arousal response' of animals when

they are faced with increased novelty and environmental complexity is crucial to the changes observed. The somewhat more popular learning and memory hypothesis argues that enrichment evokes the similar cascade of neurochemical processes that are seen during learning paradigms. This could be indicative of a priming effect on the brain, which could enhance any future memory formation. These neurochemical processes may also facilitate morphological changes in the brain that would enhance plasticity, such as synaptogenesis or neurogenesis. Much of the recent research that is being undertaken into the mechanisms underlying environmental enrichment is directly targeted at ways that it would be possible to manipulate and reproduce these mechanisms therapeutically, to enhance memory function or more frequently as a tool to combat neurodegenerative diseases.

Animals housed in enriched environments have shown improvements in memory function in both recognition and spatial memory tasks and rescuing of cognitive deficits following experimentally induced ischemia or surgical lesions (Gobbo and O'Mara, 2004, Mandolesi *et al.*, 2008). Additionally, there are many studies which link environmental enrichment with reduced anxiety in rodents (Galani *et al.*, 2007, Amaral *et al.*, 2008). These behavioural changes seem to be, at least in part, due to changes in neurotrophin levels: mice heterozygous for BDNF show increased anxiety which is rescued via housing in an enriched environment (Chourbaji et al., 2008) and BDNF increases have also been found with corresponding improvement in memory tasks (Gobbo and O'Mara, 2004). Enriched animals also demonstrate increased NGF concentration in the hippocampus and associated entorhinal cortices, together with improvements in spatial memory (Pham *et al.*, 1999a, Pham *et al.*, 1999b). The data do not always agree however, with some studies showing changes in both NGF and BDNF expression (Ickes et al., 2000), some reporting changes in BDNF and not NGF (Turner and Lewis, 2003), and some showing no change in BDNF expression (Bindu *et al.*, 2007, Kumar *et al.*, 2011). These results are most likely to do with the large variation in experimental protocol in studies of environmental enrichment; the time period over which animals are housed in enriched environments varies considerably, as does whether the enrichment is continuous (stimulation remains within the animals' home cages (eg. Bindu et al., 2007)) or non-continuous (animals are introduced to an enriched environment for a certain period of time per day (eg. Bruel-Jungerman *et al.*, 2005)). The housing of the control animals is also not always the same, with some studies comparing animals housed in isolated conditions with the effects of environmental enrichment (Nilsson *et al.*, 1999, Pham *et al.*, 2002) instead of socially housed animals

with no extra stimulation. This can be a serious confound as there is evidence that social enrichment alone can induce increases in both BDNF and NGF concentrations in the hippocampus and reduce behaviours associated with anxiety and aggression (Branchi *et al.*, 2006).

Conflicting data also hampers efforts to understand the importance of hippocampal neurogenesis in the cognitive improvements from environmental enrichment. There is a considerable amount of evidence to show that animals housed in enriched environments have significant increases in neurogenesis (Nilsson *et al.*, 1999, Kempermann *et al.*, 2002, Rossi *et al.*, 2006, Segovia *et al.*, 2006) and that behavioural improvements due to enrichment may be reliant upon neurogenesis, however some data suggest that neurogenesis may not be required for the enrichment-induced improvements in memory (Meshi *et al.*, 2006).

An additional source of variation within environmental enrichment protocols gives rise to possibly the most important confound in understanding its underlying mechanisms: the inclusion or exclusion of running wheels. Numerous studies have shown that exercise alone can induce memory improvements, upregulate neurotrophins and increase neurogenesis in animals (van Praag *et al.*, 1999, Anderson *et al.*, 2000, Farmer *et al.*, 2004, Xu *et al.*, 2006, O'Callaghan *et al.*, 2007, Wu *et al.*, 2008, Griffin *et al.*, 2009). It is not clear however, whether exercise and cognitive enrichment, whilst able to bring about similar behavioural improvements, do so via similar mechanisms of change in the brain. van Praag *et al*(1999) reported that enrichment only affected cell survival and not cell proliferation whereas exercise increased cell division and net neuronal survival in C57BL/6 mice. It has therefore been proposed that enrichment does not stimulate an increase in proliferation *per se*, but promotes increased survival of neuronal progenitor cells hence increases the number of young neurons able to functionally integrate into neuronal networks (Kempermann *et al.*, 1998, Kempermann and Gage, 1999). This would suggest that cell survival and cell proliferation may be regulated by differing mechanisms that can be affected by behavioural and environmental factors (Olson *et al.*, 2006). In developing an enrichment protocol, it is therefore crucial to recognise that the possibility of exercise-induced behavioural, neurochemical or neurogenic changes may interact with any other changes occurring due to cognitive or social enrichment.

It is becoming increasingly evident that environmental enrichment may be able to play a role in neuroprotection, with an accumulation of studies suggesting that it may be useful as a preventative tool for protection against neurodegenerative diseases such as Alzheimer's disease and dementia or indeed as a treatment for other neurological disorders such as depression (Fratiglioni *et al.*, 2004, Laviola *et al.*, 2008). Neurotrophins have been therapeutic targets for the treatment of neurodegenerative diseases since their importance in neuroplasticity and memory function has been recognised(Tapia-Arancibia *et al.*, 2008). Interestingly, BDNF expression remains relatively similar in the hippocampus throughout life (Silhol *et al.*, 2005, Kumar *et al.*, 2011), although there is evidence that BDNF serum concentrations are reduced in aged humans (Lommatzsch *et al.*, 2005a, Ziegenhorn *et al.*, 2007). BDNF has been shown to protect against the neurotoxic effects of beta-amyloid both *in vivo* and *in vitro* (Arancibia et al., 2008) and also mediate the rescue of cognitive deficits in a transgenic mouse model of Alzheimer's disease via direct infusion (Nagahara *et al.*, 2009) or the transplantation of neural stem cells (Blurton-Jones *et al.*, 2009). These improvements were not associated with a reduction in beta-amyloid plaque load. Similarly, both environmental enrichment and exercise increase hippocampal neurogenesis in a mouse model of Alzheimer's disease and do not affect the plaque load seen (Mirochnic *et al.*, 2009). They did reduce the amount of insoluble $A\beta_{1-42}$ however; this particular form of beta-amyloid is known to have neurotoxic effects. The evidence is still mixed, with some data to suggest that environmental enrichment can reduce beta-amyloid plaque load (Cracchiolo *et al.*, 2007). Environmental enrichment has also been shown to induce increases in neurogenesis and neuroplasticity in ageing as well as rescuing age-related cognitive decline (Kempermann *et al.*, 2002, Leal-Galicia *et al.*, 2008). The vast majority of these studies do include running wheels in their protocols and because exercise has been shown to prevent age-related memory deficits (O'Callaghan *et al.*, 2009), the neuroprotective effects that cognitive enrichment alone may have on the cognitive deficits associated with normal ageing or neurodegenerative diseases have yet to be elucidated. A recent study by Kumar and colleagues however, does suggest that it is the cognitive enrichment, rather than exercise which can prevent age-related decline in spatial memory retention, whereas both exercise and cognitive enrichment prevent the loss of LTP induction with age (Kumar *et al.*, 2011). They did not find an underlying mechanism for this effect and argue that it is the extra locomotor activity associated with the enriched environment which is inducing a beneficial effect on memory function. Yet they do not

show any evidence for increased locomotor activity in the enriched animals and therefore this hypothesis is lacking in any direct evidence.

The vast amount of studies published in recent years on environmental enrichment are testament to its possible therapeutic benefits, and yet how is it that two very different interventions such as physical and cognitive stimulation can elicit similar memory improvements? Dissociating these pathways and teasing out any subtle differences in cognitive improvements is crucial to further research into the benefits of environmental enrichment as a therapeutic target.

1.6 Ageing & Memory Decline

With the significant advances in modern science and medical technology over the past decades, life expectancy has increased a great deal in the western world. Indeed, there has been a fundamental shift in the distribution of age across populations, with the number of people over the age of 65 being greater than those under 15 in Europe in 2000 and it is predicted that the rest of the world will follow this pattern by 2050 (UN, 2002). This shift poses significant economic and social burdens on countries, as incidences of age-related cognitive diseases such as Alzheimer's disease are becoming increasingly common. The majority of people however, will not develop a dementia but nevertheless will undergo cognitive decline, particularly in hippocampal function, with normal ageing which can lead to severe lifestyle impairments (Plassman *et al.*, 2008).

Rats provide a useful model for measuring age-related cognitive decline because they have a relatively short lifespan (typically 2 years) which facilitates longitudinal studies. Their learning abilities have also been well-documented using a variety of behavioural tasks, particularly hippocampal-dependent tasks. Age-related deficits in spatial memory have been well documented in rats (for review, see Rosenzweig and Barnes, 2003) and humans have shown similar deficits in an equivalent spatial task (Moffat *et al.*, 2001). Whilst there is a great deal of evidence to show that lesions in the hippocampal formation cause deficits in spatial memory (Schoville and Milner, 1957, Squire, 1992, Duva *et al.*, 1997, Broadbent *et al.*, 2004), the evidence to suggest age-related memory decline is associated with region-specific volumetric reductions is inconclusive. In a meta-analysis of results from 33 studies, Van Petten found a very weak association between hippocampal size and episodic memory (Van Petten, 2004) whereas Driscoll and colleagues found deficits in spatial memory associated with reductions in hippocampal volume in aged subjects (Driscoll *et*

al., 2003). It is clear however, that there is disruption to memory with age and in particular, the hippocampus and prefrontal cortex seem to be more vulnerable to dysfunction than other cortical regions (Grady, 2008).

To date, most treatments for Alzheimer's disease and other dementias are effective only at ameliorating symptoms and not curative, therefore lifestyle factors such as exercise and cognitive stimulation may be important behavioural targets for upregulating endogenous mechanisms that can enhance cognitive function. These treatments would provide benefits to overall health, are relatively straightforward to employ and do not have any potential side-effects or interactions with any other medications that most elderly people would be prescribed (Frick and Benoit, 2010). There have been some recent studies that have addressed the impact that exercise or cognitive training may have on elderly subjects: one year of exercise training increased hippocampal volume and this volume was positively correlated with BDNF serum concentration in aged adults, however there was no significant improvement in spatial memory in the exercise group when compared with stretching controls (Erickson *et al.*, 2011). Another study addressed the concept of brain-training to improve memory in elderly adults, Mahnke and colleagues showed that 8-10 weeks of an auditory/language-based cognitive training program can improve general memory improvements in nonrelated tests and this improvement is maintained 3 months post-training (Mahncke *et al.*, 2006). Despite this, and the large number of review papers arguing for the neuroprotective benefits of environmental enrichment, there is a relative sparsity of human studies using exercise or cognitive stimulation as therapeutic interventions.

There is a significant body of evidence however, for a 'cognitive reserve', with epidemiological observations that suggest increased cognitive function and enhanced complex mental activity can provide a functional reserve with age that can compensate for pathological changes associated with neurodegenerative diseases such as Alzheimer's disease and other dementias (Valenzuela *et al.*, 2007). Valenzuela and Sachdev (2006) report that there is a significant overall reduction in the risk of developing dementia in people classed as having 'high mental activity levels' such as higher educational level, occupational complexity and increased cognitive lifestyle in their meta-analysis that incorporates 22 studies with data from over 29,000 participants.Wilson *et al*(2002) also show that frequent participation in cognitive activities reduces the risk of developing Alzheimer's disease in a longitudinal cohort study with over 800 participants. These

reports, together with animal studies, suggest that environmental enrichment could induce behavioural improvements in a 'cognitive reserve' associated manner; there can be cognitive improvements or protection against cognitive decline in the absence of any improvements in brain pathology (Mirochnic *et al.*, 2009, Nithianantharajah and Hannan, 2009). Indeed, studies suggest that it is not necessary to cause a change in Aβ plaque deposits in order to see improvements in the cognitive dysfunction seen in Alzheimer's disease (Erten-Lyons *et al.*, 2009). Additionally, it seems that although level of education may not prevent the onset of Alzheimer's disease, it may confer some protection against the development of the clinical symptoms (Letenneur *et al.*, 1999, Stern, 2002).There is still some debate regarding the causal direction of these associations however, with questions remaining about whether it is a lack of cognitive stimulation that causes an increased risk of dementia or a high level of cognitive stimulation that provides some protection against memory decline(Gallacher *et al.*, 2005). In addition, many questions still remain regarding the biological mechanisms that underlie the proposed modulating effect that environmental enrichment can have upon the development of dementia.

Animal studies tend to show a relatively clear-cut association between environmental enrichment and reduced risk of memory decline: exercise can prevent age-related cognitive decline in rats (O'Callaghan *et al.*, 2009, Kim *et al.*, 2010) and has been shown to prevent or delay cognitive decline in a number of neurodegenerative mouse models of traumatic brain injury (Griesbach *et al.*, 2009), Huntington's disease (Pang *et al.*, 2006) and Alzheimer's disease (Ke *et al.*, 2011, Liu *et al.*, 2011). Similarly environmental enrichment can prevent age-related spatial memory decline (Kempermann *et al.*, 2002, Bennett *et al.*, 2006, Leal-Galicia *et al.*, 2008, Kumar *et al.*, 2011) and delay memory deficits in mouse models of Alzheimer's disease (Berardi *et al.*, 2007, Cracchiolo *et al.*, 2007) and huntington's disease (Nithianantharajah *et al.*, 2008). Many of these studies report roles for neurotrophins, synaptogenesis or neurogenesis in these improvements (for reviews, see van Praag *et al.*, 2000, Nithianantharajah and Hannan, 2011).

Aging is also strongly associated with an increase in glial activation, with aged rodents show increases in TLR4, CD14 and MHCII, pro-inflammatory cytokines and astrocyte number with age (Hayakawa *et al.*, 2007, Letiembre *et al.*, 2007, Lynch, 2010). This is indicative of an increased inflammatory state in the aged brain, which could impact on neuronal functioning and behaviour. IL-1β in particular is known to impact on synaptic plasticity: IL-1β expression was increased in free moving rats 8 hours after LTP induction,

furthermore the induction of this LTP could be prevented with an infusion of the IL-1 receptor antagonist IL-1ra (Schneider *et al.*, 1998). IL-1 receptor knockout mice also show impaired LTP induction and spatial memory deficits (Goshen *et al.*, 2009). This might suggest that increases in IL-1β expression may have a positive impact on memory function, however this is not the case. Aged rats exhibit an increase in hippocampal IL-1β and an associated deficit in contextual fear conditioning which are ameliorated with the inhibition of caspase-1, an enzyme that proteolytically cleaves the precursor of IL-1β to generate its mature form (Gemma *et al.*, 2005). Furthermore, Alzheimer's disease is associated with elevated levels of IL-1β, TNF-α and IL-6 both in the serum and cerebrospinal fluid (Akiyama *et al.*, 2000, Shaftel *et al.*, 2008). It is therefore well recognised that whilst certain pro-inflammatory cytokines play an important role in modulating synaptic plasticity and memory functioning, a prolonged pro-inflammatory phenotype can be detrimental to brain functioning and may accelerate cognitive decline in neurodegenerative diseases (Lynch, 2010, Viviani and Boraso, 2011, Yirmiya and Goshen, 2011). Additionally, aging seems to enhance brain vulnerability to inflammatory challenges and these can interact to induce memory deficits, possibly via downregulation of BDNF (Barrientos *et al.*, 2006, Cortese *et al.*, 2011).

Vascular endothelial growth factor (VEGF) is an angiogenic trophic factor, inducing the proliferation, migration and integration of blood vessels into a connected network (Ferrara *et al.*, 2003). It is a vital factor in maintaining vascular function during development and throughout life. Given the current evidence that shows a role for cardiovascular health and cognitive decline, there has been a great deal of research into whether VEGF can be a mediator of cognitive function. The expression of VEGF is upregulated after hippocampal-dependent learning in rats and overexpression of VEGF can further improve spatial memory and contextual learning (Cao *et al.*, 2004, Licht *et al.*, 2011). Furthermore, rats treated with VEGF displayed significantly more angiogenesis and neurogenesis than control rats suggesting that, in addition to improving memory in the short-term, VEGF may also play a role in maintenance of a suitable neurogenic niche in the dentate gyrus (Licht *et al.*, 2011). However, Licht and collegues also found that inhibition of VEGF under normal conditions does not affect neurogenesis but still impaired contextual and spatial memory, although it has been shown that knocking down VEGF expression inhibits the environmental enrichment-induced increase in neurogenesis in the dentate gyrus (Cao*et al.*, 2004). It has also been found that treatment with VEGF enhanced spatial memory in a

Alzheimer's disease mouse model, coupled with an increase in angiogenesis in the hippocampus and a reduction in Aβ plaque load (Wang *et al.*, 2011). These data suggest that VEGF could be a suitable neurochemical marker to analyse local changes in angiogenesis that induce memory improvements and can also correlate with changes in blood perfusion. Given that environmental enrichment can increase the expression of VEGF in the hippocampus (Cao *et al.*, 2004), it is conceivable that environmental enrichment may prevent any age-related reductions in cerebral blood volume via a VEGF-induced maintenance of vascular health.

Aging is an inevitable part of life and as medical research prolongs our lifespan, it is becoming increasingly important to find ways to support and maintain healthy minds and bodies. Whilst there is a great deal of hope that more effective treatments and cures can be developed for many neurodegenerative diseases, environmental enrichment provides an easily implemented intervention that can be utilised both in younger and older people to prevent or ameliorate cognitive decline. Ongoing research into understanding the underlying neurochemical mechanisms associated both with age-related cognitive decline and environmental enrichment as an intervention will also provide invaluable insights into neurodegeneration and possible therapeutic targets.

1.7 The Use of Animal MRI

Studying plasticity at a cellular level is crucial in order to understand the mechanisms behind brain changes during adult life. Studying structural and neurochemical changes within the hippocampus can only be done using animal studies with invasive techniques or with analysis of brains postmortem in humans. It is not possible therefore, to conclude definitively that there are similar mechanisms occurring within human brains and animal brains when they exhibit similar cognitive improvements. Functional magnetic resonance imaging (fMRI) however, has provided neuroscientists and cognitive psychologists with a non-invasive tool that is a window into the activity of the brain during cognitive tasks. This has been invaluable for the advancement of our knowledge of which specific regions of the brain are important for particular skills. It would be unethical to test rodents in a similar way, as they would have to be fully conscious to perform the cognitive tasks and this would be distressing in the confined space of the MRI scanner. There are many different types of MRI scan that can be utilised whilst the animals are anaesthetised however, particularly to study any structural changes that may have occurred in the brain due to an

experimental intervention. There have been a limited number of fMRI studies however, which have measured changes in blood flow in the sensory cortex of rats during stimulation of whiskers or paws under light anaesthetic(Goloshevsky *et al.*, 2008, Zhao *et al.*, 2008), showing that it is possible to map relatively simple brain activity in rodents. As techniques develop further, it seems very likely that more sophisticated techniques will be utilised in order to more directly compare more complex animal and human brain activity.

It is possible to study experience-dependent plasticity changes on a regional scale: in a highly publicised study by Maguire and colleagues (2000), it was shown that London taxi drivers (who have extensive experience with spatial navigation) had significantly larger posterior hippocampi than age-matched controls that did not drive taxis. Furthermore, the posterior hippocampal volume was positively correlated with the amount of time spent working as a taxi driver. Grey matter and white matter changes have also been seen after learning particular tasks in humans and non-human primates (Draganski *et al.*, 2004, Quallo *et al.*, 2009), suggesting that experience-dependent plastic changes can be large enough to be detected using analysis of structural MRI scans. Task training in this way is a cognitive stimulation that is similar to the cognitive enrichment in environmental enrichment protocols for rodents, although at a much more complex level. As environmental enrichment has been shown to upregulate neurogenesis in the hippocampus, in the long term this may lead to grey matter changes in this region that can be analysed using MRI.

Typically, studies comparing cognitive function and grey or white matter changes concentrate on cognitively-impaired groups such as mild cognitive impairment (MCI), dementia or Alzheimer's patients. Voxel-based morphometry studies show reductions in global grey matter, with more significant changes in medial temporal regions in Alzheimer's patients (Baron *et al.*, 2001, Frisoni *et al.*, 2002, Karas *et al.*, 2003) and positive correlations between volumes of grey matter in temporal and frontal regions and memory performance (Duarte *et al.*, 2006). In normal ageing, the patterns are not as clear-cut, some studies show associations with semantic and short-term memory performance and grey matter volume in the anterior temporal lobes and hippocampus respectively (Taki *et al.*, 2011), whereas there was only a weak association between episodic memory and hippocampal volume in a meta-analysis of 33 studies (Van Petten, 2004). This suggests that only certain types of memory may be affected by regional-specific reductions in grey matter volume, indeed in MCI and Alzheimer's disease patients higher cognitive function

was associated with reduced brain volumes but an increase in brain activation (Solé-Padullés *et al.*, 2009). Interestingly, the same study also found the opposite pattern in healthy controls: higher cognitive function was correlated with a larger brain volume and reduced brain activity. This would suggest that some patients that are able to maintain higher cognitive functions can utilise compensatory mechanisms, most likely via alternative neural networks. An additional advantage of this technique is that it can enable direct comparisons of changes in regional brain volume in the same subjects over time. Kramer and colleagues found a correlation between loss of hippocampal volume and episodic memory and global cortical volume reductions and executive function in healthy elderly subjects, with no changes patterns in white matter integrity and memory function (Kramer *et al.*, 2007).

Using voxel-based morphometry in rodent studies is a relatively new area of research. To date, there are no studies that have compared the global grey matter changes with age using automated analytical techniques. Driscoll and colleagues (2006) manually measured grey matter intensity from MR images taken using a 9.4T scanner. They reported a significant reduction in hippocampal volume with age, which was correlated with performance on the Morris water maze. *In vivo* studies show that there is a reduction in neurogenesis with ageing and that this is correlated with spatial memory performance in rats (Drapeau *et al.*, 2003, McDonald and Wojtowicz, 2005). This reduction in neurogenesis is rescued by environmental enrichment (Kempermann *et al.*, 2002) and so it is possible that this rescue effect may be measurable as grey matter changes in the hippocampus, especially if the enrichment is over a long enough period.

An alternative measure to volumetric analysis that is often used in MRI studies is the assessment of changes in cerebral blood perfusion in the brain. Measuring cerebral blood perfusion can be done either by using exogenous tracers (such as bolus tracking with gadolinium chelates) or labelling endogenous arterial water (arterial spin labelling, ASL). Using exogenous tracers assumes that the blood brain barrier is intact and therefore the contrast agents only travel through the vasculature. Using this technique, it is possible to measure the flow of the blood through and within specific regions of the brain with a sensitive scanning sequence such as T_2*-weighted images (Pagani *et al.*, 2008). Using ASL to measure perfusion has the advantage over using exogenous tracers of removing the requirement for a contrast agent, and therefore reduces the stress to the animal. The theoretical models behind this analysis are more complex however, which makes

interpretation of the results more difficult. This technique labels the water molecules in the blood upstream of the region of interest by inverting their longitudinal magnetisation and then subsequently imaging the region of interest during the time that the labelled blood is within that region. This enables a measure of cerebral blood flow to be derived via calculations from the ratio of relaxation (time taken for the inverted ions to return to their relaxed state) between the labelled and control blood and the amount of magnetised blood that arrived at the region of interest (Buxton, 2005). This study uses an updated approach to ASL, bolus-tracking ASL (btASL) in which boluses of varying duration were labelled using the continuous ASL (cASL) technique (Kelly *et al.*, 2009).

It has been well-established that alterations in blood flow are a significant factor in dementia: evidence from epidemiological studies show associations between cardiovascular disease and dementia (Launer, 2002), in particular hypertension seems to play a role in cognitive decline with age. Many studies show that increases in both systolic and diastolic blood pressure in midlife are associated with lower cognitive function in later life (Launer *et al.*, 1995, Kilander *et al.*, 1998, Launer *et al.*, 2002) and therefore anti-hypertensive treatments are considered to be significant factors in the prevention of developing dementia (Launer, 2002). Elevated blood pressure can damage the blood-brain barrier and surrounding capillaries which will cause a reduction the cerebral blood flow, therefore a lack of nutrients to the brain and subsequent neuronal cell death. Recent evidence shows that cerebral blood flow is reduced in the hippocampus of aged humans and is also positively correlated with spatial memory performance (Heo *et al.*, 2010). Cerebral blood volume analysis in monkeys shows that the dentate gyrus is particularly vulnerable to reductions with age, and reductions have been correlated with reduced expression of *Arc*, the expression of which is associated with LTP, in the dentate gyrus of aged rats (Small *et al.*, 2004). Furthermore, it has been shown the cerebral blood volume is positively correlated with neurogenesis in the dentate gyrus of mice and that exercise significantly increases cerebral blood volume and correspondingly neurogenesis (Pereira *et al.*, 2007). This study also showed that exercising humans had increases in cerebral blood volume in the dentate gyrus which was positively correlated with both the fitness level of the individual and their cognitive performance on a verbal reasoning test.

1.8 Objectives

1. The first aim of this study is to investigate the minimum period of environmental enrichment, without exercise, that is needed to elicit an improvement in hippocampal-dependent memory in the rat. Furthermore this study analyses the changes in neurotrophins and neurogenesis that are associated with this memory improvement.

2. Having elucidated a role for both neurogenesis and NGF in the enrichment-induced memory improvement, this study analyses the direct effect of NGF on hippocampal-dependent memory by administering a single intracerebroventricular (i.c.v.) infusion of NGF. Following this, a chronic i.c.v. infusion of NGF for 6 weeks that directly mimics the increase in NGF observed with environmental enrichment is used to further elucidate the role that the enriched-induced NGF increase has on memory function, neurogenesis and apoptosis.

3. The aim of the final study is to investigate the neuroprotective effects of environmental enrichment, without exercise, on memory decline tested at both middle-age and old-age in rats and whether there are any associated changes in neurotrophins, neurogenesis, apoptosis, grey matter or cerebral blood volume.

Chapter 2: General Methods

2.1 Materials

2.1.1 Animals

Wistar rats (3 month old males)	Bioresources, TCD
Laboratory rat diet	Harlan
Enrichment toys	€2 euro and charity shops
Nestboxes (15cmx15cmx12cm)	Noldus

2.1.2 ELISA Kits

Human BDNF DuoSet ELISA kit	R & D Systems
Rat βNGF DuoSet ELISA kit	R & D Systems
Rat VEGF DuoSet ELISA kit	R & D Systems
Human Trk B DuoSet IC ELISA kit	R & D Systems
Substrate Solution	R & D Systems

2.1.3 General Laboratory Chemicals

Acrylamide electrophorysis reagent	Invitrogen
Ammonium Persulphate	Sigma
Aprotitin	Sigma
Bio-Rad dye reagent concentrate	Bio-Rad
Bis-acrylamide	Sigma
Bovine Serum Albumin (BSA)	Sigma
Bromopehnol Blue	Sigma
Calcium Chloride	Lennox
Dimethyl sulphoxide	Sigma
Di-Sodium Hydrogen Orthophosphate (Na_2HPO_4)	Sigma
DL-Dithiothreitol (DTT)	Sigma

Ethanol	Lennox
Glucose	Lennox
Glycerol	Sigma
Glycine	Sigma
Hydrochloric Acid	Lennox
Hydrogen Peroxide	Sigma
Heparin from bovine lung	Sigma
Isofluorane	Bioresources, TCD
Leupeptin	Sigma
Magnesium Sulphate	Sigma
Magnesium Chloride ($MgCl_2$)	Sigma
β-Mercaptoethanol	Sigma
Methanol (MeOH)	Sigma
Nitrocellulose membrane	Amersham
PAP pen for immunostaining	Sigma
Pepstatin A	Sigma
Potassium Chloride (KCl)	Sigma
Potassium Dihydrogen Orthophosphate (KH_2PO_4)	Sigma
Potassim Hydroxide	Sigma
Potassium Phosphate	Sigma
2-Propanol	Sigma
O.C.T.™ compound	Tissue Tek
Sodium Azide (NaN_3)	Sigma
Sodium Carbonate (Na_2CO_3)	Sigma
Sodium Bicarbonate ($NaHCO_3$)	Sigma
Sodium Chloride (NaCl)	Sigma

Sodium Dodecylsulphate (SDS)	Sigma
Sodium Hydrogen Carbonate	Sigma
Sodium Hydroxide (NaOH)	Lennox
Sodium Phosphate (monobasic)	Sigma
Sodium Phosphate (dibasic)	Sigma
Sodium Tetraborate	Fluka
Sucrose	Sigma
Tetramethyl-diamine (TEMED)	Sigma
Tris-base	Sigma
Tris-HCl	Sigma
Trypan blue	Sigma
Tween-20	Lennox
Xylenes	Sigma

2.1.4 General Laboratory Products and Plastics

96 Microwell ELISA plates	Nunc
Biosphere filter pipette tips	Sarstedt
Hyperfilm	Pierce
Laboratory roll	Ace Cleaners
Latex powder-free gloves	Abina
Microtest 96-well flat bottomed plates	Sarstedt
Microtubes	Sarstedt
Microscope slides twin frosted	Sparks
Needles 26G	BD Microlance
96-well optical reaction plates	Applied Biosystems
Optical adhesive covers	Applied Biosystems
PCR tubes	Sarstedt

Plastic transfer pipettes	Sarstedt
Pipette tips	Sarstedt
Standard grade No.1 filter paper	Whatman
Standard grade No.3 filter paper	Whatman
Sterile syringes (Luer 2ml)	BD Plastipak

2.1.5 Molecular Reagents

Absolute ethanol	Sigma
Agarose	Condra
Ethidium bromide	Sarstedt
High capacity cDNA reverse transcription kit	Applied Biosystems
Hematoxylin	Sigma
Molecular grade water	Sigma
RNA*later*™	Ambion
RNase-free microtubes	Ambion
RNaseZap® wipes	Ambion
Total RNA isolation kit	Macherney-Nagel
Taqman gene expression assays (see table 2b)	Applied Biosystems
Taqman universal PCR master mix	Applied Biosystems

2.1.6 Western Blotting Reagents and Antibodies

Anti-mouse (goat) IgG peroxidase conjugate	Sigma
Anti-rabbit (goat) IgG peroxidase conjugate	Sigma
Anti-Trk A rabbit polyclonal IgG	Cell Signalling Technology
Anti-Trk B rabbit polyclonal IgG	Millipore
Anti-βActin mouse monoclonal IgG	Sigma
Anti-ERK2 mouse monoclonal IgG	Santa Cruz

Anti-p44/42 MAPK (ERK1/2) rabbit polyclonal IgG	Cell Signalling Technology
Anti-$p75^{NTR}$ rabbit polyclonal antibody	Cell Signalling Technology
Anti-phospho-p44/42 MAPK (ERK1/2) rabbit monoclonal	Cell Signalling Technology
Anti-phospho-ERK mouse monoclonal antibody	Santa Cruz
Anti-synapsin I rabbit monoclonal antibody	Cell Signalling Technology
Anti-synaptophysin mouse monoclonal antibody	Millipore
Hybond-C extra nitrocellulose membrane	Amersham
Prestained dual band molecular weight standard	BioRad
ReBlot Plus strong antibody stripping solution	Chemicon
Supersignal®	Pierce

2.1.7 Immunohistochemical reagents and antibodies

Anti-mouse (Goat) IgG AlexaFluor®633 conjugate	Invitrogen
5-bromo-2'-deoxyuridine	Sigma
BrdU chicken monoclonal IgG	Abcam
BrdU rat monoclonal IgG FITC conjugate	Abcam
DAB Chromagen tablets	Dako
DPX mountant	Sigma
Hoescht	Sigma
NeuN mouse monoclonal IgG	Millipore
Normal Goat Serum	Vector
Normal Rabbit Serum	Vector
Rabbit polyclonal anti-chicken (H&L) biotinylated	AbCam
Triton® X-100	Sigma
Vectashield®	Vector
VECTASTAIN ABC kit standard	Vector

2.2 Animals

Male Wistar rats in all experiments (Bioresources Unit, TrinityCollege, Dublin). All experiments were conducted in accordance with National and European Union guidelines (European Communities [Amendment to Cruelty to Animals Act 1876] Regulations 2002 and 2005). At approximately twelve weeks of age (300-350g), rats were allocated randomly to experimental groups.

2.3 Housing Conditions

Rats in all conditions were group housed, three per cage in large, high-top standarye rat cages (59x31cm) and maintained under a 12:12 light:dark cycle with a controlled ambient temperature of between 21-24°C. Food and water was available *ad libitum*. Rats housed in enriched conditions (EE) were supplied with various different toys, nest boxes and extra bedding in their cages (figure 2a). The toys were replaced weekly with new objects and the position of the nest boxes was changed weekly.

Figure 2a.An example of the enriched housing conditions including toys, a nestbox, tunnel and extra bedding.

2.4 Assessing Memory Function

2.4.1 Anxiety Tests

To measure whether anxiety could be a significant factor in any differences in performance on memory tasks, the open field test and elevate plus maze were utilised. Animals always underwent these tests prior to exposure to any of the memory tests to reduce the amount of handling that they would have, enabling a more accurate measure of their innate anxiety levels.

Open Field Exploration

Rats explored the same circular arena, without objects, used for the NOR and OD tasks for five minutes in a dimly lit room. Using EthoVision® 3.0, the amount of time spent in the centre of the arena and around the outside of the arena (20cm corridor around the edge of the arena) was measured. The amount of time spent exploring the centre and the edge of the arena was calculated as a percentage of the time spent in the arena.

Elevated plus maze

Rats explored a '+'-shaped maze elevated 40cm off the floor placed in a dimly lit room for five minutes. Two of the arms in this maze are enclosed with high walls (40cm) and the alternate two arms are open (figure 2d). This exploration was digitally recorded and the number of times the rats entered the open and closed arms was measured.

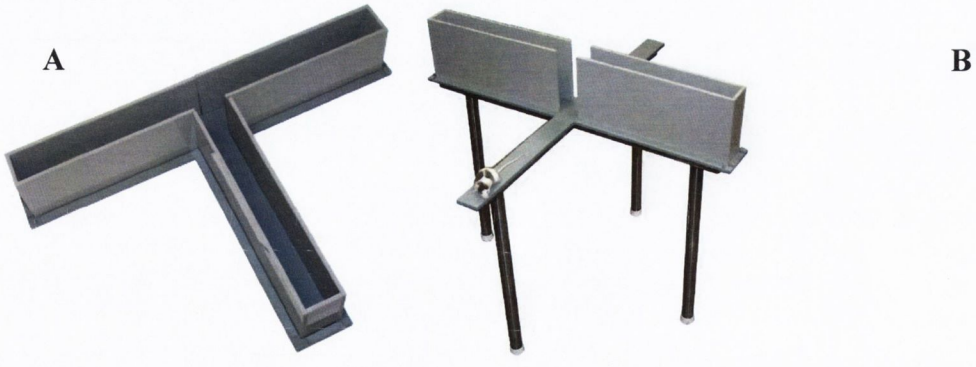


Figure 2b. An example of the structure of a T maze (A) and Elevated Plus Maze (B)(Stoelting Company, USA).

2.4.2 The NOR task

Habituation

Rats were habituated to the arena in which the task was to be performed in order to minimise anxiety and facilitate sufficient levels of exploration. The arena consisted of a black circular open field without spatial cues (diameter = 120cm, height = 35cm), placed in a dimly lit room. The rats were habituated for two days prior to the familiarisation phase of the NOR task. On the first day of habituation, the rats were introduced to the open field in pairs for a period of ten minutes. On the second day, the rats were introduced singly for a period of five minutes each.

Familiarisation

The day following habituation, different objects made of large Lego blocks were positioned in the arena, equidistant from the side and each other (figure 2b). Two variants of this task were used: two object NOR or three object NOR. The number of objects used changes the level of difficulty for the rat because the greater the number of objects, the more information the rat has to remember.

The rats were placed in the arena at random positions and allowed to explore the objects freely for three trials of five minutes, with a rest interval of five minutes in between each trial. They were placed in a holding cage during the rest period in order to minimise disturbance from home cage co-habitants. This constitutes the familiarisation phase (training) of the task.

Testing

24 hours post-training, one of the objects was replaced by a novel object in the same position. The rats were reintroduced to the arena for a single period of five minutes and allowed to explore freely.

All objects were cleaned thoroughly between each rat to eliminate olfactory cues in both the training and testing phases. The time spent exploring each object was recorded using stopwatches. Exploration had to be deemed to be active to be recorded; rats needed to be touching the object with their paws or nose and in active investigation, not solely sitting on or in the vicinity of the object. Exploration of each object was then calculated as a percentage of the total exploration of all objects.

2.4.3 The OD task

Habituation

Rats were habituated to the same arena and using the same protocol as the NOR task. Visible spatial cues were also attached to the walls of the arena in this variant of the task. Briefly, the rats were habituated for two days; on day one, the rats were introduced to the open field in pairs for ten minutes and on day two, the rats were introduced singly for five minutes each.

Familiarisation

The day following habituation, three different objects made of large Lego blocks were positioned in the arena, equidistant from the side and each other (figure 2b). Spatial cues had been positioned on the walls of the arena. The rats were placed in the arena and allowed to explore the objects freely for one trial of five minutes. This constitutes the familiarisation phase (training) of the trial. The position at which the rats were introduced to the arena was always random with respect to the other rats and groups.

Testing

24 hours post-training, one of the objects was placed in a novel position. The rats were reintroduced to the arena for a single period of five minutes and allowed to explore freely.

As with the NOR task, all objects were cleaned thoroughly between each rat to eliminate olfactory cues in both the training and testing phases. The time spent exploring each object was recorded using stopwatches. Exploration had to be deemed to be active to be recorded; rats needed to be touching the object with their paws or nose and in active investigation, not solely sitting on or in the vicinity of the object. Exploration of each object was then calculated as a percentage of the total exploration of all objects.

Figure 2c. The Open Field used in the NOR, OD and OF tasks with three examples of objects used in the NOR and OD tasks.

2.4.4 The Morris Water Maze task

Training

Rats were placed in a circular arena (diameter = 200cm, height = 35cm) filled with tepid water in a dimly lit room containing spatial cues (figure 2c), and allowed to swim around until they found a static hidden platform (diameter = 9.5cm, height = 29cm) approximately two centimetres below the water level, for five trials each day for five consecutive days. Each trial lasted a maximum of sixty seconds or until the rat had found the hidden platform. If rats had not found the platform within sixty seconds, they were led to the platform. The rats were allowed to remain on the platform for thirty seconds to enable them to orientate the position of the platform in relation to the spatial cues. Between each trial, rats were dried off and placed in a holding cage for a rest interval of sixty seconds.

Each trial was recorded and analysed using Ethovision® 3.0 (Noldus). The time spent to find the hidden platform (escape latency) and distance travelled to the platform weremeasured and mean escape latency and mean distance were calculated for each rat on each day. The pattern of exploration was also recorded.

Probe test

Two days after the final training day, the hidden platform was removed and the rats were reintroduced into the arena for a single trial of sixty seconds. Using EthoVision® 3.0, the

arena was divided into quadrants and the time the rat spent in each quadrant was recorded. The pattern of exploration was also recorded.

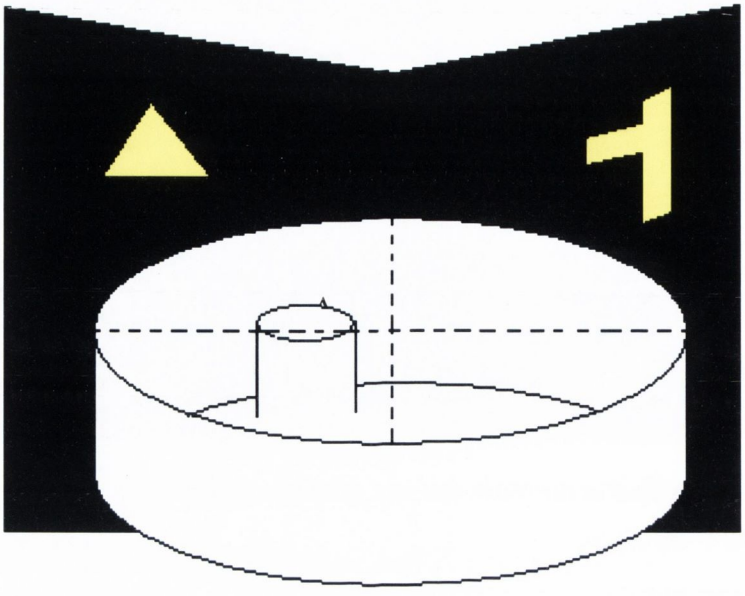


Figure 2d.A pictorial representation of the Morris water mazewith spatial cues visible around the sides of the maze and a hidden platform (A) placed 2cm below the water level in one quadrant.

2.4.5 T Maze task

Seven days prior to habituation on this task, rats were placed on a restricted diet of 85% of the suggested weight of food for rats (Wolfensohn and Lloyd, 2003). This ensured that the rats were sufficiently incentivised to search for the food reward.

Habituation

Rats were habituated to the T maze for seven days prior to training. The T maze consisted of a stem of 10 x 50cm and two arms of 10 x 40cm with walls of 20cm placed in a dimly lit room (figure 2d). During habituation, the rats were introduced to the T maze and food reward for five minutes per day. On the first day of habituation the food reward (chocolate cereal flakes) was distributed evenly throughout the T maze whilst the rats were allowed to explore freely. On the second and third day of habituation, the food reward was distributed evenly within the two arms of the T maze whilst the rats were exploring the maze. On the fourth and fifth day of habituation, the food reward was confined solely to the ends of the two arms of the T maze whilst the rats were exploring the maze and on the final two days of habituation, a single food reward (5g) was placed at the end of each of the arms of the maze whilst the rats were exploring. Throughout the habituation phase, a small amount of

the food reward was also placed in the home cages of the rats to ensure they were fully familiarised.

Training

Rats were given six trials per day for seven days. Before the trials began, one of the arms was closed off and a single food reward was placed at the end of the open arm. Rats were then placed into the stem of the maze and allowed to find the food reward in the open arm. Once the rat had eaten the reward, it was removed and placed in a holding cage for thirty seconds. Following this, both arms were opened and the reward was placed in the opposite arm to its position in the pre-trial. The rat was then returned to the stem of the maze for trial one and allowed to choose which arm to explore in search of food. Once the rat had explored its chosen arm, it was removed and placed a holding cage for a thirty second inter-trial interval. If the rat chose the arm with the food reward, in the following trial the food was positioned in the alternate arm. However, if the rat chose the arm without the food reward, then it would remain in the same arm until the rat found it on a subsequent trial. The maze was cleaned thoroughly between trials to remove any olfactory cues. The number of correct and incorrect entries into the arms was recorded, where a correct entry meant that that rat had chosen the arm with the food reward. The mean number of correct entries, as a percentage, was calculated for each day.

2.5 Neurochemical Analyses

All rats were sacrificed by decapitation and the brains were quickly removed and dissected on ice. The left hemisphere was removed and covered in Tissue-Tek® O.C.T.™ compound, flash-frozen with liquid nitrogen and stored at -80°C to preserve it for immunohistochemical analysis. The right hemisphere was sub-dissected to extract the perirhinal cortex, hippocampus proper and dentate gyrus. Tissue from each of these brain regions were homogenised or stored for RNA extraction. Trunk bloods were collected and centrifuged (20 minutes at 11,000g) after which the supernatant (serum) was collected for future analysis and stored at -20°C.

2.5.1 Preparation of Samples

Preparation of homogenate

Freshly dissected samples of the perirhinal cortex, hippocampus proper and dentate gyrus from each rat were homogenised in ice cold Krebs solution (15 strokes, 500µl)or lysis buffer (15 strokes, 500µl) using a 1ml glass homogeniser. The homogenates were then centrifuged (20 minutes at 11,000g at 4°C), supernatant taken and then stored in microtubes at -20°C until required.

Protein quantification

Protein concentrations of supernatant were quantified using the Bradford method (Bradford, 1976). Samples were analysed in triplicate (5µl/well) on a 96-well plate (microtest plate; Sarstedt, Ireland). A standard curve was prepared from a stock solution of 1000µg.ml^{-1} bovine serum albumin (BSA) diluted in deionised water ranging from 1000-15.625µg.ml^{-1} with a blank of de-ionised water also included. A 1:5 dilution of Bio-Rad reagent was filtered and added (195µl) to samples and standards. The absorbance was measured at 620nm using a 96-well plate reader (Labsystems Flouroscan Ascent FL, Medical Supplies Ltd, Ireland). The absorbance of the standards was plotted and the regression equation of the curve was used to calculate the protein concentrations of the samples. The values were then calculated as mg.ml^{-1} protein. Samples were equalised to ensure all samples had equal concentrations of protein before any further neurochemical analysis was performed.

2.5.2 SDS-PAGE and Western Immunoblotting

Preparation of samples for gel electrophoresis

100µl of equalised samples in lysis buffer were added to sample buffer (1:1 dilution) and boiled for 5 minutes in a heating block.

Gel electrophoresis

Acrylamide gels (10% or 7.5%) were cast between 2 glass plates and inserted into the electrophoresis unit (BioRad Mini-PROTEAN 3, BioRad Laboratories, Herfordshire,

England). Electrode running buffer was added to the inner and outer reservoirs. Samples prepared in sample buffer (10µl) or pre-stained molecular weight marker (5µl; BioRad) were loaded into the wells and run at 30mA (per gel) for approximately 40 minutes.

After electrophoresis, the gel was rinsed gently in transfer buffer. Nitrocellulose paper (Amersham) and filter paper (Whatman no.3 grade) were cut to the size of the gel and soaked in transfer buffer for approximately 3 minutes. A sandwich was made of one sheet of filter paper, followed by nitrocellulose paper, followed by the gel and finally a second sheet of filter paper and placed on the anode of a semi-dry blotter (Apollo Instruments, Alpha Technologies, Dublin, Ireland) that had been moistened with transfer buffer. Any air bubbles in the sandwich were removed and the lid (containing the cathode) was placed firmly on top. The transfer was carried out for 75 minutes at 225 mA.

Following this, blots were blocked for non-specific binding overnight at 4°C or 2 hours at room temperature with a solution of TBS-T (10ml; 0.05% Tween®20 in TBS) containing BSA (5%) and then probed with an antibody raised against the specific protein (table 2a). This was washed off with TBS-T (6 x 5 minute washes) and a secondary HRP-conjugated antibody was added for 1 hour at room temperature (table 2a). Immunoreactive bands were detected with HRP conjugated secondary antibody using Supersignal® West Dura chemiluminescence reagents (Pierce). The membranes were then exposed to photographic film (Hyperfilm, Amersham, UK) and developed using a Fuji Processor.

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

Primary Antibody	Dilution	Incubation	Secondary Antibody	Dilution	Incubation
Mouse Anti- ERK-2 Monoclonal IgG (Santa Cruz)	1:1000 TBS-T with BSA (2% w/v)	Overnight (4°C)	Goat anti-mouse IgG HRP (Sigma)	1:1000 TBS-T with BSA (2% w/v)	1 hour (room temperature)
Mouse Anti-phospho-ERK1/2 Polyclonal IgG (Santa Cruz)	1:2000 TBS-T with BSA (2% w/v)	Overnight (4°C)	Goat anti-mouse IgG HRP (Sigma)	1:6000 TBS-T with BSA (2% w/v)	1 hour (room temperature)
Rabbit Anti-p44/42MAPK (ERK1/2) Polyclonal IgG (Cell Signalling Technology)	1:2000 TBS	Overnight (4°C)	Goat anti-rabbit IgG HRP (Sigma)	1:6000TBS-T with BSA (2% w/v)	1 hour (room temperature)
Rabbit Anti-phospho-p44/42MAPK (ERK1/2) Monoclonal IgG (Cell Signalling Technology)	1:2000 TBS	Overnight (4°C)	Goat anti-rabbit IgG HRP (Sigma)	1:6000TBS-T with BSA (2% w/v)	1 hour (room temperature)
Rabbit Trk B Polyclonal IgG (Millipore)	1:2000 PBS with non-fat milk (2% w/v)	Overnight (4°C)	Goat anti-rabbit IgG HRP (Sigma)	1:5000 PBS with non-fat milk (2% w/v)	1 hour (room temperature)
Mouse Anti-β-Actin (Sigma)	1:2000 TBS-T with BSA (2% w/v)	Overnight (4°C)	Goat anti-mouse IgG HRP (Sigma)	1:2000 TBS-T with BSA (2% w/v)	1 hour (room temperature)
Rabbit Anti-synapsin I Monoclonal IgG (Cell Signalling Technology)	1:4000 TBS-T with BSA (2% w/v)	Overnight (4°C)	Goat anti-Rabbit IgG HRP (Sigma)	1:6000TBS-T with BSA (2% w/v)	1 hour (room temperature)
Mouse Anti-Synaptophysin Monoclonal IgG (Millipore)	1:4000 TBS-T with BSA (2% w/v)	Overnight (4°C)	Goat anti-Mouse IgG HRP (Sigma)	1:6000TBS-T with BSA (2% w/v)	1 hour (room temperature)
Mouse Anti-GAPDH Monoclonal IgG (AbCam)	1:1000 TBS-T with BSA (2% w/v)	Overnight (4°C)	Goat anti-Mouse IgG HRP (Sigma)	1:2000 TBS-T with BSA (2% w/v)	1 hour (room temperature)
Rabbit Anti-Trk A Polyclonal IgG (Cell Signalling Technology)	1:500 TBS-T with non-fat milk (2% w/v)	Overnight (4°C)	Goat anti-Rabbit IgG HRP (Sigma)	1:2000 TBS-T with non-fat milk (2% w/v)	1 hour (room temperature)
Rabbit Anti-$p75^{NTR}$ Polyclonal IgG (Cell Signalling Technology)	1:500 TBS-T with BSA (2% w/v)	Overnight (4°C)	Goat anti-Rabbit IgG HRP (Sigma)	1:2000 TBS-T with BSA (2% w/v)	1 hour (room temperature)

Table 2a. Dilutions, incubations and secondary antibodies used for Western immunoblotting

2.5.3 Polymerase Chain Reaction Analysis

Preparation of samples for RNA extraction

Freshly dissected samples of the perirhinal cortex, hippocampus and dentate gyrus from each rat were placed in RNase-free tubes containing RNA*later*™ and stored at 4°C.

Preparation of samples for polymerase chain reaction analysis

Tissue samples in RNA*later*™ were prepared by extracting RNA using a total RNA isolation kit (Macherey-Nagel). Samples were placed in a 350µl solution of β-mercaptoethanol in buffer RA1 (1:100 dilution) and homogenised using a polytron tissue disrupter (Kinetatica). Following the user manual provided with the kit, the sample homogenate was added to Nucleospin® Filter units and centrifuged for 1 minute at 11,000g. 350µl ethanol (70%) was added to the resultant sample and mixed by pipetting up and down approximately 5 times. Each sample was added to a Nucleospin® RNA II column, pipetted up and down twice and centrifuged for 30 seconds at 11,000g. Following this, 350µl Membrane Desalting Buffer (MDB) was added to each of the columns and centrifuged for 1 minute at 11,000g. 95µl DNase reaction mixture (1:10 dilution of reconstituted rDNase in Reaction buffer rDNase) was added directly into the centre of the silica membrane in each of the columns and incubated at room temperature for 15 minutes to digest DNA. 200µl buffer RA2 was then added to each of the columns and centrifuged for 30 seconds at 11,000g and the columns were placed in new collecting tubes. 600µl buffer RA3 (1:3 dilution of RA3 in ethanol) was added to each of the columns and centrifuged for 30 seconds at 11,000g and the flow-through was discarded. A final wash of 250µl buffer RA3 was added to each of the columns and centrifuged for 2 minutes at 11,000g to dry the membrane fully. Finally, the RNA was eluted by adding 60µl of H_20 (RNase-free) to each of the columns in a fresh RNase-free microtube and centrifuged for 1 minute at 11,000g. The eluted RNA was stored at -80°C for quantification and reverse transcription.

RNA quantification and reverse transcription

The RNA was quantified using a NanoDrop™ Spectrophotometer (Thermo Fisher Scientific). RNA concentrations were equalised with RNase-free H_20 in order that equal concentrations of RNA could be used as a template for cDNA transcription.

A high capacity cDNA archive kit (Applied Biosystems) was used to reverse transcribe the equalised RNA samples. 20µl of equalised RNA was mixed in a PCR mini-tube with an equal volume of 2x master mix (1:5 dilution of 10x Reverse Transcriptase 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 of H_20). Samples were then placed in a thermal cycler (PTC-200 Peltier Thermal Cycler) and incubated at 25°C for 10 minutes and 37°C for 120 minutes. The cDNA was frozen at -20°C for later real-time polymerase chain reaction (PCR) analysis.

Real-time PCR

Gene expression of targets (table 2b) was assessed using Taqman gene expression assays containing specific target primers and FAM-labelled MGB target probes. β-actin gene expression was used to normalise gene expression between samples and was quantified using a β-actin endogenous control gene expression assay containing specific primers and a VIC-labelled MGB probes for rat β-actin.

Multi-target (multiplex) Q-PCR

cDNA was diluted 1:4 with RNase-free H_20, 10µl of which was pipetted onto a PCR plate. Following this, 1:12 dilution of both target primer/probe and β-actin primer/probe in Taqman master mix was added to each well (25µl reaction volume). Electronic pipettes (EDP3 20-200µl, 2-20µl and 10-100µl) were used to ensure pipetting accuracy.

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

Real-time PCR analysis

The ΔΔCT method (Applied Biosystems 7300) was used to assess gene expression for all real-time PCR analysis. This method assesses relative gene expression by comparing the gene expression of experimental samples to the mean of control samples, instead of quantifying the exact copy number of the target gene. In this manner, the fold-change (increase-decrease) can be assessed between experimental and control samples. The fold-change is assessed using the cycle number (CT) difference between samples; a threshold for fluorescence is set, against which CT is measured. In order to accurately assess the difference between gene expression, the threshold is set when the PCR reaction is in the exponential phase.

Gene Name	Assay Number	**NCBI Gene Reference***
βNGF	Rn01533872_m1	XM_227525.3
Trk A	Rn00572130_m1	NM_021589.1
BDNF	Rn00560868_m1	----------
Trk B	Rn00820626_m1	NM_012731.1
p75NTR	Rn00561634_m1	NM_01610.2
Ki67	Rn01451446_m1	XM_225460.5
VEGF	Rn01511601_m1	NM_001110333.1
IL-1β	Rn00580432_m1	NM_031512.2
CD68	Rn01495634_g1	NM_134360.1
CD40	Rn01423583_m1	NM_001031638.1

Table 2b. 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/entrez/query.fcgi?CMD=search&DB=nucleotide

2.5.4 Enzyme Linked Immunosorbent Assay (ELISA)

An enzyme-linked immunosorbent assay (ELISA) was used to examine the concentration of BDNF, Bngf, VEGF and Trk B in samples of brain tissue homogenate. Commercially available kits were used; Human BDNF DuoSet ELISA Development system (R&D Systems Europe, Oxon, United Kingdom), rat βNGF DuoSet ELISA Development system (R&D Systems), Rat VEGF DuoSet ELISA Development system (R&D Systems) and Human Total Trk B DuoSet IC Development system. The manufacturer states that Human BDNF ELISA kit is 100% cross-reactive with rat BDNF and that Human Trk B ELISA kit is 93% cross-reactive with rat Trk B.

Human BDNF

A 96-well plate was coated with 2μg.ml^{-1} concentration of capture antibody (mouse anti-human BDNF in phosphate buffered saline (PBS; 50μl/well)) and incubated overnight at room temperature. The plate was washed with 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 hour at room temperature. After washing, the samples and standards were added (50μl/well) and incubated for 2 hours at room temperature. The plates were washed again and detection antibody (25ng.ml^{-1} biotinylated mouse anti-human BDNF in reagent diluent; 50μl/well) was added and incubated for 2 hours at room temperature. After washing, the plates were reacted with Streptavidin-HRP (50μl/well) for 20 minutes at room temperature, washed and substrate solution was added (50μl/well) and incubated in the dark at room temperature for 20 minutes. To stop the reaction, 1M H_2SO_4 was added (50μl/well). The absorbances of the samples and standards were read at 450nm in a plate reader (Labsystems Flouroscan Ascent FL, Medical Supplies Ltd, Ireland). The regression equation of the standard curve was used to calculate the BDNF concentrations of the samples.

Rat β-NGF

A 96-well plate was coated with 0.4μg.ml^{-1} concentration of capture antibody (goat anti-rat β-NGF in phosphate buffered saline (PBS; 50μl/well)) and incubated overnight at room temperature. The plate was washed with 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 hour at room temperature. After washing, the samples and standards were added (50µl/well) and incubated for 2 hours at room temperature. The plates were washed again and detection antibody (100ng.ml^{-1} biotinylated goat anti-rat β-NGF in reagent diluent; 50µl/well) was added and incubated for 2 hours at room temperature. After washing, the plates were reacted with Streptavidin-HRP (50µl/well) for 20 minutes at room temperature, washed and substrate solution was added (50µl/well) and incubated in the dark at room temperature for 20 minutes. To stop the reaction, 1M H_2SO_4 was added (50µl/well). The absorbances of the samples and standards were read at 450nm in a plate reader (Labsystems Flouroscan Ascent FL). The regression equation of the standard curve was used to calculate the β-NGF concentrations of the samples.

Rat VEGF

A 96-well plate was coated with 1µg.ml^{-1} concentration of capture antibody (mouse anti-rat VEGF in phosphate buffered saline (PBS; 50µl/well)) and incubated overnight at room temperature. The plate was washed with 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 hour at room temperature. After washing, the samples and standards were added (50µl/well) and incubated for 2 hours at room temperature. The plates were washed again and detection antibody (100ng.ml^{-1} biotinylated goat anti-rat VEGF in reagent diluent; 50µl/well) was added and incubated for 2 hours at room temperature. After washing, the plates were reacted with Streptavidin-HRP (50µl/well) for 20 minutes at room temperature, washed and substrate solution was added (50µl/well) and incubated in the dark at room temperature for 20 minutes. To stop the reaction, 1M H_2SO_4 was added (50µl/well). The absorbances of the samples and standards were read at 450nm in a plate reader (Labsystems Flouroscan Ascent FL, Medical Supplies Ltd, Ireland). The regression equation of the standard curve was used to calculate the VEGF concentrations of the samples.

Human Total Trk B

A 96-well plate was coated with 8µg.ml^{-1} concentration of capture antibody (mouse anti-human Trk B in phosphate buffered saline (PBS; 50µl/well)) and incubated overnight at

room temperature. The plate was washed with PBS-T (0.05% Tween®20 in PBS) using an automated plate washer (Columbus Plus, Tecan, Austria) and blocked with a block buffer (1% BSA, 0.05% NaN_3 in PBS; 150µl/well) for 1 hour at room temperature. After washing, the samples and standards were added (50µl/well) and incubated for 2 hours at room temperature. The plates were washed again and detection antibody (400ng.ml^{-1} biotinylated goat anti-human Trk B in reagent diluent (20 mM Tris, 137 mM NaCl, 0.05% Tween 20, 0.1% BSA, 50µl/well) was added and incubated for 2 hours at room temperature. After washing, the plates were reacted with Streptavidin-HRP (50µl/well) for 20 minutes at room temperature, washed and substrate solution was added (50µl/well) and incubated in the dark at room temperature for 20 minutes. To stop the reaction, 1M H_2SO_4 was added (50µl/well). The absorbances of the samples and standards were read at 450nm in a plate reader (Labsystems Flouroscan Ascent FL). The regression equation of the standard curve was used to calculate the β-NGF concentrations of the samples.

2.6 Immunohistochemical Analyses

Analysis of the proliferation of cells in the dentate gyrus of the rats was performed via immunohistochemical staining for the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU).

2.6.1 Preparation of Samples for Immunostaining

The left hemisphere, which had been flash froze, was sectioned at -20°C using a cryostat (Leica CM1900). For each hemisphere, 18 sections of 10µm thickness through the hippocampus were made and stored at -20°C for immunohistochemical analysis with light microscopy. 9 sections of 20µm thickness through the hippocampus were made and stored at -20°C for double immunhistochemical labelling with fluorescent antibodies.

2.6.2 Quantification of BrdU Positive Nuclei in the Dentate Gyrus

Initial quantification of the proliferation of cells in the dentate gyrus was done using 3,3'-diaminobenzidine(DAB)-linked staining. First, 10µm sections were fixed with 100% methanol (Sigma-Aldrich) for 10 minutes followed by PBS washes (3 x 3 minutes). To denature the DNA in order for the antibody to bind to the incorporated BrdU, the sections were incubated with 2N HCl (Fluka) at 37°C for 30 minutes (75µl per section). Following this, the sections were washed with 0.1M Borate buffer (0.1M sodium borate in deionised H_2O, pH 8.5; 2 x 5 minute washes) to neutralise the acid. The sections were then washed

with PBS (3 x 3 minutes). To block endogenous peroxidase, the sections were incubated in 0.3% H_2O_2 ([Sigma], 75µl per section) for 20 minutes at room temperature and then washed with PBS (3x 3 minute washes). To block non-specific binding, the sections were incubated in blocking buffer (1:5 Normal Rabbit Serum [Vector Laboratories, UK] in 1% BSA in PBS; 75µl per section) for one hour at room temperature. The sections were then incubated with chicken anti-BrdU ([AbCam], 1:200 dilution in blocking buffer; 75µl per section) overnight at 4°C. On day two, the sections were washed in PBS (3 x 3 minutes) and then the sections were incubated in the secondary antibody (rabbit anti-chicken [AbCam], 1:1000 dilution in 1% BSA in PBS; 75µl per section) for 30 minutes at room temperature. Following this, the sections were washed in PBS (3 x 3 minutes). To amplify the signal, Vectastain® ABC (Vector Laboratories) was utilised; sections were incubated in ABC solution for 30 minutes at room temperature (75µl per section) and then washed in PBS (3 x 3 minutes). Finally, the sections were reacted with DAB (Dako, Denmark) activated with 30% H_2O_2 (1:1000 dilution; 75µl per section) for approximately five minutes at room temperature. To stop the reaction, sections were immersed in PBS. Following 2 washes with H_2O, sections were counterstained with hematoxylin (Sigma) to stain all nuclei. Sections were then dehydrated using increasing concentrations of methanol (70%, 90% and then 100%), immersed in xylene and mounted with DPX mountant (Sigma). The sections were stored at room temperature until further analysis.

Nine sections of the dentate gyrus were stained per rat and then the number of BrdU positive (BrdU+) nuclei was calculated as a percentage of the number of proliferating cells. This was done by counting the total number of BrdU+ nuclei and dividing this by the total number of nuclei in the dentate gyrus of the sections stained (6 views per section at 40x on by light microscopy [Olympus CH-2, Olympus Optical Company, Japan]).

As a negative control, the above protocol was performed on three sections without the inclusion of the primary antibody. No BrdU+ nuclei were visible on any of the sections.

2.6.3 Classification of BrdU Positive Nuclei in the Dentate Gyrus

In order to classify the type of cell differentiating in the dentate gyrus of the rat, immunofluorescent antibodies were used. First, 20µm sections were fixed with 4% paraformaldehyde for 10 minutes followed by PBS washes (3 x 3 minutes). To denature the DNA in order for the antibody to bind to the incorporated BrdU, the sections were incubated with 0.5M NaOH at room temperature for 30 minutes (75µl per section) and

then washed with PBS (3 x 3 minutes). To block non-specific binding and permeabilise the tissue, the sections were incubated in blocking buffer (1:5 Normal Goat Serum [Vector Laboratories] and 1:20 0.2% Triton X-100 in PBS; 75µl per section) for two hours at room temperature (75µl per section). The sections were then incubated in rat anti-BrdU FITC-conjugated (1:50 dilution in blocking buffer; 75µl per section) overnight, in the dark, at 4°C. All the following steps took place in the dark. On day two, the sections were washed in PBS (12 x 3 minutes) and the sections were incubated in blocking buffer for two hours at room temperature. To stain for neurons, the sections were incubated in mouse anti-NeuN (1:100 dilution in blocking buffer; 75µl per section) overnight at 4°C. On day three, the sections were washed with PBS (6 x 3 minutes) and incubated in goat anti-mouse Alexa633-conjugated (1:2000 dilution in blocking buffer; 75µl per section) for 30 minutes at room temperature. Following this, the sections were washed in PBS (12 x 3 minutes). To stain for all nuclei, the sections were also incubated in Hoescht (1:1000 dilution in PBS; 75µl per section) for 10 minutes at room temperature, washed in PBS (3 x 3 minutes) and then mounted with Vectashield®. The sections were stored in the dark at 4°C until analysed using a LSM 700 Confocal Laser Scanning Microscopeand LSM software (Carl Zeiss, Germany).

Three sections of the dentate gyrus per rat were stained and the co-localisation of BrdU+ and NeuN positive (NeuN+) nuclei was analysed. The number of BrdU+/NeuN+ nuclei was calculated as a percentage of the total number BrdU+ nuclei.

2.6.4 Analysis of Apoptosis (Fluorometric TUNEL)

Levels of apoptosis in the dentate gyrus were assessed using the DeadEnd™ Fluorometric TUNEL System (Promega Corporation, Madison, USA) according to manufacturer's instructions. This system detects apoptotic cells by measuring levels of nuclear DNA fragmentation, a biochemical hallmark of apoptosis. First, 10µm sections were fixed with 4% paraformaldehyde (75µl per section) for 10 minutes followed by PBS washes (2 x 5 minutes). To permeabilise the tissue, Proteinase K (20µg.ml^{-1}; 75µl per section) for 5 minutes at room temperature followed by a 5 minute PBS wash. Sections were then fixed with 4% paraformaldehyde (75µl per section) for 5 minutes followed by a 5 minute PBS wash. The sections were then incubated in Equilibration Buffer (75µl per section) for 10 minutes. To catalytically incorporate the Fluorescein-12-dUTP at 3'OH DNA, sections were incubated in rTdT Incubation Buffer (1:1.13 Equilibration Buffer, 1:10.2 Nucleotide

Mix, 1:51 rTdT enzyme; 50µl per section) at 37°C, in the dark, for 60 minutes. To stop the reaction, sections were incubated in 2x SSC for 15 minutes at room temperature (75µl per section) followed by PBS washes (3 x 5 minutes).

To stain for all nuclei, the sections were also incubated in Hoescht (1:1000 dilution in PBS; 75µl per section) for 10 minutes at room temperature, washed in PBS (3 x 3 minutes) and then mounted with Vectashield®. The sections were stored in the dark at 4°C until analysed using a LSM 700 Confocal Laser Scanning Microscopeand LSM software (Carl Zeiss, Germany). Three sections of the dentate gyrus per rat were stained and the mean fluorescent intensity at 488nm was analysed.

2.7 MRI Analyses

Animals were first anesthetised with 5% isoflurane in 1:2 O_2:N_2 airmix. For the maintenance of anesthesia during the experiment, the isoflurane concentration was reduced to 2% and administered via a facemask at 1l.min^{-1} of 1:2 O_2:N_2 air mix. The rat's breathing and heart-rate was monitored throughout the scanning using custom hardware and software (SA Instruments Inc., Stony Brook, NY, USA). Their body temperature was controlled using a water-containing line-system (SA Instruments Inc., Stony Brook, NY, USA), and monitored throughout the experiment using a rectal probe. The rat's head was fixated using a bite-bar, a plastic head-tube and plastic ear-bars.

Animals were scanned in a 7 Tesla horizontal bore magnet (Bruker Biospin, Germany) at Trinity College Institute of Neuroscience, Trinity College Dublin. A variety of images were acquired to enable further analysis for grey matter volume differences and alterations in blood perfusion rates.

2.7.1 Voxel-Based Morphometry (VBM)

T_1-weighted MR axial images were collected using a rapid-acquisition relaxation-enhanced (RARE) sequence with the following acquisition parameters: field of view (FOV) = 4.00 x 3.00cm, image matrix = 266 x 200, 64 x 0.5mm slices, repetition time (TR) = 6.26s, echo time (TE) = 36.00ms. Images were analysed based on a fully automated voxel-based morphometry (VBM) analysis adapted from human brain analyses (Ashburner and Friston, 2000, Good et al., 2001) carried out with FSL tools (Smith et al., 2004).

Using this analysis, the structural images were first brain-extracted using a skull-stripping technique in MIPAV software (Medical Image Processing, Analysis and Visualisation;

(McAuliffe *et al.*, 2001)) followed by segmentation into the different tissue types using FSL tools. The resulting grey matter partial volume images were aligned to a standard rat brain. These normalised images were then averaged to create a study specific template to which the native grey matter images were then non-linearly re-registered. The registered partial volume images were then modulated (to correct for local expansion or contraction) by dividing by the Jacobian of the warp field. The modulated segmented images were then smoothed with an isotropic Gaussian kernel with a sigma of 2.5 in preparation for further statistical analysis (figure 2e).

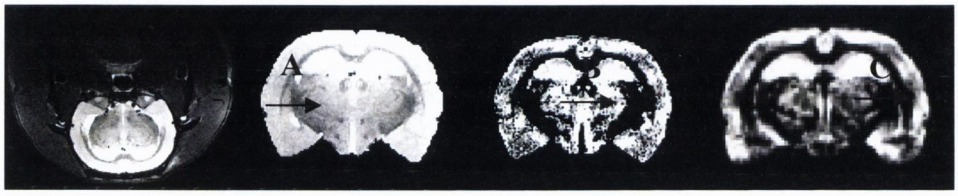


Figure 2e. Automated Voxel Based Morphometry(A) Raw images were reorientated and skull-stripped **(B)** Brains were segmented into different tissue types **(C)** Grey matter images were normalised to a standard rat brain, registered to the study-specific template and spatially smoothed.

2.7.2 Continuous ASL analysis

Axial images were obtained with a 5s preparation interval (where the magnetisation of the water molecules was inverted **(2)**) followed by a fast low angle shot (FLASH) sequence to image the flow of the 'tagged' blood with the following acquisition parameters: FOV = 3.00 x 3.00cm, image matrix = 128 x 64, 1 x 2mm slice, TR = 6.94ms, TE = 2.63mm with 22 x repetition images per slice to enable the calculation of the blood flow in that slice **(1)**. The position of the slice was selected to provide the best view of the hippocampal region. Control images were taken at the same position as the 'tagged' images to account for noise, where the magnetisation of the water molecules was not inverted **(3)** before the images were obtained **(4)** (figure 2f).

Regions of interest (ROIs) of 26 voxels in size were drawn in ImageJ (Rasband W.S., Bethesda, MD, USA). Regions were selected in the left and right hippocampus and the left and right cortex. These slices encompassed the maximum amount of hippocampal tissue. The whole brain was also used as a control image. Difference images were calculated by subtracting the control from the labelled image to give an image with signal intensities proportional to the concentration of excited spins. The ROIs were placed on these images and mean intensity measures in these ROIs were plotted against time and used to produce

intensity-time curves. Using the method described in Kelly *et al* (2009), mean transit time: time taken for the 'tagged' blood to reach the target slice (MTT) and capillary transit time: time taken for the 'tagged' blood to traverse the vasculature in the target slice (CTT) were calculated. Proportional blood flow was calculated by dividing the mean intensity measures from the ROIs by the mean cerebral blood flow (as measured from the middle cerebral arteries).

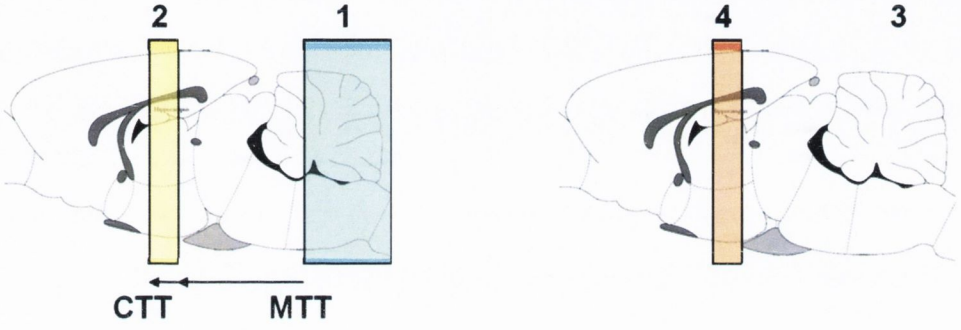


Figure 2f. Pictorial representation of continuous arterial spin labeling method (1) The water molecules in the blood are magnetised **(2)** 5 seconds later, 22 repetition images are taken at the slice of interest **(3)** For control images, no magnetisation of the water occurs and **(4)** images are taken at the same slice position. Control images are subtracted from 'tagged' images to reduce noise. MTT, CTT and proportional blood flow are calculated.

2.8. Statistical Analyses

All data are represented as mean ± standared error of the mean (SEM). Outliers were calculated as ±2 standard deviations from the mean, and excluded from any analyses. Behavioural analyses were performed in GraphPad Prism v4 with One-Way ANOVAs or in SPSS v14 with Mixed Within-Between ANOVAs. Neurochemical analyses were performed in GraphPad Prism with One-Way ANOVAs or T-tests.

Chapter 3: Evaluation of the cognitive enhancing effects of short-term environmental enrichment & assessment of the underlying mechanisms

3.1 Introduction

In the 1940s, Hebb first documented that animals housed as pets in a home secan betting exhibited behavioural improvements when compared with littermates that were kept in a laboratory setting (Hebb, 1947), but it was Rosenzweig and colleagues in the 1960s who began to study the behavioural consequences of an enriched environment as a scientific paradigm (Bennett *et al.*, 1969, Rosenzweig and Bennett, 1969, Rosenzweig *et al.*, 1969, Rosenzweig and Bennett, 1996). Since that time, environmental enrichment has emerged as a particularly useful experimental manipulation that can be utilised to facilitate memory formation and induce various neuroplastic changes in the brain, including increases in neurogenesis, synaptic plasticity and synaptogenesis (van Praag *et al.*, 2000).

Classically, environmental enrichment can be defined as any enhancement of standard laboratory housing and typically involves larger cages, increased social stimulation via the housing of larger groups together and the inclusion of extra objects in the cages such as nestboxes, extra bedding material, tunnels and toys. The objects are moved frequently to add further stimulation and the majority of enrichment protocols would also include running wheels in the animals' homecages to facilitate additional physical activity. Environmental enrichment can therefore enhance the animal's cognitive and sensory stimulation, social interactions and physical activity.

Due to the large number of factors that are associated with an enriched environment, it has been hypothesised that it is the combination of factors that is the most important aspect and that they interact to induce cognitive and neuroplastic benefits, particularly in neurodegeneration (Cracchiolo *et al.*, 2007, Mirochnic *et al.*, 2009). Studies that have tested the effects of increased social stimulation alone would suggest that this is not a major factor in the enhancement of memory (Rosenzweig *et al.*, 1978, Cracchiolo *et al.*, 2007, Zaias *et al.*, 2008), however social isolation can have detrimental effects on neurodevelopment and memory function (Bianchi *et al.*, 2006, King *et al.*, 2009, McCormick *et al.*, 2011). In contrast to this, there is a wealth of data including from our own lab showing that physical activity alone can enhance memory and increase neurogenesis, as well as being neuroprotective (Anderson *et al.*, 2000, Gobbo and O'Mara,

2005, O'Callaghan *et al.*, 2007, Griesbach *et al.*, 2009, Griffin *et al.*, 2009). Given that exercise is such a potent cognitive enhancer, some groups do argue that it is physical activity that is the key beneficial factor of environmental enrichment (van Praag *et al.*, 2000, Ehninger and Kempermann, 2003). Few studies have analysed the effect of cognitive enrichment alone as an environmental enrichment protocol for improving memory, however recent evidence suggests that it can prevent age-related cognitive decline in spatial memory (Kumar *et al.*, 2011).

Whilst significant interaction between the different factors in environmental enrichment may elicit memory improvements, the mechanisms that underlie these improvements have not been fully elucidated. Indeed, it is likely that these different interventions can induce the same behavioural improvements via dissociable pathways (Olson *et al.*, 2006). Many studies have shown that neurotrophic factors are upregulated during environmental enrichment, particularly NGF and BDNF (Ickes *et al.*, 2000, Pham *et al.*, 2002). Both NGF and BDNF are known to play important roles in synaptic plasticity and memory function (Woolf *et al.*, 2001, Alonso *et al.*, 2002, Bramham and Messaoudi, 2005, Conner *et al.*, 2009), therefore it is likely that they are associated with enrichment-induced memory improvements. Exercise in particular, has been consistently shown to increase BDNF in association with memory improvements (Griesbach *et al.*, 2009, Griffin *et al.*, 2009, Erickson *et al.*, 2011).

Additionally, environmental enrichment can increase hippocampal neurogenesis and synaptogenesis (Ehninger and Kempermann, 2003, Nithianantharajah *et al.*, 2008). Learning itself could also be argued to be a form of cognitive enrichment and performance in the Morris water maze task has been shown to induce neuronal network remodelling via increases in neurogenesis (together with some apoptosis), synaptogenesis and changes in dendritic aborisation (Dupret *et al.*, 2007, Ambrogini *et al.*, 2010, Tronel *et al.*, 2010). The potential confound of these studies is that the Morris water maze has a large physical activity component, therefore these effects could in fact be due to an interaction between the cognitive stimulation of learning and the physical stimulation of swimming.

Although it is clear that there are therapeutic benefits in environmental enrichment, the variety of different enrichment protocols makes the dissociation between the factors within an enrichment setting difficult. Certainly, it is not clear whether different neurochemical

pathways are associated with one factor over another and whether they are capable of eliciting similar behavioural improvements.

The aim of this study was to use cognitive enrichment in the absence of physical or additional social stimulation to elicit an improvement in cognitive function and assess the underlying neurochemical mechanisms associated with this improvement. This will add much needed clarification to the question of how environmental enrichment can enhance memory and begin to dissociate the different mechanisms that may be involved in this process. To date, there has been no study that assessed the minimum duration of environmental enrichment, in the absence of exercise, necessary induce a cognitive improvement. Therefore, this study assessed the effect of environmental enrichment, in the absence of exercise and for different lengths of time, cognitive function in rats. Furthermore, the underlying mechanisms of enrichment-induced improvements were analysed, especially in the context of neurotrophin expression and neurogenesis.

3.2 Methods

3.2.1 Subjects and Design

The experimental groups consisted of two groups of rats housed in standard conditions (SH, n=6) and three groups of rats housed in enriched conditions for two weeks (2wk EE, n=6), three weeks (3wk EE, n=6) and six weeks (6wk EE, n=6) respectively and then tested on the NOR task. At a later date, two groups of rats were tested on the OD task (SH: n=6; 6wk EE: n=6), and two groups of rats were tested on the T maze task (SH; n=11; 6wk EE: n=12). For a full list of all the rats used in the short-term enrichment experiments and the tasks that they performed, see table 3a.

Group	**Number of rats**	**Behavioural task performed**
2 week EE (proliferation)	6	NOR
3 week EE (proliferation)	6	NOR
6 week EE (proliferation)	6	NOR
2 & 6 week SH (proliferation)	6	NOR (2 weeks) & NOR (6 weeks)
3 week SH (proliferation)	6	NOR (3 weeks)
6 week EE (survival)	6	OD, OF
6 week SH (survival)	6	OD, OF
6 week EE (ageing)	12	T maze
6 week SH (ageing)	11	T maze

Table3a.Rats used in assessing short-term enrichment and the tasks that they performed. Proliferation = rats used in the experiment to assess the effect of environmental enrichment upon proliferation in the dentate gyrus. There were 3 different enriched housing groups in this study (2, 3 and 6 week EE) and 2 standard housing groups, with one group of SH rats being tested on the NOR task at 2 and 6 weeks, and the other SH group being tested on the NOR task at 3 weeks. Survival = rats used in the experiment to assess the effect of environmental enrichment upon survival of neurons in the dentate gyrus. Ageing = rats used in the study to assess the long-term benefits of environmental enrichment. These rats were also tested following 6 weeks of housing in their respective conditions to assess the effect of short-term enrichment upon spatial working memory.

3.2.2 BrdU Administration

Cell proliferation

To evaluate hippocampal cell proliferation, the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU [Sigma]) was administered intraperitoneally (50mg.kg^{-1}) to all rats. For the EE rats, the BrdU was administered three times weekly for the last two weeks of their enrichment period. One group of SH rats were injected three times weekly in weeks 2 and 3 of the housing. One group of SH rats were injected 3 times weekly at the same time as the 6wk EE group (figure 3a).

Cell survival

To evaluate hippocampal cell survival, BrdU (50mg.kg^{-1}; i.p) was administered to an additional group of rats (SH: n=6, EE: n=6) for 7 days prior to housing in standard or enriched housing conditions. Following six weeks of housing in their respective environments, rats were tested with the 3 object OD task (see 2.4.2) and sacrificed by decapitation. Brains were quickly removed and the left hemisphere was covered in TissueTec® OCT™ compound, flash frozen and stored at -80°C to preserve it for immunohistochemical analysis. The right hemisphere was sub-dissected to extract the perirhinal cortex, dentate gyrus and hippocampus. All tissue was prepared for further neurochemical analysis using methods described in section 2.5. The data collected from this study was analysed, under supervision, by Niamh McGarry.

3.2.3 Behavioural Testing

The hippocampal-dependent memory performance of all the rats was tested with the two object NOR task in the final two days of their enrichment period (figure 3b, see 2.4.1). Briefly, the rats had three trials of five minutes with an inter-trial interval of five minutes to explore two different novel objects in an open field (training day). Twenty-four hours post-training, one of the objects was replaced by a novel object in the same position and the rats were placed back into the open field for five minutes and allowed to explore (testing day). During both the training and testing days, the time spent exploring each object was recorded using stopwatches and calculated as a percentage of the total time spent exploring both objects.

One group of SH rats were tested at week two and six with the 2wk EE and 6wk EE rats and one group of SH rats were tested at week three with the 3wk EE rats (figure 3b). The day following the NOR test day, rats were sacrificed by decapitation and tissue was collected as described in section 2.5.1.

Two additional groups of rats (SH: n=6; EE: n=6; D on figure 3b) were tested for levels of anxiety following 6 weeks of enrichment using the OF test (see section 2.4.1). Briefly, rats were individually placed in a circular arena in a dimly lit room for 5 minutes and allowed to explore freely. Their exploratory patterns were measured using EthoVision® 3.0 and the amount of time spent in the centre of the arena versus around the outside of the arena (20cm corridor around the edge of the arena) was calculated. The amount of time spent exploring the centre and the edge of the arena was calculated as a percentage of the time spent in the arena. Following this, rats were tested with the three object OD task (figure 3b, see 2.4.2). For this task, the rats had one trial of five minutes to explore three different novel objects in an open field (training day). Twenty-four hours post-training, one of the objects was placed in a novel position and the rats were placed back into the open field for five minutes and allowed to explore freely (testing day). During both the training and testing days, the time spent exploring each object was recorded using stopwatches and calculated as a percentage of the total time spent exploring both objects.

Two additional groups of rats (SH: n=11; EE: n=12) were tested on the T maze task after 6 weeks of enrichment. This utilises working memory (figure2d, see 2.4.4) and is designed to test the rats' spontaneous alteration behaviour in search of food. Rats were placed on a reduced diet for two weeks prior to testing on this task and throughout the task itself, of approximately 85% of their recommended daily food intake for their weight. The training for this task takes 7 days and therefore rats began testing on this task in the seventh week of enrichment. The rats were given six trials per day in which they had to learn to alternate between exploration of either arm in order to obtain a food reward. The number of correct entries was recorded as a percentage of total trials per day for eight days.

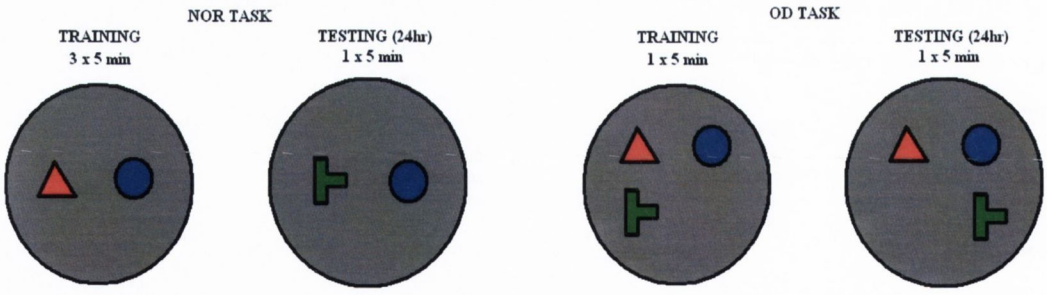


Figure 3a.Overview of the tasks used to assess novel object recognition (NOR) and spatial (OD) memory.

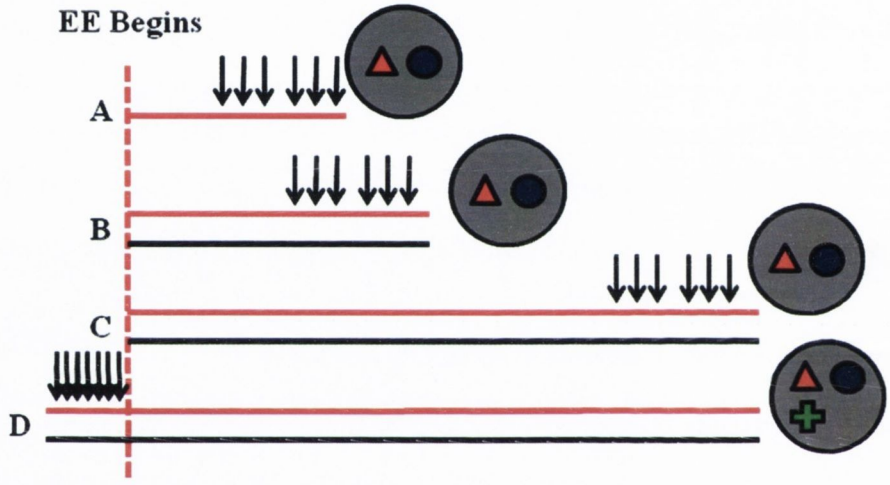


Figure 3b.The time course for assessing the effect of short-term environmental enrichment on cognitive function, cell proliferation and survival. There were three groups of EE rats: 2week EE(red line; A), 3week EE (red line; B) and 6week EE (red line; C),and two groups of SH rats (black lines; B & C). 2week EE rats were injected with BrdU (↓) on days 2, 4, 7, 9, 11 and 14. One group of SH and 3week EE rats were injected on days 9, 11, 14, 16, 18 and 21. The second group of SH rats and 6week EE were injected on days 28, 30, 32, 35, 37 and 39. 2week EE and one group of SH rats were tested on the 2 object NOR task on day 14, 6 week EE rats and the same group of SH rats were tested on the 2 object NOR task on day 42. 3 week EE and the second group of SH rats were tested on the 2 objectNOR task on day 21. To assess cell survival, an additional group of SH (black; D) and 6wk EE rats (red; D) were injected once a day for 7 days prior to housing in standard or enriched housing. At the end of six weeks, rats were tested on the 3 object OD task.

3.2.4 Analysis of the expression of NGF, BDNF, Trk A and Trk B and phosphorylation and expression of ERK

Neurotrophin expression was measured in the dentate gyrus, perirhinal cortex and hippocampus in all groups. BDNF and βNGF protein concentration were measured by ELISA (see 2.5.4) and BDNF and βNGF mRNA expression were measured using PCR analysis (see 2.5.3). Trk A and Trk B mRNA expression were also measured using PCR analysis in the dentate gyrus and hippocampus in all groups (see 2.5.3).

Trk B protein concentration was measured using western immunoblotting in the dentate gyrus and perirhinal cortex for SH and 6wk EE groups (see 2.5.2). ERK phosphorylation was measured using western immunoblotting in the dentate gyrus, hippocampus and perirhinal cortex for SH and 6wk EE groups (see 2.5.2). The phosphorylation of this protein is a downstream effect of the activation of Trk receptors in the MAPkinase pathway and therefore indicative of neurotrophin action.

3.2.5 Analysis of Synaptic Vesicle Proteins

Synapsin and synaptophysin protein concentration in the dentate gyrus and hippocampus was measured using western immunoblotting (see 2.5.2). These proteins are key modulators of neurotransmitter release and increases in these proteins are often linked with increases in the number of synapses on neuronal axons and increases in the density of synaptic vesicles within the presynaptic nerve terminals.

3.2.6 Analysis of Neurogenesis

In order to measure any changes in neuronal proliferation, immunohistochemical analysis was performed on sections of the dentate gyrus in all groups. To analyse the rate of cell proliferation in the dentate gyrus, BrdU immunostaining was performed on 10μm sections and analysed via light microscopy (see 2.6.2). The number of BrdU positive nuclei was calculated as a percentage of the total number of nuclei stained with hematoxylin. Following this, the characteristics of the BrdU positive nuclei were measured by double-labelling 20μm sections through the dentate gyrus with BrdU and NeuN antibodies in SH, 3wk EE and 6wk EE groups (see 2.6.3). The co-localisation of this staining was calculated as a percentage of the total number of BrdU positive cells.

3.2.7 Statistical Analysis

All data are expressed as mean ± standard error of the mean (SEM). Outliers were excluded from any analysis if they were ±2standard deviations away from the mean. Two-way ANOVAs were used to analyse data from the NOR, OD and OF tasks. In the T maze task, data were analysed in two day groupings so that performance was compared between days 1-2, 3-4, 5-6 and 7-8 together and analysed using a two-way repeated measures ANOVA. Post-hoc analyses were performed using either Bonferroni or Tukey multiple comparison tests.

All neurochemical and immunohistochemical data were analysed using one-way ANOVAs, except the analysis of Trk B, phosphorylated-ERK (pERK), synapsin and synaptophysin protein concentration which were analysed using unpaired t tests.

3.3 Results

3.3.1 Six weeks of environmental enrichment does not affect anxiety behaviour

Anxiety levels in the SH and 6wk EE groups were measured using the OF test. Exploration in the centre of the open field was compared to exploration around the wall (within 20cm of the wall) and both were calculated as a percentage of total exploration. There was a significant effect of zone ($F_{1,42}$ = 169.5, $p<0.001$, figure 3f), with both groups exploring around the wall significantly more than in the centre of the open field (t_{23} = 21.07, $p<0.001$). There was however, no significant effect of housing conditions ($F_{1,42}$ = 0.00, $p>0.05$) and no significant interaction ($F_{1,42}$ = 1.174, $p>0.05$). These data suggest that short-term environmental enrichment does not significantly affect anxiety levels in rats. Mean percentage exploration ± SEM: SH Centre = 20.78±3.70, SH Wall = 79.22±3.71, 6wk EE Centre = 25.27±4.47, 6wk EE Wall = 74.73±4.47).

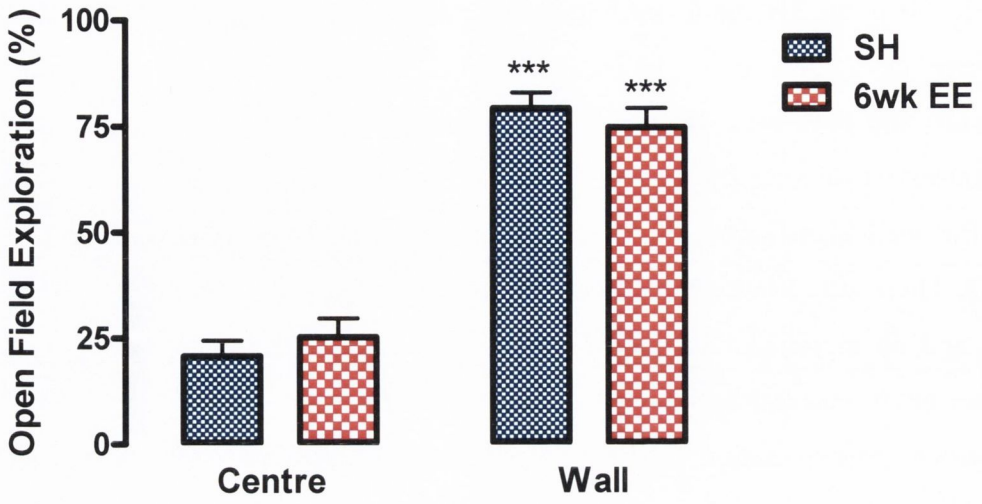


Figure 3c. Six weeks of environmental enrichment has no effect on open field exploration. There was a significant effect of zone ($F_{1,42}$ = 169.5, $p<0.001$, figure 3e), with both the SH and 6wk EE group exploring around the wall significantly more than in the centre of the open field (t_{23} = 21.07, ***<0.001). There was no effect of group. SH: n=11, 6wk EE: n=12. Data analysed as percentage of total open field exploration and expressed as mean ± SEM.

3.3.2 Environmental enrichment improves object recognition memory in a time-dependent manner

Object recognition memory was tested with the two object NOR task on SH, 2wk EE, 3wk EE and 6wk EE groups. On the training day, there was no significant difference between the exploration of the two objects A and B ($F_{1,44}$ = 0.0013, $p>0.05$). There was no significant difference in total exploration between any of the groups ($F_{3,44}$ = 0.0011, $p>0.05$) and no interaction ($F_{3,44}$ = 1.407, $p>0.05$, figure 3c.i).

On the testing day, object B was replaced with a novel object C*. There was no significant difference in total exploration between any of the groups ($F_{3,44}$ = 0.0000, $p>0.05$) but there was a significant difference between the exploration of the objects A and C* ($F_{1,44}$ = 340.5, $p<0.001$), with Bonferroni posttests showing that all groups explored the novel object C* significantly more than the familiar object A (mean percentage exploration of objects ± SEM: SH: A = 44.58±1.29, C* = 55.42±1.25, $p<0.05$; 2wk EE: A = 41.45±1.83, C* = 58.55±1.83, $p<0.001$; 3wk EE: A = 32.12±2.74, C* = 67.88±2.74, $p<0.001$; 6wk EE: A = 21.05±2.57, C* = 78.95±2.57, $p<0.001$). There was a significant interaction between the housing conditions and the exploration of objects ($F_{3,44}$ = 19.48, $p<0.001$, figure 3c.ii).

To find the source of the interaction, a one-way ANOVA was performed on the percentage exploration of the novel object C* between all the groups. This analysis revealed that there was significant difference in the exploration of object C* between all the groups ($F_{3,22}$ = 13.60, $p<0.001$). Tukey's multiple comparison test revealed that there was no significant difference in the exploration of object C* between the SH and 2wk EE group (mean difference = 3.14, $p>0.05$). There was however, a significant increase in the exploration of object C* between the SH and 3wk EE group (mean difference = 12.47, $p<0.01$) and between the SH and 6wk EE group (mean difference = 23.54, $p<0.001$). Furthermore, there was a significant increase in the exploration of object C* between the 3wk EE and 6wk EE group (mean difference = 11.07, $p<0.05$).

These data show that two weeks of environmental enrichment, in the absence of exercise, are not sufficient to induce a behavioural improvement in object recognition memory as measured by the two object NOR task. Housing rats in an enriched environment for three or six weeks is sufficient to induce a significant improvement in object recognition memory and in addition to this, rats housed in an enriched environment for six weeks have a significantly greater improvement when compared with those housed for three weeks.

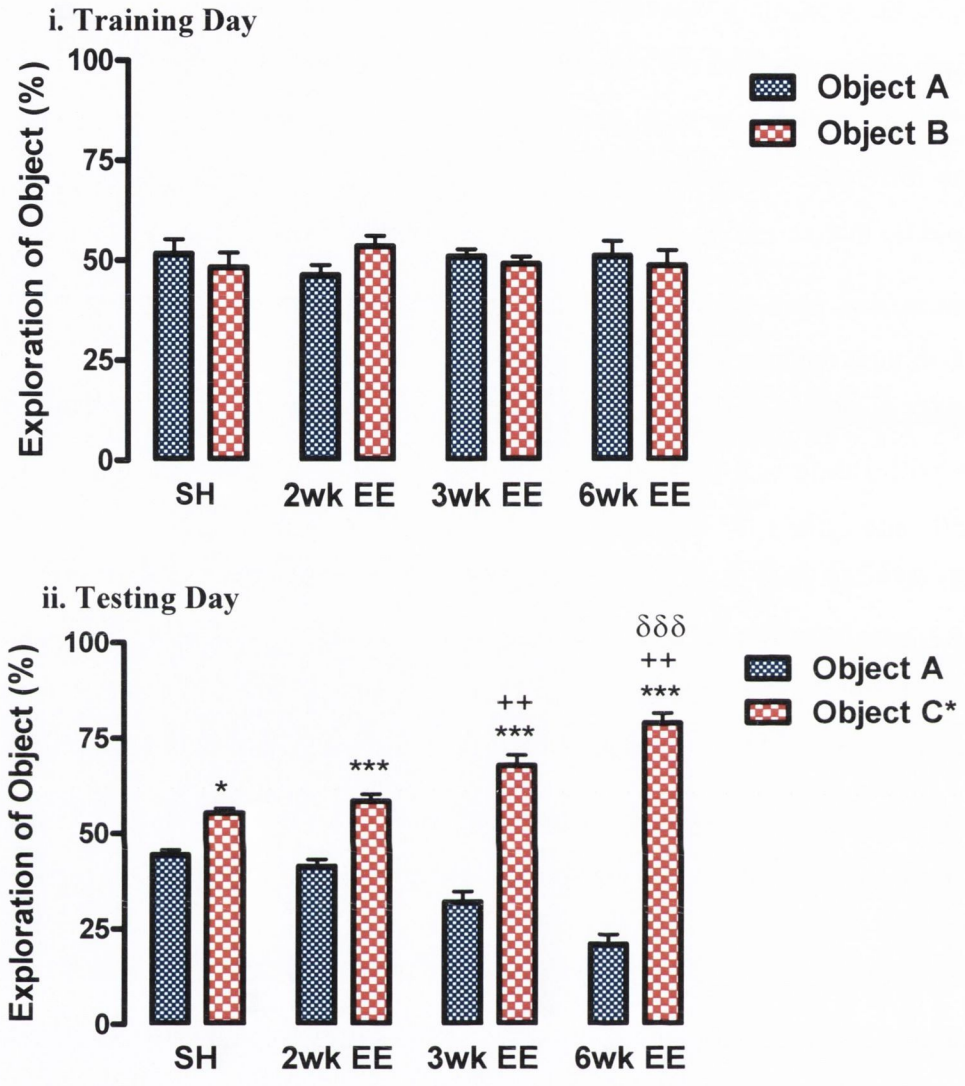


Figure 3d. Environmental enrichment improves object recognition memory in a time-dependent manner(i) There was no significant difference between the exploration of objects on the training day of the NOR task ($F_{1,44}$ = 0.0013, p>0.05). (ii) There was a significant increase in the exploration of the novel object C* when compared with the familiar object A on the testing day in all the groups ($F_{1,44}$ = 340.5, p<0.001; SH: *p<0.05, 2wk EE: ***p<0.001, 3wk EE: ***p<0.001, 6wk EE: ***p<0.001). There was also an interaction between the exploration of objects and housing conditions (F3,44 = 19.48, p<0.001) with no significant difference in the exploration of object C* between the SH and 2wk EE group but with 3wk EE and 6wk EE exploring object C* significantly more than SH ($^{++}$p<0.01), and 6wk EE object C* significantly more than 3wk EE ($^{\delta\delta\delta}$p<0.001). n=6 in all groups. Data expressed as mean ± SEM.

3.3.3 Six weeks of environmental enrichment improves spatial memory

Spatial memory in SH and 6wk EE groups was tested with the three object OD task. On the training day, there was no significant difference between the exploration of the three objects A, B and C ($F_{2,63}$ = 1.597, $p>0.05$). There was no significant difference in total exploration between the SH and 6wk EE group ($F_{1,63}$ = 0.117, $p>0.05$) and no interaction ($F_{2,63}$ = 0.557, $p>0.05$, figure 3d.i).

On the testing day, object A was moved to a novel position. There was no significant difference in total exploration between any of the groups ($F_{3,44}$ = 0.0000, $p>0.05$) but there was a significant difference between the exploration of the objects A*, B and C ($F_{2,63}$ = 32.42, $p<0.001$), with Bonferroni posttests showing that the SH group did not explore the displaced object A* significantly more than object B (mean percentage exploration of objects ± SEM: A* = 37.35±2.80, B = 34.08±2.80, $p>0.05$) but did explore the object A* more than object C (mean percentage exploration of object C ± SEM = 28.57±1.90, $p<0.05$). The 6wk EE group explored the displaced object A* significantly more than object B (mean percentage exploration of objects ± SEM: A* = 49.49±2.30, B = 30.35±2.16, $p<0.001$) and object C (mean percentage exploration of object C ± SEM = 20.17±2.23, $p<0.001$) and explored object B significantly more than object C ($p<0.01$). There was a significant interaction between the housing conditions and the exploration of objects ($F_{2,63}$= 10.25, $p<0.001$, figure 3d.ii).

To find the source of the interaction, an unpaired t test was performed on the percentage exploration of the displaced object A* between the SH and 6wk EE group. This analysis revealed that there was significant difference in the exploration of object A* between these two groups (t_{21} = 3.37, $p<0.01$).

These data show that six weeks of environmental enrichment, in the absence of exercise, can improve spatial memory in the rat as measured by the three object OD task.

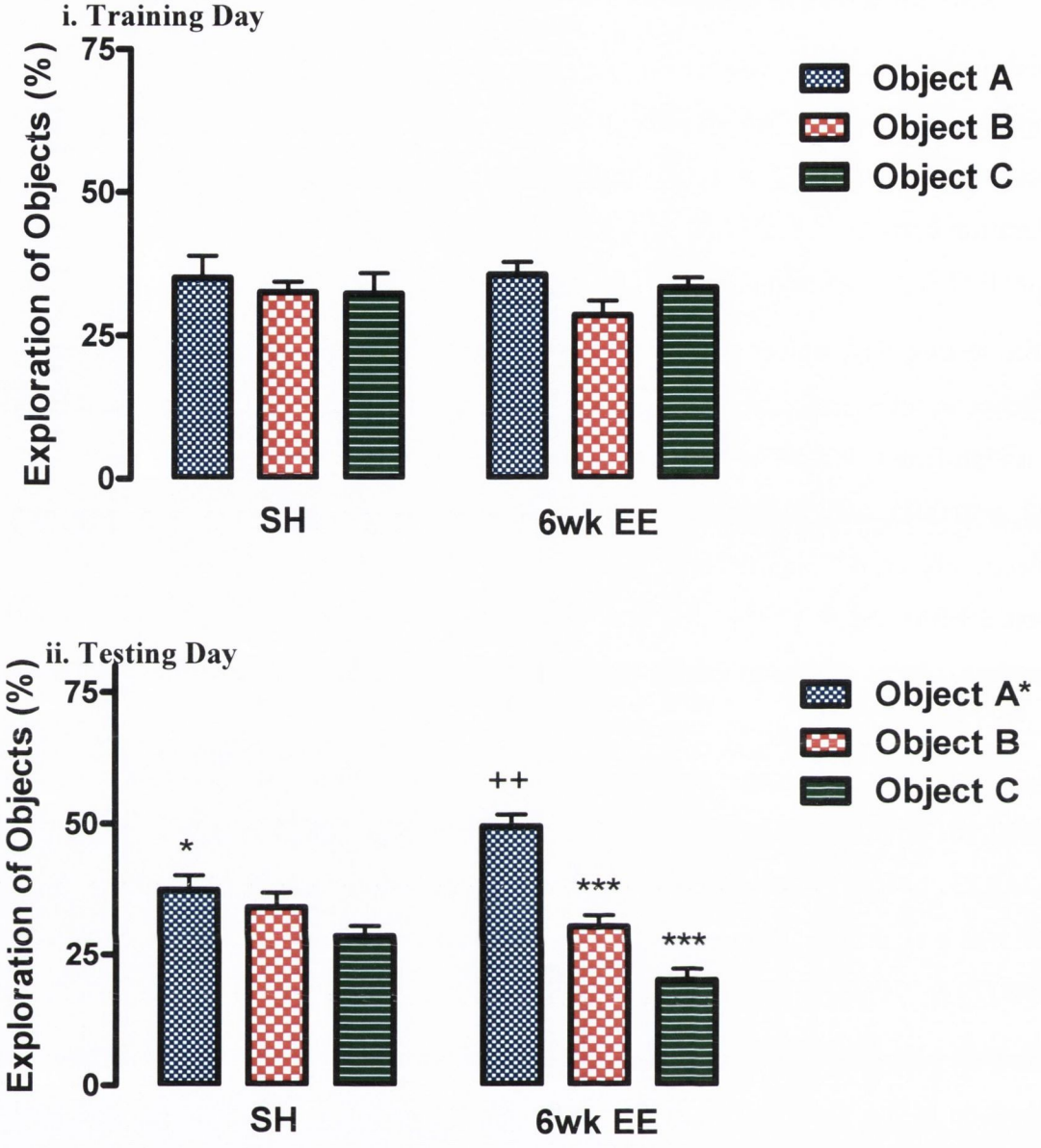


Figure 3e. Six weeks of environmental enrichment improves spatial memory(i) There was no significant difference between the exploration of objects on the training day of the OD task ($F_{2,63}$ = 1.597, p>0.05). (ii) On testing day, there was a significant difference in the exploration of the objects ($F_{2,63}$= 32.42, p<0.001), with the SH group exploring the displaced object A* more than object C (*p<0.05) and the 6wk EE group exploring object A* more than both object B and C (***p<0.001). There was also an interaction between the exploration of objects and housing conditions ($F_{2,63}$= 10.25, p<0.001) with the 6wk EE group exploring object A* significantly more than the SH group ($^{++}$p<0.01). SH: n=11, 6wk EE: n=12. Data expressed as mean ± SEM.

3.3.4 Six weeks of environmental enrichment improves working memory

Working memory in SH and 6wk EE groups was tested using the T maze task (figure 3e). There was a significant effect of time on this task ($F_{3,63}$ = 4.995, $p<0.01$). Post-hoc repeated measures ANOVA revealed that there was a significant increase in the percentage of correct arm entries between days 1-2 and 7-8 in the SH group (mean percentage entries ± SEM: days 1-2 [59.09±3.63] vs days 3-4 [63.64±3.23], $p>0.05$; days 3-4 vs days 5-6 [66.67±2.75], $p>0.05$; days 5-6 vs days 7-8 [75.00±2.51], $p>0.05$; days 1-2 vs days 7-8, $p<0.05$) and that there was a significant increase in the percentage of correct arm entries across the grouped days in the 6wk EE group (mean percentage entries ± SEM: days 1-2 [50.69±3.47] vs days 3-4 [72.22±3.60] $p<0.001$; days 3-4 vs days 5-6 [75.69±1.91], $p>0.05$; days 5-6 vs days 7-8 [87.50±1.62], $p<0.001$). There was a significant effect of housing conditions ($F_{1,63}$ = 7.520, $p<0.05$) and a significant interaction ($F_{3,63}$ = 4.995, $p<0.01$). Bonferroni post-tests revealed a significant increase in the percentage of correct arm entries in the 6wk EE group when compared with the SH group on days 7-8 (mean difference = 12.50, $p<0.05$).

These data show that rats housed in an enriched environment, in the absence of exercise, for six weeks show faster learning of the T maze task, indicative of a significant improvement in spatial working memory.

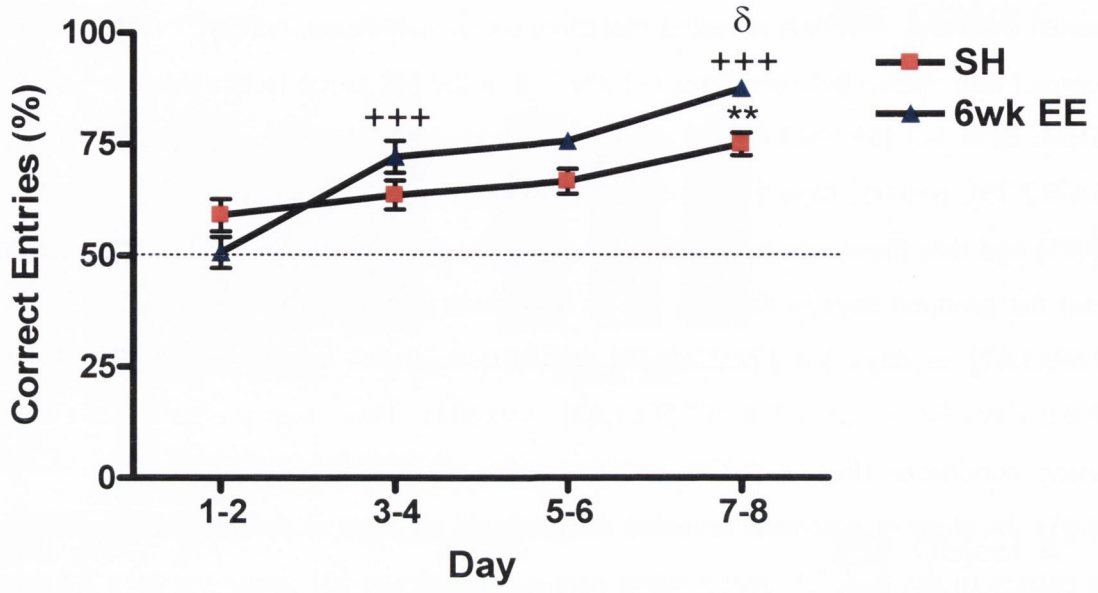


Figure 3f. Six weeks of environmental enrichment improves working memory. There was a significant increase in the percentage of correct arm entries over time ($F_{3,63} = 4.995$, $p<0.01$). In the SH group, there was a significant increase in the percentage of correct arm entries between days 1-2 and days 7-8 (**$p<0.01$). In the 6wk EE group, there was a significant increase in the percentage of correct arm entries between days 1-2 and days 3-4 ($^{+++}p<0.001$) and between days 5-6 and days 7-8 ($^{+++}p<0.001$). There was a significant interaction ($F_{3,63} = 4.995$, $p<0.01$), with a significant difference in the percentage of correct entries between the SH and 6wk EE group at days 7-8 ($^{\delta}p<0.05$). SH: n=11, 6wk EE: n=12. Data expressed as mean ± SEM.

3.4.1 Environmental enrichment increases βNGF concentration in the dentate gyrus

There was a significant difference in the concentration of βNGF in the dentate gyrus across all groups ($F_{3,24}$ = 3.521, $p<0.05$, figure 3g.i). Tukey's multiple comparison test revealed that there was a significant increase in βNGF concentration in the 6wk EE group compared with the SH group ($p<0.05$). There was no significant difference in βNGF concentration between SH and 2wk EE groups, SH and 3wk EE groups or 2wk EE and 3wk EE groups. Mean βNGF concentration ± SEM ($pg.mg^{-1}$): SH = 14.57±1.42, 2wk EE = 20.84±3.72, 3wk EE = 24.80±3.38, 6wk EE = 31.51±5.35.

There was a significant difference in the concentration of βNGF in hippocampus across all groups ($F_{3,21}$ = 9.369, $p<0.001$, figure 3g.ii). Tukey's multiple comparison test revealed that there was a significant decrease in βNGF concentration in the 3wk EE and 6wk EE groups when compared with SH and 2wk EE groups (SH vs 3wk EE, $p<0.01$; SH vs 6wk EE, $p<0.01$; 2wk EE vs 3wk EE, $p<0.05$; 2wk EE vs 6wk EE, $p<0.05$). There was no significant difference between SH and 2wk EE groups or between 3wk EE and 6wk EE groups. Mean βNGF concentration ± SEM ($pg.mg^{-1}$): SH = 20.14±0.90, 2wk EE = 17.41±2.72, 3wk EE = 9.71±1.42, 6wk EE = 8.24±3.08.

There was no significant difference in the concentration of βNGF in the perirhinal cortex across all groups ($F_{3,17}$ = 1.686, $p>0.05$, figure 3g.iii). Mean βNGF concentration ± SEM ($pg.mg^{-1}$): SH = 31.79±4.26, 2wk EE = 18.76±2.14, 3wk EE = 40.78±9.06, 6wk EE = 30.10±14.96. These data suggest that changes in βNGF concentration may be regionally specific to the hippocampus and not in associated structures such as the perirhinal cortex.

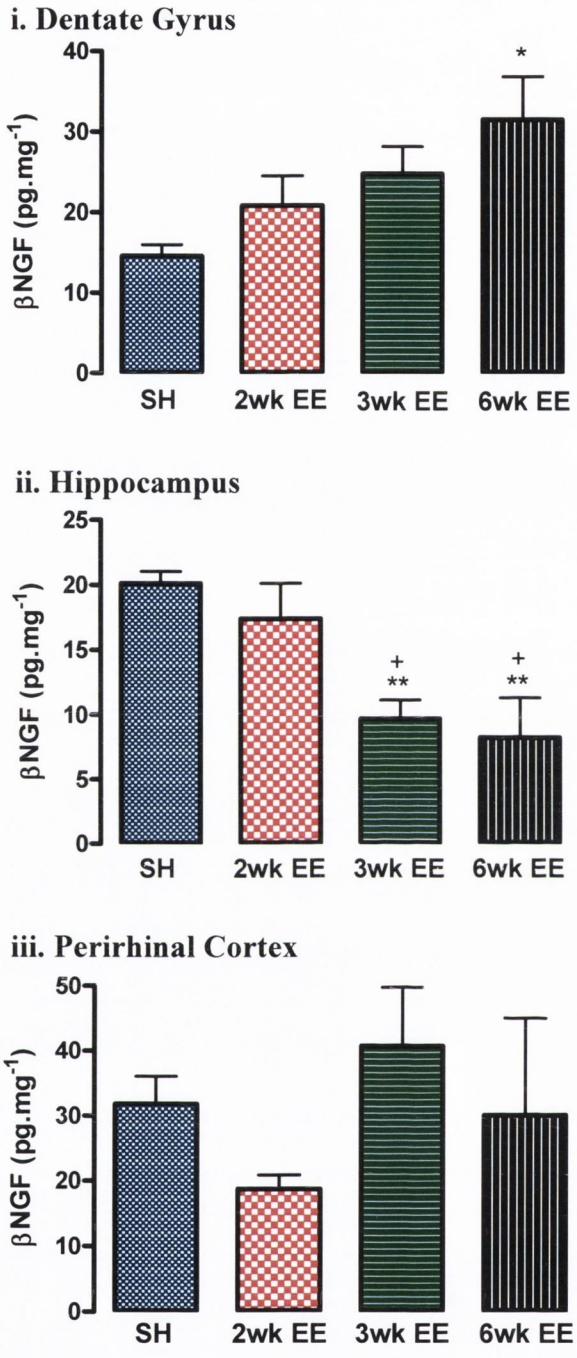


Figure 3g. Six weeks of environmental enrichment increases the concentration of βNGF in the dentate gyrus.(i) There was a significant difference in the concentration of βNGF in the dentate gyrus across all groups ($F_{3,24} = 3.521$, $p<0.05$), with a significant increase in βNGF in the 6wk EE group when compared with the SH group (*$p<0.05$). SH: n=6, 2wk EE: n=6, 3wk EE: n=7, 6wk EE: n=6 (ii) There was a significant difference in the concentration of βNGF in hippocampus across all groups ($F_{3,21} = 9.369$, $p<0.001$), with a significant decrease in βNGF between the SH and 3wk EE group (**$p<0.01$) and between the SH and 6wk EE group (**$p<0.01$). There was a significant decrease between the 2wk EE and 3wk EE group ($^{+}p<0.05$) and between the 2wk EE and 6wk EE group ($^{+}p<0.05$). SH: n=8; 2wk EE: n=6; 3wk EE, n=7; 6wk EE, n=4 (iii) There was no significant difference in the concentration of βNGF in the perirhinal cortex across all groups ($F_{3,17} = 1.686$, $p>0.05$). SH: n=5, 2wk EE: n=6, 3wk EE: n=7, 6wk EE: n=3.Data expressed as mean ± SEM.

3.4.2 Environmental enrichment induces a time-dependent decrease in BDNF concentration in the dentate gyrus, hippocampus and perirhinal cortex

There was a significant difference in the concentration of BDNF in the dentate gyrus across all groups ($F_{3,26}$ = 5.951, $p<0.01$, figure 3h.i). Tukey's multiple comparison test revealed that there was a significant decrease in BDNF concentration in the 2wk EE and 3wk EE groups when compared with the 6wk EE group (2wk EE vs 6wk EE, $p<0.01$; 3wk EE vs 6wk EE, $p<0.05$). There was no significant difference in BDNF concentration between SH and 2wk EE, 3wk EE or 6wk EE groups and no significant difference between 2wk EE and 3wk EE groups. Mean BDNF concentration ± SEM ($pg.mg^{-1}$): SH = 277.6±40.49, 2wk EE = 108.0±20.18, 3wk EE = 189.0±34.45, 6wk EE = 377.7±54.68.

There was a significant difference in the concentration of BDNF in hippocampus across all groups ($F_{3,20}$ = 11.26, $p<0.001$, figure 3h.ii). Tukey's multiple comparison test revealed that there was a significant decrease in BDNF concentration in the 3wk EE group when compared with the SH group ($p<0.05$) and when compared with the 6wk EE group ($p<0.001$). There was also a significant decrease in BDNF concentration the 2wk EE group when compared with the 6wk EE group ($p<0.01$). There was no significant difference in BDNF concentration between the SH and 6wk EE or between the 2wk EE and 3wk EE group. Mean BDNF concentration ± SEM ($pg.mg^{-1}$): SH = 138.7±13.81, 2wk EE = 83.38±12.47, 3wk EE = 68.40±3.91, 6wk EE = 188.3±26.58.

There was a significant difference in the concentration of BDNF in the perirhinal cortex across all groups ($F_{3,20}$ = 9.369, $p<0.001$, figure 3h.iii). Tukey's multiple comparison test revealed that there was a significant decrease in BDNF concentration in the 2wk EE and 3wk EE groups when compared with the SH and 6wk EE groups (SH vs 2wk EE, $p<0.05$; SH vs 3wk EE, $p<0.01$; 6wk EE vs 2wk EE, $p<0.05$; 6wk EE vs 3wk EE, $p<0.01$). There was no significant difference in BDNF concentration between the SH and 6wk EE groups or 2wk EE and 3wk EE groups. Mean BDNF concentration ± SEM ($pg.mg^{-1}$): SH = 120.1±14.55, 2wk EE = 51.58±14.53, 3wk EE = 34.73±8.60, 6wk EE = 112.1±16.88.

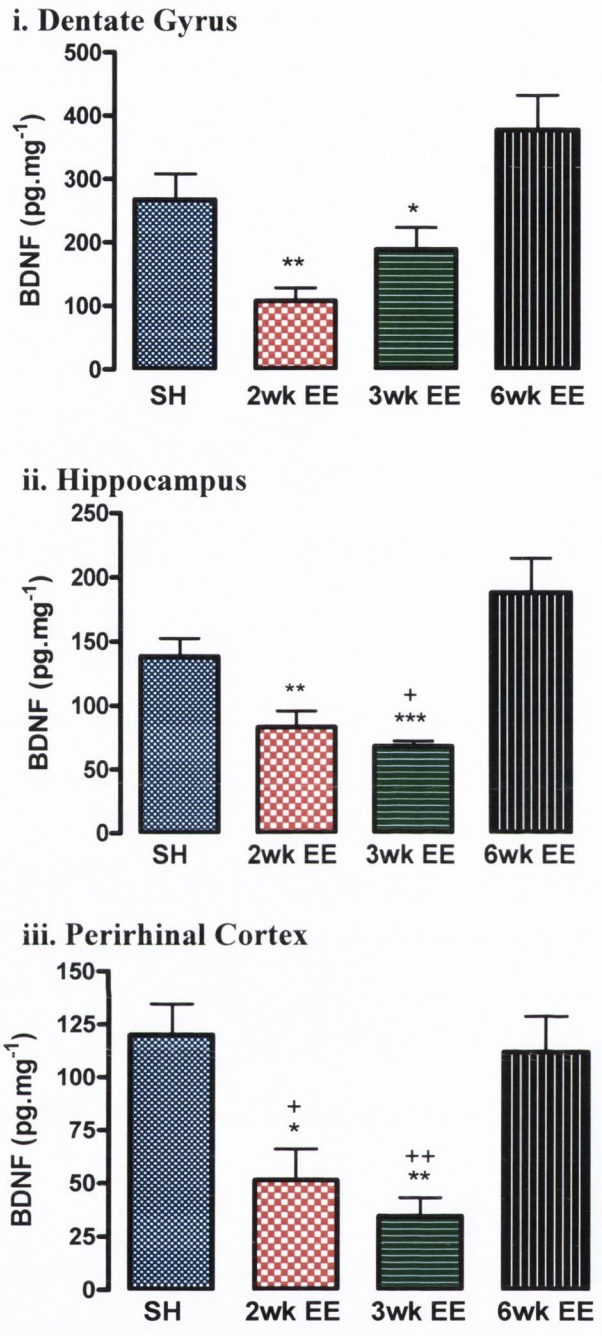


Figure 3h. Two and three weeks of environmental enrichment decrease the concentration of BDNF in the dentate gyrus, hippocampus and perirhinal cortex. (i) There was a significant difference in the concentration of BDNF in the dentate gyrus across all groups ($F3,23 = 3.521$, $p<0.05$), with a significant decrease in BDNF between the 2wk EE group and 6wk EE group ($p<0.01$[**]) and between the 3wk EE group and 6wk EE group (*$p<0.05$). SH: n=12, 2wk EE: n=6, 3wk EE: n=6, 6wk EE, n=6 (ii) There was a significant difference in the concentration of BDNF in the hippocampus across all groups ($F_{3,20} = 11.26$, $p<0.001$), with a significant decrease in BDNF between the 2wk EE and 6wk EE groups (**$p<0.0$), between the 3wk EE and 6wk EE groups (***$p<0.001$) and between the SH and 3wk EE groups ($^{+}p<0.05$). n=6 in all groups (iii) There was a significant difference in the concentration of BDNF in the perirhinal cortex across all groups ($F_{3,20} = 9.369$, $p<0.001$), with a significant decrease in BDNF between the 2wk EE and SH groups (*$p < 0.05$), between the 3wk EE and SH groups (**$p<0.01$). There is a significant decrease in BDNF between the 6wk EE and 2wk EE groups ($^{+}p<0.05$) and between the 6wk EE and 3wk EE groups ($^{+}p<0.01$). n=6 in all groups.Data expressed as mean ± SEM.

3.4.3 Two weeks of environmental enrichment upregulates βNGF mRNA expression in the hippocampus

There was no significant difference in the expression of βNGF mRNA in the dentate gyrus across all groups ($F_{3,24}$ = 2.343, $p>0.05$, figure 3i.i). Mean fold change ± SEM: SH = 1.00±0.11, 2wk EE = 0.99±0.13, 3wk EE = 1.13±0.15, 6wk EE = 1.43±0.11. Although it is not significant, these data appear to mirror the pattern of increases in NGF protein in the dentate gyrus with increasing time spent in an enriched environment.

There was a significant difference in the expression of βNGF mRNA in the hippocampus across all groups ($F_{3,25}$ = 3.507, $p<0.05$, figure 3i.ii). Tukey's multiple comparison test revealed a significant increase in the fold change of βNGF mRNA in the 2wk EE group when compared with the 6wk EE group ($p<0.05$). There was no significant difference in the fold change of βNGF mRNA between all other groups. Mean fold change ± SEM : SH = 1.00±0.18, 2wk EE = 1.94±0.55, 3wk EE = 1.04±0.19, 6wk EE = 0.81±0.15.

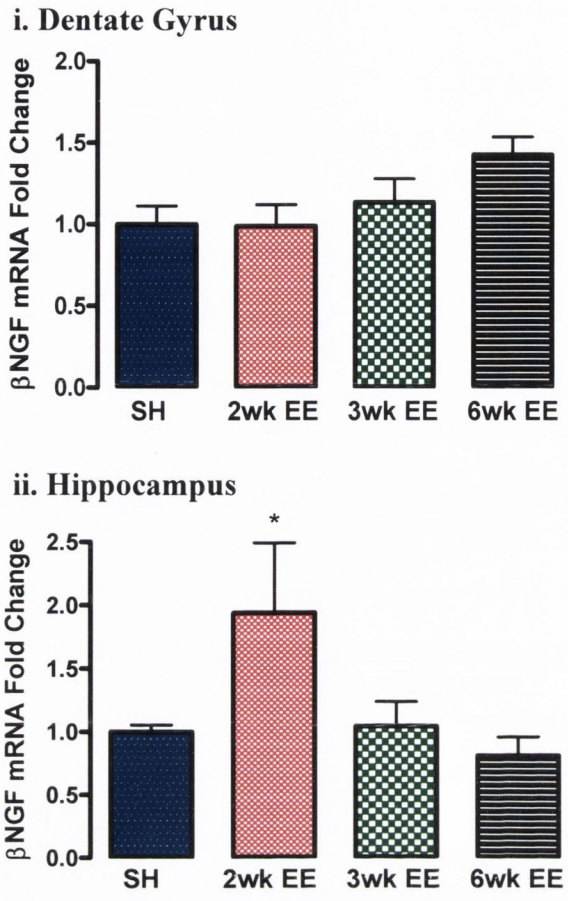


Figure 3i. Two weeks of environmental enrichment upregulates βNGF mRNA expression in the hippocampus.(i) There was no significant difference in the fold change ofβNGF mRNA in the dentate gyrus across all groups ($F_{3,24} = 2.343$, $p>0.05$). SH: n=11, 2wk EE: n=5, 3wk EE: n=6, 6wk EE: n=6 (ii) There was a significant difference in the expression of βNGF mRNA in the hippocampus across all groups ($F_{3,25} = 3.507$, $p<0.05$), with a significant increase in the fold change of βNGF mRNA in the 2wk EE compared to the 6wk EE group (*$p<0.05$). SH: n=11, 2wk EE: n=6, 3wk EE: n=6, 6wk EE: n=6. Data expressed as mean ± SEM.

3.4.4 Environmental enrichment does not affect BDNF mRNA expression in the dentate gyrus or hippocampus

There was no significant difference in the expression of BDNF mRNA in the dentate gyrus across all groups ($F_{3,23}$ = 0.7956, p>0.05, figure 3j.i). Mean fold change ± SEM: SH = 1.00±0.23, 2wk EE = 1.51±0.32, 3wk EE = 1.06±0.32, 6wk EE = 1.42±0.37.

There was no significant difference in the expression of BDNF mRNA in the hippocampus across all groups ($F_{3,23}$ = 0.5647, p>0.05, figure 3j.ii). Mean fold change ± SEM: SH = 1.00±0.76, 2wk EE = 0.65±0.17, 3wk EE = 0.70±0.13, 6wk EE = 0.90±0.29.

3.4.5 Environmental enrichment does not affect Trk A mRNA expression in the dentate gyrus or hippocampus

There was no significant difference in the expression of Trk A mRNA in the dentate gyrus across all groups ($F_{3,21}$ = 0.6426, p>0.05, figure 3k.i). Mean fold change ± SEM: SH = 1.00±0.70, 2wk EE = 1.06±0.40, 3wk EE = 1.60±0.45, 6wk EE = 1.32±0.68. There was no significant difference in the expression of Trk A mRNA in the hippocampus across all groups ($F_{3,25}$ = 2.874, p>0.05, figure 3k.ii). Mean fold change ± SEM: SH = 1.00±0.18, 2wk EE = 1.25±0.40, 3wk EE = 1.11±0.33, 6wk EE = 0.19±0.08.

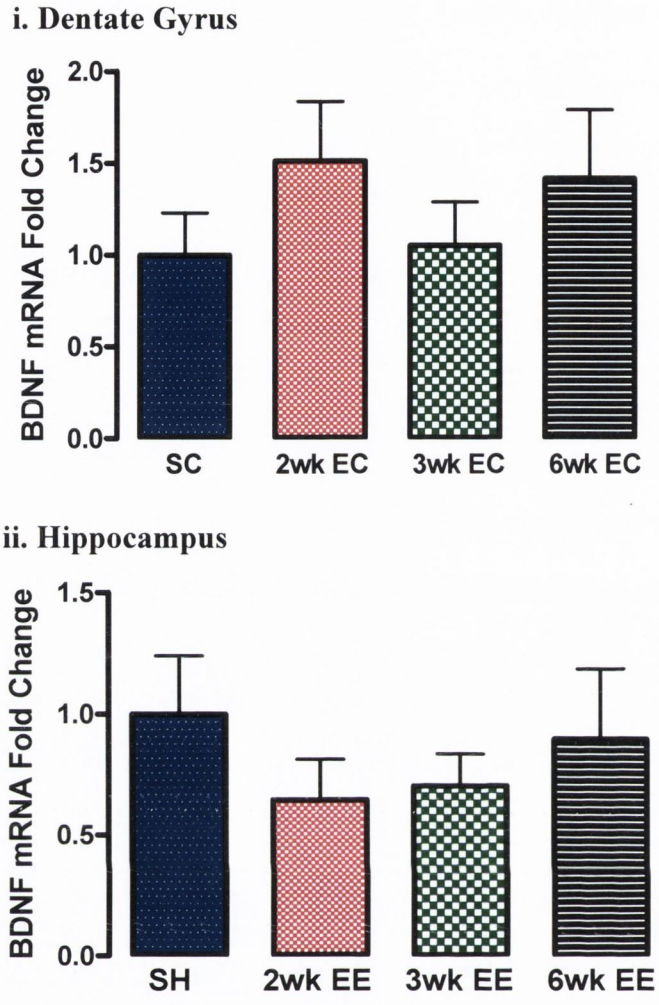


Figure 3j. There is no difference in the expression of BDNF mRNA in the dentate gyrus or hippocampus with two, three or six weeks of environmental enrichment. (i) There was no significant difference in the expression of BDNF mRNA in the dentate gyrus across all groups ($F_{3,23} = 0.7956$, $p>0.05$). SH: n=11, 2wk EE: n=5, 3wk EE: n=6, 6wk EE: n=5. (ii) There was no significant difference in the expression of BDNF mRNA in the hippocampus across all groups ($F_{3,23} = 0.5647$, $p>0.05$). SH: n=10, 2wk EE: n=6, 3wk EE: n=6, 6wk EE: n=5. Data expressed as mean ± SEM.

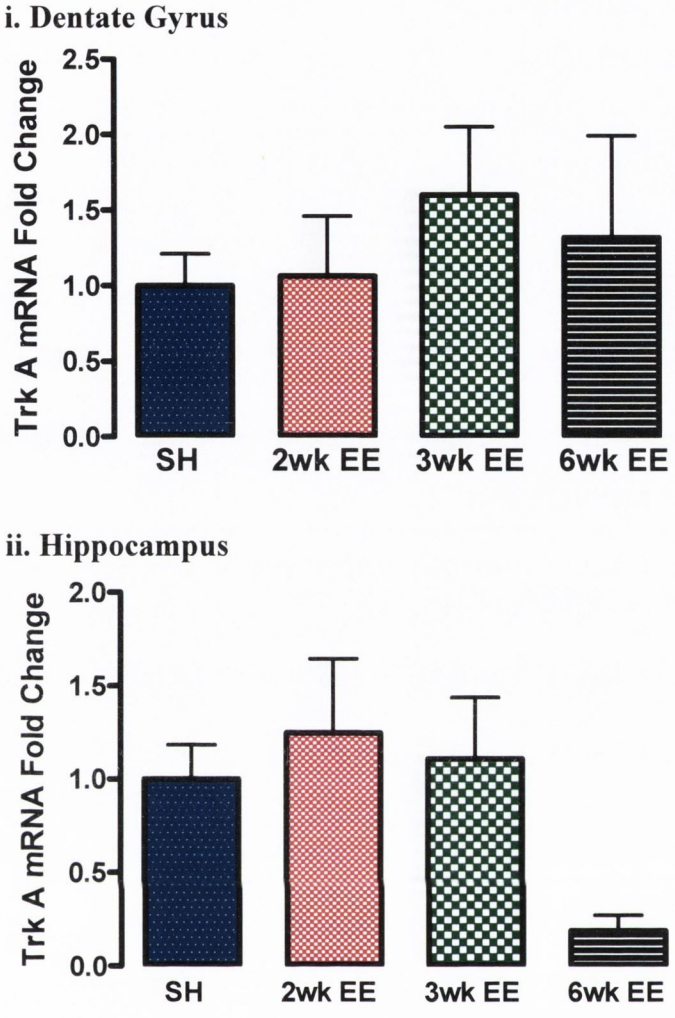


Figure 3k. There is no difference in the expression of Trk A mRNA in the dentate gyrus or hippocampus with two, three or six weeks of environmental enrichment.(i) There was no significant difference in the expression of Trk A mRNA in the dentate gyrus across all groups ($F_{3,21}$ = 0.6426, $p>0.05$). SH: n=11, 2wk EE: n=5, 3wk EE: n=6, 6wk EE: n=3. (ii) There was no significant difference in the expression of Trk A mRNA in the hippocampus across all groups ($F_{3,25}$ = 2.874, $p>0.05$). SH: n=11, 2wk EE: n=6, 3wk EE: n=6, 6wk EE: n=6. Data expressed as mean ± SEM.

3.4.6 Six weeks of environmental enrichment decreases Trk B receptor expression in the dentate gyrus but not in the perirhinal cortex

There was a significant decrease in Trk B receptor expression in the dentate gyrus after six weeks of enrichment (t_{10} = 2.28, $p<0.05$, figure 3l.i). Mean Trk B expression per βActin expression ± SEM: SH = 0.88±0.20, 6wk EE = 0.41±0.06.

There was no difference in Trk B receptor expression in the perirhinal cortex after six weeks of enrichment (t_9 = 1.06, $p>0.05$, figure 3l.ii). Mean Trk B expression per βActin expression ± SEM: SH = 0.59±0.14, 6wk EE = 0.37±0.15.

3.4.7 Environmental enrichment does not affect Trk B mRNA expression in the dentate gyrus or hippocampus

There was no significant difference in the expression of Trk B mRNA in the dentate gyrus across all groups ($F_{3,26}$ = 0.3218, $p>0.05$, figure 3m.i). Mean fold change ± SEM: SH = 1.00±0.77, 2wk EE = 0.72±0.09, 3wk EE = 0.99±0.17, 6wk EE = 0.92±0.22.

There was no significant difference in the expression of Trk B mRNA in the hippocampus across all groups ($F_{3,26}$ = 0.3218, $p>0.05$, figure 3m.ii). Mean fold change ± SEM: SH = 1.00±0.22, 2wk EE = 0.72±0.09, 3wk EE = 0.99±0.17, 6wk EE = 0.92±0.22.

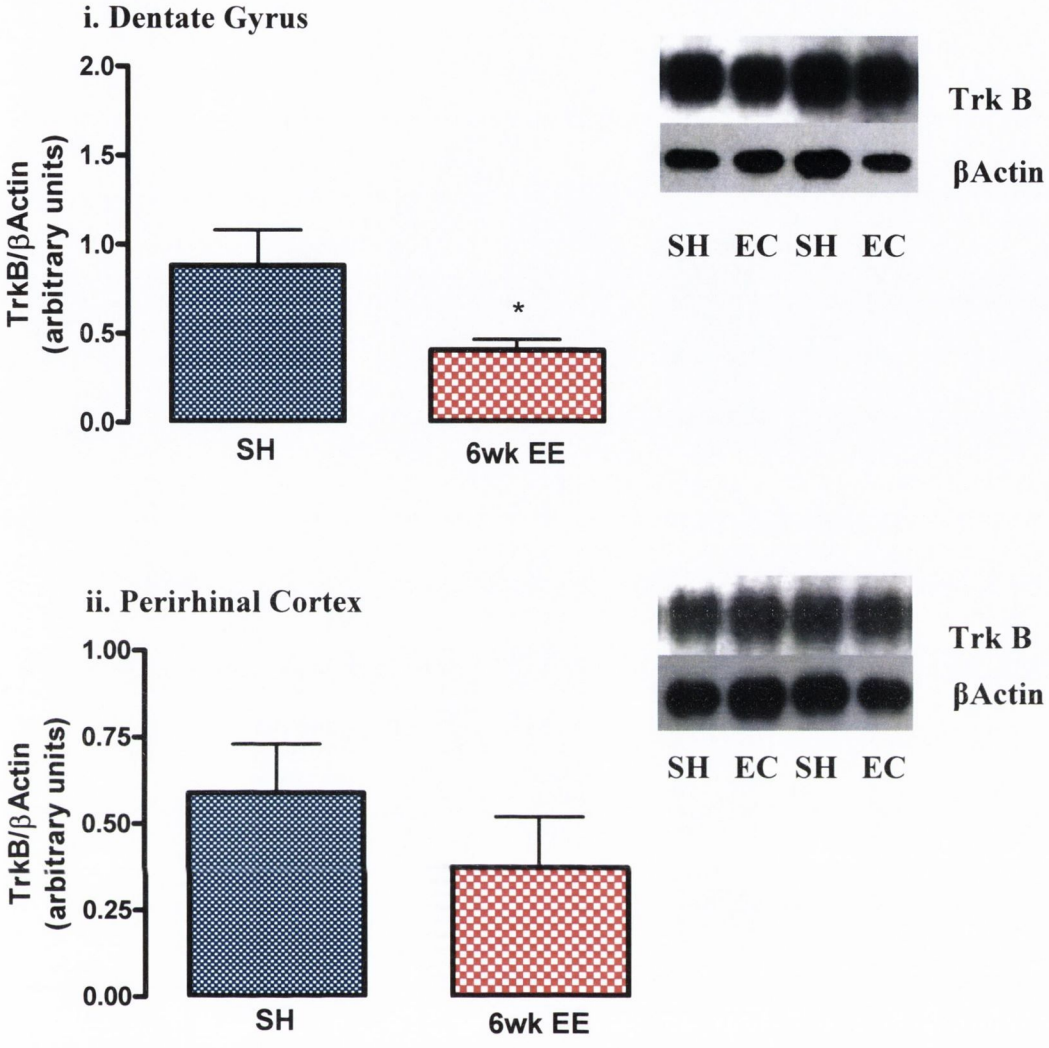


Figure 3l. Six weeks of environmental enrichment significantly decreases Trk B receptor expression in the dentate gyrus, but not in the perirhinal cortex.(i) There was a significant decrease in Trk B receptor per βActin expression in the 6wk EE group in the dentate gyrus: $t_{10} = 2.28$ (*$p<0.05$). n=6 in both groups. (ii) There was no significant difference between groups in Trk B receptor per βActin expression in the perirhinal cortex: $t_9 = 1.06$ ($p>0.05$). SH: n=6, 6wk EE: n=5. Data expressed as mean ± SEM.

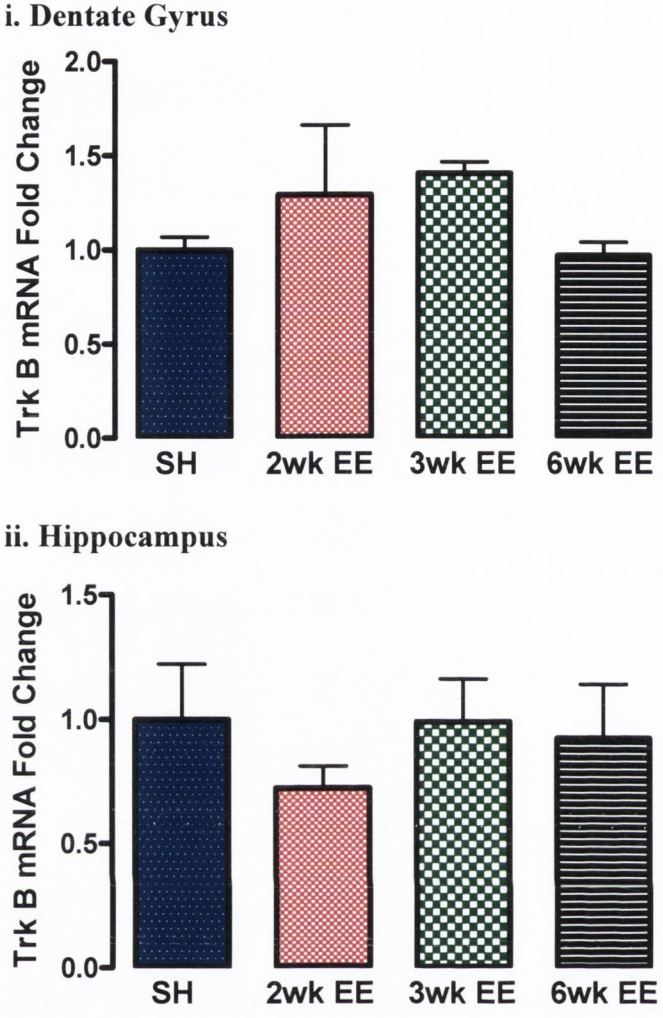


Figure 3m. There is no significant difference in the expression of Trk B in the dentate gyrus or hippocampus with two, three or six weeks of environmental enrichment. (i)There was no significant difference between groups in the expression of Trk B mRNA in the dentate gyrus ($F_{3,26}$ = 0.3218, $p>0.05$). SH: n=11, 2wk EE: n=5, 3wk EE: n=6, 6wk EE: n=6 (ii) There was no significant difference between groups in the expression of Trk B mRNA in the hippocampus ($F_{3,26}$ = 0.3218, $p>0.05$). SH: n=12, 2wk EE: n=6, 3wk EE: n=6, 6wk EE: n=6. Data expressed as mean ± SEM.

3.4.8 Six weeks of environmental enrichment does not affect ERK phosphorylation in the dentate gyrus, hippocampus or perirhinal cortex

There was no significant difference in the ratio of p42ERK to total ERK 42 protein expression in the dentate gyrus after six weeks of environmental enrichment ($t_{10} = 0.02$, $p>0.05$, figure 3n.i). Mean p42ERK expression per ERK 42 expression ± SEM: SH = 3.11±0.43, 6wk EE = 3.10±0.43.

There was no significant difference in the ratio of p42ERK to total ERK 42 protein expression in the hippocampus after six weeks of environmental enrichment ($t_{10} = 0.94$ $p>0.05$, figure 3n.ii). Mean p42ERK expression per ERK 42 expression ± SEM: SH = 1.67±0.81, 6wk EE = 0.81±0.41.

There was no significant difference in the ratio of p42ERK to total ERK 42 protein expression in the perirhinal cortex after six weeks of environmental enrichment ($t_8 = 1.46$, $p > 0.05$, figure 3n.iii). Mean p42ERK expression per ERK 42 expression ± SEM: SH = 7.87±3.28, 6wk EE = 20.09±9.26.

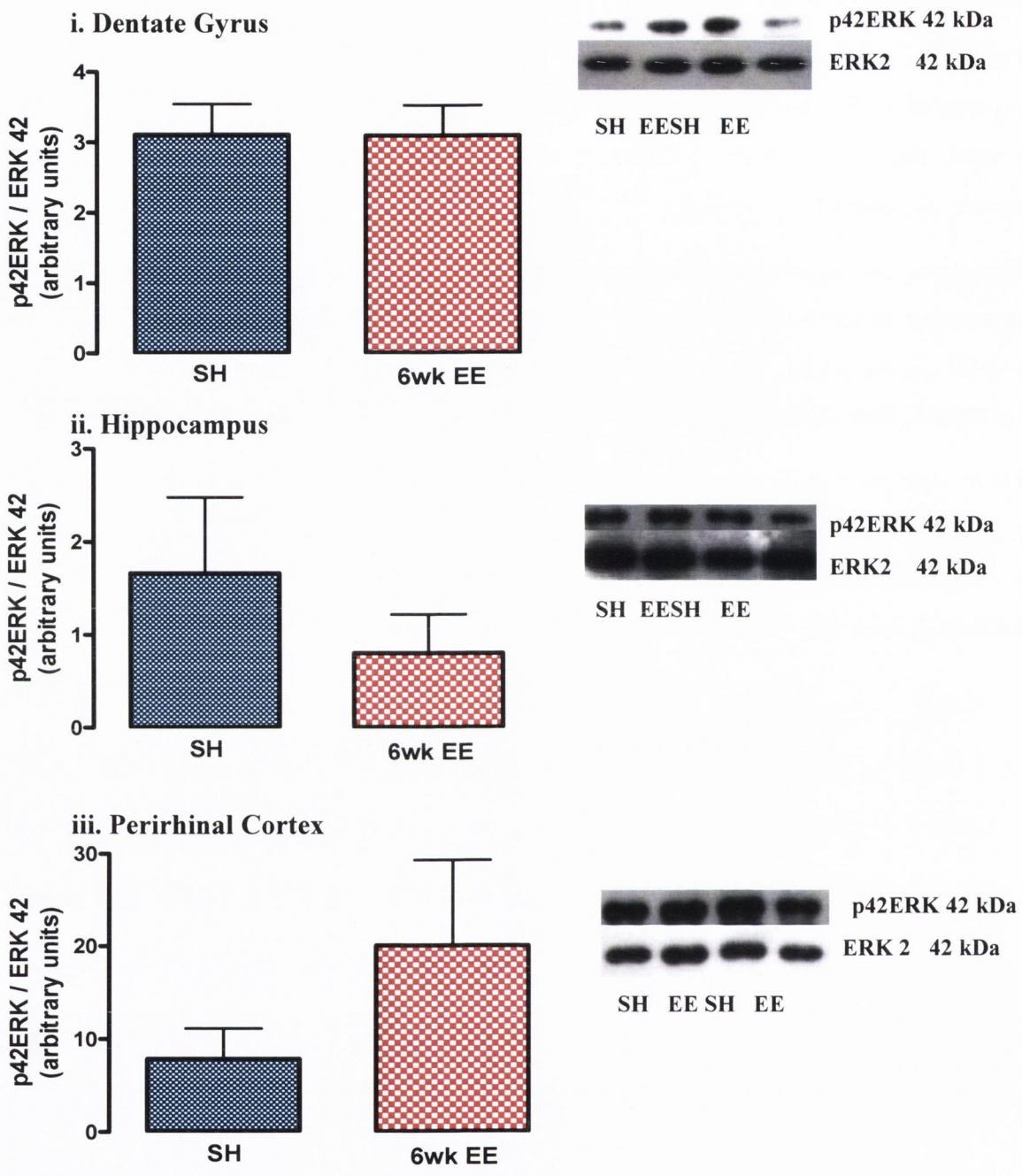


Figure 3n. There is no significant difference in p42ERK expression in the dentate gyrus, hippocampus or perirhinal cortex after six weeks of environmental enrichment. (i) p42ERK per ERK 42 expression in the dentate gyrus (t_{10} = 0.02, p>0.05). n=6 in both groups (ii) p42ERK per ERK 42 expression in the hippocampus (t_{10} = 0.94, p>0.05). n=6 in both groups (iii) p42ERK per ERK 42 expression in the perirhinal cortex (t_8 = 1.46, p>0.05). SH: n=6, 6wk EE: n=4.Data expressed as mean ± SEM.

3.4.9 Six weeks of environmental enrichment increases synaptophysin in the dentate gyrus

There was a significant increase in synaptophysin expression in the dentate gyrus after six weeks of environmental enrichment ($t_{10} = 2.629$, $p<0.05$; figure 3o.i). Mean synaptophysin per GAPDH expression ± SEM: SH = 2.37±0.20, 6wk EE = 4.28±0.63.

There was no significant increase in synaptophysin expression in the hippocampus after six weeks of environmental enrichment ($t_{10} = 0.185$, $p>0.05$; figure 3o.ii). Mean synaptophysin per GAPDH expression ± SEM: SH = 1.70±0.12, 6wk EE = 1.75±0.25.

There was no significant increase in synaptophysin expression in the perirhinal cortex after six weeks of environmental enrichment ($t_{10} = 0.104$, $p>0.05$; figure 3o.iii). Mean synaptophysin per GAPDH expression ± SEM: SH = 4.94±0.57, 6wk EE = 5.06±0.9.

3.4.10 Six weeks of environmental enrichment increases synapsin I in the dentate gyrus

There was a significant increase in synapsin I expression in the dentate gyrus after six weeks of environmental enrichment ($t_{10} = 2.578$, $p<0.05$; figure 3p.i). Mean synapsin I per GAPDH expression ± SEM: SH = 2.28±0.32, 6wk EE = 4.88±0.89.

There was no significant increase in synapsin I expression in the hippocampus after six weeks environmental enrichment ($t_{10} = 0.315$, $p>0.05$; figure 3p.ii). Mean synapsin I per GAPDH expression ± SEM: SH = 1.91±0.21, 6wk EE = 2.06±0.44.

There was no significant increase in synapsin I expression in the perirhinal cortex after six weeks environmental enrichment ($t_{10} = 1.289$, $p>0.05$; figure 3p.iii). Mean synapsin I per GAPDH expression ± SEM: SH = 9.43±1.28, 6wk EE = 7.44±0.71.

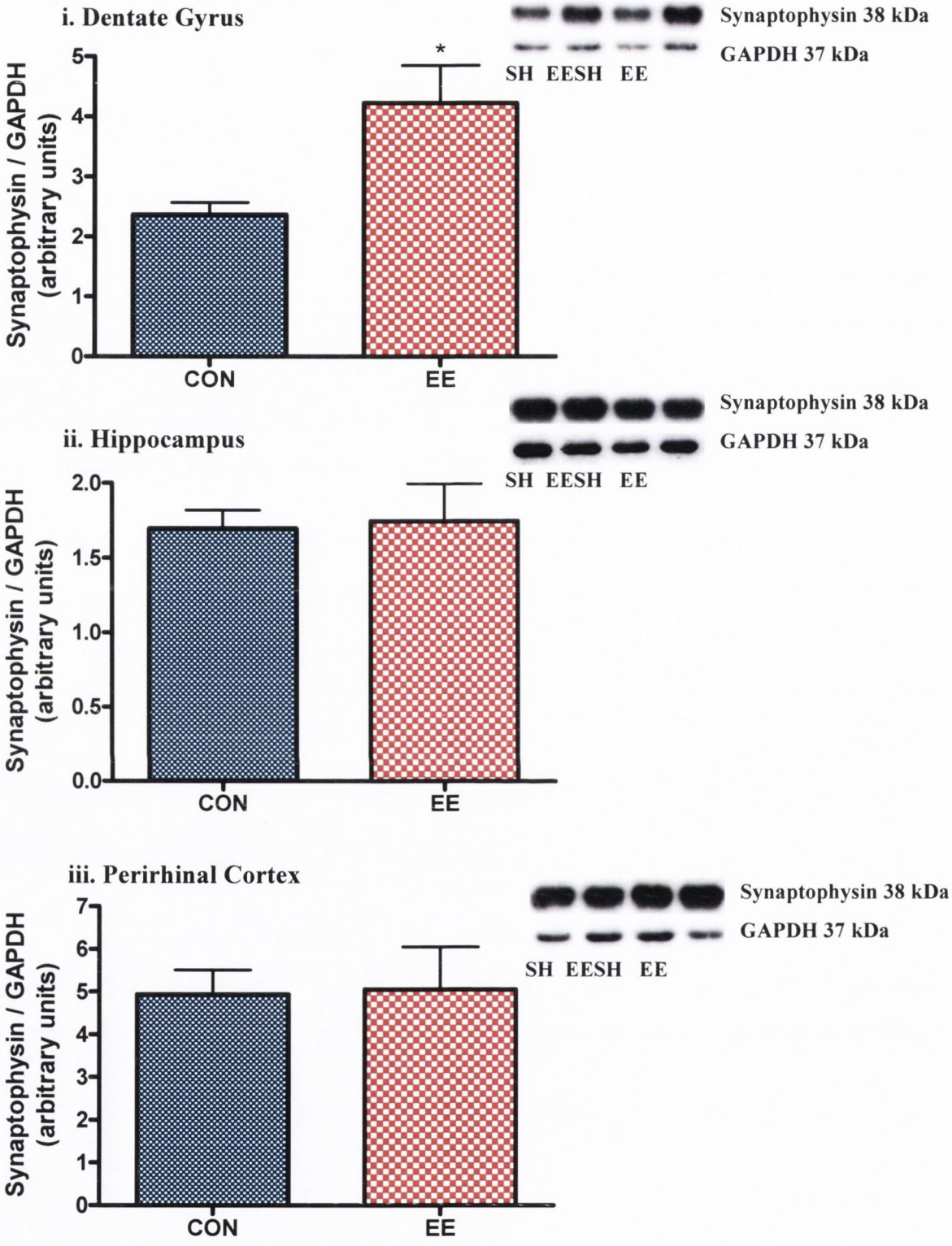


Figure 3o. There is a significant difference in synaptophysin expression in the dentate gyrus after six weeks of environmental enrichment, but not in the hippocampus or perirhinal cortex. (i) synaptophysin per GAPDH expression in the dentate gyrus ($t_{10} = 2.629$, *$p<0.05$). n=6 in both groups (ii) synaptophysin per GAPDH expression in the hippocampus ($t_{10} = 0.185$, $p>0.05$). n=6 in both groups (iii) synaptophysin per GAPDH expression in the perirhinal cortex ($t_{10} = 0.104$, $p>0.05$). n=6 in both groups. Data expressed as mean ± SEM.

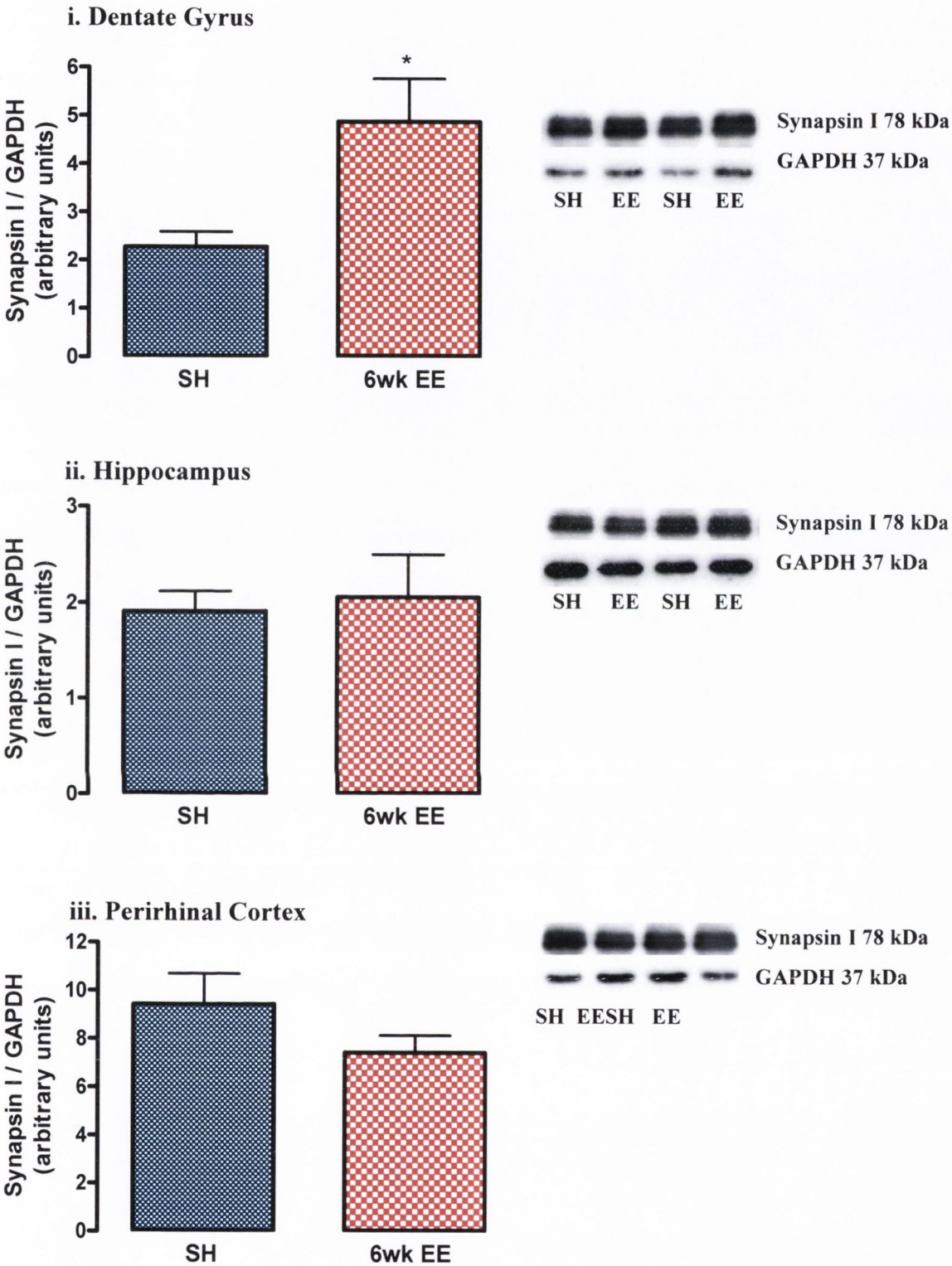


Figure 3p. There is a significant difference in synapsin I expression in the dentate gyrus after six weeks of environmental enrichment, but not in the hippocampus or perirhinal cortex. (i) synapsin I per GAPDH expression in the dentate gyrus (t_{10} = 2.578, *p<0.05). n=6 in both groups (ii) synaptophysin per GAPDH expression in the hippocampus (t_{10} = 0.315, p>0.05). n=6 in both groups (iii) synaptophysin per GAPDH expression in the perirhinal cortex (t_{10} = 1.298, p>0.05). n=6 in both groups. Data expressed as mean ± SEM.

3.5.1 Six weeks of environmental enrichment increases cell proliferation in the dentate gyrus

There was a significant difference in the number of BrdU+ nuclei across all groups injected at the end of their respective housing periods ($F_{3,20} = 9.352$, $p<0.001$, figure 3q). Tukey's multiple comparison test revealed that there was a significant increase in the number of BrdU+ nuclei in the 6 wk EE group when compared with the SH group ($p<0.001$) and compared with the 2wk EE ($p<0.01$). Mean percentage BrdU+ nuclei ± SEM: SH = 4.63±0.31, 2wk EE = 4.80±0.58, 3wk EE = 7.03±1.33, 6wk EE = 9.91±0.63.

3.5.2 Three and six weeks of environmental enrichment increase neuronal proliferation in the dentate gyrus

There was a significant difference between the BrdU+/NeuN+ labelling in the dentate gyrus across groups ($F_{2,9} = 4.609$, $p<0.05$, figure 3r). Tukey's multiple comparison test revealed that there was a significant increase in the percentage of BrdU+/NeuN+ nuclei in the 3wk EE group when compared with the SH group ($p<0.05$) and in the 6wk EE group when compared with the SH group ($p<0.05$). Mean percentage of BrdU+/NeuN+ nuclei ± SEM: SH = 48.61±4.23, 3wk EE = 57.49±1.27, 6wk EE = 58.82±0.76.

3.5.3 Six weeks of environmental enrichment does not affect long-term survival of proliferating cells in the dentate gyrus

There was no significant difference between groups in the number of BrdU+ nuclei, wheninjected with BrdU 6 weeks prior to sacrifice ($t_{10} = 0.1803$, $p>0.05$; figure 3s). Mean percentage BrdU+ nuclei ± SEM: SH = 5.36±0.45, EE = 5.488±0.53.

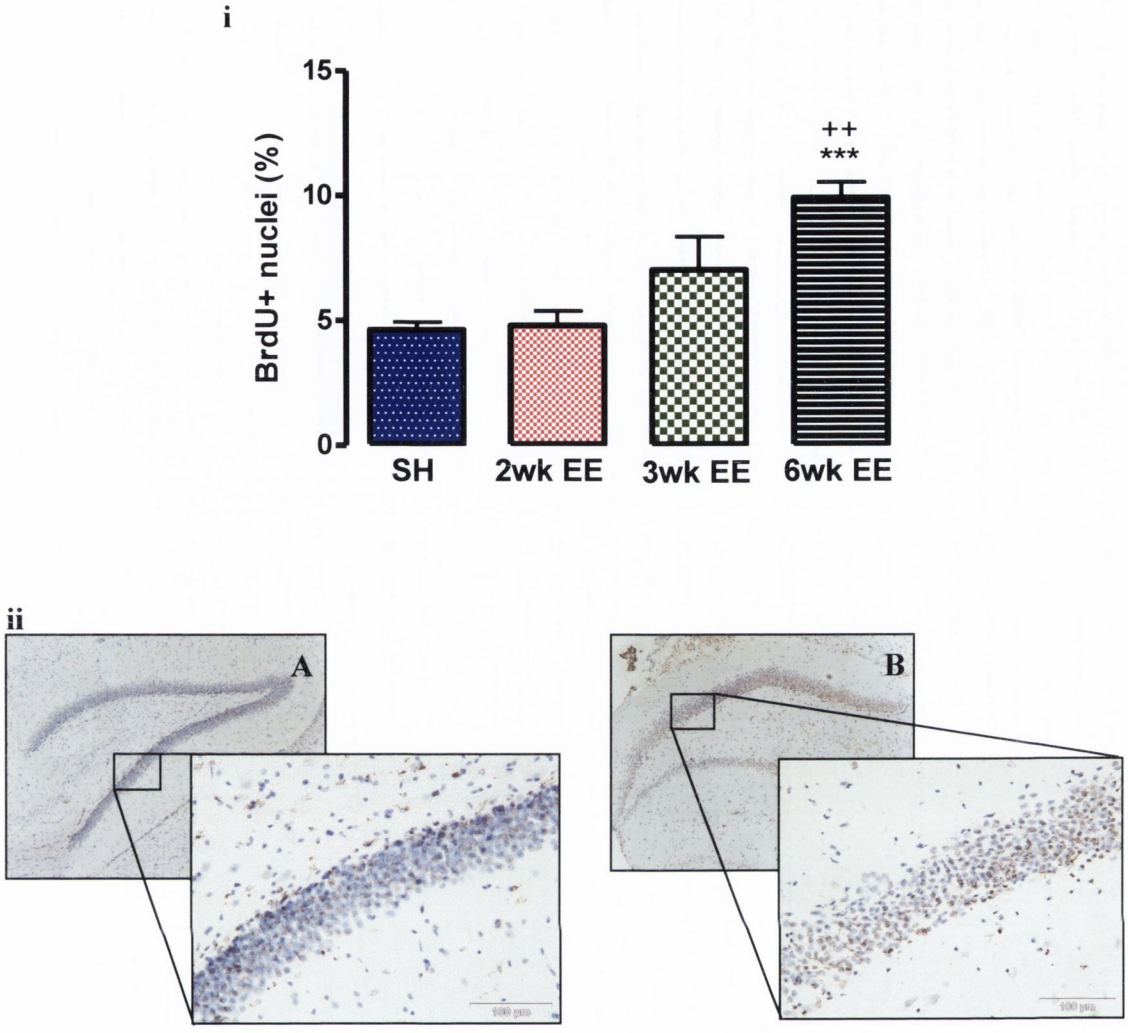


Figure 3q. Six weeks of environmental enrichment increases the number of BrdU+ nuclei in the dentate gyrus.(i) There was a difference in the number of BrdU+ nuclei across all groups ($F_{3,20}$ = 9.352, $p<0.001$), with a significant increase in BrdU+ nuclei between the 6wk EE and SH groups (***$p<0.001$) and between the 6wk EE and 2wk EE groups ($^{++}p<0.01$). SH: n=8, 2wk EE: n=5, 3wk EE: n=6, 6wk EE: n=5 (ii) Representative pictures of BrdU+ (brown) nuclei in SH (A) and 6wk EE groups (B) with hematoxylin (blue) staining of all nuclei. Data expressed as mean ± SEM.

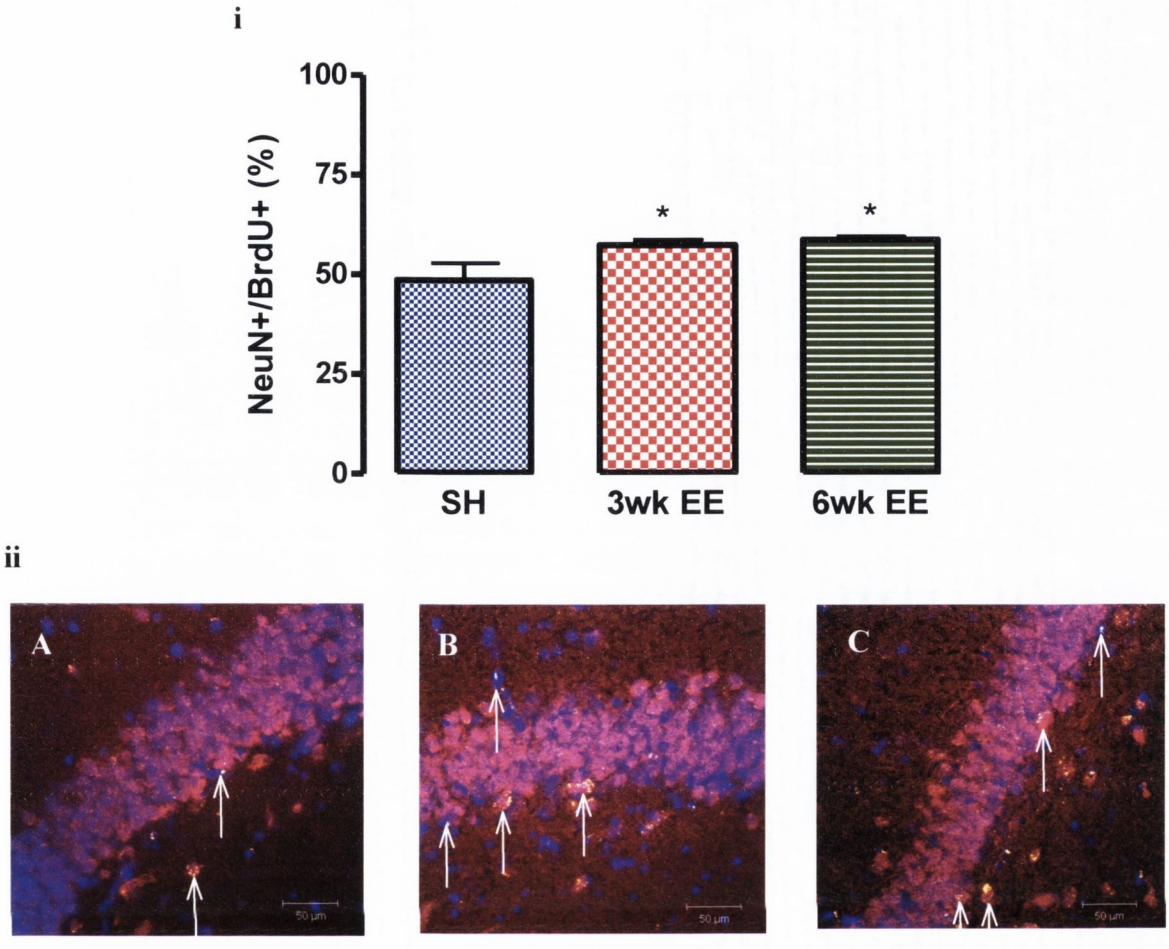


Figure 3r. Three and six weeks of environmental enrichment increase the percentage of BrdU+/NeuN+ nuclei in the dentate gyrus. (i) There was a significant difference between the BrdU+/NeuN+ labelling in the dentate gyrus across groups ($F_{2,9}$ = 4.609, $p<0.05$) with a significant increase in the percentage of BrdU+/NeuN+ nuclei in the 3wk EE group compared to the SH group (*$p<0.05$) and in the 6wk EE group compared to the SH group (*$p<0.05$). n=4 in all groups. (ii) Representative pictures of BrdU+ (yellow), NeuN+ (red) and all nuclei (blue) staining in SH (A), 3wk EE (B) and 6wk EE (C) groups. Data calculated as percentage of colocalised NeuN+/BrdU+ nuclei per total number of Brdu+ nuclei counted, and expressed **as** mean ± SEM.

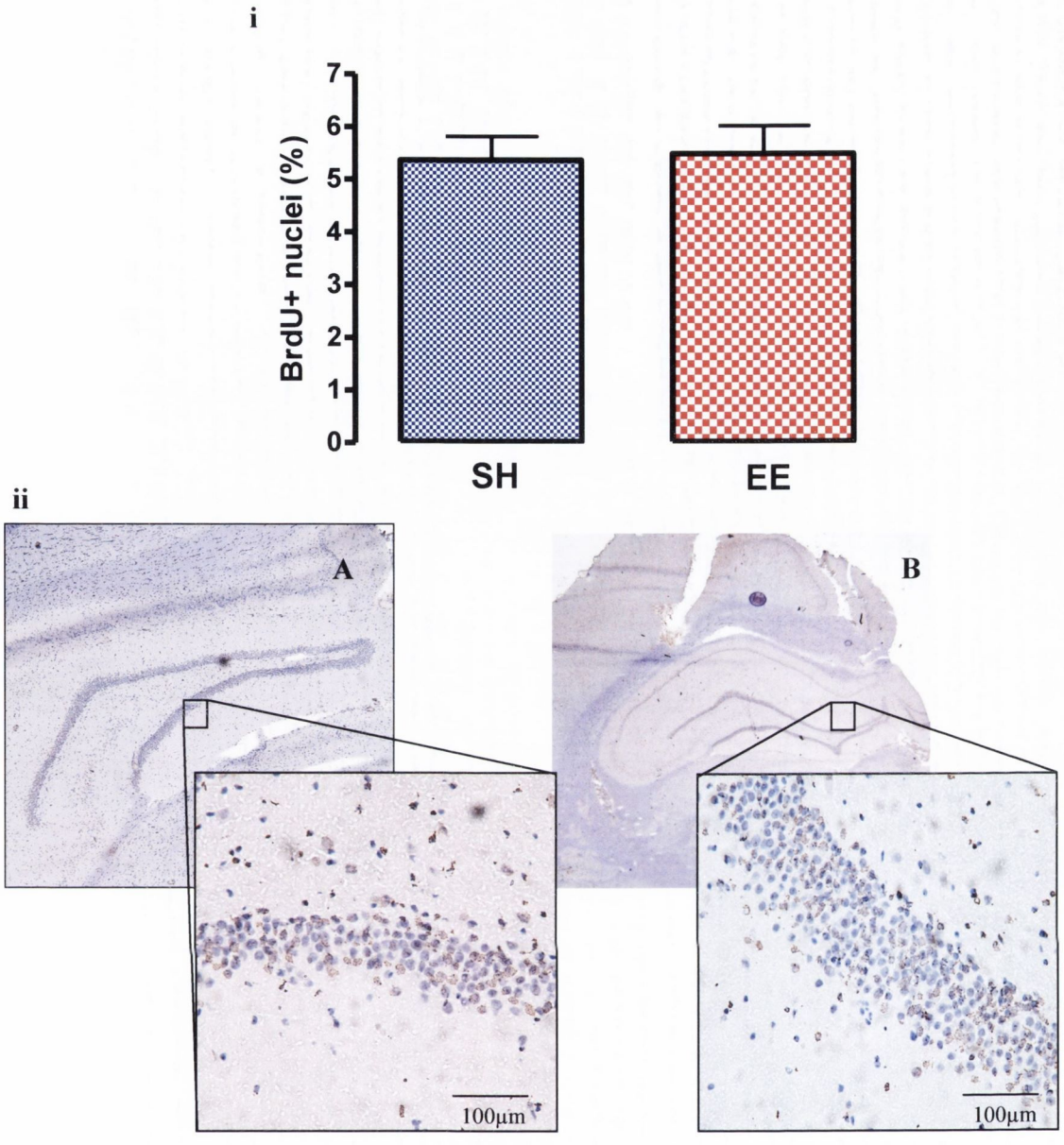


Figure 3s. Six weeks of environmental enrichment does not affect long-term survival of proliferating cells in the dentate gyrus(i) There was no significant difference in the number of BrdU+ nuclei between groups when injected with BrdU 6 weeks before sacrifice (t_{10} = 0.1803, $p>0.05$). SH: n=6, EE: n=6 (ii) Representative pictures of BrdU+ (brown) nuclei in SH (A) and 6wk EE groups (B) with hematoxylin (blue) staining of all nuclei. Data expressed as mean ± SEM

To further analyse the link between the cognitive enhancement seen after three weeks of environmental enrichment and the neurochemical and immunohistochemical results, the rats' performance on the 2 object NOR for all groups was correlated with βNGF concentration in the dentate gyrus and with the BrdU+ nuclei percentages.

3.6.1 βNGF concentration in the dentate gyrus positively correlates with performance on the 2 object NOR task

There was a significant positive correlation between the exploration of the novel object in the 2 object NOR task and the concentration of βNGF in dentate gyrus of rats ($r = 0.461$, $p<0.05$, $N = 25$, figure 3t). This suggests that an increase of βNGF in the dentate gyrus may play a role in the improvement of object recognition memory in rats.

3.6.2 The percentage of BrdU+ nuclei in the dentate gyrus positively correlates with performance on the 2 object NOR task

There was a significant positive correlation between the exploration of the novel object in the two object NOR task and the percentage of BrdU+ nuclei in the dentate gyrus of rats ($r = 0.733$, $p<0.001$, $N = 21$, figure 3u). This suggests that an increase in proliferation in the dentate gyrus may play a role in the improvement of object recognition memory in rats.

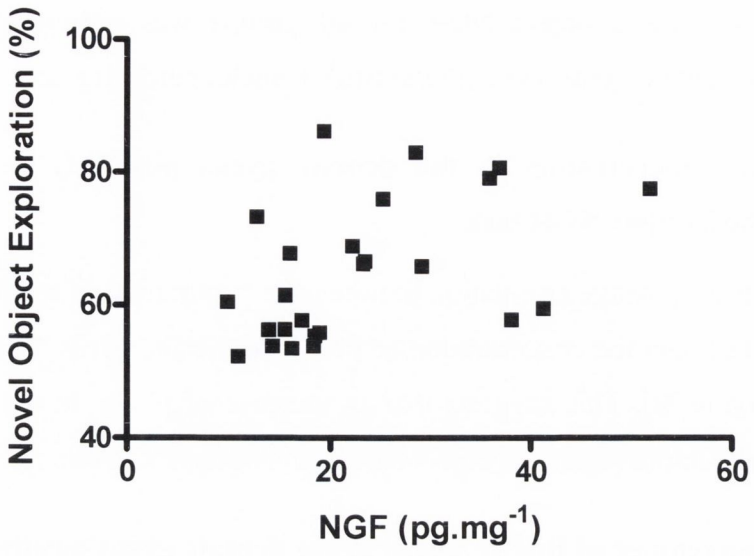


Figure 3t. There is a positive correlation between the percentage exploration of the novel object and the concentration of NGF in the dentate gyrus. There was a significant correlation across all groups between the concentration of NGF in the dentate gyrus and percentage exploration of the novel object in the two object NOR task ($r=0.461$, $p<0.05$). N=25.

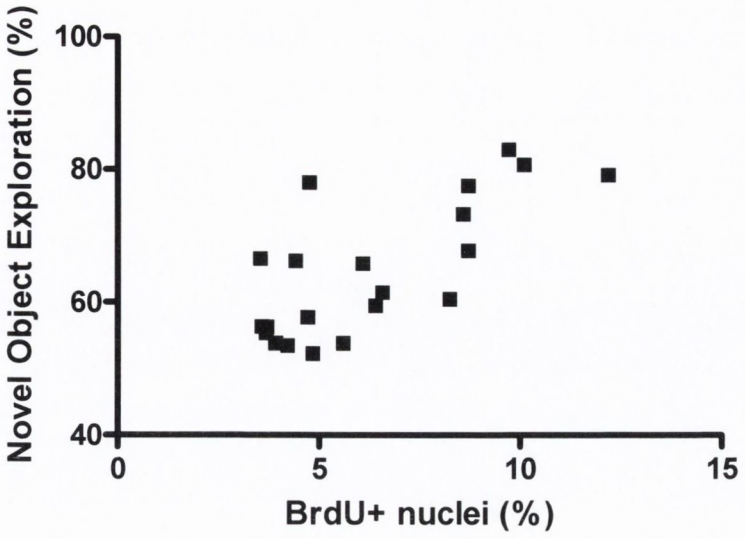


Figure 3u. There is a positive correlation between the percentage exploration of the novel object and the percentage of BrdU+ nuclei in the dentate gyrus. There was a significant correlation across all groups between the percentage of BrdU+ nuclei in the dentate gyrus and percentage exploration of the novel object in the two object NOR task ($r=0.733$, $p<0.001$). N=21.

3.6 Discussion

The aim of this study was to investigate the minimum period of environmental enrichment, in the absence of exercise, that is necessary to elicit a cognitive enhancement in the rat. The data presented here demonstrate that three weeks of housing in an enriched environment was sufficient to induce an improvement in hippocampal-dependent memory, as measured by the novel object recognition (NOR) task. Furthermore, six weeks of enriched housing induced an improvement in memory that was significantly increased when compared with animals housed for three weeks in enriched conditions.

In order to further characterise the enrichment-induced memory improvements, rats housed for six weeks in enriched conditions were tested using the spatial variant of the NOR task, the object displacement (OD) task, and the T maze task. Animals housed in the enriched environment also displayed significant improvements in these tasks when compared with animals in standard laboratory housing.

These data show that environmental enrichment, in the absence of exercise, can improve different types of memory. They also show that there is a temporal improvement in memory, with rats that were housed in the enriched environment for a longer period exhibiting improvements over the rats housed in the enriched environment for a shorter period of time. It could be argued that the enriched rats show improvements on both the NOR and OD tasks because their housing environment gives them prior exposure to the exploration of novel objects in their environment. However, the toys that are used in the enrichment protocol are always very different from the objects that are used in behavioural testing: objects used in the tasks are made from large multicoloured lego blocks whereas objects used in enrichment are typically toys from children's play kits (firetrucks, police cars and dinosaurs for example). Nevertheless, there may be a general 'priming' effect due to the extra stimulation that the rats will have with the extra objects in their homecages. This may have facilitated the improved performance that is seen on the NOR and OD tasks because they are heavily reliant on object exploration. Performance in the T maze task does not rely on object exploration and the six week enriched rats still showed an improvement in performance on this task when compared with rats housed in standard conditions, evidenced by the fact that they had a faster learning rate. The enrichment-induced improvements on this task would suggest that the cognitive improvements seen with environmental enrichment are not exclusive to tasks that rely on object exploration.

There is some evidence to suggest that enriched rats exhibit cognitive improvements on some behavioural tasks because they are less anxious (Zimmermann *et al.*, 2001, Harris *et al.*, 2009). Typical anxiolytic behaviours in rats would include a reduction in thigmotaxis and this can affect exploratory patterns within a behavioural task. Harris and colleagues argue that a reduction in thigmotaxis in enriched rats that are performing the Morris water maze sufficiently alters the exploratory pattern of the rats so that they are more likely to encounter the hidden platform on an earlier trial purely by chance and therefore they would have a greater opportunity to learn its position and hence perform better on this task. The objects in the NOR and OD tasks are placed approximately 20cm from the wall of the circular arena and therefore it may be possible that reduced levels of thigmotaxis in the enriched rats would increase the number of encounters that they had with the objects and therefore may facilitate their learning of these tasks. As a measure of anxiety that would be appropriate to the behavioural tasks that the rats have performed, the rats' open field activity was measured. The amount of thigmotaxis was analysed by calculating the percentage of time that the rats spent within a 20cm corridor around the wall of the same open field that was used in the behavioural task versus the percentage of time that they spend in the centre. This analysis showed that there was no difference in the amount of thigmotaxis between the six week enriched rats and the standard housed rats, suggesting that this enrichment paradigm does not affect levels of anxiety as measured by thigmotaxis and that differences in exploratory strategy cannot explain the improved performance on the NOR and OD tasks that is exhibited by the enriched rats.

Whilst the NOR, OD and T maze tasks test memory function that has been shown to be reliant on the hippocampus, they do so to differing extents. The OD task tests the rat's ability to remember the spatial pattern and location of the objects and is therefore heavily hippocampal-dependent. The NOR task is testing the rat's ability to distinguish between objects and recognise familiar versus novel objects and therefore, whilst there is hippocampal involvement, the perirhinal cortex plays an essential role in this type of memory(Bussey *et al.*, 1999). In fact, some studies argue that an intact hippocampus is not necessary for object recognition memory (Barker and Warburton, 2011), however at the 24 hour testing timepoint that is used in this study, there is evidence to show that the hippocampus is required for normal recognition memory (Clark *et al.*, 2000, Hammond *et al.*, 2004). Therefore, we would argue that in this particular variant of the NOR task, normal hippocampal functioning is necessary and enhancement of hippocampal function

would elicit a benefit in performance upon this task. The T maze task is designed to test the rat's spatial working memory and therefore utilises the hippocampus but it has also been shown to recruit higher cortical regions such as the prefrontal cortex because the rat must learn a rule in order to complete the task successfully. The prefrontal cortex, perirhinal cortex and hippocampus are strongly interconnected, with the perirhinal cortex connecting to the prefrontal cortex in a reciprocal manner and to the hippocampus via the entorhinal cortex. Whilst information associated with recognition memory is conveyed via perirhinal cortex connections, spatial information is typically conveyed via a non-perirhinal cortex pathway such as the postrhinal cortex (Naber *et al.*, 1997).

Studies show that further classifications are possible within the hippocampus proper with respect to its involvement in certain types of memory: the dorsal hippocampus has been heavily implicated in spatial navigation and memory, with lesions of less than 25% of the dorsal hippocampus inducing spatial memory deficits (Moser *et al.*, 1993) whereas the ventral hippocampus has been shown to be important for emotional memory and behaviour (Kjelstrup *et al.*, 2002, Bannerman *et al.*, 2004). There is a greater density of place fields in the dorsal hippocampus than the ventral hippocampus (Jung *et al.*, 1994), and human fMRI studies have shown that the posterior hippocampus (akin to the dorsal hippocampus in rodents) is preferentially activated during task involving conceptual information (Kumaran *et al.*, 2009). Conceptual information of this kind is thought to be associated with the acquisition of a set of rules that are necessary to understand a novel situation, and are similar to the kinds of rules that would be necessary to guide suitable navigational behaviour in a novel environment or to search out food sources. It is likely therefore, that dorsal hippocampal processing is also crucial for successful performance in the T maze task, as this task necessitates rule-learning of this kind. Indeed, dorsal hippocampal lesions impair performance on a radial arm maze, whereas ventral hippocampal lesions did not affect performance on this task (Pothuizen *et al.*, 2004). Due to the dissociations that are possible within the hippocampus proper, it would have been interesting to subdissect the hippocampus into the dorsal and ventral regions to compare neurochemical changes within these regions.

Whilst the dentate gyrus is not typically associated with a role in any specific type of memory, it is known to be crucial for memory consolidation (Monopoli *et al.*, 2011). Many studies also suggest that it is be important for spatial pattern separation: lesions to the dentate gyrus cause rats to be unable to discriminate between displacing the correct object

to obtain a food reward and an identical object when they were situated close together, whereas lesions to the CA1 region did not cause an impairment (Gilbert *et al.*, 2001). Recent genetic data suggests that there are distinct subpopulations of granule cells within the dentate gyrus that fire during exploration of the different environments, and the same subpopulations fired when rodents returned to the same environment twice (Chawla *et al.*, 2005). In a similar study, rats that explored the same environment twice displayed an overlap of 70% of the same granule cells being active compared with 35% of the same cells being active when rats explored different environments (Marrone *et al.*, 2011). In this context, adult neurogenesis is argued to play a particular role in pattern separation, with immature granule cells being preferentially activated during exposure to a novel environment (Kee *et al.*, 2007, Tashiro *et al.*, 2007). A reduction in neurogenesis can induce an inability to discriminate between similar contexts in a fear conditioning task and a deficit in location discrimination when objects are close together (Clelland *et al.*, 2009, Sahay *et al.*, 2011) and conversely, an enhancement in neurogenesis can improve performance on a touch screen location task and contextual fear conditioning (Creer *et al.*, 2010, Sahay *et al.*, 2011).

The neurochemical results show that there is a significant increase in βNGF, but not BDNF, in the dentate gyrus of rats following six weeks of enrichment and further analysis revealed that there was a significant positive correlation between performance on the NOR task and βNGF concentration. In addition to this there was a significant increase in neuronal proliferation in the dentate gyrus in rats following six weeks of environmental enrichment and this also positively correlates with performance on the NOR task. These data would suggest that both βNGF and neurogenesis are involved in the enrichment-induced memory improvements seen in this study. Further analysis revealed that there are increases in the synaptic vesicle proteins synapsin and synaptophysin in the dentate gyrus of enriched rats. Increases in these proteins have been linked with synaptogenesis, thus these data provide further evidence for the activity-dependent plasticity changes associated with housing in an enriched environment.

There is however, a significant reduction in βNGF concentration in the hippocampus after three and six weeks of environmental enrichment. At the mRNA level, there is also a significant increase in βNGF expression after two weeks of enrichment. These results are seemingly in conflict with the association between increased βNGF and memory improvements that has been found and may indicate that there are certain signalling and

neurotrophin transport mechanisms between the hippocampus and dentate gyrus that are being affected by environmental enrichment. Dentate gyrus granule cells send axonal projections into the hippocampus, particularly the CA3 region and therefore it is possible that environmental enrichment is stimulating an increase in the production of βNGF in the hippocampus and that this βNGF is being taken up at axon terminals in the CA3 and retrogradely transported to granule cell bodies in the dentate gyrus where it can exert its key function of regulating neuronal survival and growth (for reviews, see Lu and Chow, 1999, Poo, 2001). This may stimulate the increase in local production of βNGF in the dentate gyrus that is suggested in this study, as there is a small increase in βNGF mRNA in the dentate gyrus with increasing weeks of environmental enrichment. No changes in βNGF in the perirhinal cortex were observed in this study, suggesting that this brain region is not involved in the memory improvements that are seen with this type of environmental enrichment. Trk A is the main receptor for NGF and whilst there are no statistically significant differences between groups in Trk A mRNA expression in the dentate gyrus or hippocampus, there does seem to be a reduction in Trk A expression in the hippocampus after six weeks of enrichment. This reduction mirrors the decrease in βNGF concentration shown in the hippocampus after three and six weeks of enrichment, and therefore it is possible that there is a downregulation of Trk A in the hippocampus in direct response to the reduction of βNGF that is available to bind to it. Because there are no data available regarding the protein expression of Trk A however, it is not possible to draw a firm conclusion on this point.

The increase in NGF concentration that is found in the dentate gyrus of enriched rats in this study is complemented by data in the literature that shows increases in NGF in the hippocampus following sixty days of enrichment (Pham *et al.*, 1999b). Their enrichment protocol does include running wheels however, and there is no analysis of BDNF concentrations, which would be expected to increase following exercise. Indeed, studies from the same group show increases in hippocampal BDNF, NGF and Trk A following long-term environmental enrichment (Pham *et al.*, 1999a, Ickes *et al.*, 2000). Most studies do not subdissect the hippocampus and therefore it is not possible to pinpoint the source of neurotrophin increases within the hippocampal formation. Zhu and colleagues report that there are different baseline and experimental concentrations of BDNF and NGF in the ventral and dorsal hippocampus in mice (Zhu *et al.*, 2006), indicating that are changes in neurotrophin concentrations between these regions and that they may be differentially

affected experimentally. The data from this study add to the current literature that provides strong evidence for a role for neurotrophins in the cognitive improvements associated with environmental enrichment, and yet the mechanisms of action of BDNF and NGF as mediators of memory improvements in these paradigms are still unclear and yield conflicting evidence in the literature.

This study shows a consistent reduction in BDNF concentration after two and three weeks of environmental enrichment in the dentate gyrus, hippocampus and perirhinal cortex. There is also a significant reduction in Trk B receptor expression following six weeks of enrichment. This would suggest that BDNF is not involved in the improvements in memory that have been measured in this study. Whilst it is not clear why there would be such a consistent enrichment-induced reduction in BDNF across all these regions, some papers that also utilise enrichment protocols without exercise report no increases in BDNF concentration (Bindu *et al.*, 2007) and behavioural improvements in $BDNF^{+/-}$ heterozygous mice (Chourbaji *et al.*, 2008, Zhu *et al.*, 2009). These studies suggest that environmental enrichment can affect behaviour via a pathway that is independent of BDNF. Nevertheless, no study to date has shown decreases in BDNF following environmental enrichment although decreases in expression of BDNF have been shown after social isolation with associated increases in spatial memory (Pisu *et al.*, 2011).

There were no changes in ERK phosphorylation found in the dentate gyrus, hippocampus or perirhinal cortex. Changes in ERK phosphorylation would be indicative of activation of the MAP kinase pathway that is heavily associated with memory function and the pro-survival pathways of neurotrophins. The rats in this study were sacrificed one day after they had been tested on the NOR task and therefore it is likely that, at this time, any increased activation of the MAP kinase pathway associated with enhanced learning had decreased to baseline levels. Further analysis of other proteins associated with activation of this pathway, such as CREB phosphorylation, would confirm this. Alternatively, NGF may be eliciting memory improvements via activation of the PI3 kinase or PLC-γ pathways, therefore future studies could focus on the analysis of tissue directly after learning occurs for assessment of all downstream signalling pathways associated with Trk receptor signalling.

Interestingly, whilst there is a significant increase in neurogenesis at three and six weeks of environmental enrichment, there is no difference in the survival of these new neurons

following six weeks of enrichment. Given the importance of NGF in neuronal survival, it would be likely that an increase in NGF in the dentate gyrus would provide an enhanced neurogenic niche and stimulate an increase in the survival of adult-born neurons. This could be associated with the specific protocol that was used in this study: rats were injected with BrdU prior to housing in the enriched environment. Whilst there is a temporal increase in concentration of βNGF in the dentate gyrus over the weeks of enrichment, perhaps the proliferating cells that were BrdU-tagged in this study were born too early to benefit from the pro-survival effects of this increased βNGF. Recent studies show that neurons of approximately four weeks old or younger are vitally important for long-term memory or for the induction of LTP (Snyder *et al.*, 2001, Kee *et al.*, 2007, Deng *et al.*, 2009). Therefore, if BrdU had been injected one or two weeks into the enrichment (between three and four weeks prior to sacrifice), this may have increased the likelihood that enhanced survival of these neurons would be observed. Additionally, it is at this point during the enrichment period that the increase in NGF concentration in the dentate gyrus begins to emerge.

BrdU has a half-life of approximately 2 hours and therefore it provides an immediate measure of the rate of cell proliferation at the time of injection. Given the granule cell cycle in the rat SGZ is approximately 25 hours(Cameron and McKay, 2001), some of the cells that were labelled will be up to 14 days old, and therefore this study is more accurately measuring early progenitor cell survival rather than just increases in proliferation. Therefore the results presented here may show that NGF has a positive impact upon early cell survival as opposed to directly stimulating and increase in cell proliferation, which is more in line with current theories regarding the role of NGF in adult hippocampal neurogenesis. To fully answer this question, rats should be injected with a single higher dose of BrdU 24 hours before sacrifice. This would provide a perfect snapshot of the rate of proliferation at that point in time.

Due to time constraints in this study, it was not possible to use the standard serial counting technique for an estimation of the total number of BrdU+ cells in the dentate gyrus. Therefore the results presented here could be a biased sample of a specific section of the dentate gyrus and not representative of the whole brain region. Whilst this may be the case, every effort was made to ensure that the sections taken where at the same position in each brain and therefore the results from each rat can be grouped appropriately and results from the groups can be compared. Therefore, the differences seen do reflect differences in the

rate of neurogenesis, or early cell survival, in the dentate gyrus but these differences may only be limited to a small subsection of this region and may not reflect differences in proliferation in the whole of the dentate gyrus.

This study analysed the expression of two synaptic vesicle proteins, synapsin I and synaptophysin, and found that both were increased in the dentate gyrus of rats housed in an enriched environment for six weeks. Synaptic vesicle proteins are important for neurotransmitter release and hence can play a role in experience-dependence plasticity. Synapsin expression is also known to be increased during synaptogenesis and synaptophysin is one of the most abundant synaptic vesicle proteins (Lohmann *et al.*, 1978), therefore they are commonly used in the literature as markers of synaptogenesis (Kelsch *et al.*, 2010, Cuesto *et al.*, 2011). Increases in their expression in this study suggests that environmental enrichment can induce changes in synaptic plasticity, possibly via enhancement of synaptic vesicle availability and release during neurotransmission. Alternatively, these increases could suggest morphological changes in existing synapses, such as enlargement of the active zone and spine volume, or the formation of new synapses. A meta-analysis conducted by Marrone (2007) found increases in spine size and density of synapses in the dentate gyrus following hippocampal-dependent learning. Ambrogini and colleagues (2009) showed that learning in the Morris water maze enhanced dendritic tree complexity and increased synaptogenesis. Whilst it is possible that the physical exercise associated with performance of the Morris water maze facilitated the enhancement in synaptic and dendritic morphology, the continuous stimulation that is associated with environmental enrichment in this study could be hypothesised to stimulate changes in synaptic morphology in a manner similar to learning-induced changes.

To date, this is the first study to measure the minimum period of enrichment that is required without additional physical activity, to elicit behavioural improvements on hippocampal-dependent memory. Many of the studies that utilise similar protocols have shown that environmental enrichment can reduce anxiety levels in rodents but memory improvements are not always tested (Brenes *et al.*, 2009) and when these data are available either no memory improvement was shown or there was a very moderate effect (Galani *et al.*, 2007, Chourbaji *et al.*, 2008). Lambert and colleagues compared the effects of six weeks of daily enrichment (three hours per day of environmental enrichment) and found that it was exercise but not enrichment that induced an improvement in spatial memory (Lambert *et al.*, 2005). In contrast to this, Bruel-Jungerman and colleagues report that two

weeks of daily enrichment, without exercise, can improve object recognition memory (Bruel-Jungerman *et al.*, 2005). It is not evident therefore, if daily enrichment is more stimulating than continuous enrichment for rodents however it is clear that the frequency of novel stimulation could be a significant factor in the intensity of the intervention and its efficacy at inducing a behavioural improvement.

Most often, environmental enrichment protocols include physical activity via running wheels and typically use a housing period of over eight weeks. These studies show that young rodents exhibit spatial memory improvements and that enrichment can be neuroprotective against spatial memory decline with age and both spatial and recognition memory in a stroke model (Pham *et al.*, 1999b, Gobbo and O'Mara, 2004, Bennett *et al.*, 2006). In a further study, Gobbo and O'Mara compared the efficacy of ten weeks of voluntary exercise with ten weeks of cognitive enrichment at rescuing kainic acid-induced deficits in spatial and recognition memory and found that it was actually exercise, and not enrichment, that could ameliorate the memory impairments. They also report an increase in hippocampal BDNF concentration in the exercise group, but not in the enrichment group. This result corresponds with data from this study that show no increases in BDNF after enrichment and previous data in our lab that show that a week of treadmill exercise can improve memory and correspondingly increase serum and hippocampal BDNF concentrations in both humans and rats (Bechara & Kelly, unpublished; Griffin *et al.*, 2009, Griffin *et al.*, 2011). Further data from our lab show that exercise is a very potent memory enhancer: after a single training session of forced treadmill exercise, rats exhibit an improvement in spatial and recognition memory (McCreddin & Kelly, unpublished). Given that this study shows memory improvement following three or more weeks of environmental enrichment without exercise, it may be that cognitive stimulation is a less potent memory enhancer than exercise alone and also works via different neurochemical pathways. Nevertheless, it is clear that it can be used to enhance various types of memory and elicit neuroplastic changes in the brain.

There is strong evidence in this study to suggest that the increase neurogenesis may also be an important mechanism by which enrichment is inducing a cognitive improvement, because there is a significant positive correlation between object recognition memory and proliferation. In line with this, previous data show that daily environmental enrichment without exercise for ten days does not affect the concentration of BDNF in the hippocampus but does increase dendritic branching (Bindu *et al.*, 2007). There are also

complementary data that show an ablation of neurogenesis prevents the object recognition memory improvements that are induced via a daily enrichment protocol (Bruel-Jungerman *et al.*, 2005). In contrast, it has been suggested that neurogenesis is not required for the induction of memory improvements via enrichment and that BDNF is a crucial factor in the enrichment-induced increase in neurogenesis (Meshi *et al.*, 2006, Rossi *et al.*, 2006). The role that this increase in neurogenesis plays in memory improvements is becoming more clear, with recent data suggesting that young neurons are preferentially activated during spatial memory tasks (Kee *et al.*, 2007). Tashiro and colleagues not only showed that exposure to an enriched environment increased neurogenesis, but also that following enrichment, two week old neurons are more likely to be activated upon re-exposure to the same environment, when compared to exposure to a different environment one month later (Tashiro *et al.*, 2007). This suggests that these young neurons are capable of encoding information and being integrated into the neural circuitry in the hippocampus so that there is a representation of that information which can be re-activated upon re-exposure, hence the proposal that these young neurons are important for novel memory formation. Whilst it is not possible to conclude that this is the process by which cognitive function is enhanced in this study, the increase in the number of young neurons may boost the accuracy of an object or place representation and therefore enhance memory formation. A recent study by Kobilo and colleagues (2011) argues that it is running, via and upregulation of BDNF, that is the most crucial aspect of environmental enrichment to induce a neurogenic enhancement which is in direct contradiction to these results. Certainly, whilst it is clear that exercise is a potent cognitive enhancer, these data show that there are alternative mechanisms that can be utilised to improve memory and increase neuronal proliferation and that improvements via environmental enrichment are likely to be due to a complex interaction of factors.

In summary, this study indicates that there is a time-dependent cognitive enhancing effect of environmental enrichment that is independent of physical activity. This effect is not specific to one type of hippocampal-dependent memory as rats show an improvement in recognition, spatial and working memory following enrichment. Neurochemical data suggest that there is a role for NGF, and not BDNF, in this improvement as well long-term neuroplasticity changes such as neurogenesis and synaptogenesis. However, further studies are needed to elucidate the exact roles that NGF and the neuroplastic changes may play in

the enrichment-induced improvement of memory function, and the cellular mechanisms that underpin these improvements.

Chapter 4: An assessment of the efficacy of Nerve Growth Factor in facilitating hippocampal-dependent memory

4.1 Introduction

Nerve Growth Factor (NGF) was the first neurotrophin to be described in the literature and is classically defined as a factor important for neuronal survival and growth (Levi-Montalcini and Hamburger, 1951). It is an important growth factor during neurodevelopment, as NGF heterozygous$^{+/-}$ mice exhibit significant reductions in basal forebrain cholinergic neurons and cholinergic innervation in the hippocampus (for review, see Sofroniew *et al.*, 2001). It is also widely expressed in the mature brain, particularly by pyramidal neurons in the hippocampus and granule neurons in the dentate gyrus (French *et al.*, 1999).

Trk A binds with high affinity to βNGF, inducing autophosphorylation and the subsequent phosphorylation and activation of adaptor proteins such as Shc and PLCγ. Shc is critical for the activation of the Ras/ERK signalling cascade and can also activate the the PI3-kinase/Akt pathway, both of which promote survival and growth in neurons (Dudek *et al.*, 1997, Bonni *et al.*, 1999). Signalling in the Ras/ERK pathway leads to phosphorylation of the transcription factor CREB, which is important for long-term memory processes since deficits in CREB can lead to impairments in both spatial and contextual memory formation (Mizuno and Giese, 2005). Activation via PLCγ induces activation of Ca^{2+}-dependent pathways via IP_3, such as CAMKII which is known to play a crucial role in LTP maintenance (Miyamoto, 2006).

The high expression of βNGF in the adult hippocampus has lead to research regarding its involvement in memory processes, indeed blocking hippocampal βNGF reduces LTP and impairs spatial memory (Conner *et al.*, 2009). Whilst there is abundant evidence supporting the role of BDNF in synaptic plasticity, βNGF is known to be vital for the maintenance of LTP (Kelly *et al.*, 1998b) and an intrahippocampal infusion of βNGF can enhance memory on an inhibitory avoidance task via activation of the MAP kinase pathway (Walz *et al.*, 2000). It is also well documented that synapsin is a substrate for the ERK family of MAP kinases, through its phosphorylation via ERK, and is vital for neurotransmitter release and the maintenance of a synaptic vesicle pool (Shupliakov *et al.*, 2011). Inhibition of MEK, a kinase upstream of ERK in the Ras/ERK signalling pathway,

causes a reduction in the number of functional synapses and this is associated with a reduced phosphorylation and functional activation of synapsin (Giachello *et al.*, 2010). It is therefore likely that βNGF can stimulate enhanced neurotransmitter release via activation of the Ras/ERK pathway and also induce morphological changes to enhance synaptic plasticity in addition to its classical role of maintenance of neuronal survival and growth.

Therapeutically, βNGF and Trk A have been proposed as viable targets for neuroprotection after traumatic brain injury and stroke, and for the treatment of neurodegenerative diseases such as Alzheimer's disease. In a mouse model of Alzheimer's disease, selective Trk A agonists ameliorated the loss of short-term spatial memory observed (Aboulkassim *et al.*, 2011). Interestingly, a direct infusion of βNGF in this study did not affect memory function in the Alzheimer's model or wild type mice. An exogenous infusion of βNGF following middle cerebral artery occlusion in a rabbit model of stroke reduced levels of apoptosis and infarct volume, highlighting its role neuronal repair and survival (Yang *et al.*, 2011). Recent clinical studies show significant positive correlations between the outcome of children with traumatic brain injury and the concentration of βNGF and doublecortin in cerebrospinal fluid (Chiaretti *et al.*, 2008, Chiaretti *et al.*, 2009). These studies highlight the crucial role that βNGF plays in neuronal repair following injury. Animal studies typically observe a βNGF-induced increase in survival of neurons in the hippocampus rather than an enhancement of proliferation (Olson *et al.*, 2006, Frielingsdorf *et al.*, 2007). To date however, there has not been a study that assesses hippocampal neurogenesis following chronic βNGF administration.

Although these studies show the therapeutic potential of βNGF, the need for a central delivery method and the high dosages that are typically used present serious confounds for realistic treatment options in patients. High doses of βNGF can induce neuropathic pain (Eriksdotter Jonhagen *et al.*, 1998). Therefore utilising protocols such as exercise or environmental enrichment to upregulate endogenous neurotrophic factors can reduce the chance of side effects or interactions with other drug treatments. Data from the Chapter 3 would suggest a role for βNGF in the enrichment-induced memory improvements observed. Nevertheless, it is not possible to conclude that βNGF has a direct influence on the cognitive improvements as only a correlative link was found.

The aim of this study was to assess the efficacy of an exogenous infusion of βNGF into the lateral ventricle in enhancing hippocampal-dependent memory. The concentration of

βNGF in the dentate gyrus was observed to increase over a period of six weeks in the previous study. Thus, a continuous infusion of βNGF into the lateral ventricle for six weeks was also used in this current study in order to mimic the enrichment-induced βNGF increase and to further analyse the underlying effects that this may have on neurogenesis and synaptogenesis. The dosage we use is calculated based upon the enrichment-induced increase that is observed in the previous study, enabling a comparison of the neurochemical changes associated with both studies.

4.2 Single Intracerebroventricular βNGF Infusion Methods

4.2.1 Subjects and Design

The experimental groups in this study consisted of one group of rats injected with a single infusion of 5µl of recombinant rat βNGF into the lateral ventricle (n=3, 0.8ng.µl^{-1}, R&D Systems) and one group of rats injected with a single infusion of 5µl of cytochrome c into the lateral ventricle (n=4, 0.8ng.µl^{-1}). Following a 14 day washout period, the groups of rats were swapped over so that the group that had previously been injected with rat βNGF were now injected with cytochrome c and *vice versa*. All animals were singly housed, with food and water *ad libitum* and a 12:12 light:dark cycle. During the 14 day washout period, a cannula fell out of one of the rats and was excluded from the analysis. Therefore during the second bout of injections, the groups consisted of the βNGF group (n=4) and the cytochrome c group (n=2). Therefore by the end of the study, a total of 6 rats had been injected with both the vehicle and βNGF solution. It could be argued that the prior infusion could have an impact upon any further behavioural analysis. Because of this limitation, half the rats were infused with the vehicle solution and half with the βNGF solution first and this was reversed following the 14 day washout period. This balances any effect the previous infusion between the groups and reduces the chance that this would impact on any behavioural analysis. This also counterbalances any possibility that changes that are reported are due to a practise effect. Nevertheless, this limited the possibility of any analysis of neurochemical changes that may have occurred following the infusions, and therefore no neurochemical data was obtained from these rats.

The concentration of the recombinant βNGF solution was calculated using the increase in βNGF protein that was seen in rats housed in enriched conditions for 6 weeks in the study described in Chapter 3. From the ELISA results, mean SH value of βNGF was 14.57 pg.mg^{-1} and the mean 6wk EE value of βNGF was 31.51 pg.mg^{-1} which is an increase of 16.94 pg.mg^{-1}. The average wet weight of the hippocampus is 160 mg and therefore an additional 2.71 ng of βNGF should to be injected to mimic this increase. The solution was injected into the lateral ventricle, adjacent to the hippocampus, and so to allow for any loss, this value was rounded up to 4 ng per animal (in 5µl; 0.8ng.µl^{-1}). Cytochrome c is a commonly used control protein for neurotrophin infusions because it is an inert protein that has a similar molecular weight to βNGF (~12,000 daltons) with no known extracellular actions (Kobayashi *et al.*, 1997, Willson *et al.*, 2008).

4.2.2 Surgical Methods

Rats were placed in an anaesthetising chamber and anaesthetised with 4% isoflurane in pure 0_2. Rats were placed on a stereotaxic frame and secured with ear bars and incisor bars and injected with carprofen ($5mg.kg^{-1}$; s.c.). Once full anaesthesia was confirmed, their heads were shaved and an incision of approximately 0.5 inches long was made vertically down the centre of the skull. Mosquito clamps were used to keep the incision open whilst the skull was cleaned and sterilised with 3% H_2O_2. A single hole was drilled at the site of the right lateral ventricle (0.9mm posterior and +0.14mm lateral to bregma). A guide cannula (depth of 3.6mm) was inserted into this hole and secured using 3 screws and dental cement. Once the cannula was secure, the wound was closed using surgical staples and the animals were allowed to recover for at least 14 days before any studies began. The rats' food and water intake were monitored as a measure of their wellbeing.

4.2.3 Behavioural Testing

Rats were injected i.c.v. with either βNGF or Cytochrome c (VEH) using a Hamilton syringe at a rate of approximately $1\mu l.min^{-1}$ 30 minutes prior to training on the 3 object NOR Task, as described in 2.4.1 (figure 4.1). Briefly, the rats had three trials of five minutes with an inter-trial interval of five minutes to explore three different novel objects in an open field (training day). Twenty-four hours post-training, one of the objects was replaced by a novel object in the same position and the rats were placed back into the open field for five minutes and allowed to explore (testing day). During both the training and testing days, the time spent exploring each object was recorded using stopwatches and calculated as a percentage of the total time spent exploring all objects.

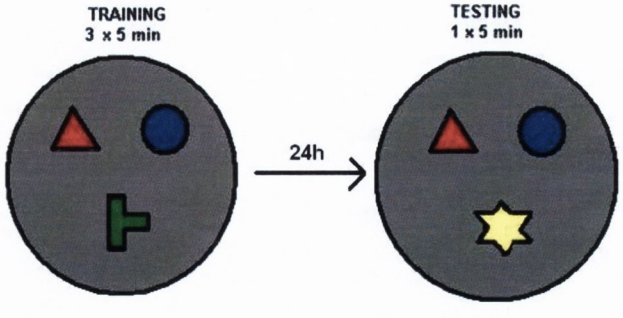


Figure 4a. The 3 object NOR Task used for behavioural analysis in both single and chronic βNGF infusion studies. During Training, rats were exposed to the objects in the circular open field for 3 x 5 minute sessions. 24 hours later, one object was replaced with a novel object and rats were re-exposed to the objects for 1 x 5 minute session. The amount of exploration on each object was measured and calculated as a percentage of total object exploration.

4.3 Chronic Intracerebroventricular βNGF Infusion Methods

4.3.1 Subjects and Design

The experimental groups consisted of two groups receiving a 42 day chronic infusion of βNGF (βNGF; n=6) or cytochrome c (VEH; n=5) into the lateral ventricle at a rate of $4ng.hr^{-1}$ using Alzet® Osmotic Pumps (2006, Charles River, Cambridge, UK). This dose was calculated so that the animals received the same amount of βNGF or cytochrome c per hour that was administered in the single infusion study. With this dose, a total of 4.032 μg of βNGF or cytochrome c was infused into the lateral ventricle of the rats by the end of the 42 day infusion. There was also one group of surgery naive rats (CON; n=6) included in the study to control for the effect that the i.c.v surgery and subsequent infusion may have on the behaviour and neurochemistry of the rats. All rats were housed 3 per cage, with food and water *ad libitum* under a 12:12 light:dark cycle. On day 36, the cannula became dislodged from one of the rats in the βNGF group. Given the short period of time left in the study, the rat completed the behavioural testing.

4.3.2 Minipump Priming and Surgical Methods

The chronic infusions were carried out using Alzet® Osmotic Pumps and Brain Infusion Kits (Charles River, Cambridge, UK). The pumps were primed in sterile conditions for 62 hours prior to surgery. First, the cannulas were adjusted to the correct depth of 3.5mm and attached to tubing that was then attached to the flow moderator (figure 4.2). Using a sterile needle and syringe, the minipump and tubing were filled with the solution containing βNGF or cytochrome c, ensuring there were no bubbles. The flow moderator was then attached to the minipump and placed in a falcon tube filled with sterile saline in an incubator at 37°C.

Once the minipumps had been primed, rats were placed in an anaesthetising chamber and anaesthetised with 4% isoflurane in pure 0_2. Rats were then placed on a stereotaxic frame and secured with ear bars and incisor bars and injected with carprofen ($5mg.kg^{-1}$; s.c.). Once full anaesthesia was confirmed, their heads were shaved and an incision of approximately 0.5 inches long was made vertically down the centre of the skull. Mosquito clamps were used to keep the incision open whilst the skull was cleaned and sterilised with 3% H_2O_2. A single hole was drilled at the site of the right lateral ventricle (0.9mm posterior and +0.14mm lateral to bregma). Following this, a pocket under the skin was

created using metzenbaum scissors from the back of the head to below the shoulder blade and the pump was inserted into the pocket. The cannula was then inserted into the drilled hole and secured using superglue. Once the cannula was secure, dental cement was used to smooth over the cannula and reduce irritation. The wound was then closed using surgical staples and the animals were allowed to fully recover from their anaesthetic before being returned to their home cages. The rats' food, water intake and weight were monitored throughout the study.

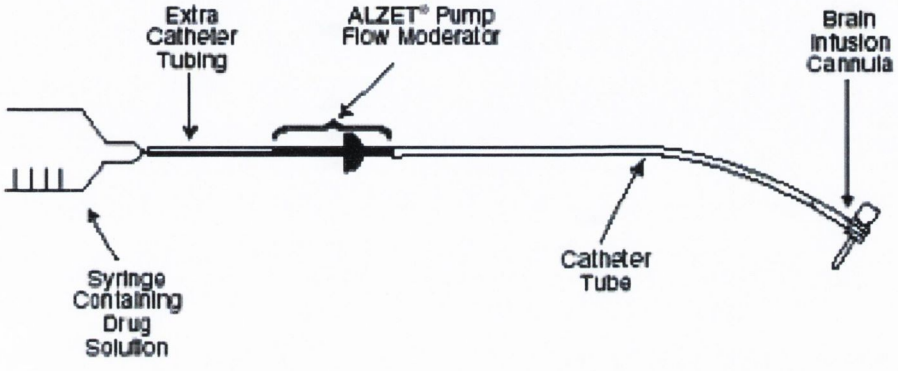


Figure 4b. ALZET® Pump Flow Moderator

4.3.3 BrdU Administration

To measure the amount of cell proliferation in dentate gyrus of the animals in this study, 5'-bromo-2'-deoxyuridine (BrdU) was injected on days 30, 32, 34, 37, 39 and 41 ($50mg.kg^{-1}$, i.p.).

4.3.4 Behavioural Testing

Rats were tested with the 3 object NOR Task, as described in 2.4.1 (figure 4.2). Briefly, the rats had three trials of five minutes with an inter-trial interval of five minutes to explore three different novel objects in an open field (training day). Twenty-four hours post-training, one of the objects was replaced by a novel object in the same position and the rats were placed back into the open field for five minutes and allowed to explore (testing day). During both the training and testing days, the time spent exploring each object was recorded using stopwatches and calculated as a percentage of the total time spent exploring all objects.

Immediately after testing, rats were sacrificed by decapitation and tissue was collected as described in section 2.5.1. Briefly, the ipsilateral hemisphere was flash frozen in liquid nitrogen to confirm the cannulation site and sectioning for neurogenesis analysis. The perirhinal cortex, dentate gyrus and hippocampus were dissected from the contralateral hemisphere for protein and mRNA analysis. Trunk bloods were collected, centrifuged at 11,000g for 20 minutes and serum was removed and stored at -20°C for later analysis.

4.3.5 Analysis of the expression of βNGF, BDNF, Trk A and Trk B and phosphorylation and expression of ERK

Neurotrophin expression was measured in supernatant samples from the dentate gyrus, perirhinal cortex and hippocampus in all groups. BDNF and βNGF protein concentration were measured by ELISA (see 2.5.4) and Trk A protein concentration was measured in the hippocampus by Western immunoblotting (see 2.5.2). BDNF, βNGF, Trk A and Trk B mRNA expression were measured using PCR analysis in the dentate gyrus and hippocampus in all groups (see 2.5.3).

ERK phosphorylation was measured using western immunoblotting in the dentate gyrus and hippocampus in all groups (see 2.5.2). The phosphorylation of this protein is a downstream effect of the activation of Trk receptors in the Ras/ERK pathway and therefore indicative of neurotrophin action.

4.3.6 Analysis of Synaptic Vesicle Proteins

Synapsin and synaptophysin protein concentration was measured using western immunoblotting in the dentate gyrus and hippocampus in all groups (see 2.5.2). These synaptic vesicle proteins are important in synaptic vesicle release and maintenance of a synaptic pool in the synaptic bouton and therefore play a role in neurotransmission. Synapsin is also linked to the cytoskeleton within the synapse, while synaptophysin is the most abundant synaptic vesicle protein. Therefore, they can be used as markers of changes in both synaptic transmission and synaptogenesis.

4.3.7 Analysis of Neurogenesis

In order to measure any changes in neuronal proliferation, immunohistochemical analysis was performed on sections of the dentate gyrus in all groups. To analyse the rate of cell proliferation in the dentate gyrus, BrdU immunostaining was performed on 10μm sections

and analysed via light microscopy (see 2.6.2). The number of BrdU positive nuclei was calculated as a percentage of the total number of nuclei stained with hematoxylin.

4.3.8 Analysis of Apoptosis

Levels of apoptosis in the dentate gyrus of all groups were assessed using the DeadEnd™ Fluorometric TUNEL System (Promega Corporation, Madison, USA) according to manufacturer's instructions (see 2.6.4). This system measures nuclear DNA fragmentation in cells, a marker of programmed cell death. Briefly, Fluorescein-12-dUTP was catalytically incorporated at 3'-OH DNA in 10μm sections using the Terminal Deoxynucleotidyl Transferase, Recombinant, enzyme (rTdT). The labelled DNA were then visualized via confocal microscopy at the 488nm wavelength. The mean intensity of fluorescence at 488nm was calculated for each animal and group means were compared.

4.3.9 Statistical Analysis

All data are expressed as mean ± standard error of the mean (SEM).Outliers were excluded from any analysis if they were ±2 standard deviations away from the mean. Repeated measures two-way ANOVA were used to analyse data from the NOR task in both studies, where the dependent variable was percentage object exploration. Because the same rats were infused with both βNGF and a vehicle solution in the single βNGF i.c.v. infusion study, the within-subjects factors were both 'Object' and 'Treatment' whereas in the chronic βNGF i.c.v. infusion, the within-subjects factor was 'Object' and the between-subjects factor was 'Group'. One-way ANOVAs were used to analyse data from the protein, mRNA and immunohistochemical analyses. Post-hoc analyses were performed using Bonferroni or Tukey multiple comparison tests.

4.4 Results

4.4.1 Chronic infusion of βNGF does not affect weight gain

A common side-effect of βNGF infusions is weight-loss therefore the rats' weights were recorded throughout the study. Repeated measures 2-way ANOVA revealed no significant difference between groups in weight across the study ($F_{1,234} = 0.9297$, $p>0.05$; figure 4c). All rats significantly gained weight through the study ($F_{26, 234} = 105.3$, $p<0.001$).

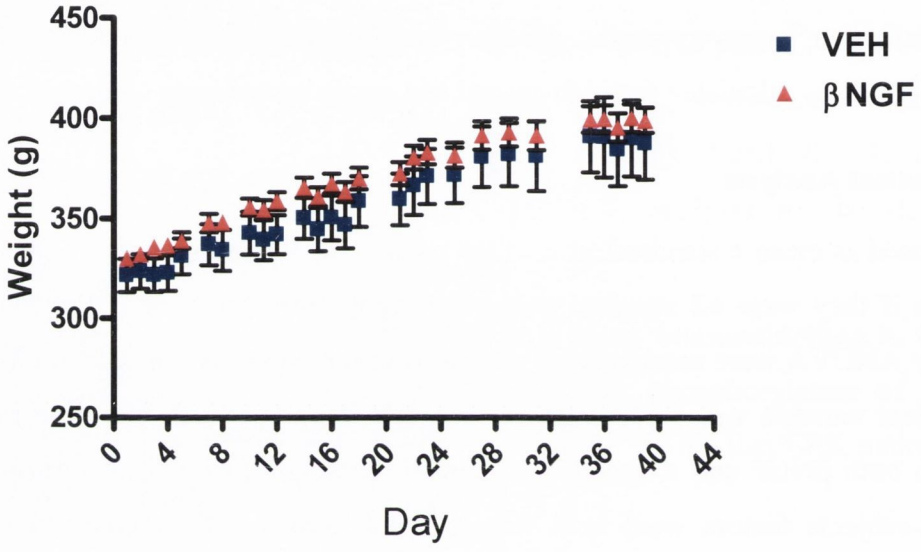


Figure 4c. Chronic infusion of βNGF does not affect weight gain. There was no significant difference between groups in weight across the study ($F_{1,234} = 0.9297$, $p>0.05$), with all rats gaining weight overall ($F_{26, 234} = 105.3$, $p<0.001$). Data is expressed as mean ± SEM.

4.4.2 Single i.c.v. infusion of βNGF improves object recognition memory

Object recognition memory was tested with the three object NOR task. On the training day, there was no significant difference between the exploration of the three objects A, B and C ($F_{2,10}$ = 1.480, p>0.05). There was no significant difference in the exploration between the treatments ($F_{1,5}$ = 0.000, p>0.05) and no interaction ($F_{2,5}$ = 0.594, p>0.05, figure 4d.i).

On the testing day, object C was replaced with a novel object D*. There was no significant difference in exploration between any of the groups ($F_{1,5}$= 0.0000, p>0.05) but there was a difference between the exploration of the objects A, B and D*, although it did not reach significance ($F_{2,10}$ = 3.934, p=0.055).

Analysis also revealed a significant interaction between the exploration of objects and treatment group ($F_{2,10}$ = 5.795, p<0.05, figure 4d.ii). To explore the source of the interaction, one-way repeated measures ANOVAs were performed for each treatment with the exploration of the objects as the within subjects factor. This analysis showed that there was no significant difference in exploration of the novel object D* and the familiar objects A or B ($F_{2,10}$ = 1.134, p>0.05; mean percentage exploration of objects ± SEM: A = 40.48±6.04, B = 24.48±5.72, D* = 35.04±6.89) in the VEH group. There was a significant difference in the exploration of the objects in the βNGF group ($F_{2,10}$ = 24.25, p<0.001) with rats exploring the novel object D* significantly more than the familiar objects A and B (mean percentage exploration of objects ± SEM: A = 27.21±1.86, B = 21.95±3.01, D* = 50.84±2.65; p<0.001).

These data show that a single i.c.v. infusion of βNGF, at a physiologically similar concentration to the increase in βNGF measured after six weeks of environmental enrichment, can induce an improvement in object recognition memory in rats as measured by the three object NOR task.

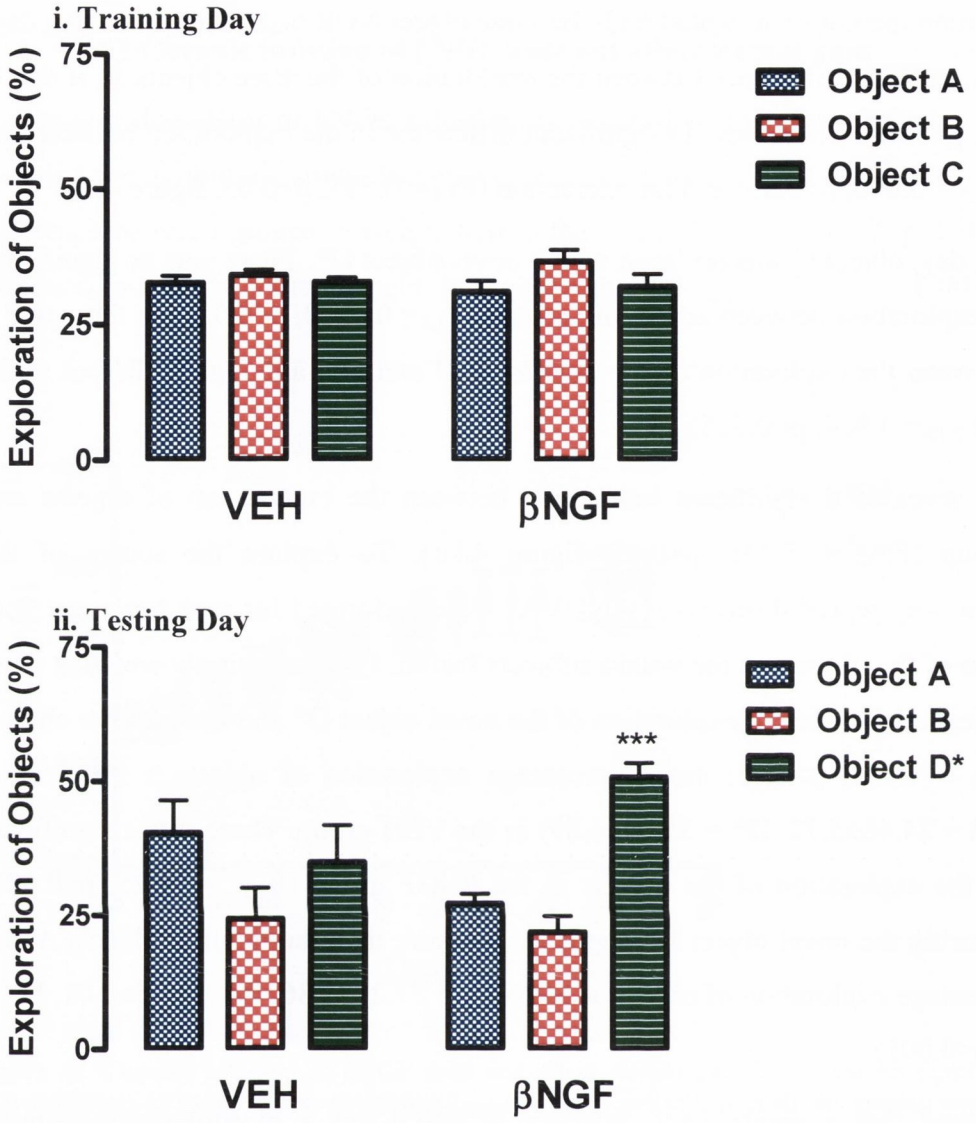


Figure 4d. Single i.c.v infusion of βNGF improves object recognition memory(i) The was no significant difference found between the exploration of the objects on training day of the NOR task ($F_{2,10}$ = 1.480, p>0.05). (ii) On testing day, there was a significant interaction between the exploration of objects and the treatment group, with no significant difference between the exploration of objects in the VEH group ($F_{2,10}$ = 1.134, p>0.05) but a significant increase in the exploration of the novel object D* when compared with the familiar objects A and B in the βNGF group ($F_{2,10}$ = 24.25, p<0.001; exploration of A vs D*: ***p<0.001, exploration of B vs D*: ***p< 0.001. n=6 in both groups. Data expressed as mean ± SEM.

4.4.3 Chronic i.c.v infusion of βNGF improves object recognition memory

Object recognition memory was tested with the three object NOR test. On training day there was no significant difference between the exploration of the objects ($F_{2,14} = 0.156$, $p>0.05$) or between the exploration of the treatment groups ($F_{2,14} = 1.355$, $p>0.05$). There was also no interaction between the exploration of the objects and treatment group ($F_{4,14} = 1.055$, $p>0.05$, figure 4e.i)

On testing day, object B was replaced with a novel object D*. There was no significant difference between the exploration of the treatment groups ($F_{2,14} = 1.443$, $p>0.05$) but there was a significant difference between the exploration of the objects ($F_{2,14} = 13.16$, $p<0.001$) and a significant interaction between the exploration of the objects and treatment group ($F_{4,14} = 3.233$, $p<0.05$, figure 4e.ii).

Bonferroni posttests revealed that the CON group explored the novel object D* significantly more than the familiar object A (mean percentage exploration of objects ± SEM: A = 23.42±2.59, D* = 37.85±3.33; $p<0.05$) but not the familiar object C (mean percentage exploration of object C = 38.73±2.14; $p>0.05$). There was no significant difference between the exploration of the two familiar objects B and C ($p>0.05$). There was no significant difference between the exploration of the objects in the VEH group (mean percentage exploration of the objects ± SEM: A = 30.77±3.02, D* = 38.47±5.86, C = 30.77±3.40; $p>0.05$). The βNGF group explored the novel object D* significantly more than both the familiar objects A and C (mean percentage exploration of objects ± SEM: A = 22.67±2.47, D* = 49.73±3.68, C = 27.61±1.66; $p<0.001$). There was no significant difference between the exploration of the two familiar objects A and C ($p>0.05$).

These data show that a six week chronic i.c.v. infusion of βNGF can induce an improvement in object recognition memory in the rat as measured by the three object NOR task.

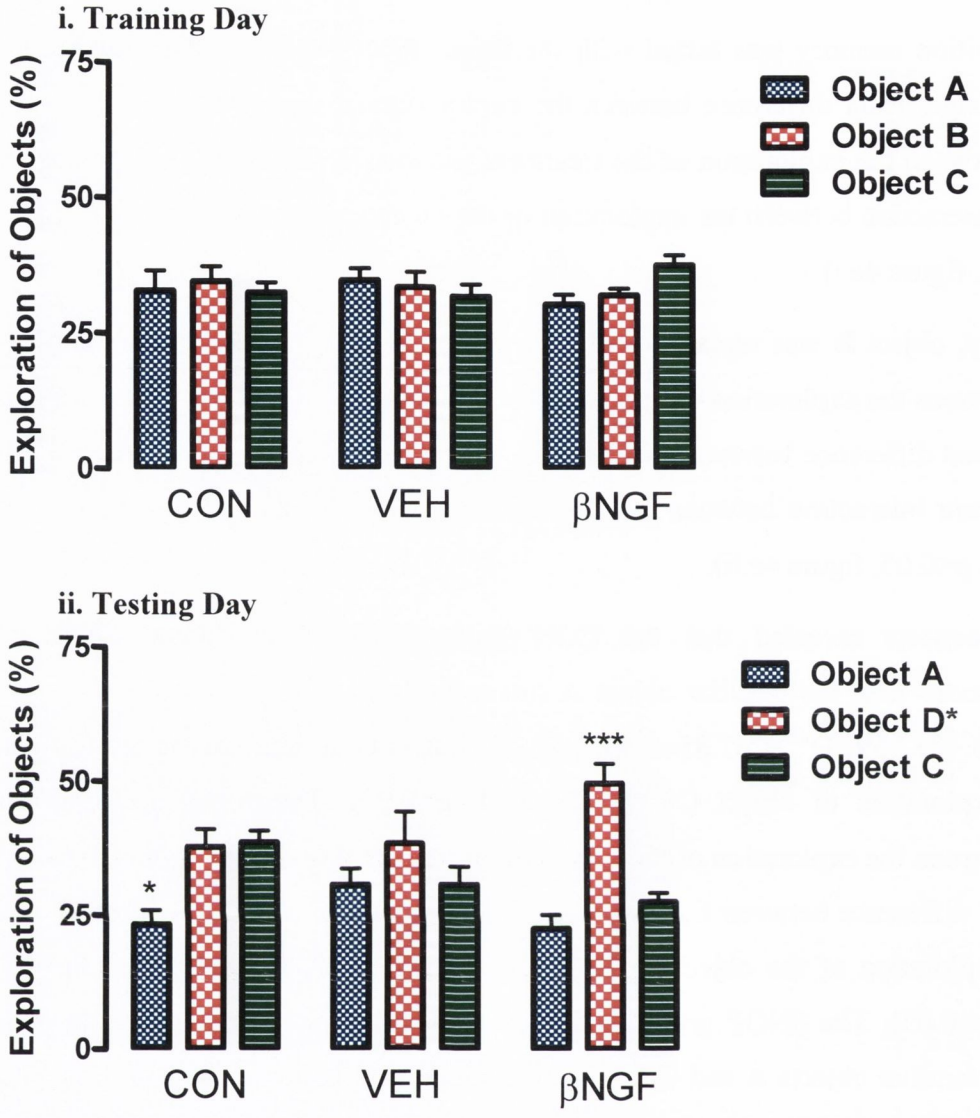


Figure 4e. Chronic i.c.v. infusion of βNGF improves object recognition memory(i) The was no significant difference found between the exploration of the objects on training day of the NOR task ($F_{2,14}$ = 0.156, $p>0.05$). (ii) On testing day, there was a significant difference in the exploration of the objects ($F_{2,14}$ = 13.16, $p<0.001$) with a significant increase in the exploration of the novel object D* when compared with the familiar objects A in the CON group (*$p<0.05$) and a significant increase in the exploration of the novel object D* when compared with the familiar objects A and C in the βNGF group (exploration of A vs D*: ***$p<0.001$, exploration of B vs D*: ***$p< 0.001$. n=6 in CON and βNGF, n=5 in VEH. Data expressed as mean ± SEM.

4.5.1 Chronic i.c.v. infusion of βNGF increases the concentration of βNGF in the contralateral hippocampus

There was no significant difference in the concentration of βNGF in the contralateral dentate gyrus between groups ($F_{2,14}$ = 2.067, $p>0.05$; figure 4f.i.). Mean βNGF concentration ± SEM ($pg.mg^{-1}$): CON = 132.0±17.62, VEH = 86.83±17.08, βNGF = 115.8±11.52.

There was a significant difference in the concentration of βNGF in the contralateral hippocampus between groups ($F_{2,14}$ = 7.74, $p<0.01$; figure 4e.ii.). Bonferroni's multiple comparison test revealed that there was a significant increase in βNGF concentration in the βNGF group when compared with the CON group ($p<0.01$) and VEH group ($p<0.05$). There was no significant difference in the concentration of βNGF between the CON and VEH groups ($p>0.05$). Mean βNGF concentration ± SEM ($pg.mg^{-1}$): CON = 81.72±14.37, VEH = 93.06±17.64, βNGF = 154.7±11.62.

There was no significant difference in the concentration of βNGF in the contralateral perirhinal cortex between groups, although the data suggest an increase in the βNGF group ($F_{2,14}$ = 3.279, $p=0.068$; figure 4f.iii.). Mean βNGF concentration ± SEM ($pg.mg^{-1}$): CON = 99.75±14.70, VEH = 144.3±16.90, βNGF = 191.9±37.53.

These data confirm that the exogenous βNGF was infusing throughout the lateral ventricle into the contralateral hippocampus, and possibly the perirhinal cortex.

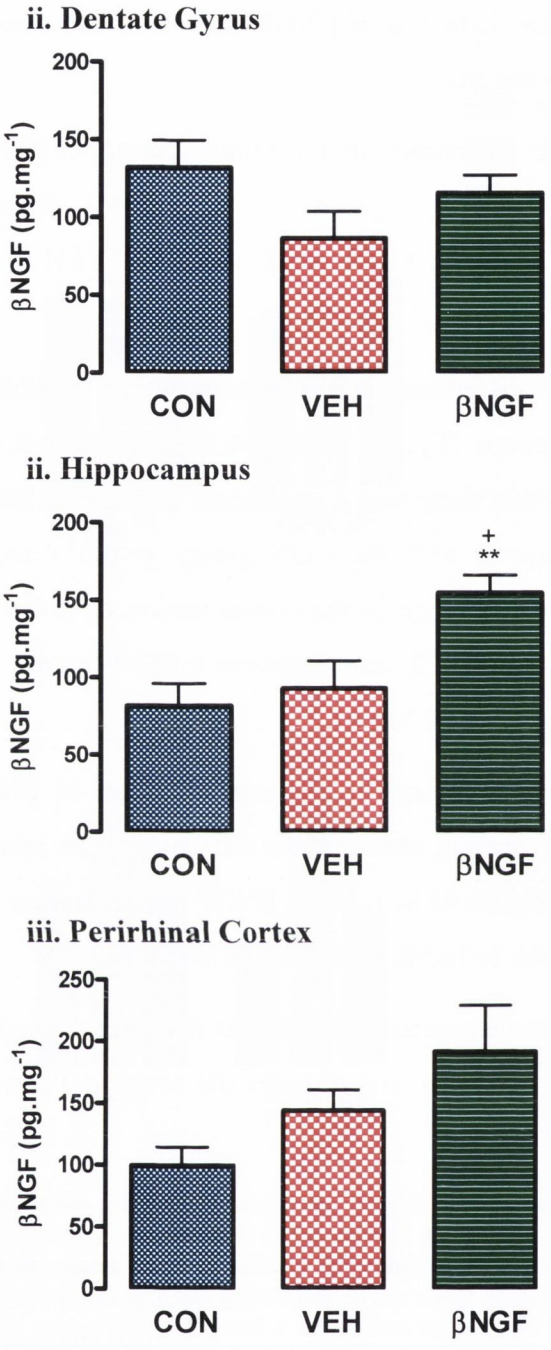


Figure 4f. Chronic i.c.v. infusion of βNGF increases the concentration of βNGF in the hippocampus(i) There was no significant difference between groups in the concentration of βNGF between groups in the dentate gyrus ($F_{2,14}$ = 2.067, $p>0.05$). (ii) The was a significant difference in the concentration of βNGF between groups in the hippocampus ($F_{2,14}$ = 7.74, $p<0.01$) with a significant increase in the βNGF group when compared with the CON (**$p<0.01$) and VEH ($^{+}p<0.05$) groups. n=6 in CON and βNGF, n=5 in VEH.(iii) There was a nonsignificant increase in βNGF concentration in the perirhinal cortex in the βNGF group, but this did not reach significance ($F_{2,14}$ = 3.279, p=0.068). n=6 in CON and βNGF, n=5 in VEH. Data expressed as mean ± SEM.

4.5.2 Chronic βNGF infusion does not affect BDNF concentration in the contralateral hippocampus, dentate gyrus or perirhinal cortex

There was no significant difference in the concentration of BDNF in the dentate gyrus between groups ($F_{2,14} = 0.599$, $p>0.05$; figure 4g.i.). Mean concentration of BDNF ± SEM ($pg.mg^{-1}$): CON = 236.5±28.22, VEH = 196.3±28.67, βNGF = 255.7±49.90.

There was no significant difference in the concentration of BDNF in the hippocampus between groups ($F_{2,13} = 0.192$, $p>0.05$; figure 4f.ii.). Mean concentration of BDNF ± SEM ($pg.mg^{-1}$): CON = 204.0±22.52, VEH = 191.1±16.33, βNGF = 183.0±29.95.

There was a significant difference in the concentration of BDNF in the perirhinal cortex between groups ($F_{2,14} = 3.849$, $p<0.05$; figure 4g.iii.). Bonferroni's multiple comparison test showed a significant increase in BDNF concentration in the VEH group when compared with the CON ($p<0.05$). There was no significant difference in the BDNF concentration between the CON and βNGF groups ($p>0.05$) or VEH and βNGF groups ($p>0.05$). Mean concentration of BDNF ± SEM ($pg.mg^{-1}$): CON = 207.7±18.02, VEH = 300.6±24.36, βNGF = 256.9±30.36.

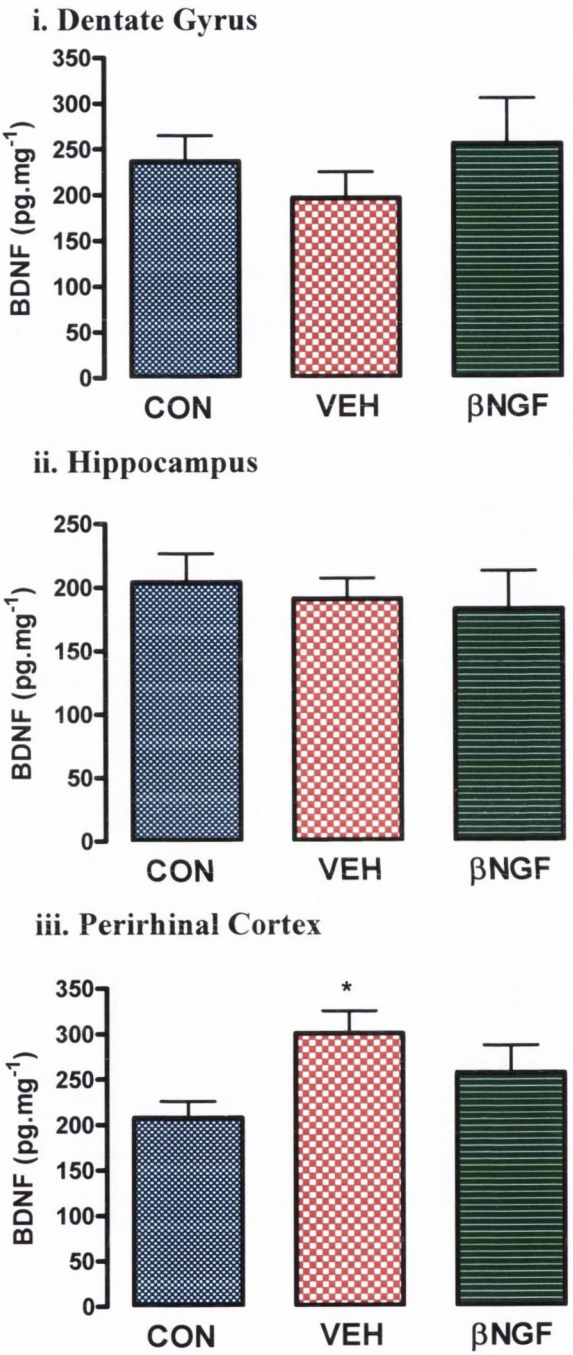


Figure 4g. Chronic i.c.v. βNGF infusion does not affect BDNF concentration in the hippocampus, dentate gyrus and perirhinal cortex(i) There was no significant difference between groups in the BDNF concentration in the dentate gyrus ($F_{2,14}$ = 0.599, $p>0.05$) (ii) There was no significant difference between groups in the BDNF concentration in the hippocampus ($F_{2,13}$ = 0.192, $p>0.05$) (iii)There was a significant difference in the BDNF concentration in the perirhinal cortex ($F_{2,14}$ = 3.849, $p<0.05$) with a significant increase in BDNF in the VEH group when compared with the CON group (*$p<0.05$). n=6 in CON and βNGF, n=5 in VEH. Data expressed as mean ± SEM.

4.5.3 Chronic βNGF infusion does not affect βNGF mRNA expression in the contralateral hippocampus, dentate gyrus or perirhinal cortex

There was no significant difference in the expression of βNGF mRNA between groups in the dentate gyrus ($F_{2,13}$ = 0.285, p>0.05; figure 4h.i.). Mean βNGF mRNA fold change ± SEM: CON = 1.00±0.12, VEH = 0.90±0.13, βNGF = 1.01±0.09.

There was no significant difference in the expression of βNGF mRNA between groups in the hippocampus ($F_{2,13}$ = 0.103, p>0.05; figure 4h.ii.). Mean βNGF mRNA fold change ± SEM: CON = 1.00±0.11, VEH = 1.01±0.12, βNGF = 0.96±0.04.

There was no significant difference in the expression of βNGF mRNA between groups in the perirhinal cortex ($F_{2,13}$ = 1.962, p>0.05; figure 4h.iii.). Mean βNGF mRNA fold change ± SEM: CON = 1.00±0.15, VEH = 1.31±0.23, βNGF = 0.86±0.10.

These data suggest that an exogenous infusion of βNGF does not induce an increase in endogenous βNGF mRNA transcription.

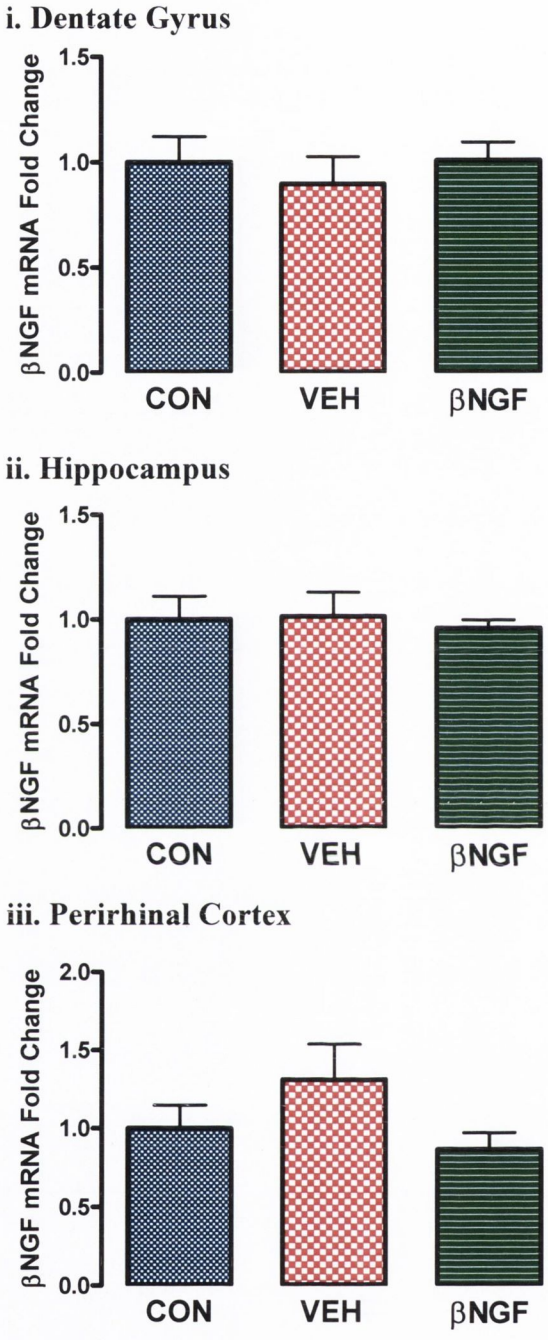


Figure 4h. Chronic i.c.v. βNGF infusion does not affect βNGF mRNA expression(i) There was no significant difference between groups in the expression of βNGF mRNA in the dentate gyrus ($F_{2,13}$ = 0.285, p>0.05) (ii) There was no significant difference between groups in the expression of βNGF mRNA in the hippocampus ($F_{2,13}$ = 0.103, p>0.05) (iii) There was no significant difference between groups in the expression of βNGF mRNA in the perirhinal cortex ($F_{2,13}$ = 1.962, p>0.05). Data expressed as fold change of control. n=6 in CON and βNGF, n=4 in VEH. Data expressed as mean ± SEM.

4.5.4 Chronic βNGF infusion does not affect BDNF mRNA expression in the contralateral hippocampus, dentate gyrus or perirhinal cortex

There was no significant difference in the expression of BDNF mRNA between groups in the dentate gyrus ($F_{2,14}$ = 0.010, p>0.05; figure 4i.i.). Mean BDNF mRNA fold change ± SEM: CON = 1.00±0.31, VEH = 1.04±0.11, βNGF = 1.03±0.20.

There was no significant difference in the expression of BDNF mRNA between groups in the hippocampus ($F_{2,14}$ = 0.565, p>0.05; figure 4i.ii.). Mean BDNF mRNA fold change ± SEM: CON = 1.00±0.30, VEH = 0.64±0.18, βNGF = 0.86±0.19.

There was no significant difference in the expression of BDNF mRNA between groups in the perirhinal cortex ($F_{2,14}$ = 3.350, p>0.05; figure 4i.iii.). Mean BDNF mRNA fold change ± SEM: CON = 1.00±0.23, VEH = 1.43±0.10, βNGF = 0.68±0.16.

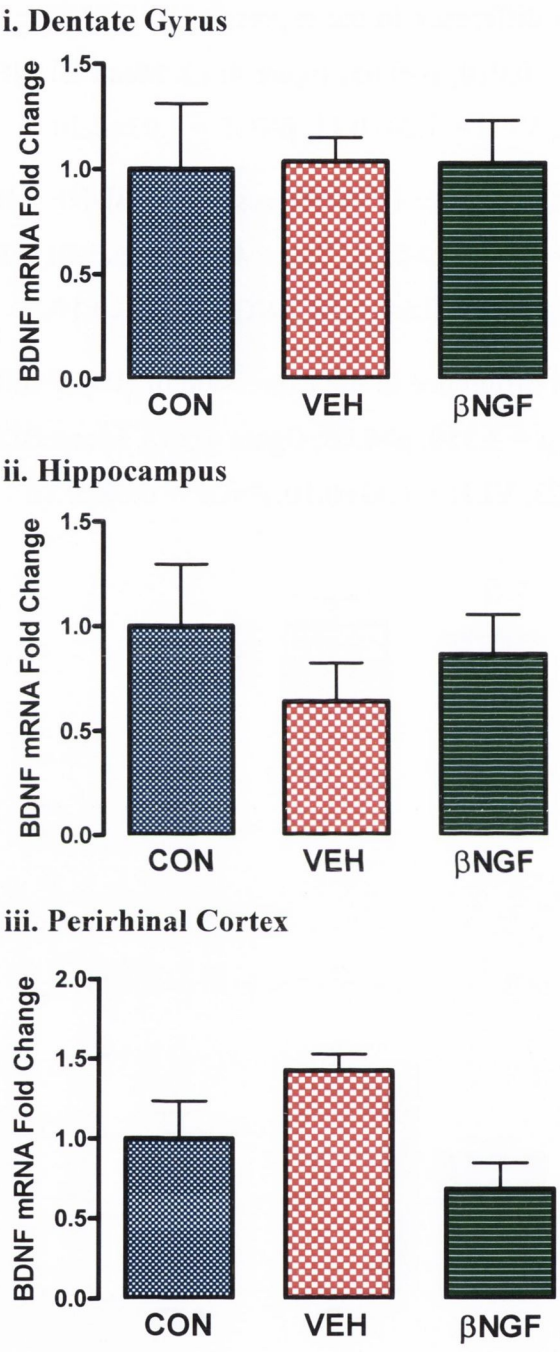


Figure 4i. Chronic i.c.v. βNGF infusion does not affect BDNF mRNA expression (i) There was no significant difference between groups in the expression of BDNF mRNA in the dentate gyrus ($F_{2,14}$ = 0.010, p>0.05) (ii) There was no significant difference between groups in the expression of BDNF mRNA in the hippocampus ($F_{2,14}$ = 0.565, p>0.05)(iii) There was no significant difference between groups in the expression of BDNF mRNA in the perirhinal cortex ($F_{2,14}$ = 3.350, p>0.05). Data expressed as fold change of control. n=6 in CON and βNGF, n=5 in VEH. Data expressed as mean ± SEM.

4.5.5 Chronic βNGF infusion increases the expression of Trk A mRNA in the contralateral hippocampus

There was no significant difference in the expression of Trk A mRNA between groups in the dentate gyrus ($F_{2,14}$ = 1.387, $p>0.05$; figure 4j.i). Mean βNGF mRNA fold change ± SEM: CON=1.00±0.24, VEH=2.99±1.21, βNGF=1.52±0.84.

There was a significant difference in the expression of Trk A mRNA between groups in the hippocampus ($F_{2,14}$ = 3.954, $p<0.05$; figure 4j.ii.). Bonferroni's multiple comparison test revealed a significant increase in the expression of Trk A mRNA in the βNGF group when compared with the CON group ($p<0.05$). Mean βNGF mRNA fold change ± SEM: CON=1.00±0.13, VEH=1.04±0.31, βNGF=2.75±0.83).

There was no significant difference in the expression of Trk A mRNA between groups in the perirhinal cortex ($F_{3,15}$ = 0.461, $p>0.05$; figure 4j.iii.). Mean βNGF mRNA fold change ± SEM: CON=1.00±0.34, VEH=1.53±0.44, βNGF=1.50±0.52.

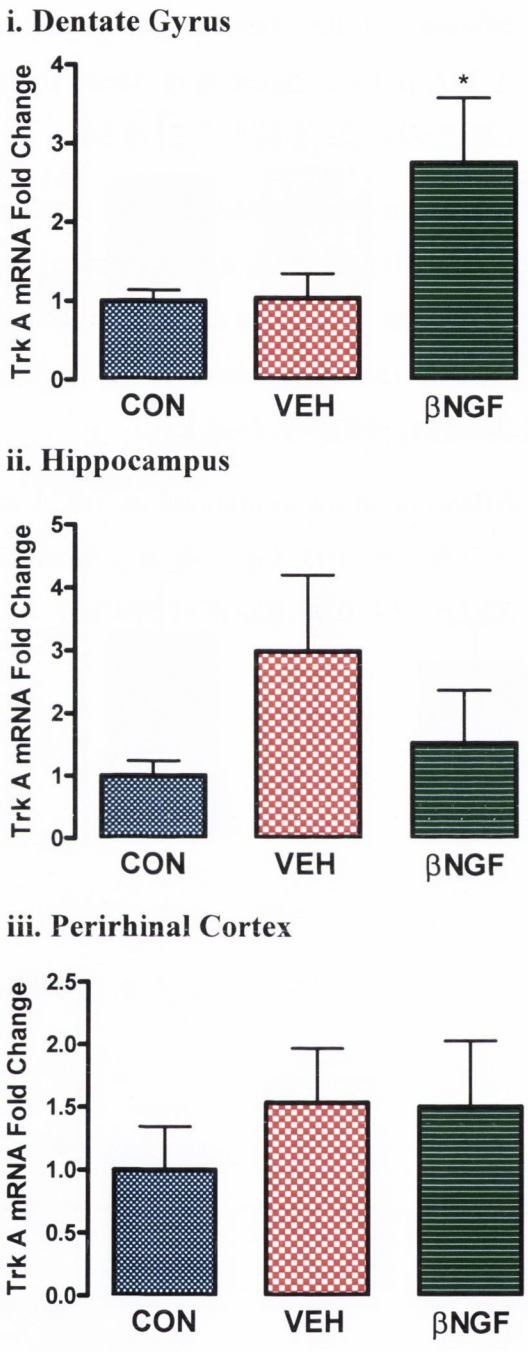


Figure 4j. Chronic i.c.v. βNGF infusion increases TrkA mRNA expression in the contralateral hippocampus(i) There was no significant difference between groups in the expression of Trk A mRNA in thehippocampus ($F_{2,14}$ = 1.387, $p>0.05$) (ii) There was a significant difference in the expression of Trk A mRNA in thedentate gyrus ($F_{2,14}$ = 3.94, $p<0.05$), with a significant increase in Trk A mRNA expression in the βNGF group when compared with the CON group (*$p<0.05$)(iii) There was no significant difference between groups in the expression of Trk A mRNA in the perirhinal cortex ($F_{3,15}$ = 0.461, $p>0.05$). Data expressed as fold change of control. n=6 in CON and βNGF, n=5 in VEH. Data expressed as mean ± SEM.

4.5.6 Chronic βNGF infusion increases Trk A protein expression in the contralateral hippocampus

There was a difference in the Trk A protein expression between groups in the hippocampus, however it did not reach significance ($F_{2,13}$ = 3.281, p=0.0702, figure 4k). Bonferroni's multiple comparison test revealed a significant increase in Trk A protein expression in the βNGF group when compared with the VEH group ($p<0.05$). Mean Trk A expression per Actin ± SEM: CON=0.48±0.07, VEH=0.32±0.11, βNGF=0.71±0.14.

4.5.7 Chronic βNGF infusion increases p44ERK phosphorylation in the contralateral dentate gyrus

There was no significant difference in the ratio of p42ERK to total ERK42 protein expression between groups in the dentate gyrus ($F_{2,14}$ = 0.43, $p>0.05$; figure 4l.i.). Mean p42ERK expression per ERK42 expression ± SEM: CON=0.07±0.01, VEH=0.06±0.01, βNGF=0.07±0.01.

There was a significant difference in the ratio of p44ERK to total ERK44 protein expression between groups in the dentate gyrus ($F_{2,14}$ = 10.57, $p<0.01$; figure 4l.ii.). Bonferroni's multiple comparison test revealed a significant increase in the ratio of p44ERK to ERK44 in the βNGF group when compared with the CON group ($p<0.01$) and VEH group ($p<0.01$). Mean p44ERK expression per ERK44 expression ± SEM: CON=0.21±0.06, VEH=0.15±0.04, βNGF=0.51±0.07.

There was no significant difference in the ratio of p42ERK to total ERK42 protein expression between groups in the hippocampus ($F_{2,14}$ = 1.22, $p>0.05$; figure 4l.iii.). Mean p42ERK expression per ERK42 expression ± SEM: CON=1.13±0.15, VEH=1.06±0.18, βNGF=1.38±0.14.

There was no significant difference in the ratio of p44ERK to total ERK44 protein expression between groups in the hippocampus ($F_{2,14}$ = 0.54, $p>0.05$; figure 4l.iv.). Mean p44ERK expression per ERK44 expression ± SEM: CON=1.05±0.13, VEH=0.87±0.15, βNGF=0.87±0.14.

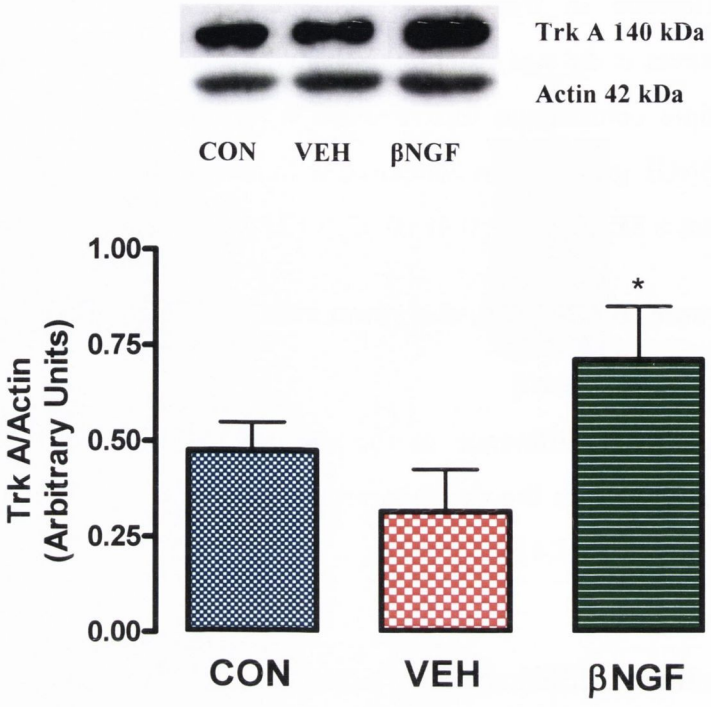


Figure 4k. Chronic i.c.v. βNGF infusion increases TrkA protein expression in the contralateral hippocampus There was no significant difference in the expression of Trk A protein in the hippocampus across all groups ($F_{2,13} = 3.28$, $p=0.07$), but there was a significant increase in Trk A protein expression in the βNGF group when compared with the VEH group (*$p<0.05$). Data are expressed per Actin. n=6 in CON and n=5 βNGF & VEH. Data expressed as mean ± SEM.

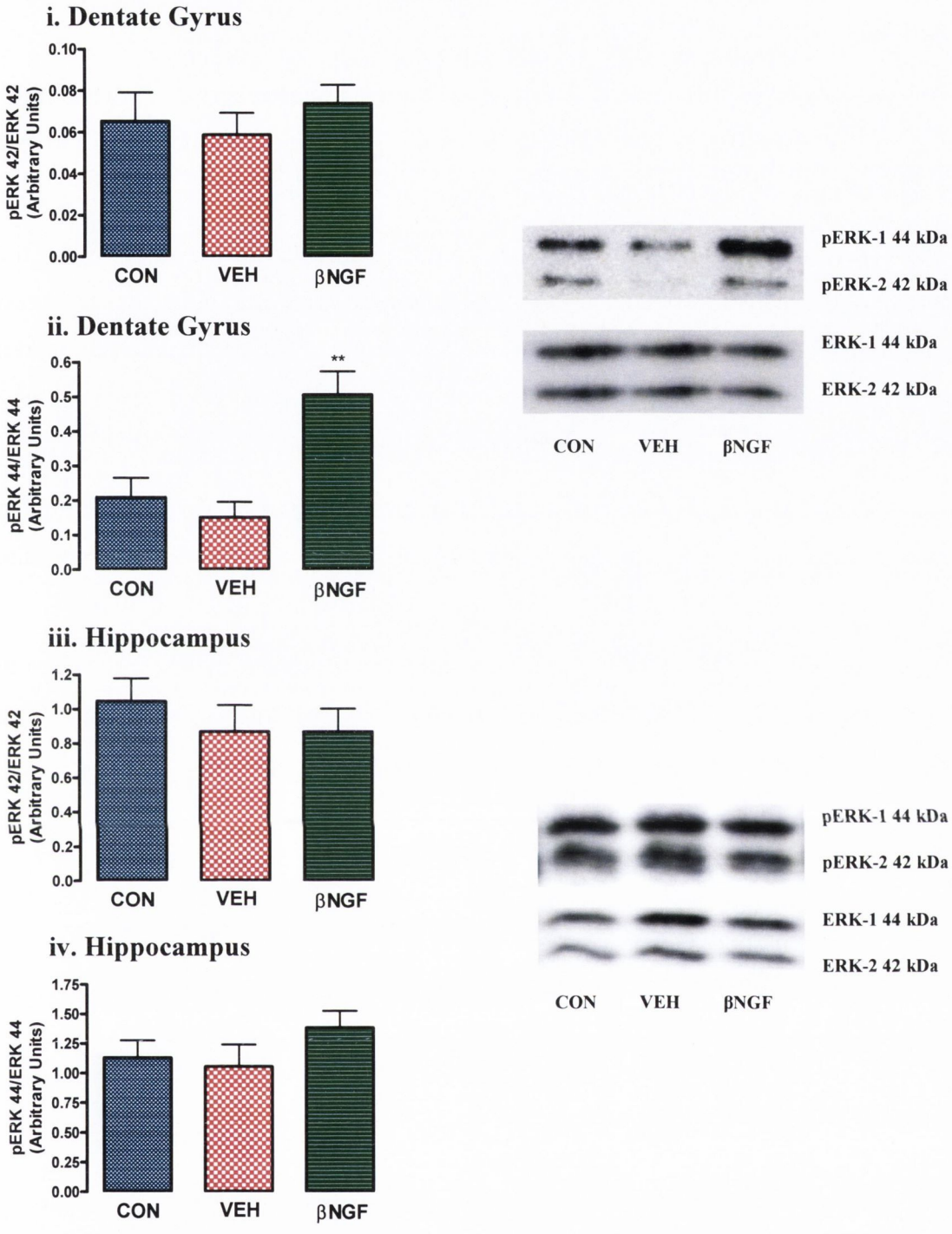


Figure 4l. Chronic i.c.v. βNGF infusion increases p44ERK phosphorylation in the contralateral dentate gyrus.(i) There was no significant difference between groups in p42ERK phosphorylation in the dentate gyrus ($F_{2,14}$ = 0.43, p>0.05) (ii) There was a significant difference p44ERK phosphorylation in the dentate gyrus ($F_{2,14}$ = 10.57, p<0.01), with a significant increase in p44ERK phosphorylation in the βNGF group when compared with CON (**p<0.01) and VEH (**p<0.01) groups (iii) There was no significant difference between groups in p42ERK phosphorylation in the hippocampus ($F_{2,14}$ = 1.22, p>0.05) (iv) There was no significant difference between groups in p44ERK phosphorylation in the hippocampus ($F_{2,14}$ = 0.54, p>0.05) Data expressed per total ERK expression. n=6 in CON and βNGF, n=5 in VEH. Data expressed as mean ± SEM.

4.5.8 Chronic βNGF infusion increases synapsin I in the contralateral hippocampus

There was no significant difference in synapsin I protein expression between groups in the dentate gyrus ($F_{2,14}$ = 0.316, $p>0.05$; figure 4m.i.). Mean synapsin I expression per GAPDH expression ± SEM: CON=0.76±0.17, VEH=0.56±0.11, βNGF=0.70±0.18.

There was a significant difference in synapsin I protein expression between groups in the hippocampus ($F_{2,14}$ = 4.885, $p<0.05$; figure 4m.ii.). Bonferroni's multiple comparison test revealed a significant increase in synapsin I expression in the βNGF group when compared with the VEH group ($p<0.05$). Mean synapsin I expression per GAPDH expression ± SEM: CON=0.64±0.09, VEH=0.58±0.05, βNGF=0.88±0.06.

There was no significant difference in synaptophysin protein expression between groups in the dentate gyrus ($F_{2,14}$ = 0.774, $p>0.05$; figure 4m.iii.). Mean synaptophysin expression per GAPDH expression ± SEM: CON=0.56±0.04, VEH=0.48±0.06, βNGF=0.60±0.08.

There was no significant difference in synaptophysin protein expression between groups in the hippocampus ($F_{2,14}$ = 0.054, $p>0.05$; figure 4m.iv.). Mean synaptophysin expression per GAPDH expression ± SEM: CON=0.64±0.14, VEH=0.59±0.11, βNGF=0.64±0.10.

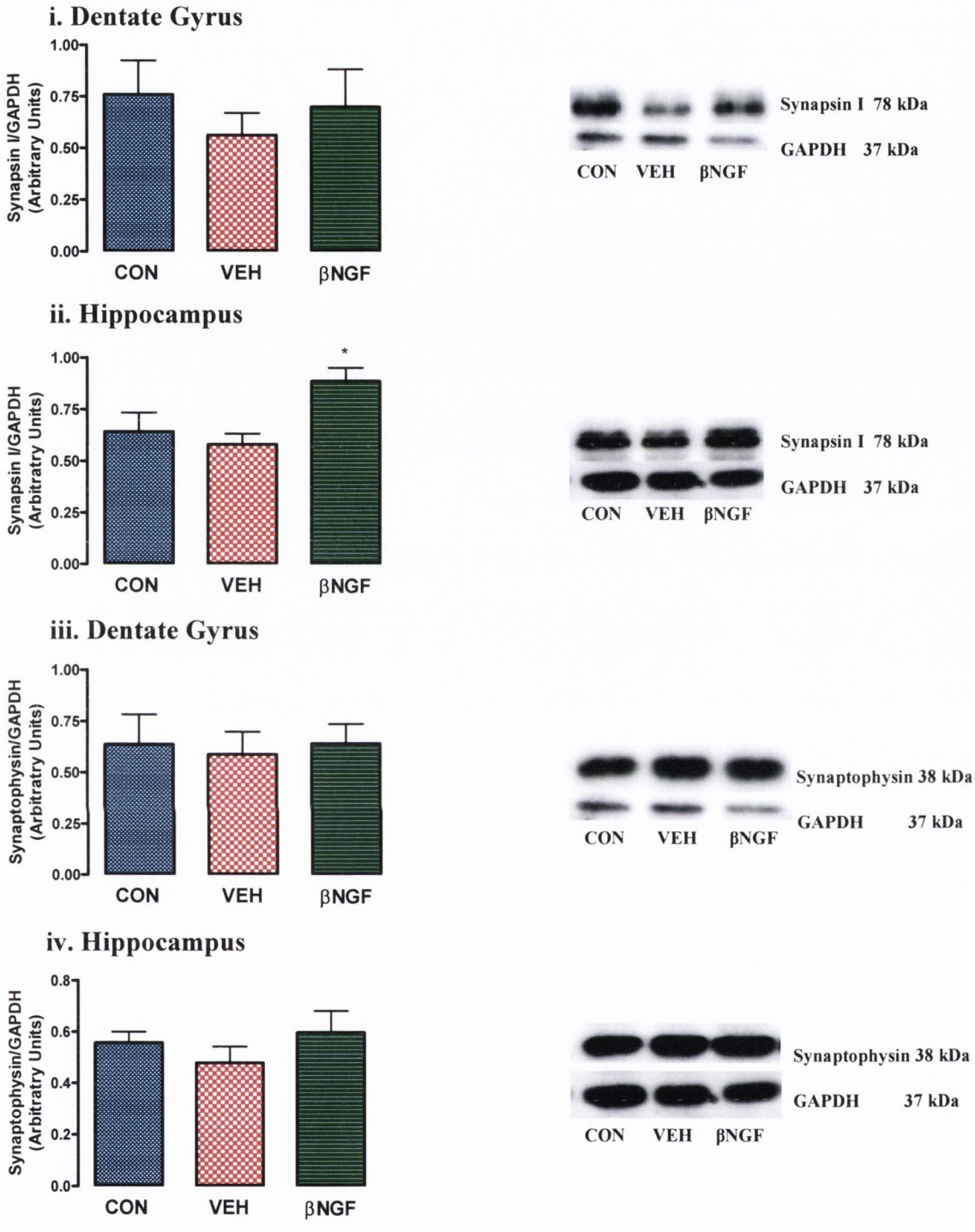


Figure 4m. Chronic i.c.v. βNGF infusion increases synapsin I expressin in the contralateral hippocampus(i) There was no significant difference between groups in synapsin I expression in the dentate gyrus ($F_{2,14}$ = 0.316, $p>0.05$) (ii) There was a significant difference in synapsin I expression in the hippocampus ($F_{2,14}$ = 4.885, $p<0.05$), with a significant increase in synapsin I in the βNGF group when compared with the VEH (*$p<0.05$) group. There was no significant difference between groups in synaptophysin expression in the (iii) dentate gyrus ($F_{2,14}$ = 0.774, $p>0.05$) or (iv) hippocampus ($F_{2,14}$ = 0.054, $p>0.05$) Data expressed per GAPDH expression. n=6 in CON and βNGF, n=5 in VEHData expressed as mean ± SEM.

4.5.9 Chronic βNGF infusion does not increase cell proliferation in the ipsilateral dentate gyrus

There was a significant difference in the percentage of BrdU+ nuclei between groups ($F_{2,14}$ = 4.043, $p<0.05$; figure 4n). Bonferroni's multiple comparison test revealed an increase in the percentage of BrdU+ nuclei in the βNGF group when compared with CON, however this did not quite reach significance ($p=0.058$). There was no significant differences in the percentage of BrdU+ nuclei between the βNGF group when compared with VEH ($p<0.05$) or between the CON and VEH groups ($p>0.05$). Mean percentage of BrdU+ nuclei ± SEM: CON=5.13±0.21, VEH=5.28±0.38, βNGF=6.20±0.30.

4.5.10Chronic βNGF infusion attenuates an increase in apoptosis in the ipsilateral dentate gyrus in response to surgery

Apoptosis was measured using the DeadEnd™ Fluorometric TUNEL System (see 2.6.4). Fragmented DNA are labelled with a marker that fluoresces when excited at 488nm. There is a significant difference in the mean fluorescent intensity at 488nm between all groups ($F_{2,14}$ = 4.361, $p<0.05$; figure 4o). Bonferroni's multiple comparison test revealed that there was a significant decrease in mean fluorescent intensity in the βNGF gourp when compared with the VEH group. There is an increase in the mean fluorescent intensity in the VEH group when compared with the CON group, however this does not reach significance ($p=0.098$). There is no significant difference in the mean fluorescent intensity between the CON and βNGF groups ($p>0.05$). Mean fluorescent intensity @ 488nm ± SEM: CON=427.4±68.83, VEH=651.2±48.73, βNGF=370.4±78.88.

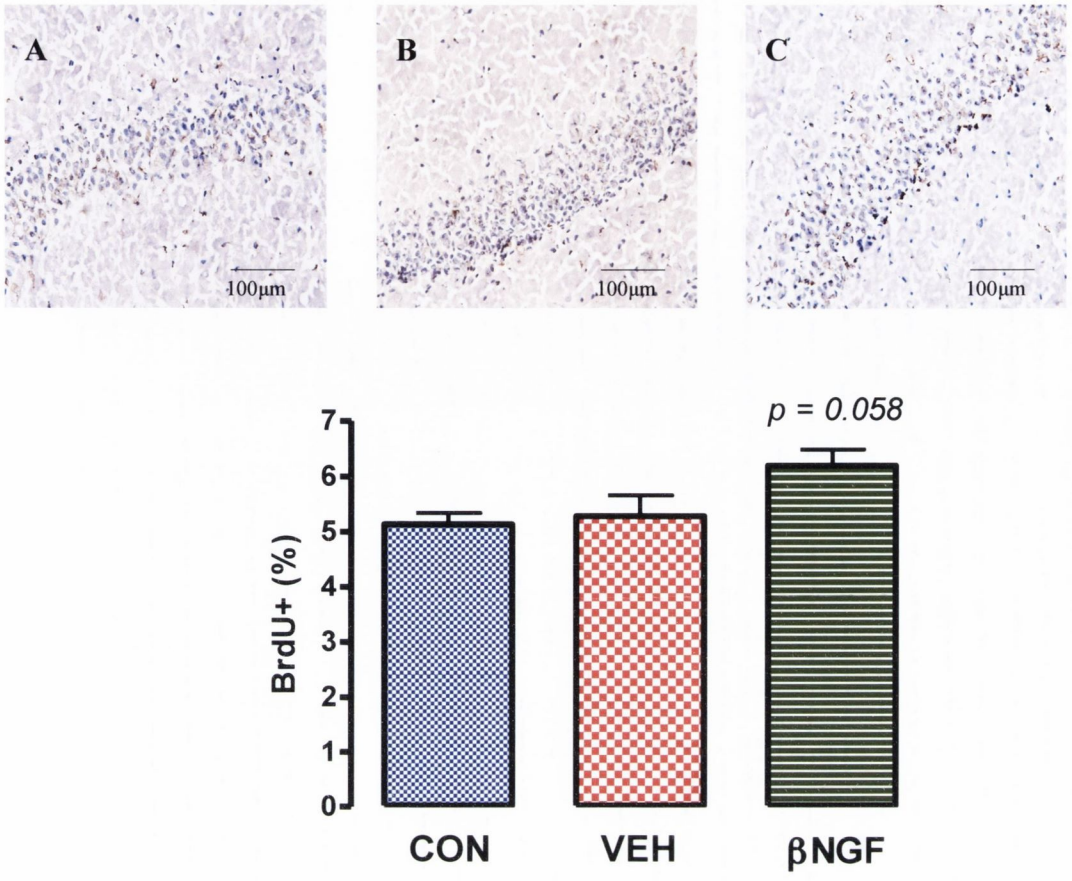


Figure 4n. Chronic i.c.v. βNGF infusion does not increase cell proliferation in the ipsilateral dentate gyrus. There was a significant difference in the percentage of BrdU+ nuclei between groups ($F_{2,14} = 4.043$, $p<0.05$). There was an increase in the percentage of BrdU+ nuclei in the βNGF group when compared with the CON group, however this did not quite reach significance ($p=0.058$). A = CON (n=6) B = VEH (n=5) C = βNGF (n=6). Blue staining = all nuclei (hematoxlin), brown staining = BrdU+ nuclei (DAB Chromagen). Data expressed as mean ± SEM.

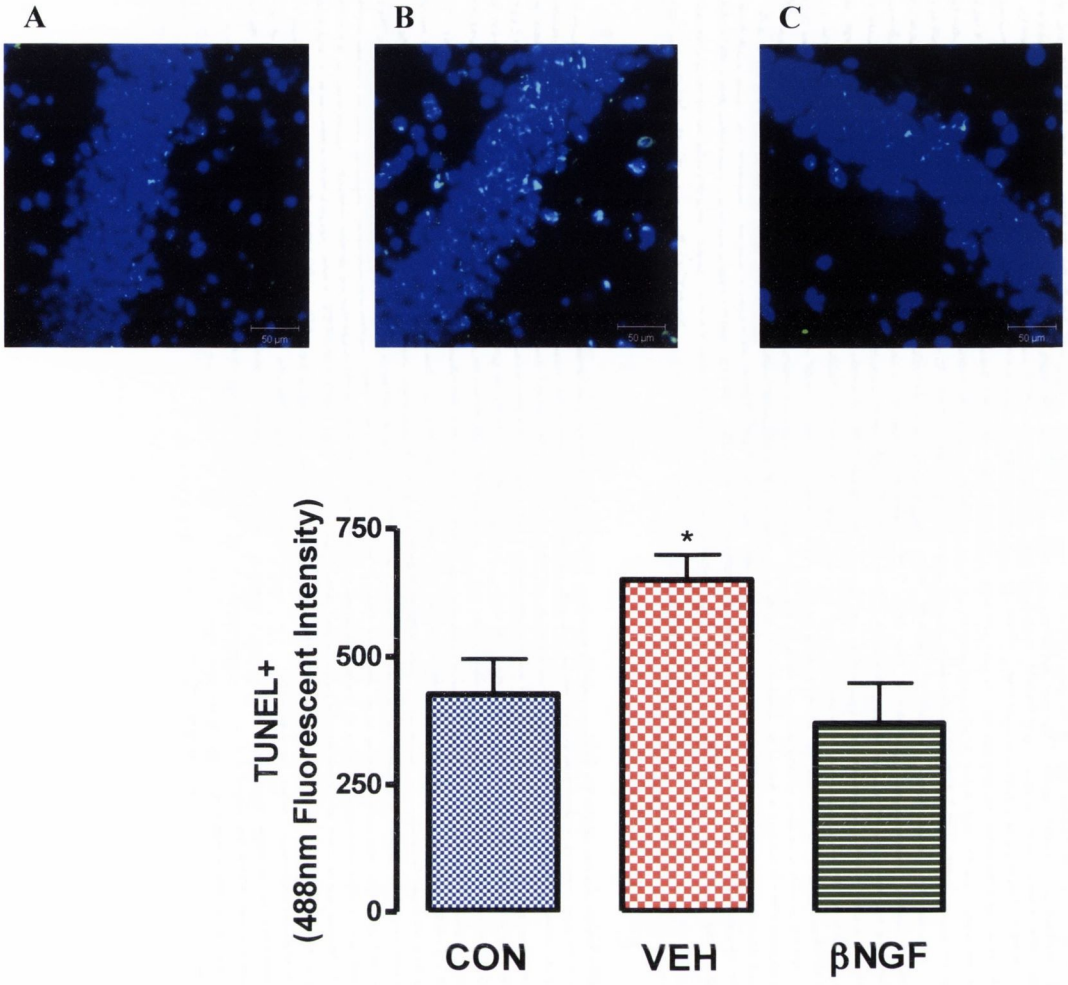


Figure 4o. Chronic i.c.v. βNGF infusion attenuates an increase in apoptosis in the ipsilateral dentate gyrus in response to surgery. There was a significant difference in the mean fluorescent intensity at 488nm between groups ($F_{2,14}$ = 4.361, $p<0.05$). There was a significant decrease in mean fluorescent intensity at 488nm in the βNGF group when compared with the VEH group (*$p<0.05$). There is an increase in mean fluorescent intensity at 488nm in the VEH group when compared with the CON group however this does not reach significance ($p=0.098$) A = CON (n=6) B = VEH (n=5) C = βNGF (n=6). Blue staining = all nuclei (Hoescht), green staining = apoptotic cells (TUNEL+ nuclei). Data expressed as mean ± SEM.

4.6 Discussion

The aim of this study was to assess the efficacy of an exogenous infusion of βNGF in enhancing memory function. Firstly, data demonstrate that a single infusion of 4ng of βNGF into the lateral ventricle can induce an improvement in object recognition memory. Furthermore, a continuous infusion of βNGF at the same dose into the lateral ventricle for 42 days also improves object recognition memory. This enhancement of memory seems to be mediated via Trk receptor activation of the Ras/ERK pathway because there is an increase in Trk A receptor expression in the hippocampus, an increase in the phosphorylation of ERK and an increase in synapsin I expression.

The NOR task used in this study is a hippocampal-dependent memory task but is also known to be dependent upon the perirhinal cortex as lesions in this region can iduce impairments in rodents and non-human primates (Brown and Aggleton, 2001). It could be argued that because the infusion of βNGF is adjacent to the hippocampus, a spatial task such as the OD task would have been more suitable since performance on this task is more heavily reliant upon the hippocampus. However, rats have excellent spatial memory and as this study was designed to measure an improvement in memory function, there was the possibility of a ceiling effect in a spatial task, with the VEH and control group performing equally as well as the βNGF group. Therefore to ensure a greater chance of detecting an improvement in memory, the 3 object NOR task was chosen because studies in our laboratory have repeatedly shown that control rats are unable to learn this task (Bechara and Kelly, unpublished). This result is confirmed with data in this chapter, as the control and VEH groups in both the single and chronic infusion studies do not explore the novel object significantly more that the two familiar objects 24 hours post-training whereas the βNGF group do explore the novel object significantly more, indicating that βNGF can induce an improvement in this type of memory.

Neurochemical analysis revealed that there was a significant increase in the concentration of βNGF in the contralateral hippocampus in βNGF group. The increase found in the hippocampus confirms the efficacy of the Alzet® Osmotic Pumps. The lateral ventricles lie adjacent to the hippocampus and therefore an increase in the concentration of βNGF in the hippocampus contralateral to the infusion would also suggest that the infused βNGF is traversing the whole ventricular system. There is also an increase in the concentration of βNGF in the contralateral perirhinal cortex in the βNGF group, although it is not

significant. Given the important role that the perirhinal cortex plays in recognition memory, this increase could be a significant factor in the improvement in the memory that is observed in this study. The perirhinal cortex makes reciprocal connections to the CA1 region of the hippocampus via the entorhinal cortex and it is possible that an increase in stimulation of the perirhinal cortex from the recognition component of the behavioural task is inducing an increase in retrograde transport of βNGF from the hippocampus into that region to facilitate learning on this task. Because the entorhinal cortex has projections from the perirhinal cortex and projects into the hippocampus, it has been argued that this region in an important gateway for hippocampal signalling and may encode for both familiarity and position of an object by incorporating information from both of these regions (Xiang and Brown, 1998). It may be interesting in a future study therefore, to examine the neurochemical changes in the entorhinal cortex in addition to the hippocampus and perirhinal cortex.

Whilst there were increases found in βNGF concentration, no differences in βNGF mRNA expression between groups were observed in the dentate gyrus, hippocampus or perirhinal cortex. These data would suggest that an exogenous infusion of βNGF cannot stimulate an endogenous increase in the transcription of βNGF. This provides further support for the therapeutic benefit of utilising environmental enrichment or exercise to induce memory improvements as they upregulate endogenous pathways to enhance production of neurotrophins such as βNGF and BDNF.

In addition to the increase in βNGF in the contralateral hippocampus in the βNGF group, there is also an increase in Trk A receptor protein and mRNA expression in the contralateral hippocampus. It is likely that this upregulation is a functional response to the increased availability of βNGF in the region and will enhance the amount of Trk A that is able to bind to βNGF and induce intracellular signalling cascades. Activated NGF-TrkA complexes can also be internalised and transported retrogradely down the axon and continue to activate downstream signalling transduction pathways whilst being transported (Bhattacharyya *et al.*, 1997, Poo, 2001). This retrograde transport of complexes may be another explanation for the increase in βNGF concentration in the perirhinal cortex, however this would have to be confirmed with analysis of Trk A protein and mRNA expression in the perirhinal cortex. Unfortunately, there is no commercially available antibody for phosphorylated Trk A and therefore it was not possible to directly measure whether there was an increase in receptor activation. A chronic infusion of NGF has been

reported to rescue reductions in Trk A expression in aged rats, but this was with a significantly higher dose of 100μg (Niewiadomska *et al.*, 2006). This study also reports that there is an improvement in spatial memory in the aged rats treated with an infusion of NGF, but NGF-treated young rats displayed no improvement in spatial memory and neither group exhibited an improvement in recognition memory. However the authors used five objects in the OD and NOR tasks which is a significantly higher memory load than the tasks used in the current study.

To further analyse whether an increase in Trk A receptor expression is indicative of an increase in the downstream signalling pathways associated with Trk activation, the phosphorylation of ERK was analysed. Data show an increase in the phosphorylation of ERK1 but not ERK2 in the dentate gyrus of the βNGF group and no difference in the phosphorylation of ERK1/2 in the hippocampus. This result is interesting because the increase in βNGF concentration is specific to the hippocampus and not the dentate gyrus and therefore it would be expected that any downstream signalling changes would be observed in the hippocampus. It is known however, that there is transport of neurotrophins from their site of uptake all along the axon and long-range signalling may be responsible for many neurotrophin actions including plasticity related changes (Poo, 2001). Therefore, this process likely plays an important role in the ERK1 phosphorylation in the dentate gyrus shown in this study. This also points to the important role that βNGF may be playing in neuronal survival and maintenance in the dentate gyrus, particularly with respect to adult born neurons. Both ERK1 and ERK2 are upregulated during memory tasks (Blum *et al.*, 1999, Huang *et al.*, 2010) but it is more frequently reported that ERK2 rather than ERK1 is crucial for memory function (English and Sweatt, 1996, Selcher *et al.*, 2001, Satoh *et al.*, 2007). These data suggest that the two ERK isoforms are differentially regulated, thus there may be an βNGF-induced specific ERK1 activation occuring in this study. Further analysis of upstream regulators of ERK would need to be analysed to confirm this, such as MEK1/2, although there are limitations regarding the specificity of blockers in this signalling pathway and these are currently hampering efforts to fully dissect the specific roles of ERK1 and ERK2 in learning and memory. Downstream substrates of ERK, such as activation of CREB, could also be analysed to further elucidate the role that this protein has in the βNGF-induced memory improvement.

Interestingly, whilst there are no differences in the concentration of BDNF in the contralateral hippocampus or dentate gyrus between groups, there is a significant increase

in BDNF concentration in the contralateral perirhinal cortex in the VEH group. This is an unusual result, as it would not be expected that an infusion of a vehicle solution would interact with neurochemical pathways in the perirhinal cortex. This increase could be associated with increased stimulation in the perirhinal cortex whilst the rats are exploring the objects during the NOR task because it has been reported that BDNF can be upregulated during training on a behavioural task (Hall *et al.*, 2000a). This upregulation would typically be associated with successful learning on a task, and whilst there is a small increase in exploration of the novel versus familiar objects in the VEH group, it is not sufficient to conclude that they have learnt the task. Thus, if the increase was due to participation in the task alone and not associated with learning, a corresponding increase should be seen in both the CON and βNGF groups because they were exposed to the objects for an equal amount of time to the VEH group.

The results from this study show that there is βNGF-induced upregulation of signalling pathways associated with memory performance (in particular the Ras/ERK pathway), but the data also suggest that chronic βNGF can induce morphological changes that may be a significant factor in memory improvement. There is a significant increase in the expression of synapsin I in the hippocampus of the βNGF group, but there are no differences in the expression of synaptophysin in the hippocampus or dentate gyrus across all groups. Synapsin is important for synaptic plasticity and neurotransmission, in particular it is important in the clustering and release of synaptic vesicles in to the active zone of the synaptic bouton (Cesca *et al.*, 2010). There are also increases in synapsin reported during synaptogenesis (Lohmann *et al.*, 1978). Synaptophysin is the most abundantly expressed synaptic vesicle protein, hence its common use as a marker for synaptogenesis. However, it is not vital for neurotransmission (McMahon *et al.*, 1996). This suggests that in this study there is an βNGF-induced enhancement of synaptic plasticity via more efficient neurotransmitter release rather than synaptogenesis. This is because it would be expected that an increase in the number of functional synapses would be associated with an increase in both synapsin and synaptophysin.

Whilst there may not be a significant increase in synaptogenesis, there is an increase in cell proliferation in the ipsilateral dentate gyrus in the βNGF group, suggesting that βNGF may play a role in enhancing adult hippocampal neurogenesis. Due to time constraints it was not possible to confirm that the proliferating cells were neurons via co-labelling, however the majority of BrdU+ nuclei were localised to the subgranular zone of the dentate gyrus

which would suggest that these cells are proliferating neurons. It has been suggested that β is important for the survival and growth of neurons rather than directly stimulating an increase in proliferation, however this is the first study to directly test this hypothesis with a chronic infusion of βNGF. Frielingsdorf and colleagues (2007) infused NGF i.c.v. for six or twenty days and found that NGF promoted increased survival of neurons following twenty days of infusion using the Alzet® Osmotic minipump. Their protocol only measures proliferation after six days of NGF infusion however, as they injected the rats with BrdU within the first week of minipump implantation. This study therefore, provides the first evidence to suggest that a chronic NGF infusion can increase neurogenesis in the dentate gyrus and this may be associated with memory improvements. The increase in proliferation is less marked than the enrichment-induced increase reported in Chapter 3, which suggests that the environmental enrichment protocol that is used in the previous chapter is likely to be inducing a number of different neurochemical pathways that may be associated with enhancing the neurogenic profile in the dentate gyrus and is not fully dependent on βNGF. It is also important to confirm that these new neurons are important in the memory improvements associated with the βNGF group, and therefore double-labelling for immediate-early genes such as *c-fos* or *Arc* and BrdU would enable identification of the proportion of these new neurons that are active during a behavioural task.

Due to the vast amount of research that studies the neuroprotective role that NGF can play in the brain, levels of apoptosis were measured in the ipsilateral dentate gyrus via TUNEL staining. Interestingly, whilst there was no difference in the amount of TUNEL+ staining between the control and βNGF group, there was a significant increase in TUNEL+ staining in the VEH group. An increase in apoptosis in this group may indicate that the i.c.v. surgery and continuous infusion has caused injury to the rats and that the βNGF group are protected against this damage. In this context, recent evidence that NGF infusion can reduce apoptosis levels and infarct volume in middle cerebral artery occlusion is of relevance (Yang *et al.*, 2011). Nevertheless, Alzet® Osmotic minipumps are extensively used in the literature and there have been no reports of increases in apoptosis following their implantation, and many studies use cytochrome c as a control molecule for neurotrophin infusions because it has no known extracellular actions (Kobayashi *et al.*, 1997, Willson *et al.*, 2008). Therefore this result is unusual, but it may provide further evidence for the neuroprotective role that βNGF has in the brain. To fully confirm this

result, it would be important to assess other markers of cellular apoptosis such as activity of members of the caspase family.

It could be argued that the VEH group show a deficit in performance on the NOR task because of this increase in apoptosis in the dentate gyrus, and therefore the results actually show that βNGF can rescue this deficit however the CON group that were surgery naive were also unable to successfully perform the NOR task and therefore βNGF is inducing an improvement in memory with respect to these rats. To further study whether it is the surgery and infusion or the vehicle solution that is causing an increase in apoptosis, an additional control group that were saline-infused could be used. To assess whether the VEH group has a deficit in their recognition memory, the 2 object NOR task could be used because control rats can successfully perform this task.

This study reports that both a single and a continuous infusion of βNGF can induce a significant improvement in recognition memory.Continuous βNGF infusion is associated with an upregulation of Trk A and synapsin in the hippocampus and an increase in ERK1 phosphorylation in the dentate gyrus. This suggests that the βNGF-induced improvement in recognition memory is mediated via the Ras/ERK signalling pathway and may enhance synaptic plasticity. In addition, there is an increase in cell proliferation in the dentate gyrus of βNGF-infused rats which provides novel evidence for a βNGF-mediated enhancement of adult hippocampal neurogenesis. Previous studies that infuse NGF use doses of between 500ng and 13μg for single infusions and 30μg and 100μg for chronic infusions ranging from 6 to 28 days (DeKosky *et al.*, 2004, Jakubowska-Dogru and Gumusbas, 2005, Frielingsdorf *et al.*, 2007, Jiang *et al.*, 2008). This study uses a significantly lower dose than this in both the single and chronic infusions of βNGF, yet still demonstrates a significant improvement in recognition memory and an associated enhancement of synaptic functioning and an increase in neurogenesis. This dose mimics the increases that are associated environmental enrichment and is therefore more physiologically relevant than previous studies. This helps to elucidate the cellular mechanisms that underpin the enrichment-induced memory improvements and confirms that they may be, at least in part, due to an increase in βNGF.

Chapter 5: Evaluation of the neuroprotective properties of long-term environmental enrichment in the ageing rat

5.1 Introduction

Age-related cognitive decline is universally accepted to occur in adults and, although not pathological, can cause loss of memory and other cognitive functions that reduce the quality of an individual's life. Due to the advances in medicine in the past decades, there has been an increase in life expectancy in the western world and with this increase there has also been a corresponding increase in age-related neurodegenerative disorders such as Alzheimer's disease in the human population. Consequently, there is a vast amount of funding being awarded for research into developing preclinical diagnostic tools and drug therapies to reduce the future public health burden of an ever increasing ageing population. Whilst the increase in the incidence of age-related neurodegenerative disorders poses a serious concern regarding the enhanced socio-economic burden for countries, the majority of people will not develop a dementia. Nevertheless, they are likely to undergo some cognitive decline, particularly in memory and executive function, with age and this can lead to severe effects upon their lifestyle (Plassman *et al.*, 2008). It is possible however, to induce cognitive improvements via simple lifestyle changes without the need for drug therapies, such as via exercise or cognitive stimulation (Fratiglioni *et al.*, 2004, Ploughman, 2008). Using such techniques to alter neurophysiology and improve cognition via our own body's natural mechanisms also has the advantage that there are likely to be no unwanted side effects or interactions with other medications because these techniques simply utilise and modulate the body's own physiological processes.

A longitudinal rat model of normal ageing enhances the power of any analyses being performed because it is possible to track changes in cognitive function throughout the rat's lifespan. Rats exhibit deficits in spatial memory with age (for review, see Rosenzweig and Barnes, 2003) and there are reports of humans showing similar deficits in an equivalent spatial task (Moffat *et al.*, 2001). Neurogenesis is also significantly reduced in the ageing brain, as shown in rodent and non-human primate studies (Kuhn *et al.*, 1996, Kempermann *et al.*, 1998, Gould *et al.*, 1999b). It has also been shown that adult hippocampal neurogenesis in the aged rat is partially predictive of performance in the Morris water maze task (Drapeau *et al.*, 2003). It has been argued that the reduction in neurogenesis is not caused by ageing itself, but that there is a loss of the neurogenic niche in the hippocampus

and this causes a reduction in the rate of neurogenesis (Klempin and Kempermann, 2007). In particular, there is a reduction in the availability of neurotrophic factors such as NGF and BDNF (Silhol *et al.*, 2005, Terry Jr *et al.*, 2011) which may impact on the ability of neural stem cells to proliferate, differentiate and survive. Terry Jr and colleagues (2011) report a reduction in NGF and the phosphorylated form of Trk A in the hippocampus and prefrontal cortex with age and an associated deficit in spatial and recognition memory. In addition, they report an increase in the proneurotrophin proNGF and the receptor $p75^{NTR}$, both of which are implicated in the pro-apoptotic signaling cascade and therefore may be a factor in loss of function. Age-related deficits in NGF are also associated with a loss of synaptic plasticity (Kelly *et al.*, 2000). There is also evidence to show that BDNF expression is altered with age, that there is a correlation between hippocampal BDNF and memory performance in aged rats and that BDNF-induced LTP is impaired with age (Schaaf *et al.*, 2001, Gooney *et al.*, 2004).

VEGF, whilst typically assumed to exert its main function upon blood vessels, has been shown to promote neurogenesis in the adult brain, possibly via enhancement of a vascular niche (Palmer *et al.*, 2000, Licht *et al.*, 2011), and is reported to have neuroprotective properties particularly after brain injury (Sun *et al.*, 2003). Gao and colleagues (2009) reported a reduction in VEGF-stimulated angiogenesis and neurogenesis in aged mice, which is possibly associated with a reduction in the expression of its main receptor, VEGF receptor 2. Over-expression of VEGF can also improve spatial and contextual memory, suggesting it may be important in hippocampal plasticity (Cao *et al.*, 2004).

Aging is also strongly associated with increased inflammation, particularly in glial activation, with aged rodents showing increases in TLR4, CD14 and MHCII, pro-inflammatory cytokines and astrocyte number with age (Hayakawa *et al.*, 2007, Letiembre *et al.*, 2007, Lynch, 2010). This increase in the inflammatory state in the brain could impact on behaviour and synaptic plasticity. Ageing can also increase the brain's vulnerability to inflammatory challenges and these can interact to induce memory deficits, possibly via downregulation of BDNF (Barrientos *et al.*, 2006, Cortese *et al.*, 2011). The pro-inflammatory cytokine IL-1β has been shown to be important for synaptic plasticity: its expression was increased in free moving rats 8 hours after LTP induction, furthermore the induction of this LTP could be prevented with an infusion of the IL-1 receptor antagonist IL-1ra (Schneider *et al.*, 1998). IL-1 receptor knockout mice also show impaired LTP induction and spatial memory deficits (Goshen *et al.*, 2009). Conversely, aged rats

exhibit an increase in hippocampal IL-1β together with a deficit in contextual fear conditioning which is prevented following the inhibition of caspase-1, an enzyme that can generate the mature form of IL-1β (Gemma *et al.*, 2005). In addition, Alzheimer's disease is associated with elevated levels of IL-1β, TNF-α and IL-6 in both the serum and cerebrospinal fluid (Akiyama *et al.*, 2000, Shaftel *et al.*, 2008). These data suggest that whilst certain pro-inflammatory cytokines, such as IL-1β, can play a significant role in modulating synaptic plasticity and memory functioning, an extended and elevated pro-inflammatory phenotype can elicit negative effects on brain functioning and in fact may be responsible for an acceleration in cognitive decline in neurodegenerative diseases (Lynch, 2010, Viviani and Boraso, 2011, Yirmiya and Goshen, 2011).

Despite the number of reviews that argue for the neuroprotective benefits of environmental enrichment, there are only a small number of human studies that test exercise or cognitive stimulation as therapeutic interventions. It has been reported that one year of exercise training increases hippocampal volume and this volume is positively correlated with BDNF serum concentration in aged adults, however there was no significant improvement in spatial memory in the exercise group when compared with stretching controls (Erickson *et al.*, 2011). Mahnke and colleagues (2006) have shown that 8-10 weeks of an auditory/language-based cognitive training program can induce general memory improvements in tests non-related to the tasks they performed during training and this improvement is maintained 3 months post-training which would suggest that there is long-lasting benefit to cognitive training. More studies are necessary however, to obtain consistent evidence of enrichment-induced memory improvements and to begin to elucidate the mechanisms that may be underlying them. Environmental enrichment has also been shown to play a role in neuroprotection and can be a treatment for other neurological disorders such as depression (Mahncke *et al.*, 2006, Berardi *et al.*, 2007, Harburger *et al.*, 2007, Laviola *et al.*, 2008).

Exercise can prevent age-related cognitive decline in rats (O'Callaghan *et al.*, 2009, Kim *et al.*, 2010) and has been shown to prevent or delay cognitive decline in a number of neurodegenerative mouse models of traumatic brain injury (Griesbach *et al.*, 2009), Huntington's disease (Pang *et al.*, 2006) and Alzheimer's disease (Ke *et al.*, 2011, Liu *et al.*, 2011). Similarly environmental enrichment can prevent age-related spatial memory decline (Kempermann *et al.*, 2002, Bennett *et al.*, 2006, Leal-Galicia *et al.*, 2008, Kumar *et al.*, 2011) and delay memory deficits in mouse models of Alzheimer's disease (Berardi *et*

al., 2007, Cracchiolo *et al.*, 2007) and Huntington's disease (Nithianantharajah *et al.*, 2008). Environmental enrichment is also associated with long-term upregulation of BDNF and NGF, increases in synaptic plasticity and neurogenesis (Pham *et al.*, 1999a, Ickes *et al.*, 2000, Kempermann *et al.*, 2002, Cracchiolo *et al.*, 2007) and both environmental enrichment and exercise have been shown to increase hippocampal neurogenesis in a mouse model of Alzheimer's disease (Mirochnic *et al.*, 2009). There is also evidence that enrichment may reduce beta-amyloid plaque load (Cracchiolo *et al.*, 2007), however this is disputed in other studies (Mirochnic *et al.*, 2009). Therefore, whilst there are limited human studies available to analyse the efficacy of cognitive stimulation, animal models are yielding a great deal of important evidence supporting a neuroprotective role for environmental enrichment.

The aim of this study was to assess the neuroprotective properties of long-term environmental enrichment, in the absence of exercise, on rats' cognitive function. Previous data from our lab shows that, whilst long-term treadmill exercise can rescue spatial memory deficits and a reduction in LTP expression in the aged rat, control rats that were exposed to a stationary treadmill for the same duration also exhibited a rescuing of spatial memory and LTP expression (O'Callaghan *et al.*, 2009). This treadmill exposure and additional handling is akin to environmental enrichment because it provides additional sensory stimulation for the rats and exposes them to different environments when compared with aged cage controls that are housed in a relatively impoverished environment. These data suggest that relatively minimal but consistent amount of additional stimulation can rescue age-related memory deficits in the long term. Thus, we hypothesise that environmental stimulation, in the absence of exercise, can be equally successful at ameliorating cognitive deficits associated with age. This study was undertaken as a longitudinal study and therefore it was possible to directly compare a rat's performance at 3, 13 and 22 months of age providing a powerful comparison of decline in cognitive function of the rat's lifespan and any neuroprotective effects that environmental enrichment may have. Furthermore, the neurochemical changes that are associated with age were assessed particularly in relation to neurotrophins, neurogenesis and inflammation. To date, there are no studies that have assessed the neuroprotective impact of long-term environmental enrichment, without exercise, and therefore this study will further enhance our understanding of the effects that cognitive stimulation alone can have in prevention of

cognitive decline with age. It will also add to the current research by further analysing the mechanisms by which cognitive enrichment can induce a neuroprotective effect.

5.2 Methods

5.2.1 Subjects and Design

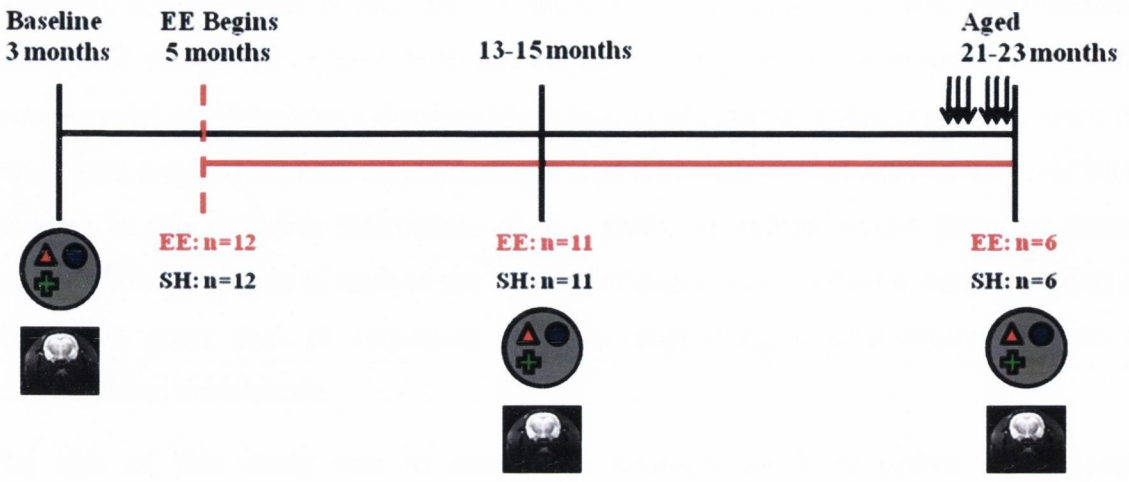


Figure 5a. Timeline for long-term environmental enrichment protocol At 3 months of age, rats were tested with a battery of behavioural tasks to obtain baseline cognitive performances and scanned in a 7T MRI scanner (SH: n=6, EE: n=7). Following this, they were randomly assigned to SH or EE housing. At 13-15 months of age, rats were tested again with the same behavioural tasks and scanned (SH: n=7, EE: n=8). At Aged, rats were again tested with the same behavioural tasks and scanned (SH: n=6, EE: n=6) and then sacrificed. All rats were administered BrdU ([↓] 50mg.kg^{-1}; i.p) 3 x weekly for 2 weeks, following final behavioural testing.

The experimental groups consisted of rats housed in standard housing conditions (SH, n=11) and rats housed in enriched conditions (EE, n=12) for approximately twenty months. All rats were tested on a variety of behavioural tests (see 4.2.2) prior to their assignment to their respective groups in order to obtain a baseline level of cognitive performance to which further behavioural results can be compared. Thirteen rats (SH, n=6; EE, n=7) were also scanned in a 7T horizontal bore magnet (Bruker) prior to their assignment to their respective groups to obtain baseline MR images. After six weeks of environmental enrichment, rats were tested on the T maze task and OF test and these data were used as part of the study described in chapter 3 (see 3.2.3).

At 13 months of age (13-15 mo; week 35 of environmental enrichment), 1 rat had died. The remaining rats were tested with the same behavioural tests that were performed at baseline (SH, n=11; EE, n=11). Fifteen rats (SH, n=7; EE, n=8) were also scanned in the 7T horizontal bore magnet to obtain 13-15 months old MR images.

At 21 months of age (Aged; week 76 of environmental enrichment), a further 10 rats had died. The remaining rats were tested again with the same behavioural tests that were

performed at baseline and 13 mo (SH, n=6; EE, n=6). All twelve rats were also scanned in the 7T horizontal bore magnet to obtain Aged MR images.

The same rats were scanned at all timepoints and any rats that died during the study were excluded from the final behavioural and MR analyses.

5.2.2 Behavioural Testing

It could be argued that rats housed in enriched environments will be more active because they have more objects in their home cage to explore. Therefore, home cage activity was recorded during their nocturnal phase in the long-term environmental enrichment study, when rats were approximately 11 months old. Species typical behaviours and general activity were measured manually for 4 x 5 minute blocks between 12:00-02:00. Mean activity scores were calculated.

The baseline hippocampal-dependent memory performance and anxiety levels of all the rats was tested with a variety of behavioural tasks prior to their assignment into standard or enriched conditions. The order of the tasks was chosen so as to minimise practise effects and prior exposure to the equipment. The rats were then tested on the same tasks at 13-15 months of age and Aged (21-23 months of age) timepoints.

The rats' anxiety levels were measured first with both the elevated plus maze test and the OF test (see 2.4.5). Briefly, the rats were placed in the centre of the elevated plus maze and allowed to explore freely for five minutes. The exploration of the rats was digitally recorded to minimise the disturbance of an experimenter's presence in the room and the number of entries into closed and open arms was measured from the videos. Entries into arms were only counted if the whole body of the rat crossed the threshold of the arm.

Five days later, the rats were placed in the open field arena for a period of five minutes each and allowed to explore freely. Thigmotaxis was measured using Ethovision 3.0® software and calculated as the time spent exploring the wall (20cm corridor from the edge). Time spent in the centre of the arena was also calculated.

Following the OF test, rats' were tested using different versions of the NOR and OD task. First, rats were tested with the three object variant of the NOR task (see 2.4.1). Rats had one trial of five minutes to explore three different novel objects in the open field (training day). Twenty-four hours post-training, one of the objects was replaced by a novel object in the same position and the rats were placed back into the open field for five minutes and

allowed to explore (testing day). During both the training and testing days, the time spent exploring each object was recorded using stopwatches and calculated as a percentage of the total time spent exploring both objects.

Seven days after performing the three object NOR task, the rats were tested with the two object variant of the NOR task as described in section 3.2.3 (see also 2.4.1). The objects used in this task did not resemble any of the objects used in the three object NOR task from the previous week. Rats had three trials of five minutes with an inter-trial interval of five minutes to explore two different novel objects in the open field (training day). Twenty-four hours post-training, one of the objects was replaced by a novel object in the same position and the rats were placed back into the open field and allowed to explore (testing day). During both the training and testing days, the time spent exploring each object was recorded using stopwatches and calculated as a percentage of the total time spent exploring both objects.

Seven days after the after performing the two object NOR task, the rats were tested with the three object OD task (see 2.4.2). The objects used in this task did not resemble any of the objects used in either the three or two object NOR tasks from the previous two weeks. Rats had three trials of five minutes with an inter-trial interval of five minutes to explore three different novel objects in the open field (training day). Twenty-four hours post-training, one of the objects was placed in a novel position and the rats were placed back into the open field for five minutes and allowed to explore freely (testing day). During both the training and testing days, the time spent exploring each object was recorded using stopwatches and calculated as a percentage of the total time spent exploring both objects.

Six days after performing the three object OD task, the rats were tested with the Morris water maze task (see 2.4.3). Rats were placed in a circular arena filled with tepid water in a dimly lit room containing spatial cues and allowed to swim until they found a static hidden platform. Rats were tested for five consecutive days with five trials each day. Each trial lasted a maximum of sixty seconds or until rats had found the hidden platform, with an inter-trial interval of sixty seconds. If rats had not found the platform within sixty seconds, they were led to the platform. Rats were allowed to remain on the platform for thirty seconds to enable spatial orientation. Each trial was recorded using Ethovision® 3.0 and the escape latency was measured. The mean escape latency was calculated for each rat on each day. The pattern of exploration was also recorded. A probe test was performed two

days after the final training day, in which the hidden platform was removed and rats were reintroduced into the arena for a single trial of sixty seconds. The arena was divided into quadrants and the time rats spent in each quadrant was recorded using Ethovision 3.0®. The pattern of exploration was also recorded.

Following 7 weeks of environmental enrichment, rats were tested on the T maze task which utilises working memory (see 2.4.4) and is designed to test the rats' spontaneous alteration behaviour in search of food. Rats were placed on a reduced diet for two weeks prior to testing on this task and throughout the task itself, of approximately 85% of their recommended daily food intake for their weight. The rats were given six trials per day in which they had to learn to alternate between exploration of either arm in order to obtain a food reward. The number of correct entries was recorded as a percentage of total trials per day for seven days. The results of this task were reported in 3.3.4. Rats were tested using the T maze task again at the 13-15 months old and Aged timepoints.

The order that the tasks were performed was fixed and therefore to reduce the chance of any practise effect with the object-based tasks, the task with the least exposure to objects (2 object NOR) was ordered first and the easiest task for the rats was ordered last (3 object OD). Following Baseline testing, rats were ranked in order of their performance on these object-based tasks and these ranks were correlated to ensure that no practise effect had occurred. There were no correlations between ranked performances on the object-based tasks (2 object NOR vs 3 object NOR: $r = -0.187$, $p>0.05$; 2 object NOR vs 3 object OD: $r = 0.239$, $p>0.05$; 3 object NOR vs 3 object OD: $r = 0.271$, $p>0.05$; figure 5b).

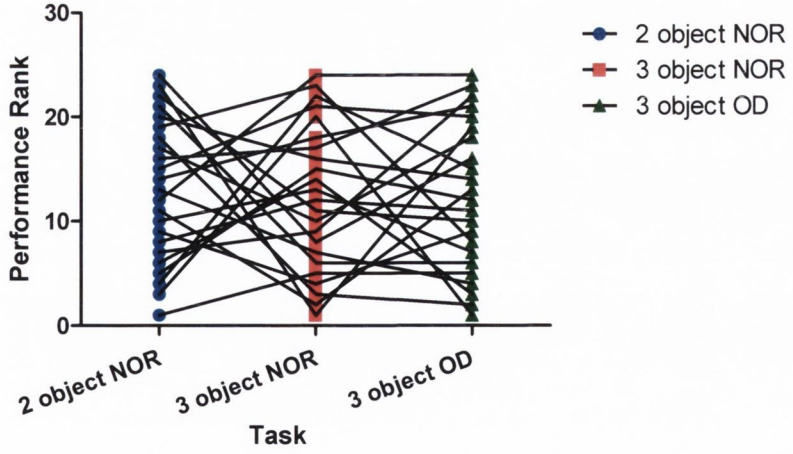


Figure 5b.Correlation of ranked performance on object-based task. There were no correlations between ranked performances on object-based tasks in rats. 2 object NOR vs 3 object NOR: $r = -0.187$, $p>0.05$; 2 object NOR vs 3 object OD: $r = 0.239$, $p>0.05$; 3 object NOR vs 3 object OD: $r = 0.271$, $p>0.05$. N=23.

Behavioural Task	Elevated Plus Maze	OF Test	2 object NOR	3 object NOR	3 object OD	MWM (acquisition)	MWM (probe)	T maze (training)	T maze (testing)
Days	1	2	3-6	13-16	23-26	32-39	42	45-52	53-60

Table 5a.The order of behavioural testing at all 3 timepoints. All rats were tested in the same orderwith rats performing the object-based task with the least exposure to objects first (2 object NOR) and easiest task (3 object OD) last in order to reduce the chance of any practise effect. All object tasks had a 1 week break in between and rats had no prior exposure to any of the objects prior to the task.

5.2.4 Neurochemical Analysis

In order to obtain baseline young control measures for neurochemical analysis, 8 young rats (250-300g; Young SH) were housed in standard conditions for the last 3 weeks of the study. Following the final MRI scans, these rats were sacrificed with the Aged SH and Aged EE rats via urethane overdose. All rats were transcardially perfused with heparinised saline for 10 minutes, decapitated and brains quickly removed. The left hemisphere was removed and flash frozen in isopentane before being covered in Tissue-Tek® OCT^{TM} compound and stored at -80^{o}C to preserve it for immunohistochemical analysis. The right hemisphere was sub-dissected to extract the perirhinal cortex, entorhinal cortex, S1 cortex, cerebellum, olfactory bulb, hippocampus and dentate gyrus. Homogenate was prepared for neurochemical analysis using the methods outlined in 2.5.1.

5.2.4 BrdU administration

To evaluate hippocampal neurogenesis, the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU [Sigma]) was administered intraperitoneally (50mg.kg^{-1}) to all rats. For all rats, the BrdU was administered three times weekly for the last two weeks of their housing period.

5.2.5 Analysis of the expression of NGF, BDNF, Trk A, Trk B and p75^{NTR}

Neurotrophin expression was measured in the dentate gyrus, perirhinal cortex and hippocampus in all groups. BDNF, βNGF and VEGF protein concentration were measured by ELISA (see 2.5.4). BDNF, βNGF, VEGF, Trk A, Trk B and p75^{NTR} mRNA expression in all groups in the dentate gyrus, hippocampus, perirhinal cortex and entorhinal cortex were measured using PCR analysis (see 2.5.3). p75^{NTR} protein concentration in the dentate gyrus and the hippocampus was measured using western immunoblotting (see 2.5.2).

5.2.6 Analysis of Synaptic Vesicle Proteins

Synapsin and synaptophysin protein concentration in the dentate gyrus and hippocampus were measured using western immunoblotting (see 2.5.2). These proteins are modulators of neurotransmitter release and are often used as markers of synaptic plasticity.

5.2.7 Analysis of Neurogenesis

In order to measure any changes in neuronal proliferation, Ki67 mRNA expression in the dentate gyrus and olfactory bulb was measured using PCR analysis (see 2.5.3) and immunohistochemical analysis was performed on sections of the dentate gyrus in all groups. To analyse the rate of cell proliferation in the dentate gyrus, BrdU immunostaining was performed on 10μm sections and analysed via light microscopy (see 2.6.2). The number of BrdU positive nuclei was calculated as a percentage of the total number of nuclei stained with hematoxylin.

5.2.7 Analysis of Apoptosis

Levels of apoptosis in the dentate gyrus of all groups were assessed using the DeadEnd^{TM} Fluorometric TUNEL System (Promega Corporation) according to manufacturer's instructions (see 2.6.4). Labelled DNA fragments were visualised via confocal microscopy at the 488nm wavelength. The mean intensity of the fluorescence at 488nm was calculated for each animal and group means were compared. The author would like to acknowledge the assistance of Alexa Pichet Binette in the analysis of this data.

5.2.8 Analysis of pro-inflammatory markers

In order to measure any changes in inflammatory markers, IL-1β, CD68 and CD40 mRNA expression in the hippocampus was measured using PCR analysis (see 2.5.3). IL-1β is a pro-inflammatory cytokine which is released as part of an inflammatory response in the body. Many studies report increases in IL-1β expression in the brain with age and it has been associated with changes in cognitive function in animal models, although its precise role as a modulator of memory abilities is unclear. Both CD68 and CD40 are expressed on microglia in an activated state and are hence used as markers of microglial activity and the pro-inflammatory response of the body. CD68 is typically associated with the phagocytic action of microglia.

5.2.9 Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM). Outliers were excluded from any analysis if they were ±2 standard deviations away from the mean. All behavioural data were analysed with a mixed between-within ANOVAwith Group as the between subjects factor and with Age and Object/Day/Quadrant as the within subjects factors. Tests for anxiety were analysed with a two-way ANOVA. Any rats that died during the longitudinal enrichment period were excluded from the final behavioural analyses. All neurochemical data were analysed with a one-way ANOVA.

5.3 Results

5.3.1 Long-term environmental enrichment enhances long-term weight gain

Rats' weights were recorded throughout their lifespan. Repeated measures 2-way ANOVA revealed that there was a significant effect of housing condition ($F_{1,660} = 7.590$, $p<0.05$; figure 5c), and a significant effect of time ($F_{60,660} = 69.91$, $p<0.001$), with all the rats gaining weight over time.

5.3.2 Envrionmental enrichment reduces home-cage activity

There was a significant decrease in rearing ($t_{20} = 2.60$, $p<0.05$; figure 5d.i) and grooming ($t_{20} = 2.57$, $p<0.05$; figure 5d.ii) in EE rats when compared with SH rats. There was no significant difference between groups in fighting bouts ($t_{20} = 1.42$, $p>0.05$; figure 5d.iii). There was a significant decrease in cage crossings in EE rats when compared with SH rats ($t_{20} = 2.34$, $p<0.05$; figure 5d.iv). These data suggest that EE rats are less active than SH rats, and therefore increase physical activity cannot be argued to account for the enrichment-induced memory improvements reported in this study.

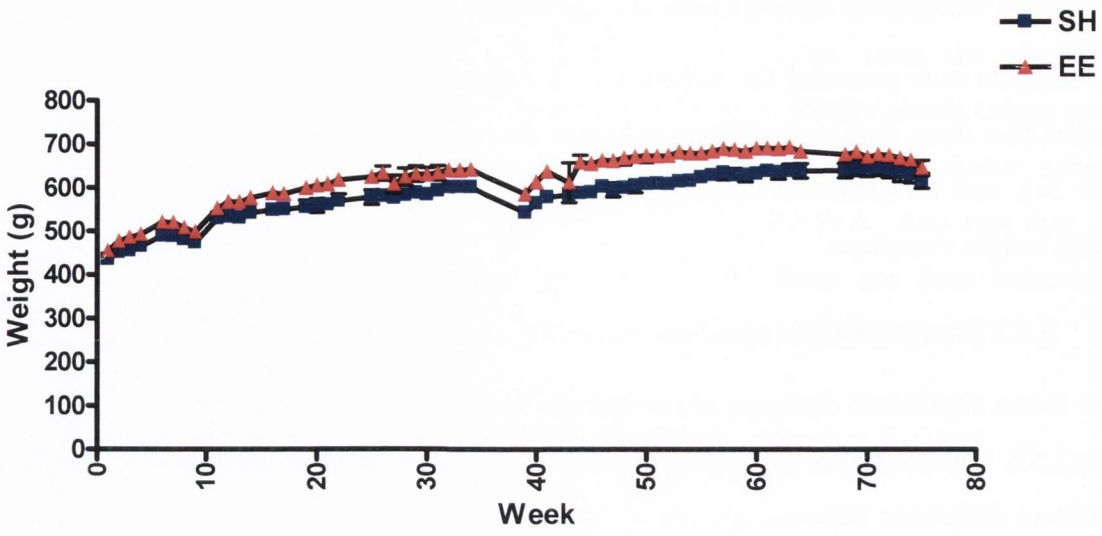


Figure 5c. Environmental enrichment enhances long-term weight gain. There was a significant effect of housing condition ($F_{1,660}$ = 7.590, $p<0.05$; figure II), and a significant effect of time ($F_{60,660}$ = 69.91, $p<0.001$), with all the rats gaining weight over time.SH: n=6; EE: n=6. Data is expressed as mean ± SEM.

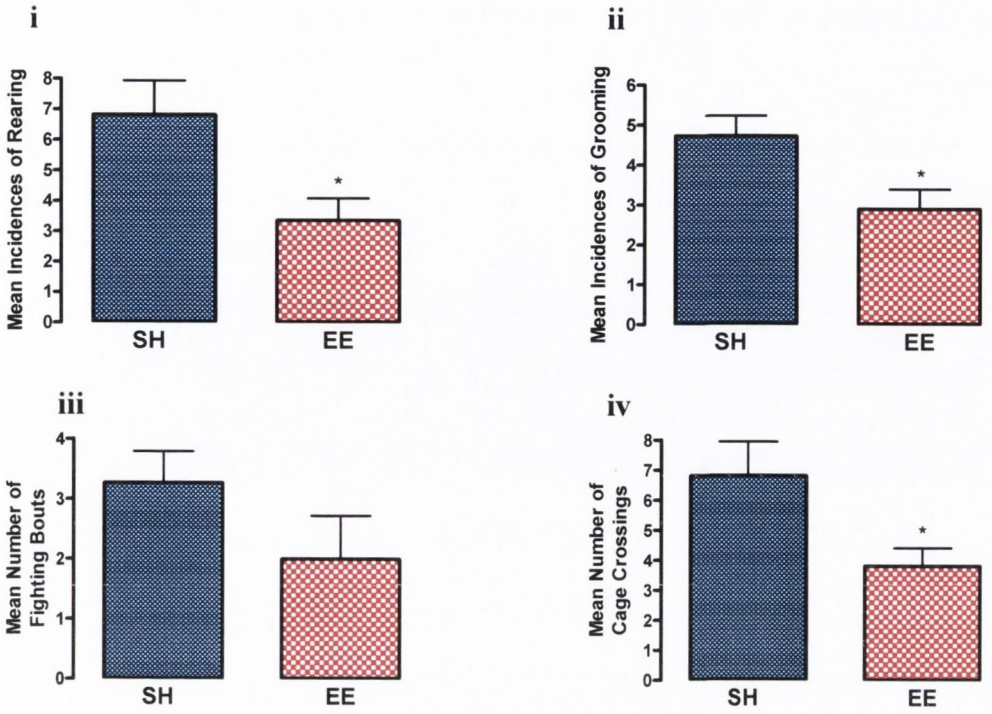


Figure 5d. Environmental enrichment reduces home-cage activity.(i)There was a significant decrease in rearing (t_{20} = 2.60, *p<0.05) and (ii) grooming (t_{20} = 2.57, p<0.05) in EE rats when compared with SH rats (iii) There was no significant difference between groups in fighting bouts (t_{20} = 1.42, p>0.05) (iv) There was a significant decrease in cage crossings in EE rats when compared with SH rats (t_{20} = 2.34, p<0.05). SH: n=11; EE: n=11

5.3.3 Environmental enrichment does not prevent an age-related increase in levels of anxiety measured by the Elevated Plus Maze

There is a significant effect age between groups on percentage of time spent in the open arms of the elevated plus maze ($F_{2,20} = 59.79$, $p<0.001$; figure 5e). There was no significant main effect of housing ($F_{1,20} = 0.165$, $p>0.05$) and no significant interaction ($F_{2,20} = 1.826$, $p>0.05$). Bonferroni posttests show a significant reduction in percentage of time spent in the open arms when both groups at MA are compared with Baseline (Baseline SH vs MA SH: ***$p<0.001$; Baseline EE vs MA EE: ***$p<0.001$) and when both groups at Aged are compared with Baseline (Baseline SH vs Aged SH: ***$p<0.001$; Baseline EE vs Aged EE: ***$p<0.001$). Mean percentage time spent in open arms ± SEM: Baseline SH = 30.94±5.23, Baseline EE = 35.06±3.30, 13-15 mo SH = 8.17±0.94, 13-15 mo EE = 7.37±1.05, Aged SH = 8.92±3.27, Aged EE = 2.41±0.86.

5.3.4 There is no age-related difference in thigmotaxis

There was no significant effect of age between groups on thigmotaxis ($F_{2,20} = 0.230$, $p>0.05$; figure 5f). There was also no significant effect of housing ($F_{1,20} = 1.897$, $p>0.05$) and no significant interaction ($F_{2,20} = 0.829$, $p>0.05$). Mean percentage thigmotaxis ± SEM: Baseline SH = 86.81±2.47, Baseline EE = 83.91±2.94, 13-15 mo SH = 80.02±9.351, 13-15 mo EE = 86.82±5.30, Aged SH = 79.57±16.03, Aged EE = 98.72±0.64.

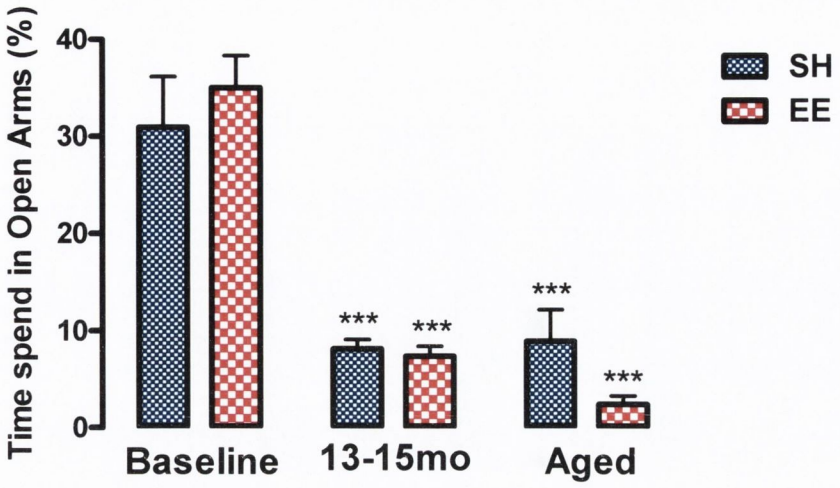


Figure 5e. Environmental enrichment does not prevent the age-related increase in levels of anxiety There is a significant effect age between groups on percentage of time spent in the open arms of the elevated plus maze ($F_{2,20}$ = 59.79, p<0.001), with a significant decrease in both groups at MA and Aged timepoints when compared with Baseline (Baseline SH vs MA SH: ***p<0.001; Baseline EE vs MA EE: ***p<0.001; Baseline SH vs Aged SH: ***p<0.001; Baseline EE vs Aged EE: ***p<0.001). SH: n=6, EE: n=6. Data expressed at mean ± SEM.

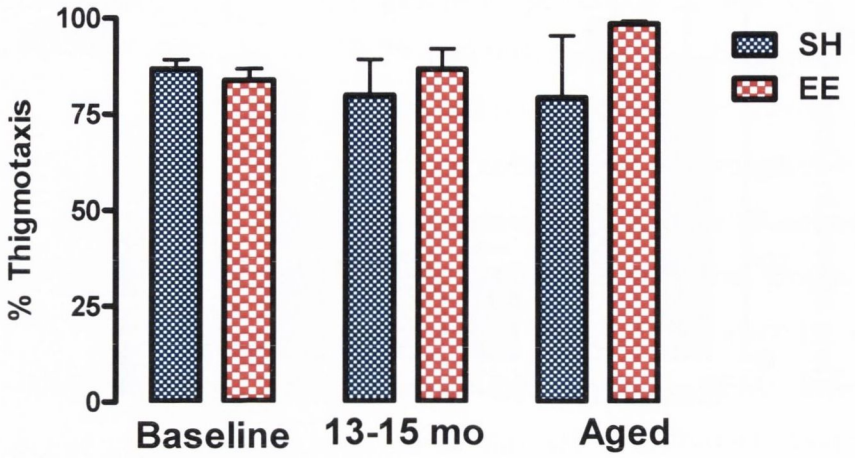


Figure 5f. There is no age-related difference in thigmotaxis There is no significant effect of age on thigmotaxis ($F_{2,20} = 0.230$, $p>0.05$), no effect of housing condition ($F_{1,20} = 1.897$, $p>0.05$) and no significant interaction ($F_{2,20} = 0.829$, $p>0.05$). SH: n=6, EE: n=6. Data expressed at mean ± SEM.

5.3.5. Environmental enrichment prevents the age-related decline in object recognition memory

Object recognition memory was tested using the 2 object NOR task at all three timepoints. On training day, there was no significant effect of age ($F_{2,28}$= 0, p>0.05), object ($F_{1,14}$ = 0.888, p>0.05) or group ($F_{1,14}$ = 0, p>0.05). There was also no interaction between object and group ($F_{1,14}$ = 2.361, p>0.05) or age ($F_{2,28}$ = 0.700, p > 0.05; figure 5g.i). Mean percentage exploration of object ± SEM : Baseline SH [Object A] = 54.39±2.08, Baseline SH [Object B] = 44.32±1.71, Baseline EE [Object A] = 46.42±3.61, Baseline EE [Object B] = 53.58±3.61, 13-15 mo SH [Object A] = 50.75±1.09, 13-15 mo SH [Object B] = 49.24±1.09, 13-15 mo EE [Object A] = 49.72±2.22, 13-15 mo EE [Object B] = 50.28±2.22, Aged SH [Object A] = 48.55±4.40, Aged SH [Object B] = 51.45±4.40, Aged EE [Object A] = 45.13±4.41, Aged EE [Object B] = 54.87±4.41.

On testing day, there was no significant effect of age ($F_{2,28}$= 0, p > 0.05) or group ($F_{1,14}$ = 0, p > 0.05). There was however, a significant main effect of object ($F_{1,14}$= 120.578, p<0.001) and a significant interaction between object and age ($F_{2,28}$ = 6.677, p<0.01). There was no significant interaction between object and group ($F_{1,14}$ = 3.646, p>0.05) or object, age and group ($F_{2,28}$ = 1.443, p>0.05; figure 5g.ii).

Pairwise comparisons on the main effect of object show that there was a significant difference between the exploration of the novel object C* and the familiar object A (p<0.001). To further explore this result, repeated measures ANOVAs were performed at each time-point and this analysis revealed a significant difference in the exploration of the objects at Baseline ($F_{1,14}$ = 35.07, p<0.001) with both groups exploring the novel object C* significantly more than the familiar object A [SH: (p<0.01); EE: (p<0.01)]. There was a significant difference in the exploration of the objects at 13-15 mo ($F_{1,14}$= 92.49, p<0.001), with both groups exploring the novel object C* significantly more than the familiar object A [SH: (p<0.001); EE: (p<0.001)]. There was also a significant difference in the exploration of the objects at Aged ($F_{1,14}$ = 6.994, p<0.05), with the EE group exploring the novel object C* significantly more than the familiar object A (p<0.01) but no difference in the exploration of the objects in the SH group (p>0.05).

To explore the significant interaction between object and age, repeated measures ANOVAs were performed for each group with age as the independent variable and exploration of the novel object C* as the dependent variable. In the EE group, there was no significant effect

of age ($F_{2,16}$ = 1.653, $p>0.05$) however in the SH group, there was a significant effect of age ($F_{2,12}$ = 9.017, $p<0.01$). Pairwise comparisons revealed that there was a significant decrease in the exploration of the novel object C* at the Aged time-point when compared with the Baseline and 13-15 mo time-point [Baseline vs Aged: $p<0.05$; 13-15 mo vs Aged: $p<0.01$)]. Mean percentage exploration of object ± SEM : Baseline SH [Object A] = 35.56±2.49, Baseline SH [Object C*] = 64.44±2.49, Baseline EE [Object A] = 35.69±3.80, Baseline EE [Object C*] = 64.32±3.80, 13-15 mo SH [Object A] = 30.28±3.65, 13-15 mo SH [Object C*] = 69.72±3.65, 13-15 mo EE [Object A] = 27.21±2.68, 13-15 mo EE [Object C*] = 72.79±2.68, Aged SH [Object A] = 48.43±3.50, Aged SH [Object C*] = 51.58±3.50, Aged EE [Object A] = 36.33±4.28, Aged EE [Object C*] = 63.67±4.28.

These results show that, at Baseline and 13-15 months old, both the SH and EE rats can distinguish the novel object from a single familiar object whereas at the Aged time-point, the SH rats cannot distinguish the novel object from the familiar object. This suggests that there is an age-related deterioration in object recognition memory which can be prevented by environmental enrichment.

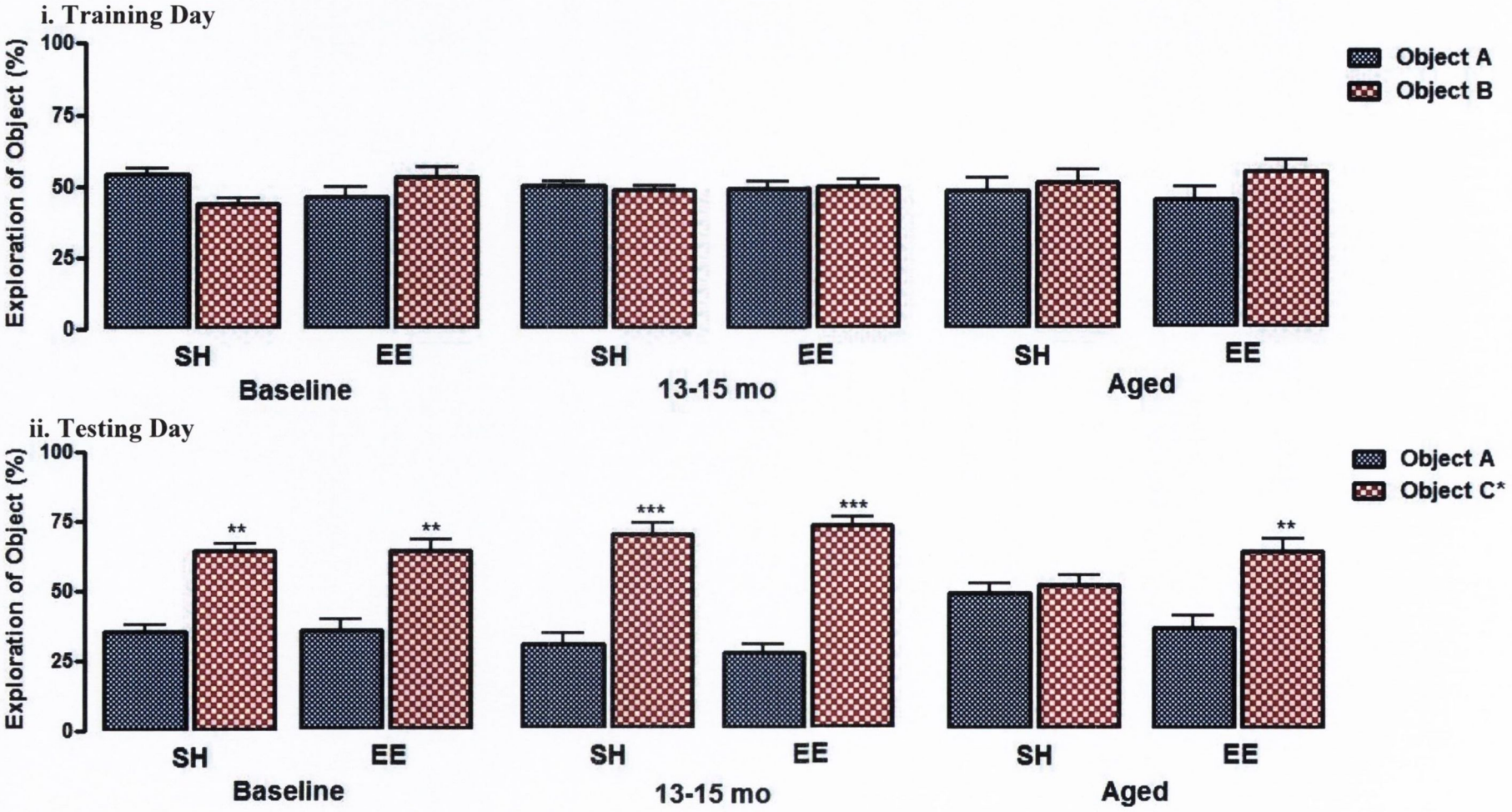


Figure 5g. Environmental prevents the age-related decline in object recognition memory(i) There was no significant effect of age ($F_{2,28}$= 0, p>0.05), object ($F_{1,14}$ = 0.888, p>0.05) or group ($F_{1,14}$ = 0, p>0.05) (ii) There is a significant effect of Object ($F_{1,14}$ = 120.578, p<0.001) and a significant interaction between object and age ($F_{2,28}$ = 6.677, p<0.01), with both groups exploring the novel object C* significantly more than the familiar object A at Baseline (***p<0.001) and 13-15 mo (***p<0.001) timepoints. At the Aged timepoint, only the EE group explore the novel object C* significantly more than the familiar object A [**], whereas the SH group explored the novel object C* significantly less than at Baseline ($^{+}$p<0.05) or 13-15 mo ($^{\&\&}$p<0.01) timepoints. SH: n=6, EE: n=6. Data is expressed as mean ± SEM.

5.3.6 Environmental enrichment improves object recognition memory throughout the lifespan

Object recognition memory was tested using the 3 object NOR task at all three timepoints. On training day, there was no significant effect of age ($F_{2,28}$= 0, $p>0.05$), object ($F_{2,28}$ = 0.888, $p>0.05$) or group ($F_{1,14}$ = 0, $p>0.05$). There was also no interaction between object and group ($F_{2,28}$ = 0.040, $p>0.05$) or age ($F_{4,56}$ = 0.516, $p>0.05$; figure 5h.i). Mean percentage exploration of object ± SEM : Baseline SH [Object A] = 34.80±1.97, Baseline SH [Object B] = 31.89±1.57, Baseline SH [Object C] = 33.30±0.86, Baseline EE [Object A] = 31.07±1.86, Baseline EE [Object B] = 35.86±1.47, Baseline EE [Object C] = 33.07±1.68, 13-15 mo SH [Object A] = 35.26±1.73, 13-15 mo SH [Object B] = 31.98±1.41, 13-15 mo SH [Object C] = 32.76±2.39, 13-15 mo EE [Object A] = 32.12±1.67, 13-15 mo EE [Object B] = 34.70±2.76, 13-15 mo EE [Object C] = 33.18±2.59, Aged SH [Object A] = 27.82±2.17, Aged SH [Object B] = 40.58±3.06, Aged SH [Object C] = 31.60±3.34, Aged EE [Object A] = 35.13±3.65, Aged EE [Object B] = 32.37±2.35, Aged EE [Object C] = 32.50±2.62.

On testing day, there was no significant effect of age ($F_{2,28}$= 0, $p>0.05$) or group ($F_{1,14}$ = 0, $p>0.05$). There was however, a significant effect of object ($F_{2,28}$ = 7.942, $p<0.01$) and a significant interaction between object and group ($F_{2,28}$ = 4.902, $p<0.01$). There was no significant interaction between object and age ($F_{4,56}$ = 1.766, $p>0.05$) or object, age and group ($F_{4,56}$ = 0.794, $p>0.05$, figure 5h.ii).

Pairwise comparisons on the effect of object show that there was a significant difference between the exploration of the novel object D* and the familiar object A ($p < 0.01$) and the familiar object B ($p<0.01$). To further explore this result, repeated measures ANOVAs were performed at each time-point and this analysis revealed no significant difference in the exploration of the objects at Baseline ($F_{2,28}$ = 0.514, $p>0.05$). There was a significant difference in the exploration of the objects at 13-15 mo ($F_{2,42}$ = 10.73, $p<0.001$), with a significant increase in the exploration of the novel object D* and the familiar objects A and B in the EE group (% Object A vs Object D* ($p<0.001$); % Object B vs Object D* ($p<0.01$) , but not in the SH group ($p>0.05$ between all groups). There was also a significant difference in the exploration of the objects at Aged ($F_{2,42}$ = 10.73, $p<0.001$), with a significant increase in the exploration of the novel object D* and the familiar

objects A and B in the EE group (% Object A vs Object D* ($p<0.01$); % Object B vs Object D* ($p<0.01$) , but not in the SH group ($p>0.05$ between all groups).

To explore the significant interaction between object and group, independent samples *t*-tests were performed between the two groups of animals for each object and at each time-point. At Baseline, there was no significant difference between the exploration of the objects. At 13-15 months old, there was no significant difference between the exploration of the familiar objects. There was however, a significant difference between the exploration of the novel object D* with the EE group exploring D* significantly more than the SH group ($^{+}p<0.05$). At Aged, there was no significant difference between the exploration of the familiar object B. There was however, a significant difference between the exploration of the familiar object with the EE group exploring A significantly less than the SH group ($p < 0.05$) and the novel object D* with the EE group exploring D* significantly more than the SH group ($^{+}p<0.05$). Mean percentage exploration of object ± SEM : Baseline SH [Object A] = 34.21±1.07, Baseline SH [Object B] = 33.44±1.29, Baseline SH [Object D*] = 32.34±1.23, Baseline EE [Object A] = 33.00±4.67, Baseline EE [Object B] = 28.51±4.34, Baseline EE [Object D*] = 38.49±4.00, 13-15 mo SH [Object A] = 29.25±3.00, 13-15 mo SH [Object B] = 34.72±2.42, 13-15 mo SH [Object D*] = 36.03±1.38, 13-15 mo EE [Object A] = 25.31±2.56, 13-15 mo EE [Object B] = 30.59±3.62, 13-15 mo EE [Object D*] = 44.11±2.41, Aged SH [Object A] = 38.67±4.19, Aged SH [Object B] = 26.13±3.71, Aged SH [Object D*] = 35.20±2.40, Aged EE [Object A] = 27.04±2.73, Aged EE [Object B] = 28.44±2.95, Aged EE [Object D*] = 44.51±2.27.

These results show that, at baseline none of the rats are able to distinguish the novel object from the familiar objects whereas at both 13-15 months old& Aged time points, the EE rats can distinguish the novel object from the two familiar objects. This supports the previous chapter's result that environmental enrichment can induce an improvement in object recognition memory and further suggests that this capacity improvement is maintained throughout the lifespan.

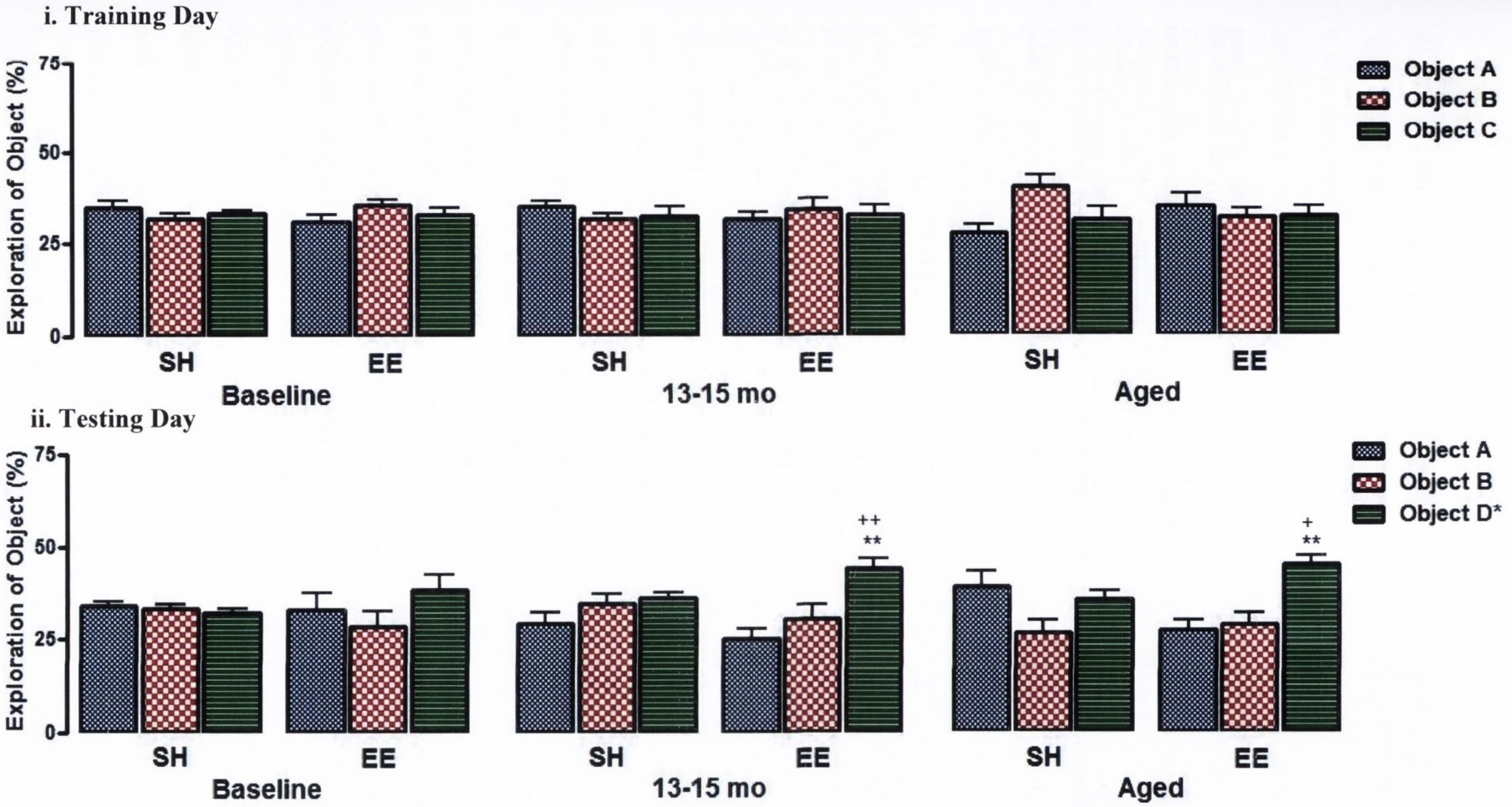


Figure 5h. Environmental enrichment improves object recognition memory throughout the lifespan(i) There was no significant effect of age ($F_{2,28}$= 0, p>0.05), object ($F_{2,28}$ = 0.888, p>0.05) or group ($F_{1,14}$ = 0, p>0.05) (ii) There is a significant effect of object ($F_{2,28}$ = 7.942, p<0.01) and a significant interaction between object and group ($F_{2,28}$ = 4.902, p<0.01), with the EE group exploring the novel object D* significantly more than the familiar objects A & B at 13-15 mo (**p<0.01) and Aged (**p<0.01) timepoints. There is also a significant increase in the exploration of the novel object D* in the EE group when compared with the SH group at both 13-15 mo ($^{+}$p<0.05) and Aged ($^{+}$p<0.05) timepoints. SH: n=6, EE: n=6. Data expressed as mean $\pm$ SEM.

5.3.7Environmental enrichment prevents the age-related decline in spatial memory

Spatial memory was tested with the three object OD task at all three timepoints. On training day, there was no significant effect of age ($F_{2,28}$= 0, $p>0.05$), object ($F_{2,28}$ = 1.520, $p>0.05$) or Group ($F_{1,14}$ = 0, $p>0.05$). There was also no interaction between object and group ($F_{2,28}$ = 0.267, $p>0.05$) or age ($F_{4,56}$ = 1.393, $p>0.05$; figure 5i.i). Mean percentage exploration of object ± SEM : Baseline SH [Object A] = 36.35±2.34, Baseline SH [Object B] = 30.90±0.94, Baseline SH [Object C] = 32.73±2.08, Baseline EE [Object A] = 39.16±1.34, Baseline EE [Object B] = 29.38±2.02, Baseline EE [Object C] = 31.46±2.74, 13-15 mo SH [Object A] = 34.05±2.81, 13-15 mo SH [Object B] = 33.36±2.52, 13-15 mo SH [Object C] = 32.59±1.20, 13-15 mo EE [Object A] = 35.19±2.13, 13-15 mo EE [Object B] = 33.61±2.18, 13-15 mo EE [Object C] = 31.21±1.44, Aged SH [Object A] = 35.88±2.12, Aged SH [Object B] = 30.32±3.30, Aged SH [Object C] = 33.79±3.41, Aged EE [Object A] = 30.80±3.42, Aged EE [Object B] = 37.08±2.62, Aged EE [Object C] = 32.12±4.09.

On testing day, there was no significant effect of age ($F_{2,28}$= 0, $p>0.05$) or group ($F_{1,14}$ = 0, $p>0.05$). There was however, a significant effect of object ($F_{2,28}$ = 70.176, $p<0.001$) and a significant interaction between object and group ($F_{2,28}$ = 7.683, $p<0.01$) and object and age ($F_{4,56}$ = 1.766, $p<0.001$). There was no significant interaction between object, age and group ($F_{4,56}$ = 0.262, $p>0.05$; figure 5i.ii).

Pairwise comparisons on the main effect of object revealed a significant difference between the exploration of the displaced object C* and the familiar object A ($p<0.05$) and the familiar object B ($p<0.01$) and between the exploration of the two familiar objects ($p<0.001$). To further explore this result, repeated measures ANOVAs were performed at each time-point and this analysis revealed a significant difference in the exploration of the objects at Baseline ($F_{2,28}$ = 71.05, $p<0.001$), with both the SH and EE group exploring the displaced object C* significantly more than the familiar objects [SH: % Object A vs Object D* ($p<0.001$); % Object B vs Object D* ($p<0.001$); EE: % Object A vs Object D* ($p<0.001$); % Object B vs Object D* ($p<0.001$)]. There was significant difference in the exploration of the objects at 13-15 mo ($F_{2,28}$ = 29.32, $p<0.001$) and also a significant interaction between Object and Group ($F_{2,42}$ = 7.861, $p<0.01$). Bonferroni posttests revealed that there was a significant increase in the exploration of the displaced object C*

and the familiar objects A and B in the EE group (% Object A vs Object D* ($p<0.001$); % Object B vs Object D* ($p<0.001$), but only a significant increase in the exploration of the displaced object C* and the familiar object B in the SH group [% Object B vs Object C* ($p < 0.05$)]. There was also a significant difference in the exploration of the objects at Aged ($F_{2,28} = 5.920$, $p<0.01$), with a significant increase in the exploration of the displaced object C* and the familiar object B in the EE group (% Object A vs Object C* ($p>0.05$); % Object B vs Object C* ($p<0.01$) , but no significant differences in the exploration of the objects in the SH group ($p>0.05$ between all groups).

To explore the significant interaction between Object and Group, Independent Samples *t*-tests were performed comparing the difference in exploration of the displaced object between the two groups at the three different time-points. At baseline, there was no significant difference in exploration ($p>0.05$) and whilst the graph would suggest that there is a reduction in exploration in the SH group when compared with the EE group at both 13-15 months old and Aged time-points, this did not reach significance (13-15 mo: $p = 0.059$; Aged: $p = 0.089$).

To explore the significant interaction between Object and Age, repeated measures ANOVAs were performed for each group with Age as the independent variable and exploration of the displaced object C* as the dependent variable. In the EE group, there was no significant effect of Age ($F_{2,16} = 3.155$, $p>0.05$) however in the SH group, there was a significant effect of Age ($F_{2,12} = 7.118$, $p<0.01$). Pairwise comparisons revealed that there was a significant decrease in the exploration of the displaced object C* at the Aged time-point when compared with the Baseline and 13-15 months old time-point [Baseline vs Aged: $p<0.05$; 13-15 mo vs Aged: $p<0.01$)] in the SH group. Mean percentage exploration of object ± SEM : Baseline SH [Object A] = 28.94±1.80, Baseline SH [Object B] = 23.92±1.77, Baseline SH [Object C*] = 47.14±2.81, Baseline EE [Object A] = 24.53±1.65, Baseline EE [Object B] = 23.63±1.56, Baseline EE [Object C*] = 51.85±2.17, 13-15 mo SH [Object A] = 32.97±1.80, 13-15 mo SH [Object B] = 29.70±1.84, 13-15 mo SH [Object C*] = 37.32±1.43, 13-15 mo EE [Object A] = 28.26±1.74, 13-15 mo EE [Object B] = 25.47±2.14, 13-15 mo EE [Object C*] = 46.27±2.12, Aged SH [Object A] = 39.29±2.26, Aged SH [Object B] = 26.80±1.80, Aged SH [Object C*] = 33.91±2.46, Aged EE [Object A] = 31.84±3.93, Aged EE [Object B] = 25.73±2.33, Aged EE [Object C] = 42.43±3.63.

These results show that, at Baseline and 13-15 months old, both the SH and EE rats can distinguish the displaced object from two familiar objects whereas at the Aged time-point, the SH rats cannot distinguish the displaced object from the familiar objects. This suggests that there is an age-related deterioration in spatial memory which can be prevented by environmental enrichment.

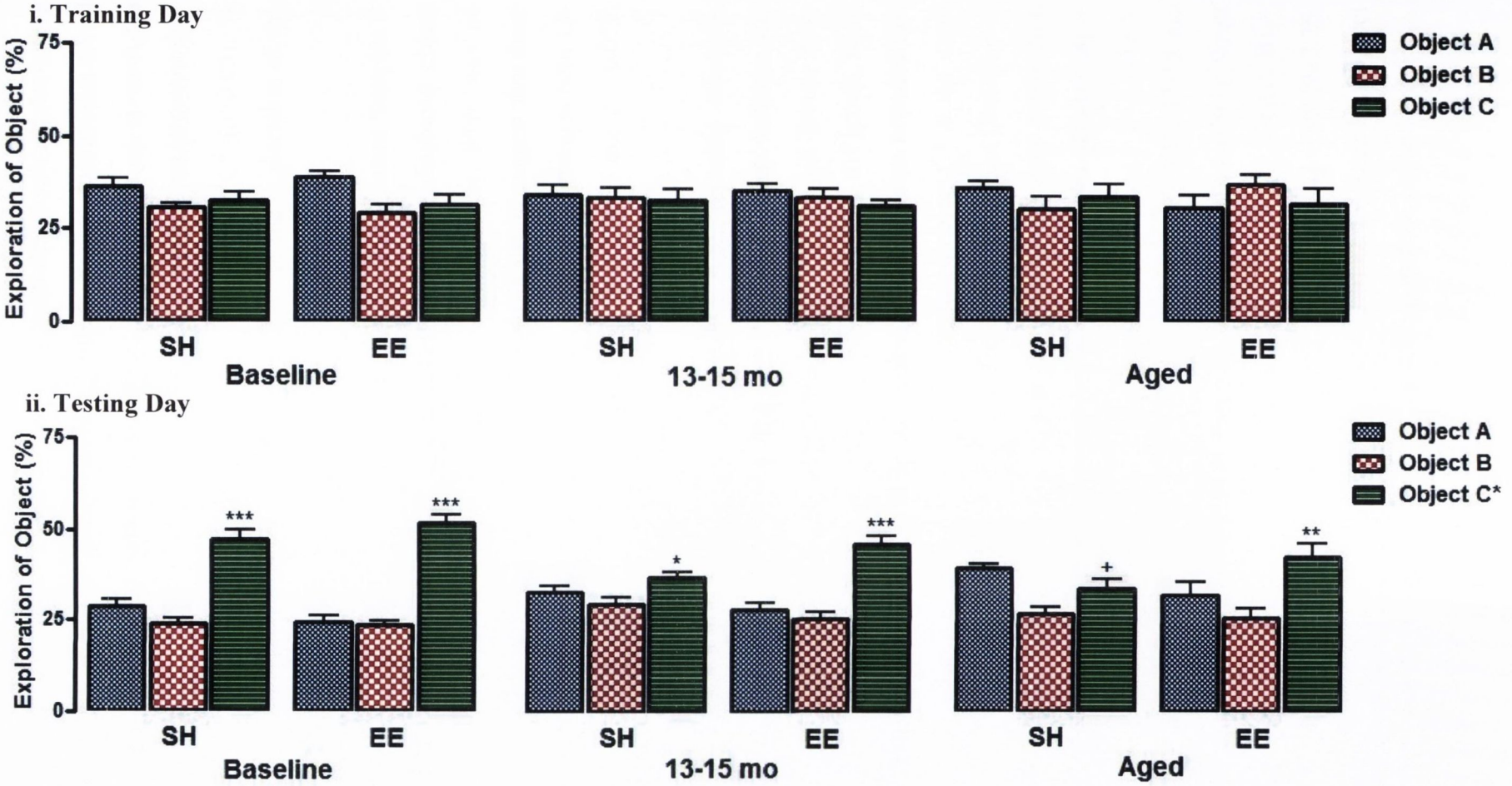


Figure 5i. Environmental enrichment prevents the age-related decline spatial memory(i) There was no significant effect of age ($F_{2,28}$= 0, p>0.05), object ($F_{2,28}$ = 1.520, p>0.05) or group ($F_{1,14}$ = 0, p>0.05) (ii) There was a significant effect of object ($F_2F_{2,28}$ = 70.176, p<0.001) and a significant interaction between object and group ($F_{2,28}$ = 7.683, p<0.01) and between object and age ($F_{4,56}$ = 1.766, p<0.001), with both groups exploring the dicplaced object C* significantly more than the familiar objects A & B at Baseline (***p<0.001). At 13-15 mo, there is a significant increase in the exploration of the displaced object C* when compared with the familiar objects A & B in the EE group (***p<0.001), whereas the SH group only explore the displaced object C* significantly more than the familiar object B (*p<0.05). At the Aged timepoint, the EE group explore the displaced object C* significantly more than the familiar objects A & B (**p<0.01), whereas the SH group explore the displaced object C* significantly less at the Aged timepoint than the Baseline or 13-15 mo timepoints ($^{+}$p<0.05). SH: n=6, EE: n=6. Data is expressed as mean $\pm$ SEM.

5.3.8Environmental enrichment prevents the age-related deficit in the retention of spatial memory

Spatial memory was tested using the Morris water maze at all three timepoints. There was a significant effect of day on latency to platform ($F_{4,40}$ = 69.346, $p<0.001$; figure 5j.i) but no significant effect of age ($F_{2,20}$ = 2.031, $p>0.05$) or group ($F_{1,10}$ = 0.287, $p>0.05$). There was also a significant interaction between day and age ($F_{8,80}$ = 2.889, $p<0.01$) but no significant interaction between age and group ($F_{2,20}$ = 0.492, $p>0.05$) or Group and Day ($F_{4,40}$ = 1.519, $p>0.05$). Pairwise comparisons revealed an overall decrease in latency to platform on consecutive days of training (Day 1 vs Day 2: $p<0.01$; Day 2 vs Day 3: $p<0.01$; Day 3 vs Day 4: $p>0.05$; Day 4 vs Day 5: $p<0.05$), indicating that both groups learnt the location of the hidden platform at each timepoint.

To further explore the interaction between day and age, posthoc repeated measures ANOVAs were used to compare the latency to platform on each day across the three different timepoints. There was no significant difference in the latency to platform on Day 1 between the three timepoints ($F_{2,22}$ 2.463, $p>0.05$). There was however, a difference in the latency to platform on Day 2 ($F_{2,22}$ 5.964, $p<0.01$) with rats at Baseline having a significantly lower latency to platform that rats at the 13-15 months old timepoint ($p<0.01$). There was no significant difference in the latency to platform on Days 3, 4 or 5.

To explore whether the latency to platform was affected by the speed of swimming, total distance swum was also measured. There was a significant effect of day on distance swum ($F_{4,40}$ = 25.659, $p<0.001$; figure j.ii) and a significant effect of age ($F_{2,20}$ = 5.301, $p<0.05$) but no significant effect of group ($F_{1,10}$ = 0.471, $p>0.05$). There were no significant interactions between factors (age*group: $F_{2,20}$ = 0.418, $p>0.05$; day*group: $F_{4,40}$ = 1.415, $p>0.05$; age*day: $F_{8,80}$ = 1.762, $p>0.05$; age*day*group: $F_{8,80}$ = 0.906, $p>0.05$). Pairwise comparisons revealed an overall decrease in distance swum in all days when compared with day 1 (Day 1 vs Day 2: $p<0.05$; Day 1 vs Day 3: $p<0.01$; Day 1 vs Day 4: $p<0.001$; Day 1 vs Day 5: $p<0.001$), but no differences on consecutive days (Day 2 vs Day 3: $p>0.05$; Day 3 vs Day 4: $p>0.05$; Day 4 vs Day 5: $p>0.05$). This would suggest that the rats were swimmer faster on consecutive days because their latency was decreasing, but their total distance swum was not. Pairwise comparisons also revealed a significant increase in the distance swum in the Aged group when compared with the 13-15 months old group

($p<0.01$), suggesting that the Aged group are swimming faster than the 13-15 month old group.

The probe test, performed two days later at each timepoint revealed that there was a significant main effect of group on the time spent in the target quadrant ($F_{1,20} = 10.58$, $p<0.01$; figure 5k.i). There was no significant effect of age ($F_{2,20} = 2.382$, $p>0.05$) and no significant interaction between age and group ($F_{2,20} = 1.058$, $p>0.05$). Bonferroni posttests revealed a significant difference in the percentage time spent in the target quadrant between the SH and EE group at the Aged timepoint ($p<0.05$[*]) Percentage time spent in target quadrant ± SEM: Baseline SH = 30.33±4.46; Baseline EE = 43.53±6.09; MA SH = 32.11±4.40, MA EE = 36.31±3.46; Aged SH = 20.05±2.22; Aged EE = 35.92±3.48.

These data suggest that there is no age-related deficit in the acquisition of the memory for the location of the hidden platform on this task but that there is an age-related deficit in the retention of this memory over 48 hours, and that environmental enrichment can prevent this deficit.

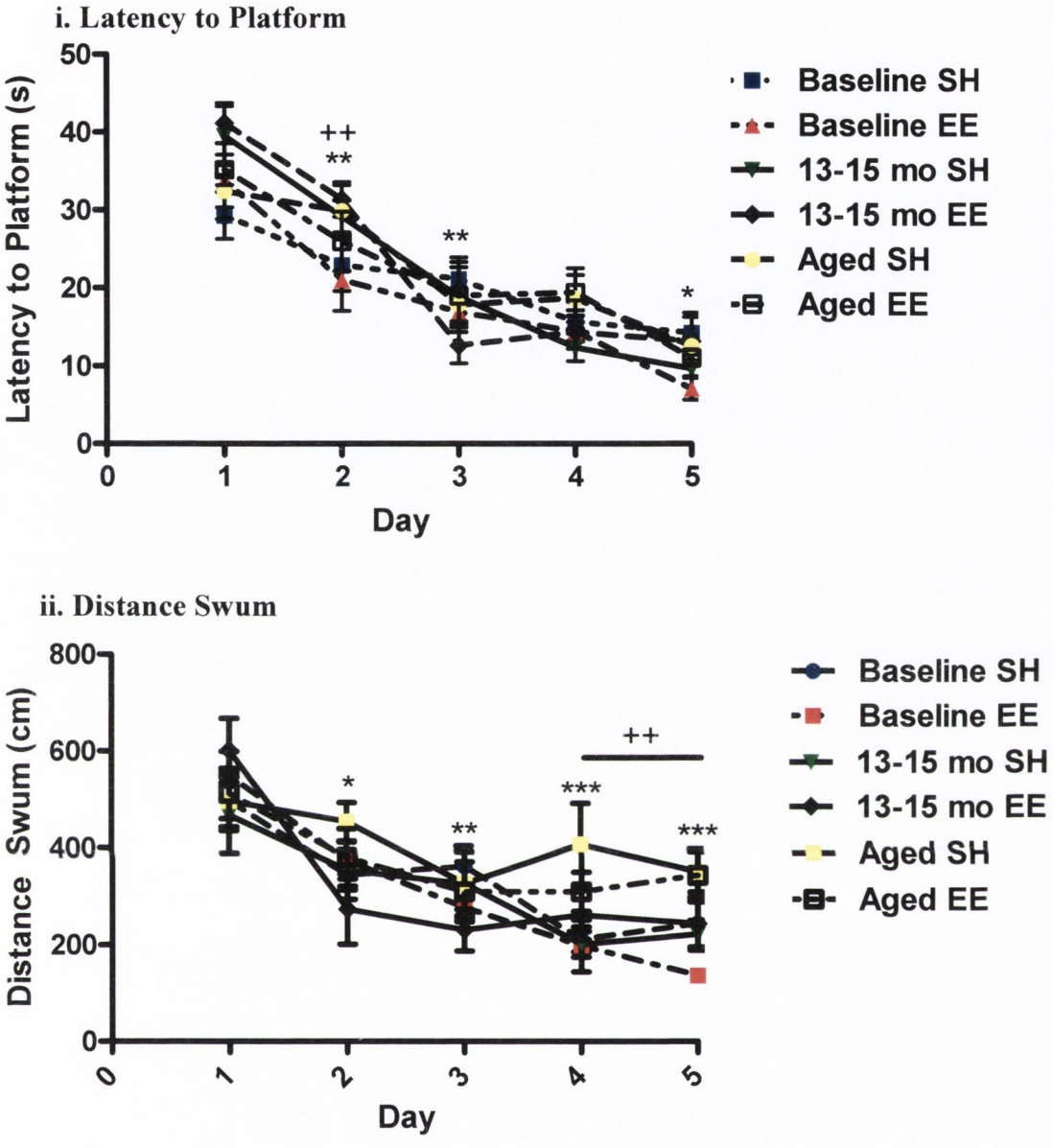


Figure 5j. Environmental enrichment does not affect memory acquisition on the Morris water maze(i) There was a significant effect of day on latency to platform ($F_{4,40} = 69.346$, $p<0.001$) with an overall decrease in latency to the platform across the five days (Day 1 vs Day 2: **$p<0.01$; Day 2 vs Day 3: **$p<0.01$; Day 3 vs Day 4: $p>0.05$; Day 4 vs Day 5: *$p<0.05$). There was no significant effect of group ($F_{1,10} = 0.287$, $p>0.05$) or age ($F_{2,20} = 2.031$, $p>0.05$). There was a significant interaction between day and age ($F_{8,80} = 2.889$, $p<0.01$), with rats at Baseline having a significantly lower latency to platform than rats at the 13-15 mo timepoint ($^{++}p<0.01$) but no significant interaction between age and group ($F_{2,20} = 0.491$,$p>0.05$) or group and day ($F_{4,40} = 1.519$, $p>0.05$) **(ii)** There was a significant effect of day on distance swum ($F_{4,40} = 25.659$, $p<0.001$) with a decrease in distance swum on all days when compared with day 1 (Day 1 vs Day 2: *$p<0.05$; Day 1 vs Day 3: **$p<0.01$; Day 1 vs Day 4: ***$p<0.001$; Day 1 vs Day 5: ***$p<0.001$), but no differences on consecutive days. There was also a significant increase in distance swum in the Aged group when compared with the 13-15mo group ($^{++}p<0.01$).

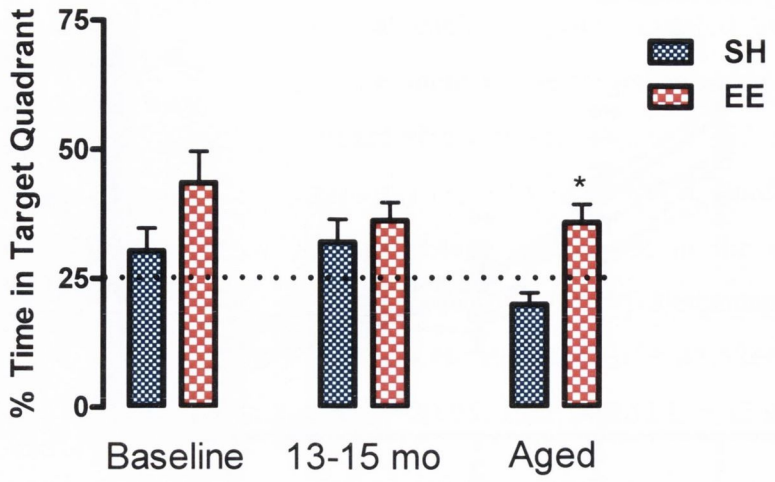


Figure 5k. Environmental enrichment prevents the age-related deficit in spatial memory retention as measured by the Morris water maze. In the probe test, there was a significant effect of group on the time spent in the target quadrant ($F_{1,20}$= 10.58, $p<0.01$) but no significant effect of age ($F_{2,20}$ = 2.382, $p>0.05$) and no significant interaction ($F_{2,20}$ = 1.058, $p>0.05$). Bonferroni posttests revealed that there was a significant decrease in the exploration of the target quadrant in SH rats when compared with EE rats at the Aged timepoint (*$p<0.05$). SH: n=6, EE: n=6. Data expressed as mean ± SEM.

5.3.9 Environmental enrichment improves working memory

Working memory in was tested using the T maze task. There was a significant effect of day ($F_{6,60}$ = 8.425, $p<0.001$), a significant effect of age ($F_{2,20}$ = 4.032, $p<0.05$) and a significant effect of group ($F_{1,10}$ = 28.017, $p<0.001$; figure 5l). There was however, no significant interaction between age and group ($F_{2,20}$ = 1.521, $p>0.05$), between day and group ($F_{6,60}$ = 1.110, $p>0.05$), between age and day ($F_{12,120}$ = 0.264, $p>0.05$) or between age, day and group ($F_{12,120}$ = 0.749, $p>0.05$).

Post-hoc pairwise comparisons revealed a significant overall increase in the percentage of correct entries in the EE group when compared with the SH group ($p<0.001$), and an overall decrease in the percentage of correct entries at the Aged timepoint when compared with the 13-15 mo timepoint ($p=0.069$).To further explore the differences between groups at each timepoint, Post-hoc repeated measures ANOVAs were performed at each timepoint.

At the Young timepoint (where the rats had been housed in EE or SH conditions for 6 weeks), there is a significant effect of group ($F_{1,10}$ = 25.60, $p<0.001$) and a significant effect of day ($F_{6,60}$ = 2.937, $p<0.05$). There was no significant interaction between group and day ($F_{6,60}$ = 1.079, $p>0.05$). Post-hoc repeated measures ANOVA revealed that there no significant difference in the percentage of correct arm entries between days in the SH group (mean percentage entries ± SEM: day 1 = 61.11±8.24, day 2 = 69.44±6.69, day 3 = 69.44±5.12, day 4 = 63.89±6.69, day 5 = 66.67±4.30, day 6 = 66.67±4.30, day 7 = 77.78±3.51). There was an increase in the percentage of correct arm entries between days 1 and 6, and between days 1 and 7 in the EE group however this does not quite reach significance (mean percentage entries ± SEM: day 1 = 58.33±3.73, day 2 = 66.67±7.45, day 3 = 69.44±5.12, day 4 = 75.00±3.73, day 5 = 77.78±7.03, day 6 = 86.11±2.78, day 7 = 86.11±2.78; day 1 vs day 6, $p=0.086$; day 1 vs day 7, $p=0.086$).

At the 13-15 mo timepoint, there is a significant effect of group ($F_{1,10}$ = 6.45, $p<0.05$) and a significant effect of day ($F_{6,60}$ = 3.58, $p<0.01$). There was no significant interaction between group and day ($F_{6,60}$ = 0.452, $p>0.05$). Post-hoc repeated measures ANOVA revealed that there no significant difference in the percentage of correct arm entries between days in the SH group (mean percentage entries ± SEM: day 1 = 62.50±3.23, day 2 = 60.42±7.51, day 3 = 64.58±3.84, day 4 = 68.75±6.25, day 5 = 68.75±6.25, day 6 = 77.08±3.84, day 7 = 75.00±6.45). There was a significant difference in the percentage of

correct arm entries between days 1 and 6 in the EE group (mean percentage entries ± SEM: day 1 = 68.75±5.35, day 2 = 77.08±5.97, day 3 = 68.75±5.35, day 4 = 81.25±34.27, day 5 = 79.17±2.64, day 6 = 83.33±2.64, day 7 = 85.12±2.08l; 1 vs 6, *$p<0.05$).

At the Aged timepoint, there is a significant effect of group ($F_{1,10} = 2.705$, $p<0.05$) and a significant effect of day ($F_{6,60} = 19.38$, $p<0.01$). There was no significant interaction between group and day ($F_{6,60} = 0.933$, $p>0.05$). Post-hoc repeated measures ANOVA revealed no significant difference in the percentage of correct arm entries between days in the SH group (mean percentage entries ± SEM: day 1 = 50.00±3.23, day 2 = 54.17±5.27, day 3 = 62.50±8.54, day 4 = 62.50±8.54, day 5 = 64.58±5.97, day 6 = 64.58±5.97, day 7 = 60.42±5.97). There is a significant increase in the percentage of correct arm entries between days 3 and 6, and between days 5 and 7 in the EE group (mean percentage entries ± SEM: day 1 = 62.50±6.46, day 2 = 70.83±6.18, day 3 = 66.67±2.64, day 4 = 75.00±7.94, day 5 = 72.92±3.84, day 6 = 81.25±4.27, day 7 = 87.5±3.23; day 3 vs day 6, $^{+}p<0.05$; day 5 vs day 7, $^{\&}p<0.05$).

These data show that there is an improvement of working memory in the enriched rats, as tested by the T maze task, and that this capacity for improvement is maintained throughout the rat's lifespan. However, the data do not suggest an age-related deficit in working memory as assessed by this task.

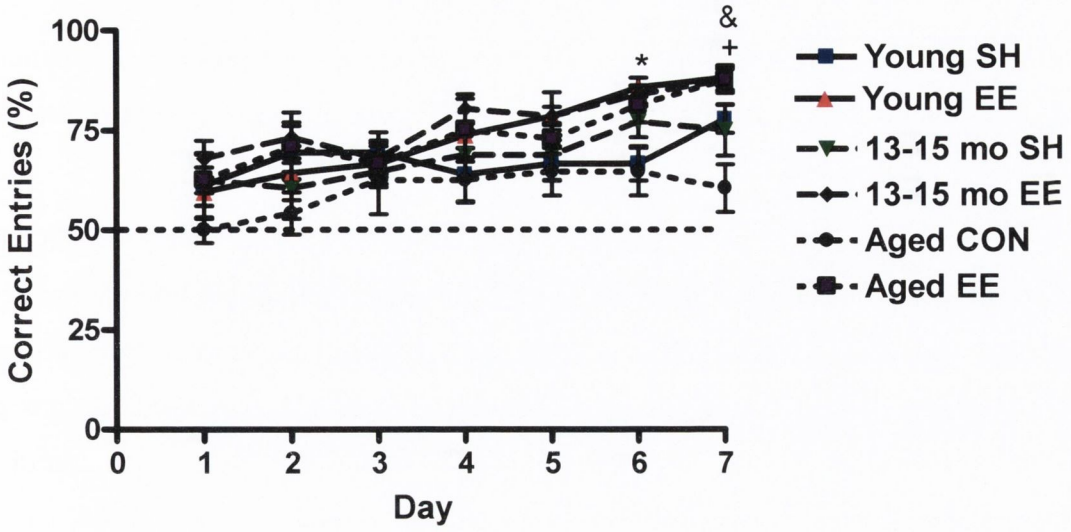


Figure 5l. Environmental enrichment improves working memory There was a significant effect of day ($F_{6,60}$ = 8.425, $p<0.001$), a significant effect of age ($F_{2,20}$ = 4.032, $p<0.05$) and a significant effect of group ($F_{1,10}$ = 28.017, $p<0.001$) but no significant interactions. Post-hoc repeated measures ANOVAs revealed that at the Young timepoint, there was no significant differences between percentage of correct entries between days in the SH group but a significant increase between day 1 & 6 in the EE group (*$p<0.05$). At the 13-15 mo timepoint, there was no significant differences in the percentage of correct entries between days in the SH group but an increase between day 1 & 6 and between day 1 & 7 in the EE group, although it does not reach significance (1 vs 6, $p=0.086$; 1 vs 7, $p=0.086$). At the Aged timepoint, there was no significant differences in the percentage of correct entries between days in the SH group but a significant increase between day 3 & 6 and day 5 & 7 in the EE group (3 vs 6, $^{+}p<0.05$; 5 vs 7, $p<0.05$[&]). SH: n=6, EE: n=6. Data expressed as mean ± SEM.

5.4.1 Environmental enrichment attenuates the age-related reduction in βNGF concentration in the hippocampus

There is no significant difference between groups in βNGF concentration in the dentate gyrus ($F_{2,17}$ = 3.011, $p>0.05$; figure 5m.i). Mean βNGF concentration ± SEM ($pg.mg^{-1}$): Young SH = 55.23±7.61, Aged SH = 83.41±10.56, Aged EE = 88.95±14.89.

There is a significant difference between groups in βNGF concentration in the hippocampus ($F_{2,17}$ = 7.355, $p<0.01$; figure 5m.ii). Bonferroni's multiple comparison test showed a significant reduction in βNGF in the Aged SH when compared with the Young SH (**$p<0.01$) but no significant difference between Young SH and Aged EE groups ($p>0.05$) or between Aged SH and Aged EE groups ($p>0.05$). Mean βNGF concentration ± SEM ($pg.mg^{-1}$): Young SH = 71.04±11.29, Aged SH = 25.31±4.44, Aged EE = 50.19±6.83.

There is no significant difference between groups in βNGF concentration in the perirhinal cortex ($F_{2,17}$ = 1.836, $p>0.05$; figure 5m.iii). Mean βNGF concentration ± SEM ($pg.mg^{-1}$): Young SH = 73.39±14.41, Aged SH = 41.13±49.09, Aged EE = 55.59±6.33.

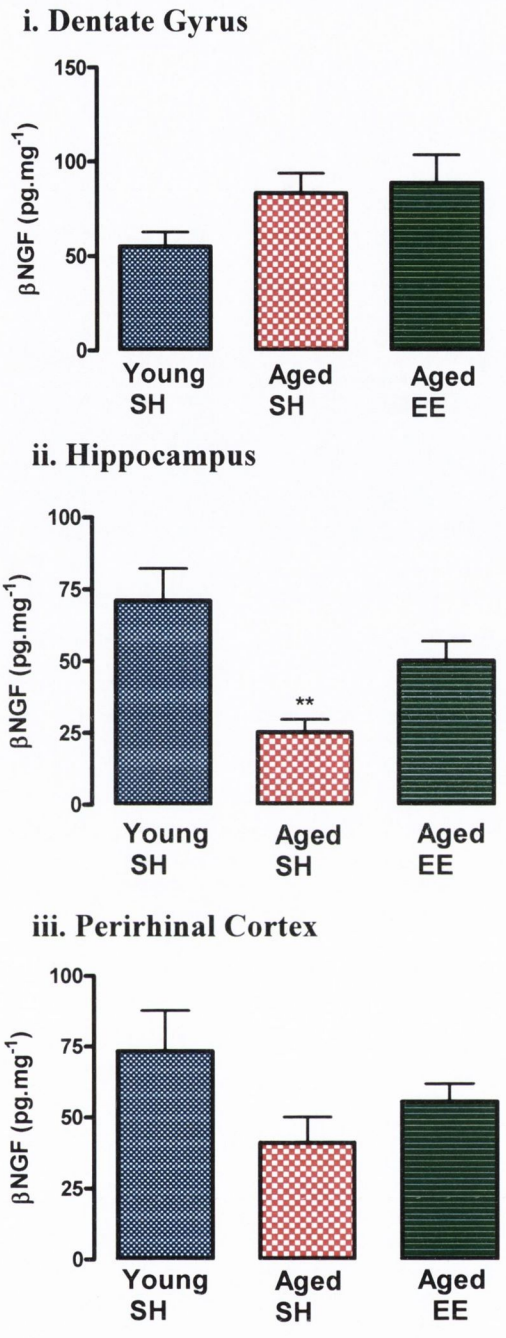


Figure 5m.Environmental enrichment attenuates the age-related reduction in βNGF concentration in the hippocampus(i) There was no significant difference between groups in the concentration of βNGF in the dentate gyrus ($F_{2,17}$ = 3.011, $p>0.05$)(ii) There was a significant difference between groups in the concentration of βNGF in the hippocampus ($F_{2,17}$ = 7.355, $p<0.01$) with a significant decrease in the Aged SH group when compared with the Young SH group (**$p<0.01$). There was no significant difference between Young SH and Aged EE in NGF concentration ($p>0.05$) or between Aged SH and Aged EE groups ($p>0.05$) (iii) There was no significant difference between groups in the concentration of βNGF in perirhinal cortex ($F_{2,17}$ = 1.836, $p>0.05$). Young SH: n=8, Aged SH: n=6, Aged EE: n=6. Data expressed as mean ± SEM.

5.4.2 Aged rats show an increase in BDNF concentration in the dentate gyrus in standard, but not enriched housing.

There is a significant difference between groups in the BDNF concentration in the dentate gyrus ($F_{2,17}$ = 15.43, $p<0.001$; figure 5n.i). Bonferroni's multiple comparison test showed a significant increase in BDNF concentration in the Aged SH group when compared with the Young SH group (***$p<0.001$) and the Aged EE group ($^{++}p<0.01$). There is no significant difference between the Young SH and Aged EE groups in BDNF concentration ($p>0.05$). Mean BDNF concentration ± SEM ($pg.mg^{-1}$): Young SH: 456.1±40.83, Aged SH: 859.7±67.45, Aged EE: 551.1±55.33.

There is no significant difference between groups in the BDNF concentration in the hippocampus ($F_{2,17}$ = 1.694, $p>0.05$; figure 5n.ii). Mean BDNF concentration ± SEM ($pg.mg^{-1}$): Young SH: 335.6±42.26, Aged SH: 250.5±10.25, Aged EE: 293.0±27.19.

There is no significant difference between groups in the BDNF concentration in the perirhinal cortex ($F_{2,17}$ = 0.339, $p>0.05$; figure 5n.iii). Mean BDNF concentration ± SEM ($pg.mg^{-1}$): Young SH: 252.2±34.88, Aged SH: 252.1±50.38, Aged EE: 235.3±71.94.

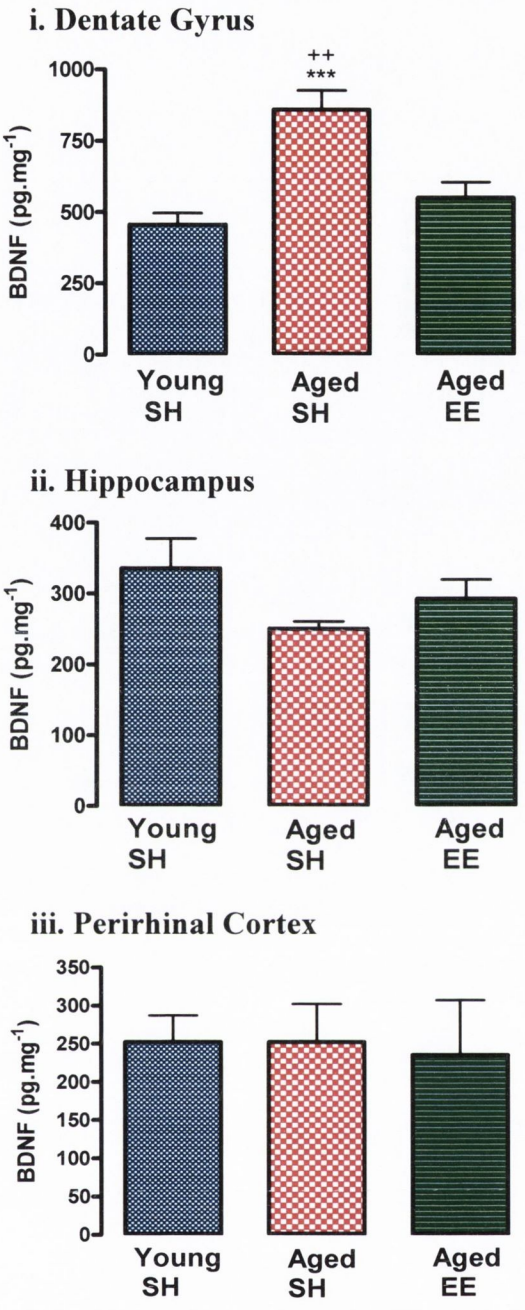


Figure 5n. There is an age-related increase BDNF concentration in the dentate gyrus in rats housed in standard, but not enriched, conditions (i) The was a significant difference between groups in the concentration of BDNF in the dentate gyrus ($F_{2,17} = 15.43$, $p<0.001$) with a significant increase in the Aged SH group when compared with the Young SH group (***$p<0.001$) and Aged EE group ($^{++}p<0.01$). There was no significant difference between Young SH and Aged EE in BDNF concentration ($p>0.05$) (ii) There was no significant difference between groups in the concentration of BDNF in the hippocampus ($F_{2,17} = 1.694$, $p>0.05$). (iii) There was no significant difference between groups in the concentration of BDNF in perirhinal cortex ($F_{2,17} = 0.339$, $p>0.05$). Young SH: n=8, Aged SH: n=6, Aged EE: n=6. Data expressed as mean ± SEM.

5.4.3 Environmental enrichment does not affect VEGF concentration in the hippocampus, dentate gyrus or perirhinal cortex

There is no significant difference between groups in the VEGF concentration in the dentate gyrus ($F_{2,17}$ = 1.875, p>0.05; figure 5o.i). Mean VEGF concentration ± SEM ($pg.mg^{-1}$): Young SH = 59.91±7.34, Aged SH = 84.04±18.96, Aged EE = 99.13±19.24.

There is a difference between groups in VEGF concentration in the hippocampus, although it does not reach significance ($F_{2,16}$ = 2.935, p=0.0821; figure 5o.ii). Bonferroni's multiple comparison test showed no significant difference between Young SH and Aged SH in the VEGF concentration (p>0.05), however there is a reduction in VEGF concentration in the Aged SH group when compared with the Aged EE group (p=0.074). There is no significant difference between Young SH and Aged EE groups (p>0.05). Mean VEGF concentration ± SEM ($pg.mg^{-1}$): Young SH (n=8) = 90.73±14.10, Aged SH (n=6) = 53.14±6.32, Aged EE (n=5) = 114.5±29.02.

There is no significant difference between groups in the VEGF concentration in the perirhinal cortex ($F_{2,17}$ = 0.094, p>0.05; figure 5o.iii). Mean VEGF concentration ± SEM ($pg.mg^{-1}$): Young SH = 105.6±20.99, Aged SH = 103.1±23.97, Aged EE = 92.29±23.55.

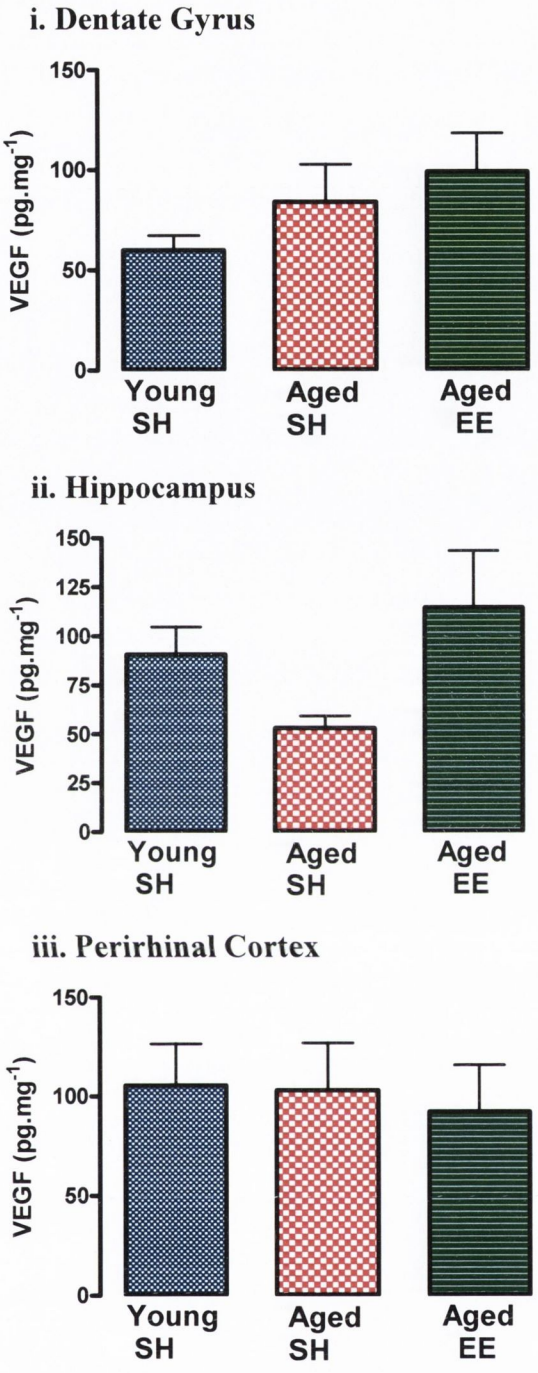


Figure 5o. Environmental enrichment does not affect VEGF concentration in the hippocampus, dentate gyrus or perirhinal cortex (i) There was no significant difference between groups in the concentration of VEGF in the dentate gyrus ($F_{2,17}$ = 1.875, p>0.05). Young SH: n=8, Aged SH: n=6, Aged EE: n=6(ii) There was a difference between groups in the concentration of VEGF in the hippocampus, although it does not reach significance ($F_{2,16}$ = 2.935, p=0.0821) with a decrease in the Aged SH group when compared with the Aged EE group (p=0.074). There was no significant difference between Young SH and Aged SH (p>0.05) or Aged EE (p>0.05) in VEGF concentration. Young SH: n=8, Aged SH: n=6, Aged EE: n=5 (iii) There was no significant difference between groups in the concentration of VEGF in perirhinal cortex ($F_{2,17}$ = 0.094, p>0.05). Young SH: n=8, Aged SH: n=6, Aged EE: n=6. Data expressed as mean ± SEM.

5.4.4 There are no age-related differences in Trk B concentration in the dentate gyrus, hippocampus or perirhinal cortex

There is no significant difference between groups in Trk B concentration in the dentate gyrus ($F_{2,16}$ = 1.514, p>0.05; figure 5p.i). Mean Trk B concentration ± SEM ($pg.mg^{-1}$): Young SH (n=8) = 157.1±26.38, Aged SH (n=5) = 139.8±41.90, Aged EE (n=6) = 218.7±32.60.

There is no significant difference between groups in Trk B concentration in the hippocampus ($F_{2,16}$ = 1.294, p>0.05; figure 5p.ii). Mean Trk B concentration ± SEM ($pg.mg^{-1}$): Young SH (n=8) = 50.95±6.54, Aged SH (n=6) = 66.34±7.04, Aged EE (n=5) = 46.98±13.57.

There is no significant difference between groups in Trk B concentration in the perirhinal cortex ($F_{2,15}$ = 0.211, p>0.05; figure 5p.iii). Mean Trk B concentration ± SEM ($pg.mg^{-1}$): Young SH (n=7) = 146.1±38.89, Aged SH (n=5) = 139.9±24.62, Aged EE (n=6) = 167.7±19.47.

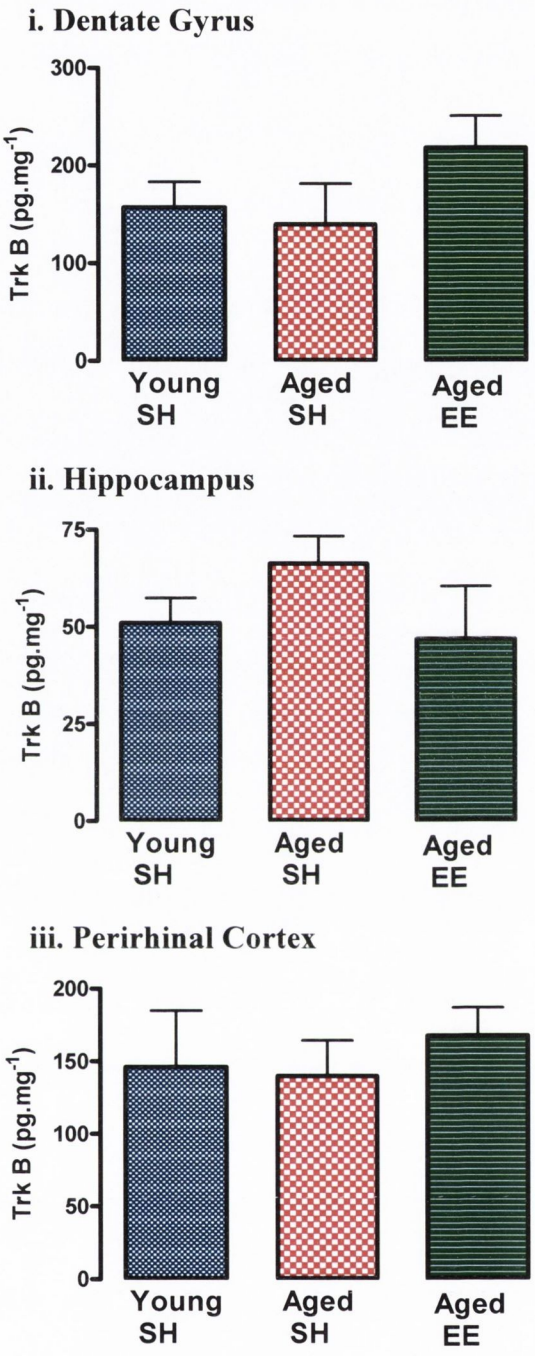


Figure 5p. There are no age-related differences in Trk B concentration in the dentate gyrus, hippocampus or perirhinal cortex (i) There was no significant difference between the groups in Trk B concentration in the dentate gyrus ($F_{2,16}$ = 1.514, $p>0.05$) Young SH: n=8, Aged SH: n=5, Aged EE: n=6 (ii) There was no significant difference between groups in the concentration of Trk B in the hippocampus ($F_{2,16}$ = 1.294, $p>0.05$). Young SH: n=8, Aged SH: n=6, Aged EE: n=5 (iii) There was no significant difference between groups in the concentration of Trk B in perirhinal cortex ($F_{2,15}$ = 0.211, $p>0.05$). Young SH: n=7, Aged SH: n=5, Aged EE: n=6. Data expressed as mean ± SEM.

5.4.5 There is a significant decrease in βNGF mRNA expression in the hippocampus following long-term environmental enrichment

There is no significant difference between groups in the expression of βNGF mRNA in the dentate gyrus ($F_{2,16} = 1.953$, $p>0.05$; figure 5q.i). Mean βNGF mRNA fold change ± SEM: Young SH (n=8) = 1.00±0.05, Aged SH (n=5) = 0.92±0.05, Aged EE (n=6) = 0.87±0.06.

There is a significant difference between groups in the expression of βNGF mRNA in the hippocampus ($F_{2,14} = 4.404$, $p<0.05$; figure 5q.ii). Bonferroni's multiple comparison test showed that there is a significant reduction in βNGF mRNA expression in the Aged EE group when compared with Young SH (*$p<0.05$). There is no difference between Aged SH and Young SH ($p>0.05$) or Aged EE ($p>0.05$). Mean βNGF mRNA fold change ± SEM: Young SH (n=8) = 1.00±0.08, Aged SH (n=5) = 0.76±0.09, Aged EE (n=4) = 0.67±0.05.

There is no significant difference between groups in the expression of βNGF mRNA in the perirhinal cortex ($F_{2,17} = 0.627$, $p>0.05$; figure 5q.iii). Mean βNGF mRNA fold change ± SEM: Young SH (n=8) = 1.00±0.07, Aged SH (n=6) = 0.93±0.08, Aged EE (n=6) = 1.09±0.14.

There is no significant difference between groups in the expression of βNGF mRNA in the entorhinal cortex ($F_{2,17} = 1.911$, $p>0.05$; figure 5q.iv). Mean βNGF mRNA fold change ± SEM: Young SH (n=8) = 1.00±0.10, Aged SH (n=6) = 0.78±0.08, Aged EE (n=6) = 0.85±0.06.

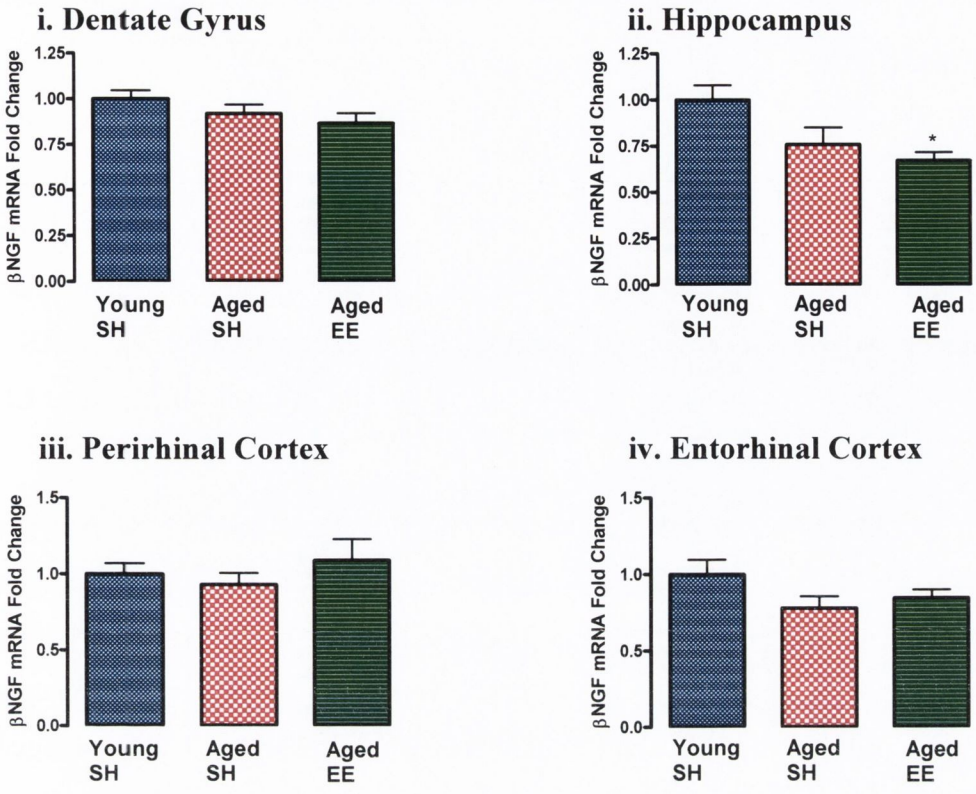


Figure 5q. There is a significant decrease in βNGF mRNA expression in the hippocampus following long-term environmental enrichment(i) There was no significant difference between groups in the βNGF mRNA expression in the dentate gyrus ($F_{2,16}$ = 1.953, p>0.05). Young SH: n=8, Aged SH: n=5, Aged EE: n=6 (ii) There was a significant difference between the groups in βNGF mRNA expression in the hippocampus ($F_{2,14}$ = 4.404, p<0.05), with a significant reduction in βNGF mRNA expression in the Aged EE group when compared with the Young SH group (*p<0.05). There was no significant differences between the Aged SH and Young SH (p>0.05) or Aged EE (p>0.05) groups. Young SH: n=8, Aged SH: n=5, Aged EE: n=4 (iii) There was no significant difference between groups in βNGF mRNA expression in perirhinal cortex ($F_{2,17}$ = 0.627, p>0.05). Young SH: n=8, Aged SH: n=6, Aged EE: n=6. (iv) There was no significant difference between groups in βNGF mRNA expression in entorhinal cortex ($F_{2,17}$ = 1.911, p>0.05). Young SH: n=8, Aged SH: n=6, Aged EE: n=6. Data expressed as mean ± SEM.

5.4.6 Long-term environmental enrichment increases BDNF mRNA expression in the hippocampus and dentate gyrus

There is a significant difference between groups in the expression of BDNF mRNA in the dentate gyrus ($F_{2,14}$ = 6.185, $p<0.05$; figure 5r.i). Bonferroni's multiple comparison test showed a significant increase in the expression of BDNF mRNA in the Aged EE group when compared with the Young SH (*$p<0.05$) and Aged SH ($^{+}p<0.05$) groups. There is no significant difference between the Young SH and Aged SH ($p>0.05$). Mean BDNF mRNA fold change ± SEM: Young SH (n=8) = 1.00±0.16, Aged SH (n=5) = 0.87±0.22, Aged EE (n=4) = 2.61±0.78.

There is a significant difference between groups in the expression of BDNF mRNA in the hippocampus ($F_{2,14}$ = 4.075, $p<0.05$; figure 5r.ii). Bonferroni's multiple comparison test showed a significant increase in the expression of BDNF mRNA in the Aged EE group when compared with the Young SH group (*$p<0.05$). There is no significant difference between the Young SH and Aged SH ($p>0.05$) groups or between the Aged SH and Aged EE groups ($p>0.05$). Mean BDNF mRNA fold change ± SEM: Young SH (n=8) = 1.00±0.21, Aged SH (n=4) = 1.07±0.22, Aged EE (n=5) = 2.76±0.89.

There is no significant difference between groups in the expression of BDNF mRNA in perirhinal cortex ($F_{2,17}$ = 0.693, $p>0.05$; figure 5r.iii). Mean BDNF mRNA fold change ± SEM: Young SH (n=8) = 1.00±0.09, Aged SH (n=6) = 0.84±0.14, Aged EE (n=6) = 1.04±0.15.

There is no significant difference between groups in the expression of BDNF mRNA in entorhinal cortex ($F_{2,17}$ = 0.672, $p>0.05$; figure 5r.iv). Mean BDNF mRNA fold change ± SEM: Young SH (n=8) = 1.00±0.21, Aged SH (n=6) = 1.11±0.27, Aged EE (n=6) = 1.37±0.18.

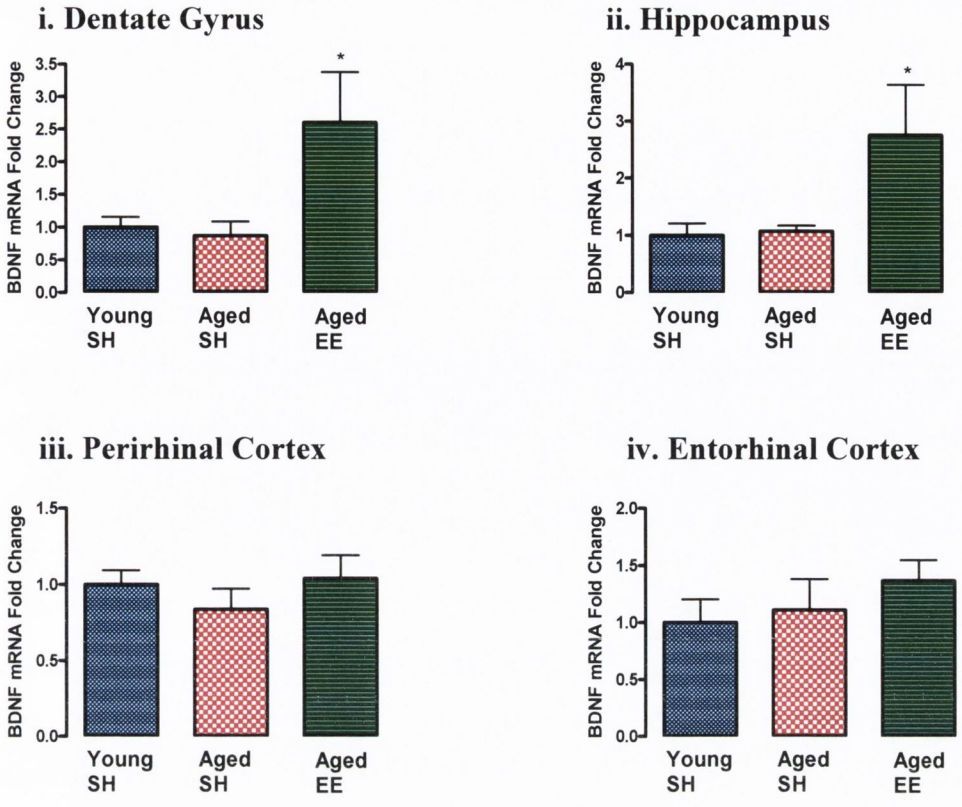


Figure 5r. Long-term environmental enrichment increases BDNF mRNA expression in the dentate gyrus and hippocampus (i) There was a significant difference between groups in BDNF mRNA expression in the dentate gyrus ($F_{2,14} = 4.075$, $p<0.05$), with a significant increase in the Aged EE group when compared with the Young SH (*$p<0.05$) and Aged SH (*$p<0.05$) groups. There was no significant difference between the Young SH and Aged SH groups ($p>0.05$). Young SH: n=8, Aged SH: n=4, Aged EE: n=5 (ii) There was a significant difference between the groups in BDNF mRNA expression in the hippocampus ($F_{2,14} = 6.185$, $p<0.05$), with a significant increase in BDNF mRNA expression in the Aged EE group when compared with the Young SH group (*$p<0.05$). There was no significant differences between the Aged SH and Young SH ($p>0.05$) or Aged EE ($p>0.05$) groups. Young SH: n=8, Aged SH: n=5, Aged EE: n=4 (iii) There was no significant difference between groups in BDNF mRNA expression in perirhinal cortex ($F_{2,17} = 0.693$, $p>0.05$). Young SH: n=8, Aged SH: n=6, Aged EE: n=6. (iv) There was no significant difference between groups in NGF mRNA expression in entorhinal cortex ($F_{2,17} = 0.672$, $p>0.05$). Young SH: n=8, Aged SH: n=6, Aged EE: n=6. Data expressed as mean ± SEM.

5.4.7 There is an age-related increase in Trk A mRNA expression in the perirhinal cortex

There is no significant difference between the groups in the expression of Trk A mRNA in the dentate gyrus ($F_{2,16}$ = 0.31, $p>0.05$; figure 5s.i). Mean Trk A mRNA fold change ± SEM: Young SH (n=8) = 1.00±0.35, Aged SH (n=5) = 0.70±0.21, Aged EE (n=6) = 1.10±0.39.

There is no difference between the groups in the expression of Trk A mRNA in the hippocampus ($F_{2,13}$ = 2.972, $p>0.05$; figure 5s.ii). Mean Trk A mRNA fold change ± SEM: Young SH (n=8) = 1.00±0.28, Aged SH (n=4) = 0.27±0.06, Aged EE (n=4) = 1.45±0.38.

There is a significant difference between the groups in the expression of Trk A mRNA expression in the perirhinal cortex ($F_{2,17}$ = 29.64, $p<0.001$; figure 5s.iii). Bonferroni's multiple comparison test revealed a significant increase in Trk A mRNA expression in the Aged SH group when compared with the Young SH group (***$p<0.001$) and in the Aged EE group when compared with the Young SH group (***$p<0.001$). There is no significant difference between the Aged SH and Aged EE groups ($p>0.05$). Mean Trk A mRNA fold change ± SEM: Young SH (n=8) = 1.00±0.16, Aged SH (n=6) = 5.53±0.62, Aged EE (n=6) = 4.05±0.54.

There is no significant difference between the groups in the expression of Trk A mRNA in the entorhinal cortex ($F_{2,17}$ = 2.391, $p>0.05$; figure 5s.iv). Mean Trk A mRNA fold change ± SEM: Young SH (n=8) = 1.00±0.30, Aged SH (n=6) = 2.05±0.48, Aged EE (n=6) = 1.63±0.26.

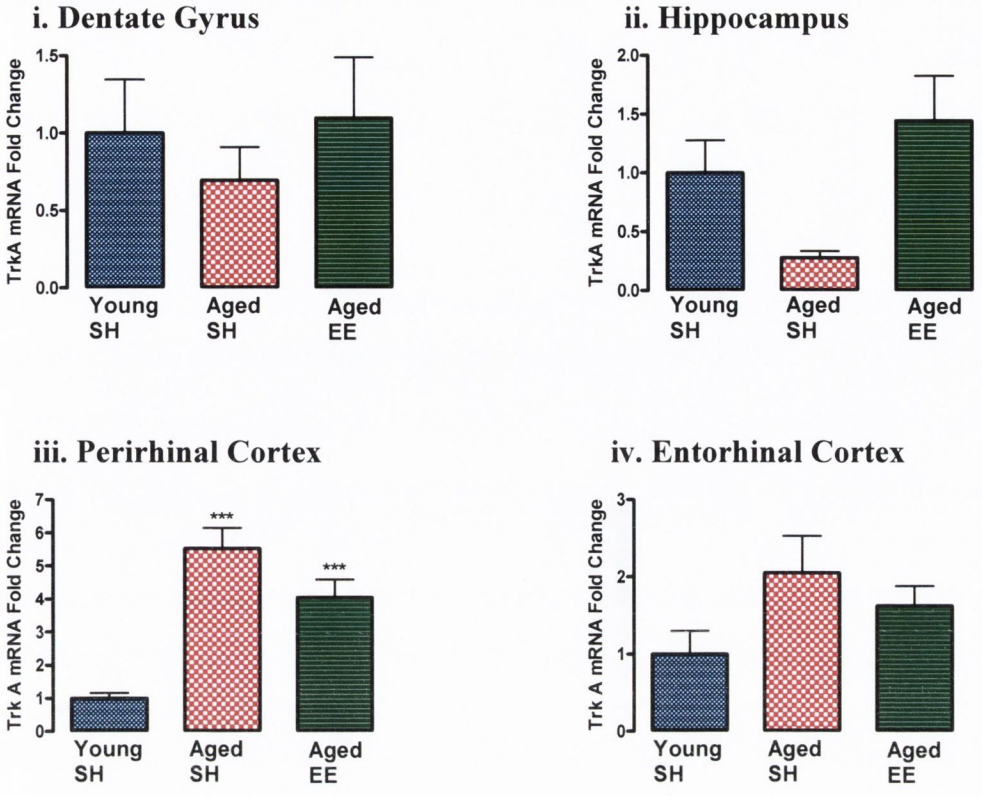


Figure 5s. There is an age-related increase in Trk A mRNA expression in the perirhinal cortex (i) There was no significant difference between groups in Trk A mRNA expression in the dentate gyrus ($F_{2,16}$ = 0.31, $p<0.05$). Young SH: n=8, Aged SH: n=5, Aged EE: n=6 (ii) There was no difference between the groups in Trk A mRNA expression in the hippocampus ($F_{2,13}$ = 2.972, $p>0.05$). Young SH: n=8, Aged SH: n=4, Aged EE: n=4 (iii) There was a significant difference between groups in Trk A mRNA expression in perirhinal cortex ($F_{2,17}$ = 29.64, $p<0.001$), with a significant increase in the Aged SH group when compared with the Young SH group (***$p<0.001$) and in the Aged EE when compared with the Young SH group (***$p<0.001$). There was no significant difference between the Aged SH and Aged EE groups ($p>0.05$). Young SH: n=8, Aged SH: n=6, Aged EE: n=6. (iv) There was no significant difference between groups in NGF mRNA expression in entorhinal cortex ($F_{2,17}$ = 2.391, $p>0.05$). Young SH: n=8, Aged SH: n=6, Aged EE: n=6. Data expressed as mean ± SEM.

5.4.8 There is an age-relate decrease in Trk B mRNA expression in the perirhinal cortex

There is no significant difference between groups in the expression of Trk B mRNA in the dentate gyrus ($F_{2,16} = 0.038$, $p>0.05$; figure 3t.i). Mean Trk B mRNA fold change ± SEM: Young SH (n=8) = 1.00±0.06, Aged SH (n=5) = 1.02±0.12, Aged EE (n=6) = 1.03±0.05.

There is no significant difference between groups in the expression of Trk B mRNA in the hippocampus ($F_{2,14} = 0.726$, $p>0.05$; figure 3t.ii). Mean Trk B mRNA fold change ± SEM: Young SH (n=8) = 1.00±0.13, Aged SH (n=5) = 1.20±0.24, Aged EE (n=4) = 0.88±0.14.

There is a significant difference between the groups in the expression of Trk B mRNA in the perirhinal cortex ($F_{2,17} = 11.35$, $p<0.001$; figure 5t.iii). Bonferroni's multiple comparison test revealed a significant decrease in Trk B mRNA expression in the Aged SH group when compared with the Young SH group (***$p<0.001$) and in the Aged EE group when compared with the Young SH group (*$p<0.05$). There is no significant difference between the Aged SH and Aged EE groups ($p>0.05$). Mean Trk B mRNA fold change ± SEM: Young SH (n=8) = 1.00±0.03, Aged SH (n=6) = 0.79±0.03, Aged EE (n=6) = 0.86±0.04.

There is no significant difference between groups in the expression of Trk B mRNA expression in the entorhinal cortex ($F_{2,17} = 0.956$, $p>0.05$; figure 3t.iv). Mean Trk B mRNA fold change ± SEM: Young SH (n=8) = 1.00±0.06, Aged SH (n=6) = 0.95±0.05, Aged EE (n=6) = 1.09±0.09.

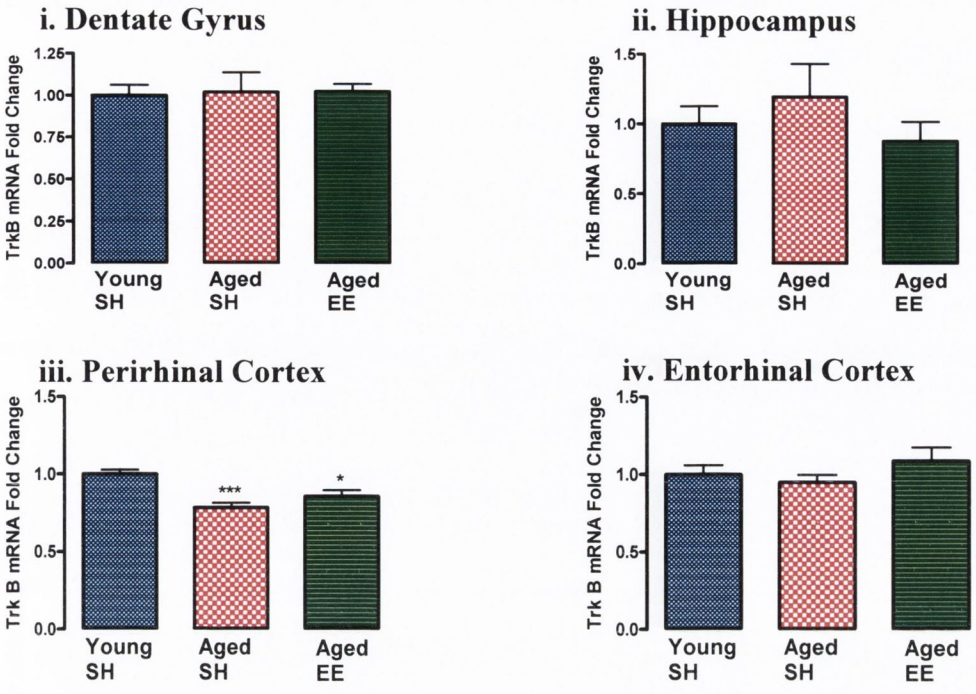


Figure 5t. There is an age-related decrease in Trk B mRNA expression in the perirhinal cortex (i) There was no significant difference between groups in Trk B mRNA expression in the dentate gyrus ($F_{2,16}$ = 0.038, $p>0.05$). Young SH: n=8, Aged SH: n=5, Aged EE: n=6 (ii) There was no significant difference between the groups in Trk B mRNA expression in the hippocampus ($F_{2,14}$ = 0.726, $p>0.05$). Young SH: n=8, Aged SH: n=5, Aged EE: n=4 (iii) There was a significant difference between groups in Trk B mRNA expression in perirhinal cortex ($F_{2,17}$ = 11.35, $p<0.001$), with a significant decrease in the Aged SH group when compared with the Young SH group (***$p<0.001$) and in the Aged EE when compared with the Young SH group (*$p<0.05$). There was no significant difference between the Aged SH and Aged EE groups ($p>0.05$). Young SH: n=8, Aged SH: n=6, Aged EE: n=6. (iv) There was no significant difference between groups in NGF mRNA expression in entorhinal cortex ($F_{2,17}$ = 0.956, $p>0.05$). Young SH: n=8, Aged SH: n=6, Aged EE: n=6. Data expressed as mean ± SEM.

5.4.9 Long-term environmental enrichment increases expression of VEGF mRNA in the dentate gyrus

There is a significant difference between groups in VEGF mRNA expression in the dentate gyrus ($F_{2,16}$ = 5.188, $p<0.05$; figure 5u.i). Bonferroni's multiple comparison test showed that there is a significant increase in VEGF mRNA expression in the Aged EE group when compared with Young SH (*$p<0.05$) and Aged SH groups (*$p<0.05$). There is no significant difference between Young SH and Aged SH groups. Mean VEGF fold change ± SEM: Young SH (n=8) = 1.00±0.44, Aged SH (n=5) = 0.97±0.08, Aged EE (n=6) = 1.26±0.09.

There is no significant difference between groups in VEGF mRNA expression in the hippocampus ($F_{2,14}$ = 0.284, $p>0.05$; figure 5u.ii). Mean VEGF fold change ± SEM: Young SH (n=6) = 1.00±0.03, Aged SH (n=6) = 1.09±0.11, Aged EE (n=5) = 1.07±0.10.

There is no significant difference between groups in VEGF mRNA expression in the perirhinal cortex ($F_{2,17}$ = 0.405, $p>0.05$; figure 5u.iii). Mean VEGF fold change ± SEM: Young SH (n=8) = 1.00±0.44, Aged SH (n=6) = 0.96±0.03, Aged EE (n=6) = 1.02±0.06.

There is no significant difference between groups in VEGF mRNA expression in the entorhinal cortex ($F_{2,17}$ = 0.817, $p>0.05$; figure 5u.iv). Mean VEGF fold change ± SEM: Young SH (n=8) = 1.00±0.06, Aged SH (n=6) = 0.99±0.07, Aged EE (n=6) = 1.12±0.10.

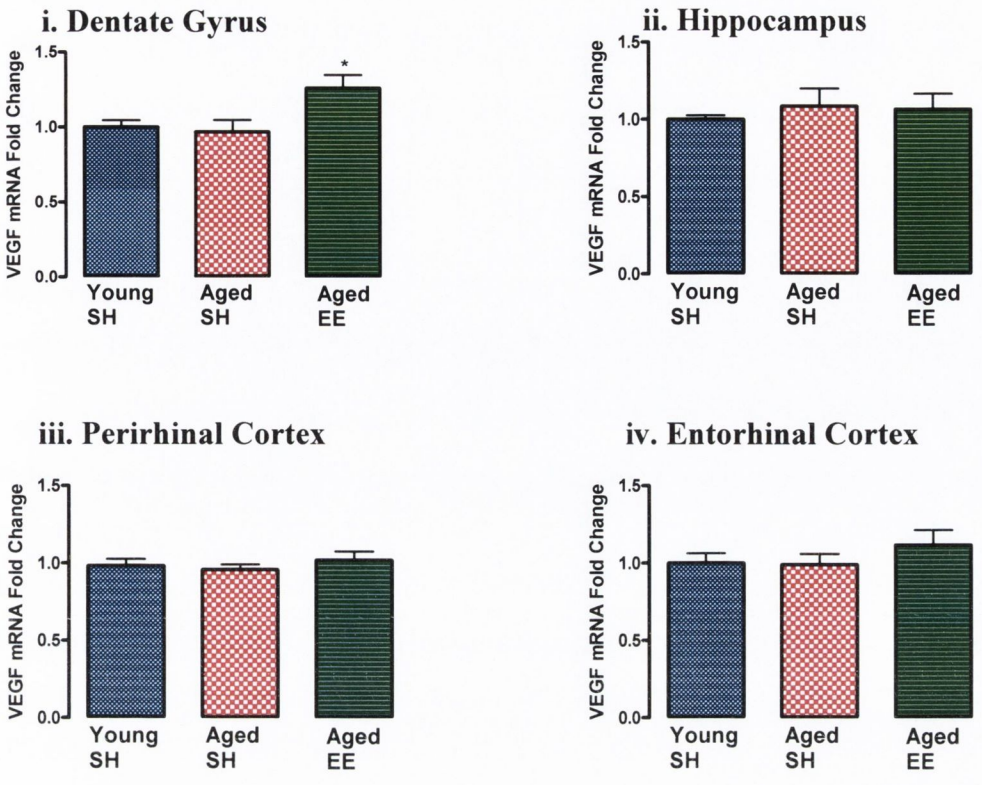


Figure 5u. Long-term environmental enrichment increases expression of VEGF mRNA in the dentate gyrus (i) There was a significant difference between groups in VEGF mRNA expression in the dentate gyrus ($F_{2,16} = 5.188$, $p<0.05$), with a significant increase in expression in the Aged EE group when compared with the Young SH (*$p<0.05$) and Aged SH (*$p<0.05$) groups. Young SH: n=8, Aged SH: n=5, Aged EE: n=6 (ii) There was no significant difference between the groups in VEGF mRNA expression in the hippocampus ($F_{2,14} = 0.284$, $p>0.05$). Young SH: n=6, Aged SH: n=6, Aged EE: n=5 (iii) There was no significant difference between groups in VEGF mRNA expression in perirhinal cortex ($F_{2,17} = 0.405$, $p<0.05$). Young SH: n=8, Aged SH: n=6, Aged EE: n=6. (iv) There was no significant difference between groups in NGF mRNA expression in entorhinal cortex ($F_{2,17} = 0.817$, $p>0.05$). Young SH: n=8, Aged SH: n=6, Aged EE: n=6. Data expressed as mean ± SEM.

5.4.10 Environmental enrichment does not affect $p75^{NTR}$ mRNA expression in the dentate gyrus, hippocampus, perirhinal cortex or entorhinal cortex

There is no significant difference between groups in $p75^{NTR}$ mRNA expression in the dentate gyrus ($F_{2,16} = 1.997$, $p>0.05$; figure 5v.i). Mean $p75^{NTR}$ fold change ± SEM: Young SH (n=8) = 1.00±0.20, Aged SH (n=5) = 1.74±0.31, Aged EE (n=6) = 1.47±0.32.

There is no significant difference between groups in $p75^{NTR}$ expression in the hippocampus ($F_{2,16} = 1.276$, $p>0.05$; figure 5v.ii). Mean $p75^{NTR}$ fold change ± SEM: Young SH (n=8) = 1.00±0.23, Aged SH (n=5) = 1.45±0.26, Aged EE (n=6) = 1.87±0.64.

There is a difference between groups in $p75^{NTR}$ expression in the perirhinal cortex but it does not quite reach significance ($F_{2,17} = 3.533$, $p=0.0521$; figure 5v.iii). Bonferroni's multiple comparison test showed that there is an increase in $p75^{NTR}$ expression in the Aged SH group when compared with the Young SH group ($p=0.053$). There is no significant differences between Aged EE and Young SH ($p>0.05$) or Aged SH groups ($p>0.05$). Mean $p75^{NTR}$ fold change ± SEM: Young SH (n=8) = 1.00±0.11, Aged SH (n=5) = 1.70±0.27, Aged EE (n=6) = 1.20±0.20.

There is no significant difference between groups in $p75^{NTR}$ expression in the entorhinal cortex ($F_{2,17}= 0.246$, $p>0.05$; figure 5v.iv). Mean $p75^{NTR}$ fold change ± SEM: Young SH (n=8) = 1.00±0.16, Aged SH (n=5) = 1.13±0.19, Aged EE (n=6) = 0.95±0.18.

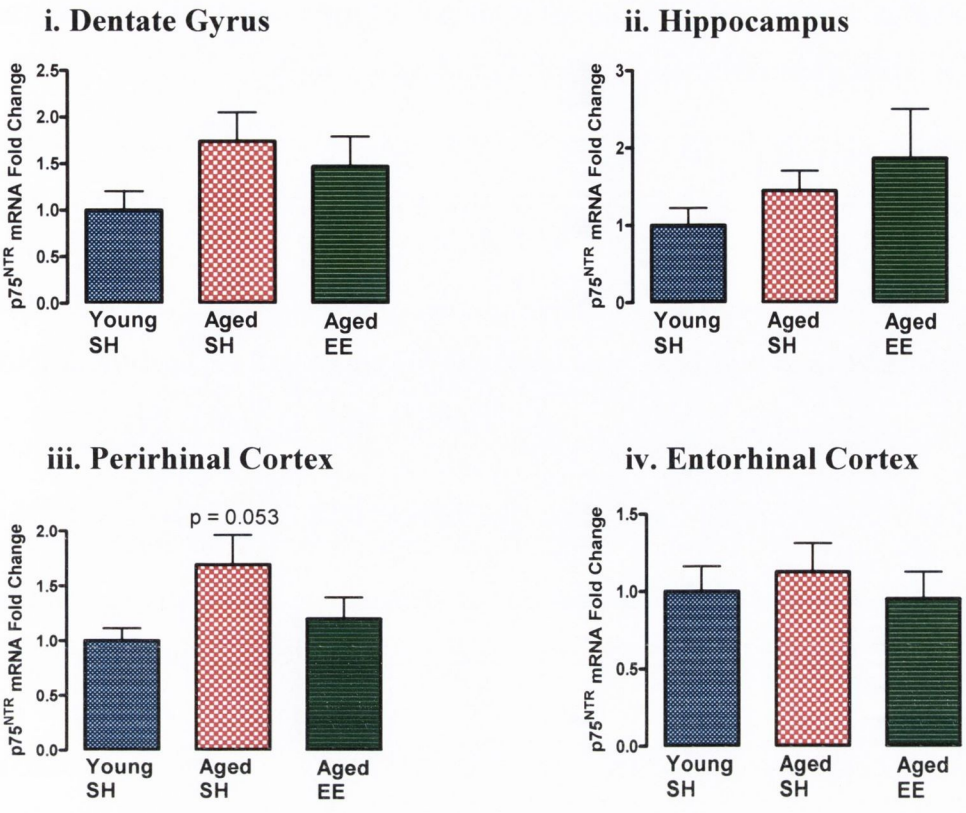


Figure 5v. Environmental enrichment attenuates the age-related increase in p75NTR in the perirhinal cortex (i) There was no significant difference between groups in $p75^{NTR}$ mRNA expression in the dentate gyrus ($F_{2,16} = 1.997$, $p>0.05$). Young SH: n=8, Aged SH: n=5, Aged EE: n=6 (ii) There was no significant difference between the groups in $p75^{NTR}$ mRNA expression in the hippocampus ($F_{2,16} = 1.276$, $p>0.05$). Young SH: n=8, Aged SH: n=5, Aged EE: n=6 (iii) There was a difference between groups in $p75^{NTR}$ mRNA expression in perirhinal cortex ($F_{2,17} = 3.533$, $p=0.0521$), with an increase in expression in the Aged SH group when compared with the Young SH group ($p=0.053$). There was no significant differences between the Aged EE group and Young SH ($p>0.05$) or Aged SH ($p>0.05$) groups. Young SH: n=8, Aged SH: n=6, Aged EE: n=6. (iv) There was no significant difference between groups in $p75^{NTR}$ mRNA expression in entorhinal cortex ($F_{2,17} = 0.246$, $p>0.05$). Young SH: n=8, Aged SH: n=6, Aged EE: n=6. Data expressed as mean ± SEM.

5.4.11 Environmental enrichment does not prevent the age-related decrease in expression of Ki67 mRNA in the dentate gyrus and olfactory bulb

There is a significant difference between groups in the expression of Ki67 mRNA in the dentate gyrus ($F_{2,14} = 55.43$, $p<0.001$; figure 5w.i). Bonferroni's multiple comparison test showed a significant decrease in Ki67 mRNA expression in the Aged SH group (***$p<0.001$) and Aged EE group (***$p<0.001$) when compared with the Young SH group. There is no significant difference between Aged SH and Aged EE in Ki67 mRNA expression ($p>0.05$). Mean Ki67 mRNA fold change ± SEM: Young SH (n=8) = 1.00±0.89, Aged SH (n=5) = 0.04±0.02, Aged EE (n=4) = 0.11±0.03.

There is a significant difference between groups in the expression of Ki67 mRNA in the olfactory bulb ($F_{2,15} = 9.075$, $p<0.01$; figure 5w.ii). Bonferroni's multiple comparison test showed a significant decrease in Ki67 mRNA expression in the Aged SH group (*$p<0.05$) and Aged EE group (**$p<0.01$) when compared with the Young SH group. There is no significant difference between Aged SH and Aged EE in Ki67 mRNA expression ($p>0.05$). Mean Ki67 mRNA fold change ± SEM: Young SH (n=8) = 1.00±0.07, Aged SH (n=5) = 0.67±0.08, Aged EE (n=5) = 0.60±0.07.

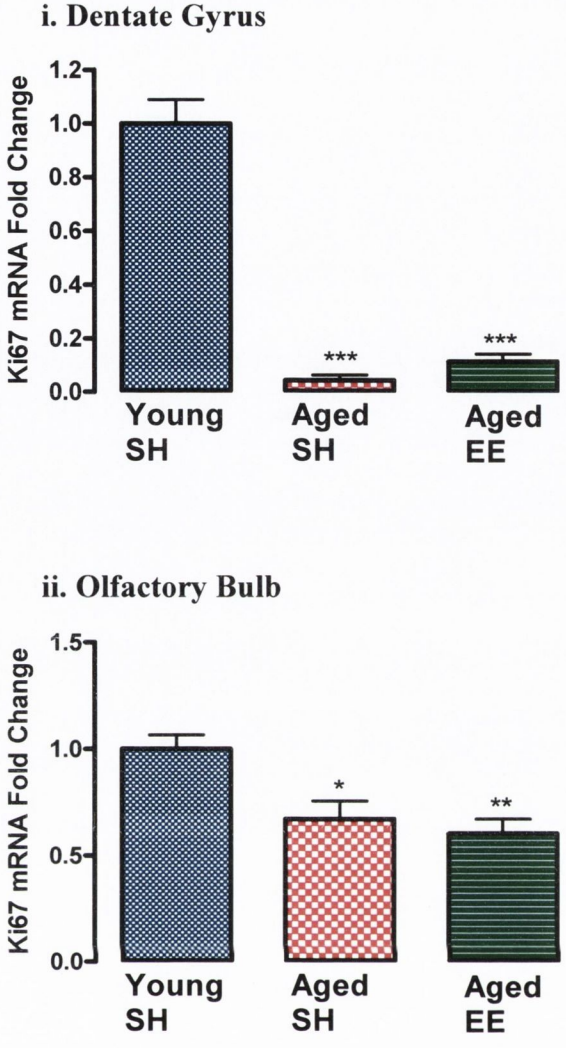


Figure 5w. Environmental enrichment does not prevent the age-related decrease in expression of Ki67 mRNA in the dentate gyrus and olfactory bulb (i) There was a significant difference between groups in Ki67 mRNA expression in the dentate gyrus ($F_{2,14} = 55.43$, $p<0.001$), with a significantly reduced expression in Aged SH (***$p<0.001$) and Aged EE (***$p<0.001$) groups when compared with the Young SH group. Young SH: n=8, Aged SH: n=5, Aged EE: n=4 (ii) There was a significant difference between groups in Ki67 mRNA expression in the olfactory bulb ($F_{2,15} = 9.075$, $p<0.01$), with a significantly reduced expression in Aged SH (*$p<0.05$) and Aged EE (**$p<0.01$) groups when compared with the Young SH group. Young SH: n=8, Aged SH: n=5, Aged EE: n=5. Data expressed as mean ± SEM.

5.4.12 Environmental enrichment attenuates the age-related increase in the expression of CD68 mRNA in the hippocampus

There is a significant difference between the groups in the expression on CD68 mRNA in the hippocampus ($F_{2,15} = 7.349$, $p<0.01$; figure 5x.i). Bonferroni's multiple comparison test showed an increase in CD68 mRNA expression in the Aged SH group when compared with the Young SH group (**$p<0.01$). There is no significant difference between Young SH and Aged EE ($p>0.05$) or Aged SH and Aged EE ($p>0.05$). Mean CD68 mRNA fold change ± SEM: Young SH (n=8) = 1.00±0.16, Aged SH (n=5) = 1.87±0.17, Aged EE (n=5) = 1.41±0.15.

There is no difference between the groups in the expression of Il-1β mRNA in the hippocampus ($F_{2,13} = 3.219$, $p>0.05$; figure 5x.ii). Mean Il-1β fold change ± SEM: Young SH (n=8) = 1.00±0.11, Aged SH (n=4) = 1.29±0.08, Aged EE (n=4) = 0.81±0.12.

There is no significant difference between the groups in the expression of CD40 mRNA in the hippocampus ($F_{2,14} = 0.54$, $p>0.05$; figure 5x.iii). Mean CD40 mRNA fold change ± SEM: Young SH (n=8) = 1.00±0.11, Aged SH (n=5) = 1.16±0.13, Aged EE (n=4) = 1.10±0.09.

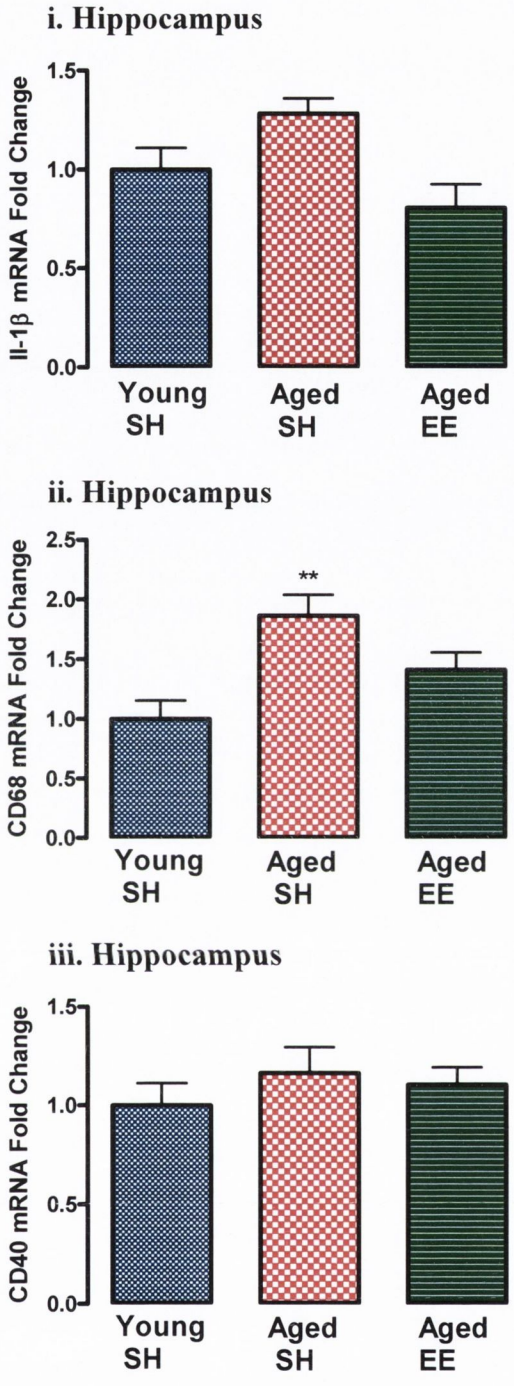


Figure 5x. Environmental enrichment attenuates an age-related in the expression of Il-1β and CD68 (i) There was no difference between groups in IL-1β mRNA expression in the hippocampus ($F_{2,13}$ = 3.219, $p>0.05$). Young SH: n=8, Aged SH: n=4, Aged EE: n=4 (ii) There was a significant difference between groups in CD68 mRNA expression in the hippocampus ($F_{2,15}$ = 7.349, $p<0.01$), with a significantly increased expression in the Aged SH group when compared with the Young SH group (**$p<0.01$). There was no significant differences between Aged EE and Young SH ($p>0.05$) or Aged SH ($p>0.05$) groups. Young SH: n=8, Aged SH: n=5, Aged EE: n=5. (iii) There was no significant difference between the groups in the expression of CD40 mRNA in the hippocampus ($F_{2,14}$ = 0.54, $p>0.05$). Young SH = 8, Aged SH = 5, Aged EE = 4. Data expressed as mean ± SEM.

5.4.13 Ageing and environmental enrichment do not affect the expression of synapsin I or synaptophysin in the hippocampus and dentate gyrus

There is no significant difference between groups in the expression of synapsin I in the dentate gyrus ($F_{2,19}$ = 0.236, p>0.05; figure 5y.i). Mean expression of synapsin I per GAPDH ± SEM: Young SH = 3.16±0.65, Aged SH = 2.95±0.59, Aged EE = 2.63±0.20.

There is no significant difference between groups in the expression of synapsin I in the hippocampus ($F_{2,19}$ = 0.137, p>0.05; figure 5y.ii). Mean expression of synapsin I per GAPDH ± SEM: Young SH = 3.70±0.37, Aged SH = 3.58±0.38, Aged EE = 3.41±0.41.

There is no significant difference between groups in the expression of synaptophysin in the dentate gyrus ($F_{2,19}$ = 0.323, p>0.05; figure 5y.iii). Mean expression of synaptophysin per GAPDH ± SEM: Young SH = 1.07±0.18, Aged SH = 1.24±0.12, Aged EE = 1.15±0.10.

There is no significant difference between groups in the expression of synaptophysin in the hippocampus ($F_{2,19}$ = 0.040, p>0.05; figure 5y.iv). Mean expression of synaptophysin per GAPDH ± SEM: Young SH = 2.08±0.25, Aged SH = 2.17±0.57, Aged EE = 2.24±0.44.

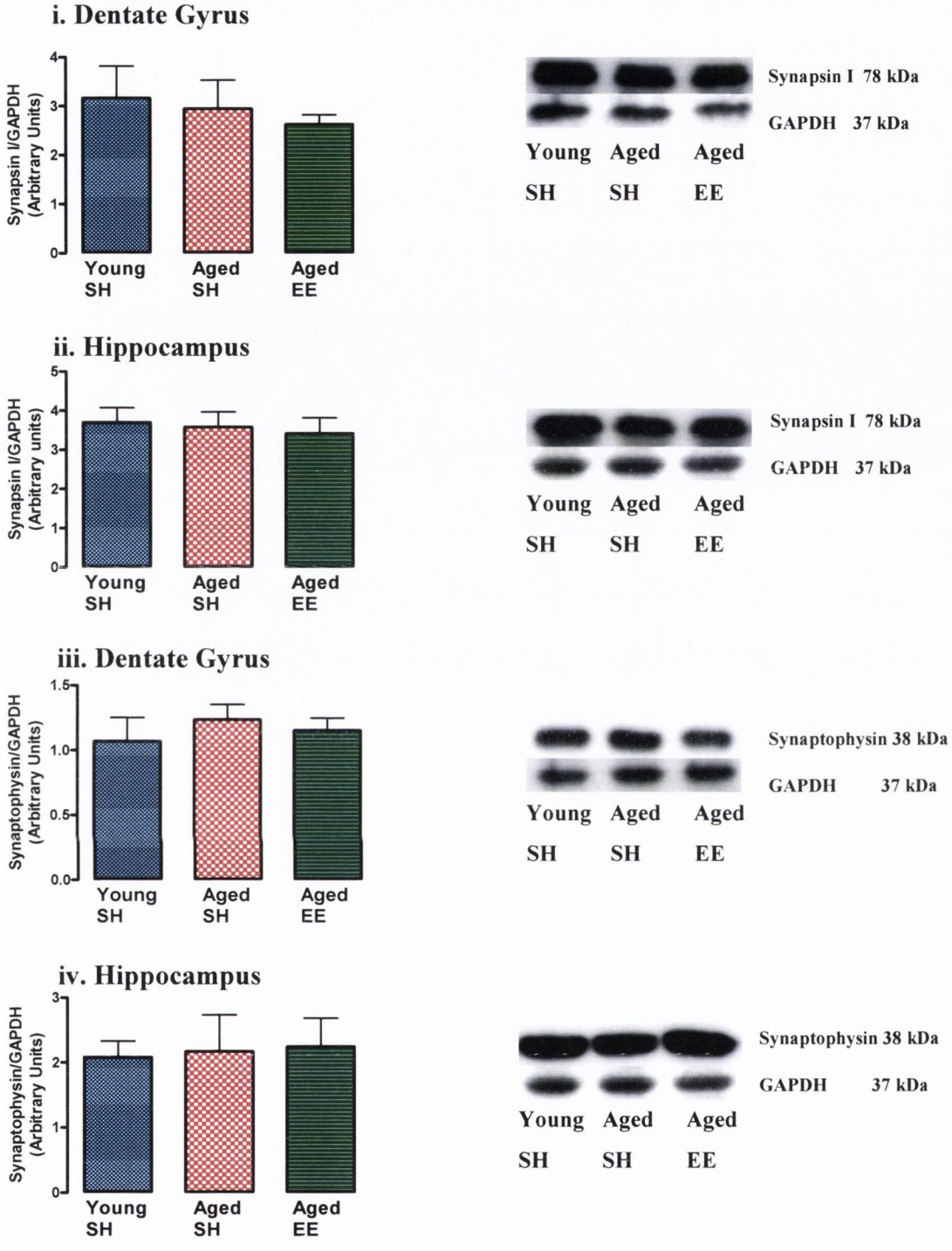


Figure 5y. Ageing and environmental enrichment do not affect expression of synapsin I or synaptophysin in the dentate gyrus and hippocampus (i) There is no significant difference between groups in the expression of synapsin I in the dentate gyrus ($F_{2,19} = 0.236$, $p>0.05$) **(ii)** There is no significant difference between groups in the expression of synapsin I in the hippocampus ($F_{2,19} = 0.137$, $p>0.05$) **(iii)** There is no significant difference between groups in the expression of synaptophysin in the dentate gyrus ($F_{2,19} = 0.323$, $p>0.05$) **(iv)** There is no significant difference between groups in the expression of synaptophysin in the hippocampus ($F_{2,19} = 0.040$, $p>0.05$). Young SH: n=8, Aged SH: n=6, Aged EE: n=6. Data expressed as mean ± SEM.

5.4.14 Environmental enrichment does not affect the expression of $p75^{NTR}$ in the hippocampus or dentate gyrus

There is no significant difference between groups in the expression of $p75^{NTR}$ in the dentate gyrus ($F_{2,18} = 0.329$, $p>0.05$; figure 5z.i). Mean expression of $p75^{NTR}$ over GAPDH ± SEM: Young SH = 3.87±0.84, Aged SH = 5.30±0.77, Aged EE = 2.63±0.33.

There is no difference between groups in the expression of $p75^{NTR}$ in the hippocampus ($F_{2,18} = 2.672$, $p>0.05$; figure 5z.ii). Mean expression of $p75^{NTR}$ over GAPDH ± SEM: Young SH = 1.56±0.31, Aged SH = 1.19±0.30, Aged EE = 1.45±0.39.

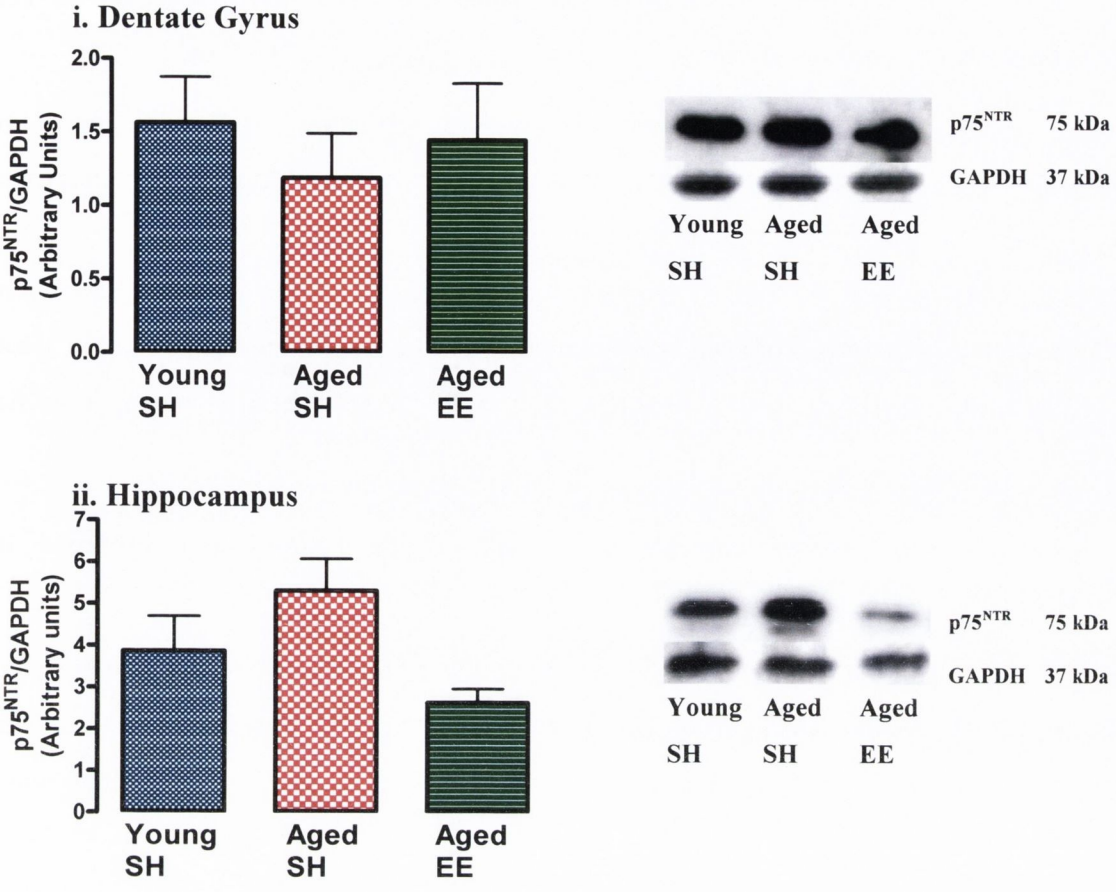


Figure 5z. Environmental enrichment reduces the expression of p75NTR in the hippocampus(i) There is no significant difference between groups in the expression of $p75^{NTR}$ in the dentate gyrus ($F_{2,18}$ = 0.329, p>0.05). (ii)There is no difference between groups in the expression of $p75^{NTR}$ in the hippocampus ($F_{2,18}$ = 2.672, p>0.05) Young SH: n=8, Aged SH: n=6, Aged EE: n=5. Young SH: n=7, Aged SH: n=6, Aged EE:, n=6. Data are expressed as mean ± SEM

5.5.1 Environmental enrichment attenuates the age-related decrease in cell proliferation in the dentate gyrus

There is a significant difference between groups in the percentage BrdU+ nuclei in the dentate gyrus ($F_{2,17}$ = 9.377, $p<0.01$; figure 5α). Bonferroni's multiple comparison test showed that there is a significant decrease in BrdU+ nuclei in the Aged SH group when compared with Young SH (**$p<0.01$) and when compared with Aged EE ($^{+}p<0.05$). There is no significant difference between the Young SH and Aged EE group ($p>0.05$). Mean percentage BrdU+ nuclei ± SEM: Young SH =5.25±0.40, Aged SH = 3.13±0.34, Aged EE = 4.10±0.25.

5.5.2 Environmental enrichment attenuates the age-related increase in apoptosis in the dentate gyrus

There is a significant difference between groups in the amount of TUNEL+ nuclei in the dentate gyrus ($F_{2,15}$ = 18.40, $p<0.001$; figure 5β). Bonferroni's multiple comparison test showed that there is a significant increase in TUNEL+ nuclei in the Aged SH group when compared with Young SH (***$p<0.001$) and when compared with Aged EE ($^{++}p<0.01$). Mean fluorescent intensity at 488nm ± SEM: Young SH=1.97±0.74, Aged SH=97.32±23.52, Aged EE=19.62±4.16.

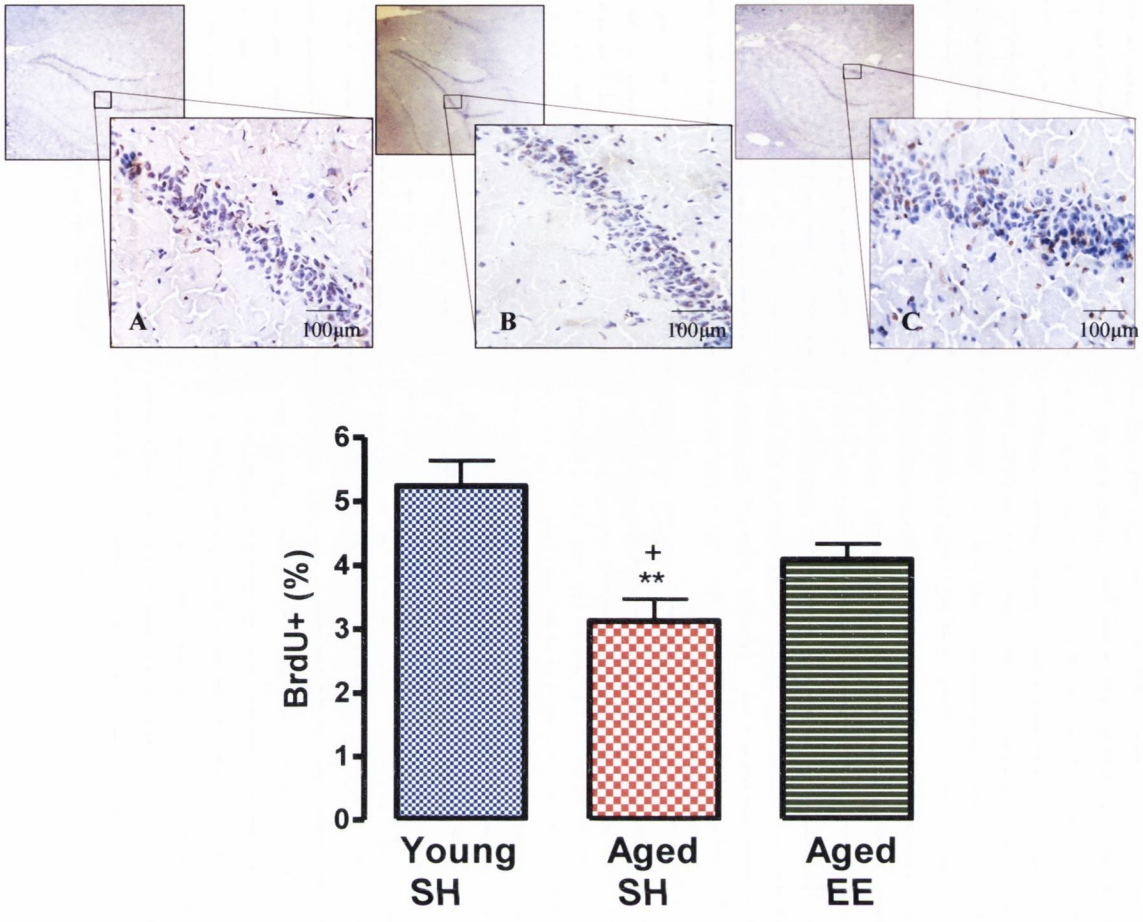


Figure 5α. Environmental enrichment attenuates the age-related decrease in cell proliferation in the dentate gyrus There is a significant difference between groups in the percentage BrdU+ nuclei in the dentate gyrus ($F_{2,17} = 9.377$, $p<0.01$ Bonferroni's multiple comparison test showed that there is a significant decrease in BrdU+ nuclei in the Aged SH group when compared with Young SH (**$p<0.01$) and when compared with Aged EE ($^{+}p<0.05$). **A** = Young SH (n=8) **B** = Aged SH (n=6) **C** = Aged EE (n=6). Blue staining = all nuclei (hematoxlin), brown staining = BrdU+ nuclei (DAB Chromagen). Data are expressed as mean ± SEM.

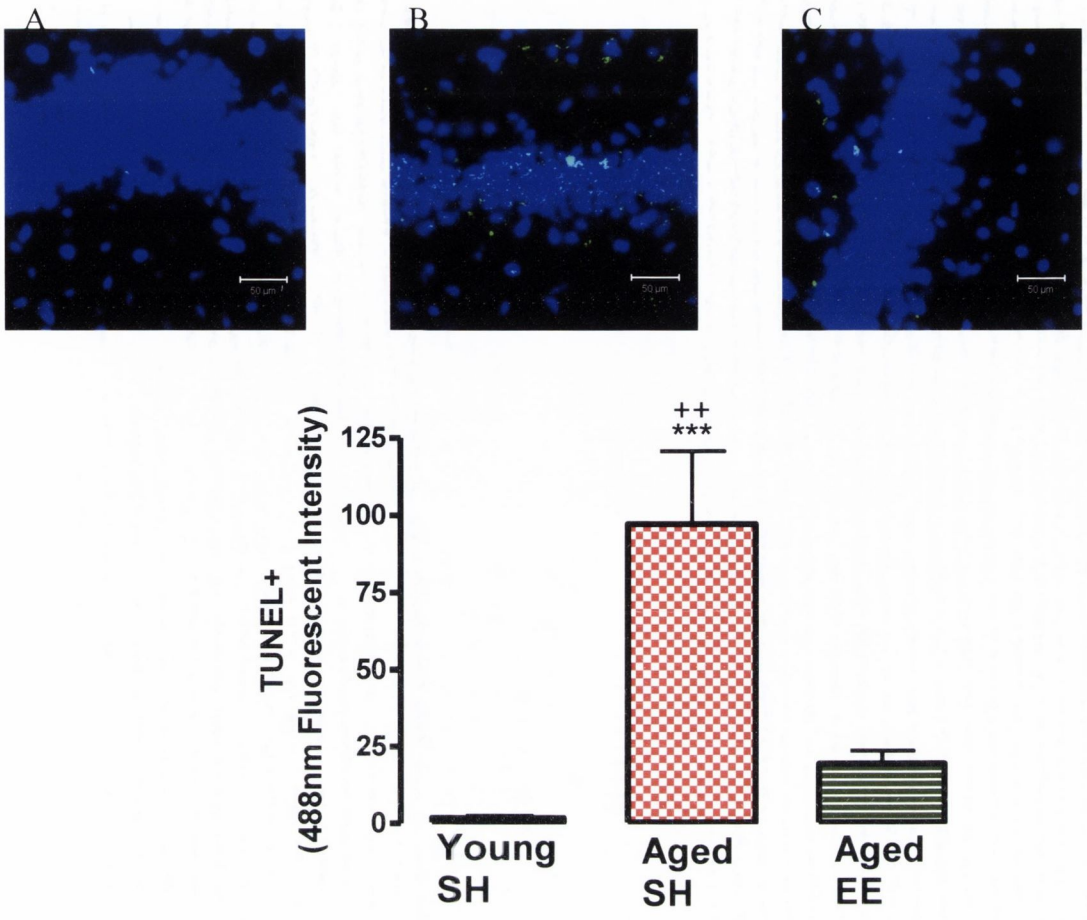


Figure 5β. Environmental enrichment attenuates the age-related increase in apoptosis in the dentate gyrus There is a significant difference between groups in the amount of TUNEL+ nuclei in the dentate gyrus ($F_{2,15}$ = 18.40, $p<0.001$). Bonferroni's multiple comparison test showed a significant increase in TUNEL+ nuclei in the Aged SH group when compared with the Young SH group (***$p<0.001$) and when compared with the Aged EE group ($^{++}p<0.01$). **A** = Young SH (n=7) **B** = Aged SH (n=5) **C** = Aged EE (n=6). Blue staining = all nuclei (Hoescht), green staining = apoptotic cells (TUNEL+ nuclei). Data are expressed as mean ± SEM.

5.6 Discussion

The aim of this study was to assess the neuroprotective effects of long-term environmental enrichment on cognitive function. Rats were housed in continuous enriched conditions for approximately 20 months and their memory was assessed at young age, prior to their housing, 13-15 mo and old age using a battery of tests designed to assess various aspects of mnemonic function; these included the two object NOR task, three object NOR task, OD task, Morris water maze and T maze task. The data in this study show that there is an age-related decline in recognition, spatial and working memory and that housing in an enriched environment prevents this cognitive decline.

Behavioural Task	**Baseline**	**13-15 months**	**Aged (22-24 months)**
2 object NOR	YES	EE = No change SH = No change	EE = No change SH = ↓
3 object NOR	NO	EE = ↑ SH = No change	EE = ↑ SH = No change
3 object OD	YES	EE = No change SH = ↓	EE = No change SH = ↓
T maze	YES	EE = ↑ SH = No change	EE = ↑ SH = ↓
MWM (acquisition)	YES	No difference	No difference
MWM (probe)	YES	No difference	EE = No change SH = ↓

Table 5b.Age-related changes in performance on all behavioural tasks At Baseline, all rats could successfully perform all tasks except the 3 object NOR. At 13-15 months of age, EE rats showed an improvement in performance on the 3 object NOR task & T maze task, whereas SH rats showed a reduction in performance on the 3 object NOR task. AT 22-24 months of age (Aged), EE rats maintained an improvement in performance on the 3 object NOR task & T maze task, whereas SH rats showed a reduction in performance on the 2 object NOR and MWM probe trial in addition to maintaining a reduction in performance on the 3 object OD task. There were no differences between groups or across the timepoints in the MWM acquisition trials. All differences are reported as compared with Baseline performance.

The two object NOR task is the simplest recognition task the rats had to perform because it requires the rats to acquire an accurate representation of only two objects during training, whereas with the three object NOR task an accurate representation of three objects is

needed. At both Baseline and 13-15 months old timepoints the rats in both groups can distinguish the novel object from the familiar objects. At the Aged timepoint however, there is a clear deficit in recognition memory of the standard housed rats as evidenced by their inability to distinguish the novel object from the familiar object. Aged EE rats do not exhibit this deficit in recognition memory however, suggesting that enrichment can protect against a loss of recognition memory with age. Performance at Baseline in the three object NOR task shows that none of the rats were able to distinguish the novel object from the two familiar objects and this persists at 13-15 months old and Aged timepoints in the standard housed rats. In contrast to this, the enriched rats show an improvement in object recognition memory at both 13-15 months old and Aged timepoints. These data support the results in Chapter 3 that show an improvement in object recognition memory with short-term environmental enrichment and taken together with the 2 object NOR task data they also clearly illustrate that enrichment can prevent a loss of recognition memory and may in fact improve memory in the long-term. These data clearly support the hypothesis that, given the appropriate stimulation, the brain retains a capacity for plasticity throughout the lifespan.

Two different tasks were used to measure spatial memory: the OD task and the Morris water maze. As rats age, they would typically reduce the amount of exploration in an open arena, hence the Morris water maze is a useful task because the rats have a strong incentive to escape from the water and therefore, to find the hidden platform. This is also a stressor for the rats however, particularly for aged animals. In the OD task, all the rats were able to distinguish the displaced object from the two familiarly positioned objects, however at 13-15 months old and Aged timepoints the rats housed in standard conditions are unable to distinguish the displaced object from the familiarly positioned objects, suggesting an age-related deficit in spatial memory. The rats housed in an enriched environment do not exhibit this deficit in spatial memory as they are able to distinguish the displaced object at both the 13-15 months old and Aged timepoint. It is interesting that the rats display a deficit in spatial memory at the 13-15 months old timepoint whereas they only begin to show a deficit in recognition memory at the Aged timepoint. This may be associated with loss of function in the hippocampus specifically because recognition memory utilises the perirhinal cortex to a greater extent than the hippocampus and therefore this memory would be preserved for longer following a reduction in hippocampal functioning.

Whilst there was no loss of performance in the Morris water maze task at any timepoint, there was an overall increase in the distance swum at the Aged timepoint when compared with 13-15 months old. This may suggest that the Aged rats are also exhibiting some deficits in acquisition on this task but they are swimming faster to compensate for this. The probe test is therefore a very powerful tool to assess whether the rats have retained the memory for the position of the hidden platform, and this test does show that there is an age-related deficit which is rescued with environmental enrichment. Therefore, whilst it is unclear whether environmental enrichment can prevent a deficit in the learning of this task, it is clear that enriched rats can retain sufficient memory for the position of the platform to search in the appropriate quadrant and the standard housed rats cannot.The discrepancy between the two tests of spatial memory may be associated with the level of difficulty of the two tests: the strong negative reinforcer element of the Morris water maze, ie. the desire to escape from the water, would facilitate faster acquisition on this task. In contrast, the OD task relies on the rats' motivation to explore objects in their environment and the absence of an external motivator is likely to make the task itself more difficult. This may mean that the water maze is not a sensitive enough task to clearly identify the deficit in spatial memory that we have detected using the OD task, but that the probe test does show a loss of long-term spatial memory which is not tested for in the OD task.

In order to target other aspects of mnemonic function, rats were tested using the T maze task. This is a test of working memory that utilises a number of different and higher-order cortical networks than those recruited to solve the Morris water maze and other tests of spatial and recognition memory. Whilst there is an overall decline in performance of this task with age, the rats housed in an enriched environment show a significant overall improvement in performance on this task at all timepoints. This would suggest that there is an age-related decline in working memory which can be prevented via enriched housing. Whether this decline is solely associated with a loss of hippocampal function or is related to a loss of connectivity between brain regions is unclear. We report a significant reduction in performance only at the Aged timepoint and therefore it may be the case that compensatory mechanisms utilising different cortical regions enable a preservation of function at 13-15 monoths old, in a similar manner to the preservation of recognition memory at 13-15 months old.

Interestingly, environmental enrichment does not prevent the age-related increase in anxiety that is shown at 13-15 months old and Aged timepoints as measured by the

Elevated Plus Maze. This mirrors the results in Chapter 3 which show that environmental enrichment does not affect levels of anxiety in the young rat and is also supported in the literature where it is reported that environmental enrichment cannot prevent an increase in anxiety that is reported in a mouse model of Alzheimer's disease (Görtz *et al.*, 2008). Leal-Galicia and colleagues (2008) report a reduced level of anxiety in 18 month old rats that had been exposed to long-term daily enrichment, measured by a reduction in thigmotaxis in the Open Field test. This is in contrast to the results reported here, which show no differences in the levels of thigmotaxis with housing condition or age. Their protocol does include running wheels however, and it has been previously shown that exercise alone can reduce anxiety behaviours in rodents (Binder *et al.*, 2004, Fulk *et al.*, 2004).

The neurochemical results suggest that the loss of cognitive function may be associated with a reduction of NGF, and possibly VEGF, concentration in the hippocampus which is attenuated with environmental enrichment. This alteration in the neurochemical balance in the hippocampus is likely to have been a significant factor in the large reduction in neurogenesis and increase in apoptosis in the dentate gyrus that is shown, which is rescued by environmental enrichment. Levels of mature NGF and Trk A expression are reduced in both Alzheimer's disease models and ageing and are often associated with corresponding increases in proNGF and $p75^{NTR}$ expression and a loss of cholinergic neurons (Salehi *et al.*, 2004, Fortress *et al.*, 2011), suggesting that with age there is an alteration in the balance between NGF signalling towards a pro-apoptotic pathway. Fortress and colleagues (2011) report that an intrahippocampal proNGF injection can increase $p75^{NTR}$ expression and reduce Trk A expression in the hippocampus, and results in degeneration of cholinergic neurons in the aged rat. Conversely, transplanting NGF secreting neural progenitor cells into the basal forebrain of middle aged or aged rats can prevent or reverse age-related spatial memory deficits and cholinergic neuronal atrophy (Martínez-Serrano *et al.*, 1995, Martínez-Serrano and Björklund, 1998). Frick and colleagues (1997) also report that a chronic NGF infusion can rescue spatial memory deficits in aged rats, and that this effect persists for at least 4 weeks post-infusion.

This study also reports attenuation of an age-related decrease in expression of Trk A mRNA in the hippocampus by environmental enrichment (although it does not quite reach statistical significance) which adds further weight to the hypothesis that there is an alteration in NGF/TrkA signalling with age that can be prevented using environmental enrichment. There is also a reduction in $p75^{NTR}$ expression in the hippocampus in the Aged

EE rats, which provides further evidence for an upregulation of the pro-survival NGF/TrkA signalling pathway. Trk A expression has been reported on immature neurons in the subventricular zone (SVZ) of non-human primates (Tonchev *et al.*, 2007), and NGF is reported to stimulate differentiation of precursor cells in the SVZ of mice with experimental autoimmune encephalomyelitis (EAE), an experimental model of multiple sclerosis (Triaca *et al.*, 2005). Thus, NGF may be directly stimulating the enrichment-induced increase in cell proliferation reported in this study. Taken together with the data presented here, these studies demonstrate that the increased cell survival, decreased apoptosis and prevention of cognitive decline reported herein may be as a result of alteration in neurotrophin stimulated signalling pathways, although further experimental investigation would be required to establish a causal link between these observations.

Many studies have reported a reduced VEGF response following injury in aged rats (Rivard *et al.*, 1999, Pola *et al.*, 2004) and an age-related reduction in VEGF responsiveness to hypoxic conditions (Rivard *et al.*, 2000). These studies point to a dysfunction in VEGF with age which could reduce responsiveness to inflammation and injury. VEGF has also been shown to enhance neurogenesis (Palmer *et al.*, 2000, Licht *et al.*, 2011) and therefore an alteration in VEGF expression with age may impact on the angiogenic niche within the dentate gyrus and reduce neurogenesis (Gao *et al.*, 2009). This study reports an age-related reduction in VEGF in the hippocampus that is rescued with environmental enrichment. There is also a corresponding increase in VEGF mRNA expression in the dentate gyrus. VEGF can enhance the angiogenic niche in the subgranular zone (SGZ) of the dentate gyrus: dividing neural stem cells and endothelial cells are clustered together at the tips of capillaries in the SGZ, dividing neural stem cells express the VEGF receptor Flk1, and neurons within the SGZ express VEGF (Palmer *et al.*, 2000). Thus, increased VEGF is likely to be associated with the enrichment-induced rescue of neurogenesis reported in this study. An exogenous infusion of VEGF can stimulate neurogenesis both *in vitro* and *in vivo*, although this seems to be associated with increased survival of the new neurons rather than increased proliferation and therefore NGF may be stimulating increased proliferation and VEGF maybe enhancing the survival of these immature neurons (Jin *et al.*, 2002, Schanzer *et al.*, 2004). Blockade of VEGF however, eliminated exercise-induced enhancements in neurogenesis, suggesting VEGF may play a significant role in the stimulation of neurogenesis itself (Fabel *et al.*, 2003). There is also evidence to suggest that NGF is an important angiogenic factor, and can

stimulate the migration of endothelial cells via VEGF stimulation (Dollé *et al.*, 2005). Moser and colleagues (2004) report increased proliferation of brain capillary endothelial cells when incubated with NGF *in vitro*, and these cells also express Trk A and secrete NGF *in vivo*. Therefore, environmental enrichment may be enhancing the signalling cross-talk between NGF and VEGF. However, it is not known whether this is via a direct stimulation of NGF which in turn stimulates VEGF or *vice versa*.

The literature points towards a strong connection between these growth factors in the adult brain and signalling between them is typical of a pro-survival, neurogenic and angiogenic phenotype. An intracerebral injection of both VEGF and NGF following cerebral ischemia inhibited caspase-3 activation more than single injections of either VEGF or NGF suggesting that in combination they can produce a more enhanced neuroprotective effect (Yang *et al.*, 2008). Therefore the prevention of the age-related decline in both of these factors with age may also be associated with the attenuation of apoptosis that is shown in this study. To further study this relationship, it would have been interesting to analyse the concentration of VEGF in the tissue of the NGF infused rats in Chapter 4, however due to time constraints and a lack of available samples, this was not possible.

There is a decrease in the NGF mRNA expression in the hippocampus of the Aged EE rats in this study. This is an unexpected result, as it does not match the pattern of NGF protein concentration in this brain region in these rats. This would suggest that there is a loss of transcription of NGF in the hippocampus with age that is not rescued with enrichment, because there is also a similar trend towards a reduction in the expression of NGF mRNA in the Aged SH rats, although it does not reach significance. However, discordance between neurotrophin mRNA expression and protein concentrations in different brain regions across the lifespan has been reported (Das *et al.*, 2001) and whilst this is an unusual result, it is the proteins themselves that exert direct actions within the brain and therefore they are more indicative of the biological effects that neurotrophins exert rather than the mRNA expression. Neurotrophin mRNA can also be transported along the neuron and translated locally at postsynaptic dendrites (Tiedge and Brosius, 1996, Tongiorgi *et al.*, 1997), however this would typically be activity-dependent and because these rats had not recently performed an experimental task it seems unlikely that this would be the case in this study. There is however, a robust increase in BDNF mRNA expression in both the dentate gyrus and hippocampus in Aged EE rats but again this does not correspond with the protein concentration, which shows an increase in BDNF in the Aged SH rats in the

dentate gyrus. Given that an increase in BDNF is typically associated with improved memory and increased proliferation (Kempermann *et al.*, 1998, Kempermann *et al.*, 2002, Choi *et al.*, 2009), this result is unusual as it is associated with a loss of hippocampal-dependent memory, decreased neurogenesis and increased apoptosis in this study. Studies have shown increased neurotrophin concentration in the cortex of aged rats which is associated with increased IL-1β concentration however, which is also seen in this study (Maher *et al.*, 2004).

Whilst there is a similar pattern in the expression of Trk A mRNA as the NGF protein concentration in the hippocampus across groups, there is a very robust increase in Trk A mRNA expression in both Aged SH and Aged EE groups in the perirhinal cortex. Conversely there is a reduction in Trk B mRNA expression in both the Aged SH and Aged EE groups in the perirhinal cortex. It may be that this is a compensatory mechanism where a downregulation of one Trk receptor is stimulating the upregulation of another. However it is typically reported in the literature that alteration in the expression of these receptors is associated with changes in $p75^{NTR}$ rather than changes between individual Trk receptors. In this study, there is a corresponding increase in $p75^{NTR}$ expression in the Aged SH rats in the perirhinal cortex that is attenuated in the Aged EE rats. It may be that the dysfunction in NGF/TrkA signalling is inducing an increase in $p75^{NTR}$/TrkA complexes that can enhance the binding of NGF to Trk A and therefore activation of downstream Trk A pathways which can be neuroprotective (Verdi *et al.*, 1994, Friedman, 2000). However, activation of $p75^{NTR}$ without colocalisation is associated with an increase in apoptosis and has been implicated in neurodegenerative diseases such as Alzheimer's disease (Friedman, 2000). From these data, it is not possible to conclude whether there is colocalisation of $p75^{NTR}$ and Trk A in the perirhinal cortex or whether there is an upregulation of Trk A as a compensatory mechanism to counteract the increase in pro-apoptotic $p75^{NTR}$ signalling, but it is clear that there is an alteration in the expression of the Trk and p75 receptors with age and that with enrichment, the altered expression profile may be promoting a pro-survival phenotype.

These results presented here also demonstrate a significant reduction in Ki67 mRNA expression in both the dentate gyrus and olfactory bulb of Aged SH and Aged EE rats. Both regions exhibit neurogenesis throughout adult life, and in this study there is clearly a marked decrease in cell proliferation with age. Neural stemsproliferate and migrate from the sub-ventricular zone to the olfactory bulb but BrdU labelling is incorporated into these

cells' DNA and therefore can still be measured in the olfactory bulb itself. Interestingly, whilst there is a significant decrease in BrdU+ cells in the dentate gyrus in the Aged SH group, this loss is attenuated in the Aged EE group suggesting that enrichment can rescue the age-related loss of neurogenesis. The contradiction between the Ki67 mRNA expression and BrdU staining may be associated with the quantification used in the two methods. Whilst both Ki67 and BrdU will label any proliferating cells, BrdU+ cells were only counted if they were within the dentate gyrus, where they are more likely to be of neuronal phenotype. It is possible that other cells types are being counted however, and therefore to fully confirm that the cells being counted were neurons a young progenitor cell marker such as doublecortin could be used. Ki67 expression was assessed in whole dentate gyrus therefore there it is impossible to pinpoint the cell type expressing Ki67. To confirm that the cells were of neuronal phenotype, a cell sorting technique such as FACS could be employed which would also have the advantage of allowing quantification of cells. It may be that there is also an alteration in the number of glial cells, particularly astrocytes, which play an important role in the trophic support of neurons (Nishimura *et al.*, 1995, Sabbatini *et al.*, 1999, Shetty *et al.*, 2005).

Previous studies report a rescue in rates of neurogenesis following environmental enrichment in aged rodents (Kempermann *et al.*, 1998, Kempermann *et al.*, 2002, Leal-Galicia *et al.*, 2008) however these studies include running wheels in their enrichment protocols. Mirochnic and colleagues (2009) have shown that enrichment without exercise can enhance neurogenesis in a mouse model of Alzheimer's disease independent of amyloid plaque load, suggesting that the increase in neurogenesis may be inducing an enhanced 'neurogenic reserve'. The neurogenic reserve hypothesis argues that increased hippocampal neurogenesis throughout life can be protective against a loss of function at a later age. Increased exposure to novel environments and learning via environmental enrichment can increase functional plasticity in the young animal, and this can be maintained during adulthood due to the integration of these neurons into functional networks. This can then be utilised in later life when there is a reduction in the levels of neurogenesis. In this study however, there is an enrichment-induced attenuation of the loss of cell proliferation which would suggest that continuous enrichment can maintain a certain level of proliferation within the dentate gyrus throughout the rat's lifespan. Alternatively, it could be proposed that there is increased survival of these newly proliferating neurons in the dentate gyrus, as results showed a marked decrease in the

amount of apoptosis in the Aged EE group when compared with the Aged SH group. However, the results in Chapter 4 would suggest that a direct infusion of NGF can increase proliferation independent of apoptosis levels. Certainly in the literature NGF is more commonly associated with a reduction in apoptosis, particularly in ageing and models of neurodegeneration (Sofroniew *et al.*, 2001, Chae and Kim, 2009, Yang *et al.*, 2011) and so this reduction in apoptosis in the Aged EE rates may be due to the increase in NGF in the hippocampus. Additionally, the increase of VEGF is likely to play a role in this neuroprotection because it is associated with increased neuronal survival and the prevention of memory loss (Sun *et al.*, 2003, Park *et al.*, 2007, Yang *et al.*, 2008, Wang *et al.*, 2011).

We observed no changes in synaptic vesicle proteins with age. This is unexpected because we report a marked increase in apoptosis with age and a significant reduction in proliferation which would suggest that there would also be a decrease in the number of synapses and therefore a loss of synaptic function. Most studies report a reduction in synaptic proteins with age or Alzheimer's disease and these are associated with a loss of synaptic plasticity (Perdahl *et al.*, 1984, Masliah *et al.*, 1989, Mullany and Lynch, 1997). However Nicolle and colleagues (1999) report no changes in synaptic proteins in aged, cognitively impaired rats. It may be the case that, whilst there is a reduction in the number of synapses, there is a compensatory increase in the size, density or active zone of existing synapses. The analysis of apoptosis does not specify the type of cell that is exhibiting fragmented DNA and therefore it may be that there is an age-related loss of other cell types such as astrocytes which would not affect the expression of synaptic proteins. To further analyse this result, expression of S100β (astrocytes) or NeuN (neurons) could be analysed simultaneously with TUNEL in order to characterise the type of cells that are undergoing apoptosis. Alternatively, a direct quantification of the synapses could be performed via electron microscopy or the expression of post-synaptic proteins, such as PSD-95 could be investigated.

These data show that there is an increase in the pro-inflammatory phenotype with age, because there is an increase in the mRNA expression of both IL-1β and CD68 in the hippocampus of the Aged SH rats and this is attenuated with enrichment. However, there are no changes in the expression of CD40 between groups in the hippocampus. Activation of this antigen is required for the activation of B cells, typically via the binding of its ligand CD40-L on activated T cells. This induces B cells to proliferate and this CD40-

dependent activation is thought to be important for the generation of memory B cells (Banchereau *et al.*, 1994). CD40 is expressed on a number of different cells inthe central nervous system, including astrocytes and microglia (Stout and Suttles, 1996, Rizvi *et al.*, 2008).The literature supports no change in the expression of this antigen with age (Colonna-Romano *et al.*, 2003), although disruption of CD40-L has been shown to rescue spatial and recognition memory in a mouse model of Alzheimer's disease (Todd Roach *et al.*, 2004) and therefore its activation may play a detrimental role in memory function with neurodegeneration. Given that expression of this antigen is associated with increased inflammatory responses,an increase in expression wouldn't necessarily be expected during normal ageing, whereas an inflammatory stimulus such as beta-amyloid plaques or infection in conjunction with age may induce an exaggerated immune response.

Cell surface expression of CD68 is increased on active macrophages and is thought to play a role in phagocytosis: the expression is increased on activated microglia that are localised around the sites of beta-amyloid plaques (Bornemann *et al.*, 2001). An increase in the expression of CD68 in the Aged SH in this study may be associated with the increase in apoptosis that is shown in the dentate gyrus, which is prevented via NGF-mediated mechanisms in the Aged EE rats. The increase in apoptosis is likely to induce an increase in the phagocytic phenotype of microglia to facilitate the clearing of debris from apoptotic cells. There is a significant increase in the proliferation of CD68+ microglia in the ipsilateral hemisphere of mice with a simulated traumatic brain injury which indicates that they can play a role recovery following injury (Rola *et al.*, 2006). Borders and colleagues (2007) report that a depletion of CD68+ microglia in the olfactory bulb of mice following a bulbectomy, but also in control mice, causes a reduction in neurogenesis and an increase in caspase activation suggesting that these cells play a role in the maintenance of normal adult neurogenesis in the absence of injury. Therefore, whilst CD68+ microglia may not directly exert negative effects on cognitive function or neurogenic processes, their increased presence is indicative of an enhanced inflammatory response which in itself may be causing detrimental effects.

In contrast, there is a great deal of literature describing the memory-modulating effects of IL-1β and the increase in its expression with normal ageing and neurodegenerative diseases (Schneider *et al.*, 1998, Maher *et al.*, 2004, Frank *et al.*, 2006, Lynch, 2010). Whilst IL-1β seems to be crucial for proper synaptic functioning and memory formation (Schneider *et al.*, 1998, Goshen *et al.*, 2009), over-expression of IL-1β is associated with deficits in

memory functioning (Gemma *et al.*, 2005, Hein *et al.*, 2010). Interestingly, it seems that the aged brain is primed towards an enhanced inflammatory phenotype which causes long-lasting impairments in memory when an exogenous inflammatory stimulus is introduced and this is associated with prolonged and exaggerated elevations in IL-1β (Barrientos *et al.*, 2006, Lynch, 2010, Tarr *et al.*, 2011). Therefore it is possible that the increase in IL-1β in the hippocampus of Aged SH rats in this study is further facilitating the cognitive deficits that are seen. Environmental enrichment prevents this increase in IL-1β, however the mechanism by which this occurs is unknown. However, NGF is expressed in a number of different glial cells, including astrocytes and microglia, under inflammatory conditions (Gadient *et al.*, 1990, Elkabes *et al.*, 1998). Both IL-1β and TNFαcan stimulate microglial NGF transcription and secretion (Gadient *et al.*, 1990, Jurič and Čarman-Kržan, 2001), and NGF can inhibit MHC II induction in microglia *in vitro*(Neumann et al., 1998),suggesting it may play a neuroprotective role during inflammatory conditions. NGF itself can also stimulate TNFα expression through Akt signalling and NFκB and this can activate a positive feedback loop within which TNFα reactivates Akt via its receptor TNFR2, promoting enhanced cell survival (Takei and Laskey, 2008). To further characterise any NGF-induced inflammatory changes, more analyses would be needed, such as examining the expression of TNFα or IL-6 or quantifying the number of glial cells via immunohistochemistry.

In recent years, there has been a great deal of emphasis upon the cognitive enhancing effect of exercise, and the role that it may play in neuroprotection. Exercise is a potent memory enhancer in normal, aged and diseased brains (O'Callaghan *et al.*, 2007, Ploughman, 2008, Griesbach *et al.*, 2009, Kim *et al.*, 2010, Liu *et al.*, 2011). It is hypothesised that exercise mediates these changes via the upregulation of neurotrophins, in particular BDNF, and hippocampal neurogenesis (van Praag *et al.*, 1999, Pereira *et al.*, 2007, Wu *et al.*, 2008, Griffin *et al.*, 2009, Griffin *et al.*, 2011). It has also been argued that it is the physical activity component of environmental enrichment protocols that is most crucial for enhancing neurogenesis and eliciting the strongest behavioural changes (Kobilo *et al.*, 2011). Previous long-term environmental enrichment studies have reported an enrichment-induced prevention of memory decline, increased neurotrophin expression or increased neurogenesis (Kempermann and Gage, 1999, Ickes *et al.*, 2000, Bennett *et al.*, 2006) but typically they have included running wheels in their enrichment protocol. More recent studies report that additional stimulation without exercise is equally as effective at

preventing age-related declines in spatial memory and synaptic plasticity (O'Callaghan *et al.*, 2009, Kumar *et al.*, 2011). This study further confirms these results, providing a comprehensive analysis of the effects of long-term environmental enrichment in the absence of exercise, upon cognitive function. It could be argued that animals housed in enriched environments will be more active due to the additional stimulation that is available to them. Therefore, we examined the home cage activity of the rats at the 13-15 months old timepoint in order to assess their natural activity levels. We report that rats housed in enriched conditions actually exhibit reduced home cage activity when compared to their standard housed counterparts. Thus we can confidently state that the enrichment-induced neuroprotection that is demonstrated in this study is not due to any additional physical activity in the environmentally enriched rats.

The aim of this study was to evaluate the neuroprotective properties of long-term environmental enrichment, in the absence of exercise, in the rat. The results presented here clearly show that this form of enrichment is highly effective at preventing an age-related decline in spatial, recognition and working memory. In agreement with the results from Chapter 3, neurochemical analysis has elucidated a role for NGF and neurogenesis in this process. Furthermore, environmental enrichment can prevent the typical inflammatory changes associated with ageing and in cell death. The mechanisms underlying these effects are not fully clear but the alteration in the expression of Trk and p75 receptors within the brain, together with the prevention of the loss of NGF expression, seems to indicate a drive towards the pro-survival pathways associated with neurotrophin signalling. To further expand upon these mechanisms, an analysis of the proportion of pro and mature forms of neurotrophins and downstream signalling molecules such as MAP kinases, JNK or NF-κB would provide a clearer picture of the signalling pathways that are being preferentially activated. Nevertheless, this study provides clear and powerful evidence that cognitive stimulation alone can prevent age-related cognitive decline and provides further support for the need for an increased number of studies assessing the efficacy of this type of intervention in humans.

Chapter 6: Evaluation of the age-related changes in grey matter volume and cerebral blood flow: a neuroprotective role for environmental enrichment?

6.1 Introduction

The use of Magnetic Resonance Imaging (MRI) to measure changes in grey matter volume and cerebral blood flow is particularly useful because it enables a direct comparison between species with the same non-invasive technique. The results from these scans can also be compared with neurochemical analyses of the same regions in animal studies in order that global markers of neurodegeneration, neuroplasticity or cognitive decline can be correlated with the underlying neurochemical changes that are occurring in the brain.

Brain structure and function change throughout the lifespan, and ageing is associated with reductions in brain volume and both grey and white matter, particularly in the frontal lobe, hippocampal formation and medial temporal lobe (Resnick *et al.*, 2003). The reasons for these reductions are more commonly associated with the loss of myelin fibres, dendritic aborisation and synapses rather than neuronal loss (Freeman *et al.*, 2008). In neurodegenerative diseases such as Alzheimer's disease and Mild Cognitive Impairment (MCI), this atrophy occurs more rapidly (West *et al.*, 1994, Buckner, 2004, Head *et al.*, 2005). The association between brain volume and cognitive decline is not straightforward however; typically the strongest correlations between cognitive function and regional grey matter loss are found in pathological conditions such as Alzheimer's disease and MCI (Van Petten, 2004, Duarte *et al.*, 2006, Zimmerman *et al.*, 2006). Studies suggest that there are compensatory mechanisms in place to maintain brain function with reduced brain volume, with patients that exhibit higher cognitive function showing reduced brain volumes but increases in brain activation (Solé-Padullés *et al.*, 2009). This suggests that there is enhanced recruitment of additional neural networks to counteract the loss of grey matter. In normal ageing, some studies to suggest that there is no clear pattern between regional grey matter volumes and cognitive function (Van Petten, 2004) whereas others show a positive correlation between episodic memory and hippocampal volume, and between global cortical volume reductions and executive function decline (Kramer *et al.*, 2007). In particular, the dentate gyrus seems to be vulnerable to age-related alterations in morphology and function; analysis in monkeys revealed that there is significant reduction in cerebral blood volume in the dentate gyrus with age and this was positively correlated with *Arc* expression (Small *et al.*, 2004).

It is possible to induce experience-dependent changes in regional grey matter: London taxi drivers (who have extensive spatial navigation training) have significantly larger posterior hippocampi than age-matched controls that do not drive taxis (Maguire *et al.*, 2000). Furthermore, in this study posterior hippocampal volume was positively correlated with the length of time spent working as a taxi driver. Quallo and colleagues (2009) report increases in grey matter (as assessed by VBM) in the somatosensory cortex, temporal sulcus and intraparietal sulcus following training on a tool-use task in macaques, suggesting that this technique can detect plasticity changes in sensory and associated motor regions following training on certain tasks. Interestingly, the monkey that was the worst performer during the training showed no changes. A similar result has been shown in humans, with increases in grey matter volume in the mid-temporal area and intrapareital sulcus following juggling training (Draganski *et al.*, 2004). Task training in this way is a cognitive stimulation that may be considered to be similar to the cognitive enrichment aspect of rodent environmental enrichment protocols, although at a much more complex level. It may be possible therefore, to detect any changes in plasticity that occur with long-term cognitive stimulation in rodent models of age and enrichment.

Cardiovascular disease is a well-established and significant risk factor in the development of dementias and stroke; hypertension, in particular, seems to play a role in cognitive decline with age and therefore anti-hypertensive treatments are often included in interventions to combat dementia (Launer, 2002). An increase in blood pressure can damage the cerebral capillaries, causing a reduction in cerebral blood flow whilst chronic hypertension is also a major risk factor in atherosclerosis which can reduce blood flow to the brain (Aram V, 1992, Nobili *et al.*, 1993). Increases in both systolic and diastolic blood pressure at midlife are associated with lower cognitive function in later life (Launer *et al.*, 1995, Kilander *et al.*, 1998, Launer *et al.*, 2002). These changes can be region-specific; Heo and colleagues (2010) report a reduction in cerebral blood flow in the hippocampus of aged subjects that was positive correlated with spatial memory performance.

Given the link between blood flow, the hippocampus and cognitive performance it is not surprising that cerebral blood volume is reported to be positively correlated with neurogenesis in the dentate gyrus of mice, both of which are increased with exercise (Pereira *et al.*, 2007). Interestingly, this study also demonstarted increases in cerebral blood volume in the dentate gyrus of exercising humans which was positively correlated with

both the fitness level of the individual and their cognitive performance on a verbal reasoning test.

Typically, measurements of cerebral blood volume are performed using exogenous tracers (such as bolus tracking with gadolinium chelates). Using exogenous tracers assumes that the blood brain barrier is intact and therefore the contrast agents can only travel through the vasculature. Alternatively endogenous arterial water can be labelled (arterial spin labelling, ASL) with radiofrequency pulses and the movement and dispersion of this labelled water can be measured. ASL is a non-invasive technique, as no exogenous tracers are needed to measure perfusion and therefore this reduces the stress to the animal. This technique labels the water molecules in the blood, upstream of the region of interest, by inverting their longitudinal magnetisation and then subsequently, images the region of interest during the time that the labelled blood is within that region. A measure of cerebral blood flow is calculated from the ratio of relaxation (time taken for the inverted ions to return to their relaxed state) between the labelled and control blood, and the amount of magnetised blood that arrived at the region of interest (Buxton, 2005). This study uses an updated approach to ASL, bolus-tracking ASL in which boluses of varying duration were labelled using the continued ASL (cASL) technique (Kelly *et al.*, 2009).

The aim of this study was to assess changes in grey matter volume and blood flow across the rats' lifespan and to evaluate whether environmental enrichment could prevent any age-related alterations. To date, there has been no study that analyses changes in grey matter volume with age in the rat and this is the first study to assess the neuroprotective properties of environmental enrichment with respect to cerebral blood flow. Therefore this study provides novel insights into the global changes in the brain that can occur with age, and the effect that enrichment may have upon them. Given the techniques used in this study, these results are also directly comparable to studies from non-human primates and humans and therefore, together with the neurochemical results, may provide biomarkers for cognitive decline that can be relatively easily measured in human studies.

6.2 Methods

6.2.1 MRI Scanning

At the Baseline timepoint, thirteen rats (SH, n=6; EE, n=7) were scanned in a 7T horizontal bore magnet (Bruker). At the 13-15 months old timepoint fifteen rats (SH, n=7; EE, n=8) were scanned in the 7T horizontal bore magnet to obtain 13-15 months old MR images. At the Aged timepoint (23 months of age) twelve rats (SH, n=6; EE, n=6) were scanned in the 7T horizontal bore magnet to obtain aged MR images. Scanning took approximately fifty minutes per rat and consisted of a variety of different sequences in order to obtain measures for cerebral blood flow and grey matter density (see 2.7). Only rats that were scanned at all three timepoints were included in the final analysis.

Voxel-Based Morphometry

Voxel-Based Morphometry (VBM) analysis was performed on T_1-weighted MR axial images (Ashburner and Friston, 2000, Good et al., 2001) with FSL tools (Smith et al., 2004) (see 2.7.1). These were collected using a RARE sequence (acquisition parameters: FOV = 4.00 x 3.00cm, image matrix = 266 x 200, 64 x 0.5mm slices, TR = 6.26s, TE = 36.00ms). Images were analysed based on a fully automated VBM analysis adapted from human brain VBM. Briefly, T_1 images were brain-extracted in MIPAV (Medical Image Processing, Analysis and Visualisation; (McAuliffe *et al.*, 2001)) followed by segmentation into the different tissue types. The resulting grey matter partial volume images were normalised to a standard rat brain and then averaged to create a study specific template to which the native grey matter images were non-linearly re-registered. The registered images were modulated (to correct for local expansion or contraction) then smoothed with an isotropic Gaussian kernel with a sigma of 2.5 in preparation for further statistical analysis (figure 6a).

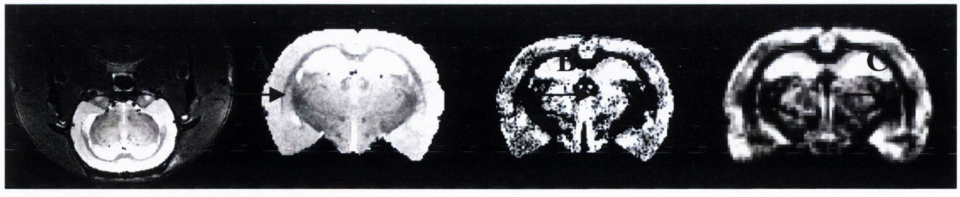


Figure 6a. Automated Voxel Based Morphometry(A) Raw images were reorientated and skull-stripped **(B)** Brains were segmented into different tissue types **(C)** Grey matter images were normalised to a standard rat brain, registered to the study-specific template and spatially smoothed.

To compare global grey matter changes between groups, independent samples t-tests were performed using Randomise v2.1. This is a permutation program that utilises a nonparametric version of the general linear model to analyse differences between voxels using Threshold-Free Cluster Enhancement, and correcting for multiple comparisons using family-wise error correction with a threshold of $p<0.01$.

Bolus-tracking Arterial Spin Labelling

Cerebral blood flow analysis was performed on Regions of Interest (ROI; 26 voxels) in the left and right hippocampus, and left and right cortex (figure 6c). Images were obtained using the btASL method developed by Kelly *et al* (2009 [see 2.7.2]). Briefly, images were taken **(2)** after a 5s preparation interval (where the magnetisation of the water molecules was inverted **(1)**) using a FLASH sequence (acquisition parameters: FOV = 3.00 x 3.00cm, image matrix = 128 x 64, 1 x 2mm slice, TR = 6.94ms, TE = 2.63mm, 22 x repetition images per slice). Control images were taken to account for noise, where the magnetisation of the water molecules was not inverted **(3)**, and images were taken in the same position as the 'tagged' images **(4)**. The position of the slice showed a clear view of the hippocampus (4mm posterior to bregma, (Paxinos and Watson, 1998)). An image with signal intensities proportional to the concentration of excited spins was calculated, and mean signal intensity measures of the ROIs were plotted against time to produce intensity-time curves. Mean Transit Time (time taken for the 'tagged' blood to reach the target slice; MTT), Capillary Transit Time (time take for the 'tagged' blood to traverse the vasculature in the target slice; CTT), and proportional blood flow were calculated (Kelly *et al.*, 2009).

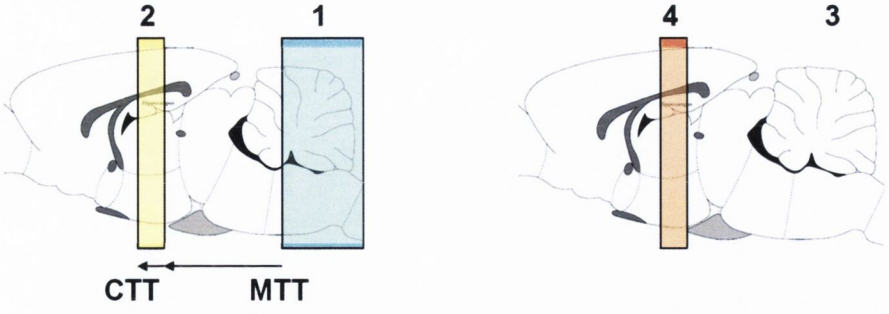


Figure 6b. Pictorial representation of bolus tracking Arterial Spin Labeling (1) The water molecules in the blood are magnetised upstream of the imaging slice **(2)** 5 seconds later, 22 repetition images are taken at the slice of interest **(3)** For control images, no magnetisation of the water occurs and **(4)** images are taken at the same slice position. Control images were subtracted from 'tagged' images to reduce noise. MTT, CTT and proportional blood flow were calculated.

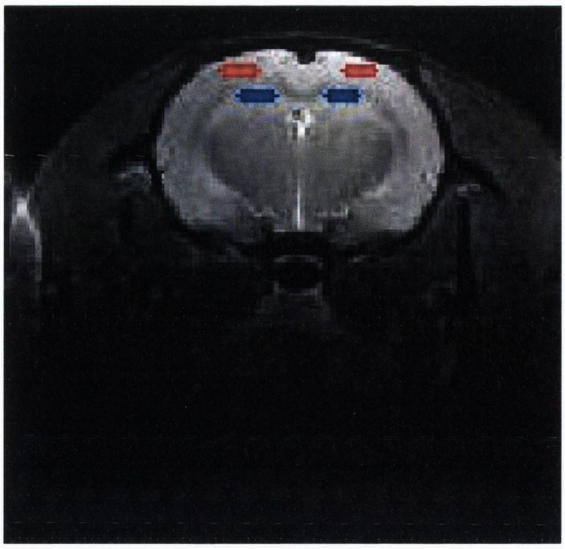


Figure 6c.Representative high resolution T1 image showing the ROIs for btASL analysis. Red represents left and right cortex, blue represents left and right hippocampus. R=L.

6.2.2 Statistical Analysis

Differences in grey matter intensity were calculated with one contrast map that shows regions of SH grey matter intensities that were greater than EE grey matter intensities and one contrast map that shows regions of EE grey matter intensities that were greater than SH grey matter intensities. To be deemed significant, the regions had to survive threshold-free cluster enhancement (tfce) and family-wise error thresholding at $p<0.01$. All proportional blood flow, MTT and CTT data were analysed using one-way ANOVAs, with Baseline SH and EE results pooled. Data are represented as mean ± standard error of the mean (SEM).

6.3 Results

6.3.1 Voxel-Based Morphometry

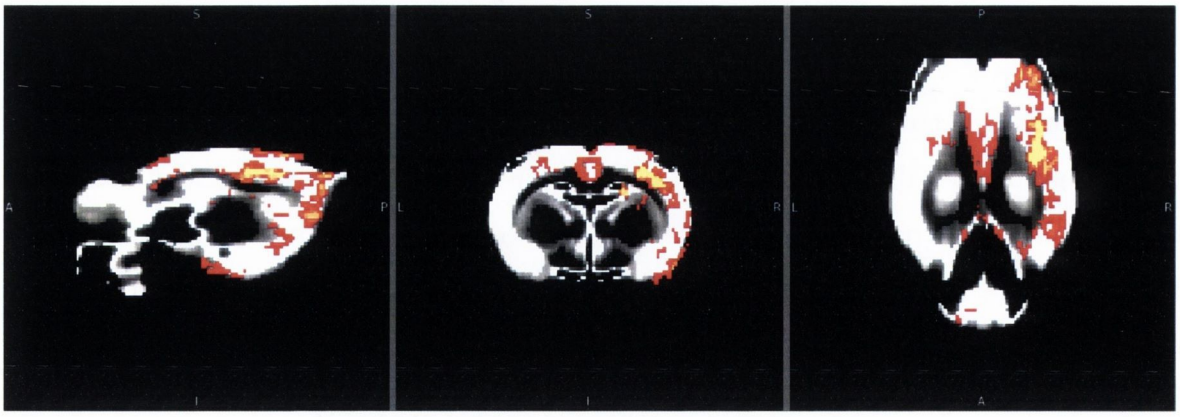
At Baseline, FSL randomise revealed no significant differences in grey matter intensity between groups. At the 13-15 months old timepoint, FSL randomise revealed that there was a significant increase in grey matter intensity in the EE group when compared with the SH group in the right somatosensory S1 cortex ($p<0.01$, corrected; figure 6d.i). There was also a significant increase in grey matter intensity in the SH group when compared with the EE group in the right hippocampus ($p<0.01$, corrected; figure 6e.i). At the Aged timepoint, FSL randomise revealed no significant differences in grey matter intensity between groups.

To further quantify the differences found between the groups at 13-15 months old, a mask was created using ROIs taken from the regions of significant difference in the contrast maps at 13-15 months old. This mask was used to obtain grey matter intensity values for all the brains at all the different timepoints, at these regions. One-way ANOVA analysis was used to compare the grey matter intensity in the right somatosensory S1 cortex ROI and right hippocampus ROI across groups.

There was a significant difference in grey matter intensity between groups in the right somatosensory S1 cortex ($F_{4,32} = 9.72$, $p<0.001$; figure 6d.ii). Bonferroni's Multiple Comparison Test showed a significant increase in grey matter intensity in the 13-15 months old EE group when compared Baseline group ($p<0.001$) and the 13-15 months old SH group ($p<0.001$). There was also a significant increase in grey matter intensity in the Aged EE group when compared with the Baseline group ($p<0.05$). Mean grey matter intensity ± SEM: Baseline = 0.55±0.03, 13-15 mo SH = 0.57±0.02, 13-15 mo EE = 0.73±0.01, Aged SH = 0.66±0.01, Aged EE = 0.66±0.02.

There was a significant difference in grey matter intensity between groups in the right hippocampus ($F_{4,32} = 6.52$, $p<0.001$; figure 6e.ii). Bonferroni's Multiple Comparison Test showed a significant increase in grey matter intensity in the 13-15 months old SH group when compared with the 13-15 months old EE group ($p<0.001$), the Aged SH group ($p<0.05$) and the Aged EE group ($p<0.05$). Mean grey matter intensity ± SEM: Baseline = 0.63±0.03, 13-15 mo SH = 0.69±0.03, 13-15 mo EE = 0.53±0.05, Aged SH = 0.57±0.02, Aged EE = 0.57±0.03.

i. 13-15 mo EE > 13-15 mo SH



ii

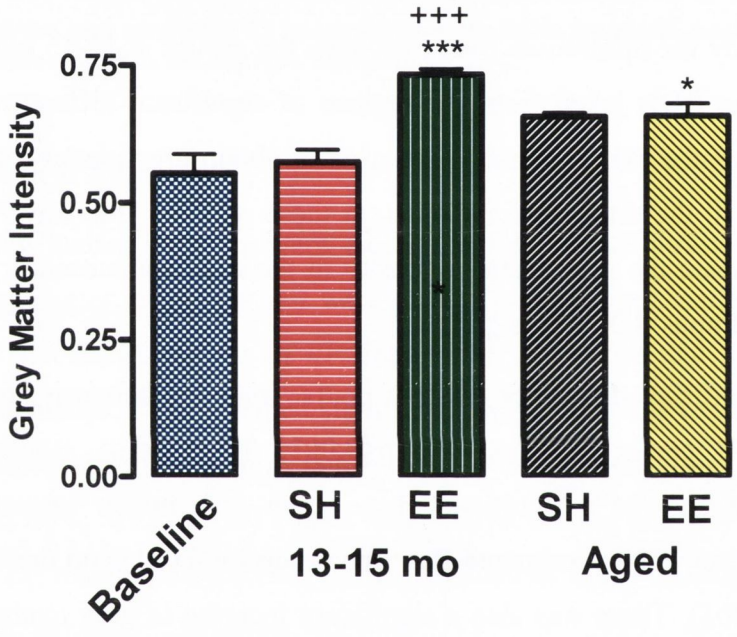
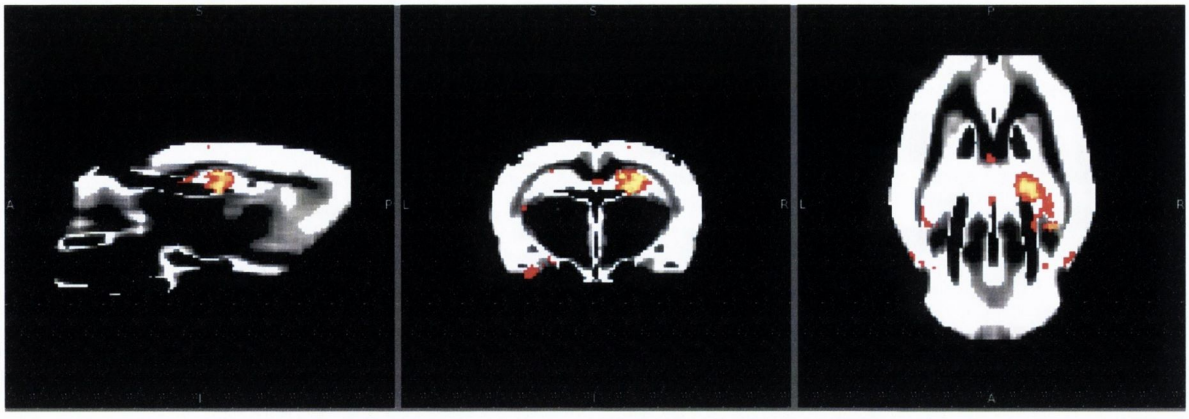


Figure 6d. Environmental enrichment increases grey matter intensity in the somatosensory S1 cortex at 13-15 months of age and the Aged timepoint (i) A contrast map showing the regions where 13-15 mo EE grey matter intensity >13-15 mo SH grey matter intensity **(ii)** There was a significant difference in grey matter intensity between groups in the right S1 cortex ($F_{4,32}$ = 9.72, $p<0.001$). Bonferroni's Multiple Comparison Test showed a significant increase in grey matter intensity in the 13-15 mo EE group when compared Baseline group (***$p<0.001$) and the 13-15 mo SH group ($^{+++}p<0.001$). There was also a significant increase in grey matter intensity in the Aged EE group when compared with the Baseline group (*$p<0.05$).Baseline: n=12, 13-15 mo SH: n=6, 13-15 mo EE: n=6, Aged SH: n=6, Aged EE: n=6.

i. 13-15 mo SH > 13-15 mo EE



ii

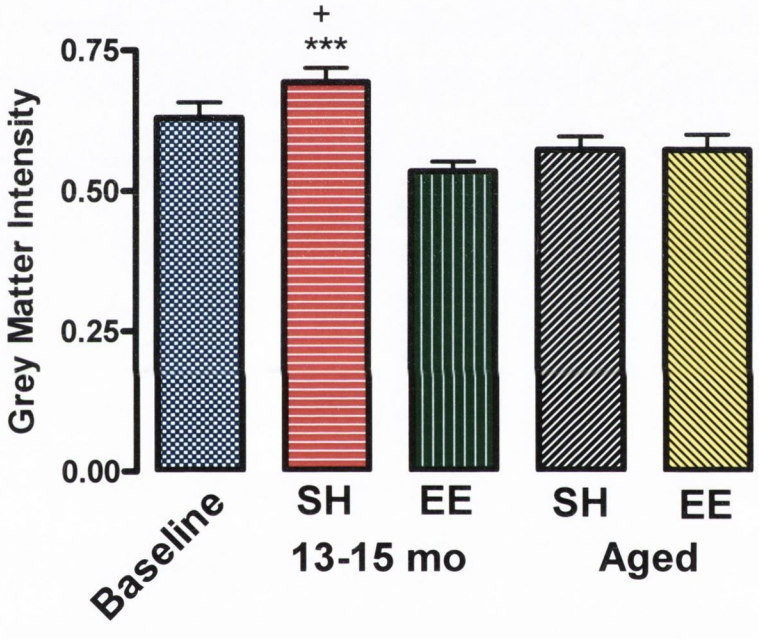


Figure 6e. There is a significant increase in grey matter intensity in the right hippocampus in standard housed rats at the 13 – 15 months of age(i) A contrast map showing the regions where 13-15 mo SH grey matter intensity >13-15 mo EE grey matter intensity **(ii)** There was a significant difference in grey matter intensity between groups in the right hippocampus ($F_{4,32} = 6.52$, $p<0.001$). Bonferroni's Multiple Comparison Test showed a significant increase in grey matter intensity in the 13-15 mo SH group when compared with the 13-15 mo EE group (***$p<0.001$), the Aged SH group ($^{+}p<0.05$) and the Aged EE group ($^{+}p<0.05$). Baseline: n=12, 13-15 mo SH: n=6, 13-15 mo EE: n=6, Aged SH: n=6, Aged EE: n=6.

6.3.2 Environmental enrichment rescues a reduction in cerebral blood volume in the hippocampus with age

There was a significant difference between groups in the proportional blood flow in the left hippocampus ($F_{4,39} = 4.14$, $p<0.001$; figure 6f.i). Bonferroni's Multiple Comparison Test revealed a significant decrease in proportional blood flow in the 13-15 month old SH group when compared with Baseline ($p<0.05$). There were no significant differences between Baseline and 13-15 month old EE ($p>0.05$), Aged SH ($p>0.05$) or Aged EE ($p>0.05$). Mean proportional blood flow ± SEM: Baseline = 0.138±0.001, 13-15 month old SH = 0.109±0.01, 13-15 month old EE = 0.115±0.01, Aged SH = 0.131±0.01, Aged EE = 0.143±0.02.

There was a significant difference between groups in the proportional blood flow in the right hippocampus ($F_{4,39} = 3.488$, $p<0.05$; figure 6f.ii). Bonferroni's Multiple Comparison Test showed a significant decrease in proportional blood flow in the 13-15 month old SH group when compared with Baseline ($p<0.05$). There were no significant differences between Baseline and 13-15 month old EE ($p>0.05$), Aged SH ($p>0.05$) or Aged EE ($p>0.05$). Mean proportional blood flow ± SEM: Baseline = 0.139±0.005, 13-15 mo SH = 0.113±0.004, 13-15 mo EE = 0.117±0.01, Aged SH = 0.130±0.01, Aged EE = 0.143±0.006.

There was no significant difference between groups in the proportional blood flow in the left cortex ($F_{4,39} = 1.567$, $p>0.05$; figure 6f.iii). Mean proportional blood flow ± SEM: Baseline = 0.133±0.004, 13-15 mo SH = 0.115±0.003, 13-15 mo EE = 0.132±0.01, Aged SH = 0.140±0.01, Aged EE = 0.143±0.001.

There was a significant difference between groups in the proportional blood flow in the right cortex ($F_{4,39} = 3.017$, $p<0.05$; figure 6f.iv), however Bonferroni's Multiple Comparison Test showed no significant differences. Mean proportional blood flow ± SEM: Baseline = 0.132±0.004, 13-15 mo SH = 0.113±0.009, 13-15 mo EE = 0.124±0.009, Aged SH = 0.137±0.01, Aged EE = 0.154±0.009.

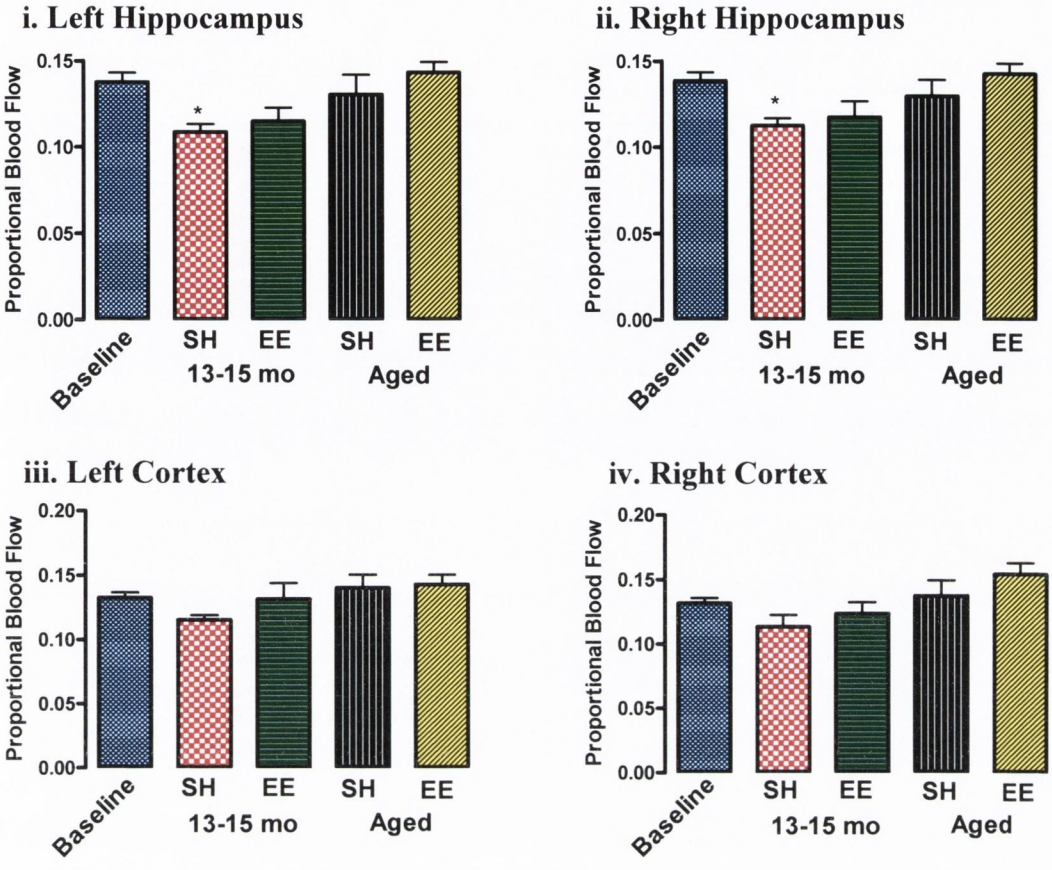


Figure 6f. Environmental enrichment rescues a reduction in proportional blood flow in the hippocampus at 13 – 15 months of age (i) There was a significant difference between groups in the proportional blood flow in the left hippocampus ($F_{4,35} = 4.14$, $p<0.001$). There was a significant decrease in proportional blood flow in the 13-15 months old SH group when compared with Baseline (*$p<0.05$) **(ii)** There was a significant difference between groups in the proportional blood flow in the left hippocampus ($F_{4,39} = 3.488$, $p<0.05$).There was a significant decrease in proportional blood flow in the 13-15 mo SH group when compared with Baseline (*$p<0.05$)**(iii)** There was no significant difference between groups in the proportional blood flow in the left cortex ($F_{4,39} = 1.567$, $p>0.05$) **(iv)** There was a significant difference between groups in the proportional blood flow in the right cortex ($F_{4,39} = 3.017$, $p<0.05$), however Bonferroni's Multiple Comparison Test showed no significant differences. Baseline: n=12, 13-15 mo SH: n=6, 13-15 mo EE: n=6, Aged SH: n=6, Aged EE: n=6.

6.3.3 Age and Environmental Enrichment do not affect Capillary Transit Time in the hippocampus or cortex

There was no significant difference between groups in the capillary transit time in the left hippocampus ($F_{2,35}$ = 1.322, p>0.05; figure 6g.i). Mean capillary transit time (s) ± SEM: Baseline = 1.70±0.04, 13-15 mo SH = 1.56±0.14, 13-15 mo EE = 1.49±0.10, Aged SH = 1.75±0.11, Aged EE = 1.60±0.08.

There was no significant difference between groups in the capillary transit time in the right hippocampus ($F_{2,35}$ = 0.421, p>0.05; figure 6g.ii). Mean capillary transit time (s) ± SEM: Baseline = 1.63±0.09, 13-15 mo SH = 1.64±0.04, 13-15 mo EE = 1.63±0.09, Aged SH = 1.76±0.09, Aged EE = 1.53±0.02.

There was no significant difference between groups in the capillary transit time in the left cortex ($F_{2,35}$ = 2.635, p>0.05; figure 6g.iii). Mean capillary transit time (s) ± SEM: Baseline = 1.71±0.08, 13-15 mo SH = 1.95±0.15, 13-15 mo EE = 1.61±0.09, Aged SH = 1.71±0.07, Aged EE = 1.47±0.04.

There was no significant difference between groups in the capillary transit time in the right cortex ($F_{2,35}$ = 0.838, p>0.05; figure 6g.iv). Mean capillary transit time (s) ± SEM: Baseline = 1.70±0.22, 13-15 mo SH = 1.77±0.13, 13-15 mo EE = 1.57±0.07, Aged SH = 2.04±0.55, Aged EE = 1.53±0.05.

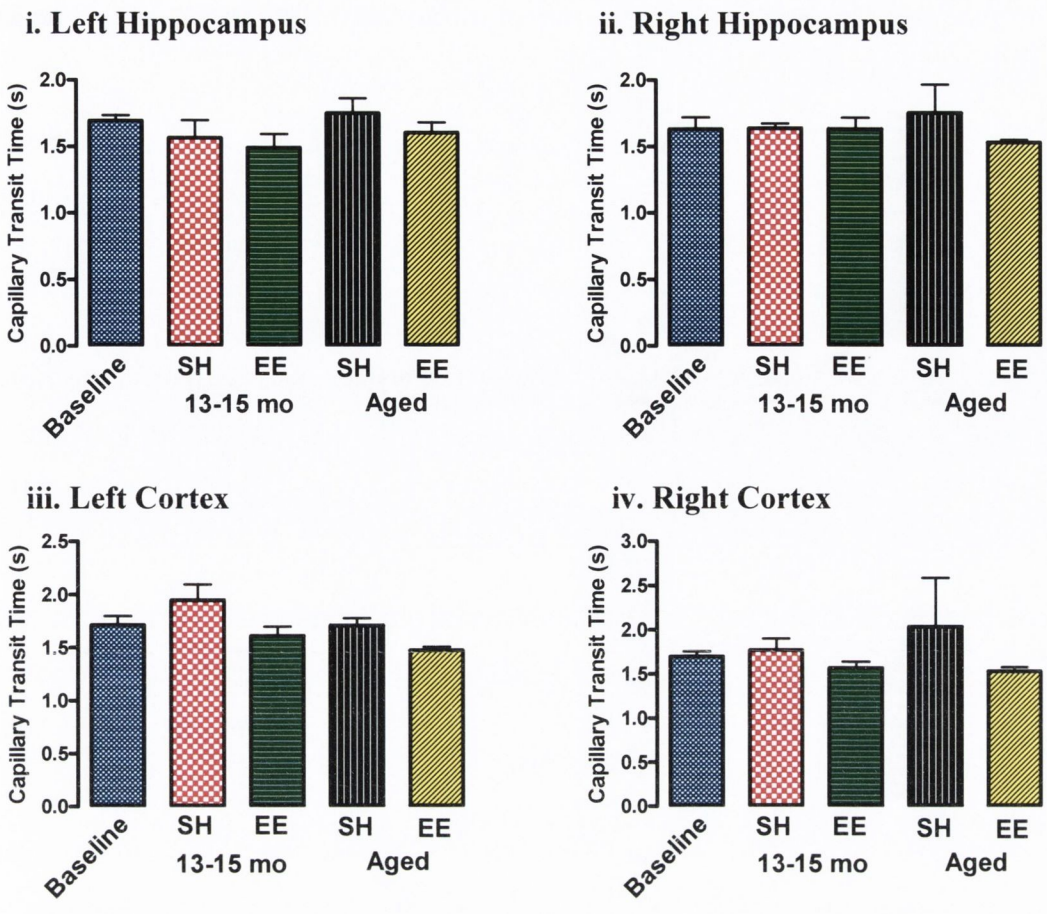


Figure 6g. Age and Environmental Enrichment do not affect Capillary Transit Time in the hippocampus or cortex (i) There was no significant difference between groups in the capillary transit time in the left hippocampus ($F_{2,35} = 1.322$, $p>0.05$) **(ii)** There was no significant difference between groups in the capillary transit time in the right hippocampus ($F_{2,35} = 0.421$, $p>0.05$) **(iii)** There was no significant difference between groups in the capillary transit time in the left cortex ($F_{2,35} = 2.635$, $p>0.05$) **(iv)** There was no significant difference between groups in the capillary transit time in the right cortex ($F_{2,35} = 0.838$, $p>0.05$), Baseline: n=12, 13-15 mo SH: n=6, 13-15 mo EE: n=6, Aged SH: n=6, Aged EE: n=6.

6.3.3 Age and Environmental Enrichment do not affect Mean Transit Time in the hippocampus or cortex

There was no significant difference between groups in the mean transit time in the left hippocampus ($F_{2,35} = 0.907$, $p>0.05$; figure 6h.i). Mean transit time (s) ± SEM: Baseline = 1.99±0.09, 13-15 mo SH = 1.97±0.06, 13-15 mo EE = 1.81±0.66, Aged SH = 1.90±0.03, Aged EE = 1.88±0.04.

There was no significant difference between groups in the mean transit time in the right hippocampus ($F_{2,35} = 0.261$, $p>0.05$; figure 6h.ii). Mean transit time (s) ± SEM: Baseline = 1.88±0.44, 13-15 mo SH = 1.91±0.62, 13-15 mo EE = 1.85±0.06, Aged SH = 1.87±0.10, Aged EE = 1.82±0.05.

There was no significant difference between groups in the mean transit time in the left cortex ($F_{2,35} = 1.985$, $p>0.05$; figure 6h.iii). Mean transit time (s) ± SEM: Baseline = 2.12±0.08, 13-15 mo SH = 2.16±0.08, 13-15 mo EE = 1.99±0.07, Aged SH = 1.95±0.08, Aged EE = 1.92±0.02.

There was no significant difference between groups in the mean transit time in the right cortex ($F_{2,35} = 1.395$, $p>0.05$; figure 6h.iv). Mean transit time (s) ± SEM: Baseline = 2.15±0.06, 13-15 mo SH = 2.13±0.07, 13-15 mo EE = 1.99±0.08, Aged SH = 2.00±0.19, Aged EE = 1.91±0.04.

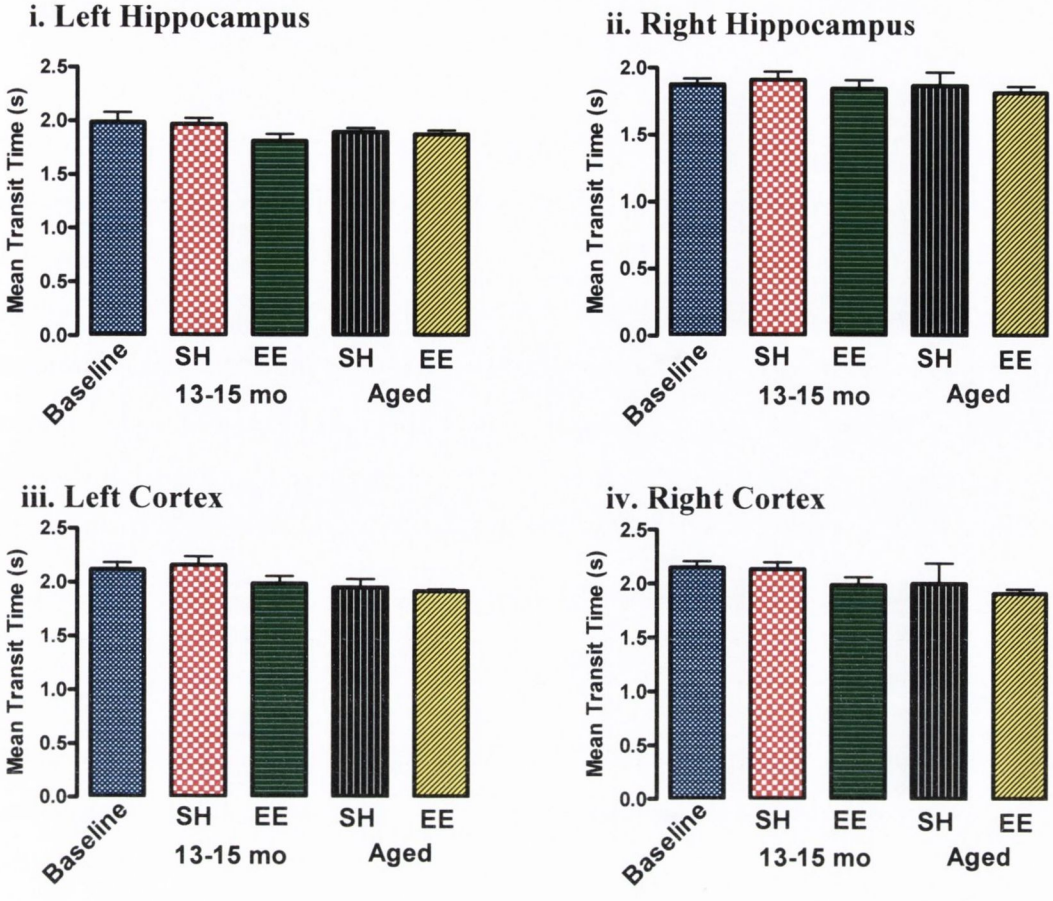


Figure 6h. Age and Environmental Enrichment do not affect Mean Transit Time in the hippocampus or cortex (i) There was no significant difference between groups in the mean transit time in the left hippocampus ($F_{2,35}$ = 0.907, p>0.05) **(ii)** There was no significant difference between groups in the mean transit time in the right hippocampus ($F_{2,35}$ = 0.261, p>0.05) **(iii)** There was no significant difference between groups in the mean transit time in the left cortex ($F_{2,35}$ = 1.985, p>0.05) **(iv)** There was no significant difference between groups in the mean transit time in the right cortex ($F_{2,35}$ = 1.395, p>0.05), Baseline: n=12, 13-15 mo SH: n=6, 13-15 mo EE: n=6, Aged SH: n=6, Aged EE: n=6.

6.4 Discussion

The aim of this study was to investigate the age-related changes in grey matter intensity and cerebral blood flow in the rat, and evaluate the effect of environmental enrichment on these changes. The VBM analysis used calculates changes in the proportion of grey matter in each voxel, and this is defined 'grey matter intensity'. This is comparable to an increase in grey matter volume because an increase in the proportion of grey matter in any given voxel is akin to an increase in grey matter volume as a voxel is a measurement of volume. Therefore, for the purposes of this discussion I will discuss the changes in grey matter intensity as volumetric changes. These results show that there is a significant increase in grey matter volume in the right somatosensory S1 cortex of enriched rats at both the 13-15 months old and Aged timepoints, suggesting enrichment-induced plasticity changes. Interestingly, there is an increase in grey matter volume in the right hippocampus in standard housed rats when compared with enriched rats at 13-15 months old but this change is transient and does not remain at the Aged timepoint. There is also significant reduction in cerebral blood flow in the hippocampus at 13-15 months old in the standard housed rats, which is partially rescued by enrichment. This pattern is also evident in the right and left cortex, however there are no significant differences. There is a gradual increase in cerebral blood flow at the Age timepoint in all regions, with the environmentally enriched rats exhibiting an increase in cerebral blood flow when compared with all other groups, although this is not significant.

The increases in grey matter in the sensory cortex that are seen in the enriched rats at both 13-15 months old and Aged timepoints would suggest that environmental stimulation can induce long-lasting neuroplastic changes in the rat brain. This complements current literature that reports that training a motor task can induce increases in grey matter in both humans and non-human primates (Draganski *et al.*, 2004, Quallo *et al.*, 2009). Boyke and colleagues (2008) also report increased grey matter in a group of elderly subjects learning to juggle in the same regions as their previous study teaching young subjects to juggle, namely the mid-temporal region and intraparietal sulcus, and additionally increases in the hippocampus and nucleus accumbens. An enriched environment provides added somatosensory stimulation for the rats and therefore, over an extended period of time, this seems to have induced increased plasticity in the primary somatosensory cortex in the rats in this study. This provides a novel insight into the plasticity of the rat brain following

complex sensory stimulation. Interestingly, there is an increase in the right hippocampus in standard housed rats at 13-15 months old. This is an unexpected result, because the majority of literature reports reductions in hippocampal volume with age in humans, non-human primates and rodents (Resnick *et al.*, 2003, Small *et al.*, 2004, Driscoll *et al.*, 2006) and these rats also exhibit impairments in spatial memory tasks at the 13-15 months old. It is also interesting, because this increase is transient as it is not evident at the Aged timepoint. It is argued that volume is not a strong predictor of functional output, because it does not correlate with synapse number or density (Anderson, 2011). This is particularly relevant in studies measuring age-related atrophy and function (Freeman *et al.*, 2008), however it may still hold true for interpreting increases in volume. There is a lack of data regarding changes in grey matter volume in rodents, particularly at middle age, and using this analysis technique. We report increased grey matter in the somatosensory cortex in environmentally enriched rats is supported with published data from human and non-human primate studies and therefore it is easier to conclude that there is an enrichment-induced alteration in plasticity in this region. However, the increase in hippocampal grey matter volume in the standard housed rats is contradictory to the vast majority of studies in humans, non-human primates and rodent studies and it is therefore difficult to come to a conclusion regarding the underlying cause. Nevertheless, there is a dramatic loss of grey matter volume from 13-15 months old to the Aged timepoint, which could be impacting cognitive function. Certainly, there needs to be more studies analysing the normal alterations in grey matter with age in the rat in order that this result can be compared to what would be typically expected.

The pattern of change in cerebral blood flow would suggest that there is a reduction in blood flow at the 13-15 months old timepoint, which partially recovers at the Aged timepoint, and that environmental enrichment can induce an increase in blood flow at this timepoint. The lack of significance in this data is likely to be due to the number of animals in this study, and this makes the results more difficult to interpret. They do however, suggest that there is cerebral blood flow dysfunction age in the rat and that this follows a U shaped curve. There were no changes seen between all groups in the mean transit time or capillary transit time. The mean transit time is a measure of the time taken for 'tagged' blood to travel to the imaging slice and capillary transit time is a measure of the time taken for the 'tagged' blood to disperse within the vasculature of the imaging plane.This would indicate that the change in blood flow is independent of these measures.

The pattern of blood flow changes in these results suggests that enrichment rescues the reduction in blood flow over time which may be a factor in the reduction in apoptosis that is shown in Chapter 5. Pereira and colleagues (2007) report a positive correlation between cerebral blood volume and neurogenesis in mice following exercise, and a positive correlation between blood volume, fitness level and cognitive function in humans following exercise. There is also a strong link between angiogenesis and neurogenesis, in particular VEGF has been shown to play a role in maintaining a positive neurogenic niche in the dentate gyrus and is associated with improvements in hippocampal dependent memory (Cao *et al.*, 2004, Licht *et al.*, 2011). Results in Chapter 5 show an amelioration of the age-related reduction in VEGF in the hippocampus with enrichment which may be preventing a loss of microvasculature in the hippocampus and therefore reducing apoptosis and enhancing neurogenesis. Increased systolic or diastolic blood pressure in middle age is a significant risk factor for dementia later in life (Kilander *et al.*, 1998, Launer *et al.*, 2002) and because the rats in this study were given food and water *ad libitum* for the majority of their lives, it is likely that they suffered from cardiovascular problems such as hypertension resulting from obesity. Therefore, it is possible that cognitive enrichment upregulated various neurochemical pathways in the brain, such as via the growth factors NGF and VEGF, that may have protected the microvasculature in regions susceptible to atrophy.

This is the first study to assess grey matter changes with age in the rat using an automated analytical technique. It shows that environmental enrichment can induce neuroplastic changes in the brain that are associated with enhanced somatosensory stimulation, and that these changes persist throughout the rat's lifespan. In addition, this study evaluates the neuroprotective role of environmental enrichment on age-related changes in cerebral blood flow. The results indicate that there is a reduction in cerebral blood flow at middle age in the hippocampus and cortex which is partially recovered at the Aged timepoint and that environmental enrichment can induce a modest increase in cerebral blood flow at both 13-15 months old and Aged timepoints. Whilst the interpretation of these data is hampered by the low n numbers, they do provide tentative evidence for environmental enrichment providing regional changes in brain neuroplasticity and an overall improvement in cerebrovascular function.

Chapter 7: Discussion

7.1 General Discussion

The main objectives of this study were to investigate the efficacy of short-term and long-term environmental enrichment, in the absence of exercise, as a cognitive enhancer and to assess the underlying mechanisms associated with any enrichment-induced changes observed. Current lifestyle trends in the U.S. show that there is an increasing shift towards a sedentary, socially inactive pattern of living, with the amount of leisure time spent watching television dramatically outstripping the amount of time spent participating in cognitively stimulating activities or exercising (U.S. Bureau of Labor Statistics, 2011). A physically and cognitively active lifestyle is crucial for neurodevelopment (Crosnoe *et al.*, 2010), and to maintain a healthy mind and body throughout life. Age-related cognitive decline is universally accepted to occur in adults, and with an ever increasing life expectancy, the socio-economic burden of a population with a high proportion of elderly people is becoming a serious global public health burden. Due to the lack of highly effective pharmacological treatments for age-related neurodegenerative diseases, there has been much recent interest in cognitive training as preventative treatment for age-related memory decline or Alzheimer's disease and other dementias (Buschert *et al.*, 2010, Martin *et al.*, 2011). Epidemiological studies also highlight the importance of enhanced cognitive stimulation throughout life can provide a 'cognitive reserve' which may protect against memory and executive function deficits associated with neurodegenerative diseases(Valenzuela *et al.*, 2007). Whilst biological mechanisms that underlie this phenomenon are yet to be elucidated, recent studies support a role for enhanced plasticity in the brain, associated with increased synaptic efficiency and an upregulation of growth factors that promote an enhanced neurogenic niche (Nithianantharajah and Hannan, 2011).

Results from the present study support the current literature in relation to the memory enhancing effects of enrichment but also provide further evidence with respect to the underlying mechanisms involved. We show the short-term efficacy of environmental enrichment, in the absence of exercise, as a cognitive enhancer &report a that minimum period of 3 weeks of continuous enrichment is necessary to induce a memory improvement in young male rats, as measured by the NOR task. Furthermore, there is a temporal improvement in memory, with rats housed in enriched conditions for 6 weeks having a significantly improved memory when compared with rats housed in enriched conditions

for 3 weeks. This improvement in memory is not selective to a single task, as rats also show improvements in the spatial variant of the NOR task, the OD task, and in the T maze task. Data from the present study also support the neuroprotective efficacy of environmental enrichment as an intervention to prevent age-related cognitive decline, as continuous long-term enrichment prevented spatial, recognition and working memory decline in aged rats. The literature reports many different protocols for environmental enrichment, including different durations in enriched housing, continuous versus daily enrichment, social enrichment and physical enrichment (Simpson and Kelly, 2011). Thus, there have been calls for a standardised environmental enrichment protocol to be utilised in order that changes in memory function are comparable between studies (Simpson and Kelly, 2011). Previous studies that use environmental enrichment without additional physical exercise report improvements in recognition and spatial memory (Nilsson *et al.*, 1999, Bruel-Jungerman *et al.*, 2005) and protection against age-related spatial memory decline (Kumar *et al.*, 2011). Whilst it is likely that different factors within environmental enrichment may interact in order to elicit memory improvements, the mechanisms underlying these improvements that are associated with the various factors has not been fully elucidated. Olson and colleagues (2006) argue that different interventions are inducing similar behavioural improvements via dissociable pathways.

This study reports a clear and consistent role for both NGF in the memory enhancement seen with environmental enrichment in this study by showing that infusions of a similar dose of exogenous NGF elicit similar cognitive improvements, and stimulate similar alterations in neuroplasticity and synaptic plasticity. In addition, this study provides novel evidence that a more physiologically relevant dose than has been used previously in literature (Jakubowska-Dogru and Gumusbas, 2005, Yang *et al.*, 2011) can enhance memory, correspondingly increase cell proliferation in the hippocampus and enhance synaptic plasticity. Thus, there is strong support for the role that βNGF plays in enrichment-induced memory improvements and increased proliferation.

A recent study by Kobilo and colleagues (2011) argue that it is exercise that is the main stimulating factor for increased neurogenesis and BDNF in the brain, and that enrichment alone has no effect upon these mechanisms.This is not in disagreement with this study; their paper reports that 30 days of enrichment alone does not increase BDNF in the brain or increase neuronal survival, both results which are mirrored in this study. Whilst they argue that enrichment alone does not enhance neurogenesis, they report this result following only

12 days of enrichment which this study shows is an insufficient time to elicit a neurogenic response. The paper also does not report any changes in the expression of NGF which I would hypothesise would be increased in the enriched group and not the exercise alone group. Interestingly, this study reports that exercise alone is more efficient at increasing BDNF and neurogenesis than both enrichment and exercise. Data from our lab suggests that exercise and enrichment together can induce a cumulative improvement in memory and neurogenesis (Bechara *et al*, personal communication). The discrepancy in these results could be associated with differing protocols: our lab utilise forced treadmill exercise in order that the amount of exercise that each animal undertakes can be controlled whereas Kobilo and colleagues (2011) use running wheels that are included in the animals' homecages. Whilst this reduces the stress to the animals, it means that there is no way to control for how much exercise each animal undertakes. It seems highly likely that animals in the exercise alone group would utilise the running wheels more than mice in the combined enrichment and exercise group because the mice in the combined group would have more stimulation from the other objects in their homecage. Therefore it is not possible to assume that the combined group are getting an accumulation of both stimuli.

Despite the suggested differences between this study and the work of Kobilo *et al*(2011), both of these studies highlight the complex nature of the mechanisms underlying enrichment-induced memory improvements. This study shows that NGF is the main factor associated with cognitive enrichment-induced memory improvements whereas previous data from our lab report that BDNF seems to be the main factor associated with exercise-induced memory improvements (Griffin *et al.*, 2009, Griffin *et al.*, 2011), results that are very well positioned within current theories regarding underlying mechanisms in literature.

The research presented here begins to further dissociate the neurochemical changes solely due to increased stimulation, which is an area that is much less analysed in the current literature when compared with the wealth of data presented in relation to the cognitive enhancing benefits of exercise. There are still a number questions that need to be answered in relation to the mechanisms that underlie the cognitive enhancing effect of environmental enrichment however the current research provides robust evidence that NGF can induce a similar behavioural improvements whether increased via exogenous or endogenous means. The correlative link between task performance and NGF concentration is highly suggestive of a direct link between these two factors. Nevertheless, it is unclear what aspect of increased stimulation could induce and increase of NGF in the first instance.

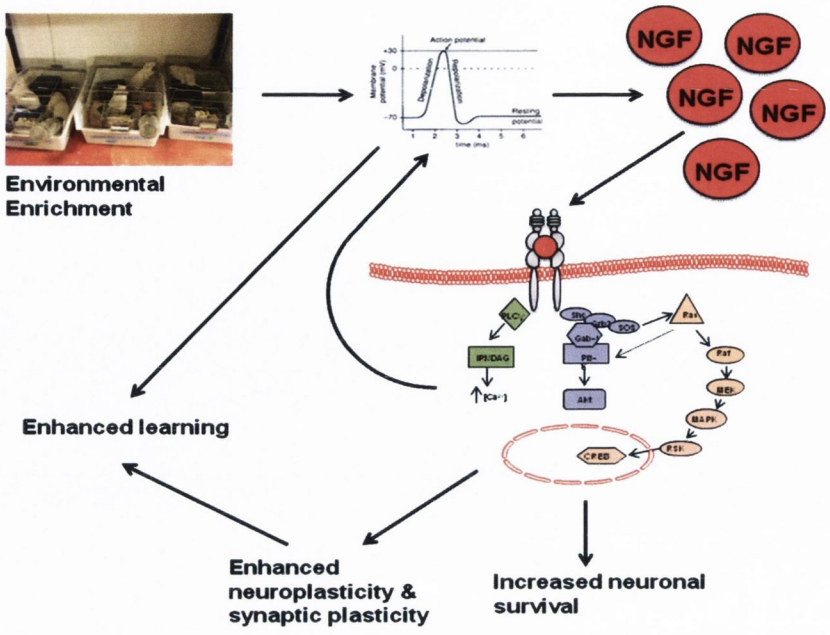


Figure 7a.The mechanisms underlying short-term environmental enrichment as a cognitive enhancer. Environmental enrichment triggers similar signalling cascades to those associated with learning, in particular it can stimulate the release of NGF which activates Ras/ERK, Plcγ and IP3/DAG pathways. Increased NGF can itself induce memory improvements and chronic upregulation of NGF lead to enhanced synaptic plasticity and increased neuronal survival.

In protocols that include exercise, there is a relationship between muscle exertion and BDNF release and whilst it is still very much debatable if or how this BDNF release can impact on central BDNF increases that are reported with exercise, this is a likely contender in the memory-enhancing effect of exercise(Pereira *et al.*, 2007, Griffin *et al.*, 2009). In the case of cognitive enrichment it is argued that the additional home-cage stimulation induces neurochemical cascades that mimic those which occur during learning paradigms.As with most therapeutic interventions like exercise or cognitive enrichment, there is a complex interaction of factors that are likely to feedback upon each other and stimulate mechanisms that can induce the same behavioural output. In the short-term, there is a stimulation of neurochemical pathways similar to those associated with learning itself, such as via the MAPKinase pathway. Learning itself can stimulate production of neurotrophins; depolarisation of neurons induces the release of both BNDF and NGF in hippocampal slices(Blöchl and Thoenen, 1995, Goodman *et al.*, 1996, Brooks *et al.*, 2000). Continuous homecage stimulation could therefore induce an upregulation of neurotrophin release that induces activation of signalling pathways such as Ras/ERK and PLCγ(Adams and Sweatt, 2002). Activation of both of these pathways has been implicated in LTP and synaptic

plasticity(English and Sweatt, 1996, Curtis and Finkbeiner, 1999), chronic stimulation being likely to induce structural, long-term changes in plasticity such as an increase in synaptic vesicle number. Chronic NGF release would also enhance neuronal survival and promote growth, possibly via the transcription of CREB and associated factors(Xing *et al.*, 1996).These changes would promote more efficient synaptic and neuroplasticity, leading to enhanced memory function (figure 7a).

This research also provides a powerful analysis of the cognitive changes that occur throughout the lifespan of Wistar rats. We report an interesting difference in the loss of the spatial versus recognition memory with age, with spatial memory declining at 13 months whereas recognition memory remains relatively intact until 22 months of age. This is most likely to be due to the heavy reliance on the hippocampus for spatial memory processing, whereas recognition memory relies more upon the perirhinal cortex. The hippocampus is particularly susceptible to volume loss with age and in neurodegenerative diseases (Yin *et al.*, 2012), and therefore spatial memory is likely to be affected earlier than other types of memory. Longitudinal human imaging studies show that there is an association between clinical decline and hippocampal activation (O'Brien *et al.*, 2010), and hippocampal volume loss is a strong predictor of cognitive decline with age (den Heijer *et al.*, 2010). Whilst there is no hippocampal volume decrease in this study, there is a clear loss of neuroplasticity and increased hippocampal apoptosis associated with cognitive decline.

The MR analyses do provide an indication of an age-associated dysfunction in blood flow, particularly at 13 months of age, that is likely to be significant factor in the cognitive decline, as numerous epidemiological studies report cerebrovascular dysfunction to be a high risk-factor for the development of dementia in later life (Launer *et al.*, 1995, Kilander *et al.*, 1998, Launer *et al.*, 2002). Of particular importance in this research is the ability to link together neurochemical and cellular changes with more global brain changes that can be directly compared with human studies. Alterations in cerebral blood flow would directly impact upon neuronal functioning and may cause neuronal damage and cell death, which is indicated in this study by an increase in DNA fragmentation and phagocytic microglia. We report an attenuation of the age-related decrease in VEGF expression in the hippocampus. VEGF is a vital factor in angiogenesis, but recent studies report it may also modulate learning and memory function and directly affect hippocampal neurogenesis (Fabel *et al.*, 2003, Cao *et al.*, 2004, Licht *et al.*, 2011, Wang *et al.*, 2011). Indirectly, VEGF may help to maintain an appropriate neurogenic niche enhancing angiogenesis within the dentate

gyrus (Palmer *et al.*, 2000). Therefore, increased VEGF expression may contribute to the partial attenuation of blood flow loss with age and additionally, may contribute, directly or indirectly, to the maintenance of hippocampal neurogenesis in the rats housed in enriched conditions.

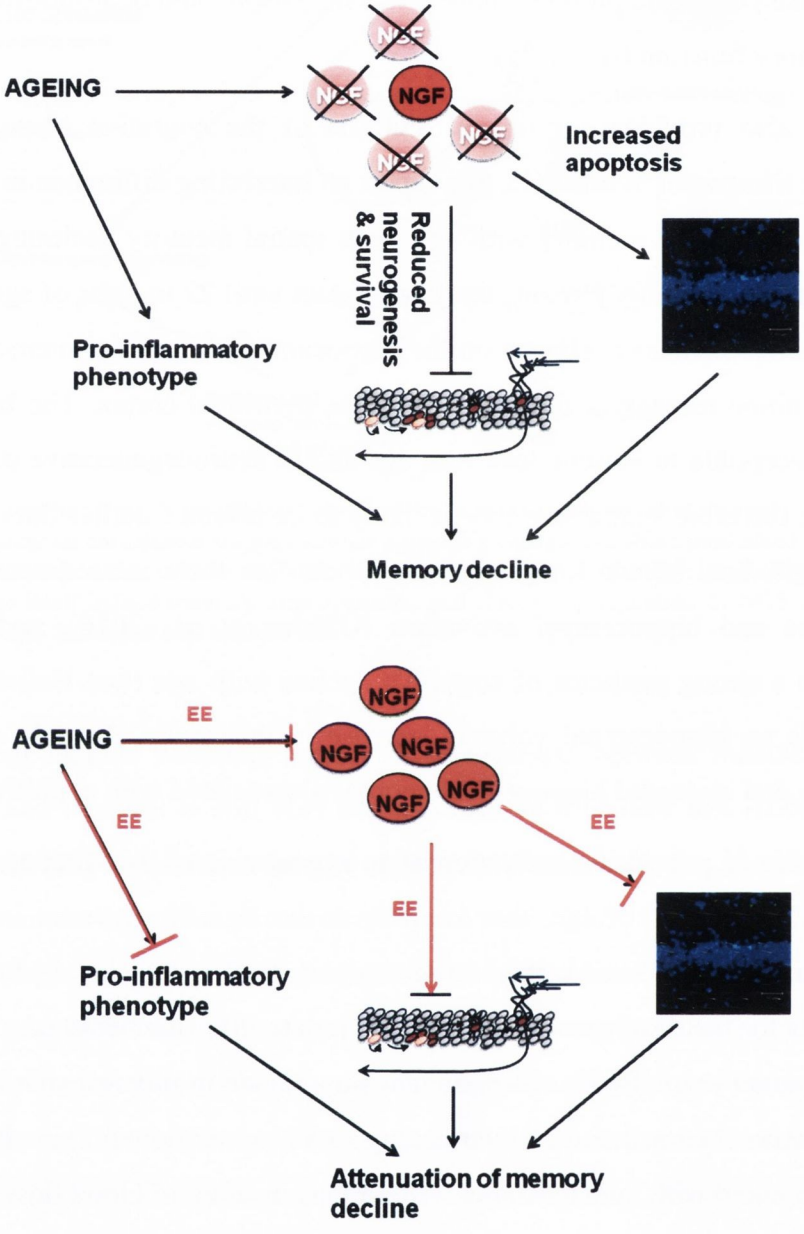


Figure 7b.Long-term environmental enrichment prevents the pro-inflammatory, pro-apoptotic phenotype and rescues memory deficits. Ageing causes a balance change in neurochemical pathways in which pro-inflammatory, pro-apoptotic cascades are upregulated and there is a reduction in the expression of pro-survival proteins, such as NGF. Environmental enrichment causes these pro-survival pathways to remain preferentially upregulated, attenuating the increase in inflammation and apoptosis and hence preventing age-related memory deficits.

Theincreased neurogenesis and early neuronal survival that is reported in this study may play different roles in young versus aged animals; recent studies demonstrate that young, immature neurons are preferentially activated during performance of a memory task (Kee *et al.*, 2007) and suggest that neurogenesis exhibited following short-term enrichment may play a direct role in enhancing memory function. Alternatively, continuous enrichment throughout the rat's lifespan may maintain this increased neurogenesis and therefore enhance the neurogenic reserve and provide neuroprotection against memory loss associated with age (Kempermann, 2008). In addition, if the enhanced synaptic plasticity that is reported in young animals following environmental enrichment continues with long-term enrichment protocols then it is very likely that this will contribute towards a 'cognitive reserve'.

Whilst this study only provides a small snapshot of the inflammatory changes that may be occurring with age and enrichment, taken together with all the results this provides a very clear indication that environmental enrichment can impact upon a number of different neurochemical pathways in the brain (figure 7b). This would suggest the involvement of a number of different factors that enrichment can effect, however consistent with the short-term enrichment data, we see a central role for NGF in these processes. In line with current literature, we see age-related memory deficits and these are coupled with alterations in the regulation of neurochemical pathways in the brain, as there is an increase in apoptosis and a more activated, inflammatory state together with a downregulation of pro-survival pathways as indicated by the reduction in NGF and Trk A expression. It is clear there are likely to be many other age-related changes in the brain, particularly in association with the changes in blood flow with age, and the results reported here are good indicators of the typical changes that are commonly seen in ageing studies. As with the short-term study, we report that environmental enrichment can rescue the reduction in expression of NGF with age, and this change is likely to have an impact upon the other age-related changes, particularly through interactions with other trophic support proteins such as VEGF, and via the activation of pro-survival pathways. Whilst it is not clear how environmental enrichment is mediating the reduction in pro-inflammatory markers, glia do express Trk A and are capable of producing NGF in response to an inflammatory stimulus (Gadient *et al.*, 1990, Elkabes *et al.*, 1998), therefore NGF may be modulating the age-related inflammatory response.

This study supports a cognitive enhancing effect of environmental enrichment, but serious consideration must also be made to the animal welfare impact of these data. Numerous studies upon a variety of species report enhanced welfare, species-specific behaviours and improved health outcomes following enriched housing (Honess and Marin, 2006, Ellis, 2009, Williams *et al.*, 2009, Robins and Waitt, 2010, Abou-Ismail and Mahboub, 2011). These studies highlight a key factor in the use of environmental enrichment interventions in animal studies to measure improvements in cognitive function: it could be argued that standard laboratory housing is impoverished when compared to the natural environment of these animals and therefore the behavioural improvements are simply ameliorating behavioural deficits associated with impoverished housing. Thus, it is crucial that all studies report the type of housing conditions of all groups of animals in studies, and there is a need for a framework for which enriched housing becomes standard for all animals. This would enable more direct comparison between animal studies and also any cognitive changes seen following additional stimulation over and above 'standard enriched housing' can be more directly compared to cognitive or physical stimulation in human studies because the standard housing would be more similar to natural environment that humans inhabit.

There are many different mechanisms reported in the literature that can account for the functional and structural changes that are seen with environmental enrichment. Of particular importance is how this enrichment can impact upon prevention of and treatment for neurodegenerative diseases. Epidemiological studies highlight the importance of environmental factors such as educational level, occupation and leisure activities in protecting against the development of age-related cognitive decline(Petrosini *et al.*, 2009). Importantly, most research to date is focussed upon targeted pharmacological treatments for cognitive decline, whereas environmental enrichment provides an alternative to this by promoting behavioural interventions to counteract loss of memory and executive function with age. This highlights the social responsibility that society must have for caring for an ever-increasing elderly population and offers an evidence-based framework for appropriate strategies that can be implemented in residential care-homes or advised by local health organisations.

Taken together, the data in this study provide strong evidence that environmental enrichment can induce memory improvements in young rats, and can prevent age-related memory loss. We consistently demonstrate that these memory improvements are

associated with an upregulation ofNGF and hippocampal neurogenesis in both the long-term and short-term.This study also demonstrates that the long-term benefitsassociated with environmental enrichmentcan ameliorate many featurestypical of the ageing brain, such as increases in apoptosis and pro-inflammatory markers. Furthermore, we provide novel data on age-related alterations in blood flow and grey matter volume in rats, and the effect environmental enrichment can have upon these measures. By dissociating the different components of environmental enrichment, it is possible to begin to elucidate the neurochemical mechanisms that may underlie the memory improvements associated with these separate factors. We argue that, whilst exercise alone is a potent memory enhancer, cognitive enrichment can also induce similar behavioural improvements and that these two interventions seem to elicit an effect via dissociable pathways; BDNF mediates the exercise-induced memory improvements whereas βNGF mediates the enrichment-induced memory improvements.

7.2 Future Directions

This study provides robust evidence for the cognitive enhancing effect of both short-term and long-term environmental enrichment, in the absence of exercise, and supports a role for βNGF and neurogenesis in this process. However, further studies are necessary to fully elucidate the roles that these factors have in enrichment-induced memory improvements.

How long do the enrichment-induced cognitive improvements persist?

Rats in this study performed behavioural tasks whilst they were housed in enriched conditions. Therefore, it would be interesting to see how long the enrichment-induced memory improvements persist after environmental enrichment is removed. This would help evaluate the robustness of the environmental enrichment to elicit long-lasting memory improvements and begin to elucidate whether these improvements are mainly associated with the increase in βNGF, which is likely to be only transiently upregulated during enrichment, or structural changes that enhance synaptic plasticity and neurotransmission.

When is the most crucial time-window for environmental enrichment in order that it is protective against age-related cognitive decline?

We report that long-term continuous environmental enrichment can protect against age-related cognitive decline, but it is not clear how long-lasting these neuroprotective effects are. Therefore it would be important to house rats in enriched conditions up to 13-15 months old, at test whether there is a long-lasting neuroprotective effect by testing the rats' cognitive performance at the aged timepoint. This will help to evaluate whether environmental enrichment does create an enhanced neurogenic reserve or continuous enrichment is necessary to elicit behavioural improvements. Additionally, rats at the aged timepoint could be housed in enriched conditions for different durations in order to assess the efficacy of environmental enrichment as therapeutic measure to ameliorate pre-existing cognitive impairments.

Is βNGF the major contributing factor in the memory improvements seen with environmental enrichment?

We have reported that a continuous infusion of βNGF, at a dose directly comparable to the increase in βNGF observed following six weeks of enrichment, can induce memory improvements and increase neurogenesis and synaptic plasticity. However, to fully confirm the role that βNGF plays in enrichment-induced memory improvements, βNGF expression would need to be blocked during environmental enrichment, and memory function assessed. This could be done utilising a technique to induce βNGF autoimmunisation in rats (Micera *et al.*, 2000, Triaca *et al.*, 2005) to neutralise endogenously produced βNGF in rats housed in enriched environments. This is more cost-effective than using a continuous infusion of βNGF antibodies with Alzet® Osmotic Pumps. To control for any detrimental effects of βNGF deprivation, there would need to be a control group that were βNGF-deprived and standard housed, as well as a standard housed saline injected control group. This study could also be done using both young and aged rats to compare any age-related differences.

Is neurogenesis necessary for enrichment-induced memory improvements?

This study reports a correlative link between enrichment-induced improvement in recognition memory and hippocampal neurogenesis, yet it is not clear whether this increase in neurogenesis is necessary for the enrichment-induced improvements. Methodological issues arise with this analysis however, because many technologies that are utilised to block or ablate neurogenesis are regionally non-specific (such as methylazoxymethanol [MAM]) and can induce inflammation (such as focal-X irradiation). Lentiviral technology is a more recent technique that could overcome these confounds. However, direct infusion into the brain would involve an invasive surgery and would necessitate single housing post-surgery for the rodents. This introduces a confounding factor of social isolation which could significantly affect the efficacy of environmental enrichment as a cognitive enhancer. However, recent conditional knockout mice have been developed that could be used to specifically knockdown neurogenesis for a certain period of time, relatively non-invasively.

What role does βNGF play in the attenuation of the age-related pro-inflammatory phenotype?

It is not clear how environmental enrichment is attenuating the age-related increase in expression of pro-inflammatory markers, but it may be that βNGF is modulating this. Comparison of the expression of Trk A upon glial cells in aged standard housed and enriched housed animals may elucidate whether glia are responding to the increased βNGF. This could be done *in vivo*, quantifying expression and colocalisation in hippocampal slices, or *ex vivo* by culturing mixed glia from standard housed and enriched housed rats and subsequently analysing different expression profiles and their response to an infusion of βNGF.

Does environmental enrichment only enhance neuronal proliferation?

We report that there is an increase in the number of BrdU+/NeuN+ cells in the dentate gyrus following short-term environmental enrichment, however this has not been analysed following long-term environmental enrichment or following chronic βNGF infusion. Therefore, it is necessary to examine the proportion of proliferating cells in these studies

that are neurons, either with a mature neuronal marker such as NeuN or an immature neuronal marker such as doublecortin. Furthermore, assessing whether environmental enrichment affects the proportion of proliferating glial cells would also be of interest. This analysis could be performed using BrdU with an astrocytic marker such as GFAP or S100β and a microglia marker such as CD11b or CD68.

Bibliography

ABOU-ISMAIL, U. A. & MAHBOUB, H. D. 2011. The effects of enriching laboratoty cages using various physical structures on multiple measures of welfare in singly-housed rats. *Laboratory Animals,* 45, 145-153.

ABOULKASSIM, T., TONG, X.-K., CHUNG TSE, Y., WONG, T.-P., WOO, S. B., NEET, K. E., BRAHIMI, F., HAMEL, E. & SARAGOVI, H. U. 2011. Ligand-Dependent TrkA Activity in Brain Differentially Affects Spatial Learning and Long-Term Memory. *Molecular Pharmacology,* 80, 498-508.

ADAMS, J. P. & SWEATT, J. D. 2002. Molecular psychology: roles for the ERK MAP kinase cascade in memory. *Annual Review of Pharmacology and Toxicology,* 42, 135-63.

AKIYAMA, H., BARGER, S., BARNUM, S., BRADT, B., BAUER, J., COLE, G. M., COOPER, N. R., EIKELENBOOM, P., EMMERLING, M., FIEBICH, B. L., FINCH, C. E., FRAUTSCHY, S., GRIFFIN, W. S. T., HAMPEL, H., HULL, M., LANDRETH, G., LUE, L.-F., MRAK, R., MACKENZIE, I. R., MCGEER, P. L., O'BANION, M. K., PACHTER, J., PASINETTI, G., PLATA-SALAMAN, C., ROGERS, J., RYDEL, R., SHEN, Y., STREIT, W., STROHMEYER, R., TOOYOMA, I., VAN MUISWINKEL, F. L., VEERHUIS, R., WALKER, D., WEBSTER, S., WEGRZYNIAK, B., WENK, G. & WYSS-CORAY, T. 2000. Inflammation and Alzheimer's disease. *Neurobiology of Aging,* 21, 383-421.

ALONSO, M., VIANNA, M. R. M., DEPINO, A. M., SOUZA, T. M. E., PEREIRA, P., SZAPIRO, G., VIOLA, H., PITOSSI, F., IZQUIERDO, I. & MEDINA, J. H. 2002. BDNF-triggered events in the rat hippocampus are required for both short- and long-term memory formation. *Hippocampus,* 12, 551-560.

AMARAL, O. B., VARGAS, R. S., HANSEL, G., IZQUIERDO, I. & SOUZA, D. O. 2008. Duration of environmental enrichment influences the magnitude and persistence of its behavioral effects on mice. *Physiology & Behavior,* 93, 388-394.

AMBROGINI, P., CUPPINI, R., LATTANZI, D., CIUFFOLI, S., FRONTINI, A. & FANELLI, M. 2009. Synaptogenesis in adult-generated hippocampal granule cells is affected by behavioral experiences. *Hippocampus,* 9999, NA.

AMBROGINI, P., CUPPINI, R., LATTANZI, D., CIUFFOLI, S., FRONTINI, A. & FANELLI, M. 2010. Synaptogenesis in adult-generated hippocampal granule cells is affected by behavioral experiences. *Hippocampus,* 20, 799-810.

AMBROGINI, P., ORSINI, L., MANCINI, C., FERRI, P., CIARONI, S. & CUPPINI, R. 2004. Learning may reduce neurogenesis in adult rat dentate gyrus. *Neuroscience Letters,* 359, 13-16.

ANDERSON, B. J. 2011. Plasticity of gray matter volume: the cellular and synaptic plasticity that underlies volumetric change. *Dev Psychobiol,* 53, 456-65.

ANDERSON, B. J., RAPP, D. N., BAEK, D. H., MCCLOSKEY, D. P., COBURN-LITVAK, P. S. & ROBINSON, J. K. 2000. Exercise influences spatial learning in the radial arm maze. *Physiology & Behavior,* 70, 425-429.

ARAM V, C. 1992. Vascular effects of systemic hypertension. *The American Journal of Cardiology,* 69, 3-7.

ARANCIBIA, S., SILHOL, M., MOULIERE, F., MEFFRE, J., HOLLINGER, I., MAURICE, T. & TAPIA-ARANCIBIA, L. 2008. Protective effect of BDNF against beta-amyloid induced neurotoxicity in vitro and in vivo in rats. *Neurobiology of Disease,* 31, 316-26.

ASHBURNER, J. & FRISTON, K. 2000. Voxel-based morphometry-the methods. *Neuroimage,* 11, 805-821.

BANCHEREAU, J., BAZAN, F., BLANCHARD, D., BRIÈ, F., GALIZZI, J. P., VAN KOOTEN, C., LIU, Y. J., ROUSSET, F. & SAELAND, S. 1994. The CD40 Antigen and its Ligand. *Annual Review of Immunology,* 12, 881-926.

BANNERMAN, D. M., RAWLINS, J. N., MCHUGH, S. B., DEACON, R. M., YEE, B. K., BAST, T., ZHANG, W. N., POTHUIZEN, H. H. & FELDON, J. 2004. Regional dissociations within the hippocampus--memory and anxiety. *Neurosci Biobehav Rev,* 28, 273-83.

BARKER, G. R. I., BIRD, F., ALEXANDER, V. & WARBURTON, E. C. 2007. Recognition Memory for Objects, Place, and Temporal Order: A Disconnection Analysis of the Role of the Medial Prefrontal Cortex and Perirhinal Cortex. *The Journal of Neuroscience,* 27, 2948-2957.

BARKER, G. R. I. & WARBURTON, E. C. 2011. When Is the Hippocampus Involved in Recognition Memory? *The Journal of Neuroscience,* 31, 10721-10731.

BARKER, P. A. 1998. p75NTR: A study in contrasts. *Cell Death & Differentiation,* 5, 346-56.

BARON, J. C., CHÉTELAT, G., DESGRANGES, B., PERCHEY, G., LANDEAU, B., DE LA SAYETTE, V. & EUSTACHE, F. 2001. In Vivo Mapping of Gray Matter Loss with Voxel-Based Morphometry in Mild Alzheimer's Disease. *Neuroimage,* 14, 298-309.

BARRIENTOS, R. M., HIGGINS, E. A., BIEDENKAPP, J. C., SPRUNGER, D. B., WRIGHT-HARDESTY, K. J., WATKINS, L. R., RUDY, J. W. & MAIER, S. F. 2006. Peripheral infection and aging interact to impair hippocampal memory consolidation. *Neurobiol Aging,* 27, 723-32.

BEKINSCHTEIN, P., CAMMAROTA, M., KATCHE, C., SLIPCZUK, L., ROSSATO, J. I., GOLDIN, A., IZQUIERDO, I. & MEDINA, J. H. 2008. BDNF is essential to promote persistence of long-term memory storage. *Proceedings of the National Academy of Sciences,* 105, 2711-2716.

BENNETT, E. L., ROSENZWEIG, M. R. & DIAMOND, M. C. 1969. Rat brain: effects of environmental enrichment on wet and dry weights. *Science,* 163, 825-6.

BENNETT, J. C., MCRAE, P. C., LEVY, L. J. & FRICK, K. M. 2006. Long-term continuous, but not daily, environmental enrichment reduces spatial memory decline in aged male mice. *Neurobiology of Aging,* 85, 139-152.

BERARDI, N., BRASCHI, C., CAPSONI, S., CATTANEO, A. & MAFFEI, L. 2007. Environmental Enrichment Delays the Onset of Memory Deficits and Reduces Neuropathological Hallmarks in a Mouse Model of Alzheimer-Like Neurodegeneration. *Journal of Alzheimer's Disease,* 11, 359-370.

BHATTACHARYYA, A., WATSON, F. L., BRADLEE, T. A., POMEROY, S. L., STILES, C. D. & SEGAL, R. A. 1997. Trk Receptors Function As Rapid

Retrograde Signal Carriers in the Adult Nervous System. *The Journal of Neuroscience,* 17, 7007-7016.

BIANCHI, M., FONE, K. F. C., AZMI, N., HEIDBREDER, C. A., HAGAN, J. J. & MARSDEN, C. A. 2006. Isolation rearing induces recognition memory deficits accompanied by cytoskeletal alterations in rat hippocampus. *European Journal of Neuroscience,* 24, 2894-2902.

BINDER, E., DROSTE, S. K., OHL, F. & REUL, J. M. H. M. 2004. Regular voluntary exercise reduces anxiety-related behaviour and impulsiveness in mice. *Behavioural Brain Research,* 155, 197-206.

BINDU, B., ALLADI, P. A., MANSOORALIKHAN, B. M., SRIKUMAR, B. N., RAJU, T. R. & KUTTY, B. M. 2007. Short-term exposure to an enriched environment enhances dendritic branching but not brain-derived neurotrophic factor expression in the hippocampus of rats with ventral subicular lesions. *Neuroscience,* 144, 412-423.

BIZON, J. L., LAUTERBORN, J. C. & GALL, C. M. 1999. Subpopulations of striatal interneurons can be distinguished on the basis of neurotrophic factor expression. *Journal of Comparative Neurology,* 408, 283-98.

BLISS, T. V. & LOMO, T. 1973. Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *Journal of Physiology,* 232, 331-56.

BLÖCHL, A. & THOENEN, H. 1995. Characterization of Nerve Growth Factor (NGF) Release from Hippocampal Neurons: Evidence for a Constitutive and an Unconventional Sodium-dependent Regulated Pathway. *European Journal of Neuroscience,* 7, 1220-1228.

BLUM, S., MOORE, A. N., ADAMS, F. & DASH, P. K. 1999. A Mitogen-Activated Protein Kinase Cascade in the CA1/CA2 Subfield of the Dorsal Hippocampus Is Essential for Long-Term Spatial Memory. *The Journal of Neuroscience,* 19, 3535-3544.

BLURTON-JONES, M., KITAZAWA, M., MARTINEZ-CORIA, H., CASTELLO, N. A., MÃLLER, F.-J., LORING, J. F., YAMASAKI, T. R., POON, W. W., GREEN, K. N. & LAFERLA, F. M. 2009. Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease. *Proceedings of the National Academy of Sciences,* 106, 13594-13599.

BODNOFF, S., HUMPHREYS, A., LEHMAN, J., DIAMOND, D., ROSE, G. & MEANEY, M. 1995. Enduring effects of chronic corticosterone treatment on spatial learning, synaptic plasticity, and hippocampal neuropathology in young and mid-aged rats. *Journal of Neuroscience,* 15, 61-69.

BONNI, A., BRUNET, A., WEST, A. E., DATTA, S. R., TAKASU, M. A. & GREENBERG, M. E. 1999. Cell Survival Promoted by the Ras-MAPK Signaling Pathway by Transcription-Dependent and -Independent Mechanisms. *Science,* 286, 1358-1362.

BORDERS, A. S., GETCHELL, M. L., ETSCHEIDT, J. T., VAN ROOIJEN, N., COHEN, D. A. & GETCHELL, T. V. 2007. Macrophage depletion in the murine olfactory epithelium leads to increased neuronal death and decreased neurogenesis. *The Journal of Comparative Neurology,* 501, 206-218.

BORNEMANN, K. D., WIEDERHOLD, K.-H., PAULI, C., ERMINI, F., STALDER, M., SCHNELL, L., SOMMER, B., JUCKER, M. & STAUFENBIEL, M. 2001. A[beta]-Induced Inflammatory Processes in Microglia Cells of APP23 Transgenic Mice. *The American Journal of Pathology,* 158, 63-73.

BOYKE, J., DRIEMEYER, J., GASER, C., BUCHEL, C. & MAY, A. 2008. Training-Induced Brain Structure Changes in the Elderly. *Journal of Neuroscience,* 28, 7031-7035.

BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem,* 72, 248-54.

BRAMHAM, C. R. & MESSAOUDI, E. 2005. BDNF function in adult synaptic plasticity: The synaptic consolidation hypothesis. *Progress in Neurobiology,* 76, 99-125.

BRANCHI, I., D'ANDREA, I., FIORE, M., DI FAUSTO, V., ALOE, L. & ALLEVA, E. 2006. Early social enrichment shapes social behavior and nerve growth factor and brain-derived neurotrophic factor levels in the adult mouse brain. *Biological Psychiatry,* 60, 690-696.

BRENES, J. C., PADILLA, M. & FORNAGUERA, J. 2009. A detailed analysis of open-field habituation and behavioral and neurochemical antidepressant-like effects in postweaning enriched rats. *Behavioural Brain Research,* 197, 125-37.

BROADBENT, N. J., SQUIRE, L. R. & CLARK, R. E. 2004. Spatial memory, recognition memory, and the hippocampus. *Proceedings of the National Academy of Sciences,* 101, 14515-20.

BROOKS, A. I., CORY-SLECHTA, D. A. & FEDEROFF, H. J. 2000. Gene-experience interaction alters the cholinergic septohippocampal pathway of mice. *Proceedings of the National Academy of Sciences,* 97, 13378-13383.

BROWN, M. W. & AGGLETON, J. P. 2001. Recognition memory: What are the roles of the perirhinal cortex and hippocampus? *Nature Reviews Neuroscience,* 2, 51-61.

BRUEL-JUNGERMAN, E., DAVIS, S. & LAROCHE, S. 2007a. Brain plasticity mechanisms and memory: a party of four. *Neuroscientist,* 13, 492-505.

BRUEL-JUNGERMAN, E., LAROCHE, S. & RAMPON, C. 2005. New neurons in the dentate gyrus are involved in the expression of enhanced long-term memory following environmental enrichment. *European Journal of Neuroscience,* 21, 513-521.

BRUEL-JUNGERMAN, E., RAMPON, C. & LAROCHE, S. 2007b. Adult hippocampal neurogenesis, synaptic plasticity and memory: Facts and hypotheses. *Reviews in the Neurosciences,* 18, 93-114.

BUCKLEY, M. J. 2005. The role of the perirhinal cortex and hippocampus in learning, memory, and perception. *The Quarterly Journal of Experimental Psychology Section B: Comparative and Physiological Psychology,* 58, 246 - 268.

BUCKNER, R. L. 2004. Memory and executive function in aging and AD: multiple factors that cause decline and reserve factors that compensate. *Neuron,* 44, 195-208.

BUSCHERT, V., BOKDE, A. L. W. & HAMPEL, H. 2010. Cognitive intervention in Alzheimer disease. *Nat Rev Neurol,* 6, 508-517.

BUSSEY, T. J., MUIR, J. L. & AGGLETON, J. P. 1999. Functionally Dissociating Aspects of Event Memory: the Effects of Combined Perirhinal and Postrhinal Cortex Lesions on Object and Place Memory in the Rat. *The Journal of Neuroscience,* 19, 495-502.

BUXTON, R. B. 2005. Quantifying CBF with arterial spin labeling. *Journal of Magnetic Resonance Imaging,* 22, 723-726.

CAMERON, H. A. & MCKAY, R. D. G. 2001. Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. *The Journal of Comparative Neurology,* 435, 406-417.

CAO, L., JIAO, X., ZUZGA, D. S., LIU, Y., FONG, D. M., YOUNG, D. & DURING, M. J. 2004. VEGF links hippocampal activity with neurogenesis, learning and memory. *Nat Genet,* 36, 827-835.

CESCA, F., BALDELLI, P., VALTORTA, F. & BENFENATI, F. 2010. The synapsins: Key actors of synapse function and plasticity. *Progress in Neurobiology,* 91, 313-348.

CHAE, C.-H. & KIM, H.-T. 2009. Forced, moderate-intensity treadmill exercise suppresses apoptosis by increasing the level of NGF and stimulating phosphatidylinositol 3-kinase signaling in the hippocampus of induced aging rats. *Neurochemistry International,* 55, 208-213.

CHAWLA, M. K., GUZOWSKI, J. F., RAMIREZ-AMAYA, V., LIPA, P., HOFFMAN, K. L., MARRIOTT, L. K., WORLEY, P. F., MCNAUGHTON, B. L. & BARNES, C. A. 2005. Sparse, environmentally selective expression of Arc RNA in the upper blade of the rodent fascia dentata by brief spatial experience. *Hippocampus,* 15, 579-586.

CHEN, K. S., NISHIMURA, M. C., ARMANINI, M. P., CROWLEY, C., SPENCER, S. D. & PHILLIPS, H. S. 1997. Disruption of a Single Allele of the Nerve Growth Factor Gene Results in Atrophy of Basal Forebrain Cholinergic Neurons and Memory Deficits. *Journal of Neuroscience,* 17, 7288-7296.

CHIARETTI, A., ANTONELLI, A., RICCARDI, R., GENOVESE, O., PEZZOTTI, P., DI ROCCO, C., TORTOROLO, L. & PIEDIMONTE, G. 2008. Nerve growth factor expression correlates with severity and outcome of traumatic brain injury in children. *European Journal of Paediatric Neurology,* 12, 195-204.

CHIARETTI, A., BARONE, G., RICCARDI, R., ANTONELLI, A., PEZZOTTI, P., GENOVESE, O., TORTOROLO, L. & CONTI, G. 2009. NGF, DCX, and NSE upregulation correlates with severity and outcome of head trauma in children. *Neurology,* 72, 609-616.

CHOI, S. H., YUN, L., PARADA, L. F. & SISODIA, S. S. 2009. Regulation of hippocampal progenitor cell survival, proliferation and dendritic development by BDNF. *Molecular Neurodegeneration,* 4.

CHOURBAJI, S., BRANDWEIN, C., VOGT, M. A., DORMANN, C., HELLWEG, R. & GASS, P. 2008. Nature vs. nurture: Can enrichment rescue the behavioural phenotype of BDNF heterozygous mice? *Behavioural Brain Research,* 192, 254-258.

CLARK, R. E., ZOLA, S. M. & SQUIRE, L. R. 2000. Impaired Recognition Memory in Rats after Damage to the Hippocampus. *The Journal of Neuroscience,* 20, 8853-8860.

CLELLAND, C. D., CHOI, M., ROMBERG, C., CLEMENSON JR, G. D., FRAGNIERE, A., TYERS, P., JESSBERGER, S., SAKSIDA, L. M., BARKER, R. A., GAGE, F. H. & BUSSEY, T. J. 2009. A functional role for adult hippocampal neurogenesis in spatial pattern separation. *Science,* 325, 210-213.

COLONNA-ROMANO, G., BULATI, M., AQUINO, A., SCIALABBA, G., CANDORE, G., LIO, D., MOTTA, M., MALAGUARNERA, M. & CARUSO, C. 2003. B cells in the aged: CD27, CD5, and CD40 expression. *Mechanisms of Ageing and Development,* 124, 389-393.

CONNER, J. M., FRANKS, K. M., TITTERNESS, A. K., RUSSELL, K., MERRILL, D. A., CHRISTIE, B. R., SEJNOWSKI, T. J. & TUSZYNSKI, M. H. 2009. NGF Is Essential for Hippocampal Plasticity and Learning. *Journal of Neuroscience,* 29, 10883-10889.

CORTESE, G. P., BARRIENTOS, R. M., MAIER, S. F. & PATTERSON, S. L. 2011. Aging and a peripheral immune challenge interact to reduce mature brain-derived neurotrophic factor and activation of TrkB, PLCgamma1, and ERK in hippocampal synaptoneurosomes. *J Neurosci,* 31, 4274-9.

CRACCHIOLO, J. R., MORI, T., NAZIAN, S. J., TAN, J., POTTER, H. & ARENDASH, G. W. 2007. Enhanced cognitive activity--over and above social or physical activity--is required to protect Alzheimer's mice against cognitive impairment, reduce A[beta] deposition, and increase synaptic immunoreactivity. *Neurobiology of Learning and Memory,* 88, 277-294.

CREER, D. J., ROMBERG, C., SAKSIDA, L. M., VAN PRAAG, H. & BUSSEY, T. J. 2010. Running enhances spatial pattern separation in mice. *Proceedings of the National Academy of Sciences of the United States of America,* 107, 2367-2372.

CROSNOE, R., LEVENTHAL, T., WIRTH, R. J., PIERCE, K. M., PIANTA, R. C. & NETWORK, N. E. C. C. R. 2010. Family Socioeconomic Status and Consistent Environmental Stimulation in Early Childhood. *Child Development,* 81, 972-987.

CUESTO, G., ENRIQUEZ-BARRETO, L., CARAMÉS, C., CANTARERO, M., GASULL, X., SANDI, C., FERRÚS, A., ACEBES, Á. & MORALES, M. 2011. Phosphoinositide-3-Kinase Activation Controls Synaptogenesis and Spinogenesis in Hippocampal Neurons. *The Journal of Neuroscience,* 31, 2721-2733.

CURTIS, J. & FINKBEINER, S. 1999. Sending signals from the synapse to the nucleus: Possible roles for CaMK, Ras/ERK, and SAPK pathways in the regulation of synaptic plasticity and neuronal growth. *Journal of Neuroscience Research,* 58, 88-95.

DAS, K. P., CHAO, S. L., WHITE, L. D., HAINES, W. T., HARRY, G. J., TILSON, H. A. & BARONE, S. 2001. Differential patterns of nerve growth factor, brain-derived neurotrophic factor and neurotrophin-3 mRNA and protein levels in developing regions of rat brain. *Neuroscience,* 103, 739-761.

DEKOSKY, S. T., ABRAHAMSON, E. E., TAFFE, K. M., DIXON, C. E., KOCHANEK, P. M. & IKONOMOVIC, M. D. 2004. Effects of post-injury hypothermia and

nerve growth factor infusion on antioxidant enzyme activity in the rat: implications for clinical therapies. *Journal of Neurochemistry,* 90, 998-1004.

DEMBER, W. N. & FOWLER, H. 1959. Spontaneous alteration after free and forced trials. *Can J Psychol,* 13, 151-4.

DEN HEIJER, T., VAN DER LIJN, F., KOUDSTAAL, P. J., HOFMAN, A., VAN DER LUGT, A., KRESTIN, G. P., NIESSEN, W. J. & BRETELER, M. M. B. 2010. A 10-year follow-up of hippocampal volume on magnetic resonance imaging in early dementia and cognitive decline. *Brain,* 133, 1163-1172.

DENG, W., SAXE, M. D., GALLINA, I. S. & GAGE, F. H. 2009. Adult-Born Hippocampal Dentate Granule Cells Undergoing Maturation Modulate Learning and Memory in the Brain. *J. Neurosci.,* 29, 13532-13542.

DENNIS, W. 1939. Spontaneous alternation in rats as an indicator of the persistence of stimulus effects. *Journal of Comparative Psychology,* 28, 305-312.

DIÓGENES, M. J., COSTENLA, A. R., LOPES, L. V., JERONIMO-SANTOS, A., SOUSA, V. C., FONTINHA, B. M., RIBEIRO, J. A. & SEBASTIAO, A. M. 2011. Enhancement of LTP in Aged Rats is Dependent on Endogenous BDNF. *Neuropsychopharmacology,* 36, 1823-1836.

DOBROSSY, M. D., DRAPEAU, E., AUROUSSEAU, C., LE MOAL, M., PIAZZA, P. V. & ABROUS, D. N. 2003. Differential effects of learning on neurogenesis: learning increases or decreases the number of newly born cells depending on their birth date. *Molecular Psychiatry,* 8, 974-982.

DOLLÉ, J.-P., REZVAN, A., ALLEN, F. D., LAZAROVICI, P. & LELKES, P. I. 2005. Nerve Growth Factor-Induced Migration of Endothelial Cells. *Journal of Pharmacology and Experimental Therapeutics,* 315, 1220-1227.

DONOVAN, M. H., YAMAGUCHI, M. & EISCH, A. J. 2008. Dynamic expression of TrkB receptor protein on proliferating and maturing cells in the adult mouse dentate gyrus. *Hippocampus,* 18, 435-439.

DRAGANSKI, B., GASER, C., BUSCH, V., SCHUIERER, G., BOGDAHN, U. & MAY, A. 2004. Neuroplasticity: Changes in grey matter induced by training. *Nature,* 427, 311-312.

DRAPEAU, E., MAYO, W., AUROUSSEAU, C., LE MOAL, M., PIAZZA, P.-V. & ABROUS, D. N. 2003. Spatial memory performances of aged rats in the water maze predict levels of hippocampal neurogenesis. *Proceedings of the National Academy of Sciences of the United States of America,* 100, 14385-14390.

DRISCOLL, I., HAMILTON, D. A., PETROPOULOS, H., YEO, R. A., BROOKS, W. M., BAUMGARTNER, R. N. & SUTHERLAND, R. J. 2003. The Aging Hippocampus: Cognitive, Biochemical and Structural Findings. *Cerebral Cortex,* 13, 1344-1351.

DRISCOLL, I., HOWARD, S. R., STONE, J. C., MONFILS, M. H., TOMANEK, B., BROOKS, W. M. & SUTHERLAND, R. J. 2006. The aging hippocampus: A multi-level analysis in the rat. *Neuroscience,* 139, 1173-1185.

DUARTE, A., HAYASAKA, S., DU, A., SCHUFF, N., JAHNG, G.-H., KRAMER, J., MILLER, B. & WEINER, M. 2006. Volumetric correlates of memory and

executive function in normal elderly, mild cognitive impairment and Alzheimer's disease. *Neuroscience Letters,* 406, 60-65.

DUDEK, H., DATTA, S. R., FRANKE, T. F., BIRNBAUM, M. J., YAO, R., COOPER, G. M., SEGAL, R. A., KAPLAN, D. R. & GREENBERG, M. E. 1997. Regulation of Neuronal Survival by the Serine-Threonine Protein Kinase Akt. *Science,* 275, 661-665.

DUPRET, D., FABRE, A., DOBROSSY, M. D., PANATIER, A., RODRIGUEZ, J. J., LAMARQUE, S., LEMAIRE, V., OLIET, S. H. R., PIAZZA, P. V. & ABROUS, D. N. 2007. Spatial learning depends on both the addition and removal of new hippocampal neurons. *Plos Biology,* 5, 1683-1694.

DUVA, C. A., FLORESCO, S. B., WUNDERLICH, G. R., LAO, T. L., PINEL, J. P. & PHILLIPS, A. G. 1997. Disruption of spatial but not object-recognition memory by neurotoxic lesions of the dorsal hippocampus in rats. *Behavioral Neuroscience,* 111, 1184-96.

EHNINGER, D. & KEMPERMANN, G. 2003. Regional effects of wheel running and environmental enrichment on cell genesis and microglia proliferation in the adult murine neocortex. *Cereb Cortex,* 13, 845-51.

EHNINGER, D. & KEMPERMANN, G. 2008. Neurogenesis in the adult hippocampus. *Cell and Tissue Research,* 331, 234-250.

EICHENBAUM, H., YONELINAS, A. P. & RANGANATH, C. 2007. The Medial Temporal Lobe and Recognition Memory. *Annual Review of Neuroscience,* 30, 123-152.

ELKABES, S., PENG, L. & BLACK, I. B. 1998. Lipopolysaccharide differentially regulates microglial trk receptor and neurotrophin expression. *Journal of Neuroscience Research,* 54, 117-122.

ELLIS, S. L. H. 2009. Environmental enrichment: Practical strategies for improving feline welfare. *Journal of Feline Medicine & Surgery,* 11, 901-912.

ENGLISH, J. D. & SWEATT, J. D. 1996. Activation of p42 Mitogen-activated Protein Kinase in Hippocampal Long Term Potentiation. *Journal of Biological Chemistry,* 271, 24329-24332.

ENNACEUR, A., NEAVE, N. & AGGLETON, J. P. 1996. Neurotoxic lesions of the perirhinal cortex do not mimic the behavioural effects of fornix transection in the rat. *Behavioural Brain Research,* 80, 9-25.

ERICKSON, K. I., VOSS, M. W., PRAKASH, R. S., BASAK, C., SZABO, A., CHADDOCK, L., KIM, J. S., HEO, S., ALVES, H., WHITE, S. M., WOJCICKI, T. R., MAILEY, E., VIEIRA, V. J., MARTIN, S. A., PENCE, B. D., WOODS, J. A., MCAULEY, E. & KRAMER, A. F. 2011. Exercise training increases size of hippocampus and improves memory. *Proceedings of the National Academy of Sciences,* 108, 3017-22.

ERIKSDOTTER JONHAGEN, M., NORDBERG, A., AMBERLA, K., BACKMAN, L., EBENDAL, T., MEYERSON, B., OLSON, L., SEIGER, A., SHIGETA, M., THEODORSSON, E., VIITANEN, M., WINBLAD, B. & WAHLUND, L.-O. 1998. Intracerebroventricular Infusion of Nerve Growth Factor in Three Patients

with Alzheimer's Disease. *Dementia and Geriatric Cognitive Disorders,* 9, 246-257.

ERTEN-LYONS, D., WOLTJER, R. L., DODGE, H., NIXON, R., VOROBIK, R., CALVERT, J. F., LEAHY, M., MONTINE, T. & KAYE, J. 2009. Factors associated with resistance to dementia despite high Alzheimer disease pathology. *Neurology,* 72, 354-60.

ESPÓSITO, M. S., PIATTI, V. C., LAPLAGNE, D. A., MORGENSTERN, N. A., FERRARI, C. C., PITOSSI, F. J. & SCHINDER, A. F. 2005. Neuronal Differentiation in the Adult Hippocampus Recapitulates Embryonic Development. *The Journal of Neuroscience,* 25, 10074-10086.

FABEL, K., FABEL, K., TAM, B., KAUFER, D., BAIKER, A., SIMMONS, N., KUO, C. J. & PALMER, T. D. 2003. VEGF is necessary for exercise-induced adult hippocampal neurogenesis. *European Journal of Neuroscience,* 18, 2803-2812.

FAHNESTOCK, M., MICHALSKI, B., XU, B. & COUGHLIN, M. D. 2001. The Precursor Pro-Nerve Growth Factor Is the Predominant Form of Nerve Growth Factor in Brain and Is Increased in Alzheimer's Disease. *Molecular and Cellular Neuroscience,* 18, 210-220.

FARMER, J., ZHAO, X., VAN PRAAG, H., WODTKE, K., GAGE, F. H. & CHRISTIE, B. R. 2004. Effects of voluntary exercise on synaptic plasticity and gene expression in the dentate gyrus of adult male Sprague-Dawley rats in vivo. *Neuroscience,* 124, 71-79.

FERRARA, N., GERBER, H.-P. & LECOUTER, J. 2003. The biology of VEGF and its receptors. *Nat Med,* 9, 669-676.

FORTRESS, A. M., BUHUSI, M., HELKE, K. L. & GRANHOLM, A. C. 2011. Cholinergic Degeneration and Alterations in the TrkA and p75NTR Balance as a Result of Pro-NGF Injection into Aged Rats. *Journal of Aging Research,* 2011, 460543.

FRANK, M. G., BARRIENTOS, R. M., BIEDENKAPP, J. C., RUDY, J. W., WATKINS, L. R. & MAIER, S. F. 2006. mRNA up-regulation of MHC II and pivotal pro-inflammatory genes in normal brain aging. *Neurobiol Aging,* 27, 717-22.

FRANKLAND, P. W., O'BRIEN, C., OHNO, M., KIRKWOOD, A. & SILVA, A. J. 2001. [alpha]-CaMKII-dependent plasticity in the cortex is required for permanent memory. *Nature,* 411, 309-313.

FRATIGLIONI, L., PAILLARD-BORG, S. & WINBLAD, B. 2004. An active and socially integrated lifestyle in late life might protect against dementia. *Lancet Neurology,* 3, 343-353.

FREEMAN, S. H., KANDEL, R., CRUZ, L., ROZKALNE, A., NEWEL, K., FROSCH, M. P., HEDLEY-WHYTE, E. T., LOCASCIO, J. J., LIPSITZ, L. A. & HYMAN, B. T. 2008. Preservation of Neuronal Number Despite Age-Related Cortical Brain Atrophy in Elderly Subjects Without Alzheimer Disease. *Journal of Neuropathology and Experimental Neurology,* 67, 1205-1212.

FRENCH, S. J., HUMBY, T., HORNER, C. H., SOFRONIEW, M. V. & RATTRAY, M. 1999. Hippocampal neurotrophin and trk receptor mRNA levels are altered by local

administration of nicotine, carbachol and pilocarpine. *Molecular Brain Research,* 67, 124-36.

FRICK, K. M. & BENOIT, J. D. 2010. Use It or Lose It: Environmental Enrichment as a Means to Promote Successful Cognitive Aging. *TheScientificWorldJOURNAL,* 10, 1129-1141.

FRICK, K. M., PRICE, D. L., KOLIATSOS, V. E. & MARKOWSKA, A. L. 1997. The effects of nerve growth factor on spatial recent memory in aged rats persist after discontinuation of treatment. *J Neurosci,* 17, 2543-50.

FRIEDMAN, W. J. 2000. Neurotrophins induce death of hippocampal neurons via the p75 receptor. *Journal of Neuroscience,* 20, 6340-6346.

FRIEDMAN, W. J., OLSON, L. & PERSSON, H. 1991. Cells that Express Brain-Derived Neurotrophic Factor mRNA in the Developing Postnatal Rat Brain. *European Journal of Neuroscience,* 3, 688-697.

FRIELINGSDORF, H., SIMPSON, D. R., THAL, L. J. & PIZZO, D. P. 2007. Nerve growth factor promotes survival of new neurons in the adult hippocampus. *Neurobiology of Disease,* 26, 47-55.

FRISONI, G. B., TESTA, C., ZORZAN, A., SABATTOLI, F., BELTRAMELLO, A., SOININEN, H. & LAAKSO, M. P. 2002. Detection of grey matter loss in mild Alzheimer's disease with voxel based morphometry. *Journal of Neurology, Neurosurgery & Psychiatry,* 73, 657-664.

FULK, L. J., STOCK, H. S., LYNN, A., MARSHALL, J., WILSON, M. A. & HAND, G. A. 2004. Chronic Physical Exercise Reduces Anxiety-Like Behavior In Rats. *Int J Sports Med,* 25, 78,82.

GADIENT, R. A., CRON, K. C. & OTTEN, U. 1990. Interleukin-1 β and tumor necrosis factor-α synergistically stimulate nerve growth factor (NGF) release from cultured rat astrocytes. *Neuroscience Letters,* 117, 335-340.

GALANI, R., BERTHEL, M. C., LAZARUS, C., MAJCHRZAK, M., BARBELIVIEN, A., KELCHE, C. & CASSEL, J. C. 2007. The behavioural effects of enriched housing are not altered by serotonin depletion but enrichment alters hippocampal neurochemistry. *Neurobiology of Learning and Memory,* 88, 1-10.

GALLACHER, J., BAYER, A. & BEN-SHLOMO, Y. 2005. Commentary: Activity each day keeps dementia away—does social interaction really preserve cognitive function? *International Journal of Epidemiology,* 34, 872-873.

GAO, P., SHEN, F., GABRIEL, R. A., LAW, D., YANG, E., YANG, G.-Y., YOUNG, W. L. & SU, H. 2009. Attenuation of Brain Response to Vascular Endothelial Growth Factor-Mediated Angiogenesis and Neurogenesis in Aged Mice. *Stroke,* 40, 3596-3600.

GE, S., GOH, E. L., SAILOR, K. A., KITABATAKE, Y., MING, G. L. & SONG, H. 2006. GABA regulates synaptic integration of newly generated neurons in the adult brain. *Nature,* 439, 589-93.

GE, S., YANG, C.-H., HSU, K.-S., MING, G.-L. & SONG, H. 2007. A Critical Period for Enhanced Synaptic Plasticity in Newly Generated Neurons of the Adult Brain. *Neuron,* 54, 559-566.

GEMMA, C., FISTER, M., HUDSON, C. & BICKFORD, P. C. 2005. Improvement of memory for context by inhibition of caspase-1 in aged rats. *European Journal of Neuroscience,* 22, 1751-1756.

GIACHELLO, C. N. G., FIUMARA, F., GIACOMINI, C., CORRADI, A., MILANESE, C., GHIRARDI, M., BENFENATI, F. & MONTAROLO, P. G. 2010. MAPK/Erk-dependent phosphorylation of synapsin mediates formation of functional synapses and short-term homosynaptic plasticity. *Journal of Cell Science,* 123, 881-893.

GILBERT, P. E., KESNER, R. P. & LEE, I. 2001. Dissociating hippocampal subregions: A double dissociation between dentate gyrus and CA1. *Hippocampus,* 11, 626-636.

GOBBO, O. L. & O'MARA, S. M. 2004. Impact of enriched-environment housing on brain-derived neurotrophic factor and on cognitive performance after a transient global ischemia. *Behavioural Brain Research,* 152, 231-241.

GOBBO, O. L. & O'MARA, S. M. 2005. Exercise, but not environmental enrichment, improves learning after kainic acid-induced hippocampal neurodegeneration in association with an increase in brain-neurotrophic factor. *Behavioural Brain Research,* 159, 21-26.

GOLOSHEVSKY, A. G., SILVA, A. C., DODD, S. J. & KORETSKY, A. P. 2008. BOLD fMRI and somatosensory evoked potentials are well correlated over a broad range of frequency content of somatosensory stimulation of the rat forepaw. *Brain Research,* 1195, 67-76.

GOOD, C. D., JOHNSRUDE, I. S., ASHBURNER, J., HENSON, R. N. A., FRISTON, K. & FRACKOWIAK, R. S. J. 2001. A Voxel-Based Morphometric Study of Ageing in 465 Normal Adult Brains. *Neuroimage,* 14, 21-36.

GOODMAN, L. J., VALVERDE, J., LIM, F., GESCHWIND, M. D., FEDEROFF, H. J., GELLER, A. I. & HEFTI, F. 1996. Regulated Release and Polarized Localization of Brain-Derived Neurotrophic Factor in Hippocampal Neurons. *Molecular and Cellular Neuroscience,* 7, 222-238.

GOONEY, M., MESSAOUDI, E., MAHER, F. O., BRAMHAM, C. R. & LYNCH, M. A. 2004. BDNF-induced LTP in dentate gyrus is impaired with age: analysis of changes in cell signaling events. *Neurobiol Aging,* 25, 1323-31.

GÖRTZ, N., LEWEJOHANN, L., TOMM, M., AMBRÉE, O., KEYVANI, K., PAULUS, W. & SACHSER, N. 2008. Effects of environmental enrichment on exploration, anxiety, and memory in female TgCRND8 Alzheimer mice. *Behavioural Brain Research,* 191, 43-48.

GOSHEN, I., AVITAL, A., KREISEL, T., LICHT, T., SEGAL, M. & YIRMIYA, R. 2009. Environmental Enrichment Restores Memory Functioning in Mice with Impaired IL-1 Signaling via Reinstatement of Long-Term Potentiation and Spine Size Enlargement. *The Journal of Neuroscience,* 29, 3395-3403.

GOULD, E., BEYLIN, A., TANAPAT, P., REEVES, A. & SHORS, T. J. 1999a. Learning enhances adult neurogenesis in the hippocampal formation. *Nature Neuroscience,* 2, 260-265.

GOULD, E., REEVES, A. J., FALLAH, M., TANAPAT, P., GROSS, C. G. & FUCHS, E. 1999b. Hippocampal neurogenesis in adult Old World primates. *Proceedings of the National Academy of Sciences,* 96, 5263-5267.

GRADY, C. L. 2008. Cognitive Neuroscience of Aging. *Annals of the New York Academy of Sciences,* 1124, 127-144.

GRAY, S. W. & KLAUS, R. A. 1965. An Experimental Preschool Program for Culturally Deprived Children. *Child Development,* 36, 887-898.

GREWAL, S. S., YORK, R. D. & STORK, P. J. 1999. Extracellular-signal-regulated kinase signalling in neurons. *Current Opinion in Neurobiology,* 9, 544-53.

GRIESBACH, G. S., HOVDA, D. A. & GOMEZ-PINILLA, F. 2009. Exercise-induced improvement in cognitive performance after traumatic brain injury in rats is dependent on BDNF activation. *Brain Research,* 1288, 105-115.

GRIFFIN, É. W., BECHARA, R. G., BIRCH, A. M. & KELLY, Á. M. 2009. Exercise enhances hippocampal-dependent learning in the rat: Evidence for a BDNF-related mechanism. *Hippocampus,* 19, 973-980.

GRIFFIN, É. W., MULLALLY, S., FOLEY, C., WARMINGTON, S. A., O'MARA, S. M. & KELLY, Á. M. 2011. Aerobic exercise improves hippocampal function and increases BDNF in the serum of young adult males. *Physiology & Behavior,* In Press, Corrected Proof.

HALL, C. S. 1934. Emotional behavior in the rat. I. Defecation and urination as measures of individual differences in emotionality. *Journal of Comparative Psychology,* 18, 385-403.

HALL, J., THOMAS, K. L. & EVERITT, B. J. 2000a. Rapid and selective induction of BDNF expression in the hippocampus during contextual learning. *Nat Neurosci,* 3, 533-5.

HALL, J., THOMAS, K. L. & EVERITT, B. J. 2000b. Rapid and selective induction of BDNF expression in the hippocampus during contextual learning. *Nat Neurosci,* 3, 533-535.

HAMMOND, R. S., TULL, L. E. & STACKMAN, R. W. 2004. On the delay-dependent involvement of the hippocampus in object recognition memory. *Neurobiology of Learning and Memory,* 82, 26-34.

HARBURGER, L. L., CHINONYERE, K. N. & FRICK, K. M. 2007. Single Enrichment Variables Differentially Reduce Age-Related Memory Decline in Female Mice. *Behavioral Neuroscience,* 121, 679-688.

HARRIS, A. P., D'EATH, R. B. & HEALY, S. D. 2009. Environmental enrichment enhances spatial cognition in rats by reducing thigmotaxis (wall hugging) during testing. *Animal Behaviour,* 77, 1459-1464.

HATTORI, S., R., H., MIYAKAWA, T., YAMANAKA, H., MAENO, H., WADA, K. & KUNUGI, H. 2007. Enriched environments influence depression-related behavior in adult mice and the survival of newborn cells in their hippocampi. *Behavioural Brain Research,* 180, 69-76.

HAYAKAWA, N., KATO, H. & ARAKI, T. 2007. Age-related changes of astorocytes, oligodendrocytes and microglia in the mouse hippocampal CA1 sector. *Mechanisms of Ageing and Development,* 128, 311-316.

HEAD, D., SNYDER, A. Z., GIRTON, L. E., MORRIS, J. C. & BUCKNER, R. L. 2005. Frontal-hippocampal double dissociation between normal aging and Alzheimer's disease. *Cereb Cortex,* 15, 732-9.

HEBB, D. O. 1947. The effects of early experience on problem solving in maturity. *American Psychologist,* 2, 306-307.

HEBB, D. O. 1949. *The Organization of Behavior: A Neuropsychological Theory*, Psychology Press.

HEESE, K., FIEBICH, B. L., BAUER, J. & OTTEN, U. 1997. Nerve growth factor (NGF) expression in rat microglia is induced by adenosine A2a-receptors. *Neuroscience Letters,* 231, 83-6.

HEIN, A. M., STASKO, M. R., MATOUSEK, S. B., SCOTT-MCKEAN, J. J., MAIER, S. F., OLSCHOWKA, J. A., COSTA, A. C. S. & O'BANION, M. K. 2010. Sustained hippocampal IL-1[beta] overexpression impairs contextual and spatial memory in transgenic mice. *Brain, Behavior, and Immunity,* 24, 243-253.

HELDT, S. A., STANEK, L., CHHATWAL, J. P. & RESSLER, K. J. 2007. Hippocampus-specific deletion of BDNF in adult mice impairs spatial memory and extinction of aversive memories. *Molecular Psychiatry,* 12, 656-670.

HENNIGAN, A., O'CALLAGHAN, R. M. & KELLY, Á. M. 2007. Neurotrophins and their receptors: roles in plasticity, neurodegeneration and neuroprotection. *Biochemical Society Transactions,* 35, 424-427.

HEO, S., PRAKASH, R. S., VOSS, M. W., ERICKSON, K. I., OUYANG, C., SUTTON, B. P. & KRAMER, A. F. 2010. Resting hippocampal blood flow, spatial memory and aging. *Brain Research,* 1315, 119-27.

HOFER, M., PAGLIUSI, S. R., HOHN, A., LEIBROCK, J. & BARDE, Y. A. 1990. Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. *EMBO Journal,* 9, 2459-64.

HOLGADO-MADRUGA, M., MOSCATELLO, D. K., EMLET, D. R., DIETERICH, R. & WONG, A. J. 1997. Grb2-associated binder-1 mediates phosphatidylinositol 3-kinase activation and the promotion of cell survival by nerve growth factor. *Proceedings of the National Academy of Sciences,* 94, 12419-12424.

HOLTZMAN, D. M. & LOWENSTEIN, D. H. 1995. Selective inhibition of axon outgrowth by antibodies to NGF in a model of temporal lobe epilepsy. *Journal of Neuroscience,* 15, 7062-7070.

HONESS, P. E. & MARIN, C. M. 2006. Enrichment and agression in primates. *Neuroscience and biobehavioural reviews,* 30, 413-436.

HUANG, C.-H., CHIANG, Y.-W., LIANG, K.-C., THOMPSON, R. F. & LIU, I. Y. 2010. Extra-cellular signal-regulated kinase 1/2 (ERK1/2) activated in the hippocampal CA1 neurons is critical for retrieval of auditory trace fear memory. *Brain Research,* 1326, 143-151.

ICKES, B. R., PHAM, T. M., SANDERS, L. A., ALBECK, D. S., MOHAMMED, A. H. & GRANHOLM, A. C. 2000. Long-term environmental enrichment leads to regional increases in neurotrophin levels in rat brain. *Experimental Neurology,* 164, 45-52.

JAKUBOWSKA-DOGRU, E. & GUMUSBAS, U. 2005. Chronic intracerebroventricular NGF administration improves working memory in young adult memory deficient rats. *Neurosci Lett,* 382, 45-50.

JESSBERGER, S., CLARK, R. E., BROADBENT, N. J., CLEMENSON JR., G. D., CONSIGLIO, A., CHICHUND LIE, D., SQUIRE, L. R. & GAGE, F. H. 2009. Dentate gyrus-specific knockdown of adult neurogenesis impairs spatial and object recognition memory in adult rats. *Learning & Memory,* 16, 147-154.

JIANG, H., TIAN, S.-L., ZENG, Y., LI, L.-L. & SHI, J. 2008. TrkA pathway(s) is involved in regulation of TRPM7 expression in hippocampal neurons subjected to ischemic-reperfusion and oxygen-glucose deprivation. *Brain Research Bulletin,* 76, 124-130.

JIN, K., ZHU, Y., SUN, Y., MAO, X. O., XIE, L. & GREENBERG, D. A. 2002. Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. *Proc Natl Acad Sci U S A,* 99, 11946-50.

JOVANOVIC, J. N., BENFENATI, F., SIOW, Y. L., SIHRA, T. S., SANGHERA, J. S., PELECH, S. L., GREENGARD, P. & CZERNIK, A. J. 1996. Neurotrophins stimulate phosphorylation of synapsin I by MAP kinase and regulate synapsin I-actin interactions. *Proc Natl Acad Sci U S A,* 93, 3679-83.

JOVANOVIC, J. N., CZERNIK, A. J., FIENBERG, A. A., GREENGARD, P. & SIHRA, T. S. 2000. Synapsins as mediators of BDNF-enhanced neurotransmitter release. *Nat Neurosci,* 3, 323-329.

JUNG, M. W., WIENER, S. I. & MCNAUGHTON, B. L. 1994. Comparison of spatial firing characteristics of units in dorsal and ventral hippocampus of the rat. *J Neurosci,* 14, 7347-56.

JURIČ, D. M. & ČARMAN-KRŽAN, M. 2001. Interleukin-1β, but not IL-1α, mediates nerve growth factor secretion from rat astrocytes via type I IL-1 receptor. *International Journal of Developmental Neuroscience,* 19, 675-683.

KARAS, G. B., BURTON, E. J., ROMBOUTS, S. A. R. B., VAN SCHIJNDEL, R. A., O'BRIEN, J. T., SCHELTENS, P. H., MCKEITH, I. G., WILLIAMS, D., BALLARD, C. & BARKHOF, F. 2003. A comprehensive study of gray matter loss in patients with Alzheimer's disease using optimized voxel-based morphometry. *Neuroimage,* 18, 895-907.

KAREGE, F., SCHWALD, M. & CISSE, M. 2002. Postnatal developmental profile of brain-derived neurotrophic factor in rat brain and platelets. *Neurosci Lett,* 328, 261-4.

KATOH-SEMBA, R., SEMBA, R., TAKEUCHI, I. K. & KATO, K. 1998. Age-related changes in levels of brain-derived neurotrophic factor in selected brain regions of rats, normal mice and senescence-accelerated mice: a comparison to those of nerve growth factor and neurotrophin-3. *Neurosci Res,* 31, 227-34.

KE, H.-C., HUANG, H.-J., LIANG, K.-C. & HSIEH-LI, H. M. 2011. Selective improvement of cognitive function in adult and aged APP/PS1 transgenic mice by continuous non-shock treadmill exercise. *Brain Research,* 1403, 1-11.

KEE, N., TEIXEIRA, C. M., WANG, A. H. & FRANKLAND, P. W. 2007. Preferential incorporation of adult-generated granule cells into spatial memory networks in the dentate gyrus. *Nat Neurosci,* 10, 355-362.

KELLY, A., CONROY, S. & LYNCH, M. A. 1998a. Evidence that nerve growth factor plays a role in long-term potentiation in the rat dentate gyrus. *Neuropharmacology,* 37, 561-570.

KELLY, A., CONROY, S. & LYNCH, M. A. 1998b. Evidence that nerve growth factor plays a role in long-term potentiation in the rat dentate gyrus. *Neuropharmacology,* 37, 561-70.

KELLY, A., MAGUIRE, C. & LYNCH, M. A. 2000. Deficits in nerve growth factor release and tyrosine receptor kinase phosphorylation are associated with age-related impairment in long-term potentiation in the dentate gyrus. *Neuroscience,* 95, 359-65.

KELLY, M. E., BLAU, C. W. & KERSKENS, C. M. 2009. Bolus-tracking arterial spin labelling: theoretical and experimental results. *Physics in Medicine and Biology,* 1235.

KELSCH, W., SIM, S. & LOIS, C. 2010. Watching Synaptogenesis in the Adult Brain. *Annual Review of Neuroscience,* 33, 131-149.

KEMPERMANN, G. 2008. The neurogenic reserve hypothesis: what is adult hippocampal neurogenesis good for? *Trends in Neurosciences,* 31, 163-169.

KEMPERMANN, G. & GAGE, F. H. 1999. Experience-dependent regulation of adult hippocampal neurogenesis: Effects of long-term stimulation and stimulus withdrawal. *Hippocampus,* 9, 321-332.

KEMPERMANN, G., GAST, D. & GAGE, F. H. 2002. Neuroplasticity in old age: Sustained fivefold induction of hippocampal neurogenesis by long-term environmental enrichment. *Annals of Neurology,* 52, 135-143.

KEMPERMANN, G., KUHN, H. G. & GAGE, F. H. 1998. Experience-induced neurogenesis in the senescent dentate gyrus. *Journal of Neuroscience,* 18, 3206-3212.

KILANDER, L., NYMAN, H., BOBERG, M., HANSSON, L. & LITHELL, H. 1998. Hypertension Is Related to Cognitive Impairment : A 20-Year Follow-up of 999 Men. *Hypertension,* 31, 780-786.

KIM, S.-E., KO, I.-G., KIM, B.-K., SHIN, M.-S., CHO, S., KIM, C.-J., KIM, S.-H., BAEK, S.-S., LEE, E.-K. & JEE, Y.-S. 2010. Treadmill exercise prevents aging-induced failure of memory through an increase in neurogenesis and suppression of apoptosis in rat hippocampus. *Experimental Gerontology,* 45, 357-365.

KING, M. V., SEEMAN, P., MARSDEN, C. A. & FONE, K. C. F. 2009. Increased dopamine D2High receptors in rats reared in social isolation. *Synapse,* 63, 476-483.

KJELSTRUP, K. G., TUVNES, F. A., STEFFENACH, H. A., MURISON, R., MOSER, E. I. & MOSER, M. B. 2002. Reduced fear expression after lesions of the ventral hippocampus. *Proc Natl Acad Sci U S A,* 99, 10825-30.

KLEMPIN, F. & KEMPERMANN, G. 2007. Adult hippocampal neurogenesis and aging. *Eur Arch Psychiatry Clin Neurosci,* 257, 271-80.

KOBAYASHI, S., ÖGREN, S. O., EBENDAL, T. & OLSON, L. 1997. Intraventricular injection of NGF, but not BDNF, induces rapid motor activation that is inhibited by nicotinic receptor antagonists. *Experimental Brain Research,* 116, 315-325.

KOBILO, T., LIU, Q.-R., GANDHI, K., MUGHAL, M., SHAHAM, Y. & VAN PRAAG, H. 2011. Running is the neurogenic and neurotrophic stimulus in environmental enrichment. *Learning & Memory,* 18, 605-609.

KORNBLUM, H. I., SANKAR, R., SHIN, D. H., WASTERLAIN, C. G. & GALL, C. M. 1997. Induction of brain derived neurotrophic factor mRNA by seizures in neonatal and juvenile rat brain. *Molecular Brain Research,* 44, 219-28.

KRAMER, J., MUNGAS, D., REED, B. R., WETZEL, M. E., BURNETT, M. M., MILLER, B., WEINER, M. & CHUI, H. C. 2007. Longitudinal MRI and Cognitive Change in Healthy Elderly. *Neuropsychology,* 21, 412-418.

KUHN, H. G., DICKINSON-ANSON, H. & GAGE, F. H. 1996. Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J Neurosci,* 16, 2027-33.

KUMAR, A., RANI, A., TCHIGRANOVA, O., LEE, W.-H. & FOSTER, T. C. 2011. Influence of late-life exposure to environmental enrichment or exercise on hippocampal function and CA1 senescent physiology. *Neurobiology of Aging,* In Press, Corrected Proof.

KUMARAN, D., SUMMERFIELD, J. J., HASSABIS, D. & MAGUIRE, E. A. 2009. Tracking the emergence of conceptual knowledge during human decision making. *Neuron,* 63, 889-901.

KWON, S. E. & CHAPMAN, E. R. 2011. Synaptophysin regulates the kinetics of synaptic vesicle endocytosis in central neurons. *Neuron,* 70, 847-54.

LALONDE, R. 2002. The neurobiological basis of spontaneous alternation. *Neuroscience and Biobehavioural Reviews,* 26, 91-104.

LAMBERT, T. J., FERNANDEZ, S. M. & FRICK, K. M. 2005. Different types of environmental enrichment have discrepant effects on spatial memory and synaptophysin levels in female mice. *Neurobiology of Learning and Memory,* 83, 206-216.

LARKIN, A. E., FAHEY, B., GOBBO, O., CALLAGHAN, C. K., CAHILL, E., O'MARA, S. M. & KELLY, Á. M. 2008. Blockade of NMDA receptors pre-training, but not post-training, impairs object displacement learning in the rat. *Brain Research,* 1199, 126-132.

LAUNER, L. J. 2002. Demonstrating the case that AD is a vascular disease: epidemiologic evidence. *Ageing Research Reviews,* 1, 61-77.

LAUNER, L. J., MASAKI, K., PETROVITCH, H., FOLEY, D. & HAVLIK, R. J. 1995. The association between midlife blood pressure levels and late-life cognitive function. The Honolulu-Asia Aging Study. *JAMA,* 274, 1846-51.

LAUNER, L. J., ROSS, G. W., PETROVITCH, H., MASAKI, K., FOLEY, D., WHITE, L. R. & HAVLIK, R. J. 2002. Midlife blood pressure and dementia: the Honolulu-Asia aging study[small star, filled]. *Neurobiology of Aging,* 21, 49-55.

LAUTERBORN, J. C., TRAN, T. M., ISACKSON, P. J. & GALL, C. M. 1993. Nerve growth factor mRNA is expressed by GABAergic neurons in rat hippocampus. *Neuroreport,* 5, 273-6.

LAVIOLA, G., HANNAN, A. J., MACRÌ, S., SOLINAS, M. & JABER, M. 2008. Effects of enriched environment on animal models of neurodegenerative diseases and psychiatric disorders. *Neurobiology of Disease,* 31, 159-168.

LEAL-GALICIA, P., CASTAÑEDA-BUENO, M., QUIROZ-BAEZ, R. & ARIAS, C. 2008. Long-term exposure to environmental enrichment since youth prevents recognition memory decline and increases synaptic plasticity markers in aging. *Neurobiology of Learning and Memory,* 90, 511-518.

LEE, K. S., SCHOTTLER, F., OLIVER, M. & LYNCH, G. 1980. Brief bursts of high-frequency stimulation produce two types of structural change in rat hippocampus. *Journal of Neurophysiology,* 44, 247-258.

LEE, R., KERMANI, P., TENG, K. K. & HEMPSTEAD, B. L. 2001. Regulation of Cell Survival by Secreted Proneurotrophins. *Science,* 294, 1945-1948.

LETENNEUR, L., GILLERON, V., COMMENGES, D., HELMER, C., ORGOGOZO, J. M. & DARTIGUES, J. F. 1999. Are sex and educational level independent predictors of dementia and Alzheimer's disease? Incidence data from the PAQUID project. *J Neurol Neurosurg Psychiatry,* 66, 177-83.

LETIEMBRE, M., HAO, W., LIU, Y., WALTER, S., MIHALJEVIC, I., RIVEST, S., HARTMANN, T. & FASSBENDER, K. 2007. Innate immune receptor expression in normal brain aging. *Neuroscience,* 146, 248-254.

LEVI-MONTALCINI, R. & HAMBURGER, V. 1951. Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. *Journal of Experimental Zoology,* 116, 321-61.

LI, Y., LUIKART, B., BIRNBAUM, S., CHEN, J., KWON, C.-H., KERNIE, S. G., BASSEL-DUBY, R. & PARADA, L. F. 2009. TrkB Regulates Hippocampal Neurogenesis and Governs Sensitivity to Antidepressive Treatment. *Neuron,* 59, 399-412.

LICHT, T., GOSHEN, I., AVITAL, A., KREISEL, T., ZUBEDAT, S., EAVRI, R., SEGAL, M., YIRMIYA, R. & KESHET, E. 2011. Reversible modulations of neuronal plasticity by VEGF. *Proceedings of the National Academy of Sciences,* 108, 5081-5086.

LINDEFORS, N., ERNFORS, P., FALKENBERG, T. & PERSSON, H. 1992. Septal cholinergic afferents regulate expression of brain-derived neurotrophic factor and beta-nerve growth factor mRNA in rat hippocampus. *Experimental Brain Research,* 88, 78-90.

LIU, H.-L., ZHAO, G., CAI, K., ZHAO, H.-H. & SHI, L.-D. 2011. Treadmill exercise prevents decline in spatial learning and memory in APP/PS1 transgenic mice through improvement of hippocampal long-term potentiation. *Behavioural Brain Research,* 218, 308-314.

LOEB, D. M., MARAGOS, J., MARTIN-ZANCA, D., CHAO, M. V., PARADA, L. F. & GREENE, L. A. 1991. The trk proto-oncogene rescues NGF responsiveness in mutant NGF-nonresponsive PC12 cell lines. *Cell,* 66, 961-6.

LOHMANN, S. M., UEDA, T. & GREENGARD, P. 1978. Ontogeny of synaptic phosphoproteins in brain. *Proc Natl Acad Sci U S A,* 75, 4037-41.

LOMMATZSCH, M., ZINGLER, D., SCHUHBAECK, K., SCHLOETCKE, K., ZINGLER, C., SCHUFF-WERNER, P. & VIRCHOW, J. C. 2005a. The impact of age, weight and gender on BDNF levels in human platelets and plasma. *Neurobiology of Aging,* 26, 115-23.

LOMMATZSCH, M., ZINGLER, D., SCHUHBAECK, K., SCHLOETCKE, K., ZINGLER, C., SCHUFF-WERNER, P. & VIRCHOW, J. C. 2005b. The impact of age, weight and gender on BDNF levels in human platelets and plasma. *Neurobiol Aging,* 26, 115-23.

LU, B. & CHOW, A. 1999. Neurotrophins and hippocampal synaptic transmission and plasticity. *Journal of Neuroscience Research,* 58, 76-87.

LYNCH, M. A. 2010. Age-related neuroinflammatory changes negatively impact on neuronal function. *Frontiers in Aging Neuroscience,* 1, 1-8.

LYNCH, M. A., VOSS, K. L., RODRIGUEZ, J. & BLISS, T. V. P. 1994. Increase in synaptic vesicle proteins accompanies long-term potentiation in the dentate gyrus. *Neuroscience,* 60, 1-5.

MAGUIRE, E. A., GADIAN, D. G., JOHNSRUDE, I. S., GOOD, C. D., ASHBURNER, J., FRACKOWIAK, R. S. J. & FRITH, C. D. 2000. Navigation-related structural change in the hippocampi of taxi drivers. *Proceedings of the National Academy of Sciences of the United States of America,* 97, 4398-4403.

MAHER, F. O., MARTIN, D. S. D. & LYNCH, M. A. 2004. Increased IL-1[beta] in cortex of aged rats is accompanied by downregulation of ERK and PI-3 kinase. *Neurobiology of Aging,* 25, 795-806.

MAHNCKE, H. W., CONNOR, B. B., APPELMAN, J., AHSANUDDIN, O. N., HARDY, J. L., WOOD, R. A., JOYCE, N. M., BONISKE, T., ATKINS, S. M. & MERZENICH, M. M. 2006. Memory enhancement in healthy older adults using a brain plasticity-based training program: A randomized, controlled study. *Proceedings of the National Academy of Sciences,* 103, 12523-12528.

MANDOLESI, L., DE BARTOLO, P., FOTI, F., GELFO, F., FEDERICO, F., LEGGIO, M. G. & PETROSINI, L. 2008. Environmental enrichment provides a cognitive reserve to be spent in the case of brain lesion. *Journal of Alzheimers Disease,* 15, 11-28.

MARRONE, D. F. 2007. Ultrastructural plasticity associated with hippocampal-dependent learning: A meta-analysis. *Neurobiology of Learning and Memory,* 87, 361-371.

MARRONE, D. F., ADAMS, A. A. & SATVAT, E. 2011. Increased pattern separation in the aged fascia dentata. *Neurobiology of Aging,* 32, 2317.e23-2317.e32.

MARTIN, M., CLARE, L., ALTGASSEN, A. M., CAMERON, M. H. & ZEHNDER, F. 2011. Cognition-based interventions for healthy older people and people with mild cognitive impairment. *Cochrane Database of Systematic Reviews,* 1.

MARTIN, S. J. & MORRIS, R. G. M. 2002. New life in an old idea: The synaptic plasticity and memory hypothesis revisited. *Hippocampus,* 12, 609-636.

MARTÍNEZ-SERRANO, A. & BJÖRKLUND, A. 1998. Ex vivo nerve growth factor gene transfer to the basal forebrain in presymptomatic middle-aged rats prevents the development of cholinergic neuron atrophy and cognitive impairment during aging. *Proceedings of the National Academy of Sciences,* 95, 1858-1863.

MARTÍNEZ-SERRANO, A., FISCHER, W. & BJÖRKLUND, A. 1995. Reversal of age-dependent cognitive impairments and cholinergic neuron atrophy by NGF-secreting neural progenitors grafted to the basal forebrain. *Neuron,* 15, 473-484.

MASLIAH, E., TERRY, R. D., DETERESA, R. M. & HANSEN, L. A. 1989. Immunohistochemical quantification of the synapse-related protein synaptophysin in Alzheimer disease. *Neuroscience Letters,* 103, 234-239.

MAZZONI, I. E., SAïD, F. A., ALOYZ, R., MILLER, F. D. & KAPLAN, D. 1999. Ras Regulates Sympathetic Neuron Survival by Suppressing the p53-Mediated Cell Death Pathway. *The Journal of Neuroscience,* 19, 9716-9727.

MCAULIFFE, M. J., LALONDE, F. M., MCGARRY, D., GANDLER, W., CSAKY, K. & TRUS, B. L. 2001. Medical Image Processing, Analysis & Visualization In Clinical Research. *IEEE Computer-based Medical Systems.* CBMS.

MCCORMICK, C. M., THOMAS, C. M., SHERIDAN, C. S., NIXON, F., FLYNN, J. A. & MATHEWS, I. Z. 2011. Social instability stress in adolescent male rats alters hippocampal neurogenesis and produces deficits in spatial location memory in adulthood. *Hippocampus,* n/a-n/a.

MCDONALD, H. Y. & WOJTOWICZ, J. M. 2005. Dynamics of neurogenesis in the dentate gyrus of adult rats. *Neuroscience Letters,* 385, 70-75.

MCGAURAN, A.-M. T., MOORE, J. B., MADSEN, D., BARRY, D., O'DEA, S., MAHON, B. P. & COMMINS, S. 2008. A possible role for protein synthesis, extracellular signal-regulated kinase, and brain-derived neurotrophic factor in long-term spatial memory retention in the water maze. *Behavioral Neuroscience,* 122, 805-815.

MCMAHON, H. T., BOLSHAKOV, V. Y., JANZ, R., HAMMER, R. E., SIEGELBAUM, S. A. & SÜDHOF, T. C. 1996. Synaptophysin, a major synaptic vesicle protein, is not essential for neurotransmitter release. *Proceedings of the National Academy of Sciences,* 93, 4760-4764.

MESHI, D., DREW, M. R., SAXE, M., ANSORGE, M. S., DAVID, D., SANTARELLI, L., MALAPANI, C., MOORE, H. & HEN, R. 2006. Hippocampal neurogenesis is not required for behavioral effects of environmental enrichment. *Nature Neuroscience,* 9, 729-731.

MESHUL, C. K. & HOPKINS, W. F. 1990. Presynaptic ultrastructural correlates of long-term potentiation in the CA1 subfield of the hippocampus. *Brain Research,* 514, 310-319.

MICERA, A., PROPERZI, F., TRIACA, V. & ALOE, L. 2000. Nerve growth factor antibody exacerbates neuropathological signs of experimental allergic encephalomyelitis in adult Lewis rats. *Journal of Neuroimmunology,* 104, 116-123.

MIROCHNIC, S., WOLF, S., STAUFENBIEL, M. & KEMPERMANN, G. 2009. Age effects on the regulation of adult hippocampal neurogenesis by physical activity and environmental enrichment in the APP23 mouse model of Alzheimer disease. *Hippocampus,* 19, 1008-1018.

MIYAMOTO, E. 2006. Molecular Mechanism of Neuronal Plasticity: Induction and Maintenance of Long-Term Potentiation in the Hippocampus. *Journal of Pharmacological Sciences,* 100, 433-442.

MIZUNO, K. & GIESE, K. P. 2005. Hippocampus-Dependent Memory Formation: Do Memory Type-Specific Mechanisms Exist? *Journal of Pharmacological Sciences,* 98, 191-197.

MOFFAT, S. D., ZONDERMAN, A. B. & RESNICK, S. M. 2001. Age differences in spatial memory in a virtual environment navigation task. *Neurobiology of Aging,* 22, 787-796.

MONGIAT, L. A. & SCHINDER, A. F. 2011. Adult neurogenesis and the plasticity of the dentate gyrus network. *European Journal of Neuroscience,* 33, 1055-1061.

MONOPOLI, M. P., RAGHNAILL, M. N., LOSCHER, J. S., O'SULLIVAN, N. C., PANGALOS, M. N., RING, R. H., VON SCHACK, D., DUNN, M. J., REGAN, C. M., PENNINGTON, S. & MURPHY, K. J. 2011. Temporal proteomic profile of memory consolidation in the rat hippocampal dentate gyrus. *PROTEOMICS,* 11, 4189-4201.

MORA, F., SEGOVIA, G. & DEL ARCO, A. 2007. Aging, plasticity and environmental enrichment: Structural changes and neurotransmitter dynamics in several areas of the brain. *Brain Research Reviews,* 55, 78-88.

MORRIS, R. 1984. Developments of a water-maze procedure for studying spatial learning in the rat. *Journal of Neuroscience Methods,* 11, 47-60.

MOSER, E., MOSER, M. B. & ANDERSEN, P. 1993. Spatial learning impairment parallels the magnitude of dorsal hippocampal lesions, but is hardly present following ventral lesions. *Journal of Neuroscience,* 13, 3916-25.

MOSER, K. V., REINDL, M., BLASIG, I. & HUMPEL, C. 2004. Brain capillary endothelial cells proliferate in response to NGF, express NGF receptors and secrete NGF after inflammation. *Brain Research,* 1017, 53-60.

MUFSON, E. J., IKONOMOVIC, M. D., STYREN, S. D., COUNTS, S. E., WUU, J., LEURGANS, S., BENNETT, D. A., COCHRAN, E. J. & DEKOSKY, S. T. 2003. Preservation of Brain Nerve Growth Factor in Mild Cognitive Impairment and Alzheimer Disease. *Arch Neurol,* 60, 1143-1148.

MULLANY, P. & LYNCH, M. A. 1997. Changes in protein synthesis and synthesis of the synaptic vesicle protein, synaptophysin, in entorhinal cortex following induction of long-term potentiation in dentate gyrus: an age-related study in the rat. *Neuropharmacology,* 36, 973-80.

MUMBY, D. G. 2001. Perspectives on object-recognition memory following hippocampal damage: lessons from studies in rats. *Behavioural Brain Research,* 127, 159-181.

NABER, P. A., CABALLERO-BLEDA, M., JORRITSMA-BYHAM, B. & WITTER, M. P. 1997. Parallel input to the hippocampal memory system through peri- and postrhinal cortices. *Neuroreport,* 8, 2617-21.

NAGAHARA, A. H., MERRILL, D. A., COPPOLA, G., TSUKADA, S., SCHROEDER, B. E., SHAKED, G. M., WANG, L., BLESCH, A., KIM, A., CONNER, J. M., ROCKENSTEIN, E., CHAO, M. V., KOO, E. H., GESCHWIND, D., MASLIAH, E., CHIBA, A. A. & TUSZYNSKI, M. H. 2009. Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. *Nature Medicine,* 15, 331-337.

NANDA, S. A. & MACK, K. J. 2000. Seizures and sensory stimulation result in different patterns of brain derived neurotrophic factor protein expression in the barrel cortex and hippocampus. *Molecular Brain Research,* 78, 1-14.

NEMANIC, S., ALVARADO, M. C. & BACHEVALIER, J. 2004. The hippocampal/parahippocampal regions and recognition memory: insights from visual paired comparison versus object-delayed nonmatching in monkeys. *Journal of Neuroscience,* 24, 2013-26.

NEUMANN, H., MISGELD, T., MATSUMURO, K. & WEKERLE, H. 1998. Neurotrophins inhibit major histocompatibility class II inducibility of microglia: Involvement of the p75 neurotrophin receptor. *Proceedings of the National Academy of Sciences,* 95, 5779-5784.

NICOLLE, M. M., GALLAGHER, M. & MCKINNEY, M. 1999. No loss of synaptic proteins in the hippocampus of aged, behaviorally impaired rats. *Neurobiol Aging,* 20, 343-8.

NIEWIADOMSKA, G., BAKSALERSKA-PAZERA, M., GASIOROWSKA, A. & MIETELSKA, A. 2006. Nerve Growth Factor Differentially Affects Spatial and Recognition Memory in Aged Rats. *Neurochemical Research,* 31, 1481-1490.

NILSSON, M., PERFILIEVA, E., JOHANSSON, U., ORWAR, O. & ERIKSSON, P. S. 1999. Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory. *Journal of Neurobiology,* 39, 569-578.

NISHIMURA, A., UEDA, S., TAKEUCHI, Y., SAWADA, T. & KAWATA, M. 1995. Age-related decrease of serotonergic fibres and S-100 beta immunoreactivity in the rat dentate gyrus. *Neuroreport,* 6, 1445-8.

NITHIANANTHARAJAH, J., BARKUS, C., MURPHY, M. & HANNAN, A. J. 2008. Gene-environment interactions modulating cognitive function and molecular correlates of synaptic plasticity in Huntington's disease transgenic mice. *Neurobiology of Disease,* 29, 490-504.

NITHIANANTHARAJAH, J. & HANNAN, A. J. 2009. The neurobiology of brain and cognitive reserve: Mental and physical activity as modulators of brain disorders. *Progress in Neurobiology,* 89, 369-382.

NITHIANANTHARAJAH, J. & HANNAN, A. J. 2011. Mechanisms mediating brain and cognitive reserve: Experience-dependent neuroprotection and functional compensation in animal models of neurodegenerative diseases. *Progress in Neuro-Psychopharmacology and Biological Psychiatry,* 35, 331-339.

NOBILI, F., RODRIGUEZ, G., MARENCO, S., DE CARLI, F., GAMBARO, M., CASTELLO, C., PONTREMOLI, R. & ROSADINI, G. 1993. Regional cerebral blood flow in chronic hypertension. A correlative study. *Stroke,* 24, 1148-1153.

O'BRIEN, J. L., O'KEEFE, K. M., LAVIOLETTE, P. S., DELUCA, A. N., BLACKER, D., DICKERSON, B. C. & SPERLING, R. A. 2010. Longitudinal fMRI in elderly reveals loss of hippocampal activation with clinical decline. *Neurology,* 74, 1969-76.

O'CALLAGHAN, R. M., GRIFFIN, É. W. & KELLY, Á. M. 2009. Long-term treadmill exposure protects against age-related neurodegenerative change in the rat hippocampus. *Hippocampus,* 19, 1019-1029.

O'CALLAGHAN, R. M., OHLE, R. & KELLY, Á. M. 2007. The effects of forced exercise on hippocampal plasticity in the rat: A comparison of LTP, spatial- and non-spatial learning. *Behavioural Brain Research,* 176, 362-366.

OLSON, A. K., EADIE, B. D., ERNST, C. & CHRISTIE, B. R. 2006. Environmental enrichment and voluntary exercise massively increase neurogenesis in the adult hippocampus via dissociable pathways. *Hippocampus,* 16, 250-260.

PAGANI, E., BIZZI, A., DI SALLE, F., DE STEFANO, N. & FILIPPI, M. 2008. Basic concepts of advanced MRI techniques. *Neurological Sciences,* 29, 290-295.

PALMER, T. D., WILLHOITE, A. R. & GAGE, F. H. 2000. Vascular niche for adult hippocampal neurogenesis. *The Journal of Comparative Neurology,* 425, 479-494.

PANG, T. Y. C., STAM, N. C., NITHIANANTHARAJAH, J., HOWARD, M. L. & HANNAN, A. J. 2006. Differential effects of voluntary physical exercise on behavioral and brain-derived neurotrophic factor expression deficits in huntington's disease transgenic mice. *Neuroscience,* 141, 569-584.

PARK, H.-J., KIM, M. N., KIM, J.-G., BAE, Y.-H., BAE, M.-K., WEE, H.-J., KIM, T.-W., KIM, B.-S., KIM, J.-B., BAE, S.-K. & YOON, S. 2007. Up-regulation of VEGF expression by NGF that enhances reparative angiogenesis during thymic regeneration in adult rat. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research,* 1773, 1462-1472.

PATAPOUTIAN, A. & REICHARDT, L. F. 2001. Trk receptors: mediators of neurotrophin action. *Curr Opin Neurobiol,* 11, 272-80.

PATEL, M. N. & MCNAMARA, J. O. 1995. Selective enhancement of axonal branching of cultured dentate gyrus neurons by neurotrophic factors. *Neuroscience,* 69, 763-770.

PAUL, C.-M., MAGDA, G. & ABEL, S. 2009. Spatial memory: Theoretical basis and comparative review on experimental methods in rodents. *Behavioural Brain Research,* 203, 151-164.

PAXINOS, G. & WATSON, C. 1998. *The Rat Brain in Stereotaxic Coordinates,* London, Academic Press.

PELLOW, S., CHOPIN, P., FILE, S. E. & BRILEY, M. 1985. Validation of open : closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *Journal of Neuroscience Methods,* 14, 149-167.

PERDAHL, E., ADOLFSSON, R., ALAFUZOFF, I., ALBERT, K. A., NESTLER, E. J., GREENGARD, P. & WINBLAD, B. 1984. Synapsin I (protein I) in different brain regions in senile dementia of Alzheimer type and in multiinfarct dementia. *Journal of Neural Transmission,* 60, 133-141.

PEREIRA, A. C., HUDDLESTON, D. E., BRICKMAN, A. M., SOSUNOV, A. A., HEN, R., MCKHANN, G. M., SLOAN, R., GAGE, F. H., BROWN, T. R. & SMALL, S. A. 2007. An *in vivo* correlate of exercise-induced neurogenesis in the adult dentate gyrus. *Proceedings of the National Academy of Sciences of the United States of America,* 104, 5638-5643.

PETROSINI, L., DE BARTOLO, P., FOTI, F., GELFO, F., CUTULI, D., LEGGIO, M. G. & MANDOLESI, L. 2009. On whether the environmental enrichment may provide cognitive and brain reserves. *Brain Research Reviews,* 61, 221-239.

PHAM, T. M., ICKES, B., ALBECK, D., SODERSTROM, S., GRANHOLM, A. C. & MOHAMMED, A. H. 1999a. Changes in brain nerve growth factor levels and nerve growth factor receptors in rats exposed to environmental enrichment for one year. *Neuroscience,* 94, 279-286.

PHAM, T. M., SODERSTROM, S., WINBLAD, B. & MOHAMMED, A. H. 1999b. Effects of environmental enrichment on cognitive function and hippocampal NGF in the non-handled rats. *Behavioural Brain Research,* 103, 63-70.

PHAM, T. M., WINBLAD, B., GRANHOLM, A. C. & MOHAMMED, A. H. 2002. Environmental influences on brain neurotrophins in rats. *Pharmacology Biochemistry and Behavior,* 73, 167-175.

PISU, M. G., DORE, R., MOSTALLINO, M. C., LOI, M., PIBIRI, F., MAMELI, R., CADEDDU, R., SECCI, P. P. & SERRA, M. 2011. Down-regulation of hippocampal BDNF and Arc associated with improvement in aversive spatial memory performance in socially isolated rats. *Behavioural Brain Research,* 222, 73-80.

PLASSMAN, B. L., LANGA, K. M., FISHER, G. G., HEERINGA, S. G., WEIR, D. R., OFSTEDAL, M. B., BURKE, J. R., HURD, M. D., POTTER, G. G., RODGERS, W. L., STEFFENS, D. C., MCARDLE, J. J., WILLIS, R. J. & WALLACE, R. B. 2008. Prevalence of Cognitive Impairment without Dementia in the United States. *Annals of Internal Medicine,* 148, 427-434.

PLOUGHMAN, M. 2008. Exercise is brain food: The effects of physical activity on cognitive function. *Developmental Neurorehabilitation,* 11, 236-240.

POLA, R., APRAHAMIAN, T. R., BOSCH-MARCÉ, M., CURRY, C., GAETANI, E., FLEX, A., SMITH, R. C., ISNER, J. M. & LOSORDO, D. W. 2004. Age-dependent VEGF expression and intraneural neovascularization during regeneration of peripheral nerves. *Neurobiology of Aging,* 25, 1361-1368.

POO, M.-M. 2001. Neurotrophins as synaptic modulators. *Nature Reviews Neuroscience,* 2, 24-32.

POTHUIZEN, H. H., ZHANG, W. N., JONGEN-RELO, A. L., FELDON, J. & YEE, B. K. 2004. Dissociation of function between the dorsal and the ventral hippocampus in spatial learning abilities of the rat: a within-subject, within-task comparison of reference and working spatial memory. *Eur J Neurosci,* 19, 705-12.

POUCET, B. 1989. Object exploration, habituation, and response to a spatial change in rats following septal or medial frontal cortical damage. *Behavioral Neuroscience,* 103, 1009-16.

PRUSKY, G. T., DOUGLAS, R. M., NELSON, L., SHABANPOOR, A. & SUTHERLAND, R. J. 2004. Visual memory task for rats reveals an essential role for hippocampus and perirhinal cortex. *Proceedings of the National Academy of Sciences of the United States of America,* 101, 5064-8.

QUALLO, M. M., PRICE, C. J., UENO, K., ASAMIZUYA, T., CHENG, K., LEMON, R. N. & IRIKI, A. 2009. Gray and white matter changes associated with tool-use learning in macaque monkeys. *Proceedings of the National Academy of Sciences,* 106, 18379-18384.

RAMOS, A. 2008. Animal models of anxiety: do I need multiple tests? *Trends in Pharmacological Sciences,* 29, 493-8.

RESNICK, S. M., PHAM, D. L., KRAUT, M. A., ZONDERMAN, A. B. & DAVATZIKOS, C. 2003. Longitudinal Magnetic Resonance Imaging Studies of Older Adults: A Shrinking Brain. *The Journal of Neuroscience,* 23, 3295-3301.

REX, C. S., LAUTERBORN, J. C., LIN, C.-Y., KRAMÁR, E. A., ROGERS, G. A., GALL, C. M. & LYNCH, G. 2006. Restoration of Long-Term Potentiation in Middle-Aged Hippocampus After Induction of Brain-Derived Neurotrophic Factor. *Journal of Neurophysiology,* 96, 677-685.

RICHTER-LEVIN, G., CANEVARI, L. & BLISS, T. V. 1998. Spatial training and high-frequency stimulation engage a common pathway to enhance glutamate release in the hippocampus. *Learning & Memory,* 4, 445-450.

RIVARD, A., BERTHOU-SOULIE, L., PRINCIPE, N., KEARNEY, M., CURRY, C., BRANELLEC, D., SEMENZA, G. L. & ISNER, J. M. 2000. Age-dependent Defect in Vascular Endothelial Growth Factor Expression Is Associated with Reduced Hypoxia-inducible Factor 1 Activity. *Journal of Biological Chemistry,* 275, 29643-29647.

RIVARD, A., FABRE, J.-E., SILVER, M., CHEN, D., MUROHARA, T., KEARNEY, M., MAGNER, M., ASAHARA, T. & ISNER, J. M. 1999. Age-Dependent Impairment of Angiogenesis. *Circulation,* 99, 111-120.

RIZVI, M., PATHAK, D., FREEDMAN, J. E. & CHAKRABARTI, S. 2008. CD40–CD40 ligand interactions in oxidative stress, inflammation and vascular disease. *Trends in Molecular Medicine,* 14, 530-538.

ROBINS, J. G. & WAITT, C. D. 2010. Improving the Welfare of Captive Macaques (Macaca sp.) Through the Use of Water as Enrichment. *Journal of Applied Animal Welfare Science,* 14, 75-84.

RODGERS, R. J. & DALVI, A. 1997. Anxiety, defence and the elevated plus-maze. *Neuroscience and Biobehavioural Reviews,* 21, 801-10.

ROLA, R., MIZUMATSU, S., OTSUKA, S., MORHARDT, D. R., NOBLE-HAEUSSLEIN, L. J., FISHMAN, K., POTTS, M. B. & FIKE, J. R. 2006. Alterations in hippocampal neurogenesis following traumatic brain injury in mice. *Experimental Neurology,* 202, 189-199.

ROSENZWEIG, E. S. & BARNES, C. A. 2003. Impact of aging on hippocampal function: plasticity, network dynamics, and cognition. *Progress in Neurobiology,* 69, 143-179.

ROSENZWEIG, M. R. & BENNETT, E. L. 1969. Effects of differential environments on brain weights and enzyme activities in gerbils, rats, and mice. *Dev Psychobiol,* 2, 87-95.

ROSENZWEIG, M. R. & BENNETT, E. L. 1996. Psychobiology of plasticity: Effects of training and experience on brain and behavior. *Behavioural Brain Research,* 78, 57-65.

ROSENZWEIG, M. R., BENNETT, E. L., DIAMOND, M. C., WU, S. Y., SLAGLE, R. W. & SAFFRAN, E. 1969. Influences of environmental complexity and visual stimulation on development of occipital cortex in rat. *Brain Res,* 14, 427-45.

ROSENZWEIG, M. R., BENNETT, E. L., HEBERT, M. & MORIMOTO, H. 1978. Social grouping cannot account for cerebral effects of enriched environments. *Brain Research,* 153, 563-76.

ROSSI, C., ANGELUCCI, A., COSTANTIN, L., BRASCHI, C., MAZZANTINI, M., BABBINI, F., FABBRI, M. E., TESSAROLLO, L., MAFFEI, L., BERARDI, N. & CALEO, M. 2006. Brain-derived neurotrophic factor (BDNF) is required for the enhancement of hippocampal neurogenesis following environmental enrichment. *European Journal of Neuroscience,* 24, 1850-1856.

ROUX, P. P. & BARKER, P. A. 2002. Neurotrophin signaling through the p75 neurotrophin receptor. *Progress in Neurobiology,* 67, 203-233.

SABBATINI, M., BARILI, P., BRONZETTI, E., ZACCHEO, D. & AMENTA, F. 1999. Age-related changes of glial fibrillary acidic protein immunoreactive astrocytes in the rat cerebellar cortex. *Mechanisms of Ageing and Development,* 108, 165-172.

SAHAY, A., SCOBIE, K. N., HILL, A. S., O'CARROLL, C. M., KHEIRBEK, M. A., BURGHARDT, N. S., FENTON, A. A., DRANOVSKY, A. & HEN, R. 2011. Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. *Nature,* 472, 466-470.

SALEHI, A., DELCROIX, J. D. & SWAAB, D. F. 2004. Alzheimer's disease and NGF signaling. *Journal of Neural Transmission,* 111, 323-345.

SATOH, Y., ENDO, S., IKEDA, T., YAMADA, K., ITO, M., KUROKI, M., HIRAMOTO, T., IMAMURA, O., KOBAYASHI, Y., WATANABE, Y., ITOHARA, S. & TAKISHIMA, K. 2007. Extracellular Signal-Regulated Kinase 2 (ERK2) Knockdown Mice Show Deficits in Long-Term Memory; ERK2 Has a Specific Function in Learning and Memory. *The Journal of Neuroscience,* 27, 10765-10776.

SAXE, M. D., BATTAGLIA, F., WANG, J.-W., MALLERET, G., DAVID, D. J., MONCKTON, J. E., GARCIA, A. D. R., SOFRONIEW, M. V., KANDEL, E. R., SANTARELLI, L., HEN, R. & DREW, M. R. 2006. Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. *Proceedings of the National Academy of Sciences,* 103, 17501-17506.

SCHAAF, M. J., WORKEL, J. O., LESSCHER, H. M., VREUGDENHIL, E., OITZL, M. S. & DE KLOET, E. R. 2001. Correlation between hippocampal BDNF mRNA expression and memory performance in senescent rats. *Brain Res,* 915, 227-33.

SCHANZER, A., WACHS, F. P., WILHELM, D., ACKER, T., COOPER-KUHN, C., BECK, H., WINKLER, J., AIGNER, L., PLATE, K. H. & KUHN, H. G. 2004. Direct stimulation of adult neural stem cells in vitro and neurogenesis in vivo by vascular endothelial growth factor. *Brain Pathol,* 14, 237-48.

SCHARFMAN, H., GOODMAN, J., MACLEOD, A., PHANI, S., ANTONELLI, C. & CROLL, S. 2005. Increased neurogenesis and the ectopic granule cells after intrahippocampall BDNF infusion in adults rats. *Experimental Neurology,* 192, 348-356.

SCHMIDT-HIEBER, C., JONAS, P. & BISCHOFBERGER, J. 2004. Enhanced synaptic plasticity in newly generated granule cells of the adult hippocampus. *Nature,* 429, 184-187.

SCHMITT, U., TANIMOTO, N., SEELIGER, M., SCHAEFFEL, F. & LEUBE, R. E. 2009. Detection of behavioral alterations and learning deficits in mice lacking synaptophysin. *Neuroscience,* 162, 234-243.

SCHNEIDER, H., PITOSSI, F., BALSCHUN, D., WAGNER, A., DEL REY, A. & BESEDOVSKY, H. O. 1998. A neuromodulatory role of interleukin-1β in the hippocampus. *Proceedings of the National Academy of Sciences,* 95, 7778-7783.

SCHOVILLE, W. B. & MILNER, B. 1957. Loss Of Recent Memory After Bilateral Hippocampal Lesions. *Journal of Neurology, Neurosurgery & Psychiatry,* 20, 11-21.

SEGAL, R. A. 2003. Selectivity in neurotrophin signaling: theme and variations. *Annu Rev Neurosci,* 26, 299-330.

SEGOVIA, G., YAGÜE, A. G., GARCÍA-VERDUGO, J. M. & MORA, F. 2006. Environmental enrichment promotes neurogenesis and changes the extracellular concentrations of glutamate and GABA in the hippocampus of aged rats. *Brain Research Bulletin,* 70, 8-14.

SELCHER, J. C., NEKRASOVA, T., PAYLOR, R., LANDRETH, G. E. & SWEATT, J. D. 2001. Mice lacking the ERK1 isoform of MAP kinase are unimpaired in emotional learning. *Learn Mem,* 8, 11-9.

SHAFTEL, S. S., GRIFFIN, W. S. T. & O'BANION, M. K. 2008. The role of interleukin-1 in neuroinflammation and Alzheimer disease: an evolving perspective. *Journal of Neuroinflammation,* 5.

SHETTY, A. K., HATTIANGADY, B. & SHETTY, G. A. 2005. Stem/progenitor cell proliferation factors FGF-2, IGF-1, and VEGF exhibit early decline during the course of aging in the hippocampus: Role of astrocytes. *Glia,* 51, 173-186.

SHIHABUDDIN, L. S., HORNER, P. J., RAY, J. & GAGE, F. H. 2000. Adult spinal cord stem cells generate neurons after transplantation in the adult dentate gyrus. *Journal of Neuroscience,* 20, 8727-35.

SHORS, T. J., TOWNSEND, D. A., ZHAO, M. R., KOZOROVITSKIY, Y. & GOULD, E. 2002. Neurogenesis may relate to some but not all types of hippocampal-dependent learning. *Hippocampus,* 12, 578-584.

SHUPLIAKOV, O., HAUCKE, V. & PECHSTEIN, A. 2011. How synapsin I may cluster synaptic vesicles. *Seminars in Cell & Developmental Biology,* In Press, Corrected Proof.

SILHOL, M., BONNICHON, V., RAGE, F. & TAPIA-ARANCIBIA, L. 2005. Age-related changes in brain-derived neurotrophic factor and tyrosine kinase receptor isoforms in the hippocampus and hypothalamus in male rats. *Neuroscience,* 132, 613-624.

SIMPSON, J. & KELLY, J. P. 2011. The impact of environmental enrichment in laboratory rats--Behavioural and neurochemical aspects. *Behavioural Brain Research,* 222, 246-264.

SMALL, S. A., CHAWLA, M. K., BUONOCORE, M., RAPP, P. R. & BARNES, C. A. 2004. Imaging correlates of brain function in monkeys and rats isolates a hippocampal subregion differentially vulnerable to aging. *Proceedings of the National Academy of Sciences of the United States of America,* 101, 7181-7186.

SMITH, S. M., JENKINSON, M., WOOLRICH, M. W., BECKMANN, C. F., BEHRENS, T. E. J., JOHANSEN-BERG, H., BANNISTER, P. R., DE LUCA, M., DROBNJAK, I., FLITNEY, D. E., NIAZY, R. K., SAUNDERS, J., VICKERS, J., ZHANG, Y. Y., DE STEFANO, N., BRADY, J. M. & MATTHEWS, P. M. 2004. Advances in functional and structural MR image analysis and implementation as FSL. *Neuroimage,* 23, S208-S219.

SNYDER, J. S., KEE, N. & WOJTOWICZ, J. M. 2001. Effects of Adult Neurogenesis on Synaptic Plasticity in the Rat Dentate Gyrus. *Journal of Neurophysiology,* 85, 2423-2431.

SOFRONIEW, M. V., HOWE, C. L. & MOBLEY, W. C. 2001. Nerve growth factor signaling, neuroprotection, and neural repair. *Annual Review of Neuroscience,* 24, 1217-81.

SOLÉ-PADULLÉS, C., BARTRÉS-FAZ, D., JUNQUÉ, C., VENDRELL, P., RAMI, L., CLEMENTE, I. C., BOSCH, B., VILLAR, A., BARGALLÓ, N., JURADO, M. A., BARRIOS, M. & MOLINUEVO, J. L. 2009. Brain structure and function related to cognitive reserve variables in normal aging, mild cognitive impairment and Alzheimer's disease. *Neurobiology of Aging,* 30, 1114-1124.

SQUIRE, L. R. 1986. Mechanisms of memory. *Science,* 232, 1612-9.

SQUIRE, L. R. 1992. Memory and the hippocampus: A synthesis from findings with rats, monkeys, and humans. *Psychological Review,* 99, 195-231.

STATISTICS, B. O. L. 2011. American Time User Survey 2009. United States Department of Labor.

STERN, Y. 2002. What is cognitive reserve? Theory and research application of the reserve concept. *J Int Neuropsychol Soc,* 8, 448-60.

STOUT, R. D. & SUTTLES, J. 1996. The many roles of CD40 in cell-mediated inflammatory responses. *Immunology Today,* 17, 487-492.

SUN, Y., JIN, K., XIE, L., CHILDS, J., MAO, X. O., LOGVINOVA, A. & GREENBERG, D. A. 2003. VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. *The Journal of Clinical Investigation,* 111, 1843-1851.

TAKEI, Y. & LASKEY, R. 2008. Interpreting crosstalk between TNF-alpha and NGF: potential implications for disease. *Trends Mol Med,* 14, 381-8.

TAKI, Y., KINOMURA, S., SATO, K., GOTO, R., WU, K., KAWASHIMA, R. & FUKUDA, H. 2011. Correlation between gray/white matter volume and cognition in healthy elderly people. *Brain and Cognition,* 75, 170-176.

TALIAZ, D., STALL, N., DAR, D. E. & ZANGEN, A. 2010. Knockdown of brain-derived neurotrophic factor in specific brain sites precipitates behaviors associated with depression and reduces neurogenesis. *Mol Psychiatry,* 15, 80-92.

TAPIA-ARANCIBIA, L., ALIAGA, E., SILHOL, M. & ARANCIBIA, S. 2008. New insights into brain BDNF function in normal aging and Alzheimer disease. *Brain Research Reviews,* 59, 201-20.

TARR, A. J., MCLINDEN, K. A., KRANJAC, D., KOHMAN, R. A., AMARAL, W. & BOEHM, G. W. 2011. The effects of age on lipopolysaccharide-induced cognitive deficits and interleukin-1[beta] expression. *Behavioural Brain Research,* 217, 481-485.

TASHIRO, A., MAKINO, H. & GAGE, F. H. 2007. Experience-Specific Functional Modification of the Dentate Gyrus through Adult Neurogenesis: A Critical Period during an Immature Stage. *J. Neurosci.,* 27, 3252-3259.

TERRY JR, A. V., KUTIYANAWALLA, A. & PILLAI, A. 2011. Age-dependent alterations in nerve growth factor (NGF)-related proteins, sortilin, and learning and memory in rats. *Physiology & Behavior,* 102, 149-157.

THOENEN, H., ZAFRA, F., HENGERER, B. & LINDHOLM, D. 1991. The synthesis of nerve growth factor and brain-derived neurotrophic factor in hippocampal and cortical neurons is regulated by specific transmitter systems. *Ann N Y Acad Sci,* 640, 86-90.

TIEDGE, H. & BROSIUS, J. 1996. Translational Machinery in Dendrites of Hippocampal Neurons in Culture. *The Journal of Neuroscience,* 16, 7171-7181.

TODD ROACH, J., VOLMAR, C.-H., DWIVEDI, S., TOWN, T., CRESCENTINI, R., CRAWFORD, F., TAN, J. & MULLAN, M. 2004. Behavioral effects of CD40-CD40L pathway disruption in aged PSAPP mice. *Brain Research,* 1015, 161-168.

TONCHEV, A. B., BONEVA, N. B., KAPLAMADZHIEV, D. B., KIKUCHI, M., MORI, Y., SAHARA, S. & YAMASHIMA, T. 2008. Expression of neurotrophin receptors by proliferating glia in postischemic hippocampal CA1 sector of adult monkeys. *J Neuroimmunol,* 205, 20-4.

TONCHEV, A. B., YAMASHIMA, T., GUO, J., CHALDAKOV, G. N. & TAKAKURA, N. 2007. Expression of angiogenic and neurotrophic factors in the progenitor cell niche of adult monkey subventricular zone. *Neuroscience,* 144, 1425-35.

TONGIORGI, E., RIGHI, M. & CATTANEO, A. 1997. Activity-Dependent Dendritic Targeting of BDNF and TrkB mRNAs in Hippocampal Neurons. *The Journal of Neuroscience,* 17, 9492-9505.

TREIT, D. & FUNDYTUS, M. 1988. Thigmotaxis as a test for anxiolytic activity in rats. *Pharmacology Biochemistry and Behavior,* 31, 959-962.

TRIACA, V., TIRASSA, P. & ALOE, L. 2005. Presence of nerve growth factor and TrkA expression in the SVZ of EAE rats: evidence for a possible functional significance. *Exp Neurol,* 191, 53-64.

TRONEL, S., FABRE, A., CHARRIER, V., OLIET, S. H., GAGE, F. H. & ABROUS, D. N. 2010. Spatial learning sculpts the dendritic arbor of adult-born hippocampal neurons. *Proc Natl Acad Sci U S A,* 107, 7963-8.

TURNER, C. A. & LEWIS, M. H. 2003. Environmental enrichment: effects on stereotyped behavior and neurotrophin levels. *Physiology & Behavior,* 80, 259-266.

TYLER, W. J., ALONSO, M., BRAMHAM, C. R. & POZZO-MILLER, L. D. 2002. From acquisition to consolidation: On the role of brain-derived neurotrophic factor signaling in hippocampal-dependent learning. *Learning & Memory,* 9, 224-237.

UN 2002. World Population Ageing: 1950-2050. *In:* DESA (ed.). New York.

VALENZUELA, M. J., BREAKSPEAR, M. & SACHDEV, P. 2007. Complex mental activity and the aging brain: Molecular, cellular and cortical network mechanisms. *Brain Research Reviews,* 56, 198-213.

VALENZUELA, M. J. & SACHDEV, P. 2006. Brain reserve and dementia: a systematic review. *Psychol Med,* 36, 441-54.

VAN PETTEN, C. 2004. Relationship between hippocampal volume and memory ability in healthy individuals across the lifespan: review and meta-analysis. *Neuropsychologia,* 42, 1394-1413.

VAN PRAAG, H., CHRISTIE, B. R., SEJNOWSKI, T. J. & GAGE, F. H. 1999. Running enhances neurogenesis, learning, and long-term potentiation in mice. *Proceedings of the National Academy of Sciences of the United States of America,* 96, 13427-13431.

VAN PRAAG, H., KEMPERMANN, G. & GAGE, F. H. 2000. Neural consequences of environmental enrichment. *Nature Reviews Neuroscience,* 1, 191-198.

VAN PRAAG, H., SCHINDER, A. F., CHRISTIE, B. R., TONI, N., PALMER, T. D. & GAGE, F. H. 2002. Functional neurogenesis in the adult hippocampus. *Nature,* 415, 1030-1034.

VERDI, J. M., BIRREN, S. J., IBANEZ, C. F., PERSSON, H., KAPLAN, D. R., BENEDETTI, M., CHAO, M. V. & ANDERSON, D. J. 1994. p75LNGFR regulates Trk signal transduction and NGF-induced neuronal differentiation in MAH cells. *Neuron,* 12, 733-45.

VICTOR, M. & AGAMANOLIS, D. 1990. Amnesia due to Lesions Confined to the Hippocampus: A Clinical-Pathologic Study. *Journal of Cognitive Neuroscience,* 2, 246-257.

VIVIANI, B. & BORASO, M. 2011. Cytokines and neuronal channels: A molecular basis for age-related decline of neuronal function? *Experimental Gerontology,* 46, 199-206.

VOLOSIN, M., TROTTER, C., CRAGNOLINI, A., KENCHAPPA, R. S., LIGHT, M., HEMPSTEAD, B. L., CARTER, B. D. & FRIEDMAN, W. J. 2008. Induction of Proneurotrophins and Activation of p75NTR-Mediated Apoptosis via Neurotrophin Receptor-Interacting Factor in Hippocampal Neurons after Seizures. *Journal of Neuroscience,* 28, 9870-9879.

WALSH, R. N. & CUMMINS, R. A. 1975. Mechanisms mediating the production of envirionmentally induced brain changes. *Psychological Bulletin,* 82, 986-1000.

WALZ, R., LENZ, G., ROESLER, R., VIANNA, M. M. R., MARTINS, V., BRENTANI, R., RODNIGHT, R. & IZQUIERDO, I. 2000. Time-dependent enhancement of inhibitory avoidance retention and MAPK activation by post-training infusion of nerve growth factor into CA1 region of hippocampus of adult rats. *European Journal of Neuroscience,* 12, 2185-2189.

WANG, D. D., KRUEGER, D. D. & BORDEY, A. 2003. GABA depolarizes neuronal progenitors of the postnatal subventricular zone via GABAA receptor activation. *J Physiol,* 550, 785-800.

WANG, P., XIE, Z.-H., GUO, Y.-J., ZHAO, C.-P., JIANG, H., SONG, Y., ZHU, Z.-Y., LAI, C., XU, S.-L. & BI, J.-Z. 2011. VEGF-induced angiogenesis ameliorates the memory impairment in APP transgenic mouse model of Alzheimer's disease. *Biochemical and Biophysical Research Communications,* 411, 620-626.

WANG, S., SCOTT, B. W. & WOJTOWICZ, J. M. 2000. Heterogenous properties of dentate granule neurons in the adult rat. *Journal of Neurobiology,* 42, 248-257.

WEBSTER, M. J., HERMAN, M. M., KLEINMAN, J. E. & SHANNON WEICKERT, C. 2006. BDNF and trkB mRNA expression in the hippocampus and temporal cortex during the human lifespan. *Gene Expr Patterns,* 6, 941-51.

WEST, M. J., COLEMAN, P. D., FLOOD, D. G. & TRONCOSO, J. C. 1994. Differences in the pattern of hippocampal neuronal loss in normal ageing and Alzheimer's disease. *The Lancet,* 344, 769-772.

WILLIAMS, T. D., READMAN, G. D. & OWEN, S. F. 2009. Key issues concerning environmental enrichment for laboratory-held fish species. *Laboratory Animals,* 43, 107-120.

WILLSON, M. L., MCELNEA, C., MARIANI, J., LOHOF, A. M. & SHERRARD, R. M. 2008. BDNF increases homotypic olivocerebellar reinnervation and associated fine motor and cognitive skill. *Brain,* 131, 1099-1112.

WILSON, R. S., MENDES DE LEON, C. F., BARNES, L. L., SCHNEIDER, J. A., BIENIAS, J. L., EVANS, D. A. & BENNETT, D. A. 2002. Participation in Cognitively Stimulating Activities and Risk of Incident Alzheimer Disease. *JAMA: The Journal of the American Medical Association,* 287, 742-748.

WINTERS, B. D. & BUSSEY, T. J. 2005a. Glutamate receptors in perirhinal cortex mediate encoding, retrieval, and consolidation of object recognition memory. *Journal of Neuroscience,* 25, 4243-51.

WINTERS, B. D. & BUSSEY, T. J. 2005b. Transient inactivation of perirhinal cortex disrupts encoding, retrieval, and consolidation of object recognition memory. *Journal of Neuroscience,* 25, 52-61.

WOLFENSOHN, S. & LLOYD, M. 2003. *Handbook of Laboratory Animal Management and Welfare,* Oxford, Blackwell Publishing Ltd.

WOOLF, N. J., MILOV, A. M., SCHWEITZER, E. S. & ROGHANI, A. 2001. Elevation of Nerve Growth Factor and Antisense Knockdown of TrkA Receptor during Contextual Memory Consolidation. *Journal of Neuroscience,* 21, 1047-1055.

WU, C.-W., CHANG, Y.-T., YU, L., CHEN, H.-I., JEN, C. J., WU, S.-Y., LO, C.-P. & KUO, Y.-M. 2008. Exercise enhances the proliferation of neural stem cells and

neurite growth and survival of neuronal progenitor cells in dentate gyrus of middle-aged mice. *Journal of Applied Physiology,* 105, 1585-1594.

XIANG, J. Z. & BROWN, M. W. 1998. Differential neuronal encoding of novelty, familiarity and recency in regions of the anterior temporal lobe. *Neuropharmacology,* 37, 657-676.

XING, J., GINTY, D. D. & GREENBERG, M. E. 1996. Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science,* 273, 959-63.

XU, W.-P., SHAN, L.-D., GONG, S., CHEN, L., ZHANG, Y.-J., YIN, Q.-Z., HISAMITSU, T., JIANG, X.-H. & GUO, S.-Y. 2006. Forced running enhances neurogenesis in the hippocampal dentate gyrus of adult rats and improves learning ability. *Acta Physiologica Sinica,* 58, 415-420.

YANG, J.-P., LIU, H.-J., YANG, H. & FENG, P.-Y. 2011. Therapeutic time window for the neuroprotective effects of NGF when administered after focal cerebral ischemia. *Neurological Sciences,* 32, 433-441.

YANG, J.-P., LIU, X.-F., LIU, H.-J., XU, G.-L. & MA, Y.-P. 2008. Extracellular signal-regulated kinase involved in NGF/VEGF-induced neuroprotective effect. *Neuroscience Letters,* 434, 212-217.

YIN, J.-X., TURNER, G. H., LIN, H.-J., COONS, S. W. & SHI, J. 2012. Deficits in Spatial Learning and Memory is Associated with Hippocampal Volume Loss in Aged Apolipoprotein E4 Mice. *Journal of Alzheimer's Disease,* 27, 89-98.

YING, S. W., FUTTER, M., ROSENBLUM, K., WEBBER, M. J., HUNT, S. P., BLISS, T. V. & BRAMHAM, C. R. 2002. Brain-derived neurotrophic factor induces long-term potentiation in intact adult hippocampus: requirement for ERK activation coupled to CREB and upregulation of Arc synthesis. *Journal of Neuroscience,* 22, 1532-40.

YIRMIYA, R. & GOSHEN, I. 2011. Immune modulation of learning, memory, neural plasticity and neurogenesis. *Brain, Behavior, and Immunity,* 25, 181-213.

ZAFRA, F., CASTREN, E., THOENEN, H. & LINDHOLM, D. 1991. Interplay between glutamate and gamma-aminobutyric acid transmitter systems in the physiological regulation of brain-derived neurotrophic factor and nerve growth factor synthesis in hippocampal neurons. *Proceedings of the National Academy of Sciences of the United States of America,* 88, 10037-41.

ZAIAS, J., QUEENEY, T. J., KELLEY, J. B., ZAKHAROVA, E. S. & IZENWASSER, S. 2008. Social and Physical Environmental Enrichment Differentially Affect Growth and Activity of Preadolescent and Adolescent Male Rats. *Journal of the American Association for Laboratory Animal Science,* 47, 30-34.

ZHAO, C., TENG, E. M., SUMMERS, R. G., MING, G.-L. & GAGE, F. H. 2006. Distinct Morphological Stages of Dentate Granule Neuron Maturation in the Adult Mouse Hippocampus. *The Journal of Neuroscience,* 26, 3-11.

ZHAO, F., ZHAO, T., ZHOU, L., WU, Q. & HU, X. 2008. BOLD study of stimulation-induced neural activity and resting-state connectivity in medetomidine-sedated rat. *NeuroImage,* 39, 248-260.

ZHU, S.-W., CODITA, A., BOGDANOVIC, N., HJERLING-LEFFLER, J., ERNFORS, P., WINBLAD, B., DICKINS, D. W. & MOHAMMED, A. H. 2009. Influence of environmental manipulation on exploratory behaviour in male BDNF knockout mice. *Behavioural Brain Research,* 197**,** 339-346.

ZHU, S. W., YEE, B. K., NYFFELER, M., WINBLAD, B., FELDON, J. & MOHAMMED, A. H. 2006. Influence of differential housing on emotional behaviour and neurotrophin levels in mice. *Behavioural Brain Research,* 169**,** 10-20.

ZIEGENHORN, A. A., SCHULTE-HERBRUGGEN, O., DANKER-HOPFE, H., MALBRANC, M., HARTUNG, H. D., ANDERS, D., LANG, U. E., STEINHAGEN-THIESSEN, E., SCHAUB, R. T. & HELLWEG, R. 2007. Serum neurotrophins--a study on the time course and influencing factors in a large old age sample. *Neurobiology of Aging,* 28**,** 1436-45.

ZIMMERMAN, M. E., BRICKMAN, A. M., PAUL, R. H., GRIEVE, S. M., TATE, D. F., GUNSTAD, J., COHEN, R. A., ALOIA, M. S., WILLIAMS, L. M., CLARKE, C. R., WHITFORD, T. J. & GORDON, E. 2006. The relationship between frontal gray matter volume and cognition varies across the healthy adult lifespan. *American Journal of Geriatric Psychiatry,* 14**,** 823-833.

ZIMMERMANN, A., STAUFFACHER, M., LANGHANS, W. & WÜRBEL, H. 2001. Enrichment-dependent differences in novelty exploration in rats can be explained by habituation. *Behavioural Brain Research,* 121**,** 11-20.

ZOLA-MORGAN, S., SQUIRE, L. R. & AMARAL, D. G. 1986. Human amnesia and the medial temporal region: enduring memory impairment following a bilateral lesion limited to field CA1 of the hippocampus. *Journal of Neuroscience,* 6**,** 2950-67.

Appendix I

Solutions Used

Electrode Running Buffer

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

Krebs Solution

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

Lysis Buffer

NP-40	1% (v/v)
Tris base	20mM, pH8.0
NaCl	137mM
Glycerol	10% (v/v)
EDTA	2mM
Activated NaN_3	1mM
Aproptinin	23mM
Leupeptin	1.54mM
Distilled Water	

Phosphate Buffered Saline (PBS, pH 7.4)

NaCl	100mM
Na_2HPO_4	80mM
NaH_2PO_4	20mM
Distilled water	

Sample Buffer

Tris-HCl	0.5mM, pH 6.8
Glycerol	10% (v/v)
SDS	0.05% (w/v)
β-mercaptoethanol	5% (v/v)
Bromophenol blue	0.05% (w/v)
Distilled Water	

Separating Gel 7.5%

Bisacrylamide	25% (w/v)
Tris-HCl	1.5M, pH 8.8
SDS	1% (w/v)
Ammonium persulphate	0.5% (w/v)
TEMED	0.1% (v/v)
Distilled water	

Separating Gel 10%

Bisacrylamide	33% (w/v)
Tris-HCl	1.5M, pH 8.8
SDS	1% (w/v)
Ammonium persulphate	0.5% (w/v)
TEMED	0.1% (v/v)
Distilled water	

Stacking Gel

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

Transfer Buffer (pH 8.3)

Tris base	25mM
Glycine	192mM
Methanol	20% (v/v)
SDS	0.05% (w/v)
Distilled Water	

TBS-Tween wash buffer

Tris-HCl	20mM
NaCl	150mM
Tween-20	0.05% (v/v)
Distilled Water	

Publications

Griffin, É. W., Bechara, R. G., Birch, A. M. & Kelly, Á. M. 2009. Exercise enhances hippocampal-dependent learning in the rat: Evidence for a BDNF-related mechanism. *Hippocampus,* 19, 973-980

Birch, A. M., Griffin, É., Kelly, Á. M. 2008. Short-term environmental enrichment enhances object recognition memory and increases the concentration of Nerve Growth Factor, but not Brain Derived Neurotrophic Factor in the dentate gyrus of male Wistar rats. *Irish Journal of Medical Sciences*, 177, S368-369 (Abstract only).

In Preparation

Birch, A. M. & Kelly, Á. M. Chronic intracerebroventricular βNGF infusion enhances recognition memory and hippocampal neuroplasticity in the rat via MAPKinase pathway activation.

Birch, A. M., Griffin, É. W. & Kelly, Á. M. Short-term environmental enrichment, in the absence of exercise, enhances hippocampal-dependent memory and increases βNGF expression, neurogenesis and synaptogenesis in the dentate gyrus of the rat.

Birch, A. M., Pichet-Binette, A., Kelly, Á. M. Long-term environmental enrichment protects against age-related cognitive decline in rat and attenuates reductions in expression ofhippocampal growth factors and neurogenesis.

Poster/Oral Presentations

Birch, A. M., Griffin, É., Kelly, Á. M. Short-term environmental enrichment enhances object recognition memory and increases the concentration of Nerve Growth Factor, but not Brain Derived Neurotrophic Factor in the dentate gyrus of male Wistar rats

> *Oral presentation, Royal Academy of Medicine Ireland: Biomedical Sciences 2008 (3rd prize, Donegan Medal competition)*

Birch, A. M., Kelly, Á. M. Environmental Enrichment in the absence of exercise enhances cognitive function in the male Wistar rat

> *Poster presentation, Royal Academy of Medicine Ireland: Biomedical Sciences 2009*

Birch, A. M., Kelly, Á. M. Environmental Enrichment in the absence of exercise enhances cognitive function in the male Wistar rat

Oral presentation, Neuroscience Ireland 2009 (1st prize, best oral presentation)

Birch, A. M., Kelly, Á. M. Short-term environmental enrichment, in the absence of exercise, improves cognition and increases neurogenesis in the dentate gyrus in the male Wistar rat

Poster presentation, Federation of European Neurosciences 2010

Birch, A. M., Kelly, Á. M. Chronic intracerebroventricular βNGF infusion improves hippocampal-dependent memory

Poster presentation, International Brain Research Organisation 2011

Birch, A. M., Pichet Binette, A., Kelly, Á. M. An evaluation of the neuroprotective properties of long-term environmental enrichment in the ageing rat

Oral presentation, 4th School of Medicine Postgraduate Research Day, Trinity College Dublin 2011