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Exercise and TrkB activation as neuroprotectants in mouse models of age and neuroinflammation: an assessment of underlying mechanisms

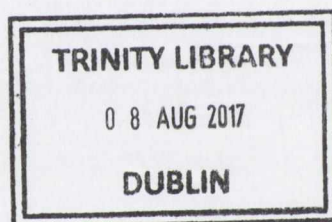
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Thesis submitted for the degree of Doctor of Philosophy
at the University of Dublin, Trinity College.

2017

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Declaration

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Abstract

The processes of learning and memory concern themselves primarily with the hippocampus - one of two exclusive cortical regions where neurogenesis has been found to take place. These cognitive processes are themselves affected in various conditions such as depression, brain injury, neuroinflammation, Alzheimer's disease, and in the process of aging in general. Physical activity has been duly recognised as having a role in the induction of neurogenesis - a process resulting in the generation of new neurons, through the up-regulation of Brain-derived Neurotrophic Factor (BDNF). A neurochemical cascade triggered by physical exercise results in the generation of new neurons in the dentate gyrus of the hippocampus, providing an opportunity to counteract and recover from distress within the CNS caused by disorder- or disease-induced deficits in cognitive function, or indeed by the neurodegenerative effects of aging alone.

Numerous studies have demonstrated the success of exercise in inducing cognitive enhancement, generating new neurons, and increasing expression of BDNF, when testing is carried out immediately following cessation of exercise. Increased circulating concentration of BDNF acts via TrkB receptor activation to induce various changes in cell signalling that can influence neuronal proliferation, neuronal survival, and neuronal death. In a bid to mimic the physiological response to exercise, the TrkB receptor has been targeted by pharmacological intervention in the form of 7,8-DHF – a synthetically derived flavonoid with selective agonistic activity at the TrkB receptor.

Recent studies have demonstrated the potential for 7,8-DHF to imitate endogenous BDNF through cognitive enhancement, which has been observed in conjunction with increased TrkB phosphorylation, activation of the MAPK/ERK and PI3K/Akt intracellular pathways, and changes in neuronal proliferation. This research hopes to replicate this enhancement using physical or pharmaceutical intervention, in a mouse model subject to CNS insult or injury. With a view to determine if neurogenesis is related to cognitive function in mouse models of neuroinflammation and age, this work was designed to test cognitive function at a time point that coincides with the integration of new neurons into the circuitry, as they reach a stage of functional maturity.

At a behavioural level, we observed an exercise-induced enhancement in hippocampus-dependent spatial memory in a C57/BL6 mouse that had been subject to experimentally-induced neuroinflammation. Furthermore, we replicated this improvement in cognitive function in mice receiving 7,8-DHF, who were exposed to the same inflammatory induction, and thus we confirm the mimetic activity of 7,8-DHF at the behavioural level. This cognitive enhancement is reported without current recognition of the pathway through which this effect was exerted, as we did not observe a significant LPS-induced change in mRNA expression of IL1 β and TNF α when exercised and 7,8-DHF animals were compared to their sedentary and vehicle counterparts. To date we report no significant change in cell number in the dentate gyrus.

In a long-term study of cognitive function and the potential for 7,8-DHF to rescue cognitive decline in aged animals, we observed a persistent enhancement in spatial and episodic memory over nine months of behavioural testing that was not sustained in vehicle treated controls of the same age, and without any discernible alteration in mRNA expression of key components of the pathways linking TrkB activation with the elicited cognitive improvement.

Taken together, our results demonstrate the protective effect of 7,8-DHF on cognitive function, and suggest a number of avenues for further research in an effort to connect this behavioural response with its molecular effectors.

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List of Abbreviations

7,8-DHF	7,8-dihydroxyflavone
AD	Alzheimer’s disease
Akt	/PKB – protein kinase B
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of Variance
BBB	blood brain barrier
BCA	bicinchoninic acid
BDNF	Brain-Derived Neurotrophic Factor
BrdU	5’-bromo-2’-deoxyuridine
BSA	bovine serum albumin
°C	degrees Celsius
CA1	<i>Cornu ammonis</i> 1
CA2	<i>Cornu ammonis</i> 2
CA3	<i>Cornu ammonis</i> 3
CA4	<i>Cornu ammonis</i> 4
Ca ²⁺	calcium
CaCl ₂	calcium chloride
CaMKII	calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
cDNA	complimentary deoxyribonucleic acid
CNS	central nervous system
CREB	cAMP response-element binding protein
C _T	cycle threshold
DAG	diacylglycerol
DCX	doublecortin
dH ₂ O	distilled water
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay

ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
Ex	exercised
g	gram
G ₁ phase	growth 1/gap 1 phase (of cell cycle)
G ₂ phase	pre-mitotic phase (of cell cycle)
GAB 1/2	Grb2-associated-binding protein 1/2
GABA	γ-amino butyric acid
GCL	granule cell layer
GDP	guanosine diphosphate
Grb2	growth factor receptor-bound protein 2
GTP	guanosine triphosphate
H ₂ O ₂	hydrogen peroxide
H ₂ SO ₄	sulphuric acid
HCl	hydrochloric acid
Hipp	hippocampus
HRP	horseradish peroxidase
hrs	hours
IgG	immunoglobulin G
IL-1	interleukin 1
IL-6	interleukin 6
i.p.	intraperitoneal
IP ₃	inositol 1, 4, 5-trisphosphate
KCl	potassium chloride
K _d	dissociation constant
kDa	kiloDalton
kg	kilogram
km	kilometre
LTP	long term potentiation
M	molar mass
MAPK	mitogen-activated protein kinase
MBD	membrane-desalting buffer
MEK 1 / 2	mitogen-activated protein kinase kinase 1 / 2

Mg ²⁺	magnesium
μg	microgram
μg/ml	microgram/ millilitre
mg	milligram
mg/kg	milligram per kilogram
min	minute
ml	millilitre
MTL	medial temporal lobe
mRNA	messenger ribonucleic acid
mTOR	mechanistic target of rapamycin
MW	molecular weight
Na ⁺	sodium
NaCl	sodium chloride
ng	nanogram
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NOR	novel object recognition
NPC	neural progenitor cell
NSC	neural stem cell
NT-3	neurotrophin-3
NT-4	neurotrophin-4
O ₂	oxygen
OCT	optimal cutting temperature
OD task	object displacement task
OD	optical density
p75 ^{NTR}	low-affinity nerve growth factor receptor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline + Tween
PCR	polymerase chain reaction
pg	picogram
pg/mg	picogram/milligram
PI3-K	phosphoinositide 3-kinase

PIP ₂	phosphatidylinositol 4, 5-bisphosphate
PIP ₃	phosphatidylinositol (3,4,5)-trisphosphate
PKA	protein kinase A (also known as cAMP-dependent protein kinase)
PKC	protein kinase C
PLC γ	phospholipase C gamma
PSD-95	postsynaptic density protein 95
PNS	peripheral nervous system
Raf	rapidly accelerated fibrosarcoma
Ras	rat sarcoma
Rheb	Ras homolog enriched in brain
RNA	ribonucleic acid
rpm	rotations per minute
Rq	relative quantification
RT-PCR	real time PCR
Sed	sedentary
SD	standard deviation
SDS	sodium dodecyl sulphate
sec	second
SED	sedentary group
SEM	standard error of the mean
SGZ	subgranular zone
Shc	Src homology 2 domain-containing
SOS	son of sevenless
S-phase	synthesis phase (of cell cycle)
Src	proto-oncogene tyrosine-protein kinase
SVZ	subventricular zone
t $\frac{1}{2}$	half life
TBS	Tris buffered saline
TBS-T	Tris buffered saline- Tween
TEMED	N,N,N',N'-Tetramethylethylenediamine
TMB	3,3',5,5'-Tetramethylbenzidine
TNF α	tumour necrosis factor α
Trk	tropomyosin receptor kinase

Trk A	tropomyosin receptor kinase A
Trk B	tropomyosin receptor kinase B
Trk C	tropomyosin receptor kinase C
TRPC 3 / 6	transient receptor potential cation channel 3 / 6
TSC 1 / 2	tuberous sclerosis complex 1 / 2
Veh	vehicle
VEx	voluntary exercise

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Chapter 1

Introduction

1.1 Introduction

Evolution and survival are processes of the organism that revolve around a vital function – the capacity to learn, and with it; to remember. Together, these abilities provide us with the tools to thrive within our environment. The brain can be compared to a computer – a vessel of information processing, intricate networks and functional execution. Cognitive function involves all of the cerebral activities that lead to knowledge; attention, thought, and memory, and because of this, higher cognitive function encompasses a myriad of structural components within the body working in unison. It has become the goal of many to pinpoint the exact mechanisms involved in this crucial process (Deutsch, 2012, Markowitsch, 1995, Russell, 1971), because of the many scenarios that threaten the ability to navigate and decipher our surroundings. The most common of these threats is aging, and therefore investigation into the consequences of aging and how its impact can be diminished is a field of research that is consistently studied.

1.2 Aging

1.2.1 Aging and cognitive impairment

Aging is defined as the process of becoming older, and involves the accumulation of minor bodily injury and degeneration, resulting in a decrease in functionality of the organism as a whole. With this degeneration comes a plethora of biological changes; loss of skin elasticity, loss of hair pigmentation, weaker bones, aching joints, increased blood pressure, waning eyesight – all signs that the body requires much more energy to maintain normal function. Many faculties suffer as a result of prioritisation. Possibly the most debilitating of these changes is the decline in cognitive function. As with all of these characteristics of aging, cognitive decline affects individual organisms in varying degrees of severity. Mild cognitive impairment (MCI) is classified as a condition that causes a mild but noticeable decline in memory and thought skills, to the point where it is evident to the person themselves, but does not interfere greatly with every-day life (Petersen *et al.*, 1997). The Alzheimer's Association estimates that 15 – 20% of those aged 65 and older may have some form of MCI (Roberts and Knopman, 2013). Dementia is a more pronounced form of cognitive decline - a deterioration beyond that which may be seen with normal aging. The World Health Organisation (WHO) states that dementia is characterised by disturbances in comprehension and thought, orientation and calculation, language, judgement, and learning and memory capacity, without affecting the state of consciousness. There are a number of causes for dementia, including

ischemic attack, Parkinson's disease, Huntington's disease, and Alzheimer's disease (AD). Worldwide, there are approximately 47.5 million cases of dementia, and AD accounts for about 60-70% of these (WHO statistics, April 2016).

1.2.2 The aging population

Aside from cases of early-onset AD - which account for about 5-10% of all instances of AD, and occur in patients under the age of 65, (Zhu *et al.*, 2015), Alzheimer's is an affliction of the elderly, and so with an increasingly aged population comes an increase in cases of AD. In 2015, the WHO estimated that between the years 2015 and 2050 the proportion of the population aged 65 years and older will rise from 900 million (12% of population) to 2 billion (22%). Of this, 125 million and 434 million, respectively, are and will be over the age of 80. This shows how rapidly the world's demographics are changing and how quickly we need to adapt to and prepare for this inevitability.

Despite an abundance of research focusing on the study of aging and cognition, the exact mechanisms, and interaction of these mechanisms, is something that will continue to be studied extensively in a bid to enhance the quality of life of the elderly. Every year, billions of euro goes into neuroscience-based research, and to date there have been many advances made in quite a short period of time. Finding cures, preventions, or even just aids in managing cognitive decline means that quality of life for an individual, and for the people around them, can be greatly enhanced. The use of mammalian models closely related to the human species means that many debilitating diseases can be replicated both *in vivo* and *in vitro* using a range of physical or pharmacological measures that can restore cognitive ability.

1.3 Learning and Memory

The processes of learning and memory are not mutually exclusive. These aspects of cognitive function are integral to functional living, and refer to the process of acquiring information from an experience or event (learning), storing that information within the brain (memorising), and being able to recall that information at a later time-point (remembering) – be that ten hours, or ten years after the information was first encountered. Respectively, these steps can be referred to as acquisition, consolidation, and recall.

1.3.1 Memory classification

Memory may be categorised under three subtypes – short-term, long-term, and working memory. Short-term memory lasts a matter of seconds or minutes. This information is not required again, the memory is not reinforced, and so the information is forgotten. Repetition of an event establishes familiarity and unconsciously the information will be stored as a long-term memory. Short-term memory is quickly accessible, but is limited and temporary (Cowan, 2008).

Working memory has some overlap with short-term memory (D'Esposito *et al.*, 1995). It is classed as the active use of details that have been remembered to carry out something in the short term - the manipulation of memory for short-term execution. Working memory can use either long-term or short-term memories, but the fact that these memories are being used in an immediate situation is the reason there is an overlap between working and short-term memory.

Long-term memories are information that has been consolidated for storage for a much longer period of time, so that it can be recalled hours, or years later. This is a much more intricate system of sorting memories, and thus there are many different types of long-term memory (Fig. 1.1). Initially, long-term memory is subdivided into explicit (conscious) memory and implicit (unconscious) memory. These types themselves are further divided.

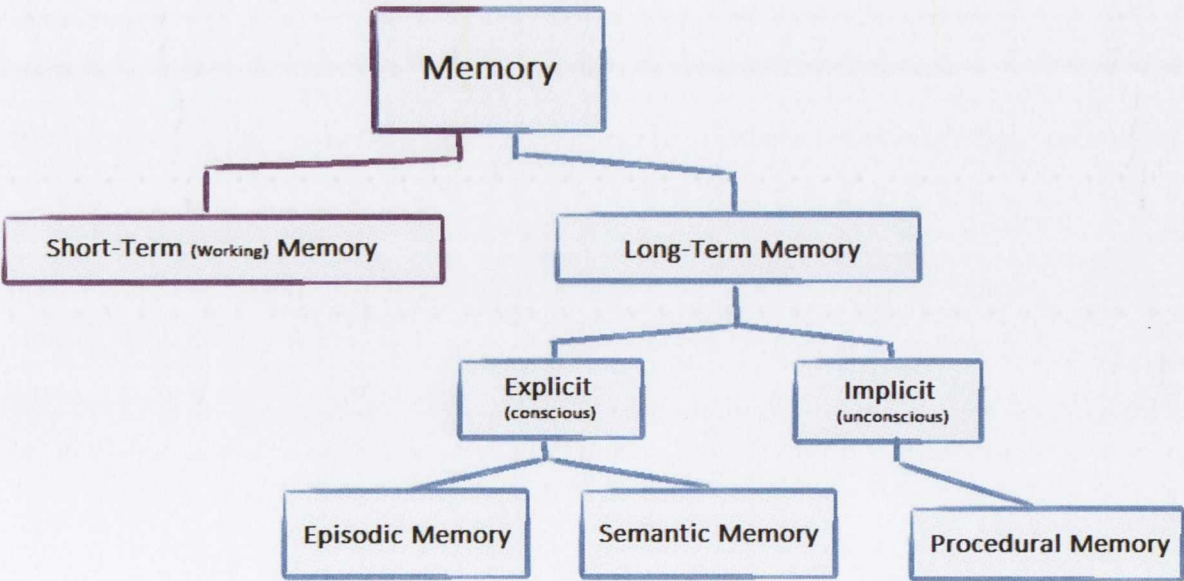


Fig. 1.1 – Types of memory
A schematic of the distinguishable kinds of memory. Adapted from: www.brainhq.com

Explicit memory encompasses episodic and semantic memory. Conscious memory elicits an awareness of itself and is actively recounted. Episodic memory relates to past personal experience; it is autobiographical in nature. This includes anything that has emotional connotation, such as life events, and the details of the context in which they happened. Semantic memory applies to all of the other things that are consciously known, but which are common knowledge. They have been actively learned at some point in life. This can include the names of colours, elements of the periodic table, the history of a country, and in general does not include an emotional connection.

Implicit memory refers to procedural memory. This is performance of everyday tasks that don't require thought to be executed. Driving a car, eating with a knife and fork, tying shoelaces – these are all activities that are almost reflex. They required learning at some point, and the learning was repeated again and again, but are now at the stage where the activity does not require an active recall of the steps involved. Often, it would be difficult to explain to others how something is done, when for you the ability just exists. This can be referred to as expertise-induced amnesia (Oliveira and Goodman, 2004), when one is so accustomed to doing something that when asked to recall how it was done they simply cannot, like playing the piano. It is at this point that explicit memory may be employed in an effort to explain.

1.3.2 Memory impairment

As mentioned, memory is integral to functional life, and can be affected in numerous ways. Restoring, maintaining, and enhancing cognitive function is a topic of research under continuous investigation, primarily in rodents (Means *et al.*, 1993, Latimer *et al.*, 2014) and humans (Albert *et al.*, 1995, Atkinson *et al.*, 2007). As discussed, the loss in the ability to learn and memorise can be consequent of various diseases and traumas. AD is a neurodegenerative disease associated with a loss of neurons and breakdown in the synaptic connectivity of the brain. This happens primarily in the hippocampus and other temporal lobe structures, leaving patients forgetful and usually unable to form new memories. Often comprehension of speech is a difficulty, as is the ability to explain oneself properly. This is a severe form of cognitive decline; the most debilitating of all forms of dementia.

Brain injury can be another cause of impaired cognitive function. Classification of an injury depends on lack of memory following the trauma, degree of unconsciousness, and verbal and motor skills. Both learning and memory can be greatly affected in traumatic brain injury, because in this type of injury the hippocampus is the area of the brain most at risk of damage (Sun *et al.*, 2007), and thus

the memory centres are severely impinged upon. Sun *et al.* (2007) induced lateral fluid percussion injury in rats, and reported that the production of new neurons in the dentate gyrus is substantially increased following brain injury, as BrdU tagged cells are abundant in this area following testing. Performance in the Morris Water Maze was also monitored, and it was found that learning and memory had improved. They concluded that neurogenesis had restored much of the cell loss following the trauma. Working with the ability of existing neurons to up-regulate their plasticity, it was observed that many new cells were fully integrated into the neuronal circuitry approximately 70 days post-injury.

1.3.3 Testing memory

It is known that the hippocampus is concerned with spatial memory and object recognition (Barker and Warburton, 2011), and assigning emotional markers to particular events (LeDoux, 1993). In this way a subject can recall a particular object or place by distinction of smell, as well as visual indicators that help with spatial awareness. In animal studies, an object displacement task may be used to test spatial memory, and a novel object recognition task may be used to test memory when presented with a new object. Animals are known for natural curiosity, and it is this fact that makes these kinds of tasks so effective in testing learning and memory. During their first exposure it is expected that all objects should be explored equally, as they are equally novel to the subject. The task can be varied in difficulty by adding more objects, or limiting acquisition time, so they are quite easily adapted to a particular testing paradigm.

1.4 Hippocampus

The hippocampus is a forebrain structure concerned primarily with the processes of learning and memory. This structure is highly susceptible to the effects of aging, as seen in the general reduction in volume of hippocampal tissue in the elderly (Burke and Barnes, 2006, Driscoll *et al.*, 2006). A reduction in hippocampal volume has also been reported as a marker for AD (Convit *et al.*, 1997) and was observed to coincide with major depressive disorder (MacQueen *et al.*, 2003), post-traumatic stress disorder (Bonne *et al.*, 2001), and childhood maltreatment (Teicher *et al.*, 2012).

1.4.1 Hippocampal function

Located within the medial temporal lobe, spanning both hemispheres, and joined by the hippocampal commissure, the hippocampus (Fig.1.2) is an integral structure in relation to learning and memory. It is listed as part of the Limbic system - a collection of structures concerned mainly with processing emotion, located either side of the thalamus. The limbic system also includes the amygdala, cingulate gyrus, fornix, mammillary bodies of the hypothalamus, and olfactory bulbs.

The hippocampus itself deals with episodic and spatial memory (Smith and Mizumori, 2006, Fortin *et al.*, 2002). Episodic memory is drawn from individual experience; personal details or events, and the context in which they happened, as opposed to semantic memory which refers mainly to general knowledge that has no personal connotation. The spatial memory aspect of the hippocampus is vital for navigation of spaces that have been experienced, and registers information about surroundings, forming a kind of cognitive map (O'Keefe and Dostrovsky, 1971). The hippocampus consolidates new information into long term memories, storing them appropriately in the brain, and retrieving these memories when needed (Leon *et al.*, 2010). For this reason, it is known to play an important role in long term potentiation. Hippocampal degeneration with age, and particularly neurodegenerative diseases such as AD, are thus characterised by general disorientation, and an incapability to form new memories, so that recent events cannot be recalled. This is known as anterograde amnesia.

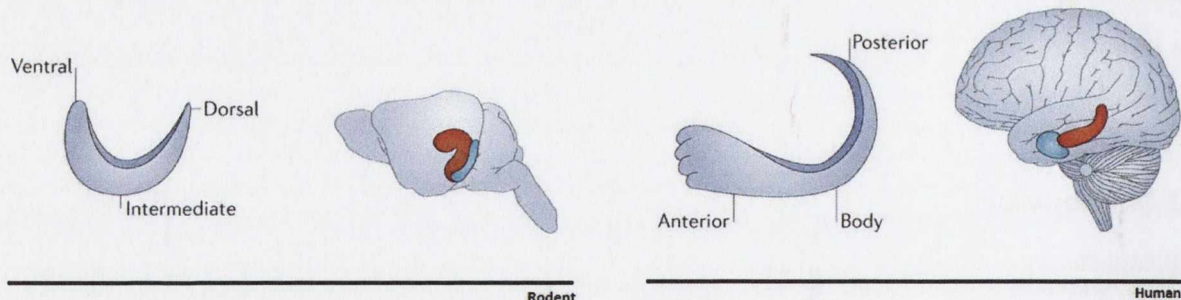


Fig. 1.2 - Location of the Hippocampus

Hippocampal formation (in red) in both the rodent and human brain.

The axis of the hippocampus is ventrodorsal in rodents and anteroposterior in the human brain.

The dorsal and posterior hippocampus concerns itself with spatial navigation and memory, while the ventral and anterior hippocampus is involved in emotion and anxiety.

Adapted from Figure 1: Cross-species comparison of hippocampal anatomy. (Strange et al., 2014)

The importance of the hippocampus in memory is probably most widely known through the study of patient Henry Molaison, who suffered with epilepsy. He underwent experimental bilateral removal of the hippocampus and some associated cortical regions to relieve his epileptic seizures, and developed loss of recent memory (Scoville and Milner, 1957). The brains of AD patients have shown large decreases in hippocampal volume (Bobinski *et al.*, 1995, Convit *et al.*, 1997).

1.4.2 Anatomy of the hippocampal formation

The hippocampal formation is the collective term for the subiculum, the hippocampus proper, and the dentate gyrus (Giap *et al.*, 2000). Each are composed of three layers; a polymorphic layer, molecular layer, and pyramidal/granular layer. The subiculum has a polymorphic layer of interneurons, and a pyramidal layer. The hippocampus proper is subdivided into regions CA1 - CA4 (*cornus ammonis*) (Fig. 1.3) and is composed mainly of GABAergic interneurons and pyramidal cells. The dentate gyrus has a granular layer of excitatory interneurons.

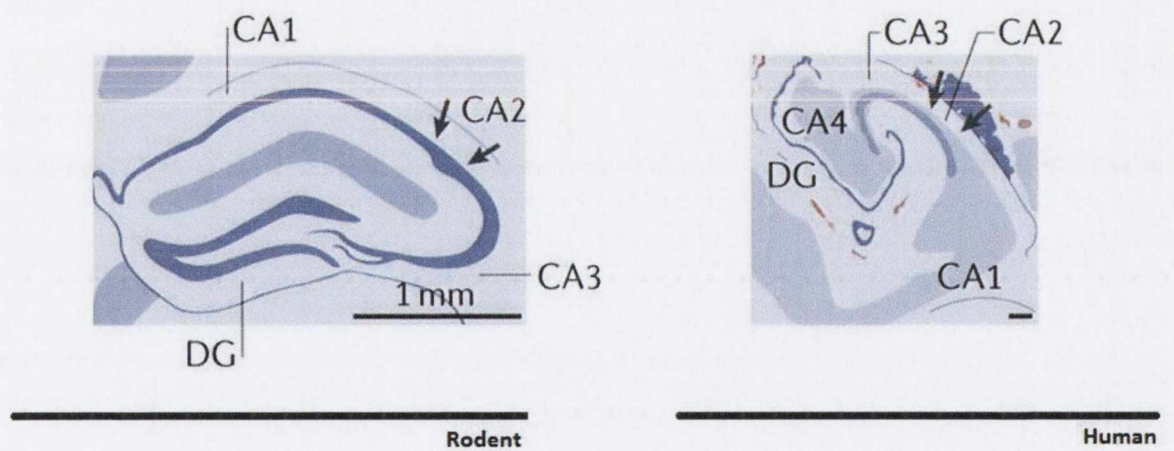


Fig. 1.3 - The Hippocampus

A coronal section of the hippocampal formation shows CA1 – CA4 and dentate gyrus regions in both the rodent and human brain.

Adapted from Figure 1: Cross-species comparison of hippocampal anatomy. (Strange *et al.*, 2014)

The dentate gyrus is the area most importantly involved in hippocampus-dependent memory formation (Kesner, 2007). Damage to neurons of the dentate gyrus has resulted in observed deficits in spatial memory (Colicos and Dash, 1996). It is recognised in two parts; the fascia dentate, and the hilus, which is a continuation from the CA4 region of the hippocampus proper. These small glutamatergic interneurons receive input from the entorhinal cortex, and their mossy fibres synapse onto pyramidal cells and interneurons in the CA3 region of the hippocampus proper. The granule cell layer is one of two specialised areas of the cortex where new neurons can be generated in the adult brain. This process is known as neurogenesis, and facilitates long term potentiation, and thus creation of new memories (Derrick *et al.*, 2000, Snyder *et al.*, 2005).

Although neurogenesis is known to occur throughout the entire nervous system during development, it has been found that adult (postnatal) neurogenesis occurs only in specific regions of the brain that contain neuronal progenitor cells. These are the subventricular zone (SVZ) which lines the lateral ventricles and projects to the olfactory bulb, and the subgranular zone (SGZ) (Curtis *et al.*, 2012). The SGZ is located between the hilus and the granule cell layer of the dentate gyrus. Progenitor cells migrate to the granule cell layer, and as they mature they travel away from this zone to form connections with other regions.

1.4.3 Neurogenesis in the adult dentate gyrus

Since the 1990s, a lot of progress has been made in recognising the existence of mammalian adult neurogenesis (Kuhn *et al.*, 1996, van Praag *et al.*, 1999a). Until this point, it was generally believed that neurons were generated exclusively during development, and this number then declined from birth.

In the 1960s, Joseph Altman suggested otherwise (Altman, 1963). His work produced results that were indicative of the induction of new glial cells into the mammalian brain, presenting the possibility that “new neurons may be formed in forebrain structures” (Altman, 1963). He based this hypothesis on work in which young adult rat and cat subjects were injected with radioactively labelled Thymidine at various time points, and one week following administration all subjects were killed. Staining for microscopic detection of labelled Thymidine in brain slices showed its presence in various areas throughout the brain, including the thalamus, corpus callosum and the dentate gyrus of the hippocampus. With the knowledge that thymidine is a DNA nucleoside, the radioactively labelled thymidine (H^3 -dT) could only have been incorporated into cells that were undergoing cell division, or consequently in cells that were the product of this cell division.

The 1980s brought further research that reported the presence of newly generated neurons in the dentate gyrus of the adult male rat when assessed at various ages from 1 to 12 months old (Bayer *et al.*, 1982, Bayer, 1982). In the late 1990s, an abundance of scientific investigation solidified the basis for the original proposal of mammalian adult neurogenesis, following the discovery of a new, non-radioactive labelling technique used in mice involving Bromodeoxyuridine (BrdU); a thymidine analogue, therefore capable of competing with thymidine and integrating in its place, and thus detectable in new cells that had incorporated it into their nuclei during proliferation (Corotto *et al.*, 1993). In rodent studies, work by H. Georg Kuhn and Fred Gage (Kuhn *et al.*, 1996) showed neurogenesis in the dentate gyrus of the aged rat, and reported a decline with age when rats aged 12 – 27 months were compared to 6 month old controls. Along with Gerd Kempermann, Kuhn and Gage also showed that numbers of BrdU positive cells were increased in the dentate gyrus of environmentally enriched 8- and 20-month old mice when compared to their age-matched controls (Kempermann *et al.*, 1998). Henriette van Praag, Kempermann and Gage reported an increase in BrdU positive neurons, but not BrdU labelled glia, in groups of either exercised or environmentally enriched 3-5 month old mice when compared to controls (van Praag *et al.*, 1999b). In non-rodent research, Peter Eriksson - also with Fred Gage, studied human cancer patients post-mortem (average age of 64.4 ± 2.9 years) and found BrdU positive cells in the dentate gyrus that could express a neuronal phenotype, similar in nucleic morphology and location to the nuclei of cells found in the dentate gyrus of adult rats and mice (Eriksson *et al.*, 1998). Elizabeth Gould reported the presence of BrdU positive neurons, both mature and immature, in the hippocampus of non-human adult primates aged between 5 and 23 years (Gould *et al.*, 1999).

This collection of work paved the way for a rapidly progressing discipline of study in developmental and disease models in neuroscience, as well as the general acceptance of the phenomenon of adult neurogenesis. More recent studies in humans have concluded that there is substantial neurogenesis in the adult hippocampus throughout life (Spalding *et al.*, 2013), and in depth research in rodent mammalian models identifies an extensive range of intrinsic and extrinsic factors influencing adult neurogenesis.

In addition to investigation in the SGZ of the dentate gyrus and the SVZ of the lateral ventricles, reports of neurogenesis in alternate regions of the brain are emerging. A study of treatment with the selective-serotonin reuptake inhibitor; fluoxetine has shown neurogenesis in the cortex of adult mice (Ohira *et al.*, 2013). Human study has shown the presence of newly generated interneurons in the adult striatum, thought to have projected there from the SVZ (Ernst *et al.*, 2014). In rodents, cells migrate from the SVZ to the olfactory bulb, but this activity is deemed almost non-existent in

humans (Bergmann *et al.*, 2012). However, in support of this finding in humans, BrdU positive cells were reported in the striatum of adult zebra finch birds (Kubikova *et al.*, 2014), and preceding these findings, it was shown that inducing ischemia in the adult rat could trigger migration of new cells from the SVZ to the striatum (Arvidsson *et al.*, 2002).

1.4.3.1 Timeframe of neurogenesis

The steps from neural stem cell to functionally mature neuron include self-renewal, fate specification, migration, maturation, and synaptic integration into the circuitry (Fig. 1.4).

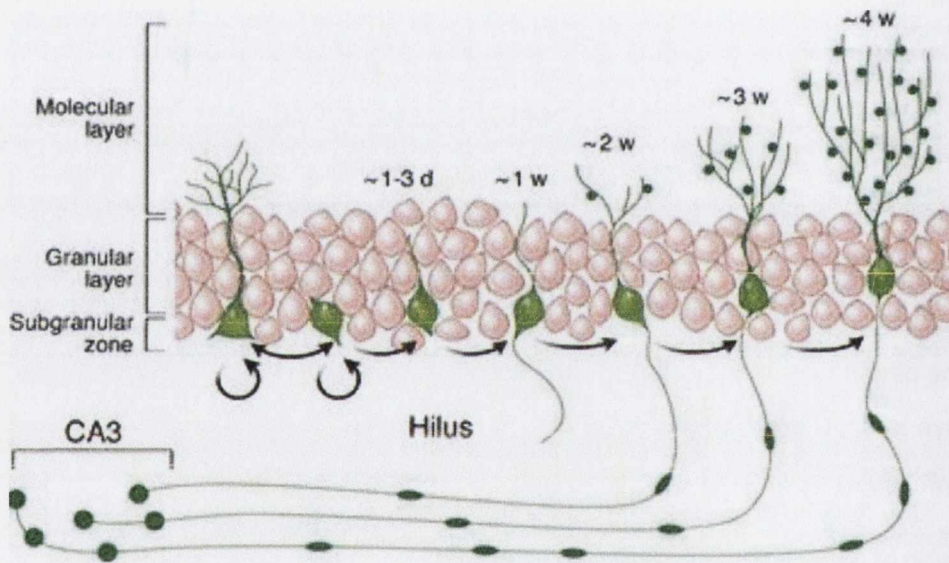


Fig. 1.4 - Stem Cell Lineage

An illustration of the steps from neural stem cell to mature neuron in the dentate gyrus.
Sourced from (Toni and Sultan, 2011).

Neural stem cells originating in the SGZ migrate to the granule cell layer and incorporate themselves as granule cells before they undergo differentiation. The new neurons are at first quite immature and will take time to transform into a fully functional neuron. It has been reported that the process of maturation takes about three weeks (Cameron *et al.*, 1993) and that once this time point is reached, any cells that are going to survive will have survived (Kempermann *et al.*, 2004).

The SGZ is often referred to as a neurogenic and angiogenic niche (Palmer *et al.*, 2000, Alvarez-Buylla and Lim, 2004) because it is a kind of ambient microenvironment that contains everything required for neuronal development, and orchestrates interaction among stem cells and with neurotransmitters, growth factors, and other essential influences. Radial glial cells assist in translocation of neural stem cells from SGZ to the granule cell layer, where they are termed progenitor cells. Despite not having yet made any synaptic connections, progenitor cells will be activated continuously by localized release of GABA from established neurons. Its effect at this stage of development is the opposite of its relationship with mature cells; this inhibitory neurotransmitter will excite, rather than inhibit neural progenitor cells (Deng *et al.*, 2010).

Eventually the granule cells will begin to form polarized axons. Still lacking dendritic spines, and thus glutamatergic connections, they continue to be excited by input from GABA. This input is vital to both the survival and differentiation of the juvenile neurons (Deng *et al.*, 2010, Kempermann *et al.*, 2004).

In their third week following cell division, they will start to become more structurally similar to mature neurons but with an increased potential for excitation. By now, their processes will have made afferent and efferent connections into the circuitry, and newly formed dendritic spines will allow them to receive input from glutamate. Concurrently, the excitatory input they will have received from GABA up until this point reverts to its naturally inhibitory effect (Ge *et al.*, 2006). The following weeks see their transformation into much more strongly active neurons. It is probable that their highly excitable nature at this stage contributes to their added involvement in plasticity-related learning and memory processes. In fact, it has been found that hippocampal-dependent learning enhances the potential for dentate granule cell survival during the earliest weeks of development (Epp *et al.*, 2011).

1.4.3.2 Neurogenesis and memory

Of the types of memory that involve hippocampal function, many have been shown to be either enhanced or diminished by an increase or decrease in neurogenesis, respectively. Reports show that a reduced number of newly generated neurons impairs hippocampal-dependent trace learning in the adult rat (Shors *et al.*, 2001). Similarly in the adult mouse, it was reported that numbers of newly generated neurons in the dentate gyrus were positively correlated with the ability to learn the Morris water maze task (Kempermann and Gage, 2002), and that pattern separation assessed

by a radial arm maze was detrimentally affected in adult mice subject to ablation of hippocampal neurogenesis (Clelland *et al.*, 2009).

1.4.3.3 Neurogenesis and neurodegenerative disease

Neurodegenerative diseases are, by their nature, conditions that deplete the numbers of neurons in particular brain structures leading to a deficit in functionality of the organism, as determined by the purpose of that area of the brain. Parkinson's disease (PD), Huntington's disease (HD), and AD are among these diseases, and ones that have been shown to be linked with levels of neurogenesis in the SVZ and SGZ. In PD, the natural depletion of dopamine is seen to coincide with a reduction in levels of neurogenesis, but proliferation in the SVZ was restored by agonist activity at the D2-like receptor in rodent subjects (Hoglinger *et al.*, 2004). The R6/1 transgenic mouse model of HD is reported to have fewer BrdU positive hippocampal cells when compared to control subjects (Lazic *et al.*, 2004), but this same group of researchers observed higher levels of BrdU positive neural precursor cells in the dentate gyrus of environmentally enriched R6/1 mice when compared to non-enriched transgenic subjects (Lazic *et al.*, 2006). AD is probably most relevant to the research outlined in this thesis, as it is characterised by a decline in cognitive function. A study of the triple transgenic mouse model of AD (3xTg-AD) in comparison to age matched controls from 2 to 12 months of age reported that both Tg and non-Tg mice showed a decline in levels of hippocampal neurogenesis with age, but that 3xTg-AD mice had significantly lower levels by 9 months of age, and neurogenesis had been completely depleted in these animals by 12 months of age (Rodríguez *et al.*, 2008).

1.5 Long term potentiation (LTP)

Long term potentiation refers to the reinforcement of synapses to ensure their lasting viability. It was discovered by Terje Lømo in 1966, and later described in detail (Lømo, 2003). The discovery came from his work on anaesthetized rabbits and the observation that the efficiency of synaptic transmission in cells of the granule layer could be increased substantially, and the effects lasted for hours. The relationship between a pre- and postsynaptic neuron is strengthened by continuous use, thus confirming its importance to the circuitry. Before the idea of neurogenesis was accepted, it seemed likely that reinforcing synapses could explain our ability to form memories (Cajal, 1894).

Agreeing with Cajal, Donald Hebb released *The Organization of Behaviour* in 1949 (Hebb, 1949), and discussed the need for reverberation of stimuli to trigger continued firing and induce structural changes at a synapse, which in themselves will reinforce the repetition of the stimulus. In tandem, these activities lead to the formation of a lasting memory.

1.5.1 Synaptogenesis

There is not much yet known about the exact time frame of adult synaptogenesis. The process is characterised by the formation of new connections between neurons. An environment populated by many newly born neurons is a highly plastic one and synaptogenesis has the potential to be up-regulated. This function lends itself to the process of long term potentiation. Synaptogenesis has been shown to have a link with enhanced cognitive function (Birch *et al.*, 2013). Most research into this process has been focused on environmental enrichment, but studies have shown that physical activity, as a form of enrichment, can also influence the generation of new synapses and/or the up-regulation of synaptic proteins, both in the hippocampus (Hu *et al.*, 2009), and in various motor areas of the brain (Ferreira *et al.*, 2010). Exercise regulates levels of the pre-synaptic protein synapsin-1, in the hippocampus through a rise in BDNF levels (Vaynman *et al.*, 2003). It has also been shown that pre-synaptic density is increased, and correlates to improvement in spatial memory in aged exercised rats (Siette *et al.*, 2012). Postsynaptic density protein 95 (PSD-95) is a postsynaptic protein that is upregulated in tandem with increased levels of BDNF expression and N-Methyl-D-aspartate (NMDA) receptor activation (Yoshii and Constantine-Paton, 2007).

Following synaptogenesis, synaptic density will be high. The synapses themselves will have an increased number of synaptic vesicles and a larger surface area. At this stage the axons making these connections will not possess any specificity, but as they differentiate, they eliminate synaptic connections that are not being utilized. In this way, active learning should make use of these new, and plentiful connections, and memory consolidation should be enhanced.

1.5.2 Early stages of LTP

The process of LTP has two stages, early and late, and can last from minutes to days. Signals from the entorhinal cortex arrive at granule cells of the dentate gyrus, from where they are passed onto the CA3 pyramidal cells, along the Schaffer Collateral axons of these pyramidal cells, to then

synapse onto pyramidal cells of the CA1. The Schaffer Collaterals are axons specific to the CA3 region that project to the CA1.

Post-synaptic NMDA receptors are excited by binding of glutamate, glycine, or D-serine. In the case of glutamate, synaptic vesicles release this neurotransmitter into the CA3-CA1 synaptic cleft as action potentials arrive from the Schaffer Collateral axons. This release of glutamate allows it to bind to post-synaptic NMDA and AMPA (α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors. AMPA receptors will freely open upon binding to permit an influx on Na^+ ions. The NMDA receptor has an Mg^{2+} plug that blocks the receptor from opening even when bound extracellularly by glutamate. Therefore, low levels of glutamate release will not induce synaptic plasticity. However, when a burst of action potentials arrive at the pre-synaptic terminal at high frequency, there is a greater release of glutamate, causing AMPA receptors to remain open for a longer period of time, allowing a greater influx of Na^+ ions. The high levels of Na^+ intracellularly repel the Mg^{2+} blockade, and open the NMDA receptor channel. NMDA receptors permit influx of both Na^+ and Ca^{2+} , and with continuous firing, more Ca^{2+} can enter the CA1 pyramidal cells (Lu *et al.*, 2001).

Calcium ions trigger a range of different events in the post-synaptic environment. In the case of early LTP, the rise in intracellular Ca^{2+} causes an increase in binding of CaMKII (Ca^{2+} /calmodulin-dependent protein kinase), which triggers the translocation of intracellular AMPA receptors to the post-synaptic membrane (Kauer and Malenka, 2006). This increase in AMPA receptor availability only further upregulates the potential for glutamate binding in that moment, and during future instances of depolarization. This stage also results in an increase in protein kinase C (PKC) activation, which acts to further maintain the LTP response (Linden and Routtenberg, 1989) by phosphorylating AMPA receptors and assisting in their translocation to the post-synaptic membrane. Both CaMKII and PKC can interact with MAP kinases, such as ERK (extracellular-signal-regulated kinases) (Kelleher III *et al.*, 2004).

1.5.3 Late stages of LTP

Early LTP will take place within a matter of minutes. Late LTP takes longer to exert its effect as it is triggered by transcription factor expression and protein synthesis in the post-synaptic CA1 neuron. The prolonged increase in localized Ca^{2+} ions activates CREB (cAMP response element-binding protein), which induces these transcriptional changes, and leads to synthesis of growth factors and more AMPA receptors, among other proteins. ERK, activated earlier, can assist with these transcriptional signals. Growth factors can cause the development of new synapses in the area.

There is a theory that retrograde signalling has a part to play in modulating the postsynaptic response to LTP. Neurotransmitters released from the postsynaptic membrane traverse the synaptic cleft to bind to the pre-synaptic membrane. This could be to relay information on the induction of LTP, or to encourage greater glutamate secretion for continued activation of post-synaptic NMDA and AMPA receptors. Although an exact candidate for the role has not been settled upon (Williams *et al.*, 1993), it does seem plausible that this would be taking place. As an overall result of post-synaptic activity and growth-factor induced plasticity, consolidation and long-term retention of new memories is possible.

1.5.4 The impairment of LTP

Richard Morris used the Morris Water Maze (MWM) to demonstrate the importance of LTP in hippocampal-dependent learning in the rat (Morris *et al.*, 1986). This study involved the blockade of the NMDA-receptor, and thus the prevention of hippocampal LTP, as determined by a spatial memory task in the MWM. This task involves a submerged platform in a pool of opaque water, and following a training session, requires the subject to find the platform again in a testing session. By blocking NMDA receptor activity, Morris found that subjects were significantly impaired in their ability to find the platform again, demonstrating a deficit in spatial memory - a process dependent upon the hippocampus.

As is expected, LTP is also susceptible to the detrimental effects of aging (Gooney *et al.*, 2004, Blank *et al.*, 2003, O'Donnell *et al.*, 2000). Gooney *et al.* used infusion of BDNF to trigger LTP and found that when compared to young adults, aged subjects displayed an impairment in TrkB receptor activation, ERK phosphorylation, and glutamate release. Aged controls showed a significant difference in TrkB and ERK activation.

1.6 Neurotrophin signalling

As mentioned, it has been hypothesised that there is a retrograde cascade of signalling taking place across the synaptic cleft in response to LTP. The Trk receptor family have been found to be expressed both pre- and post-synaptically (Aoki *et al.*, 2000, Drake *et al.*, 1999). Both BDNF and its receptor TrkB are reported to have a role in LTP (Gooney *et al.*, 2004).

1.6.1 Tropomyosin receptor kinase

The tropomyosin receptor kinases (Trk) are a family of three tyrosine receptors; TrkA, TrkB, and TrkC. In the periphery, these receptors are found in many tissues, including the heart, lungs, kidneys, and skeletal muscle (Shibayama and Koizumi, 1996, Shelton *et al.*, 1995). However, they are found in larger quantities in the brain, reported to be uniformly distributed in neurons (Kryl *et al.*, 1999), and found on non-neuronal cells, such as microglia, in their truncated isoform. TrkA receptors are found primarily in the striatum and basal forebrain, while TrkB receptors are abundant in most neurons and glia, as well as in the ependymal cells that line the ventricles (Yan *et al.*, 1994). They are each bound by one or more of the neurotrophin family; nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) (Fig. 1.5) (Huang and Reichardt, 2003). They are homomers which dimerize and upon ligand binding, phosphorylate each other, and thereby enhance catalytic activity of the kinase itself.

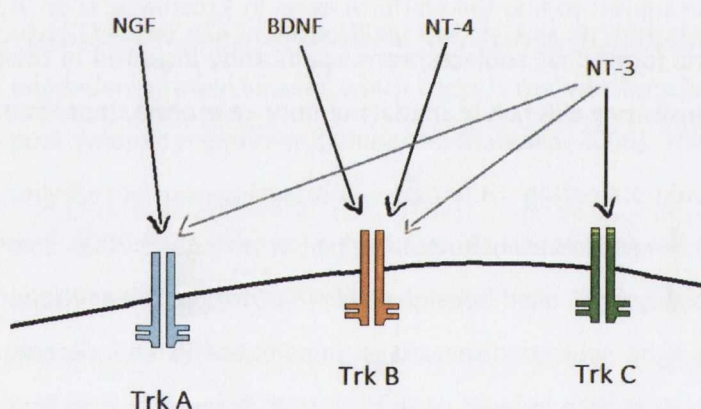


Fig. 1.5 - Trk receptors
Trk family, and their associated ligands.
Adapted from:
<http://www.wako-chem.co.jp/>

The *trkA* gene was the first to be discovered – it was the proto-oncogene of an isolated oncogene containing seven of the first eight exons of non-muscle tropomyosin fused to the transmembrane and cytoplasmic domains of a novel tyrosine kinase (Martin-Zanca *et al.*, 1986). It was named so as it was discovered at the same time as this oncogene, and the *trkB* and *trkC* genes were found to have a high sequence homology to *trkA*. All of these have a part to play in regulation of neuronal growth, proliferation, and survival.

TrkA is bound by NGF and is also very weakly bound by NT-3 (Cordon-Cardo *et al.*, 1991). When bound by NGF, TrkA is internalized and can be targeted for ubiquitination and subsequent destruction (Takahashi *et al.*, 2011). This is thought to be a way of determining the survival of a neuron. Nedd4-2, a ubiquitin ligase enzyme, was found to regulate this behaviour, and upon its depletion there is an increase in TrkA activity and survival of NGF-dependent dorsal root ganglion neurons (Yu *et al.*, 2011). TrkA has been shown to activate the Ras-ERK mitogen activated protein kinase (MAPK) pathway (Segal, 2003).

TrkC is bound by NT-3. Upon ligand binding, it dimerizes and the homomers phosphorylate each other. But TrkC can also phosphorylate substrates within the cell, such as phosphoinositide 3-kinase (PI 3-K). This enzyme can also be phosphorylated by NMDA-activation induced CaMKII activity. Once phosphorylated, it goes on to activate Akt, and recruits mTOR and PSD-95 downstream, among other proteins.

TrkB has an affinity for both BDNF and NT-4, but also weak binding from NT-3. There are different isoforms of the TrkB receptor – one that has the full length intracellular tyrosine kinase structure (TK+), and possibly two that have a truncated intracellular structure (TK-). All three neurotrophins will bind to the normal isoform, TK+, but the TK- isoform is not bound by NT-3 or NT-4, and has decreased BDNF binding affinity (Boeshore *et al.*, 1999). The full isoform carries BDNF’s signal to induce the Ras-ERK (like TrkA), PI 3-K (like TrkC), and PLCγ pathways.

To a lesser extent, all four neurotrophins mentioned here will bind to the p75^{NTR} (neurotrophin receptor) (Chao, 1994), and p75 has been shown to signal in the absence of Trk receptors (Ye *et al.*, 1999). It works with NFκB to induce neural apoptosis, but the presence of TrkA, B, and C receptors on the same cells are anti-apoptotic in nature and are likely to counteract the effect when bound. It is thought that p75 therefore modulates the extent, or onset of apoptosis in cells that are signalling through neurotrophin binding.

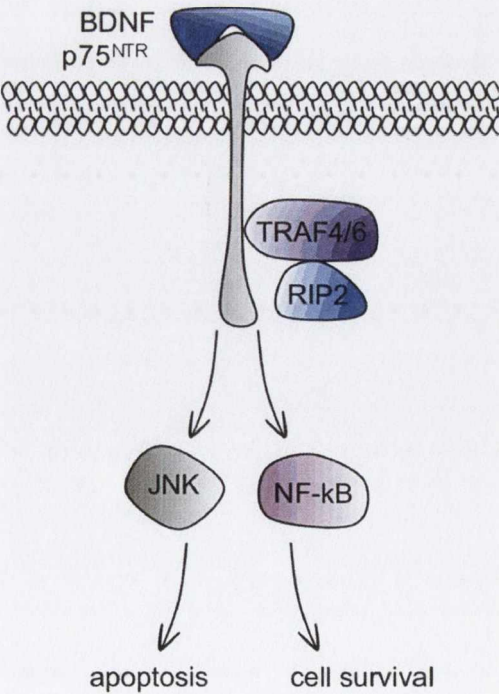


Fig. 1.6 – BDNF signalling – p75
 p75^{NTR} activation via BDNF binding.
Sourced from A simple role for BDNF in learning and memory? (Cunha et al., 2010)

1.6.2 Brain-derived neurotrophic factor (BDNF)

BDNF is the most abundantly expressed and widely investigated neurotrophin. As mentioned, it is a ligand at the TrkB receptor. It is expressed throughout the central and peripheral nervous systems, but is localised abundantly in the pyramidal neurons of the CA1 and CA3 of the hippocampus – areas critical in learning and memory. It is produced in ribosomes of the endoplasmic reticulum – as are most proteins, and is carried around the cell in vesicles. It is secreted from the post-synaptic membrane where it will act upon localised TrkB. Upon binding to TrkB, BDNF stimulates cell survival, differentiation, and plasticity (Svensson *et al.*, 2014). The TrkB receptor is expressed in almost all hippocampal neurons, including CA1, CA3, and dentate gyrus granule cells (Huang and Reichardt, 2001). Upon binding, two homomers of TrkB come together to form a dimer, and phosphorylate each other to further catalyse the signal that BDNF has triggered.

BDNF is integral in supporting neuronal survival, and enhancing growth and differentiation of new neurons also. The essential role it plays in LTP can be seen in BDNF knockout mice (Korte *et al.*, 1995, Korte *et al.*, 1998), where protein synthesis-dependent LTP is greatly reduced. Further investigation into BDNF's effects on LTP showed that its expression was upregulated in tandem with phosphorylation of the NMDA receptor subunit 1 (NR1) (Slack *et al.*, 2004), and that both the ERK and PKC intracellular signalling pathways were involved, albeit in individual capacities (Suen *et al.*, 1997), when BDNF bound to TrkB extracellularly.

1.6.2.1 BDNF signal transduction

When BDNF binds to the TrkB receptor, a series of signalling pathways can be activated within the neuron (Fig. 1.7) (Cunha *et al.*, 2010). Short-term functions of BDNF include neurotransmitter release, and the activation of glutamate receptors in the synapse. The long-term consequences of BDNF binding at the TrkB receptor cause the activation of signalling molecules that trigger intracellular pathways, which modify gene expression downstream in the nucleus.

Autophosphorylation of TrkB dimers induces phosphorylation of phospholipase C gamma (PLC γ) by the phospho-tyrosine 785 residue on TrkB's intracellular domain. PLC γ cleaves membrane bound phospholipid Phosphatidylinositol 4, 5-bisphosphate (PIP $_2$) to produce inositol 1, 4, 5-trisphosphate (IP $_3$) and diacylglycerol (DAG); both second messengers in this pathway. IP $_3$ is released from the membrane and travels to the endoplasmic reticulum (ER) to induce secretion of intracellular stores of Ca $^{2+}$ through IP $_3$ -dependent channels in the ER membrane. DAG remains membrane-bound, and in conjunction with the released calcium, activates PKC, which goes on to phosphorylate various

proteins, and allow even more calcium into the neuron. Upregulated levels of intracellular calcium affect synaptic plasticity and LTP, as described.

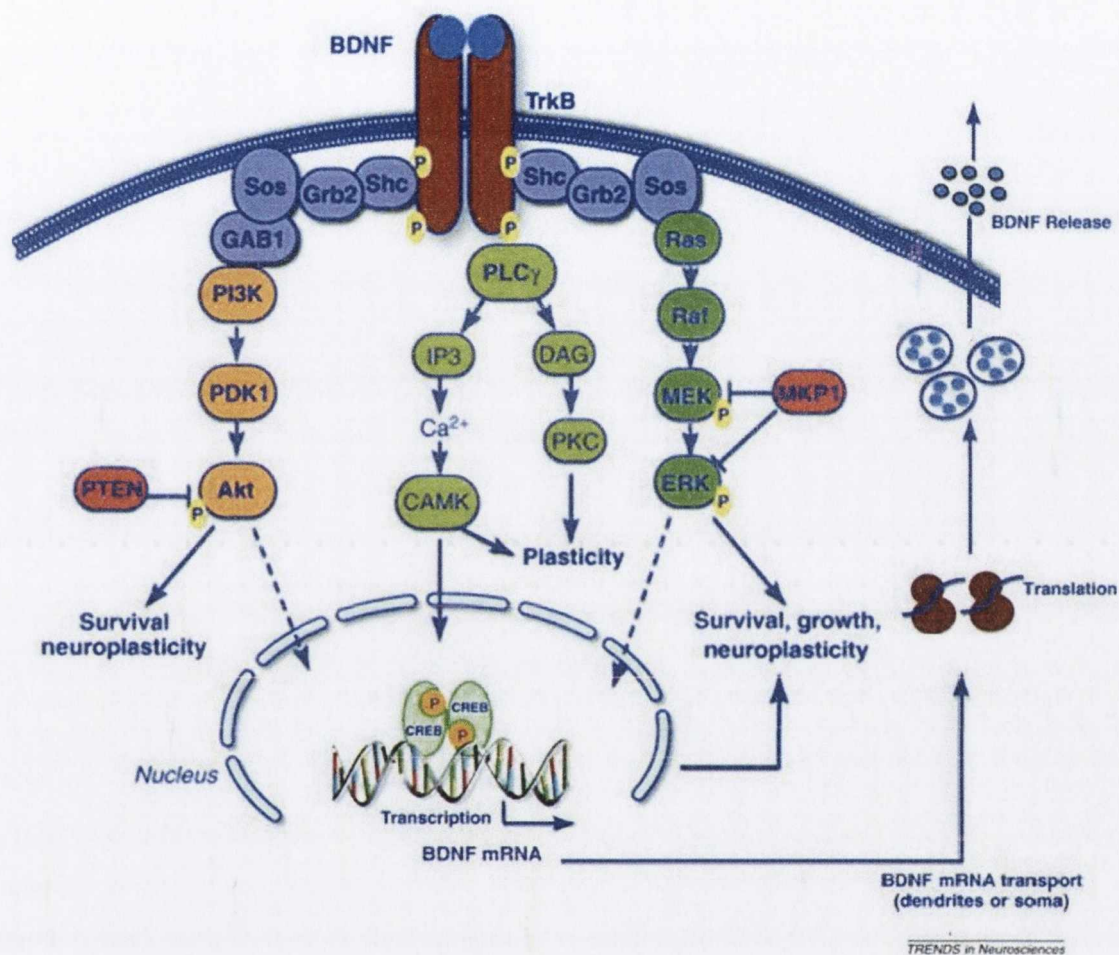


Fig. 1.7 – BDNF signalling - TrkB
 TrkB activation triggers signalling via PLCγ, PI3-K, and ERK.
 Adapted from (Duman and Voleti, 2012)

The phospho-tyrosine 515 residue can phosphorylate the Src homology (Shc) adaptor protein, which recruits the Grb2/SOS complex (growth factor receptor-bound protein/sons of sevenless). This complex induces phosphorylation of membrane-bound Ras. Inactive Ras is bound to GDP (guanosine di-phosphate), but when phosphorylated by Grb2/SOS, is bound to GTP (guanosine tri-phosphate) and is activated. Ras can then exert its effects by activating Raf. Raf phosphorylates and activates MEK 1 and 2, and MEK 1 and 2 phosphorylate and activate ERK 1 and 2. ERK goes on to phosphorylate CREB (cAMP response element-binding protein). CREB is also upregulated by increased intracellular levels of calcium. It is a transcription factor, and can regulate the

transcription of the BDNF gene. In this way, BDNF is signalling for the upregulation of its own synthesis. Through this interaction, CREB is known to be of importance in long-term, but not short-term memory (Silva *et al.*, 1998).

Activation of the Grb2/SOS complex can also activate the PI3-K/Akt pathway through the recruitment of Grb2-associated binder-1 (GAB1). PI3-K has been found to assist in inserting AMPA receptors into the post-synaptic membrane during neurotransmission of glutamate (Man *et al.*, 2003). Active PI3-K translocates to the cell membrane to phosphorylate membrane-bound PIP₂, transforming it to PIP₃. In this phosphorylated state, PIP₃ can activate PDK1 (Phosphoinositide-dependent kinase-1), resulting in the activation of the kinase Akt, which in turn blocks the inhibitory activity of the TSC1/TSC2 complex on Rheb and mTOR, allowing for their action (not shown in Fig. 1.7). This PI3-K/Akt/mTOR pathway has been found to be vital to the proliferation of adult neural stem cells (Peltier *et al.*, 2007), as it plays a part overall in cellular proliferation, survival, and apoptosis. Peltier *et al.* also found that down regulation of Akt limited the activity of CREB. Overexpression of CREB can dangerously increase the rate of proliferation, so this pathway is thought to regulate neuronal propagation.

1.6.2.2 BDNF and memory

BDNF is intrinsic in many processes that go on to influence learning and memory in various ways. This could be with a decrease in peripheral levels of BDNF in depressed patients (Karege *et al.*, 2002), or decreased levels of cortical BDNF in patient in the pre-clinical stages of AD (Peng *et al.*, 2005). When functioning properly, the system has an overall positive effect by stimulating protective pathways and limiting damage to neurogenesis and long-term potentiation (Almeida *et al.*, 2005, Radecki *et al.*, 2005). It is for these reasons that so much research has gone into enhancing BDNF signalling throughout the nervous system.

Performance in a spatial memory task was found to be positively correlated to hippocampal BDNF concentration (Molteni *et al.*, 2002). Bekinschtein *et al.* showed that intra-hippocampal infusion of BDNF reversed deficits in the consolidation of long-term memories, caused by inhibition of hippocampal protein synthesis (Bekinschtein *et al.*, 2008). Heldt *et al.* silenced BDNF specifically in the hippocampus and found it to have a detrimental effect on spatial memory (Heldt *et al.*, 2007), and Callaghan and Kelly showed that blocking the Trk receptors manifested an impairment in both long-term and short-term memory processes (Callaghan and Kelly, 2013). The literature solidifies the importance of the role BDNF plays in cognitive function.

1.6.2.3 BDNF and neurogenesis

The direct involvement of TrkB receptors in embryonic neurogenesis has been documented (Bartkowska *et al.*, 2007), and in adult neurogenesis deletion of full-length TrkB receptors has been reported to directly compromise cell survival and impair neurogenesis-dependent LTP (Bergami *et al.*, 2008). Intra-hippocampal infusion of BDNF increased neurogenesis in the adult rat (Scharfman *et al.*, 2005), and the literature consistently reports a simultaneous increase (Marlatt *et al.*, 2012) or decrease (Kikusui *et al.*, 2009) in both BDNF and neurogenesis when either one is targeted for therapeutic effect. Thus, the intricate relationship between BDNF/TrkB signalling and neurogenesis is undisputed in the field of cognitive function.

1.7 The immune response

The immune system harnesses a network of cells, tissues, and organs working in tandem to protect the body from the threat of disease. In the event that a foreign microorganism has gained entry into the body, and is distinguished as being different to the body’s own healthy tissue, the immune system orchestrates a response to eradicate any threat that they may pose. The initial reaction is called the innate immune response, and is rapid and non-specific, in an effort to quickly remove any foreign pathogens. This response is induced when pattern-sensing macrophages detect an indicator of stress from damaged or infected cells, move to the infected area and commence phagocytic ingestion of the foreign microorganism. During this process, the macrophage presents antigens that signal to other cells and trigger the migration of cytokines, chemokines, prostaglandins and interferons to the site of the problem. This process generates inflammation in the area (Kawai and Akira, 2006).

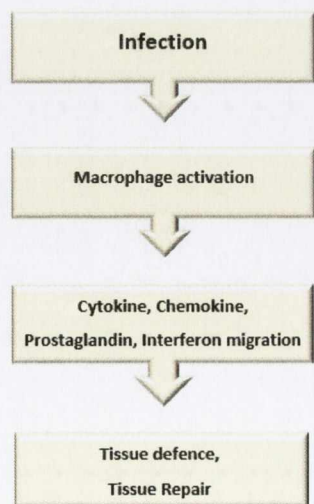


Fig. 1.8 – Innate immune response
Arrival of a foreign microorganism triggers the innate immune response to prevent further damage from the invasion, and repair tissue.

1.7.1 Inflammation

Inflammation is a protective response of the body characterised by redness, heat, and swelling, and is an indication that the immune system is functioning correctly. This is as a result of the increase of blood-flow to the infected area, which is required to carry white blood cells (leukocytes) and antibody-rich plasma to the affected tissue in a bid to clear away any damaged or necrotic cells, and facilitate the onset of tissue repair (Le *et al.*, 2004).

Acute inflammation is an immediate response to infection and can last from a few hours in duration to a few days, beginning to subside once the intrusion has been eliminated. Chronic inflammation manifests as a result of persistent acute inflammation, triggered by frequent infection, or an upregulation in cell death, among other things. Chronic inflammation can last for months, and even years.

1.7.2 Inflammation and aging

Unfortunately, inflammation and aging are intricately linked (Cevenini *et al.*, 2010), in a relationship now widely referred to as 'inflammaging' (Franceschi and Campisi, 2014). As the body ages, there is a natural loss of functionality in organs of the body brought on by a decline in the cellular population, and translating to an increase in apoptotic cells (Jenny, 2012). Without adequate clearance, there can be a build-up of cellular debris, reactive oxygen species and free radicals, triggering an exaggerated response for their elimination, and this is noted as one of many causes for a persistence of low-level inflammation in these areas of the body. Its persistence could also be attributed to a leakage of microbiota from the gut, or an upregulation in cellular senescence with age (Franceschi and Campisi, 2014).

Although the process of innate immunity is intended to be protective and of benefit to the body, an excessive inflammatory response can cause or contribute to tissue damage and disease pathology (Lyman *et al.*, 2014). With age, there are many conditions characterised by low-grade inflammation, including arthritis, atherosclerosis, type-2 diabetes, PD, and AD (Reale, 2014).

1.7.3 Blood Brain Barrier (BBB)

The brain is protected from substances circulating in the peripheral vasculature by the presence of the blood-brain barrier. This is made of an intricate network of vessels so tightly lined with endothelial cells that the membrane is almost entirely impenetrable. Its discovery came in 1885, when Paul Ehrlich injected a Trypan blue dye into the bloodstream of mice, and found that everywhere in the body was dyed blue, with the exception of the brain (Ehrlich, 1885). Following this, his student injected the same Trypan blue dye directly into the brain, and observed that the dye had been restricted to the brain and spinal cord, and all other tissue remained unstained (Goldmann, 1913).

1.7.4 Immunity in the central nervous system

With the exception of tiny and/or fat soluble molecules, few molecules can gain entry into the brain from the periphery. For this reason, the brain requires its own immune system. The first layer of protection for the central nervous system (CNS) is the BBB itself, just as the skin and mucosa work to keep microbes out of the periphery. If a pathogen does gain entry to the brain, via protein carriers or active transport, then the CNS has its own resident population of immune cells. Microglia, astrocytes, and oligodendrocytes work in tandem as a secondary barrier in the CNS, just as macrophages work in the periphery.

In the normal, healthy brain, microglia exist primarily in a state of surveillance. They account for approximately 10% of the CNS cell population, and their quiescent role is the maintenance of homeostasis in and around cells, synapses, and myelin (Benarroch, 2013). Their 'resting' form allows for this; a ramified shape, with many processes reaching out and investigating the environment. During times of injury in the CNS, microglia alter their morphology to an amoeboid state, using pseudopodia to engulf foreign organisms and remove them from sites of infection, reminiscent of macrophage activity in the peripheral immune system (Ferreira and Bernardino, 2015). However, because immunity in the brain is so tightly controlled, microglia are extremely sensitive to even minor changes in the system that could be interpreted as detrimental to the healthy brain.

The state of microglial activation coincides with an increase in proinflammatory cytokine secretion (Liu and Hong, 2003), signalling to nearby cells to mobilize and assist in protection. Interleukin-1 (IL1) and Tumour Necrosis Factor (TNF) are examples of proinflammatory cytokines (Fig 1.9). The presence of ionized calcium-binding adapter molecule-1 (Iba1) has been found to distinguish

between the active (amoeboid shape) and inactive (ramified shape) state of microglia (Deininger *et al.*, 2002).

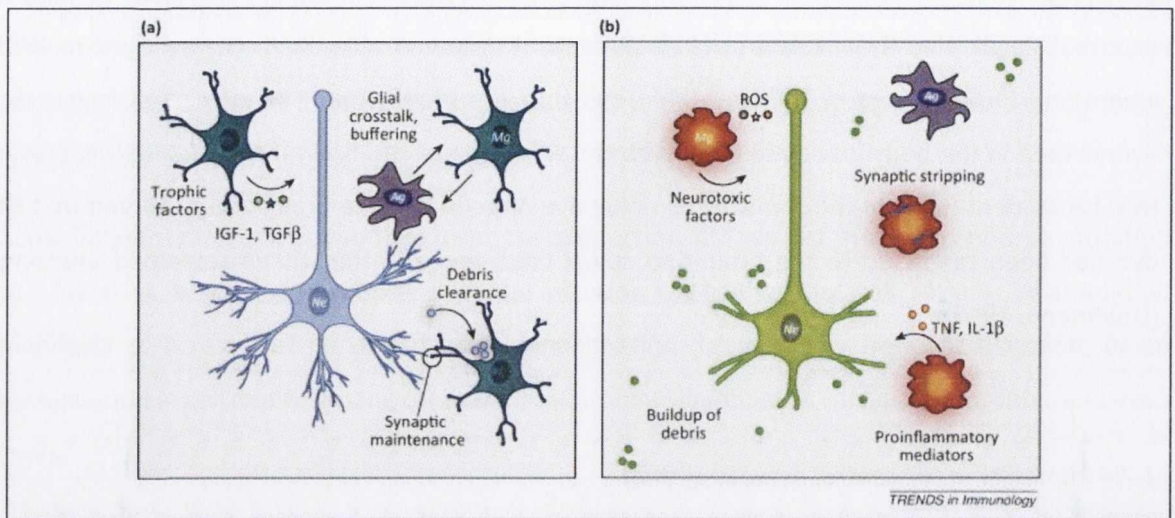


Fig. 1.9 – Microglial function

a) In their quiescent (ramified) state, microglia maintain homeostasis in the brain, carrying out functions including phagocytosis of neuronal debris, secretion of trophic factors, and synaptic maintenance.

b) In response to injury or infection, microglia become amoeboid in shape and secrete pro-inflammatory factors and reactive oxygen species

Sourced from (Derecki *et al.*, 2013)

1.7.5 LPS and neuroinflammation

Lipopolysaccharide (LPS) is a gram-negative endotoxin, found as a component of bacterial cell walls (Lyman *et al.*, 2014). It is frequently used to trigger inflammation in animal models (Hoshino *et al.*, 1999, Saban *et al.*, 2001) due to its affinity for Toll-like receptor 4 (TLR4) (Politorak *et al.*, 1998). TLR4 is expressed abundantly on the surface of astrocytes and microglia, and so is vital in the activation of the innate immune response by triggering the release of IL1 β and TNF α and thus modulating proinflammatory mechanisms. LPS has been shown to exert its neuro-inflammatory effect through synaptic dysfunction, neuronal death, prevention of neurogenesis (Wu *et al.*, 2012), and ultimately impairment in cognitive function.

IL1 β activity is mediated through binding of the IL-1 receptor-1. In rodents, this receptor is found on the surface of neurons and glial cells throughout the brain. Neuronal expression is primarily in the dentate gyrus of the hippocampus, with some cells found in the amygdala and hypothalamus. Glial expression is focused along major brain barriers such as the choroid plexus, meninges, and

endothelial cells of brain capillaries (Ban, 1994). Signalling cascades triggered by binding at the IL-1R include the activation of p38 MAPK (mitogen-activated protein kinase) (Ridley *et al.*, 1997), a kinase that is involved in apoptosis and autophagy, and activation of nuclear factor κ B (NF κ B) which is important in cytokine induction and cell survival (Basu *et al.*, 1998).

Upon binding to its receptor; TNF receptor-1, TNF α triggers downstream signalling in reactions that alter gene transcription, which in turn activate transcription factors such as NF κ B and cause further upregulation in inflammation, apoptosis, and neurodegeneration (Hohmann *et al.*, 1990). These receptors are found throughout the brain on the cell surface of neurons, astrocytes, microglia, and endothelial cells (Botchkina *et al.*, 1997). The expression of both IL-R1 and TNF-R1 receptors along the fluid barriers into the brain means that they are in a position to respond quickly to insult or injury to the CNS.

1.7.6 Neuroinflammation, cognition and BDNF

An exaggerated immune response has been documented in the aged brain of rodent models (Kohman and Rhodes, 2013, Chen *et al.*, 2008), and has been shown to impair cognitive function in younger animals as tested by spatial memory tasks in genetically modified mice with prolonged expression of IL1 β , and in LPS-treated rats (Moore *et al.*, 2009, Rosi *et al.*, 2006). A comparison of young and aged mice showed that aged animals had a significantly increased susceptibility to cognitive impairment triggered by LPS administration (Sparkman *et al.*, 2005).

It is known that both resting and activated microglia can express BDNF (Patterson, 2015), and selective activation of microglia to produce BDNF without the concurrent release of cytokines has also been demonstrated (Nakajima *et al.*, 2002). There are studies that report a decrease in BDNF expression following LPS treatment (Guan and Fang, 2006), while others report no change in its expression, even when cognitive impairment was observed (Zhu *et al.*, 2014).

1.8 Enrichment; environmental and physical

1.8.1 Environmental enrichment

The stimulation of a diverse environment is crucial for the brain to carry out its proper functions (Falkenberg *et al.*, 1992). In many cases of young children it has been found that abandonment,

abuse, and neglect will impact negatively on their cognitive ability later in life, and can quite easily manifest as something much more severe as they develop fully (Dubner and Motta, 1999). The case is quite similar when it comes to the adult stages of life, even though this is not strictly a time concerned with development. Social isolation, especially in the elderly, is reported to result in a greater decline in cognitive ability (Fratiglioni *et al.*, 2000, Wang *et al.*, 2002), and physiological health in general (Shankar *et al.*, 2011) when compared to the effects of normal aging. However, this effect has been shown to be blunted in patients who had reached a higher level of education, or worked in careers that were mentally demanding, and thus stimulating. Lower levels of education are reported to result in poorer cognitive performance in old age (Lee *et al.*, 2003). Being engaged sociably, or playing games that are mentally invigorating are found to have a positive effect. An active life full of mental enrichment can assist in slowing down the onset of diseases like Alzheimer's (Archer, 2011), but also in easing the decline in cognitive ability with aging as a whole.

1.8.2 Exercise

In concordance with the positive effects of enrichment, it has been found that physical exercise throughout adult life and into old age has a beneficial effect by slowing the onset of dementia and other neurodegenerative diseases (Radak *et al.*, 2010) as well as enhancing neurogenesis (van Praag *et al.*, 2005). Stimulation is found to rescue this depletion of neurons. The idea of leading an active and socially integrated life has been investigated from various angles (Fratiglioni *et al.*, 2004), and conclusions have been drawn as to the link between both stimuli in relation to slowing down cognitive decline. It has been reported that a combination of physical exercise and other environmentally enriching factors have an additive effect (Fabel *et al.*, 2009). Similar results were also obtained in studies with animal models benefitting from either one of these factors alone, or a mixture of both simultaneously (Simpson and Kelly, 2011). In addition to this, it has been reported that exercise has not only an acute effect on cognitive function, but a long term one too (Kempermann *et al.*, 2002) indicating that the beneficial effects of physical activity have the capacity to persist.

Numerous studies have indicated that a generally enriched quality of life; with regard to physical activity and the setting in which subjects live (and thus, the mental stimulation available) has the ability to prevent age-related decline in learning and memory, induced by an increase in neurogenesis and enhanced neuronal plasticity and synaptogenesis (Fabel *et al.*, 2009, Kempermann *et al.*, 2002).

Aside from the physical benefits of strength and cardiovascular fitness to the body as a whole, exercise also increases blood and oxygen flow to the brain, supplying its high oxygen demand (Doering *et al.*, 1998). Improvements in cardiovascular health also reduce the risks of myocardial infarction, as well as cerebral infarction, or stroke (Sargent *et al.*, 2004, Daviglus *et al.*, 2003, Strandberg *et al.*, 2004).

1.8.2.1 Types of exercise

There have been a number of methods used to induce, and thus quantify, changes in the number of newly born neuronal cells in animal subjects. Depending on the investigation, researchers have been known to utilize a forced swim technique, based in a Morris Water Maze, but without a platform for the mice to rest upon (Llorens-Martin *et al.*, 2007). This approach would generally be used in determining a correlation between exercise, stress, and neurogenesis. Rodent subjects would find having to either stay afloat or swim to be rather stressful and so generally this protocol is employed as an experimental stressor as opposed to a form of physical activity.

Running is the most commonly utilised mode of physical exercise. Rodent subjects are assigned to exercising and non-exercising groups. Running animals are placed on a treadmill (Kim *et al.*, 2010) and will run for a set time every day. In order to encourage movement, the end of the treadmill belt is equipped with looped wires that emit a mild shock if animals cease activity and sit on the treadmill long enough that they are carried backwards to these wires. Simultaneously, the non-exercising animals will remain on a stationary treadmill for the same length of time. This ensures they are exposed to the same environment as the exercised animals, but without exercising, and excludes any underlying effect of exposure to a novel environment on a daily basis.

Alternatively, voluntary running can be employed, by means of a running wheel installed in, or adjacent to, the subjects' cages (Bednarczyk *et al.*, 2009, Kitamura and Sugiyama, 2006, Kohman *et al.*, 2012). Rotations of the wheel are counted, and distance run can be calculated by a formula unique to each brand of wheel. In this way, exercise is chosen to be undertaken by the subject, maybe with breaks in between, and at night time when they are most likely to be active. This is found to add to the environmental enrichment they experience, as opposed to the effects of a stress-inducing one where they are forced to run or swim. Unless stress was being investigated, this could negatively skew results, as stress is known to reduce the level of proteins such as BDNF (Altar, 1999), that trigger neurogenesis. However, the problem that comes with exercising by choice is that animals would have to be housed separately during their exercise regime to allow for precise

monitoring of each animal's individual distance. Being subjected to solitary housing is a known stressor (Heinrichs and Koob, 2006, Marchlewska-Koj, 1997).

1.8.2.2 Limiting exercise

The effect of a lack of exercise has been investigated in the adult rat using hind-limb suspension, leaving the subject unable to move properly, and so incapable of exercising during the experimental timeline and at risk of depression (Yasuhara *et al.*, 2007). The results from this study showed a marked decrease in neurogenesis, as determined by BrdU tagging of newly synthesised neurons. It was observed that the number of BrdU positive neurons was significantly different in comparison to control subjects who had not had their hind-legs suspended. Stress was ruled out as a contributing factor using a measure of plasma corticosterone levels that showed no significant changes in levels of this glucocorticoid in hind-limb suspended subjects when compared to controls. This successfully displayed that neurogenesis was much lower in incapacitated subjects compared to those that were free moving. Alternatively, cessation of exercise following a physically active period could replace the model of hind-leg suspension (Nishijima *et al.*, 2013) and was shown to impair hippocampal neurogenesis in the adult mouse. Elderly human subjects who received rehabilitation therapy had more success in recovery than those who remained bedridden (Tinetti *et al.*, 2002).

1.8.2.3 The anti-depressive effects of exercise

Depression and the formation of new neurons have been shown to be intricately linked (Surget *et al.*, 2008), with depression impairing levels of newly generated neurons in the adult mammalian dentate gyrus (Simon *et al.*, 2005), and treatment with exercise quite substantially alleviating the symptoms of acute depression (Brene *et al.*, 2007). Physical activity has been shown to retain an anti-depressive effect by inducing cell proliferation in the hippocampus (Bjornebekk *et al.*, 2005). Loss of hippocampal volume is one feature of depression (Hattori *et al.*, 2007) and so it may be the case that recovering this loss can assist in combating the disorder itself. As is a major trend in treatment thus far, Hattori *et al.* (2007) have reported that environmental enrichment in general will further enhance any anti-depressive benefits. However, patients do report that a relapse into depression is less likely to occur if they engage in exercise as an antidepressant as opposed to when they take antidepressant drugs and attend psychotherapy. Exercise has been found to be just as

beneficial as taking antidepressants (Brene *et al.*, 2007) and as it is a naturally rewarding behaviour, is capable of influencing changes in circuitry of the brain without the side-effects of potential dependency. The effects of stress and anxiety; being similar in ways to depression, and quite often leading to the development of some form of depressive behaviour, could also be mitigated by the positive effects of exercise (Mitra *et al.*, 2006).

1.8.2.4 Exercise and aging

Exercise increases a capacity for learning, as has been reported (Ploughman, 2008). Although there have been fewer studies investigating the effects of exercise in older rodents, it was found that just one month of voluntary wheel running could increase neurogenesis and enhance cognitive function as tested by MWM in aged mice, even though they had been sedentary until 19 months of age (Van Praag *et al.*, 2005). Similarly, 23 month old rats were subject to treadmill exercise for 7 weeks and displayed an improvement in cognitive ability when tested in the MWM (Albeck *et al.*, 2006). Kim *et al.* compared 5 and 24 month old rats after 6 weeks of treadmill running, and spatial memory testing showed a marked decrease in cognitive decline in the exercised aged animals (Kim *et al.*, 2010). Also of note is the effect of exercise both centrally and peripherally on levels of inflammatory markers. A decrease was observed in the activation of hippocampal microglia observed in 22 month old mice who had undertaken 10 weeks of wheel running (Kohman *et al.*, 2013). An epidemiological study reported an increase in inflammatory cytokine concentration in the blood plasma of less physically active individuals when comparisons were made across a group whose age ranged from 70 – 79 years old (Taaffe *et al.*, 2000), and a study of patients with chronic heart failure showed a marked reduction in levels of peripheral inflammatory markers following a 12-week programme of physical exercise (Adamopoulos *et al.*, 2001).

1.8.2.5 Exercise and BDNF

There are numerous reports of increases in BDNF being linked to enhancements in cognitive function, as a result of various types of exercise ranging in intensity and length of experimental intervention. However, the exact mechanisms by which this change in BDNF concentration is mediated are still being debated. As BDNF is so abundantly expressed in the hippocampus, it is inevitable that acute modulation of its concentration would correlate with alterations in hippocampal-dependent processes. This was demonstrated using a single nucleotide

polymorphism in human BDNF that interfered with normal BDNF function, and correlated with diminished hippocampal-dependent cognition (Hariri *et al.*, 2003).

Linking a reduction in hippocampal volume in old age with spatial memory deficits and an increased risk of dementia, Erickson *et al.* showed that aerobic exercise was able to increase hippocampal volume by 2%, enhance spatial memory, and increase serum BDNF concentration in a human study of 120 older adults (Erickson *et al.*, 2011). A human study of younger adults showed an increase in serum BDNF levels in an exercise intensity-dependent manner (Ferris *et al.*, 2007). An increase in hippocampal BDNF mRNA expression in the rat after just six hours of wheel running has been observed, and again reported a positive correlation with distance run (Oliff *et al.*, 1998). In adolescent rats, six weeks of either voluntary wheel running or involuntary treadmill running were both found to increase levels of hippocampal BDNF, together with an improvement in cognitive function when both exercise groups were compared to sedentary subjects (Uysal *et al.*, 2015). More recently, an increase in BDNF mRNA and protein levels were observed in the dentate gyrus of mice that had taken part in four weeks of either running wheel or treadmill exercise, and coincided with a change in astrocytic morphology and orientation of their projections towards granule cells of the dentate gyrus (Fahimi *et al.*, 2016). This was reported in tandem with an increase in levels of TrkB receptors on astrocytes themselves, and could be an interesting avenue of investigation if the results discussed from Nakajima *et al.* (2002) are also taken into account - a mechanism of BDNF and TrkB signalling that is modulated by microglia and astrocytes.

1.9 Mimicking the effects of exercise

Exercise is not a viable option for everybody. Physical or mental disability could render one incapable of benefiting from activity to the same extent as someone both physically and mentally healthy. Society also makes it increasingly more convenient to forgo physical activity altogether, which does not bode well for the aging populations of Western Europe and North America. Optimistically in response more-so to the former issue than the latter, there is a need for the possibility to benefit from the positive effects of exercise, without requiring actual exercise. As described, BDNF exerts the effects of exercise throughout the nervous system, so it makes sense that this protein could be targeted to mimic these effects. By targeting this physiological response, there is an opportunity to enhance quality of life for people with disabilities, neurodegenerative diseases, and the aging population as a whole.

A simple answer would be to administer BDNF itself in order to upregulate its availability within the nervous system. Unfortunately, proteins are difficult to get to their desired location fully intact. Neurotrophins are notorious for having a short *in vivo* half-life (this can range from 1 to 10 minutes, and sometimes even less), poor bioavailability once they have been proteolysed or hydrolysed, and limited permeability at biological barriers, such as the BBB (Géral *et al.*, 2013, Ochs *et al.*, 2000). If BDNF were given orally, it would be indistinguishable from any other protein taken orally, for example in ingested meat, and would be broken down shortly after consumption. Géral *et al.* also state that injecting a neurotrophin will also encounter problems, and it would be wise to further investigate some kind of protein carrier that could protect BDNF from degradation before it even gets the opportunity to carry out its therapeutic effect, but even the use of carriers only extended the half-life without affecting BBB penetration. Therefore, there is much interest in finding a compound that can exert the same physiological effects as BDNF, but which could also satisfy the criteria for successful delivery (Stranahan *et al.*, 2009).

The discovery of many compounds that boast ease of delivery and TrkB-specific activation have been reported, (O'Leary and Hughes, 2003, Longo and Massa, 2006, Massa *et al.*, 2010, Zeng *et al.*, 2012b), and among them is the small peptide, 7,8-dihydroxyflavone (7,8-DHF), discovered by Jang *et al.* in 2010. They screened 2000 compounds from the Spectrum Collection Library, searching for a small biologically active compound to mimic the effects of exercise through TrkB activation, in place of BDNF (Jang *et al.*, 2010). They created a cell line; T48, that would over-express TrkB. T48 cells were generated by fusing murine olfactory bulb neurons with N18TG2 neuroblastoma cells to create a hybrid; SN56, which expressed negligible levels of TrkB. These cells were then transfected with rat TrkB, to produce TrkB expressing T48 cells. Using this cell line, they found 66 compounds that protected T48 cells from apoptosis, but did not protect cells from the parental line SN56. 5 of these 66 compounds were some form of flavone, and upon further investigation, 7,8-DHF was found to be the strongest suppressor of apoptosis in the T48 cell line, and with an EC₅₀ of 35nM it was even stronger than BDNF. 7,8-DHF was also found to induce phosphorylation of the Y490 tyrosine residue of TrkB to the same extent as BDNF, and even Akt and ERK1/2 were robustly activated downstream.

Shortly after the discovery of 7,8-DHF, the same lab observed the effects of 7,8-DHF *in vivo* (Andero *et al.*, 2011) and reported an activation of TrkB receptors in the mouse amygdala, along with an enhancement in both the acquisition and extinction of fear in the fear conditioning test.

1.9.1 TrkB agonist; 7,8-DHF

7,8-DHF is a small synthetic peptide extracted from leaves of the *Primula* tree in the form of *Godmania aesculifolia* (Colombo *et al.*, 2014), and the flowering plant classified as a weed; *Tridax procumbens*, or tridax daisy (Abubakar *et al.*, 2012). These plants grow abundantly in both Central and South America. Flavone consumption in the human diet is estimated at about 20 – 50mg per day (Cermak and Wolfram, 2006), but this quantity is much too low to exert any therapeutic effect.

7,8-DHF is an agonist at the TrkB receptor, thus mimicking the binding of BDNF. It shows no binding affinity for either TrkA or TrkC receptors, much like BDNF (Liu *et al.*, 2016). To date, its potential to weakly bind to the p75^{NTR} receptor has not been investigated. The catechol group C₆H₄(OH)₂ (Fig. 1.10) is critical for 7,8-DHF's agonistic activity (Liu *et al.*, 2012), and in particular the presence of a hydroxyl group on the 7th carbon. Its small size and lipophilic nature are therapeutically ideal in comparison to cumbersome BDNF molecules, and these characteristics help 7,8-DHF to cross the BBB. It also has a higher percentage of oral-bioavailability than BDNF. With a K_d constant of 320 nM, 7,8-DHF has a much higher affinity for TrkB binding compared to BDNF's K_d of 1.7 nM (Jang *et al.*, 2010, Liu *et al.*, 2016).

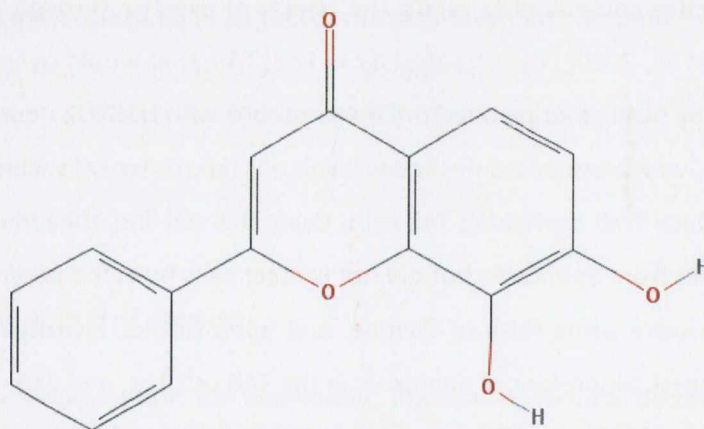


Fig. 1.10 - 7,8-DHF

IUPAC Name: 7,8-dihydroxy-2-phenylchromen-4-one, molecular formula: C₁₅H₁₀O₄

Source: <http://pubchem.ncbi.nlm.nih.gov>

1.9.2 7,8-DHF and cognitive function

Just as with BDNF, 7,8-DHF binds and causes dimerization and autophosphorylation of TrkB homomers, inducing intracellular signalling cascades (Jang *et al.*, 2010). Jang *et al.* reported its ability to protect wild-type, but not TrkB-deficient neurons from apoptosis. As mentioned, 7,8-DHF was found to activate TrkB receptors in the amygdala, and to enhance fear conditioning, but also assist in its extinction (Andero *et al.*, 2011) - demonstrating its effectiveness in emotional learning. This was also found in aged rats, showing how 7,8-DHF could rescue age-related deficits in emotional learning (Zeng *et al.*, 2012a). Zeng *et al.* also reported that 7,8-DHF reversed age-related decline in spatial memory and synaptic plasticity, though the treatment had a more profound effect on rats at 22 months of age when compared to advanced aging at 30 months (Zeng *et al.*, 2012b). This supports the benefit of early treatment, as the therapeutic effect is more pronounced.

With regard to neurodegenerative disease, 7,8-DHF was found to improve spatial memory in the APPswe/PS1dE9 mouse model of AD (Bollen *et al.*, 2013). This study also reported a similar enhancement to spatial memory in healthy controls, leading them to conclude that the acute effect may be just that, as opposed to having a curing effect. However, scopolamine-induced Alzheimer's symptoms were also found to be improved following 7,8-DHF administration – reducing A β (amyloid beta) deposition, oxidative stress, synaptic dysfunction, and cognitive defects (Chen *et al.*, 2014).

7,8-DHF seems to be promising on all fronts so far.

1.10 Overall objective of this research

As our population ages, and the causes and aetiology of neurodegenerative diseases remain somewhat elusive, there is increasingly more truth in the fact that prevention is better than cure. There has been a lot of research into long-term protocols for modulating the beneficial effects of BDNF, but acute effects of short-term modulation, or discerning changes at a significant time-point for neurogenesis will provide key information that is certainly just as important in enhancing cognitive function.

Neurogenesis is an increasingly important area of research and the fact that it has only been so recently recognised makes it an exciting topic. The various methods that have been developed to quantify levels of new neurons, from the H³Thymidine analogue to the more frequently used BrdU

tagging, mean that it is easily monitored in different experimental scenarios. Synaptogenesis and LTP are crucially important during critical periods of life. Once these connections have been upregulated, they need to be reinforced to ensure their survival. Otherwise, they will be lost through synaptic pruning - a process that removes any connections that are unused, and consequently are an unnecessary expenditure to maintain. This demonstrates the necessity for continued stimulation.

The use of a pharmacomimetic to deliver the same physiological benefits of physical activity follows a natural progression in our need to gain a better understanding of and more control over the challenges we will face as we age, hopefully ensuring a better quality of life for all, and exposing those incapable of sufficient physical activity to the benefits of BDNF also.

The research in our laboratory to date has focused on work with rats. Unfortunately, transgenic models in the rat are not yet as advanced as they are in the mouse. As we progress into work with mice we hope to replicate some of our findings so far, but most importantly we hope to broaden our understanding of the manipulation of exercise and 7,8-DHF administration (or similar) in both young and adult subjects.

1.10.1 Hypotheses

1. We hypothesise that one month of treadmill exercise can protect against experimentally-induced neuroinflammation in the mouse, and will rescue disturbances in cognitive function triggered by this acute inflammatory response.
2. We hypothesise that one month's administration of the TrkB agonist; 7,8-DHF, will replicate the neuroprotective effects of exercise that have been reported in a mouse model treated with LPS, and will rescue disturbances in cognitive function caused by this inflammatory response.
3. We hypothesise that long-term administration of the TrkB agonist; 7,8-DHF, will protect the mouse throughout a 9 month study of the effects of aging on spatial and episodic memory.

Chapter 2

Materials and Methods

2.1 Materials

Animals

C57/BL6 mice (male, 3-12months old)	Comparative Medicine, TCD
Irradiated animal pellets	LabDiet, St. Louis, MO, USA
Tecniplast 1285LN ventilated cages	Tecniplast UK Ltd, Kettering, UK

Animal Treatments

5-bromo-2'-deoxyuridine (BrdU)	Sigma Aldrich, Wicklow, Ireland
7, 8-Dihydroxyflavone hydrate	Tokyo Chemical Industry Ltd, Oxford, UK
Lipopolysaccharide from <i>E. Coli</i>	Sigma Aldrich, Wicklow, Ireland

General Laboratory Chemicals

Acrylamide	Sigma Aldrich, Wicklow, Ireland
Ammonium persulfate	Sigma Aldrich, Wicklow, Ireland
Aprotinin	Sigma Aldrich, Wicklow, Ireland
BCA Protein Assay Kit	Thermo Fisher Scientific Inc.
Bovine Serum Albumin (BSA)	Sigma Aldrich, Wicklow, Ireland
Bromophenol blue	Sigma Aldrich, Wicklow, Ireland
Buffer, reference standard; pH 4, 7, 10	Sigma Aldrich, Wicklow, Ireland
Calcium chloride (CaCl ₂)	VWR International Ltd., Dublin, Ireland
Dimethyl sulphoxide (DMSO)	Sigma Aldrich, Wicklow, Ireland
EDTA	Sigma Aldrich, Wicklow, Ireland
Ethanol	Sigma Aldrich, Wicklow, Ireland

Gelatin	Sigma Aldrich, Wicklow, Ireland
Glucose	Lennox, Dublin, Ireland
Glycerol	Sigma Aldrich, Wicklow, Ireland
Glycine	Sigma Aldrich, Wicklow, Ireland
Hydrochloric acid (HCl)	Sigma Aldrich, Wicklow, Ireland
Hydrogen peroxide (H ₂ O ₂)	Sigma Aldrich, Wicklow, Ireland
Leupeptin	Sigma Aldrich, Wicklow, Ireland
Magnesium sulfate (MgSO ₄)	Sigma Aldrich, Wicklow, Ireland
β-Mercaptoethanol	Sigma Aldrich, Wicklow, Ireland
Methanol	Sigma Aldrich, Wicklow, Ireland
2-Methylbutane (isopentane)	Sigma Aldrich, Wicklow, Ireland
N, N' – Methylenebisacrylamide	Sigma Aldrich, Wicklow, Ireland
NP-40	Sigma Aldrich, Wicklow, Ireland
Pentobarbital sodium (Euthatal)	Comparative Medicine, TCD
Potassium bicarbonate (KHCO ₃)	Sigma Aldrich, Wicklow, Ireland
Potassium chloride (KCl)	Sigma Aldrich, Wicklow, Ireland
Potassium chromium (III) sulfate dodecahydrate	Sigma Aldrich, Wicklow, Ireland
Potassium phosphate, dibasic (KH ₂ PO ₄)	Sigma Aldrich, Wicklow, Ireland
2-Propanol	Sigma Aldrich, Wicklow, Ireland
Sodium azide (NaN ₃)	Sigma Aldrich, Wicklow, Ireland
Sodium chloride (NaCl)	Sigma Aldrich, Wicklow, Ireland
Sodium dodecylsulfate (SDS)	Sigma Aldrich, Wicklow, Ireland
Sodium hydrogen carbonate (NaHCO ₃)	Sigma Aldrich, Wicklow, Ireland

Sodium hydroxide (NaOH)	Lennox, Dublin, Ireland
Sodium orthovanadate (Na ₃ VO ₄)	Sigma Aldrich, Wicklow, Ireland
Sodium phosphate, dibasic (Na ₂ HPO ₄)	Sigma Aldrich, Wicklow, Ireland
Sodium phosphate, monobasic (NaH ₂ PO ₄)	Sigma Aldrich, Wicklow, Ireland
Sodium tetraborate (Na ₂ B ₄ O ₇)	Sigma Aldrich, Wicklow, Ireland
N, N, N', N' – Tetramethylethylene-diamine (TEMED)	Sigma Aldrich, Wicklow, Ireland
Tris-Base	Sigma Aldrich, Wicklow, Ireland
Tris-HCl	Sigma Aldrich, Wicklow, Ireland
Triton™ X-100	Sigma Aldrich, Wicklow, Ireland
Tween	Sigma Aldrich, Wicklow, Ireland

General Laboratory Products

Biosphere filter pipette tips

- 10µl (clear), 200µl (yellow), 1000µl (blue)

Sarstedt, Nümbrecht, Germany

Cork discs

R.A. Lamb Ltd., Sussex, UK

Cover Glass 22 x 50mm

VWR International Ltd., Dublin, Ireland

Cryostat blades c35

PFM Medical UK, Cheshire, England

Eppendorfs - 0.5ml, 1.5ml, 2ml

Sarstedt, Nümbrecht, Germany

Falcon Tubes - 15ml, 50ml

Sarstedt, Nümbrecht, Germany

Fisherbrand Multichannel pipette

Thermo Fisher Scientific Inc.

Gel running apparatus -

Combs, Short plates, 1mm Spacer plates, Tank

Bio-Rad Laboratories, California, USA

Microscope slide (76x26mm) frosted 1-1.2mm thick

Thermo Fisher Scientific Inc.

Lens-cleaning wipes

Whatman Ltd., UK

Liquid Blocker Super PAP pen	Sigma Aldrich, Wicklow, Ireland
Microlance needles (25g ⁵ / ₈ inch)	BD, Oxford, UK
Nitrocellulose Membrane (Hybond-c Extra)	GE Healthcare, Buckinghamshire, UK
Optical Adhesive Film	Applied Biosystems, Dublin, Ireland
Parafilm	Thermo Fisher Scientific Inc.
Pipetman pipettes - 2µl, 10µl, 20µl, 200µl, 1000µl	Gilson, Inc., Middleton, USA
Plastic Bulb pipettes (5ml)	Sarstedt, Nümbrecht, Germany
Scalpels (disposable)	Swann-Morton, Sheffield, UK
Scienceware® Disposable Labmat	Sigma Aldrich, Wicklow, Ireland
Standard grade no.3 filter paper	Whatman Ltd, UK
Sterile syringes	BD Plastipak, Oxford, UK
96-well plates	Sarstedt, Nümbrecht, Germany
96-well semi-skirted Plates (Fast PCR)	Thermo Fisher Scientific Inc.

Immunohistochemistry

Alexa Fluor 633 goat anti-chicken IgG	Invitrogen, Bio-sciences, Dublin, Ireland
Alexa Fluor 488 goat anti-mouse IgG	Invitrogen, Bio-sciences, Dublin, Ireland
Chicken anti-BrdU monoclonal IgG	Abcam, Cambridge, UK
DAPI dihydrochloride	Sigma Aldrich, Wicklow, Ireland
Mouse anti-NeuN monoclonal IgG	Millipore, Cork, Ireland
Normal goat serum	Vector Labs Ltd, Peterborough, UK
Rabbit anti-chicken IgG	Abcam, Cambridge, UK
Tissue Tek OCT compound	VWR International Ltd., Dublin, Ireland

VectaShield mounting medium

Vector Labs Ltd, Peterborough, UK

Laboratory Machinery

Analytical Balance MH-214

Thermo Fisher Scientific Inc.

Binder incubator

Binder GmbH, Germany

BioTek Synergy HT plate reader

BioTek Instruments Ltd., UK

Duomax 1030 Rocking Platform Shaker

Heidolph, Schwabach, Germany

Hotplate & Stirrer - AGB 1000

AGB Scientific Ltd, Ireland

LAS 3000 Intelligent Dark Box

Fujifilm

Leica CM1850 cryostat

Leica Biosystems

Microscope; upright BX51

Olympus

Nanodrop 2000 spectrophotometer

Thermo Fisher Scientific Inc.

pH210 Microprocessor pH meter

Hanna Instruments, Ireland

Polytron PT 2100 homogenizer

VWR International Ltd., Dublin, Ireland

Power Station 300

Labnet International, Inc., N. Jersey, USA

PTC-200 Peltier Thermal Cycler

Bio-Rad Laboratories, California, USA

Semi-Dry Electrophoretic blotter

Apollo™ Instrumentation, CLP

Sorvall Legend RT centrifuge

Thermo Fisher Scientific Inc.

Spectrafuge 24D

Labnet International, Inc., N. Jersey, USA

Spectrafuge Mini centrifuge

Labnet International, Inc., N. Jersey, USA

StepOnePlus Real-Time PCR machine

Applied Biosystems, Dublin, Ireland

TE212 Lab Balance Scales

Sartorius Ireland Ltd, Dublin 11, Ireland

Treadmill, Exer 3/6

Linton Instrumentation, UK

Treadmill Simplex II	Columbus Instruments Int, Ohio, USA
Vortex	Labnet International, Inc., N. Jersey, USA

PCR Reagents

High Capacity cDNA reverse transcription kit	Applied Biosystems, Dublin, Ireland
Molecular-grade water	Sigma Aldrich, Wicklow, Ireland
Nucleospin RNA II isolation kit	Macherey-Nagel, Dublin, Ireland
RNAlater	Ambion, Dublin, Ireland
RNase away	Ambion, Dublin, Ireland
RNase-free tubes	Ambion, Dublin, Ireland
RNaseZap wipes	Ambion, Dublin, Ireland
TaqMan Fast Advanced Master Mix	Applied Biosystems, Dublin, Ireland
TaqMan Gene Expression Assays	Applied Biosystems, Dublin, Ireland

Western Immunoblotting reagents

Anti-mouse (goat) IgG conjugate	Sigma, Wicklow, Ireland
Anti-rabbit (goat) IgG conjugate	Sigma, Wicklow, Ireland
Magic Mark™ XP Western Protein Standard	Invitrogen, Bio-sciences, Dublin, Ireland
Mouse anti – β -Actin	Sigma, Wicklow, Ireland
PrecisionPlus Protein (Dual Colour) Standards	Bio-Rad, Laboratories, California, USA
Rabbit anti- Akt IgG	Cell Signalling Technology, Mass., USA
Rabbit anti - CREB IgG	Cell Signalling Technology, Mass., USA
Rabbit anti- ERK1/2 IgG	Cell Signalling Technology, Mass., USA

Rabbit anti- pAkt IgG	Cell Signalling Technology, Mass., USA
Rabbit anti - pCREB IgG	Cell Signalling Technology, Mass., USA
Rabbit anti- pERK1/2 IgG	Cell Signalling Technology, Mass., USA
ReBlot Plus Strong Antibody Stripping Solution	Millipore, Cork, Ireland
SuperSignal West Dura (Luminol)	Thermo Fisher Scientific Inc.

2.2 Animals

Male C57/BL6 Jax mice from a Charles River (UK) line were obtained from the Comparative Medicine unit, Trinity College Dublin (TCD) and used in all experimental procedures. When experiments began, animals were three months old, with a body mass ranging from 20 – 25g. Subjects were kept in the Comparative Medicine unit, Trinity Biomedical Sciences Institute, TCD up to one month prior to commencement of experiments and remained there until each was euthanized.

Male mice were chosen based on the length of the studies performed and to control for the effects of hormonal fluctuation that may require consideration when working with females. There is also the possibility that gender difference could require an increase in subject numbers.

The decision to use the C57/BL6 Jax strain of mouse was influenced by access to these mice and studies which report an intermediate level of running in this strain of mouse when comparisons were made across a variety of strains (Lerman *et al.*, 2002, Massett and Berk, 2005).

All experimentation followed National and European directives on the protection of animals (European Union (Protection of Animals used for Scientific Purposes) Regulations 2012 (SI 543/2012), and European Union (Protection of Animals used for Scientific Purposes) (Amendment) Regulations 2013 (SI 434/2013)), and received approval from the Animal Research Ethics Committee, TCD.

2.3 Animal housing

Animals were group housed in groups of three to seven mice. Cages were of standard size (W x D x H - 396 x 215 x 172 mm) and maintained in a facility offering a constant temperature of 22°C ($\pm$ 2°C) and humidity (55% $\pm$ 5%) on a 12hr light/dark cycle (lights on a.m., lights off p.m.).

2.4 Exercise protocol

Animals were randomly assigned to either Sedentary (Sed) or Exercise (Ex) groups. Exerciser treadmills (Linton Instrumentation) (Fig 2.1) with facilities for six mice simultaneously were used to run animals for 30min per day, five days per week, for one month. The week before exercise began, animals were habituated to the exercise room and running apparatus over two consecutive days. Each day, treadmill speed was initially set at 300m/hr and was increased incrementally to a maximum speed of 600m/hr. If animals stopped running, they were encouraged with a gentle hand; there was no electrical current set on the treadmills to provoke continuous movement. Sed animals were placed on a stationary treadmill in the same room for the same period of time to control for exposure to a novel environment. Tail markings were used to identify individual subjects, and general activity was noted.

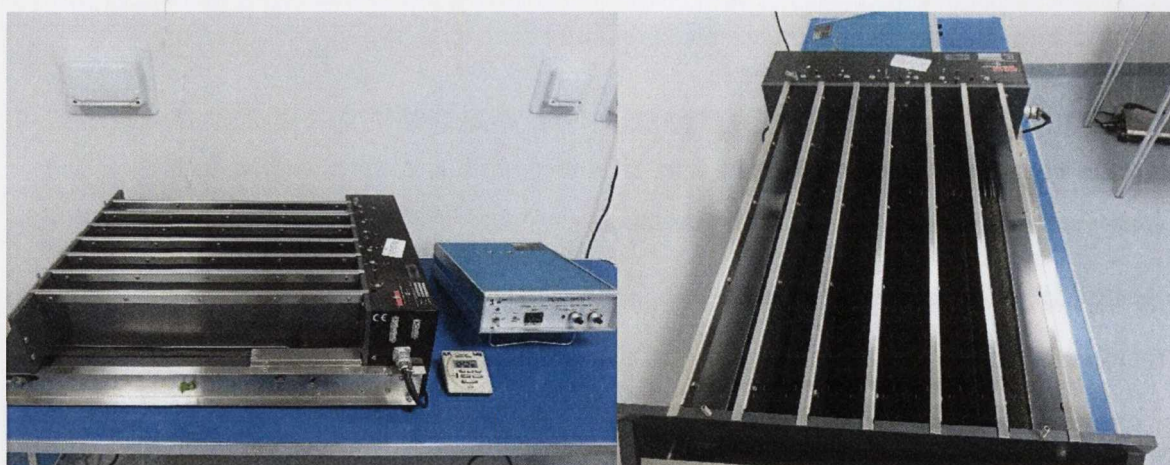


Fig. 2.1 – Linton Instrumentation Treadmill

Each treadmill was connected to a Columbus Instruments Treadmill Simplex II controller that allowed adjustment of the treadmill's belt speed. Lane dividers split the treadmill into six lanes so that six mice could use the machine at once, and were easily removed so that the belt could be cleaned. Sed animals were also placed on a divided treadmill at the same time, and the power source was switched off.

2.5 7,8-dihydroxyflavone administration

The small molecule TrkB receptor agonist 7,8-DHF (100mg) was dissolved in dimethyl sulfoxide (DMSO) (100µl). This stock solution was added to dH₂O (100ml) containing sucrose (1g) and mixed until the solution was uniform. Through measurement prior to drug delivery, it was found that mice would drink approximately 7ml standard water per day. Measurement once administration began found that this consumption was not affected by additives in the drinking water. This brought the final concentration, based on 7ml per day, to approximately 23 mg/kg of 7,8-DHF, 0.1% DMSO, 1% sucrose. Control animals received a vehicle solution (0.1% DMSO, 1% sucrose, dH₂O). Animals were randomly assigned to Vehicle (Veh) and 7, 8-DHF groups, but allocation was on a cage by cage basis. Water bottles were changed every two to three days.

Solution preparation and dose were based on existing studies using a similar dose of approximately 22 mg/kg 7,8-DHF (Johnson *et al.*, 2012, Tian *et al.*, 2015), and the reports of a pilot study that tested 5, 10, 20, and 50 mg/kg of 7,8-DHF and found that 20 mg/kg provided greater protection against behavioural deficits in a mouse model of traumatic brain injury (Wu *et al.*, 2014). Sucrose was added to disguise any residual taste of DMSO.

2.6 BrdU administration

BrdU was administered via intraperitoneal injection, starting four weeks prior to sacrifice, for seven consecutive days (50 mg/kg BrdU made up in .89% w/v Saline solution). The timing of these injections were determined by the decision to analyse the presence of BrdU in mature neurons, which would not be seen until approximately three to four weeks following administration of this compound (von Bohlen Und Halbach, 2007). The dose of 50 mg/kg is one that is frequently utilized throughout the literature, and has been used previously in this laboratory (Birch *et al.*, 2013). Each day, within one hour of exercise, if applicable, animals received the i.p injection. Approximately 200µl was administered per animal.

Injections were carried out by licensed individuals, in accordance with procedural guidelines.

2.7 LPS administration

LPS was administered via intraperitoneal injection three or four hours prior to behavioural training. Animals were assigned to receive either saline (0.89% NaCl in dH₂O) or a subseptic dose of *E. Coli* LPS (100 µg/kg) in saline. Approximately 200-300µl, determined by body mass, was administered to each animal. This dose of LPS is frequently used, and was chosen based on previous work carried out in this laboratory (Hennigan *et al.*, 2007).

Injections were carried out by licensed individuals, in accordance with procedural guidelines.

2.8 Behavioural assessment

The purpose of the behavioural procedure was to assess hippocampus-dependent learning and memory abilities in all animals. Recognition memory was tested using a novel object recognition (NOR) task, and spatial memory was tested using an object displacement (OD) task. Recognition and spatial memory concern themselves with the hippocampus in particular.

The protocol for these tasks were adapted for testing in mice from work carried out in this laboratory when testing non-spatial (recognition) (O'Callaghan *et al.*, 2007), and spatial memory (Bechara *et al.*, 2014) in the rat.

2.8.1 Novel Object Recognition task

The NOR task (Fig. 2.2) is based on the idea that animals will naturally and preferentially explore a novel object. Two objects were placed in a darkened open field (cylindrical, black plastic, (H x W – 35 x 50cm)) in a dimly-lit room. Animals like the safety of corners, so a circular open field encourages exploration. The arena contained two objects built with standard Lego bricks of various colours. Each object was fixed in place using white-tack and was positioned equidistant from the walls of the circle and the other object in the arena. Spatial cues were fixed to the walls of the open field and remained there throughout all behavioural assessments, so subjects could learn about the location of objects in relation to these cues. Once animals had explored the field, they were tested to see if they could recall it. This is done by replacing one of the objects with a different one, previously unseen by the test subject. If the subject successfully recalls the open field and its

objects, then it will recognise that one of the objects has been changed and will spend more time exploring it due to its novel nature.

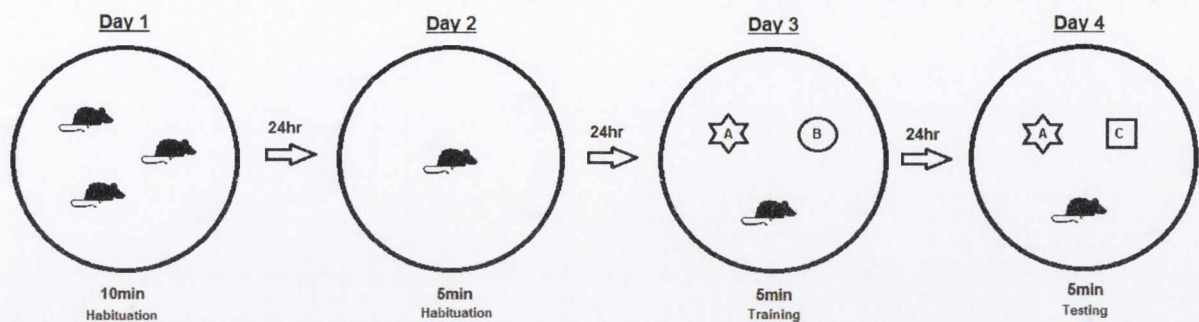


Fig. 2.2 - Timeline of events in NOR task

2.8.1.1 Habituation

For two days prior to the NOR task all animals were habituated to the open field. On day one, animals from each cage were placed in the open field together with their cage-mates for ten minutes. On day two each animal was allowed to explore the open field alone for five minutes. The field was wiped down with 70% ethanol between cages.

2.8.1.2 Training

On day three of exposure to the open field each subject was placed by the experimenter in the centre of the open field and allowed to explore alone for five minutes. On this occasion two objects of similar size and colour were placed into the open field (Fig. 2.3a), also containing a spatial cue (a cross) positioned on one wall. Two stopwatches, one designated to each object, were used to track the amount of time spent exploring each object. In this way the exploration of each object could later be expressed as a percentage of total exploration time. Between subjects, both the field and all objects were sprayed and wiped down in order to remove any olfactory cues. Animals were returned to their home cage after the five minute trial.



Fig. 2.3a - NOR Task; Training
The open field contained two different objects and was equipped with a spatial cue.

2.8.1.3 Testing

The final day in the open field was used to test whether subjects had learned from the time spent in the field during training. A delay of 24hrs between training and testing was used. One object was replaced with a previously unseen object, and the familiar object, as well as spatial cues, remained in the same position (Fig. 2.3b). The protocol used was unchanged from training. Upon completion of the five minute task, animals were removed from the open field and returned to their home-cage. The arena and objects were cleaned with 70% alcohol again before the next subject was placed into the open field.

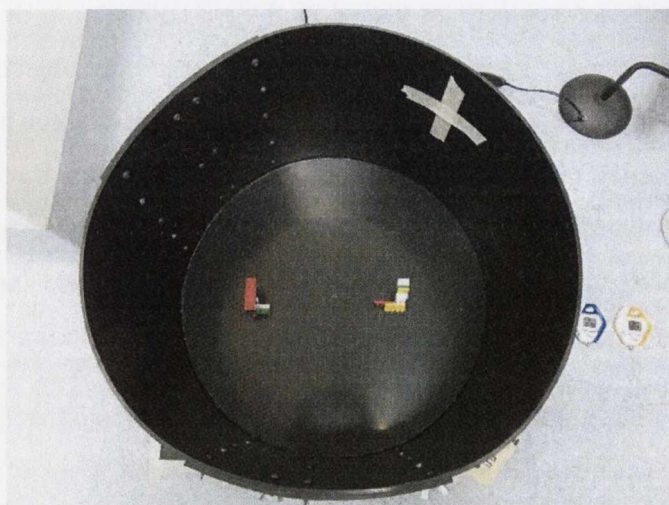


Fig. 2.3b - NOR Task; Testing
An image of the set up for testing memory recall in subjects. Object B was replaced.

2.8.2 Object Displacement task

The purpose of the OD task (Fig. 2.4) is to assess spatial learning and memory in both control and treated animals, at various ages, using a task designed to test this hippocampal-dependent memory. Three objects were placed in a darkened open field (cylindrical, black plastic, (H x W – 35 x 50cm)) in a dimly-lit room, similar to the set up for the NOR task. The arena contained three objects built with standard Lego bricks of various colours. Each object was fixed in place using white-tack and was positioned in one of three quadrants of the circle. Spatial cues were fixed to the walls of the open field and remained there throughout all behavioural assessments, so subjects could learn about the location of objects in relation to these cues. Once animals have explored the field, they can be tested to see if they recall the spatial orientation of objects. This is done by moving one of the objects to a different, previously empty part of the arena. If the subject successfully recalls the open field and its objects, then it will recognise that one of the objects has been moved and will spend more time exploring it due to its novel nature.

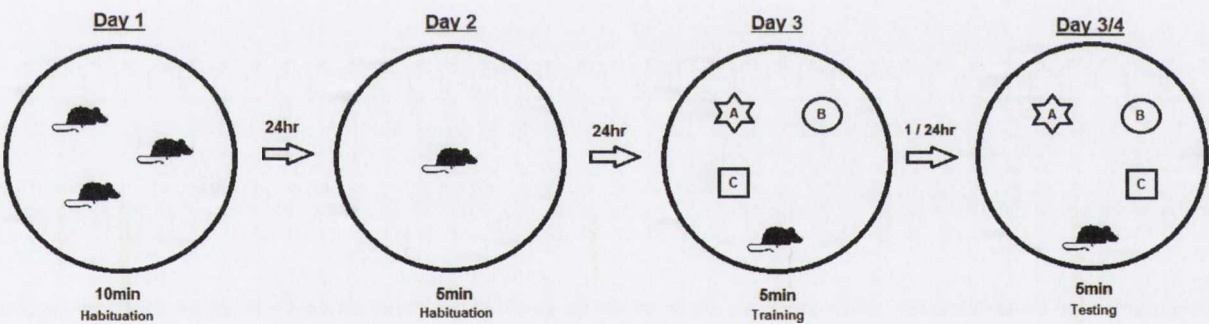


Fig. 2.4 - Timeline of events in OD task

2.8.2.1 Habituation

For two days prior to the OD task all animals were habituated to the open field. On day one, animals from each cage were placed in the open field together with their cage-mates for ten minutes. On day two each animal was allowed to explore the open field alone for five minutes. The field was wiped down with 70% ethanol between cages.

2.8.2.2 Training

On day three of exposure to the open field each subject was placed by the experimenter in the centre of the arena and allowed to explore alone for five minutes. On this occasion three objects of similar size and colour were placed into the open field (Fig. 2.5a), also containing a spatial cue (a cross) positioned on one wall. Three stopwatches, one designated to each object, were used to track the amount of time spent exploring each object. In this way the exploration of each object could later be expressed as a percentage of total exploration time. Between subjects, both the field and all objects were sprayed and wiped down in order to remove any olfactory cues. Animals were returned to their home cage after the five minute trial.



Fig. 2.5a - OD Task; Training

The open field was filled with three different objects and equipped with spatial cues.

2.8.2.3 Testing

The final day in the open field was used to test whether subjects had learned from the time spent in the field during training. A delay of either one or 24hrs between training and testing was used, depending on the study being undertaken. One object was moved to a previously empty quadrant of the arena, and the remaining objects, as well as spatial cues, remained in the same position (Fig. 2.5b). The protocol used was unchanged from training. Upon completion of the five minute task, animals were removed from the open field and returned to their home-cage. The arena and objects were cleaned with 70% alcohol again before the next subject was placed into the open field.

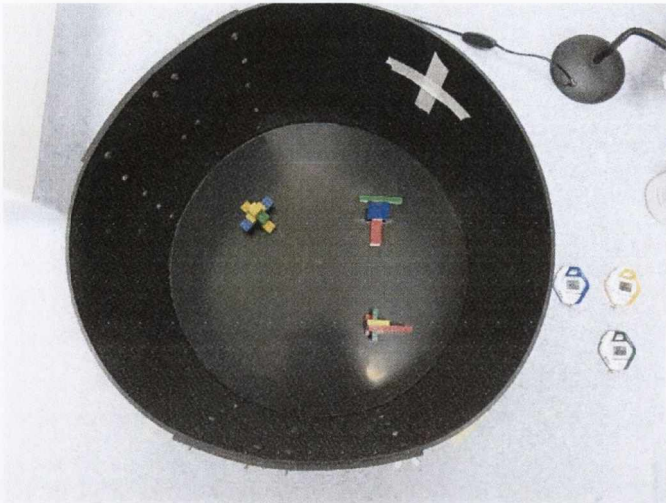


Fig. 2.5b - OD Task; Testing

An image of the set up for testing memory recall in subjects. Object C was relocated.

2.9 Collection of tissue samples

Following the final session of behavioural testing animals were sacrificed via cervical dislocation - where the spinal column is separated from the skull by applying pressure to the neck and quickly pulling the tail, or via transcardial perfusion - where saline is injected into the left ventricle of the heart, flushed through the circulatory system, and released from a tear made in the right atrium of the heart, followed by injecting a fixative solution into the circulatory system to preserve the shape and integrity of the brain.

2.9.1 Tissue collection

Subjects were deeply anaesthetised with an intraperitoneal injection of pentobarbital sodium (1g/kg), and once a reaction to foot pinching had diminished, a blood sample was collected via cardiac puncture. The blood was incubated at room temperature for at least 30min to allow for clotting, and then centrifuged at 4°C for 20min at 1500 x g. The separated serum was collected from each sample and stored at -20°C until required for further analysis.

Cervical dislocation was used to gain access to the skull, and the brain was dissected free. The left and right prefrontal cortex were removed with a scalpel and collected in individual Eppendorf tubes. The remaining brain was hemisected on ice and dissected further to expose the hippocampus. Using a closed curved forceps, the left and right hippocampi were gently rolled free from the cortices and collected into individual Eppendorfs.

All tissue samples were immediately snap-frozen on dry ice and then moved to -80°C storage until required for further analysis. The rapid post-mortem degradation of RNA was not of concern as RNA in the brain degrades much more slowly than in other tissues and can be frozen up to 48hrs post-mortem without significant degradation (Johnson *et al.*, 1986, Inoue *et al.*, 2002). Tissue here was snap-frozen within five to ten minutes of death.

2.9.2 Whole-brain fixation

Subjects were anaesthetised with an intraperitoneal injection of pentobarbital sodium (1g/kg). They were observed closely to ensure persistence of a heartbeat, but absence of reflex when a foot-pinch was administered. The beating heart was exposed by removing the ribcage, and a needle and syringe were inserted into the left ventricle to flood the circulatory system with PBS. The right atrium was punctured to allow PBS to freely flow through, and out of, the system. When the blood supply had been drained, the syringe was exchanged for one containing freshly prepared, chilled 4% paraformaldehyde (PFA), and this also was flushed through the circulatory system. The lower right limb was not pinned down so that arrival of PFA at the extremities could be observed, indicating successful perfusion of the brain. Decapitation allowed for careful removal of the intact brain, which was moved to chilled 4% PFA and kept at 4°C for 24 hrs.

Following this, brains were submerged in a freshly prepared cryoprotective solution (dH₂O, 30% sucrose, 0.05% sodium azide) and stored at 4°C until required for tissue slicing.

2.10 Tissue preparation

Snap-frozen tissue samples were homogenised by hand using a manual homogeniser in 500µl of freshly prepared buffer. All samples were kept on ice throughout the process and recollected into labelled 1500µl Eppendorf tubes. Once suspended in buffer these samples could be kept in standard freezing conditions of between -14 and -18°C.

2.10.1 Assessment of protein concentration

In order to analyse tissue samples within a working range their protein concentration must be equalized. This was done using the BCA Method; the Bicinchoninic Acid Assay. The procedure detects the total amount of protein in a sample via a green – purple colour change. Protein reduces Cu^{2+} (cupric ion) to Cu^+ (cuprous ion) in an alkaline environment – higher levels of protein reduce more cupric ions, and thus produce higher levels of cuprous ions. Bicinchoninic acid reacts with the cuprous ions to produce a strong purple colour. The darker the purple colour of the solution, the more protein there is in the tissue sample.

A green-coloured Working Reagent was freshly prepared of 50:1 parts Reagent A: Reagent B. Nine protein standards diluted from a stock concentration produced a standard curve against which tissue sample absorbance could be compared, and concentration values for each sample could be calculated using the equation of the line; $y=mx+c$. Samples were added in duplicate to obtain an average concentration per sample. A plate reader was used to measure concentration in each well at ~562nm absorbance.

Following analysis, the samples could be equalised. This was done by dividing each sample by the lowest sample reading for protein concentration, and then bringing each sample up to a total volume of 200µl using the appropriate buffer as a diluent.

2.11 Polymerase Chain Reaction (PCR)

In order to establish possible changes in messenger ribonucleic acid (mRNA) levels of certain corresponding proteins in animal subjects, it is necessary to isolate the RNA from tissue samples obtained from hippocampal and cortical tissue. Purifying the ribonucleic acid (RNA) is a process that employs homogenisation of the tissue, followed by various steps involving filtration, and buffers that remove unwanted cellular structures.

Using the Nucleospin RNA kit and vigilant sterile techniques, RNA is extracted from tissue samples. Complementary DNA (cDNA) is then synthesized from the purified RNA using a thermal cycler. Finally, cDNA is amplified and detected during PCR.

2.11.1 Total RNA extraction

In an RNase-free working area cleaned with RNaseZap wipes, tissue samples were homogenized in lysis buffer (350µl RA1 buffer, 3.5µl β-mercaptoethanol) using a polytron tissue grinder. Both RNase Away and RNase-free water were used to clean the polytron between samples. The homogenate was filtered using centrifugation (11000g for one minute) through a violet-ringed filter and into a sterile collection tube. This lysate was then mixed with 350µl ethanol (70%) to alter the RNA binding conditions, and centrifuged through a blue-ringed sterile filter (11000g for 30sec). The RNA was bound to the silica membrane of the filter. The bound RNA was then de-salted using 350µl membrane desalting buffer (MDB, provided in kit), and centrifuged at 11000g for one minute to dry.

Contaminating DNA present in the homogenate was digested using rDNase (10µl rDNase in 90µl rDNase Reaction Buffer, both provided in kit). 95µl of this buffer was pipetted into the centre of the filter, while avoiding touching the filter itself. This digestion took place over a 15min incubation at room temperature.

RA2 buffer (provided) was used to wash any digested DNA from the silica membrane; 200µl onto the filter, centrifuged at 11000g for 30sec. The filter was moved to a new sterile collection tube. Two washes with RA3 buffer were carried out - 600µl onto the membrane, centrifuged at 11000g for 30sec, 250µl onto the filter, centrifuged at 11000g for two minutes. This extra time in the centrifuge dried the membrane.

40-60µl RNase-free water was used to remove and elute the RNA from the membrane, where it was collected in a new sterile collection tube after centrifugation at 11000g for one minute. The eluted RNA was aliquoted into PCR tubes and kept at -80°C until it was to be quantified.

2.11.2 RNA quantification

A Nanodrop spectrophotometer was used to determine purity and concentration of RNA in each sample. 1µl RNase-free water was placed on the platform to produce a baseline reading. After vortexing, 1µl of each sample was pipetted onto the platform and a reading was produced. RNA concentrations were equalised to one another using RNase-free water to be within a similar working range. Equalised samples were kept at -20°C until required for cDNA synthesis.

2.11.3 cDNA synthesis

In order to work with samples of purified RNA, they need to be stored in a more stable format. The process of cDNA synthesis utilizes a thermal cycler, which incubates the samples at various temperatures optimal to each step of the procedure. A High Capacity cDNA reverse transcription kit provides the necessary components for the process of reverse transcription of total RNA to single-stranded cDNA.

10µl of equalised RNA was mixed with 10µl Reverse Transcriptase master mix (2µl 10x reverse transcriptase buffer, 0.8µl 25x deoxyribonucleotide triphosphates (dNTPs), 2µl random primers, 1µl multiscribe reverse transcriptase, 4.2µl nuclease-free H₂O). The PCR tubes were vortexed and briefly centrifuged to mix the solutions, then placed in the thermal cycler. Reaction volume was set to 20µl, with heated lids, and the cycler was set to go through four stages; ten minutes @ 25°C, 120min @ 37°C, five minutes @ 85°C, and infinite time @ 4°C, until the tubes were removed from the cycler. During this time, the primers attached to the RNA, the reverse transcriptase attached where the primers were, and moved along the strand lining up nucleotide triphosphates to create a complimentary strand. Heating the solution then deactivated the reverse transcriptase and the process was complete.

cDNA was diluted one in five with RNase-free H₂O, and stored at -20°C.

2.11.4 Duplex real-time PCR (RT-PCR)

Utilizing thermal cycling, repetitive heating and cooling of cDNA allows it to be exponentially amplified at a specific gene of interest. TaqMan gene expression assays contain target primers and FAM-labelled MGB target probes (Fluorescein amidite (FAM) is a green fluorescent dye that labels the MGB (minor groove binder) probe). VIC-labelled MGB target probes quantified β-Actin, which was used as the endogenous control for all target genes (VIC is a yellow fluorescent dye). Each well containing a sample was incubated with the target primer for one gene of interest, and the target primer for β-Actin, thus making it a duplex procedure. Genes of interest are listed in Table 2.1.

Table 2.1 - Gene expression assays used in PCR

Target Gene	TaqMan Assay
BDNF	Mm04230607_s1
TrkB (NtrK2)	Mm00435422_m1
Ki67	Mm01278617_m1
CREB	Mm00501607_m1
IL1 β	Mm00434228_m1
TNF α	Mm00443258_m1
Iba1	Mm00479862_g1
Cd44	Mm01277163_m1

Each well of a 96-well plate contained 4 μ l cDNA sample, and 6 μ l PCR mastermix (5 μ l FAST TaqMan (AmpliTa[®] Fast DNA Polymerase, Uracil-N glycosylase (UNG), dNTPs with dUTP, ROX™ dye (passive reference), Optimized buffer components), 0.5 μ l target gene primer (BDNF, TrkB, etc.), 0.5 μ l β -Actin primer). The plate was sealed and centrifuged for one minute @ 800rpm.

Step One Plus™ Software (version 2.1) was used to design a run method for the thermal cycler. Samples entered a holding stage of two minutes @ 50°C, 20sec @ 95°C, and then completed 40 cycles of one second @ 95°C, 20sec @ 60°C. Temperatures of 95°C cause denaturation of the cDNA, and ensures that the cDNA is all single stranded. Temperatures of 60°C cause primers to attach to single strands, and as the temperature rises the dNTPs align to elongate the complimentary strand so that the DNA becomes double-stranded. The temperature returns to 95°C to split this new double strand, and the process repeats, resulting in exponential amplification of the target gene.

The $\Delta\Delta C_T$ method was used to quantify gene expression. This is determined from the C_T readings produced during the reaction. The C_T value varies depending on when an amplicon was detected, so the lower the C_T value, the earlier it was detected, and so the level of amplicon in that well was higher than the level of amplicon in another well that has a higher C_T value, and thus was detected during a later cycle. For analysis, the difference in C_T for the target gene and the endogenous control in the same well is calculated. This is called ΔC_T . The difference in ΔC_T of a sample compared to the overall reference sample run on that plate is called $\Delta\Delta C_T$. A reference sample is decided on at the start of the experiment, and is usually a sample from a control group. Determining the relative quantity (Rq) of target gene in each well is calculated using $2^{-\Delta\Delta C_T}$, as a difference in one cycle is equal to a two-fold difference in levels of amplified target sequence (due to the exponential nature of the process). Rq values can be plotted and compared for statistically significant differences.

2.12 Western Immunoblotting

Western blot analysis is used to determine the presence of a particular protein within a sample. It employs the methods of Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE), electroblotting, blocking of non-specific binding, antibody probing, and detection of proteins using chemiluminescent imaging.

2.12.1 Sample preparation

Previously equalised samples stored in freshly prepared lysis buffer (NP-40 (1% w/v), 50mM Tris-Base (pH 8.0), 150mM NaCl, glycerol (10% w/v), 2mM EDTA, 1mM Na₃VO₄, aprotinin, leupeptin) were boiled with sample buffer (100µl equalised tissue sample, 100µl sample buffer (dH₂O, Tris-HCl (pH 6.8), Glycerol, 10% SDS, β-mercaptoethanol, 10x Bromophenol blue)). After five minutes at approximately 100°C, Eppendorfs were removed from dry heating block and allowed to stand at room temperature before loading. Samples were stored long-term at -20°C.

2.12.2 SDS PAGE

10% separating gels (dH₂O, Bisacrylamide, Tris-HCl (pH 8.8), SDS, APS, TEMED) were prepared and set for approximately 30min between Bio Rad electrophoresis plates. A small amount of 2-propanol was poured on top while the gels set to prevent them from drying out. Varying percentages of separating gel can be prepared to accommodate proteins of different molecular weights. Following this, a similar solution of stacking gel (dH₂O, Bisacrylamide, Tris-HCl (pH 6.8), SDS, APS, TEMED) was prepared. 2-propanol was removed and stacking gel was loaded above the separating gel. Bio Rad gel combs were inserted between the plates to create the shape of ten or fifteen wells in the stacking gel. The wells allow samples to be loaded into designated lanes for analysis.

An electrophoresis rig was set up containing four gels and the rig was flooded with 1x Electrode Running Buffer (125mM Tris Base, 960mM Glycine, 0.5% SDS, dH₂O; dilute 5x ERB to 1x). Combs were removed to expose defined wells and lanes were loaded with tissue samples in sample buffer (10 or 15µl in each lane, depending on number of wells in each gel). Each sample was vortexed before it was pipetted into its designated well. The first lane held a visual molecular weight marker and the final lane held a fluorescent molecular weight marker (5µl each). The rig was set at 120mA (30mA per gel) and allowed to run for 40 – 60min. Proteins move through the SDS gel from cathode to anode (- to +) and are separated out in bands by descending molecular size and weight.

2.12.3 Electroblotting

Once gels had run, the rig was disconnected from the power source and plates were removed from the rig. The plates were pried apart and stacking gel was removed from the separating gel. A 'sandwich' was then constructed using filter paper and nitrocellulose membrane. All components were soaked in transfer buffer (25mM Tris Base, 192mM Glycine, dH₂O, 20% MeOH, 0.05% SDS) before being layered; starting with filter paper, nitrocellulose membrane, SDS gel, and a final sheet of filter paper on top.

Each 'sandwich' was covered in enough transfer buffer to keep it moist, placed on a semi-dry blotter, and the lid was screwed into place ready for transfer. The unit was connected to a power source activating both anode (base) and cathode (lid), and allowed to transfer for 75min at 225mA. This caused protein bands separated on the gel to transfer down onto the nitrocellulose membrane, away from the cathode and towards the anode.

2.12.4 Blocking and probing

Nitrocellulose membranes were removed from the sandwich and placed into individual lidded dishes. They were then incubated in a blocking solution of 5% BSA made up in TBS-T (20mM Tris-HCl (pH 7.5), 150mM NaCl, 0.05% Tween, dH₂O) for two hours at room temperature with agitation. A shaker platform was used to ensure every part of the membrane was exposed to the solution. Blocking prevents any nonspecific binding that may occur when each membrane is incubated with primary antibody designed to bind to the protein under investigation. The blocking solution prevents the antibody from binding to the membrane in random places and producing a 'dirty' unclear image when analysed.

Incubating with primary antibody is a process specific to each particular protein that may be present in the tissue sample. The antibody is usually suspended in 2% BSA in TBS-T, but its dilution (1:500, 1:1000, 1:2000) varies depending on the antibody (Table 2.2). Once the blocking buffer was removed, the membranes were incubated with primary antibody with agitation, either at room temperature for up to four hours, or at 4°C overnight in a cold room.

Primary antibody could be recollected and frozen after use. Each membrane was then washed with TBS-T four times for ten minutes, and incubated with a secondary antibody (in 2% BSA in TBS-T) for one hour on the platform shaker at room temperature. Dilution factors were varied (Table 2.2).

Table 2.2 - Primary and Secondary Antibodies

Antibodies and their secondary conjugates, used in protein detection by Western Blot.

1° Antibody	Dilution	Incubation	2° Antibody	Dilution	Incubation
Mouse anti - β -Actin Monoclonal IgG (Sigma)	1 in 1000 in 2%(w/v) BSA in 1x TBS-T	4°C, over night	Goat anti- Mouse (Sigma)	1 in 2000 in 2%(w/v) BSA in 1x TST-T	1 hour, room temp
Rabbit anti - Akt Polyclonal IgG (Cell Signalling Tech.)	1 in 1000 in 2%(w/v) BSA in 1x TBS-T	4°C, over night	Goat anti- Rabbit (Sigma)	1 in 2000 in 2%(w/v) BSA in 1x TST-T	1 hour, room temp
Rabbit anti - CREB Monoclonal IgG (Cell Signalling Tech.)	1 in 1000 in 2%(w/v) BSA in 1x TBS-T	4°C, over night	Goat anti- Rabbit (Sigma)	1 in 2000 in 2%(w/v) BSA in 1x TST-T	1 hour, room temp
Rabbit anti - ERK 1/2 Polyclonal IgG (Cell Signalling Tech.)	1 in 1000 in 2%(w/v) BSA in 1x TBS-T	4°C, over night	Goat anti- Rabbit (Sigma)	1 in 2000 in 2%(w/v) BSA in 1x TST-T	1 hour, room temp
Rabbit anti - pAkt Polyclonal IgG (Cell Signalling Tech.)	1 in 1000 in 2%(w/v) BSA in 1x TBS-T	4°C, over night	Goat anti- Rabbit (Sigma)	1 in 2000 in 2%(w/v) BSA in 1x TST-T	1 hour, room temp
Rabbit anti - pCREB Polyclonal IgG (Cell Signalling Tech.)	1 in 1000 in 2%(w/v) BSA in 1x TBS-T	4°C, over night	Goat anti- Rabbit (Sigma)	1 in 2000 in 2%(w/v) BSA in 1x TST-T	1 hour, room temp
Rabbit anti - pERK 1/2 Polyclonal IgG (Cell Signalling Tech.)	1 in 1000 in 2%(w/v) BSA in 1x TBS-T	4°C, over night	Goat anti- Rabbit (Sigma)	1 in 2000 in 2%(w/v) BSA in 1x TST-T	1 hour, room temp

2.12.5 Detection and imaging

The membrane was washed with TBS-T (four times for ten minutes each) to remove any unbound secondary antibody, and incubated in a darkened room for five minutes at room temperature with agitation in a chemiluminescent solution (SuperSignal®). Care had to be taken to ensure the SuperSignal® was not exposed to light in the laboratory, and once made up the solution was kept wrapped in tinfoil at 4°C. Membranes were placed into a Fujifilm LAS-3000 dark box, and the LAS-3000 programme was used to adjust shelf height for clarity and focus, and to expose the membrane for varying amounts of time (average; 30sec) to produce the best image possible for analysis.

2.12.6 Re-probing

If the membrane was to be used again to detect a different protein, it could be washed twice for five minutes, then incubated for three minutes in a 1x stripping solution (1ml ReBlot Plus Strong Stripping Solution, 9ml dH₂O). This removed any antibody bound to the membrane. Two more five minute washes removed the detached antibodies, and the membrane was incubated for 60min at room temperature in blocking buffer so as to prevent non-specific binding of the next antibody. The membrane could be stored in 1x TBS at 4°C for up to one week before reprobing with a new primary antibody, and the protocol was carried out as before.

2.12.7 Protein quantification

The software programme, ImageJ (version 1.48v), was used to measure levels of protein in each sample, using the .tif format image produced by the LAS-3000 programme. By encircling protein bands on the image, a spiked wave of colour intensity was produced, varying in strength based upon protein concentration. The area of the spike was highlighted, generating a measurement of area, and calculations were exported into Microsoft Excel (2013). From here proteins could be compared to housekeeping proteins, and expressed as a percentage of total protein.

2.13 Immunohistochemistry

Animals were injected with BrdU so that it would be available for incorporation into the subject's DNA when cells were dividing and thus new, BrdU-containing cells were produced. These cells, with BrdU in their nuclei, can then be detected using immunostaining – a method that utilizes antibody detection of specific antigens in a cell, or on a cell's surface. In this case, an anti-BrdU antibody could be used to detect BrdU. Chicken anti-BrdU primary antibody attaches to BrdU, a fluorescent secondary anti-chicken antibody attaches to the primary antibody, and thus the antigen can be detected using fluorescent microscopy.

2.13.1 Cryoprotecting tissue samples

In each experiment, whole brains were perfused *in situ*, stored in 4% PFA, and then kept in a cryoprotecting sucrose solution at 4°C. Before slicing, brains were coated in a medium that would maintain them at an optimum cutting temperature (OCT), and moved to -80°C to allow the OCT to set completely. OCT medium is liquid at room temperature, but freezes very quickly. This coating was carried out in the cryostat at -20°C, and was prepared inside a cylindrical piece of aluminium foil, ensuring the brain was uniformly coated in OCT on all sides, and the brain was set with the prefrontal cortex facing upwards. This meant that every brain was sure to be facing the same direction for slicing. The OCT maintained an even temperature when the tissue was moved from -80°C to the cryostat, which was kept at a working temperature of -20°C.

2.13.2 Subbing microscope slides

Before tissue slices can be placed onto microscope slides for analysis, the microscope slides are coated with a chrome-gelatin solution that will remove any interference of repulsive forces between the glass and the tissue. The chrome-gelatin solution is thus used as an adhesive to keep the tissue in place during staining protocols. Up to 50 glass microscope slides at a time are fitted into a rack, and submerged in 100% ethanol to ensure they are clean, allowed to dry, and then immersed in a solution of chrome-gelatin (2.5g gelatin, 0.25g chromium (III) potassium sulphate, 500ml dH₂O) heated to 60°C. The rack is then removed, covered in aluminium foil to protect from any dust, and left to dry at room temperature overnight.

2.13.3 Slicing tissue

The OCT'ed tissue was allowed to come to -20°C in the cryostat and was fixed onto a cork disc using some more OCT. The cork-disc was then mounted onto a chuck, which was brought incrementally closer to the cutting blade. The cryostat was set to 40µm increments, so that every time the chuck moved closer it moved 40µm. This meant the slices of tissue removed were 40µm thick.

Slices were taken coronally from front to back of the brain. Once cutting into the brain had begun, care was taken to detect the beginning of the hippocampus. At this stage, slices were transferred onto the subbed slides by holding the slide over the tissue slice. Because the slide was at room temperature, the cold tissue stuck to the surface. A small detail brush was used to make sure the tissue had not rolled up when the anti-roll glass plate was lifted. When sliced, the tissue moved off

the blade and under the anti-roll plate to prevent it from rolling up tightly. Tissue can be damaged by unrolling it to lie flat.

It was decided that a 'one-in-six' series counting protocol would be utilized, so once the first slice of tissue was collected, the following slices were collected on five different slides, and so on. Each slide then holds four slices that are each six slices (240µm) apart. This is represented in Fig 2.6 below.

Slide 1 1 7 13 19	Slide 2 2 8 14 20	Slide 3 3 9 15 21	Slide 4 4 10 16 22	Slide 5 5 11 17 23	Slide 6 6 12 18 24
Slide 7 25 31 37 43	Slide 8 26 32 38 44	Slide 9 27 33 39 45	Slide 10 28 34 40 46	Slide 11 29 35 41 47	Slide 12 30 36 42 48
Slide 13 49 55 61 67	Slide 14 50 56 62 68	Slide 15 51 57 63 69	Slide 16 52 58 64 70	Slide 17 53 59 65 71	Slide 18 54 60 66 72

Fig 2.6 - Coronal brain slice collection
A schematic of the order in which slices were collected, and the slice numbers that are found on each slide.

Once all slices were collected, the slides were briefly kept at room temperature to allow the OCT to dry, and then slides were stored in labelled slide boxes and kept at -20°C until the tissue was to be stained.

2.13.4 Fluorescent double staining

A double stain can be used to detect two components at the same time. In this case, BrdU and NeuN were fluorescently detected in the dentate gyrus.

Slides were removed from the freezer and brought to room temperature just before the staining protocol was to commence. During acclimatisation, slices were encircled with a liquid blocker marker, which would keep any treatment concentrated on the slices, without it running over the entire slide.

Slices were washed three times for five minutes each with PBS (NaCl , Na_2HPO_4 , NaH_2PO_4 , dH_2O), and then their DNA was denatured so that antibodies can access any BrdU present in their nuclei. This was done by adding HCl (2N) to each slice, and slides were incubated for 45min at room temperature. Slices were washed again three times for five minutes each with PBS, and were then blocked for non-specific binding using 3% normal goat serum (1x PBS, 0.5% Triton-X, 3% serum) for one hour at room temperature. Primary antibody was made up with 3% normal goat serum, chicken anti-BrdU (1:100), and mouse anti-NeuN (1:200). Slices were covered and incubated overnight at 4°C.

The next day, slides were washed four times for ten minutes each with PBS, and then once for ten minutes with dH_2O . They were then incubated in the dark for 90min at room temperature with secondary antibody made up in PBS (Alexa Fluor 633 goat anti-chicken (1:200), Alexa Fluor 488 goat anti-mouse (1:200)). This was followed by two washes for five minutes each with PBS, and then incubation with DAPI. DAPI is a blue fluorescent stain that binds to DNA, and so detects all cells in the tissue. DAPI was dissolved in 1x PBS (1:500) and slides were incubated in the dark at room temperature for five minutes. There were two final five minute washes with PBS, and then slides were dried, and coverslips were placed onto the slides with hard-set mounting medium (Vectashield). Slides were kept at 4°C in a darkened box to protect them from light until imaged. Generally, this is carried out as soon as possible, as DAPI will begin to fade and the nucleic stain won't be as prominent.

Negative control staining was carried out to validate the antibodies being used. Slices were treated as above, but stored overnight in 3% goat serum that did not contain any primary antibody, and then incubated the following day with secondary antibody and DAPI stain. Imaging showed that there was no labelled BrdU or NeuN in slices incubated without these primary antibodies (Fig. 2.7).

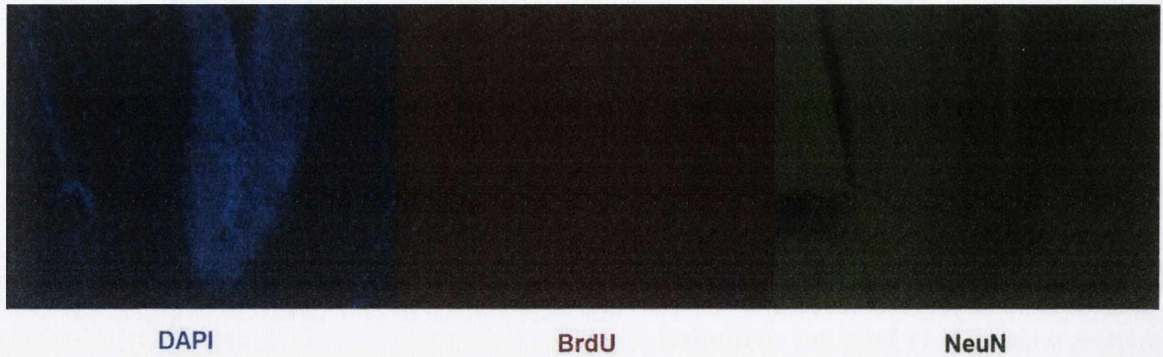


Fig 2.7 – Negative control for Chicken Anti-BrdU and Mouse-Anti NeuN primary antibodies

2.13.5 Microscope imaging

The Olympus Upright BX51 microscope was used for fluorescent imaging in the SBI Microscopy Facility, Trinity Biomedical Sciences Institute, TCD. Its LED light source stimulates light frequencies at 365nm, 535nm, and 635nm, which allowed for detection of DAPI, Alexa 488, and Alexa 633-stained cells, respectively.

Snapshots of the dentate gyrus region were taken and saved in .tif file format for further analysis. At a x4 magnification, DAPI stained images of the whole dentate gyrus were obtained, from both sides of the brain, and in all four slices on each slide. These were used to measure dentate gyrus volume.

Then at x20 magnification, DAPI, NeuN, and BrdU stained images were obtained by exhaustively saving images section by section until the whole dentate gyrus was imaged at a higher magnification. Moving systematically through these images would allow for all cells to be counted, as opposed to a sample frame from one or two sections out of the entire dentate gyrus structure.

2.13.6 Image Analysis

Cell counts were performed using image manipulation in the Image J programme. Here, there is a plug-in that enables grid structures to be imposed over the images to assist in unbiased stereological volume estimation, as demonstrated in Fig 2.8.

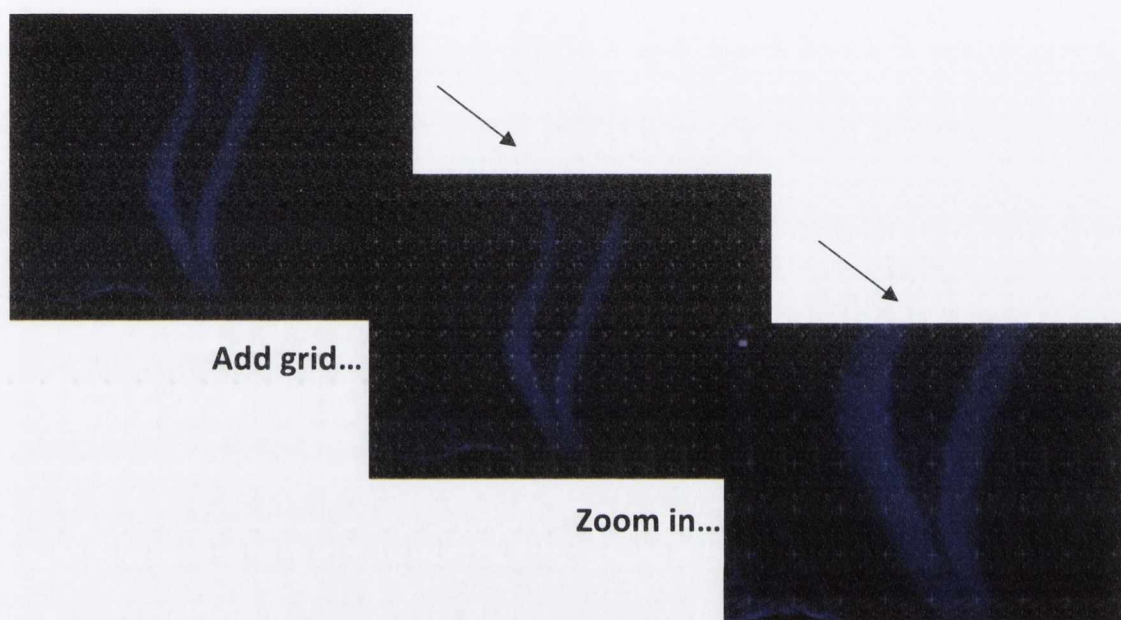


Fig 2.8 - The Cavalieri estimator; point grid overlay

The Cavalieri method is based on Cavalieri's Principle, by 17th Century Italian mathematician Bonaventura Cavalieri (Rosen and Harry, 1990). This method is used to obtain an unbiased estimate of volume. Each image is taken at the same resolution and magnification, and the entire dentate gyrus structure is imaged. A point grid, with crosses 2000 pixels apart, was imposed with random offset over each image. If a cross falls inside the dentate gyrus in its entirety, it is counted. If a cross is partially inside the dentate gyrus, then it is counted if it meets a chosen criteria, eg: top right quadrant is entirely inside the dentate gyrus, regardless of whether all other quadrants are partially or completely inside. This process of elimination is adhered to for all images in that study.

An estimate of the volume of the dentate gyrus is determined with the following equation;

$$V = T. (a/p). \Sigma P$$

The distance between sections (T) is constant through-out. A one in six series, with 40µm thick slices, gives a distance of 240µm between sections.

The length of the scale bar and length from the centre of one cross to the centre of the next cross are measured on screen at the same magnification, and converted to μm . The actual length of the scale bar on screen is divided by the length stated on the scale bar, and this gives the magnification factor (M). This value is then squared (M^2) to obtain an absolute value, regardless of negative or positive value. The distance in μm between the centres of two crosses (Δx) is also squared (Δx^2). This value is then divided by the magnification ($\Delta x^2 / M^2$) to obtain the area per point (a/p) in μm^2 . This value is constant through all volumetric analysis.

The only variable factor in this process is the sum of the points (ΣP) counted in all sections of dentate gyrus measured for a given brain. The volume of the dentate is calculated, in μm^3 .

Next, the numbers of BrdU^{+ve} cells are counted in all dentate gyrus images. ImageJ allows for manipulation of brightness and contrast to enhance cell visibility. Again, a chosen criteria determines if a cell is counted or not, but all cells must lie within the dentate gyrus or along the subgranular zone (SGZ) to be considered as a newly generated cell that could be expressing BrdU.

Finally, the total number of cells are expressed as the number of BrdU^{+ve} cells per μm^3 .

Image J can separate out fluorescent images into colour channels, and then combine them for positive double staining. If a cell is positive for both BrdU (red) and NeuN (green) then it will turn yellow when colour channels are combined, and then these cells can be counted as new neurons that were generated and have incorporated BrdU into their DNA when BrdU was being injected into the periphery.

2.14 Statistical Analysis

All data was analysed and grouped using Microsoft Excel (2013), and then imported into Graphpad Prism (version 5.01) where column statistics examined the data for outliers, and these were omitted in the case of values lying two standard deviations above or below the mean value. Data were represented as mean $\pm$ SEM.

Analysis was carried out using student t-test - when one factor is compared in two groups, one-way ANOVA (Analysis of Variance) - when one factor is compared among three or more groups, and two-way ANOVA - when two or more factors are compared in two or more groups. In addition to this, data was further analysed using Bonferroni post hoc tests.

Statistical significance was determined based upon these results, and assigned to differences with a p value less than 0.05.

A copy of the raw data produced from this research was issued to the Internal Examiner. This data can be obtained directly from Department of Physiology, TCD.

Chapter 3

An investigation into the effect of exercise and 7,8-DHF on cognition and related neuronal physiology in a model of neuroinflammation.

3.1 Introduction

Exercise has been well-established as having a role in the induction of neurogenesis, providing an opportunity for recovery, and potential to counteract distress within the CNS caused by age-related and disease-induced deficits in cognitive function. This has been shown in mouse studies reporting an LPS-induced reduction in hippocampal neurogenesis that was rescued by a five week programme of exercise, along with enhanced spatial memory (Littlefield *et al.*, 2015), and in a rat model of Vascular Dementia where exercise-induced neurogenesis delayed the onset of cognitive decline (Choi *et al.*, 2016).

Numerous studies have demonstrated the success of exercise in inducing cognitive enhancement when tested immediately following cessation of exercise. Studies with rats have shown that, along with this enhancement in cognition, and the generation of new neurons, there is an up-regulation in hippocampal expression of both BDNF and synaptic proteins, when analysed shortly after the exercise protocol has reached completion (Griffin *et al.*, 2009, Bechara *et al.*, 2014).

There is much in the literature outlining the negative interaction between inflammation and neurogenesis, whether exerted through the activity of microglia (Ekdahl *et al.*, 2003), their release of cytokines (Villeda *et al.*, 2011), or the effect of various other markers of inflammation. Chronic inflammation has been linked to cognitive decline, and it is proposed that this is due to the regulation of neurogenesis by the immune system (Kohman and Rhodes, 2013). Persistent activation of microglia, the resident immune cells of the brain, can cause immunologically-induced neurodegeneration, and the death of these neurons triggers local responses that further activate inflammatory markers (Gebicke-Haerter, 2001). Neuroinflammation is involved in the process of aging in general, and provides a link to age-related neurodegenerative diseases, such as AD (Chung *et al.*, 2009). Chung *et al.* outline the age-related inflammatory hypothesis based on two processes – the dysregulation of the immune system with age, and a weakened anti-oxidative defence system.

LPS is a molecule found in the outer membrane of gram-negative bacteria. When administered to animals, it binds to a complex of toll-like receptor 4 (TLR 4) and MD-2 (a lymphocyte) in the brain, in conjunction with CD14 and LPS-binding protein (Thomas *et al.*, 2002, Akashi *et al.*, 2003). Through the aggregation of these proteins, LPS can induce a strong immune response, and so can be used to trigger experimentally-induced neuroinflammation.

Exercise has been shown to protect against inflammation in the periphery (Pinto *et al.*, 2012), and physical inactivity is reported to induce chronic disease through mechanisms mediated by peripheral inflammation (Handschin and Spiegelman, 2008).

The current study had several aims. With exercise as an intervention, we hoped to replicate the success of previous studies carried out in the laboratory, but in a model designed to test cognitive function four weeks after exercise has begun; a time point that coincides with the integration of new neurons into the circuitry, as they reach a stage of functional maturity (Cameron *et al.*, 1993). Concurrently, we hoped to observe similar results in an investigation of the acute effect of one month of exposure to the pharmacomimetic; 7,8-DHF.

We compared our findings with data from sedentary animals exposed to stationary treadmills, or animals receiving a vehicle treatment in their drinking water while remaining in their home-cages. In these same animals, we investigated the acute effect of experimentally-induced neuroinflammation using a subseptic dose of LPS, and whether a regimen of either exercise or 7,8-DHF treatment could protect subjects from cognitive disturbance when tested in a spatial memory-dependent behavioural task.

One week of daily i.p. injections of BrdU were administered to allow us to investigate the presence of newly generated hippocampal cells. We endeavour to probe the mechanisms underlying the measured cognitive differences between groups with the quantification of BrdU⁺ cells in the dentate gyrus, the concentration of inflammatory markers, and the modulation of neurotrophic factors.

3.2 Methods

3.2.1 Animals

A total of 120 male C57BL/6 Jax mice were obtained from the Comparative Medicine unit, TCD, from a line bred from Charles River Laboratories (UK) and maintained in the Comparative Medicine unit, Trinity Biomedical Sciences Institute, TCD. Animals were 12 - 14 weeks old, with a body mass ranging from 20 – 25g, at the beginning of the experimental protocol.

3.2.2 Animal housing

Animals were group housed, with between three and seven animals per cage. Tecniplast 1285LN individually ventilated cages (W x D x H - 396 x 215 x 172 mm) were used to house subjects, and they were maintained in a facility offering a constant temperature of 22°C ($\pm$ 1°C), humidity of 55% ($\pm$ 5%), and on a 12hr light/dark cycle (lights on a.m., lights off p.m.). Food and water were available *ad libitum*. While drug was being administered in drinking water, water-bottle levels were monitored to ensure that animal hydration was unaffected.

Mice were allowed at least one week acclimatization to extended surroundings upon arrival into the research unit before experimental proceedings began.

3.2.3 Experimental design

Animals were randomly assigned to either sedentary, exercise, vehicle or 7,8-DHF groups, and undertook their designated protocol for a period of one month. Where applicable, animals received either vehicle or 7,8-DHF in their drinking water.

Animals were subject to cognitive testing via the OD task.

Before training in the OD task, treatment groups were further subdivided at random and all animals received a single intraperitoneal injection of either saline or LPS (n=20 from each group) to assess the effects of exercise or 7,8-DHF administration in a mouse model of neuroinflammation.

Tissue was collected immediately following the testing phase of the OD task.

3.2.3.1 Experiment I – Can exercise protect against experimentally-induced neuroinflammation in the C57/BL6 mouse: assessment at 24hrs post-learning?

Animals were randomly assigned to either Sed or Ex groups. Exerciser treadmills with facilities for six mice simultaneously were used to run animals for 30min per day, five days per week, for one month. Sedentary animals were placed on a stationary treadmill in the same room for the same period of time to control for exposure to a novel environment. After one month, animals were further divided into Saline (Sed_Sal, Ex_Sal) or LPS (Sed_LPS, Ex_LPS) treatment groups (n = 10). Each subject received an i.p. injection of saline or LPS following a strict timetable, and exactly three hours later each animal was trained in the OD task. Exactly 24hrs after training, each animal was tested in the OD task and sacrificed within one hour of completion. This study looked at a timeframe of behavioural testing that had been used many times in our lab.

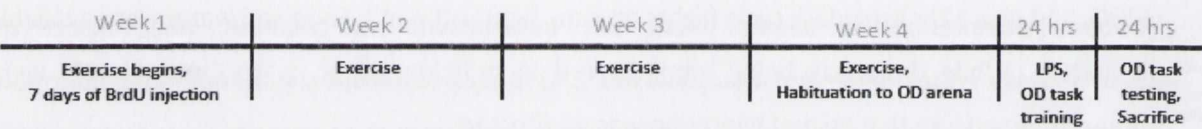


Fig 3.2.1 - Timeline of Experiment I

3.2.3.2 Experiment II – Can exercise protect acutely against experimentally-induced neuroinflammation in the C57/BL6 mouse: assessment at 1 hour post-learning?

Animals were randomly assigned to either Sed or Ex groups. Exerciser treadmills with facilities for six mice simultaneously were used to run animals for 30min per day, five days per week, for one month. Sedentary animals were placed on a stationary treadmill in the same room for the same period of time to control for exposure to a novel environment. After one month, animals were further divided into Saline (Sed_Sal, Ex_Sal) or LPS (Sed_LPS, Ex_LPS) treatment groups (n = 10). Each subject received an i.p. injection of either saline or LPS following a strict timetable, and exactly four hours later each animal was trained in the OD task. Exactly one hour after training, each animal was tested in the OD task and sacrificed within one hour of completion.

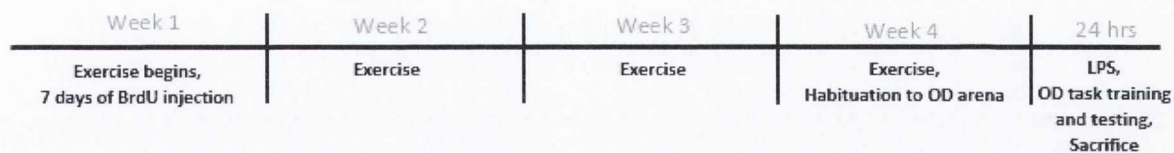


Fig 3.2.2 - Timeline of Experiment II

3.2.3.3 Experiment III – Can 7,8-DHF protect acutely against experimentally-induced neuroinflammation in the C57/BL6 mouse?

Animals were randomly assigned to either Veh or 7,8-DHF (DHF) treated groups. Vehicle or 7,8-DHF stock solutions were added to drinking water, and bottles were changed every two to three days for one month. After this time, animals were further divided into Saline (Veh_Sal, DHF_Sal) or LPS (Veh_LPS, DHF_LPS) treatment groups (n = 10). Each subject received an i.p. injection of either saline or LPS following a strict timetable, and exactly four hours later each animal was trained in the OD task. Exactly one hour after training, each animal was tested in the OD task and sacrificed within one hour of completion.



Fig 3.2.3 - Timeline of Experiment III

3.2.4 Exercise protocol

Animals were randomly assigned to either sedentary or exercise groups. Exerciser treadmills with facilities for six mice simultaneously were used to run animals for 30min per day, five days per week, for one month. Each day, treadmill speed was initially set at 300m/hr and was increased incrementally to a maximum speed of 600m/hr. If animals stopped running, they were encouraged with a gentle hand; there was no electrical current set on the treadmills to provoke continuous movement. Sedentary animals were placed on a stationary treadmill in the same room for the same period of time to control for exposure to a novel environment.

3.2.5 7,8-DHF administration

The small molecule TrkB receptor agonist 7,8-DHF (100mg) was dissolved in DMSO (100µl). This stock solution was added to dH₂O (100ml) containing sucrose (1g) (0.1% DMSO, 1% sucrose) and mixed until the solution was uniform. This brought the final concentration to approximately 23mg/kg of 7,8-DHF, per animal, per day. Control animals received a vehicle solution (0.1% DMSO, 1% sucrose, dH₂O). Animals were randomly assigned to Veh and 7, 8-DHF groups, but allocation was on a cage by cage basis. Water bottles were changed every two to three days, before 7,8-DHF started to come out of solution. Levels of water consumption were monitored to ensure there was no aversion to taste, which could result in dehydration in the animals. Subjects were also weighed individually on a weekly basis to ensure that there was no drastic fluctuation in body mass of any one specific animal as a result of dehydration, but which may have been masked by water consumption of the cage as a whole.

3.2.6 BrdU administration

Starting four weeks prior to sacrifice, subjects received a daily intraperitoneal injection of BrdU (50mg/kg BrdU made up in .89% w/v Saline solution) for seven consecutive days. Each day, within one hour of exercise, if applicable, animals received the i.p injection. Approximately 200µl was administered per animal.

Injections were carried out by licensed individuals, in accordance with procedural guidelines.

3.2.7 LPS administration

Prior to behavioural training, animals received a single i.p. injection of either saline (0.89% NaCl in dH₂O) or a subseptic dose of *E. Coli* LPS (100µg/kg) in saline. Approximately 200-300µl was administered to each animal, as determined by individual body mass.

Injections were carried out by licensed individuals, in accordance with procedural guidelines.

3.2.8 Behavioural assessment - Object Displacement

The purpose of the object displacement task is to assess spatial learning and memory in all animals, using a task designed to test this hippocampal-dependent memory. To perform testing, a cylindrical open field, 50cm in diameter, was positioned in a dimly-lit room. For two days prior to the task all animals were habituated to the open field – the first day in groups on a cage-by-cage basis for ten minutes, the second day individually for five minutes.

On day three of exposure to the open field each mouse was placed by the experimenter in the centre of the open field and allowed to explore alone for five minutes. On this occasion, three objects of similar size and colour (constructed from standard Lego blocks) were placed into the open field and a spatial cue was placed in a fixed position on the wall of the open field. Time spent exploring each object was recorded. All equipment was cleaned between subjects to remove any olfactory cues. Either one or 24hrs (depending on experiment) following training, one object was moved to the previously empty quadrant of the circle while the other two objects, as well as the spatial cue, remained in the same position. Upon completion of the five minute task, animals were removed from the arena and euthanized within 60mins.

3.2.9 Tissue collection and preparation

Following behavioural testing, animals were sacrificed via either cervical dislocation or transcardial perfusion, as described in section 2.9. The former was used for collection of fresh hippocampal and cortical tissue, and the latter for *in situ* whole-brain fixation for immunohistochemical analysis. All samples were stored at -80°C until required for further analysis.

Fresh tissue samples were homogenised by hand using a manual homogeniser in 500µl of freshly prepared lysis buffer (for western blot), or Krebs buffer (for ELISA). Once suspended in buffer these samples were kept in standard freezing conditions of between -14 and -18°C. Concentration of total protein in fresh tissue homogenates was measured using the BCA assay, as described in section 2.10.1.

Additional fresh tissue samples were mechanically homogenized in lysis buffer. RNA was extracted, cDNA was synthesized from the purified mRNA, and cDNA was amplified and detected during the polymerase chain reaction.

Whole-brain fixation was carried out via transcardial perfusion, and these brains were stored in a cryoprotective sucrose solution at 4°C until required for further analysis.

3.2.10 Analysis of mRNA expression by Polymerase Chain Reaction

Expression of various neurotrophic factors, receptors and endogenous markers of proliferation; such as BDNF, TrkB, and Ki67, and inflammatory cytokines, such as IL1 β and TNF α , in the hippocampus and cortex were assessed using PCR, as described in section 2.11. β -Actin was used as an endogenous control in calculation of Rq values.

3.2.11 Analysis of protein expression by Western Immunoblotting

To assess the effect of exercise or 7,8-DHF on important signalling proteins downstream of TrkB receptor activation, expression of phosphorylated and total Akt, CREB, and ERK1/2 were measured in hippocampal and cortical tissue using western blot, as described in section 2.12. β -Actin was used as an endogenous control for total protein concentration.

3.2.12 Quantification of BrdU⁺ cells in dentate gyrus

Fixed, whole brains were sliced, and stained for BrdU⁺ cells using fluorescent double labelling with NeuN, as described in section 2.13.

3.2.13 Statistical analysis

All data was analysed and grouped using Microsoft Excel (2013), and then imported into Graphpad Prism (version 5.01) where column statistics were used to examine the data for outliers, and these were omitted in the case of values lying two standard deviations above or below the mean value. All data were represented as mean $\pm$ SEM.

Analysis was carried out using unpaired student t-test - when one factor is compared in two groups, one-way ANOVA - when one factor is compared three or more groups, and two-way ANOVA - when two or more factors are compared in two or more groups, as well as performing Bonferroni post hoc tests. Statistical significance was determined at $p < 0.05$ based upon these results.

3.3 Results

3.3.1 Experiment 1 – Can exercise protect against experimentally-induced subacute neuroinflammation in the C57/BL6 mouse: assessment at 24hrs post-learning?

We hypothesise that one month of treadmill exercise can protect against experimentally-induced neuroinflammation in the mouse, and will rescue disturbances in cognitive function triggered by this acute inflammatory response. We aim to assess the following:

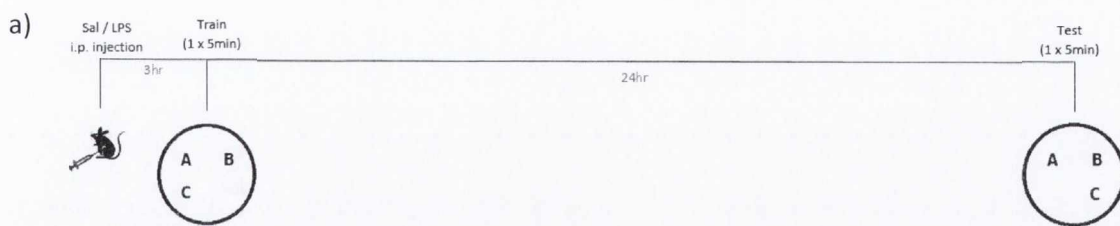
- The effect of running exercise on spatial learning in the mouse when compared to sedentary controls.
- The effect of running exercise in conjunction with LPS administration on spatial memory in the mouse, when compared to LPS-treated sedentary controls and Sal-treated running mice.
- The effect of running exercise on expression and activation of signalling proteins from the ERK and Akt signalling pathways when compared to sedentary controls.
- The effect of running exercise in conjunction with LPS administration on expression and activation of signalling proteins from the ERK and Akt signalling pathways, when compared to LPS-treated sedentary controls and Sal-treated running mice.
- The effect of running exercise on mRNA expression of genes related to TrkB receptor activation when compared to sedentary controls.
- The effect of running exercise in conjunction with LPS administration on mRNA expression of genes related to TrkB receptor activation, when compared to LPS-treated sedentary controls and Sal-treated running mice.
- The effect of running exercise on mRNA expression of markers of inflammation and microglial activation when compared to sedentary controls.
- The effect of running exercise in conjunction with LPS administration on mRNA expression of markers of inflammation and microglial activation, when compared to LPS-treated sedentary controls and Sal-treated running mice.
- The effect of running exercise on dentate gyrus volume and numbers of BrdU⁺ cells in the dentate gyrus when compared to sedentary controls.

3.3.1.1 The effect of one month of exercise on total exploration in OD task, 27 hours after experimentally-induced neuroinflammation.

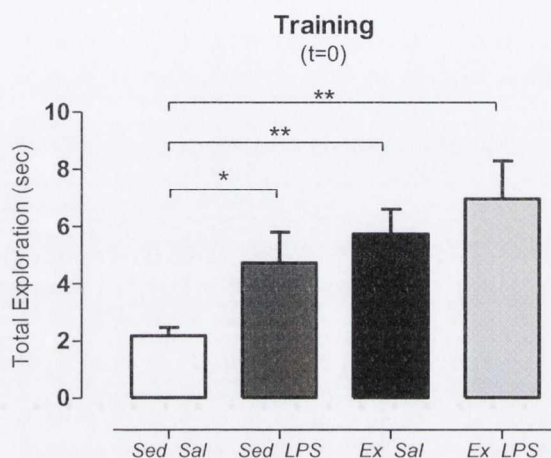
As described, subjects received an i.p. injection of either Sal or LPS, were trained in the OD task, and testing in this task took place 24hrs later (Fig 3.3.1a). Once habituated, training involved acquisition of memory with regard to the layout of the open field in which the task took place. During five minutes of exposure, a record of time spent investigating each of the three objects was kept, and total investigation calculated. Analysis of total exploration during the training session (Fig 3.3.1b) showed that Sed_Sal animals explored significantly less than Sed_LPS ($p = 0.0427$), Ex_Sal ($p = 0.0017$), and Ex_LPS animals ($p = 0.0036$) ($Sed_Sal = 2.180 \pm 0.2844$; $Sed_LPS = 4.721 \pm 1.068$; $Ex_Sal = 5.725 \pm 0.8643$; $Ex_LPS = 6.979 \pm 1.319$).

During the testing phase (Fig 3.3.1c), it was found that Sed_Sal subjects explored significantly less than Sed_LPS ($p = 0.0128$), Ex_Sal ($p = 0.0346$), and Ex_LPS ($p = 0.0036$) groups ($Sed_Sal = 2.489 \pm 0.4470$; $Sed_LPS = 4.931 \pm 0.7282$; $Ex_Sal = 7.240 \pm 1.915$; $Ex_LPS = 6.465 \pm 1.039$).

Assessed by unpaired t-test and/or one-way ANOVA and Bonferroni post-hoc test ($n=10$). Expressed as mean $\pm$ SEM.



b)



c)

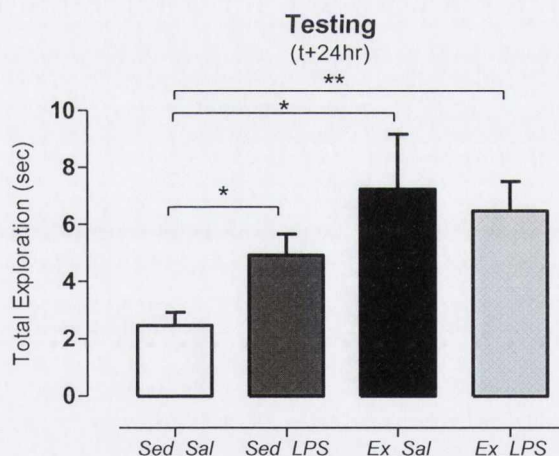


Fig. 3.3.1 - Effect of exercise and/or LPS on exploration

(a) timeline of testing, from LPS administration to OD task.

(b) shows total exploration during 5min training trial. Sed_Sal animals explored significantly less than Sed_LPS ($p = 0.0427$), Ex_Sal ($p = 0.0017$), and Ex_Sal animals ($p = 0.0346$). Exercised animals explored more as a whole.

(c) shows total exploration during 5min test. Sed_Sal animals explored significantly less than Sed_LPS ($p = 0.0128$), Ex_Sal ($p = 0.0346$), and Ex_LPS animals ($p = 0.0036$).

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. * $p < 0.05$, ** $p < 0.01$.

All data expressed as mean $\pm$ SEM. ($n = 10$).

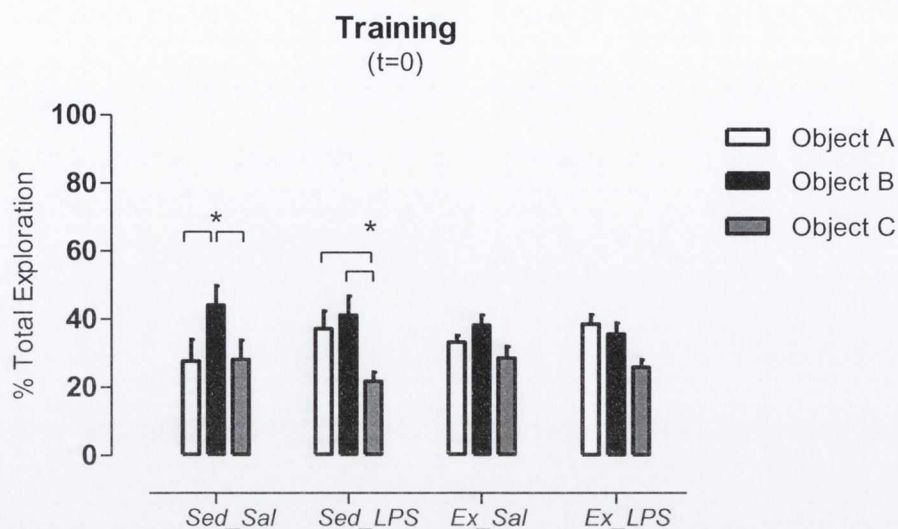
3.3.1.2 The effect of one month of exercise on behaviour in OD task, 27 hours after experimentally-induced neuroinflammation.

During the acquisition stage of the OD task (Fig 3.3.2a), there was a significant difference in object exploration within sedentary groups (*Sed_Sal*, Object A % = 27.622 ± 6.297763 , Object B % = 44.156 ± 5.593137 , Object C % = 28.223 ± 5.551255 ; *Sed_LPS*, Object A % = 37.185 ± 5.217654 , Object B % = 41.132 ± 5.614373 , Object C % = 21.681 ± 2.712403) ($n = 10$) but not within exercised groups (*Ex_Sal*, Object A % = 33.196 ± 1.987584 , Object B % = 38.198 ± 2.980479 , Object C % = 28.609 ± 3.3202 ; *Ex_LPS*, Object A % = 38.486 ± 2.804158 , Object B % = 35.627 ± 3.156799 , Object C % = 25.888 ± 2.1133) ($n = 10$).

During the memory recall phase of the OD task (Fig 3.3.2b), object C was moved to the previously empty quadrant of the arena. There was no significant difference in object exploration within either sedentary group (*Sed_Sal*, Object A % = 28.056 ± 5.042817 , Object B % = 36.596 ± 4.744203 , Object C % = 35.347 ± 3.618155 ; *Sed_LPS*, Object A % = 32.965 ± 5.522654 , Object B % = 30.145 ± 4.330857 , Object C % = 36.891 ± 4.169271) ($n = 10$), but both exercised groups spent significantly more time exploring the displaced object; C (*Ex_Sal*, Object A % = 26.995 ± 6.399475 , Object B % = 22.071 ± 2.881054 , Object C % = 50.936 ± 6.67540 ; *Ex_LPS*, Object A % = 27.427 ± 4.518898 , Object B % = 25.253 ± 4.26658 , Object C % = 47.318 ± 6.533616) ($n = 10$).

Assessed by two-way ANOVA and Bonferroni post-hoc test. Expressed as mean $\pm$ SEM.

a)



b)

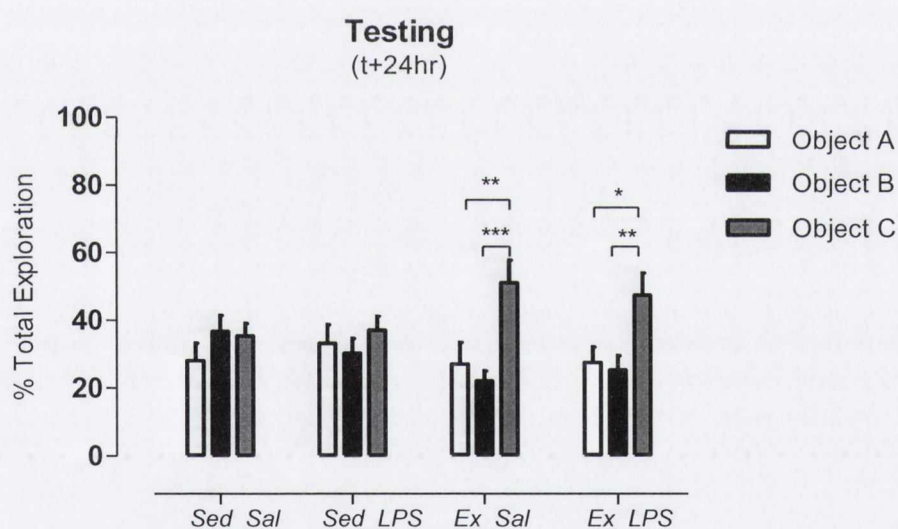


Fig. 3.3.2 – Object Displacement Task

(a) shows exploration during a single 5min trial. There was no significant difference in object exploration in exercise groups. Sed_Sal subjects explored Object B significantly more than Objects A or C, and Sed_LPS subjects explored Object C significantly less than Objects A or B.

(b) shows exploration during a single 5min test. All exercised animals displayed a significant preference for the displaced object; C.

Assessed by two-way ANOVA and Bonferroni post-hoc analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All data expressed as mean $\pm$ SEM. (n = 10).

3.3.1.3a The effect of one month of exercise on mRNA expression of BDNF, TrkB, Ki67 and CREB, 27 hours after experimentally- induced neuroinflammation.

PCR analysis showed there was no significant difference in BDNF mRNA expression in the hippocampus when Rq measurement was compared across all treatment groups ($Sed_Sal = 0.7455 \pm 0.1059$; $Sed_LPS = 0.7407 \pm 0.08162$; $Ex_Sal = 0.6636 \pm 0.05206$; $Ex_LPS = 0.7806 \pm 0.0741$). ($p = 0.7411$, $n = 5$). (Fig 3.3.3)

PCR analysis showed there was no significant difference in TrkB mRNA expression in the hippocampus when Rq measurement was compared across all treatment groups. ($Sed_Sal = 1.089 \pm 0.07904$; $Sed_LPS = 0.8737 \pm 0.05567$; $Ex_Sal = 1.098 \pm 0.0775$; $Ex_LPS = 1.037 \pm 0.04737$). ($p = 0.0896$, $n = 5$). (Fig 3.3.4)

PCR analysis showed there was no significant difference in Ki67 mRNA expression in the hippocampus when Rq measurement was compared across all treatment groups ($Sed_Sal = 1.201 \pm 0.08611$; $Sed_LPS = 1.526 \pm 0.09879$; $Ex_Sal = 1.514 \pm 0.1473$; $Ex_LPS = 1.636 \pm 0.2437$). ($p = 0.3514$, $n = 5$). (Fig 3.3.5)

PCR analysis showed there was no significant difference in CREB mRNA expression in the hippocampus when Rq measurement was compared across all treatment groups ($Sed_Sal = 1.314 \pm 0.3535$; $Sed_LPS = 1.068 \pm 0.1601$; $Ex_Sal = 1.661 \pm 0.5183$; $Ex_LPS = 1.904 \pm 0.4356$). ($p = 0.4631$, $n = 5$). (Fig 3.3.6)

Data analysed by unpaired t-test and/or one-way ANOVA and Bonferroni post-hoc test, expressed as mean $\pm$ SEM.

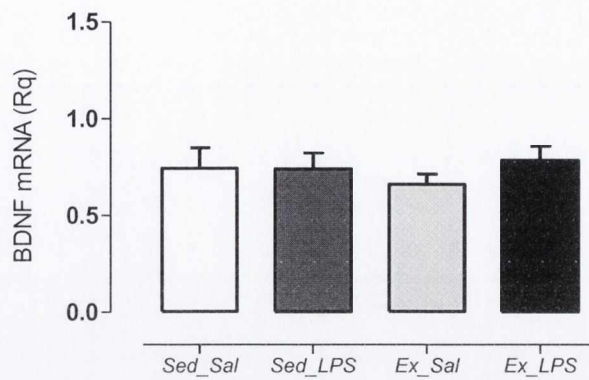


Fig. 3.3.3 – Effect of exercise and/or LPS on BDNF mRNA expression

Shows Rq of BDNF mRNA expression in hippocampal tissue. There was no significant difference in BDNF expression between subject groups

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. $p>0.05$.

Data expressed as mean $\pm$ SEM (n=5).

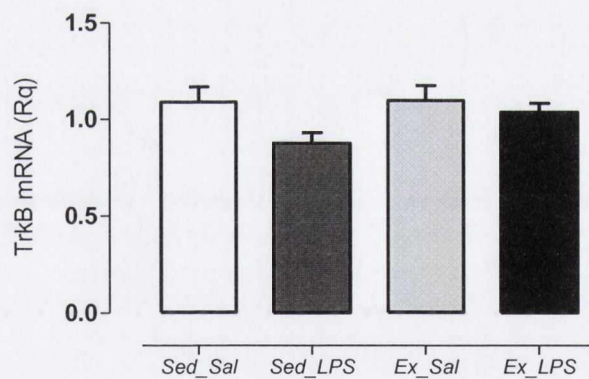


Fig. 3.3.4 – Effect of exercise and/or LPS on TrkB mRNA expression

Shows Rq of TrkB mRNA expression in hippocampal tissue. There was no significant difference in TrkB expression between subject groups

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. $p>0.05$.

Data expressed as mean $\pm$ SEM (n=5).

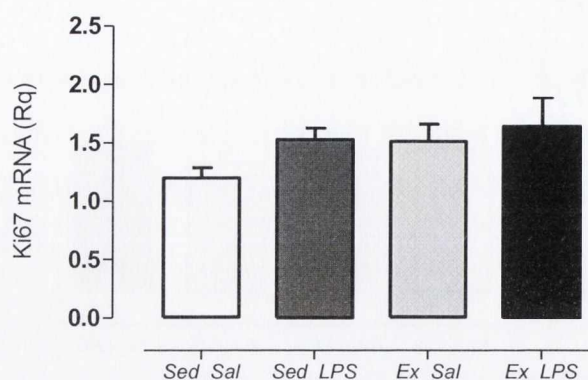


Fig. 3.3.5 – Effect of exercise and/or LPS on Ki67 mRNA expression

Shows Rq of Ki67 mRNA expression in hippocampal tissue. There was no significant difference in Ki67 expression between subject groups

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. $p > 0.05$.

Data expressed as mean $\pm$ SEM (n=5).

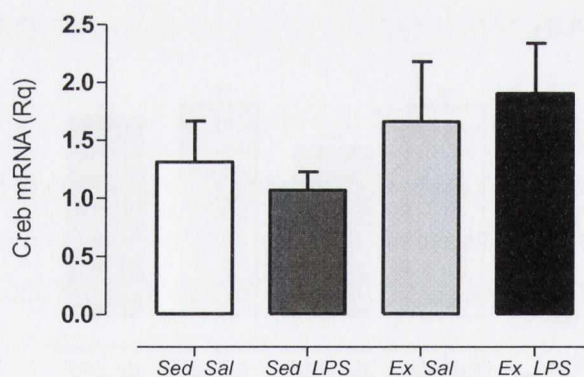


Fig. 3.3.6 – Effect of exercise and/or LPS on CREB mRNA expression

Shows Rq of CREB mRNA expression in hippocampal tissue. There was no significant difference in CREB expression between subject groups

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. $p > 0.05$.

Data expressed as mean $\pm$ SEM (n=5).

3.3.1.3b The effect of one month of exercise on mRNA expression of IL1 β TNF α , Iba1 and CD44, 27 hours after experimentally-induced neuroinflammation.

PCR analysis showed there was a significant difference in IL1 β mRNA expression in the hippocampus when Rq measurement from Sed_LPS subjects was compared to the Sed_Sal treatment group ($p=0.0035$) and when Rq of Ex_LPS was compared to that of Ex_Sal ($p=0.0002$), but not when Sed_LPS and Ex_LPS groups were analysed against each other ($p = 0.161$). ($Sed_Sal = 0.7981 \pm 0.09399$; $Sed_LPS = 5.197 \pm 0.8964$; $Ex_Sal = 0.5720 \pm 0.1084$; $Ex_LPS = 3.632 \pm 0.4721$). ($p < 0.0001$, $n = 5$). (Fig 3.3.7)

PCR analysis showed there was a significant difference in TNF α mRNA expression in the hippocampus when Rq measurement from Sed_LPS subjects was compared to the Sed_Sal treatment group ($p=0.0016$), but not when Sed_LPS was compared to Ex_LPS ($p = 0.6564$) ($Sed_Sal = 0.7214 \pm 0.2647$; $Sed_LPS = 6.437 \pm 0.9925$; $Ex_Sal = 1.353 \pm 0.4775$; $Ex_LPS = 5.442 \pm 1.911$). ($p = 0.0073$, $n = 5$). (Fig 3.3.8)

PCR analysis showed there was no significant difference in Iba1 mRNA expression in the hippocampus when Rq measurement was compared across all treatment groups. ($Sed_Sal = 1.044 \pm 0.03857$; $Sed_LPS = 1.122 \pm 0.01436$; $Ex_Sal = 0.9639 \pm 0.06470$; $Ex_LPS = 1.012 \pm 0.03023$). ($p = 0.0869$, $n = 5$). (Fig 3.3.9)

PCR analysis showed there was no significant difference in CD44 mRNA expression in the hippocampus when Rq measurement was compared across all treatment groups ($Sed_Sal = 0.8102 \pm 0.2771$; $Sed_LPS = 0.9078 \pm 0.1592$; $Ex_Sal = 0.7832 \pm 0.1578$; $Ex_LPS = 0.6662 \pm 0.105$). ($p = 0.7939$, $n = 5$). (Fig 3.3.10).

Data analysed by unpaired t-test and/or one-way ANOVA and Bonferroni post-hoc test, expressed as mean $\pm$ SEM.

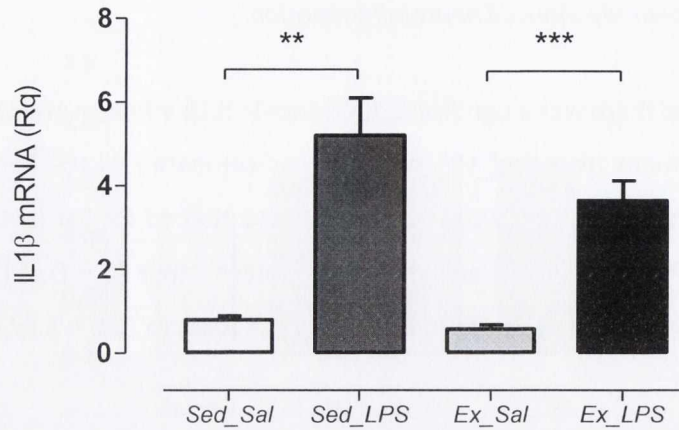


Fig. 3.3.7 – Effect of exercise and/or LPS on IL1β mRNA expression

Shows Rq of IL1β mRNA expression in hippocampal tissue. There was a significant difference in IL1β expression when comparing Sed_LPS subjects to the Sed_Sal group ($p=0.0035$). This was also observed when comparing Ex-LPS with Ex_Sal ($p=0.0002$).

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. ** $p<0.01$, *** $p<0.001$.

Data expressed as mean $\pm$ SEM ($n=5$).

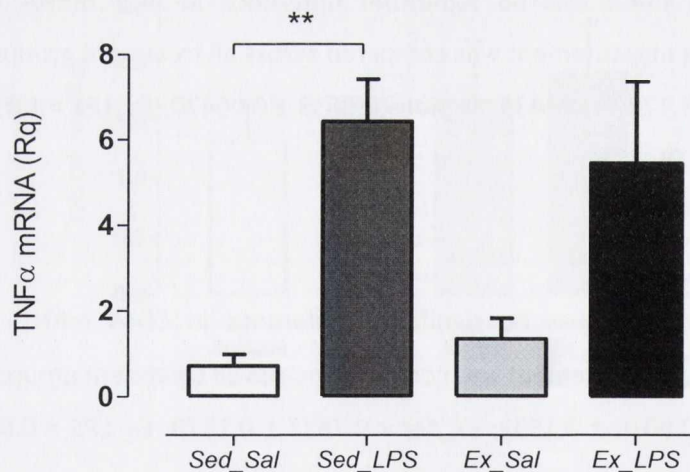


Fig. 3.3.8 – Effect of exercise and/or LPS on TNFα mRNA expression

Shows Rq of TNFα mRNA expression in hippocampal tissue. . There was a significant difference in TNFα expression when comparing Sed_LPS to Sed_Sal subjects ($p=0.0016$).

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. ** $p<0.01$.

Data expressed as mean $\pm$ SEM ($n=5$).

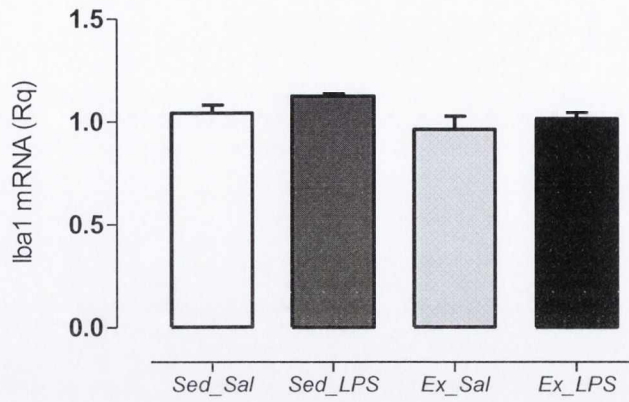


Fig. 3.3.9 – Effect of exercise and/or LPS on Iba1 mRNA expression

Shows Rq of Iba1 mRNA expression in hippocampal tissue. There was no significant difference in Iba1 expression between subject groups

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. $p > 0.05$.

Data expressed as mean $\pm$ SEM (n=5).

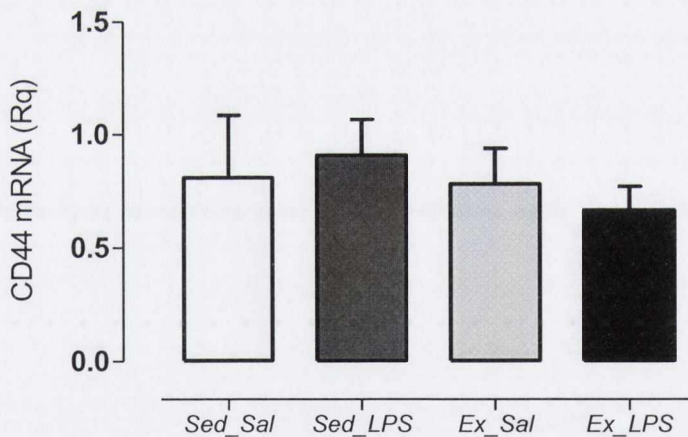


Fig. 3.3.10 – Effect of exercise and/or LPS on CD44 mRNA expression

Shows Rq of CD44 mRNA expression in hippocampal tissue. There was no significant difference in CD44 expression between subject groups

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. $p > 0.05$.

Data expressed as mean $\pm$ SEM (n=5).

3.3.1.4a The effect of one month of exercise on expression and activation of ERK, 27 hours after experimentally-induced neuroinflammation.

Western immunoblotting analysis of hippocampal tissue showed a significant difference in total ERK 1 expression relative to β -Actin (Fig 3.3.11a) in Ex_LPS subjects when compared to Sed_LPS animals ($p = 0.0018$), but not when compared to Ex_Sal ($p = 0.0869$). There was a significant difference in expression when Ex_Sal was compared to Sed_Sal ($p = 0.0271$). ($Sed_Sal = 0.2218 \pm 0.03169$; $Sed_LPS = 0.3509 \pm 0.1448$; $Ex_Sal = 0.7726 \pm 0.1727$; $Ex_LPS = 1.167 \pm 0.1047$) ($n = 5$).

There was no significant difference in phosphorylated ERK 1 expression relative to total ERK 1 in the hippocampus (Fig 3.3.11b) in any of the groups analysed. ($Sed_Sal = 1.295 \pm 0.7629$; $Sed_LPS = 1.692 \pm 0.9023$; $Ex_Sal = 0.9251 \pm 0.3398$; $Ex_LPS = 1.236 \pm 0.7668$) ($p = 0.8954$, $n = 5$).

There was no significant difference in total ERK 2 expression relative to β -Actin in the hippocampus (Fig 3.3.12a) in any of the groups analysed. ($Sed_Sal = 0.4184 \pm 0.08292$; $Sed_LPS = 0.6163 \pm 0.05200$; $Ex_Sal = 0.6307 \pm 0.07842$; $Ex_LPS = 0.5329 \pm 0.05993$) ($p = 0.1766$, $n = 5$).

There was no significant difference in phosphorylated ERK 2 expression relative to total ERK 2 in the hippocampus (Fig 3.3.12b) in any of the groups analysed. ($Sed_Sal = 0.6635 \pm 0.3490$; $Sed_LPS = 0.5655 \pm 0.2443$; $Ex_Sal = 0.5186 \pm 0.1285$; $Ex_LPS = 0.4388 \pm 0.2390$) ($p = 0.9328$, $n = 5$).

Data analysed by unpaired t-test and/or one-way ANOVA and Bonferroni post-hoc test, expressed as mean $\pm$ SEM.

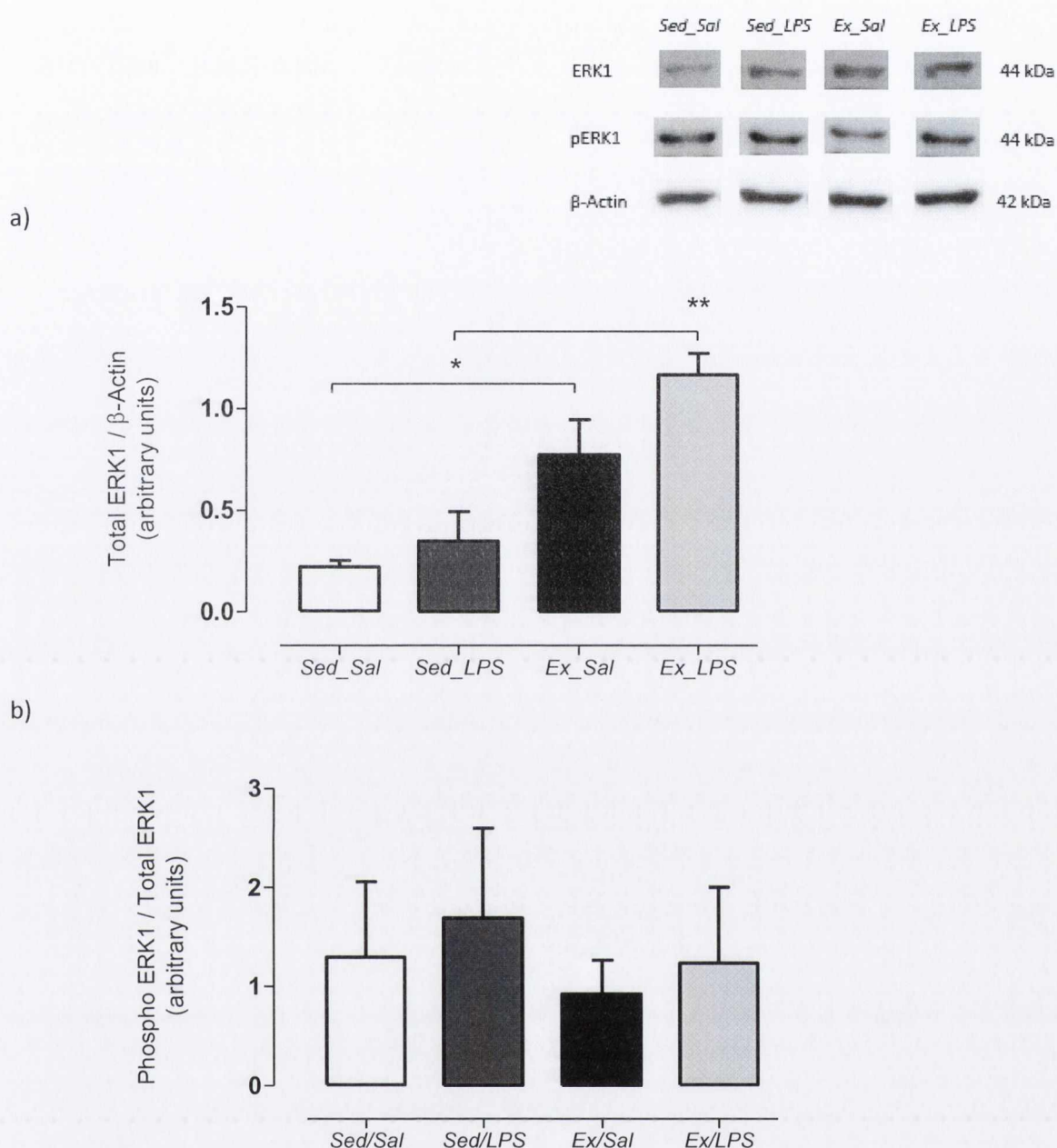


Fig. 3.3.11 – ERK1 protein concentration in hippocampus

A representative blot of ERK1 and its activated form phospho ERK1, found at approximately 44kDa, and β-Actin protein as an endogenous marker of protein content.

(a) shows a significant difference in total ERK1 expression in Ex_LPS subjects when compared Sed_LPS animals ($p=0.0018$), and a difference in Ex_Sal when compared to Sed_Sal ($p=0.0271$).

(b) shows no significant difference in phosphorylated ERK1 expression among subjects.

Assessed by one-way ANOVA and Bonferroni post-hoc analysis, * $p<0.05$, ** $p<0.01$.

Data expressed as mean $\pm$ SEM ($n=5$).

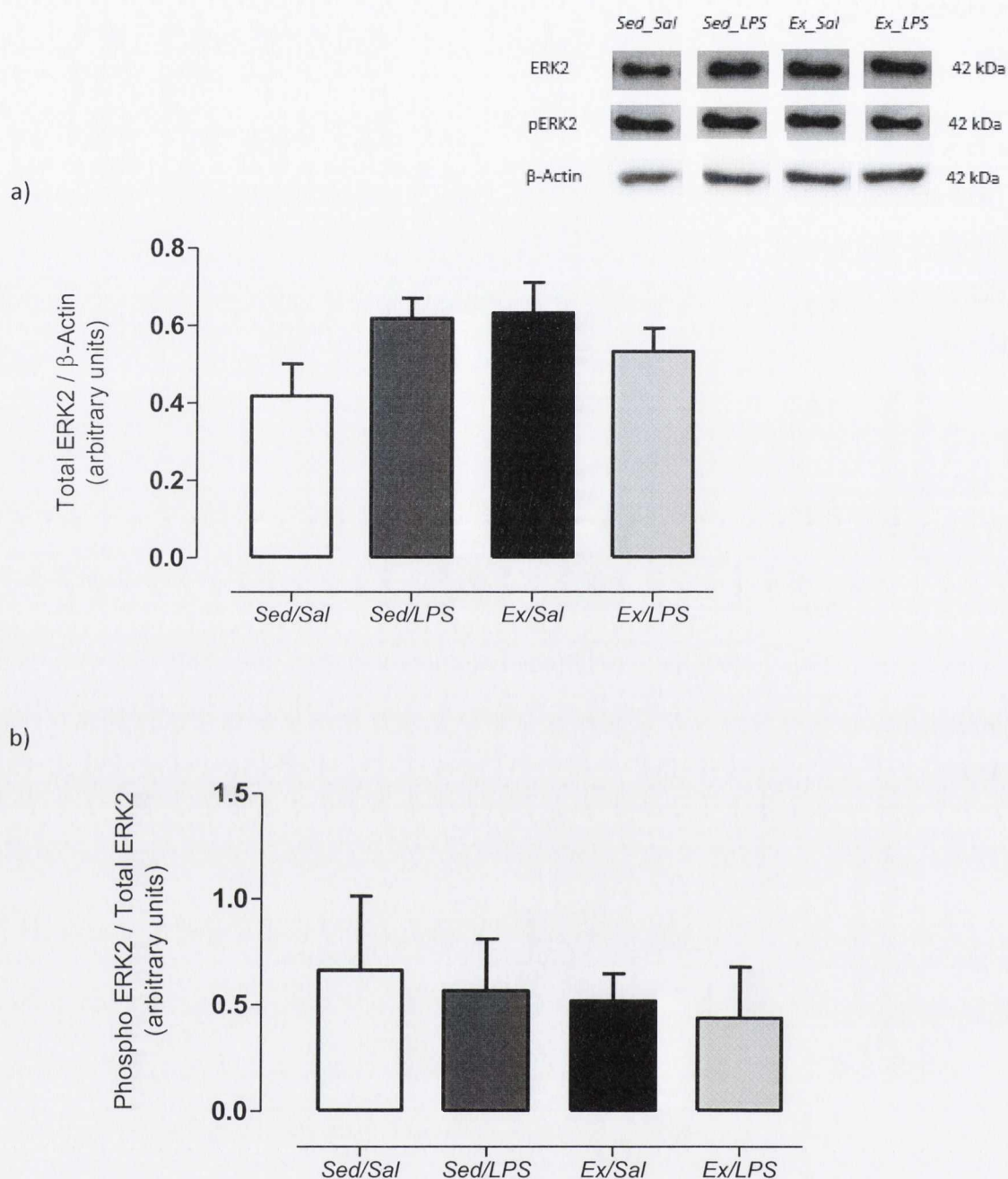


Fig. 3.3.12 – ERK2 protein concentration in hippocampus

A representative blot of ERK2 and its activated form phospho ERK2, found at approximately 42kDa, and β-Actin protein as an endogenous marker of protein content.

(a) shows no significant difference in ERK2 expression among subjects.

(b) shows no significant difference in phosphorylated ERK2 expression among subjects.

Assessed by one-way ANOVA and Bonferroni post-hoc analysis, $p > 0.05$.

Data expressed as mean $\pm$ SEM (n=5).

3.3.1.4b The effect of one month of exercise on expression and phosphorylation of Akt and CREB, 27 hours after experimentally-induced neuroinflammation.

There was no significant difference in total Akt expression relative to β -Actin in the hippocampus (Fig 3.3.13a) in any of the groups analysed. (*Sed_Sal* = 1.346 ± 0.2727 ; *Sed_LPS* = 1.180 ± 0.06399 ; *Ex_Sal* = 1.728 ± 0.2715 ; *Ex_LPS* = 1.527 ± 0.3676) ($p = 0.5129$, $n = 5$).

There was no significant difference in phosphorylated Akt expression relative to total Akt in the hippocampus (Fig 3.3.13b) in any of the groups analysed. (*Sed_Sal* = 0.9067 ± 0.1181 ; *Sed_LPS* = 0.7619 ± 0.06802 ; *Ex_Sal* = 0.7756 ± 0.04149 ; *Ex_LPS* = 0.8154 ± 0.1400) ($p = 0.7544$, $n = 5$).

There was no significant difference in total CREB expression relative to β -Actin in the hippocampus (Fig 3.3.14a) in any of the groups analysed. (*Sed_Sal* = 2.789 ± 0.5170 ; *Sed_LPS* = 3.040 ± 1.125 ; *Ex_Sal* = 3.001 ± 0.7567 ; *Ex_LPS* = 2.204 ± 0.1124) ($p = 0.8017$, $n = 5$).

There was no significant difference in phosphorylated CREB expression relative to total CREB in the hippocampus (Fig 3.3.14b) in any of the groups analysed. (*Sed_Sal* = 0.5951 ± 0.08398 ; *Sed_LPS* = 0.5407 ± 0.1660 ; *Ex_Sal* = 0.7029 ± 0.05456 ; *Ex_LPS* = 0.7142 ± 0.05833) ($p = 0.5748$, $n = 5$).

Data analysed by unpaired t-test and/or one-way ANOVA and Bonferroni post-hoc test, expressed as mean $\pm$ SEM.

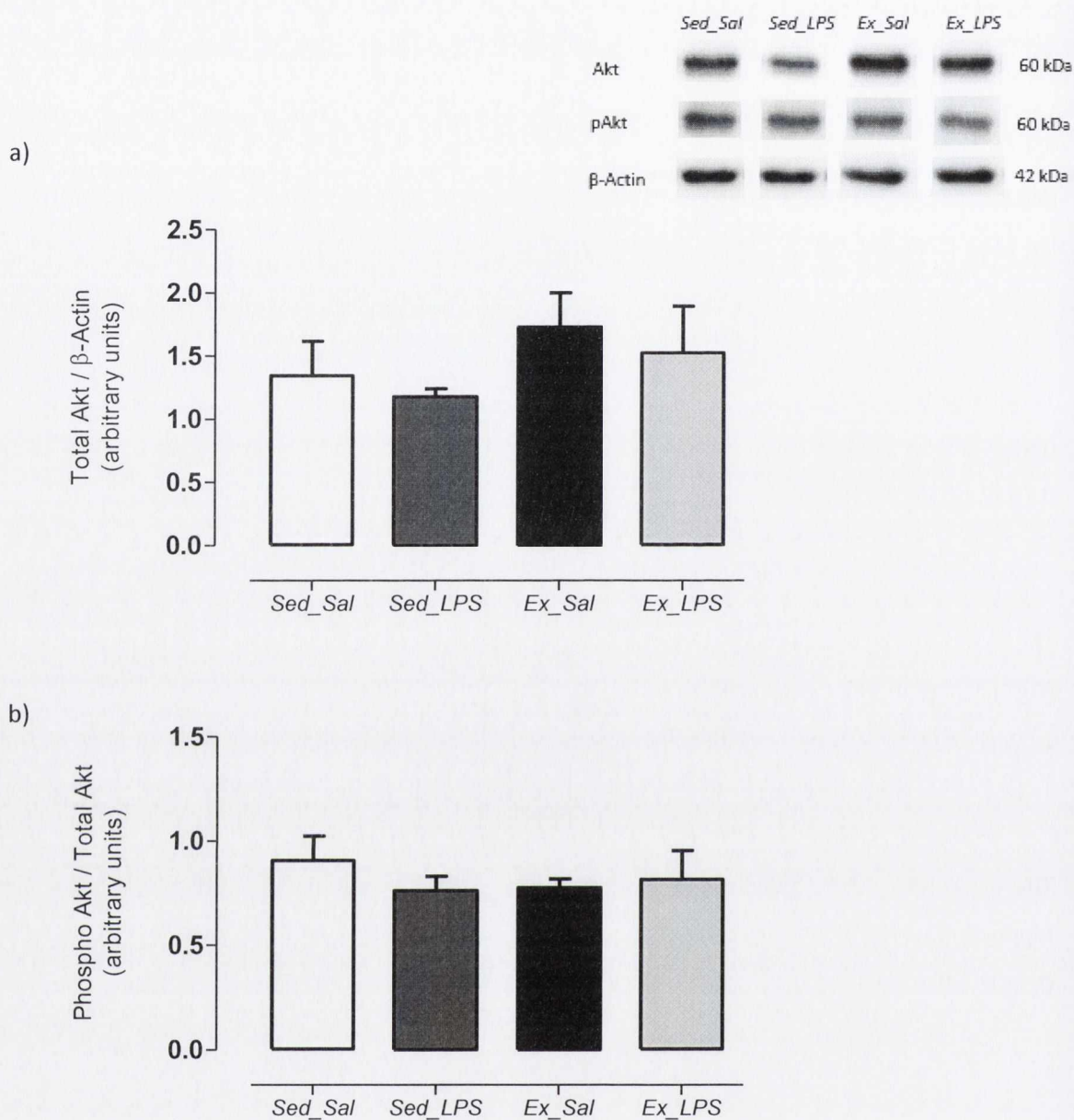


Fig. 3.3.13 – Akt protein concentration in hippocampus

A representative blot of Akt and its activated form phospho Akt, found at approximately 60kDa, and β -Actin protein as an endogenous marker of protein content.

(a) shows no significant difference in total Akt expression among subjects.

(b) shows no significant difference in phosphorylated Akt expression among subjects.

Assessed by one-way ANOVA and Bonferroni post-hoc analysis, $p > 0.05$.

Data expressed as mean $\pm$ SEM ($n=5$).

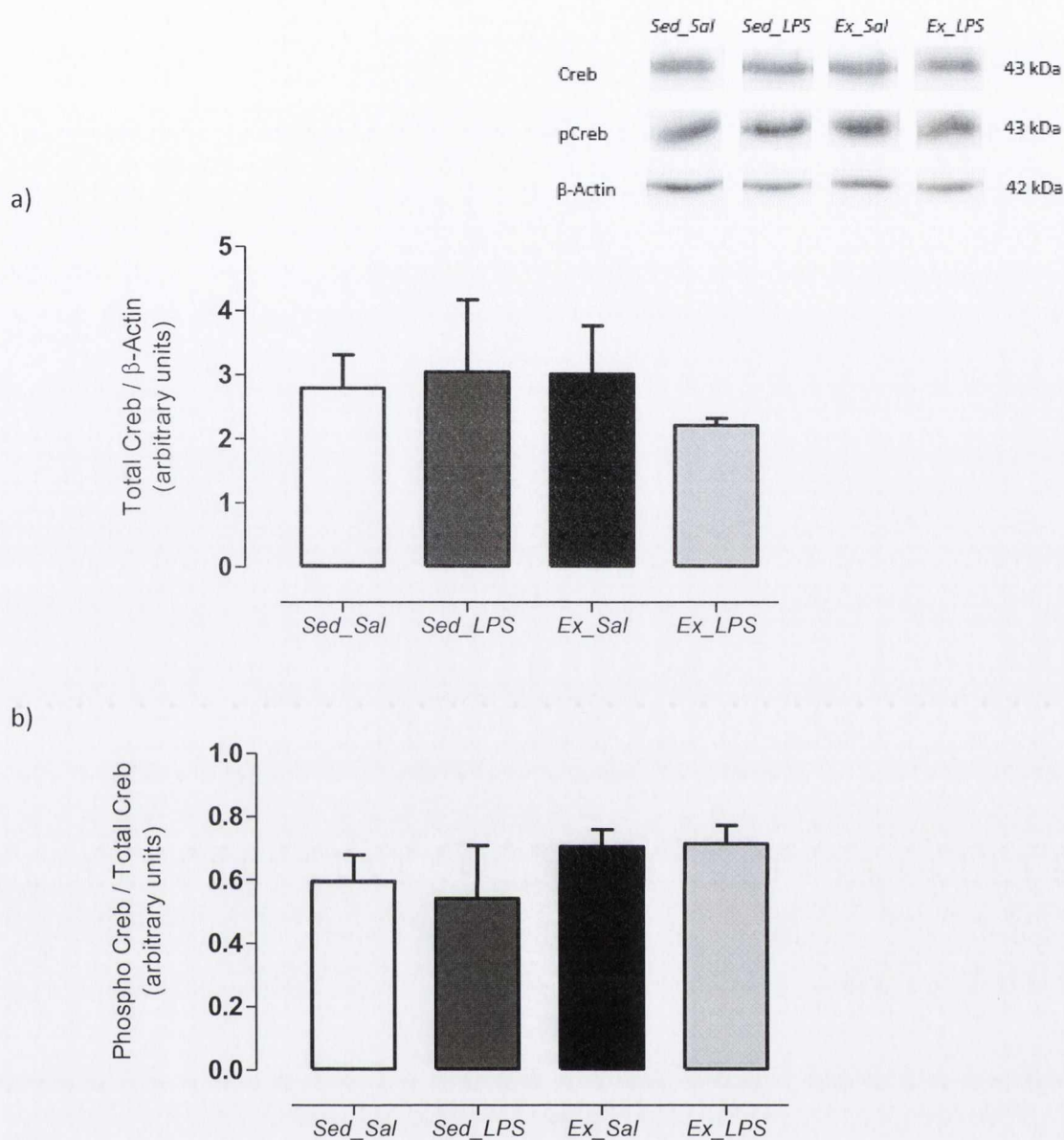


Fig. 3.3.14 – CREB protein concentration in hippocampus

A representative blot of CREB and its activated form phospho CREB, found at approximately 43kDa, and β -Actin protein as an endogenous marker of protein content.

(a) shows no significant difference in total CREB expression among subjects.

(b) shows no significant difference in phosphorylated CREB expression among subjects.

Assessed by one-way ANOVA and Bonferroni post-hoc analysis, $p > 0.05$.

Data expressed as mean $\pm$ SEM (n = 5).

3.3.1.5 The effect of one month of exercise on dentate gyrus volume, and number of BrdU⁺ cells.

Immunohistochemical staining and stereological estimation techniques show that there was no significant difference in dentate gyrus volume (Fig 3.3.15) when Sedentary animals were compared to Exercised animals, regardless of Saline or LPS administration (Sedentary = 0.5384 ± 0.02318 ; Exercise = 0.4913 ± 0.03168) ($p = 0.2507$, $n = 8$).

There was no significant difference in BrdU⁺ cell numbers (Fig 3.3.16) in animals that had undertaken four weeks of running exercise when compared to sedentary animals (Sedentary = 24.48 ± 3.120 ; Exercise = 31.70 ± 3.741) ($p = 0.1064$, $n = 8$).

Data analysed using unpaired t-test, expressed as mean $\pm$ SEM.

Figure 3.3.17 shows an example of images obtained from Sedentary (a, c) and Exercised (b, d) animals.

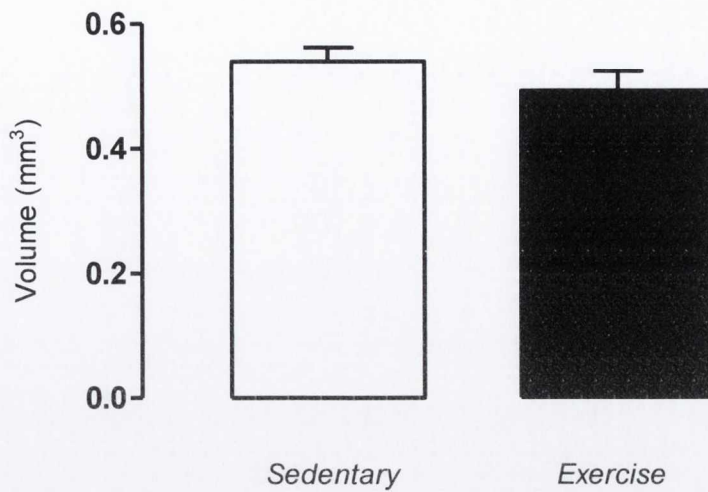


Fig. 3.3.15 – The effect of four weeks of running exercise on dentate gyrus volume
There was no significant difference in overall volume of dentate gyrus as measured using the Cavalieri method of volume estimation.
Assessed by unpaired t test ($p=0.2507$). Data expressed as mean $\pm$ SEM ($n=8$).

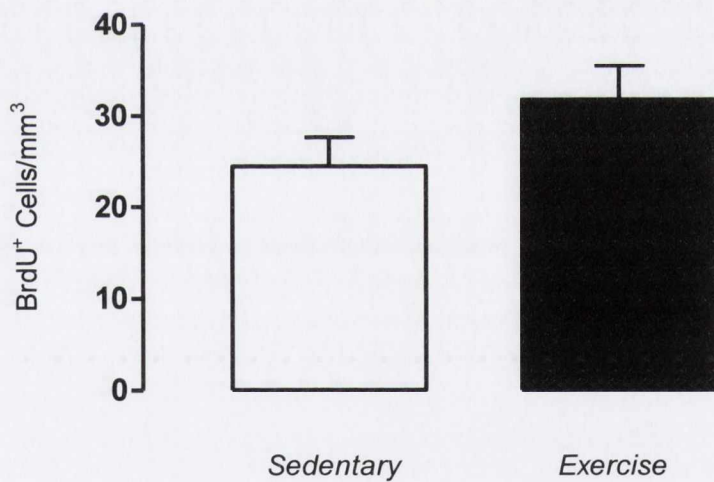


Fig. 3.3.16 - The effect of four weeks of running exercise on BrdU+ cells in the dentate gyrus
There was no significant difference in the number of BrdU+ cells in the dentate gyrus of exercised animals.
Assessed by unpaired t test ($p=0.1607$). Data expressed as mean $\pm$ SEM ($n=8$).

Sedentary

Exercise

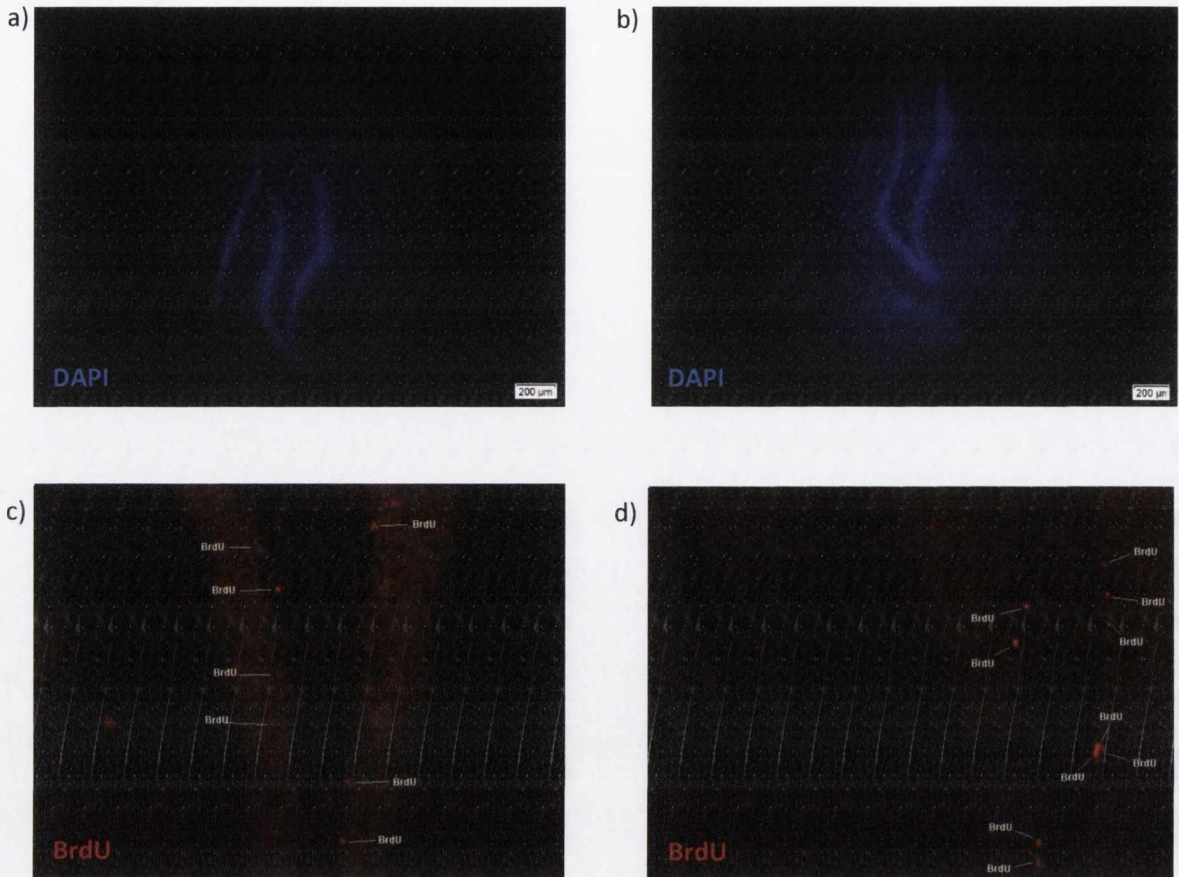


Fig. 3.3.17 - BrdU⁺ cells in the dentate gyrus

Images obtained at x4 (DAPI) and x20 (BrdU) magnification of Sedentary (a, c) and Exercise (b, d) subjects.

BrdU⁺ cells are indicated with white marker.

3.3.2 Experiment II – Can exercise protect against experimentally-induced acute neuroinflammation in the C57/BL6 mouse: assessment at 1 hour post-learning?

We hypothesise that one month of treadmill exercise can protect against experimentally-induced neuroinflammation in the mouse, and will rescue disturbances in cognitive function triggered by this acute inflammatory response when spatial memory is tested one hour after learning in the OD task. We aim to assess the following:

- The effect of running exercise on spatial learning in the mouse when compared to sedentary controls.
- The effect of running exercise in conjunction with LPS administration on spatial memory in the mouse, when compared to LPS-treated sedentary controls and Sal-treated running mice.
- The effect of running exercise on mRNA expression of genes related to TrkB receptor activation when compared to sedentary controls.
- The effect of running exercise in conjunction with LPS administration on mRNA expression of genes related to TrkB receptor activation, when compared to LPS-treated sedentary controls and Sal-treated running mice.
- The effect of running exercise on mRNA expression of markers of inflammation and microglial activation when compared to sedentary controls.
- The effect of running exercise in conjunction with LPS administration on mRNA expression of markers of inflammation and microglial activation, when compared to LPS-treated sedentary controls and Sal-treated running mice.
- The effect of running exercise on dentate gyrus volume and numbers of BrdU⁺ cells in the dentate gyrus when compared to sedentary controls.

3.3.2.1 *The effect of one month of exercise on total exploration in OD task, 5 hours after experimentally-induced neuroinflammation.*

As described, subjects received an i.p. injection of either Sal or LPS, were trained in the OD task, and testing in this task took place one hour later (Fig 3.3.18a). Once habituated, training involved acquisition of memory with regard to the layout of the open field in which the task took place. During five minutes of exposure, a record of time spent investigating each of the three objects was kept, and total investigation calculated. Analysis of total exploration during the training session (Fig 3.3.18b) showed there was no significant effect of either exercise or LPS within or between groups ($Sed_Sal = 9.159 \pm 1.598$; $Sed_LPS = 7.454 \pm 1.374$; $Ex_Sal = 12.91 \pm 2.318$; $Ex_LPS = 9.038 \pm 1.094$). (One-way ANOVA, $p = 0.1376$).

During the testing phase (Fig 3.3.18c), it was found that Sed_LPS subjects explored significantly less than Sed_Sal ($p = 0.0287$) and Ex_LPS ($p = 0.0493$) groups. Sed_Sal and Ex_LPS exploration was similar ($p = 0.855$). ($Sed_Sal = 7.723 \pm 1.228$; $Sed_LPS = 4.337 \pm 0.7200$; $Ex_Sal = 12.51 \pm 2.471$; $Ex_LPS = 7.401 \pm 1.226$). (One-way ANOVA, $p = 0.2762$).

Assessed by unpaired t-test and/or one-way ANOVA and Bonferroni post-hoc test ($n=10$). Expressed as mean $\pm$ SEM.

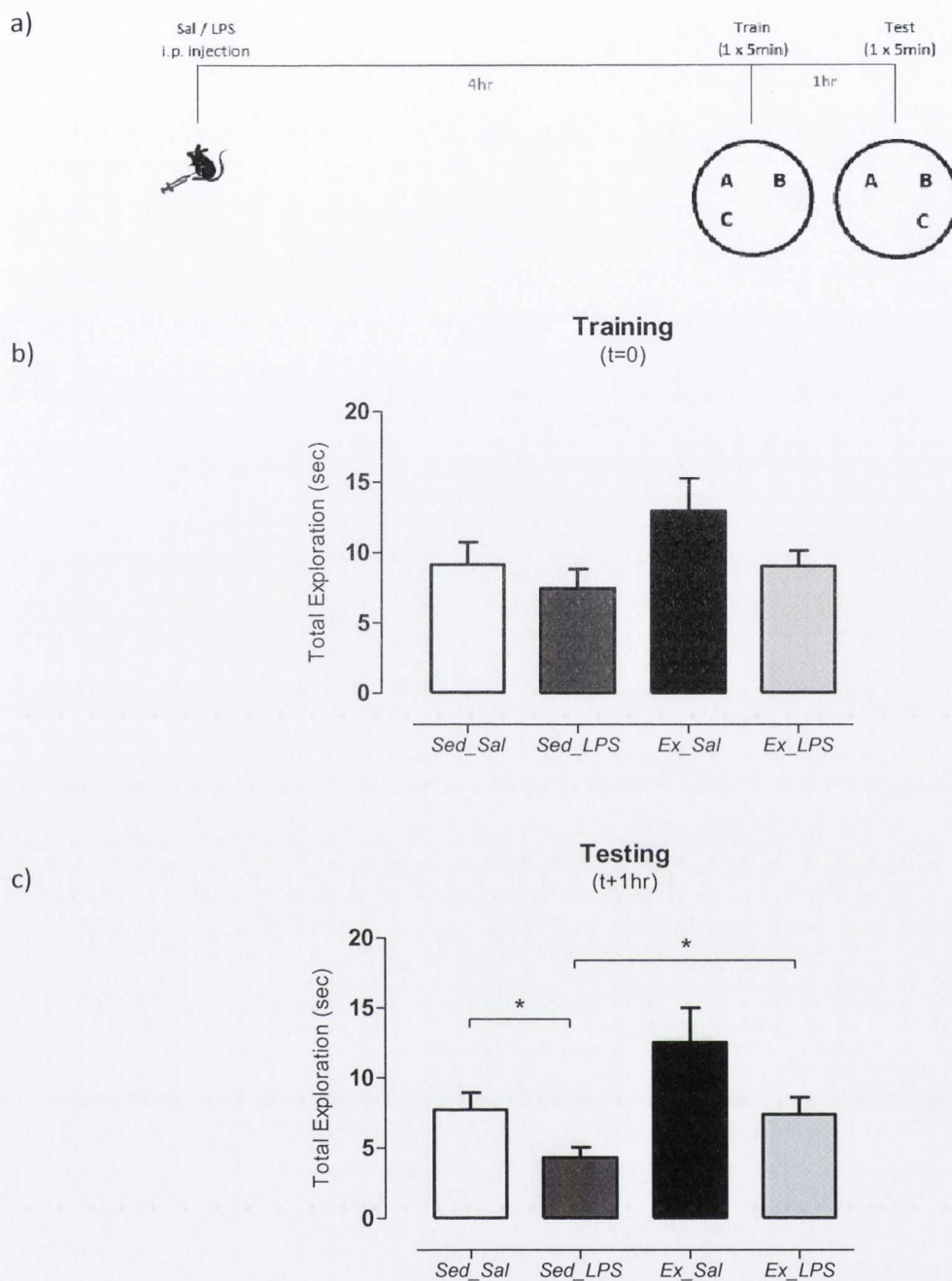


Fig. 3.3.18 - Effect of exercise and/or LPS on exploration

(a) timeline of testing, from LPS administration to OD task.

(b) shows total exploration during 5min training trial. There was no significant effect of exercise or LPS.

(c) shows total exploration during 5min test. There was a significant difference in total exploration time between Sed_LPS and Sed_Sal ($p = 0.0287$), and Sed_LPS and Ex_LPS animals ($p = 0.0493$).

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. * $p < 0.05$.

All data expressed as mean $\pm$ SEM. ($n = 10$).

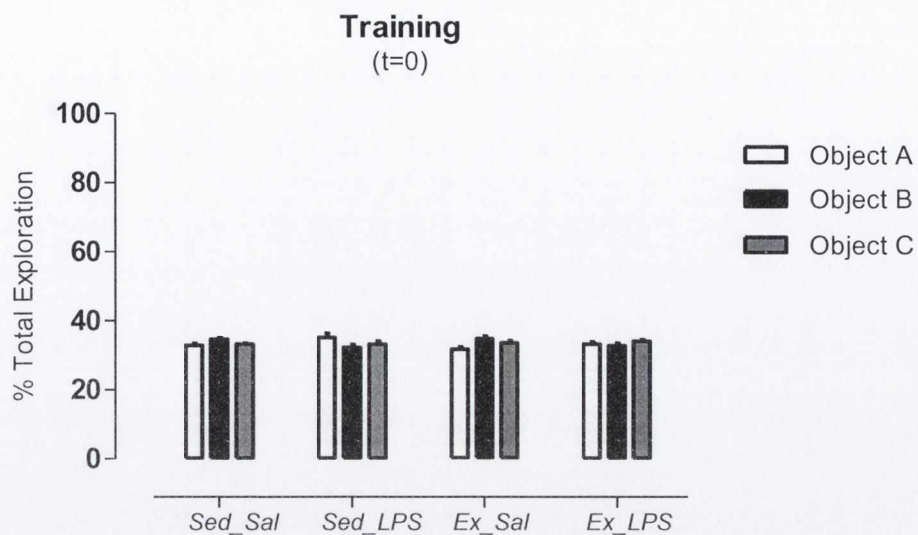
3.3.2.2 The effect of one month of exercise on behaviour in OD task, 5 hours after experimentally-induced neuroinflammation.

During the acquisition stage of the OD task (Fig 3.3.19a), there was no significant difference in object exploration across all groups (*Sed_Sal*, Object A % = 32.6311 ± 0.5915 , Object B % = 34.3764 ± 0.5986 , Object C % = 32.9923 ± 0.32449 ; *Sed_LPS*, Object A % = 34.9358 ± 1.2081 , Object B % = 32.0606 ± 0.8677 , Object C % = 33.003 ± 0.8942 ; *Ex_Sal*, Object A % = 31.6845 ± 0.64612 , Object B % = 34.7254 ± 0.7782 , Object C % = 33.59 ± 0.60459 ; *Ex_LPS*, Object A % = 33.1687 ± 0.6283 , Object B % = 32.7715 ± 0.6953 , Object C % = 34.0597 ± 0.407) (n = 10).

During the memory recall phase of the OD task (Fig 3.3.19b), object C was moved to the previously empty quadrant of the arena. There was no significant difference in object exploration in *Sed_LPS* animals (*Sed_LPS*, Object A % = 31.8188 ± 1.13441 , Object B % = 34.0386 ± 1.73437 , Object C % = 34.1425 ± 0.7341) (n = 10), but *Sed_Sal*, and both exercised groups spent significantly more time exploring the displaced object; C (*Sed_Sal*, Object A % = 30.5186 ± 0.77659 , Object B % = 29.1742 ± 0.72432 , Object C % = 40.307 ± 1.0549 ; *Ex_Sal*, Object A % = 25.3365 ± 1.5567 , Object B % = 25.2739 ± 1.1569 , Object C % = 49.3896 ± 2.0394 ; *Ex_LPS*, Object A % = 27.4404 ± 1.07913 , Object B % = 25.7665 ± 1.63731 , Object C % = 46.7929 ± 1.93774) (n = 10).

Assessed by two-way ANOVA and Bonferroni post-hoc test. Expressed as mean $\pm$ SEM.

a)



b)

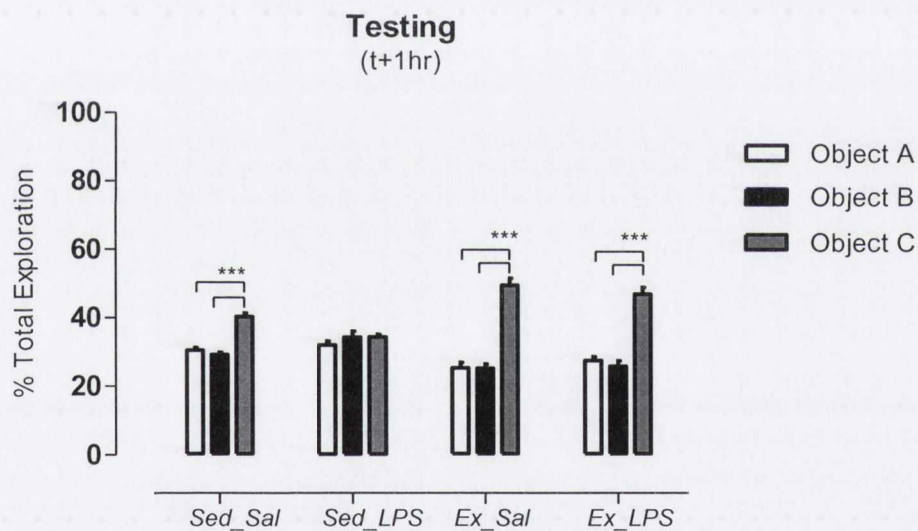


Fig. 3.3.19 – Object Displacement Task

(a) shows exploration during a single 5min trial. There was no significant difference in object exploration.

(b) shows exploration during a single 5min test. Sed_Sal, Ex_Sal, and Ex_LPS animals displayed a significant preference for the displaced object; C.

Assessed by two-way ANOVA and Bonferroni post-hoc analysis. ***p<0.001.

All data expressed as mean $\pm$ SEM. (n = 10).

3.3.2.3a The effect of one month of exercise on mRNA expression of BDNF and TrkB in cortex and hippocampus, 5 hours after experimentally-induced neuroinflammation.

PCR analysis showed there was an insignificant difference in BDNF mRNA expression in the cortex when Rq measurement in Sed_LPS was compared to Sed_Sal ($p = 0.0865$). ($Sed_Sal = 0.9914 \pm 0.05245$; $Sed_LPS = 0.8037 \pm 0.08055$; $Ex_Sal = 1.173 \pm 0.1381$; $Ex_LPS = 0.9850 \pm 0.08126$). ($p = 0.0915$, $n = 5$). (Fig 3.3.20a)

PCR analysis showed there was no significant difference in BDNF mRNA expression in the hippocampus when Rq measurement was compared across all treatment groups. ($Sed_Sal = 0.7982 \pm 0.09180$; $Sed_LPS = 0.7633 \pm 0.1121$; $Ex_Sal = 0.7497 \pm 0.07837$; $Ex_LPS = 0.8195 \pm 0.04289$) ($p = 0.9238$, $n = 5$). (Fig 3.3.20b)

PCR analysis showed there was no significant difference in TrkB mRNA expression in the cortex when Rq measurement was compared across all treatment groups. ($Sed_Sal = 0.9259 \pm 0.03739$; $Sed_LPS = 1.040 \pm 0.05126$; $Ex_Sal = 1.101 \pm 0.07842$; $Ex_LPS = 1.070 \pm 0.07241$). ($p = 0.2785$, $n = 5$). (Fig 3.3.21a)

PCR analysis showed that TrkB mRNA was significantly lower in Sed_LPS subjects compared to Ex_LPS ($p = 0.0122$). ($Sed_Sal = 0.9847 \pm 0.01465$; $Sed_LPS = 0.9296 \pm 0.02737$; $Ex_Sal = 0.9741 \pm 0.05854$; $Ex_LPS = 1.066 \pm 0.03263$). ($p = 0.0874$, $n = 5$). (Fig 3.3.21b)

Data analysed by unpaired t-test and/or one-way ANOVA and Bonferroni post-hoc test, expressed as mean $\pm$ SEM.

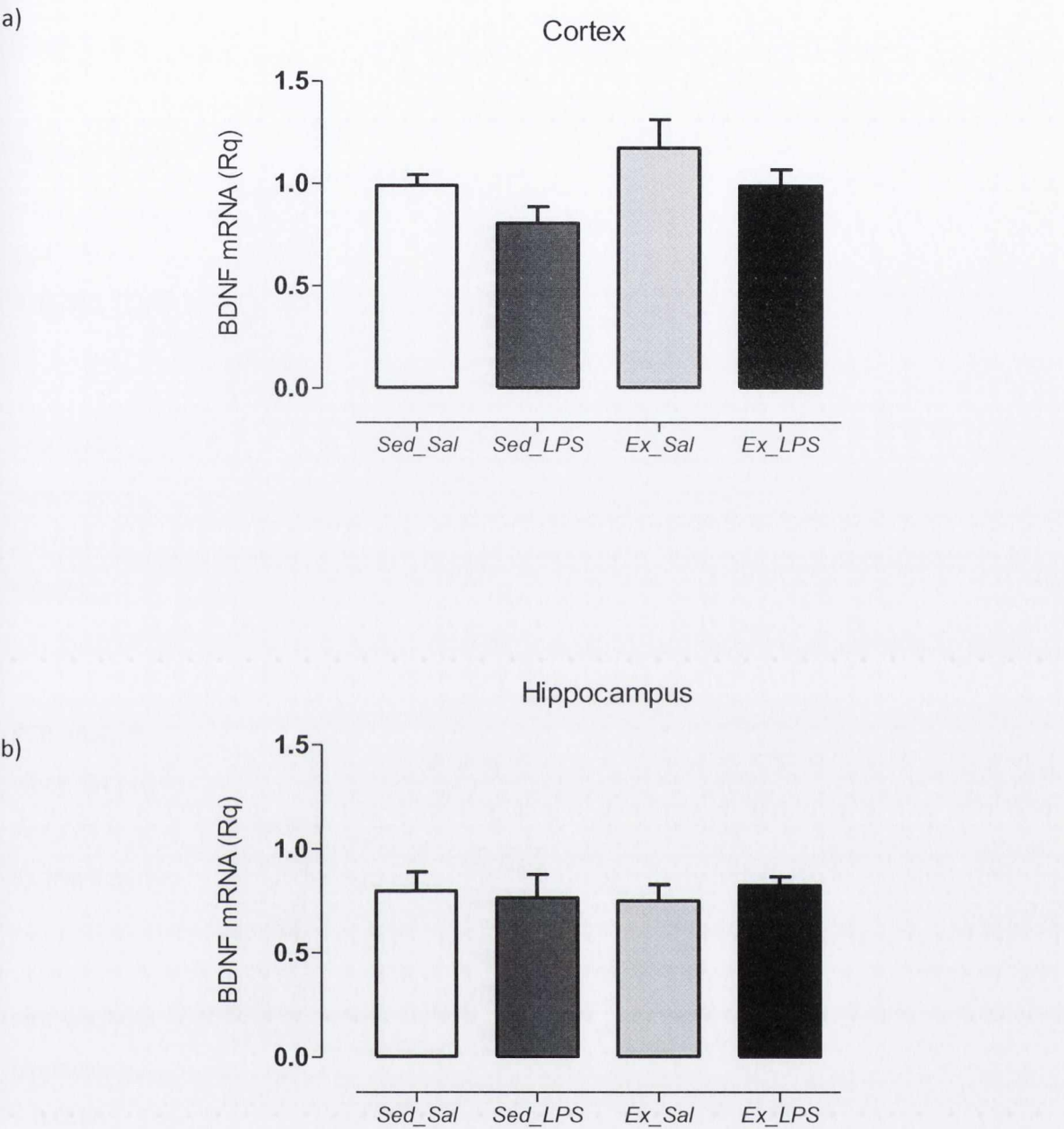
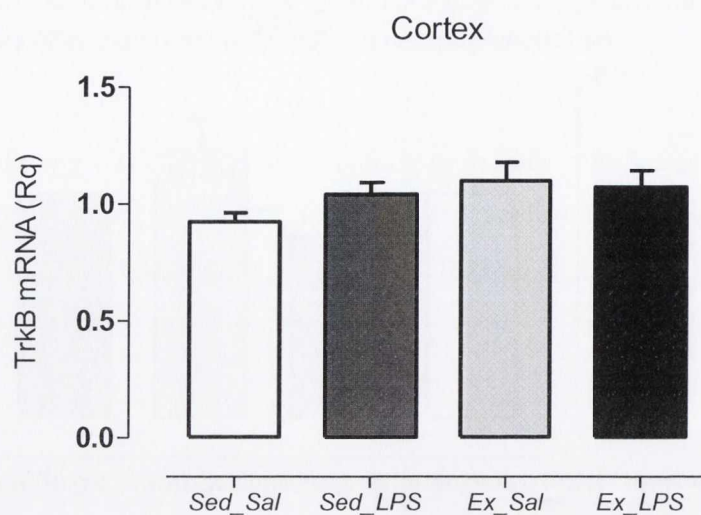


Fig. 3.3.20 – Effect of exercise and/or LPS on BDNF mRNA expression
(a) shows Rq of BDNF mRNA expression in cortical tissue. There was no significant difference in BDNF expression between subject groups.
(b) shows Rq of BDNF mRNA expression in hippocampal tissue. There was no significant difference in BDNF expression between subject groups.
Assessed by one-way ANOVA and Bonferroni post-hoc analysis. $P>0.05$.
Data expressed as mean $\pm$ SEM (n=5).

a)



b)

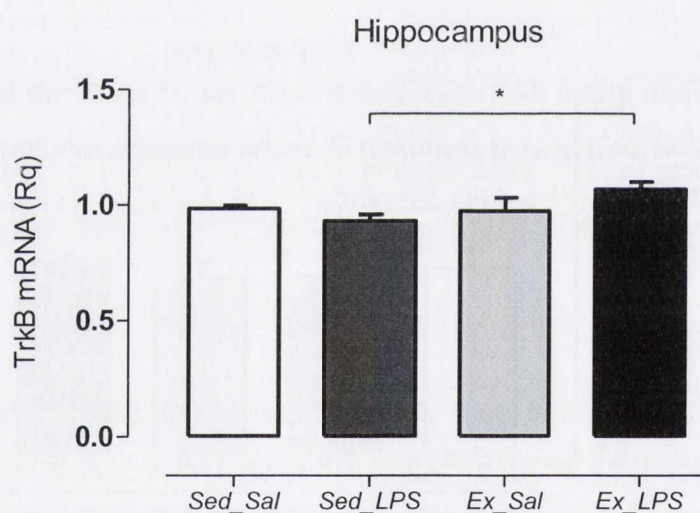


Fig. 3.3.21 – Effect of exercise and/or LPS on TrkB mRNA expression

(a) shows Rq of TrkB mRNA expression in cortical tissue. There was no significant difference in TrkB expression between subject groups.

(b) shows Rq of TrkB mRNA expression in hippocampal tissue. There was a significant difference in TrkB expression between Sed_LPS and Ex_LPS subject groups ($p=0.0122$)

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. * $p<0.05$.

Data expressed as mean $\pm$ SEM ($n=5$).

3.3.2.3b The effect of one month of exercise on mRNA expression of Ki67 and CREB in cortex and hippocampus, 5 hours after experimentally-induced neuroinflammation.

PCR analysis showed there was no significant difference in Ki67 mRNA expression in the cortex when Rq measurement was compared across all treatment groups. ($Sed_Sal = 0.9415 \pm 0.1307$; $Sed_LPS = 0.8348 \pm 0.06561$; $Ex_Sal = 0.9109 \pm 0.1575$; $Ex_LPS = 0.8131 \pm 0.04085$). ($p = 0.7925$, $n = 5$). (Fig 3.3.22a)

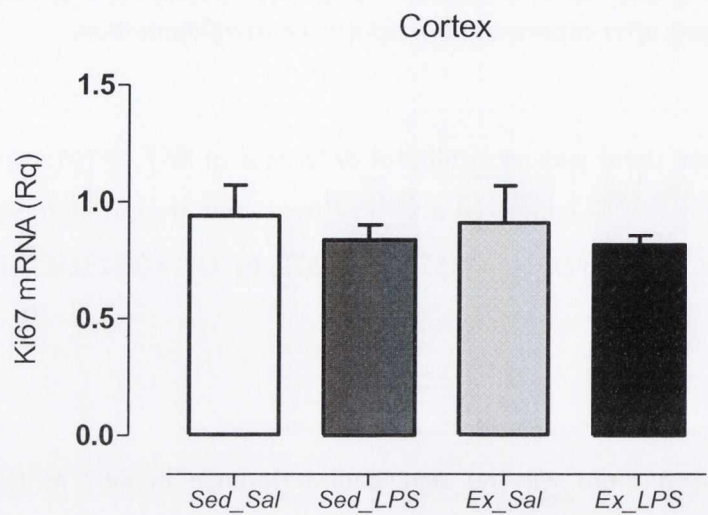
PCR analysis showed there was no significant difference in Ki67 mRNA expression in the hippocampus when Rq measurement was compared across all treatment groups. ($Sed_Sal = 0.9027 \pm 0.06272$; $Sed_LPS = 0.8110 \pm 0.02316$; $Ex_Sal = 1.149 \pm 0.3026$; $Ex_LPS = 1.018 \pm 0.2257$). ($p = 0.6676$, $n = 5$). (Fig 3.3.22b)

PCR analysis showed there was no significant difference in CREB mRNA expression in the cortex when Rq measurement was compared across all treatment groups. ($Sed_Sal = 0.6826 \pm 0.1435$; $Sed_LPS = 0.5794 \pm 0.1592$; $Ex_Sal = 0.5128 \pm 0.1047$; $Ex_LPS = 0.4866 \pm 0.08854$). ($p = 0.6890$, $n = 5$). (Fig 3.3.23a)

PCR analysis showed there was no significant difference in CREB mRNA expression in the hippocampus when Rq measurement was compared across all treatment groups. ($Sed_Sal = 1.019 \pm 0.05603$; $Sed_LPS = 1.142 \pm 0.06954$; $Ex_Sal = 1.099 \pm 0.1182$; $Ex_LPS = 1.100 \pm 0.1067$). ($p = 0.8320$, $n = 5$). (Fig 3.3.23b)

Data analysed by unpaired t-test and/or one-way ANOVA and Bonferroni post-hoc test, expressed as mean $\pm$ SEM.

a)



b)

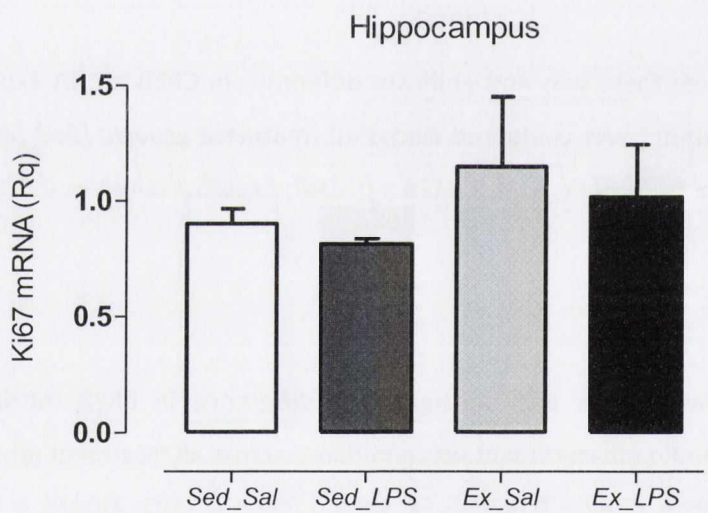


Fig. 3.3.22 – Effect of exercise and/or LPS on Ki67 mRNA expression

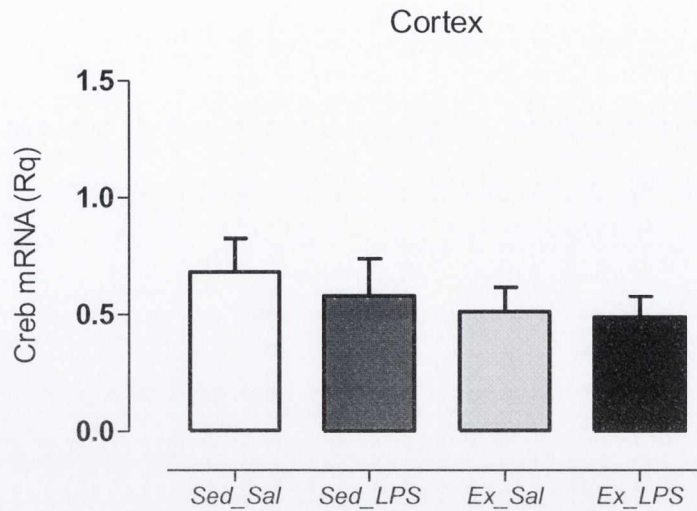
(a) shows Rq of Ki67 mRNA expression in cortical tissue. There was no significant difference in Ki67 expression between subject groups.

(b) shows Rq of Ki67 mRNA expression in hippocampal tissue. There was no significant difference in Ki67 expression between subject groups.

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. $P>0.05$.

Data expressed as mean $\pm$ SEM (n=5).

a)



b)

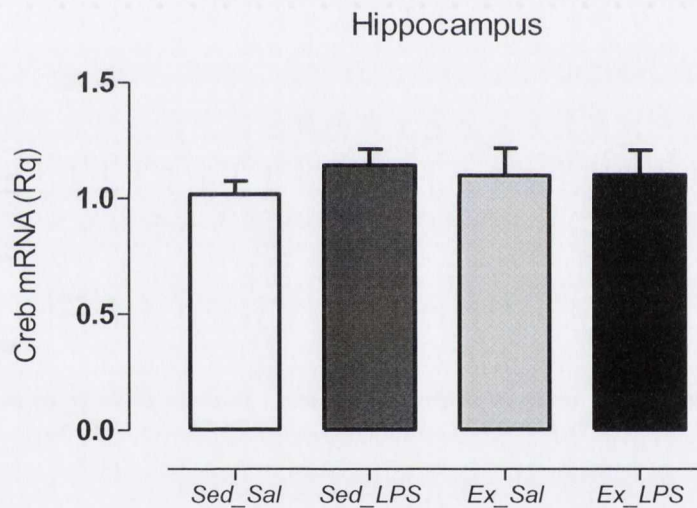


Fig. 3.3.23 – Effect of exercise and/or LPS on CREB mRNA expression

(a) shows Rq of CREB mRNA expression in cortical tissue. There was no significant difference in CREB expression between subject groups.

(b) shows Rq of CREB mRNA expression in hippocampal tissue. There was no significant difference in CREB expression between subject groups.

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. $P > 0.05$.

Data expressed as mean $\pm$ SEM (n=5).

3.3.2.3c The effect of one month of exercise on mRNA expression of IL1 β and TNF α in cortex and hippocampus, 5 hours after experimentally-induced neuroinflammation.

PCR analysis showed there was a significant difference in IL1 β mRNA expression in the cortex when Rq measurement in Sed_LPS was compared to Sed_Sal ($p = 0.0378$), and when Ex_LPS was compared to Ex_Sal ($p < 0.0001$). ($Sed_Sal = 1.107 \pm 0.2067$; $Sed_LPS = 0.8037 \pm 0.08055$; $Ex_Sal = 1.173 \pm 0.1381$; $Ex_LPS = 10.03 \pm 0.9341$). ($p < 0.0001$, $n = 5$). (Fig 3.3.24a)

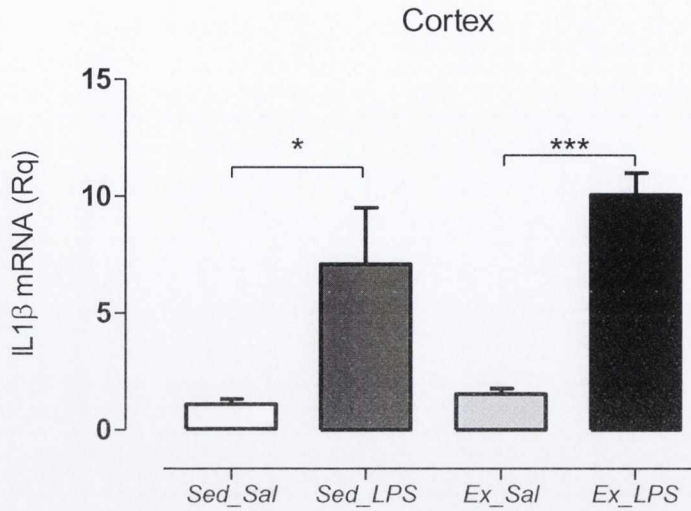
PCR analysis showed there was a significant difference in IL1 β mRNA expression in the hippocampus when Rq measurement in Sed_LPS was compared to Sed_Sal ($p = 0.0175$), and when Ex_LPS was compared to Ex_Sal ($p = 0.0006$). ($Sed_Sal = 0.9473 \pm 0.1547$; $Sed_LPS = 8.367 \pm 2.482$; $Ex_Sal = 2.029 \pm 0.3973$; $Ex_LPS = 13.72 \pm 2.038$). ($p < 0.0001$, $n = 5$). (Fig 3.3.24b)

PCR analysis showed there was a significant difference in TNF α mRNA expression in the cortex when Rq measurement in Sed_LPS was compared to Sed_Sal ($p = 0.0197$), and when Ex_LPS was compared to Ex_Sal ($p = 0.001$). ($Sed_Sal = 1.230 \pm 0.2722$; $Sed_LPS = 9.052 \pm 2.678$; $Ex_Sal = 1.522 \pm 0.1833$; $Ex_LPS = 11.33 \pm 1.841$). ($p < 0.0001$, $n = 5$). (Fig 3.3.25a)

PCR analysis showed there was a significant difference in TNF α mRNA expression in the hippocampus when Rq measurement in Sed_LPS was compared to Sed_Sal ($p = 0.0168$). ($Sed_Sal = 1.012 \pm 0.1581$; $Sed_LPS = 7.459 \pm 2.135$; $Ex_Sal = 6.089 \pm 2.712$; $Ex_LPS = 8.158 \pm 1.379$). ($p = 0.001$, $n = 5$). (Fig 3.3.25b)

Data analysed by unpaired t-test and/or one-way ANOVA and Bonferroni post-hoc test, expressed as mean $\pm$ SEM.

a)



b)

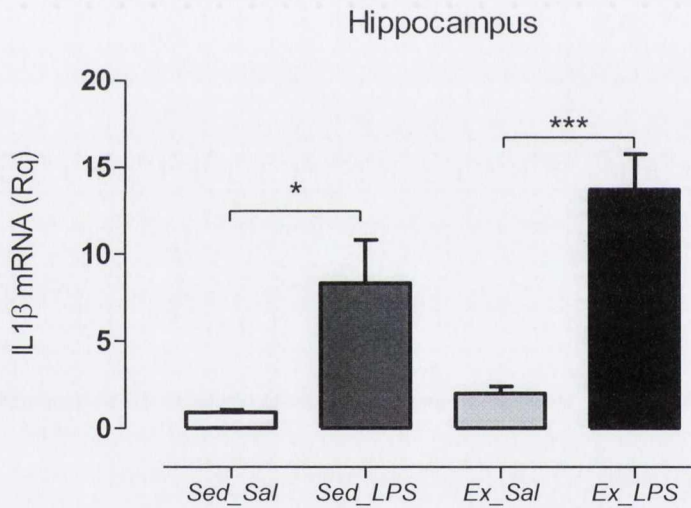


Fig. 3.3.24 – Effect of exercise and/or LPS on IL1β mRNA expression

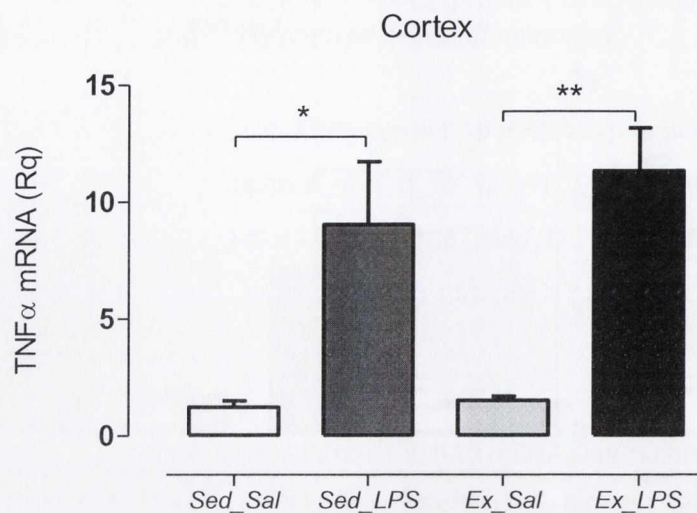
(a) shows Rq of IL1β mRNA expression in cortical tissue. There was a significant difference in IL1β expression when Sed_LPS is compared to Sed_Sal ($p = 0.0378$), and also when Ex_LPS is compared to Ex_Sal ($p < 0.0001$).

(b) shows Rq of IL1β mRNA expression in hippocampal tissue. There was a significant difference in IL1β expression when Sed_LPS is compared to Sed_Sal ($p = 0.0175$), and also when Ex_LPS is compared to Ex_Sal ($p = 0.0006$).

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. * $P < 0.05$, *** $P < 0.001$

Data expressed as mean $\pm$ SEM ($n=5$).

a)



b)

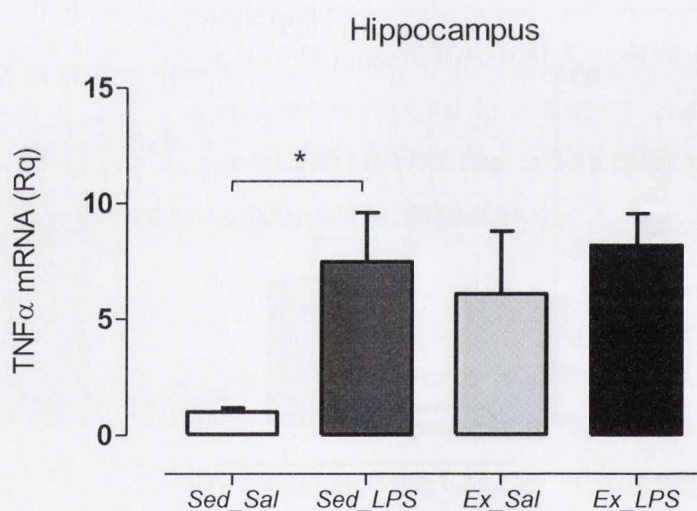


Fig. 3.3.25 – Effect of exercise and/or LPS on TNFα mRNA expression

(a) shows Rq of TNFα mRNA expression in cortical tissue. There was a significant difference in TNFα expression when Sed_LPS is compared to Sed_Sal ($p = 0.0197$), and also when Ex_LPS is compared to Ex_Sal ($p = 0.001$).

(b) shows Rq of TNFα mRNA expression in hippocampal tissue. There was a significant difference in TNFα expression when Sed_LPS is compared to Sed_Sal ($p = 0.0168$).

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. * $p < 0.05$, ** $p < 0.01$

Data expressed as mean $\pm$ SEM ($n=5$).

3.3.2.3d The effect of one month of exercise on mRNA expression of Iba1 and CD44 in the cortex and hippocampus, 5 hours after experimentally-induced neuroinflammation.

PCR analysis showed there was no significant difference in Iba1 mRNA expression in the cortex when Rq measurement was compared across all treatment groups. (*Sed_Sal* = 0.9738 ± 0.02095 ; *Sed_LPS* = 0.9182 ± 0.04076 ; *Ex_Sal* = 0.9850 ± 0.03199 ; *Ex_LPS* = 0.9547 ± 0.06301). ($p = 0.7535$, $n = 5$). (Fig 3.3.26a)

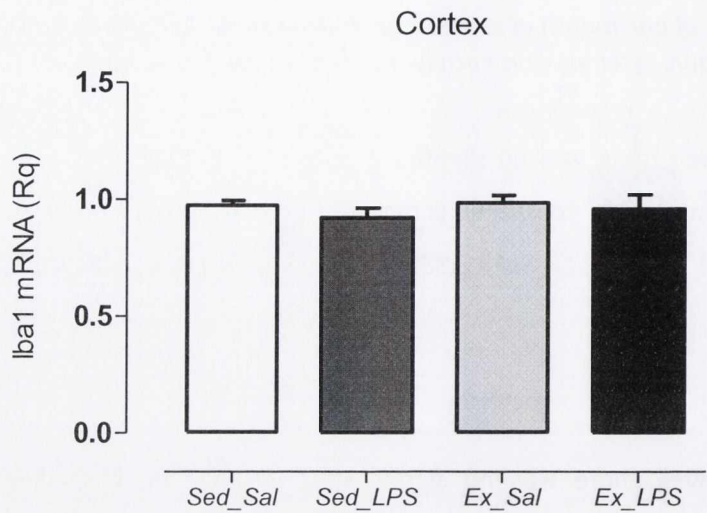
PCR analysis showed there was no significant difference in Iba1 mRNA expression in the hippocampus when Rq measurement was compared across all treatment groups. (*Sed_Sal* = 0.9585 ± 0.02969 ; *Sed_LPS* = 0.9402 ± 0.02949 ; *Ex_Sal* = 0.9814 ± 0.06331 ; *Ex_LPS* = 0.9939 ± 0.02571). ($p = 0.7653$, $n = 5$). (Fig 3.3.26b)

PCR analysis showed there was no difference in CD44 mRNA expression in the cortex when Rq measurement comparisons were made across all treatment groups. (*Sed_Sal* = 0.6747 ± 0.1672 ; *Sed_LPS* = 1.192 ± 0.3197 ; *Ex_Sal* = 0.8349 ± 0.1174 ; *Ex_LPS* = 1.755 ± 0.4212). ($p = 0.0823$, $n = 5$). (Fig 3.3.27a)

PCR analysis showed there was a significant difference in CD44 mRNA expression in the cortex when Rq measurement in *Ex_LPS* was compared to *Ex_Sal* ($p = 0.0425$), but not when comparisons were made across other treatment groups. (*Sed_Sal* = 0.6745 ± 0.09865 ; *Sed_LPS* = 0.9288 ± 0.2248 ; *Ex_Sal* = 0.7473 ± 0.1154 ; *Ex_LPS* = 1.386 ± 0.2257). ($p = 0.0472$, $n = 5$). (Fig 3.3.27b)

Data analysed by unpaired t-test and/or one-way ANOVA and Bonferroni post-hoc test, expressed as mean $\pm$ SEM.

a)



b)

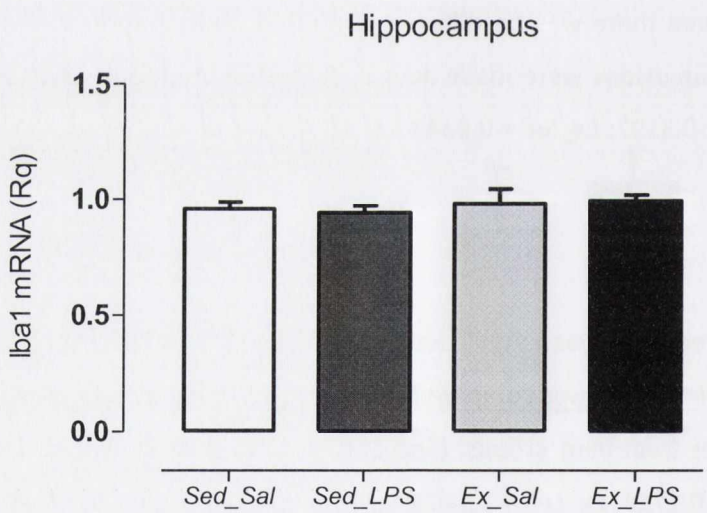


Fig. 3.3.26 – Effect of exercise and/or LPS on Iba1 mRNA expression

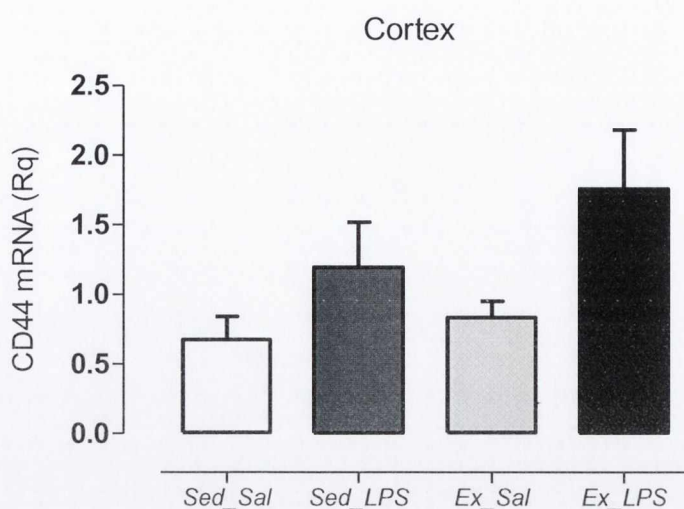
(a) shows Rq of Iba1 mRNA expression in cortical tissue. There was no significant difference in Iba1 expression between subject groups.

(b) shows Rq of Iba1 mRNA expression in hippocampal tissue. There was no significant difference in Iba1 expression between subject groups.

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. $p>0.05$.

Data expressed as mean $\pm$ SEM (n=5).

a)



b)

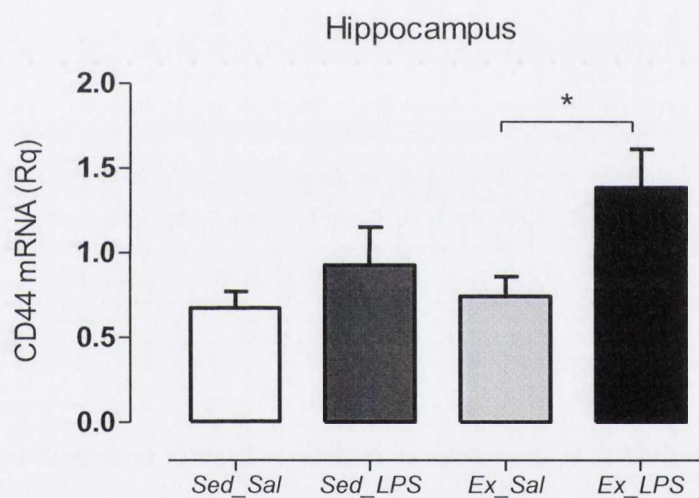


Fig. 3.3.27 – Effect of exercise and/or LPS on CD44 mRNA expression

(a) shows Rq of CD44 mRNA expression in cortical tissue. There was no significant difference in CD44 expression between subject groups.

(b) shows Rq of CD44 mRNA expression in hippocampal tissue. There was a significant difference in CD44 expression when Ex_Sal and Ex_LPS groups when compared ($p=0.0425$).

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. * $p<0.05$.

Data expressed as mean $\pm$ SEM ($n=5$).

3.3.2.4 The effect of one month of exercise on dentate gyrus volume, and number of BrdU⁺ cells.

Immunohistochemical staining and stereological estimation techniques show that there was no significant difference in dentate gyrus volume (Fig 3.3.28) when Sedentary animals were compared to Exercised animals, regardless of Saline or LPS administration (Sedentary = 0.5608 ± 0.02106 ; Exercise = 0.5051 ± 0.03404) ($p = 0.1860$, $n = 8$).

There was no significant difference in BrdU⁺ cell numbers (Fig 3.3.29) in animals that had undertaken four weeks of running exercise when compared to sedentary animals (Sedentary = 22.75 ± 1.761 ; Exercise = 24.72 ± 2.863) ($p = 0.5676$, $n = 8$).

Data analysed using unpaired t-test, expressed as mean $\pm$ SEM.

Figure 3.3.30 shows an example of images obtained from Sedentary (a, c) and Exercised (b, d) animals.

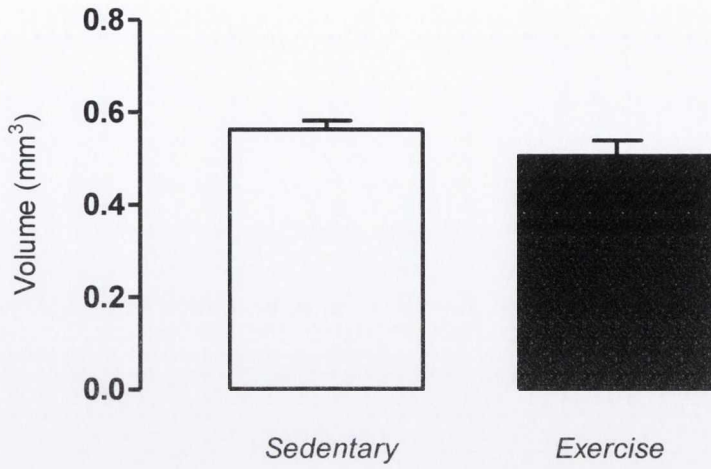


Fig. 3.3.28 – The effect of four weeks of running exercise on dentate gyrus volume

There was no significant difference in overall volume of dentate gyrus as measured using the Cavalieri method of volume estimation.

Assessed by unpaired t test ($p=0.1860$). Data expressed as mean $\pm$ SEM ($n=8$).

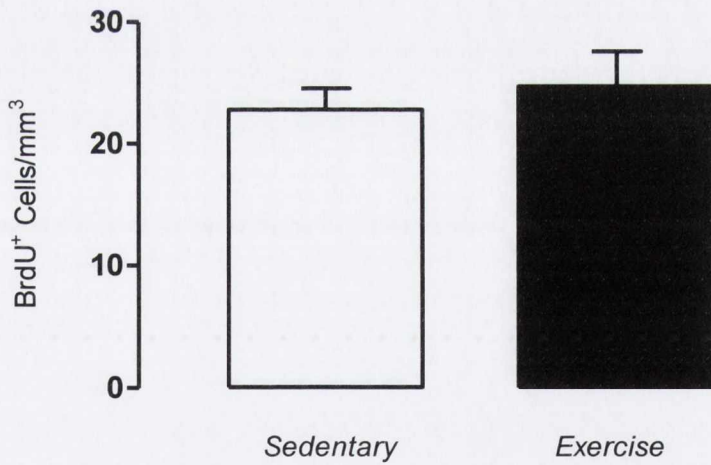


Fig. 3.3.29 - The effect of four weeks of running exercise on BrdU+ cells in the dentate gyrus

There was no significant difference in the number of BrdU+ cells in the dentate gyrus of exercised animals.

Assessed by unpaired t test ($p=0.5676$). Data expressed as mean $\pm$ SEM ($n=8$).

Sedentary

Exercise

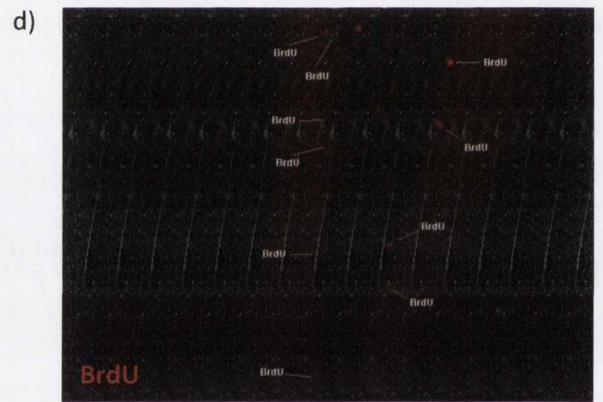
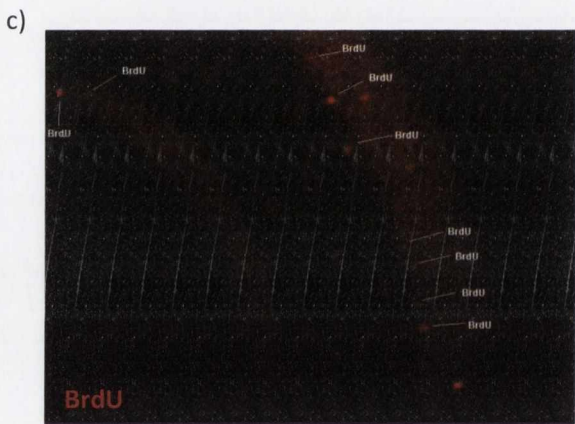
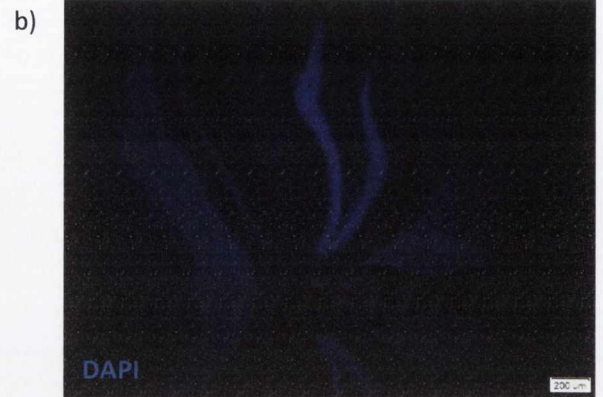


Fig. 3.3.30 - BrdU⁺ cells in the dentate gyrus

Images obtained at x4 (DAPI) and x20 (BrdU) magnification of Sedentary (a, c) and Exercise (b, d) subjects.

BrdU⁺ cells are indicated with white marker.

3.3.3 Experiment III – Can 7,8-DHF protect against experimentally-induced acute neuroinflammation in the C57/BL6 mouse?

We hypothesise that one month of 7,8-DHF administration can protect against experimentally-induced neuroinflammation in the mouse, and will rescue disturbances in cognitive function triggered by this acute inflammatory response when spatial memory is tested one hour after learning in the OD task. We aim to assess the following:

- The effect of 7,8-DHF administration on spatial learning in the mouse when compared to vehicle-treated controls.
- The effect of 7,8-DHF administration in conjunction with LPS administration on spatial memory in the mouse, when compared to LPS-treated vehicle-treated controls and Sal-treated 7,8-DHF mice.
- The effect of 7,8-DHF administration on mRNA expression of genes related to TrkB receptor activation when compared to vehicle-treated controls.
- The effect of 7,8-DHF administration in conjunction with LPS administration on mRNA expression of genes related to TrkB receptor activation, when compared to LPS-treated vehicle-treated controls and Sal-treated 7,8-DHF mice.
- The effect of 7,8-DHF administration on mRNA expression of markers of inflammation and microglial activation when compared to vehicle-treated controls.
- The effect of 7,8-DHF administration in conjunction with LPS administration on mRNA expression of markers of inflammation and microglial activation, when compared to LPS-treated vehicle-treated controls and Sal-treated 7,8-DHF mice.
- The effect of 7,8-DHF administration on dentate gyrus volume and numbers of BrdU⁺ cells in the dentate gyrus when compared to vehicle-treated controls.

3.3.3.1 The effect of one month of 7,8-DHF administration on total exploration in OD task, 5 hours after experimentally-induced neuroinflammation.

As described, subjects received an i.p. injection of either Sal or LPS, were trained in the OD task, and testing in this task took place one hour later (Fig 3.3.31a). Once habituated, training involved acquisition of memory with regard to the layout of the open field in which the task took place. During 5mins of exposure, a log of time spent investigating each of the three objects was kept, and total investigation calculated. Analysis of total exploration during the training session (Fig 3.3.31b) showed that there was no significant difference in overall exploration ($Veh_Sal = 11.51 \pm 2.365$; $Veh_LPS = 8.557 \pm 1.173$; $DHF_Sal = 9.186 \pm 1.534$; $DHF_LPS = 7.965 \pm 1.129$). ($p = 0.4464$, $n = 10$).

During the testing phase (Fig 3.3.31c), it was found that Veh_LPS subjects explored significantly less than DHF_LPS animals ($p = 0.0106$). ($Veh_Sal = 7.684 \pm 1.029$; $Veh_LPS = 5.701 \pm 0.8756$; $DHF_Sal = 11.62 \pm 2.806$; $DHF_LPS = 10.63 \pm 1.490$). ($p = 0.0772$, $n = 10$).

Assessed by unpaired t-test and/or one-way ANOVA and Bonferroni post-hoc test ($n=10$).
Expressed as mean $\pm$ SEM.

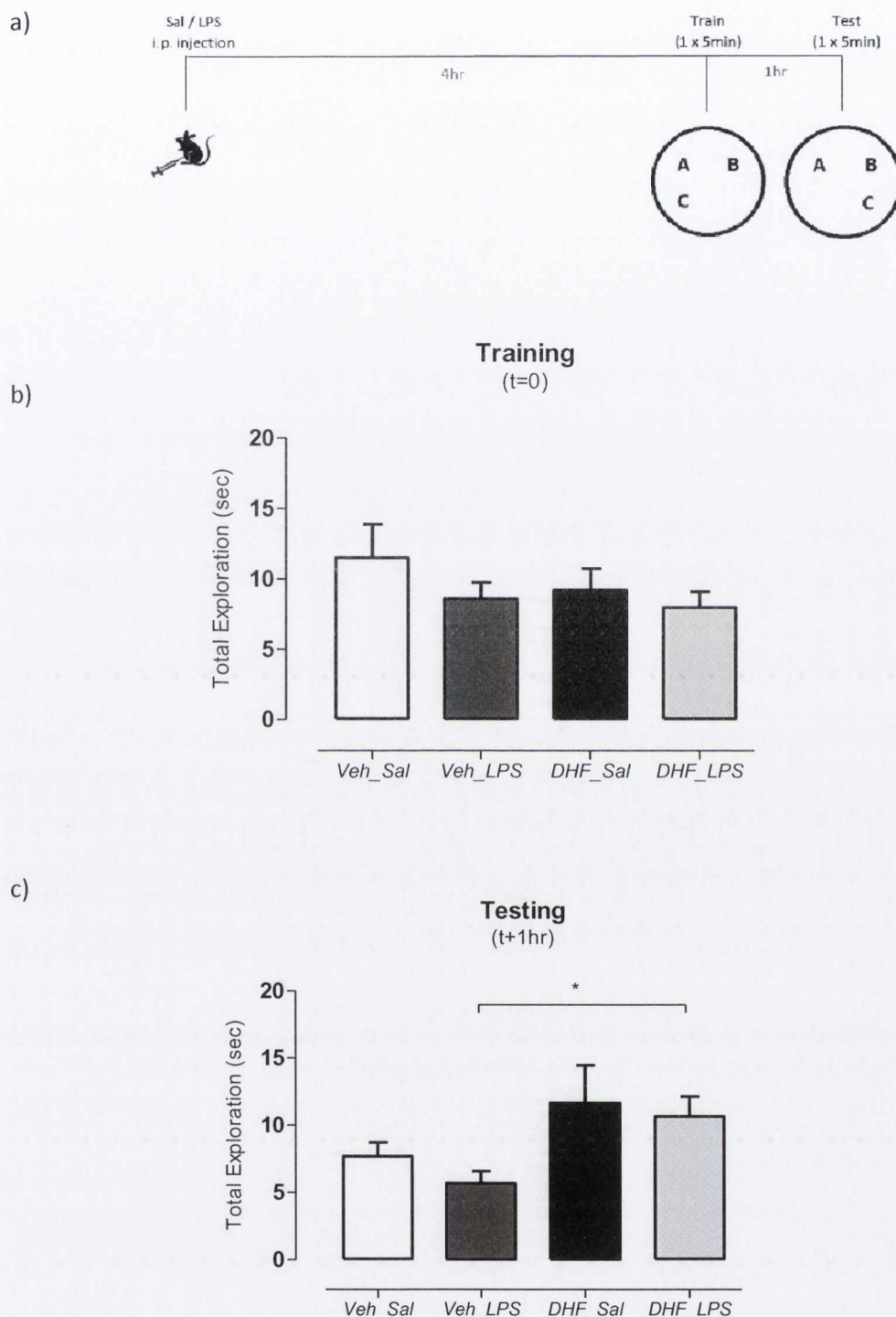


Fig. 3.3.31 - Effect of 7,8-DHF and/or LPS on exploration

(a) timeline of testing, from LPS administration to OD task.

(b) shows total exploration during 5min training trial. There was no significant effect of 7,8-DHF or LPS.

(c) shows total exploration during 5min test. There was a significant difference in exploration when Veh_LPS was compared to DHF_LPS ($p=0.0106$).

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. * $p<0.05$.

All data expressed as mean $\pm$ SEM. ($n = 10$).

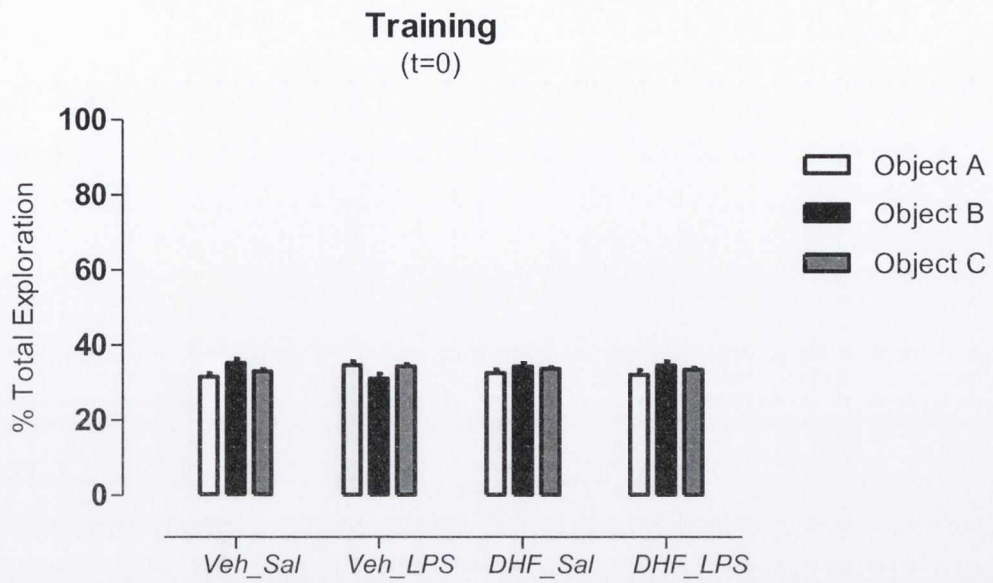
3.3.3.2 The effect of one month of 7,8-DHF administration on behaviour in OD task, 5 hours after experimentally-induced neuroinflammation.

During the acquisition stage of the OD task (Fig 3.3.32a), there was no significant difference in object exploration across all groups (*Veh_Sal*, Object A % = 31.5812 ± 0.99168 , Object B % = 35.3434 ± 1.10585 , Object C % = 33.0752 ± 0.59105 ; *Veh_LPS*, Object A % = 34.5942 ± 1.0015 , Object B % = 31.10463 ± 1.1832 , Object C % = 34.301 ± 0.6051 ; *DHF_Sal*, Object A % = 32.3931 ± 0.85455 , Object B % = 34.1463 ± 0.9974 , Object C % = 33.4604 ± 0.45498 ; *DHF_LPS*, Object A % = 32.1122 ± 1.2143 , Object B % = 34.5071 ± 1.0535 , Object C % = 33.3805 ± 0.54107) (n = 10).

During the memory recall phase of the OD task (Fig 3.3.19b), object C was moved to the previously empty quadrant of the arena. There was no significant difference in object exploration in any of the vehicle treated animals (*Veh_Sal*, Object A % = 33.2044 ± 1.00 , Object B % = 33.1073 ± 0.67 , Object C % = 33.68819 ± 0.996 ; *Veh_LPS*, Object A % = 33.6738 ± 1.8765 , Object B % = 36.9325 ± 2.21862 , Object C % = 29.3935 ± 1.0474) (n = 10), but both 7,8-DHF treated groups spent significantly more time exploring the displaced object; C (*DHF_Sal*, Object A % = 22.0724 ± 2.1702 , Object B % = 31.1797 ± 2.19 , Object C % = 46.7478 ± 2.6584 ; *DHF_LPS*, Object A % = 26.148 ± 2.2945 , Object B % = 25.5304 ± 2.0515 , Object C % = 48.3215 ± 2.7673) (n = 10).

Assessed by two-way ANOVA and Bonferroni post-hoc test. Expressed as mean $\pm$ SEM.

a)



b)

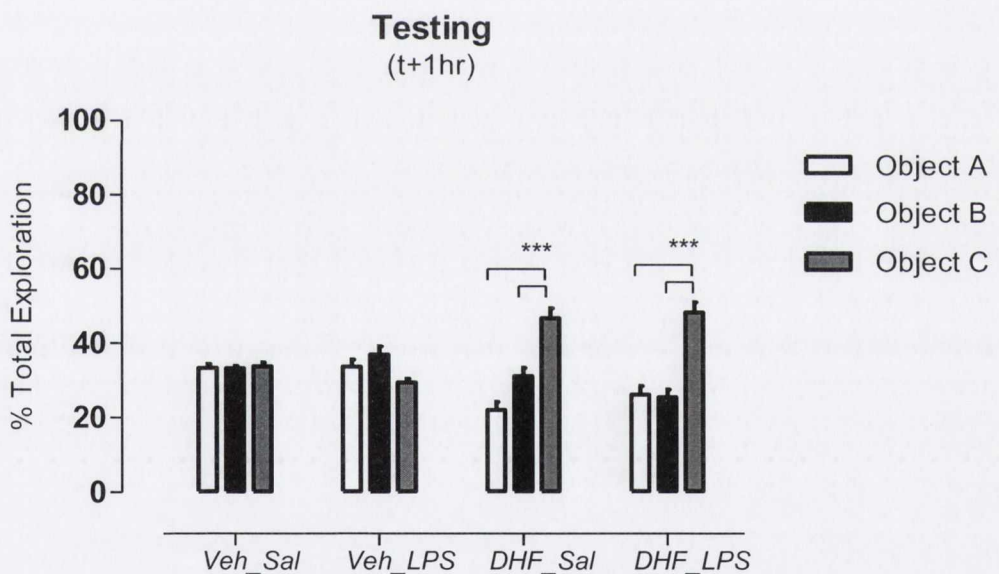


Fig. 3.3.32 – Effect of 7,8-DHF and/or LPS on Object Displacement Task

(a) shows exploration during a single 5min trial. There was no significant difference in object exploration.

(b) shows exploration during a single 5min test. Sed_Sal, Ex_Sal, and Ex_LPS animals displayed a significant preference for the displaced object; C.

Assessed by two-way ANOVA and Bonferroni post-hoc analysis. *** $p < 0.001$.

All data expressed as mean $\pm$ SEM. (n = 10).

3.3.3.3a The effect of one month of 7,8-DHF administration on mRNA expression of BDNF and TrkB in cortex and hippocampus, 5 hours after experimentally-induced neuroinflammation.

PCR analysis showed there was a significant difference in BDNF mRNA expression in the cortex when Rq measurement in Veh_LPS was compared to DHF_LPS ($p = 0.0477$), and insignificantly when compared to Veh_Sal ($p = 0.0853$). ($Veh_Sal = 1.128 \pm 0.0612$; $Veh_LPS = 0.9267 \pm 0.0823$; $DHF_Sal = 1.479 \pm 0.1527$; $DHF_LPS = 1.406 \pm 0.1881$). ($p = 0.0323$, $n = 5$). (Fig 3.3.33a)

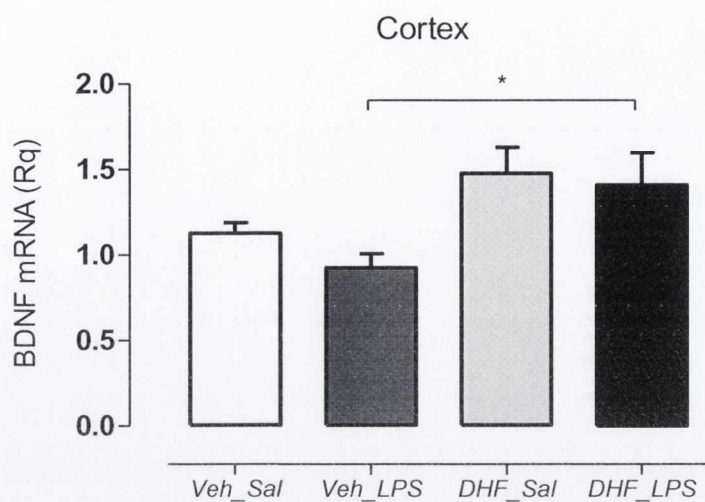
PCR analysis showed there was no significant difference in BDNF mRNA expression in the hippocampus when Rq measurement was compared across all groups. ($Veh_Sal = 0.6936 \pm 0.08348$; $Veh_LPS = 0.9587 \pm 0.1444$; $DHF_Sal = 0.7116 \pm 0.1013$; $DHF_LPS = 0.6509 \pm 0.08302$). ($p = 0.6599$, $n = 5$). (Fig 3.3.33b)

PCR analysis showed there was no significant difference in TrkB mRNA expression in the cortex when Rq measurement was compared across all groups. ($Veh_Sal = 0.8555 \pm 0.0582$; $Veh_LPS = 0.8578 \pm 0.09658$; $DHF_Sal = 0.9420 \pm 0.04002$; $DHF_LPS = 0.8712 \pm 0.04106$). ($p = 0.2634$, $n = 5$). (Fig 3.3.34a)

PCR analysis showed there was no significant difference in TrkB mRNA expression in the hippocampus when Rq measurement was compared across all groups. ($Veh_Sal = 0.8009 \pm 0.05553$; $Veh_LPS = 0.8304 \pm 0.04652$; $DHF_Sal = 0.8895 \pm 0.04668$; $DHF_LPS = 0.8434 \pm 0.03763$). ($p = 0.6153$, $n = 5$). (Fig 3.3.34b)

Data analysed by unpaired t-test and/or one-way ANOVA and Bonferroni post-hoc test, expressed as mean $\pm$ SEM.

a)



b)

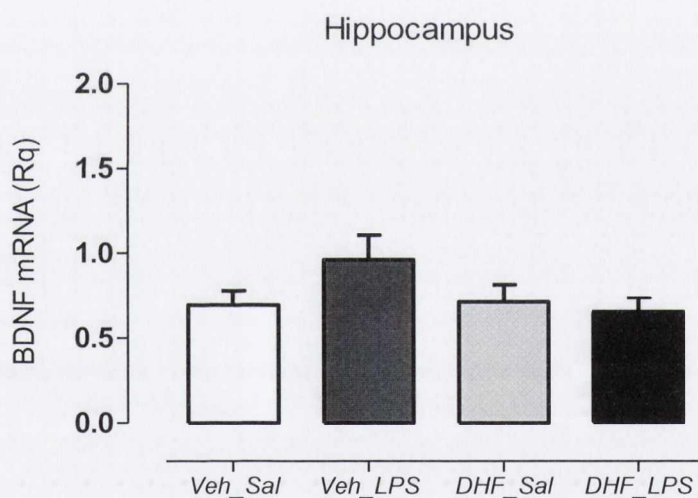


Fig. 3.3.33 – Effect of 7,8-DHF and/or LPS on BDNF mRNA expression

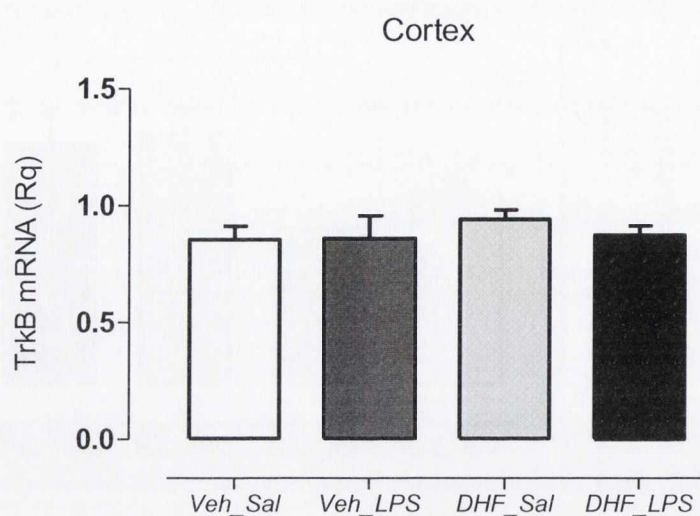
(a) shows Rq of BDNF mRNA expression in cortical tissue. There was a significant difference in BDNF expression between Veh_LPS and DHF_LPS subject groups ($p=0.0477$).

(b) shows Rq of BDNF mRNA expression in hippocampal tissue. There was no significant difference in BDNF expression between subject groups.

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. * $p<0.05$.

Data expressed as mean $\pm$ SEM ($n=5$).

a)



b)

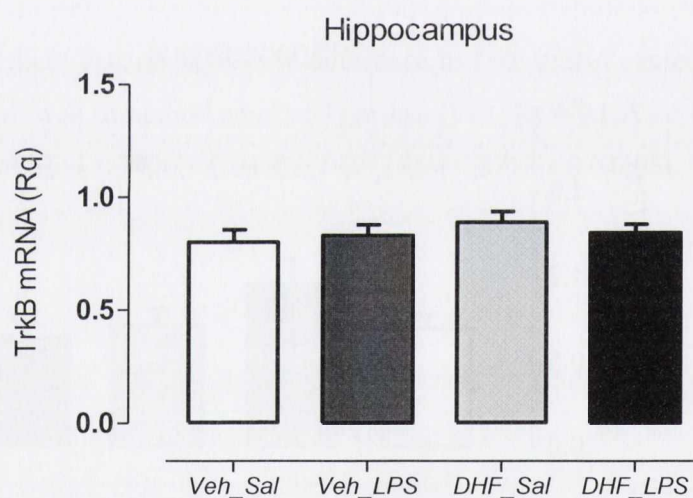


Fig. 3.3.34 – Effect of 7,8-DHF and/or LPS on TrkB mRNA expression

(a) shows Rq of TrkB mRNA expression in cortical tissue. There was no significant difference in TrkB expression between subject groups.

(b) shows Rq of TrkB mRNA expression in hippocampal tissue. There was no significant difference in TrkB expression between subject groups.

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. $p > 0.05$.

Data expressed as mean $\pm$ SEM ($n=5$).

3.3.3.3b The effect of one month of 7,8-DHF administration on mRNA expression of Ki67 and CREB in cortex and hippocampus, 5 hours after experimentally-induced neuroinflammation.

PCR analysis showed there was no significant difference in Ki67 mRNA expression in the cortex when Rq measurement was compared across all groups. (*Veh_Sal* = 1.047 ± 0.1636 ; *Veh_LPS* = 1.104 ± 0.06718 ; *DHF_Sal* = 1.099 ± 0.1827 ; *DHF_LPS* = 1.002 ± 0.1482). ($p = 0.3484$, $n = 5$). (Fig 3.3.35a)

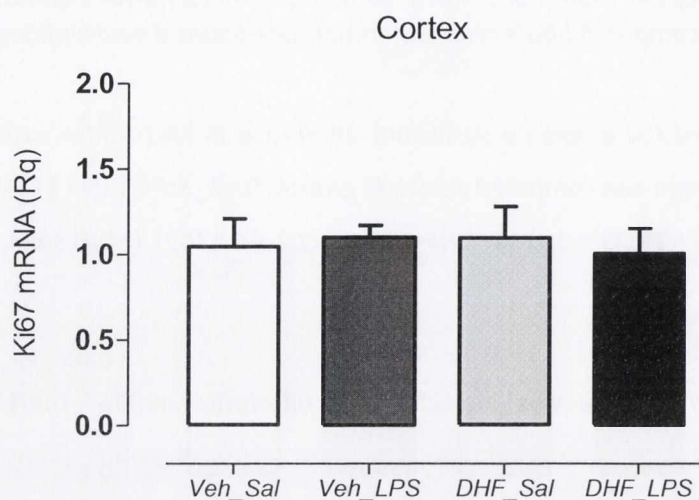
PCR analysis showed there was no significant difference in Ki67 mRNA expression in the hippocampus when Rq measurement was compared across all groups. (*Veh_Sal* = 1.142 ± 0.2125 ; *Veh_LPS* = 1.175 ± 0.1618 ; *DHF_Sal* = 1.113 ± 0.06251 ; *DHF_LPS* = 1.056 ± 0.07502). ($p = 0.9434$, $n = 5$). (Fig 3.3.35b)

PCR analysis showed there was no significant difference in CREB mRNA expression in the cortex when Rq measurement was compared across all groups. (*Veh_Sal* = 1.197 ± 0.1105 ; *Veh_LPS* = 1.838 ± 0.3437 ; *DHF_Sal* = 1.709 ± 0.2618 ; *DHF_LPS* = 2.318 ± 0.5785). ($p = 0.2305$, $n = 5$). (Fig 3.3.36a)

PCR analysis showed there was no significant difference in CREB mRNA expression in the hippocampus when Rq measurement was compared across all groups. (*Veh_Sal* = 0.6509 ± 0.09013 ; *Veh_LPS* = 0.7527 ± 0.09463 ; *DHF_Sal* = 0.7317 ± 0.1067 ; *DHF_LPS* = 0.6311 ± 0.06744). ($p = 0.7339$, $n = 5$). (Fig 3.3.36b)

Data analysed by unpaired t-test and/or one-way ANOVA and Bonferroni post-hoc test, expressed as mean $\pm$ SEM.

a)



b)

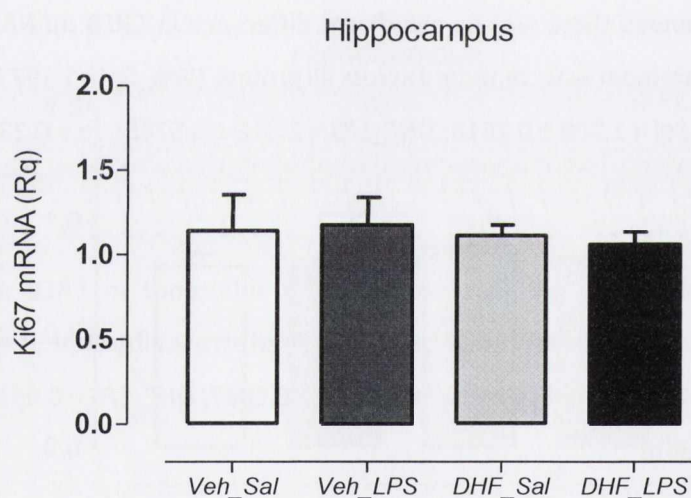


Fig. 3.3.35 – Effect of 7,8-DHF and/or LPS on Ki67 mRNA expression

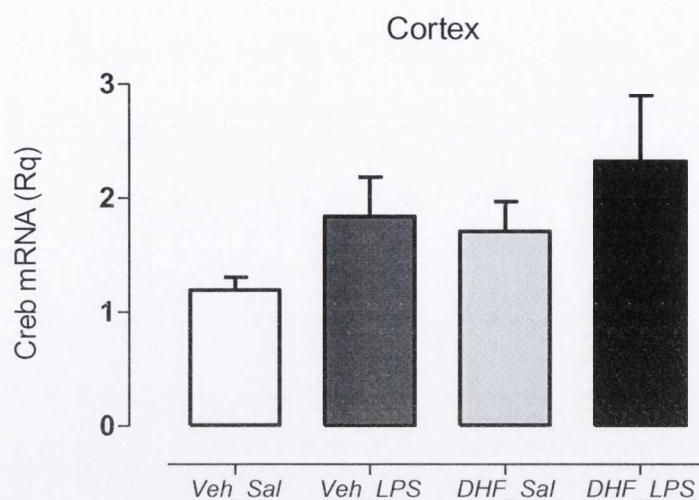
(a) shows Rq of Ki67 mRNA expression in cortical tissue. There was no significant difference in Ki67 expression between subject groups.

(b) shows Rq of Ki67 mRNA expression in hippocampal tissue. There was no significant difference in Ki67 expression between subject groups.

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. $p > 0.05$.

Data expressed as mean $\pm$ SEM (n=5).

a)



b)

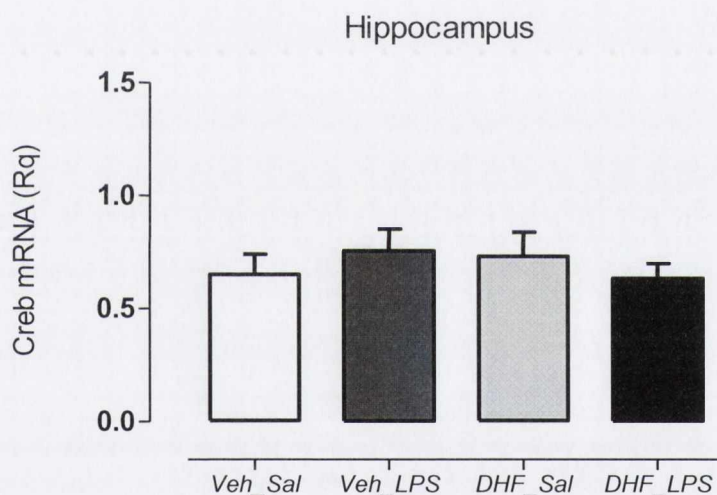


Fig. 3.3.36 – Effect of 7,8-DHF and/or LPS on CREB mRNA expression

(a) shows Rq of CREB mRNA expression in cortical tissue. There was no significant difference in CREB expression between subject groups.

(b) shows Rq of CREB mRNA expression in hippocampal tissue. There was no significant difference in CREB expression between subject groups.

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. $p > 0.05$.

Data expressed as mean $\pm$ SEM (n=5).

3.3.3.3c The effect of one month of 7,8-DHF administration on mRNA expression of IL1 β and TNF α in cortex and hippocampus, 5 hours after experimentally-induced neuroinflammation.

PCR analysis showed there was an insignificant difference in IL1 β mRNA expression in the cortex when Rq measurement in Veh_LPS was compared to Veh_Sal ($p = 0.0838$), and also when DHF_LPS was compared to DHF_Sal ($p = 0.0959$). ($Veh_Sal = 0.8328 \pm 0.4226$; $Veh_LPS = 2.111 \pm 0.4905$; $DHF_Sal = 0.7662 \pm 0.4273$; $DHF_LPS = 1.879 \pm 0.3604$). ($p = 0.0912$ $n = 5$). (Fig 3.3.37a)

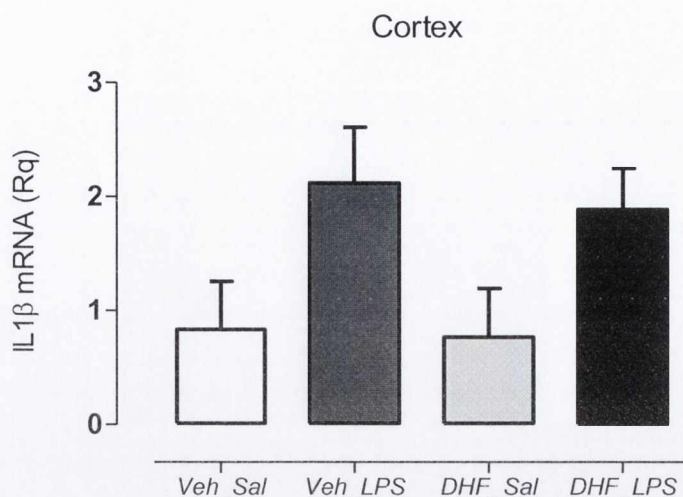
PCR analysis showed there was a significant difference in IL1 β mRNA expression in the hippocampus when Rq measurement in Veh_LPS was compared to Veh_Sal ($p = 0.0059$), and also when DHF_LPS was compared to DHF_Sal ($p = 0.0003$). ($Veh_Sal = 1.175 \pm 0.1593$; $Veh_LPS = 14.87 \pm 3.677$; $DHF_Sal = 1.894 \pm 0.435$; $DHF_LPS = 13.16 \pm 1.806$). ($p < 0.0001$, $n = 5$). (Fig 3.3.37b)

PCR analysis showed there was a significant difference in TNF α mRNA expression in the cortex when Rq measurement in Veh_LPS was compared to Veh_Sal ($p = 0.0499$). There was an insignificant difference when DHF_LPS was compared to DHF_Sal ($p = 0.0685$). ($Veh_Sal = 1.181 \pm 0.495$; $Veh_LPS = 3.540 \pm 0.8949$; $DHF_Sal = 1.489 \pm 0.7889$; $DHF_LPS = 3.449 \pm 0.1997$). ($p = 0.0487$ $n = 5$). (Fig 3.3.38a)

PCR analysis showed there was a significant difference in TNF α mRNA expression in the hippocampus when Rq measurement in Veh_LPS was compared to Veh_Sal ($p < 0.0001$), and also when DHF_LPS was compared to DHF_Sal ($p = 0.0005$). ($Veh_Sal = 1.546 \pm 0.1459$; $Veh_LPS = 10.01 \pm 1.094$; $DHF_Sal = 1.593 \pm 0.1794$; $DHF_LPS = 7.521 \pm 1.027$). ($p < 0.0001$, $n = 5$). (Fig 3.3.38b)

Data analysed by unpaired t-test and/or one-way ANOVA and Bonferroni post-hoc test, expressed as mean $\pm$ SEM.

a)



b)

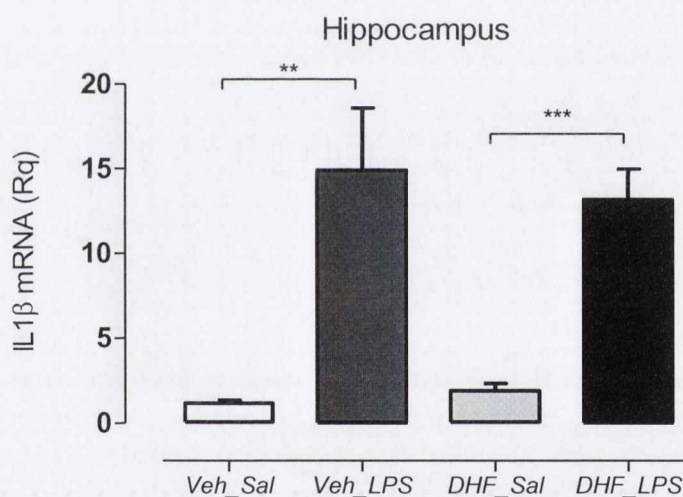


Fig. 3.3.37 – Effect of 7,8-DHF and/or LPS on IL1β mRNA expression

(a) shows Rq of IL1β mRNA expression in cortical tissue. There was an insignificant difference in IL1β expression within subject groups when Saline and LPS treated animals were compared.

(b) shows Rq of IL1β mRNA expression in hippocampal tissue. There was a significant difference in IL1β expression when Veh_LPS is compared to Veh_Sal ($p=0.0059$), and also when DHF_LPS is compared to DHF_Sal ($p=0.0003$).

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. ** $p<0.01$, *** $p<0.001$.

Data expressed as mean $\pm$ SEM ($n=5$).

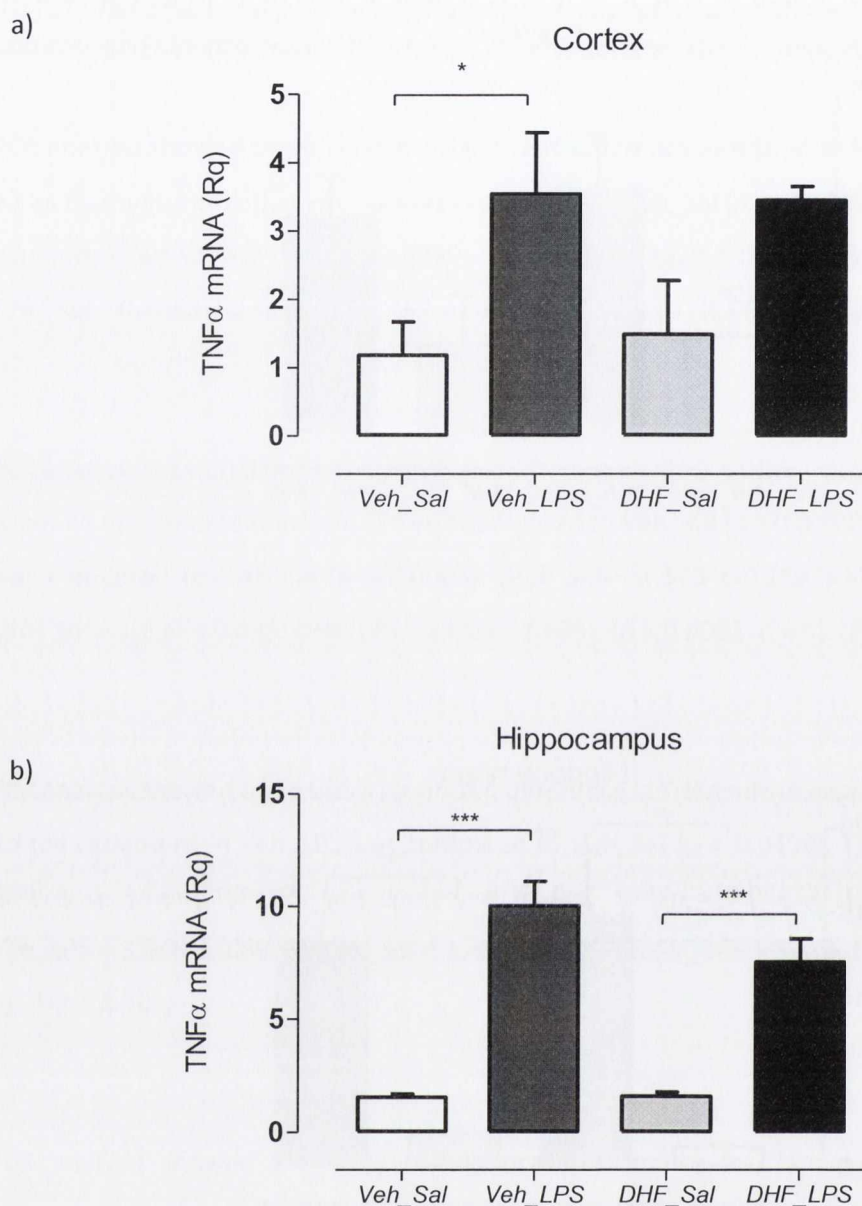


Fig. 3.3.38 – Effect of 7,8-DHF and/or LPS on TNF α mRNA expression

(a) shows Rq of TNF α mRNA expression in cortical tissue. There was a significant difference in TNF α expression between Veh_Sal and Veh_LPS subject groups ($p = 0.0499$).

(b) shows Rq of TNF α mRNA expression in hippocampal tissue. There was a significant difference in TNF α expression when Veh_LPS is compared to Veh_Sal ($p < 0.0001$), and also when DHF_LPS is compared to DHF_Sal ($p = 0.0005$).

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. * $p < 0.05$, *** $p < 0.001$.

Data expressed as mean $\pm$ SEM ($n=5$).

3.3.3.3d The effect of one month of 7,8-DHF administration on mRNA expression of Iba1 and CD44 in cortex and hippocampus, 5 hours after experimentally-induced neuroinflammation.

PCR analysis showed there was a significant difference in Iba1 mRNA expression in the cortex when Rq measurement in DHF_LPS was compared to Veh_LPS ($p = 0.0429$). ($Veh_Sal = 0.9744 \pm 0.03246$; $Veh_LPS = 0.9143 \pm 0.05437$; $DHF_Sal = 1.018 \pm 0.03848$; $DHF_LPS = 1.085 \pm 0.04598$). ($p = 0.0776$ $n = 5$). (Fig 3.3.39a)

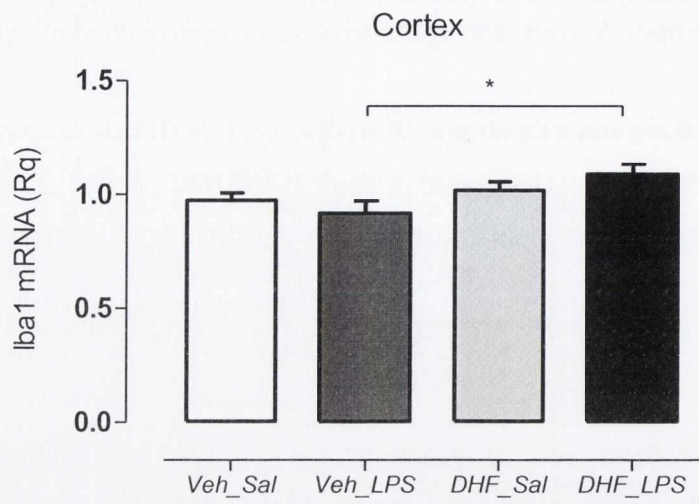
PCR analysis showed there was no significant difference in Iba1 mRNA expression in the hippocampus when Rq measurement was compared across all groups. ($Veh_Sal = 0.8630 \pm 0.0475$; $Veh_LPS = 0.9733 \pm 0.0604$; $DHF_Sal = 0.8504 \pm 0.06785$; $DHF_LPS = 0.8237 \pm 0.06867$). ($p = 0.363$, $n = 5$). (Fig 3.3.39b)

PCR analysis showed there was no significant difference in CD44 mRNA expression in the cortex when Rq measurement was compared across all groups. ($Veh_Sal = 1.341 \pm 0.1938$; $Veh_LPS = 1.500 \pm 0.1819$; $DHF_Sal = 1.652 \pm 0.3170$; $DHF_LPS = 2.995 \pm 1.041$). ($p = 0.1809$, $n = 5$). (Fig 3.3.40a)

PCR analysis showed there was a significant difference in CD44 mRNA expression in the hippocampus when Rq measurement in Veh_LPS was compared to Veh_Sal ($p = 0.0385$), and also when DHF_LPS was compared to DHF_Sal ($p = 0.025$). ($Veh_Sal = 1.695 \pm 0.2068$; $Veh_LPS = 3.302 \pm 0.6156$; $DHF_Sal = 1.609 \pm 0.2338$; $DHF_LPS = 3.002 \pm 0.4493$). ($p = 0.0177$, $n = 5$). (Fig 3.3.40b)

Data analysed by unpaired t-test and/or one-way ANOVA and Bonferroni post-hoc test, expressed as mean $\pm$ SEM.

a)



b)

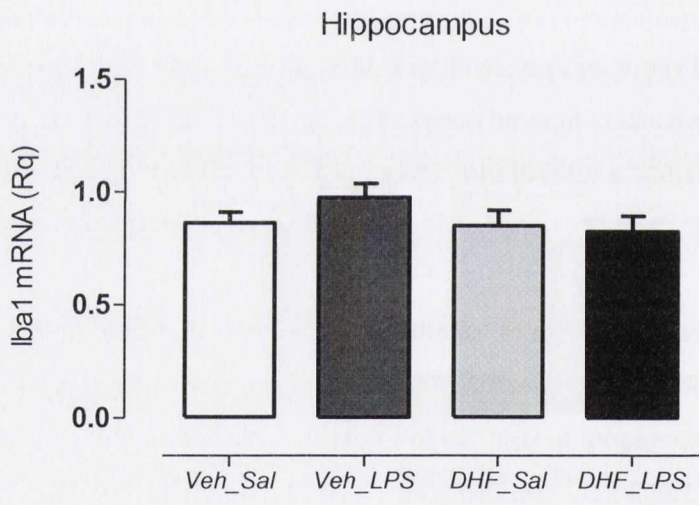
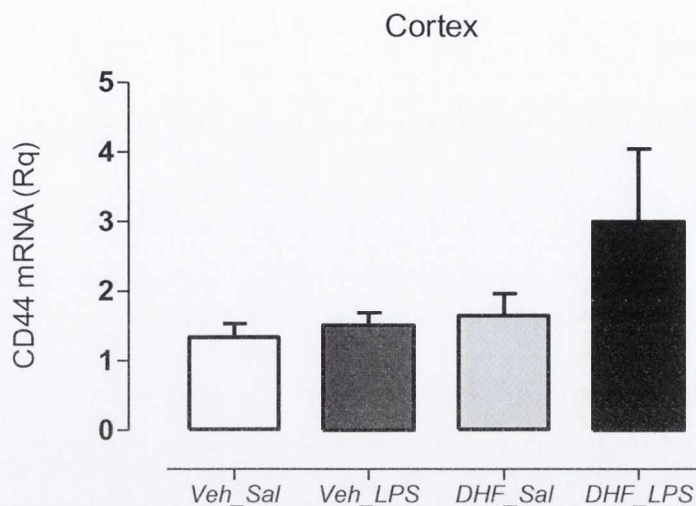


Fig. 3.3.39 – Effect of 7,8-DHF and/or LPS on Iba1 mRNA expression
(a) shows Rq of Iba1 mRNA expression in cortical tissue. There was a significant difference in Iba1 expression between Veh_LPS and DHF_LPS subject groups ($p = 0.0429$).
(b) shows Rq of Iba1 mRNA expression in hippocampal tissue. There was no significant difference in Iba1 expression between subject groups.
Assessed by one-way ANOVA and Bonferroni post-hoc analysis. * $p < 0.05$.
Data expressed as mean $\pm$ SEM ($n=5$).

a)



b)

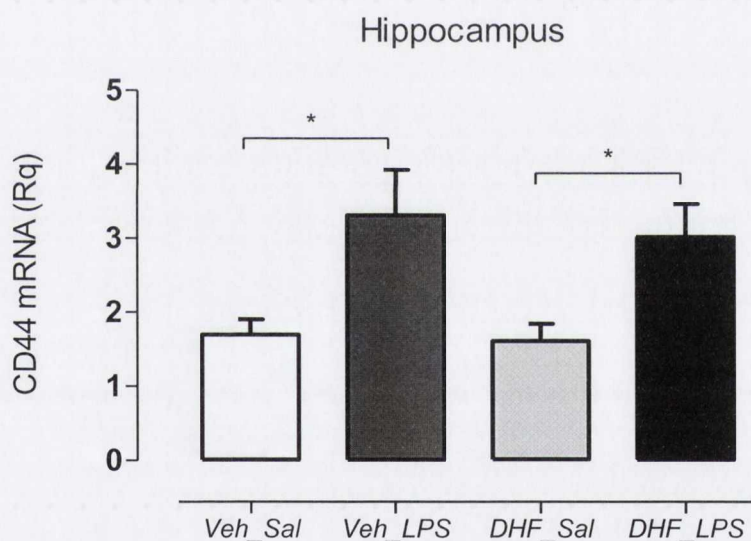


Fig. 3.3.40 – Effect of 7,8-DHF and/or LPS on CD44 mRNA expression

(a) shows Rq of CD44 mRNA expression in cortical tissue. There was no significant difference in CD44 expression between subject groups.

(b) shows Rq of CD44 mRNA expression in hippocampal tissue. There was a significant difference in CD44 expression between Veh_Sal and Veh_LPS ($p = 0.0385$), and between DHF_Sal and DHF_LPS subject groups ($p = 0.025$).

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. $*p < 0.05$.

Data expressed as mean $\pm$ SEM ($n=5$).

3.3.3.4 The effect of one month of 7,8-DHF administration on dentate gyrus volume, and number of BrdU⁺ cells.

Immunohistochemical staining and stereological estimation techniques show that there was a significant difference in dentate gyrus volume (Fig 3.3.41) when Vehicle animals were compared to 7,8-DHF treated animals, regardless of Saline or LPS administration (Vehicle = 0.5196 ± 0.02198 ; 7,8-DHF = 0.5929 ± 0.01714) ($p = 0.0198$, $n = 8$).

There was no significant difference in BrdU⁺ cell numbers (Fig 3.3.42) in animals that were administered 7,8-DHF for four weeks when compared to Vehicle treated animals (Vehicle = 22.92 ± 1.419 ; 7,8-DHF = 19.44 ± 1.709) ($p = 0.1395$, $n = 8$).

Data analysed using unpaired t-test, expressed as mean $\pm$ SEM.

Figure 3.3.43 shows an example of images obtained from Vehicle (a, c) and 7,8-DHF (b, d) treated animals.

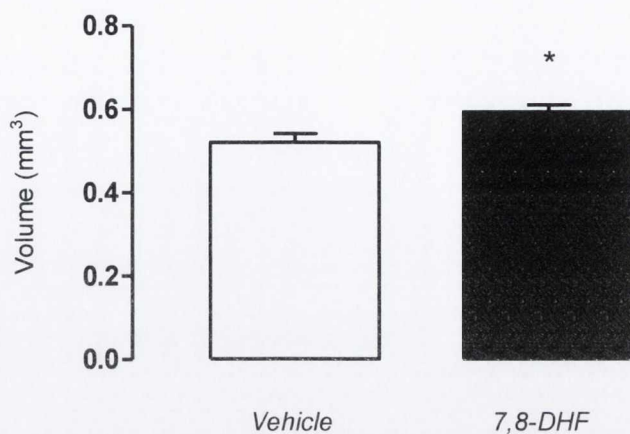


Fig. 3.3.41 – The effect of four weeks of 7,8-DHF administration on dentate gyrus volume

There was a significant difference in overall volume of dentate gyrus as measured using the Cavalieri method of volume estimation ($p=0.0198$)

Assessed by unpaired t test. * $p<0.05$. Data expressed as mean $\pm$ SEM ($n=8$).

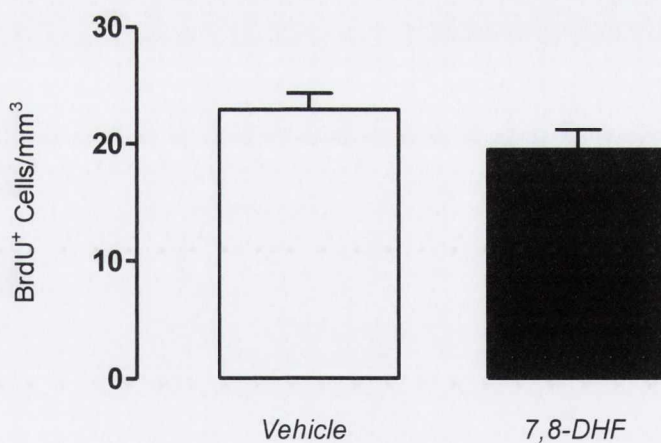


Fig. 3.3.42 - The effect of four weeks 7,8-DHF administration on BrdU⁺ cells in the dentate gyrus

There was no significant difference in the number of BrdU⁺ cells in the dentate gyrus of 7,8-DHF treated animals.

Assessed by unpaired t test ($p=0.1395$). Data expressed as mean $\pm$ SEM ($n=8$).

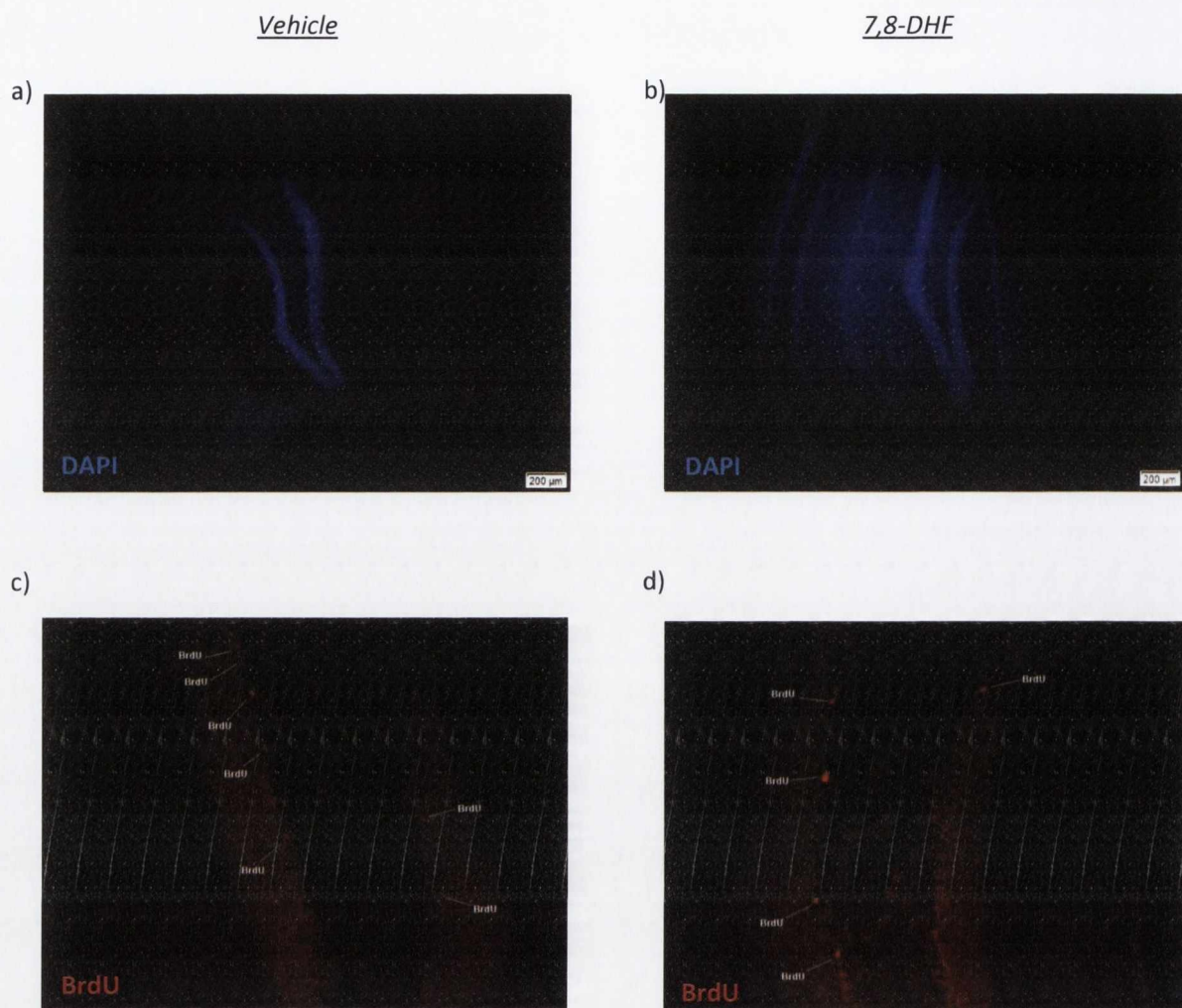


Fig. 3.3.43 - BrdU⁺ cells in the dentate gyrus

Images obtained at x4 (DAPI) and x20 (BrdU) magnification of Vehicle (a, c) and 7,8-DHF (b, d) treated subjects.

BrdU⁺ cells are indicated with white marker.

3.4 Discussion

3.4.1 Experiment I – Can exercise protect against experimentally-induced neuroinflammation in the C57/BL6 mouse: assessment at 24hrs post-learning?

3.4.1.1 Behavioural analysis

During the training phase of the OD task, one-way ANOVA showed that Sed_Sal animals spent significantly less time exploring in the open field when compared to Sed_LPS, Ex_Sal, and Ex_LPS animals. During the testing phase, this significant difference was observed again when compared to all other groups. This difference in exploratory behaviour may be due to random behavioural differences between animals. We have not observed a general lack of interest in exploration with our animal studies before this occasion, as rodents are known to be naturally inquisitive. On the other hand, when we compare the remaining treatment groups, we see no difference in exploratory behaviour. If we look at our Ex_LPS animals we see that their mean exploration was in fact higher than Ex_Sal animals during the training session of this task. We can report that LPS administration did not have a negative effect on object exploration in general.

During the training session, two-way ANOVA showed that Ex_Sal and Ex_LPS animals explored all three objects equally, as is expected from initial exposure to three novel objects. Object location was rotated after each animal underwent training, so we could eliminate the combined effect of a fixed object in a fixed location and the possibility that this might not be attractive for all animals. Despite this, Sed_Sal showed a preference for, and Sed_LPS groups an avoidance of, one object during the training session. Again, this is likely due to random behavioural variation between animals.

During testing, Sed_Sal and Sed_LPS animals showed no difference in object exploration, and Ex_Sal and Ex_LPS animals showed a preference for the displaced object; object C. From this, we can conclude that sedentary animals as a whole were unable to successfully complete the OD task. In agreement with existing reports (Griffin *et al.*, 2009, Uysal *et al.*, 2005), our month-long exercise programme had a beneficial effect on performance in this test of spatial memory. However, we cannot confirm that exercise rescues an LPS-induced inability to perform this task, as our Sedentary healthy controls were also unable to complete it successfully. However, previous studies from our laboratory indicated that this dose of LPS inhibits object recognition memory in the rat (Hennigan *et al.*, 2007).

3.4.1.2 Analysis of BDNF and its receptor; TrkB

PCR analysis of mRNA expression showed no difference in BDNF concentration in hippocampal tissue across all groups. Previous studies in our laboratory observed an increase in BDNF concentration immediately after the cessation of exercise (Bechara and Kelly, 2013, Griffin *et al.*, 2009, O'Callaghan *et al.*, 2007), but as our study required completion of exercise 48hrs before sacrifice to allow for behavioural testing, there is a possibility that an increase in mRNA expression was not being stimulated at this time. Indeed, the exercise-induced increase in BDNF has been shown to be transient (Bechara *et al.*, 2014). The lack of difference in expression means we can conclude there was no disruptive effect of LPS administration on levels of BDNF.

Similarly, there was no difference across all groups in expression of TrkB receptor mRNA at this time point, but reports on this are mixed. It has been shown that TrkB expression is increased after exercise (Liu *et al.*, 2008), unchanged after exercise (Macias *et al.*, 2007), and is not always altered in line with BDNF upregulation (Klintsova *et al.*, 2004). It is possible that TrkB receptor phosphorylation as assessed by protein analysis, as opposed to mRNA analysis carried out here, might show some difference in activation of the receptor. However, without a difference in BDNF expression being observed at this time point, it is unlikely that TrkB receptors would be activated at varying levels throughout our treatment groups. Again, we can conclude that LPS administration had no effect on TrkB mRNA expression.

3.4.1.3 Analysis of Ki67 expression

In analysing expression of Ki67 mRNA, we can deduce whether or not cells are actively proliferating at the time of tissue collection. Ki67 is an endogenous marker of cellular proliferation, and is thought to be required for a cell's progression through the cell cycle (Scholzen and Gerdes, 2000). However, Ki67 is not specific to neurons, and glial cell proliferation is also measured by this method. Ki67 is present during all active stages of the cell cycle. It is present in the nucleus during interphase (stages G₁, S, and G₂), and present on the surface of chromosomes during mitosis. The only time Ki67 is not expressed is when cells are inactive, or resting (stage G₀). So its expression is a direct measure of the level of cellular proliferation. As was observed with expression of BDNF and TrkB mRNA, Ki67 mRNA concentration was not changed across all treatment groups. It is possible that cell proliferation was not stimulated at the time of tissue collection. In agreement with this, it has been shown that Ki67 expression peaked after ten days of exercise, but returned to baseline levels

in mice that were exercising for 32 days (Kronenberg *et al.*, 2006). Again, we did not see any effect of LPS treatment on expression of this marker.

3.4.1.4 Analysis of CREB expression

PCR analysis showed no significant difference in expression of CREB mRNA in the hippocampus of either sedentary or exercising mice. This would agree with evidence that shows CREB expression to rise and fall parallel to BDNF expression in animals that have been subject to exercise (Vaynman *et al.*, 2004b, Gómez-Pinilla *et al.*, 2002). Both of these studies found an increase in BDNF and CREB expression after exercise programmes lasting between three and ten days, so perhaps this increase was not sustained for the duration of our intervention. We did not see any effect of LPS treatment on expression of this transcription factor, so again we cannot conclude that exercise was protective against the effects of LPS.

3.4.1.5 Analysis of proinflammatory cytokines; IL1 β and TNF α

As expected, we observed a significant difference in mRNA expression of IL1 β in both Sed_LPS and Ex_LPS treatment groups when compared to their saline-treated counterparts. IL1 β is a proinflammatory cytokine released from microglia as a response to infection, and the administration of LPS triggered a significant difference in its release and subsequent upregulation in mRNA expression. The difference between Sed_LPS and Ex_LPS groups is insignificant, but there is a trend towards significantly lower expression, so perhaps further analysis with an increase in n number could separate these treatment groups more definitively. This is of course purely speculative, and as of yet we cannot credit exercise with displaying a protective effect, but there have been studies that have shown a reduction in IL1 β concentration attributed to exercise in the periphery in the rat (Lira *et al.*, 2009, Smith *et al.*, 2000) and in the brain in mice (Souza *et al.*, 2013).

Similar to PCR analysis of IL1 β , mRNA expression of TNF α was higher in both Sed_LPS and Ex_LPS groups, but a significant difference was observed in Sed_LPS animals only, when compared to saline treated sedentary subjects. TNF α expression has been shown to be lower in exercising animals, in the periphery in the rat (Lira *et al.*, 2009, Smith *et al.*, 2000) and centrally in the mouse (Souza *et al.*, 2013), but from our results we cannot conclude that exercise has a tendency to protect against an LPS-induced increase in TNF α expression. It may be true that the ability of exercise to protect against LPS-induced cognitive impairment is independent of IL1 β and TNF α .

3.4.1.6 Analysis of Iba1 expression

Our results show no change in mRNA expression of Iba1 (Ionized calcium-binding adapter molecule 1) across all treatment groups. Iba1 expression is upregulated during infection-induced microglial activation and was found to be increased along with IL1 β expression (Autieri *et al.*, 2000, Fukui *et al.*, 2012), so it would be expected that its expression would be upregulated at some point in the inflammatory response, but that does not necessarily translate to an increase in its mRNA expression at this particular time point. Studies have shown that exercise decreases its expression (Gerecke *et al.*, 2013, Yoo *et al.*, 2015). We cannot conclude that Iba1 expression is altered by either exercise or LPS treatment.

3.4.1.7 Analysis of CD44 expression

CD44 is an adhesion molecule expressed on the surface of memory T cells. CD44 assists in mediating protection from re-infection, and so its expression is usually upregulated and persists long after the infection has been cleared (Baaten *et al.*, 2010). Exercise has been shown to reduce the expression of CD44 in aged mice (Kohut *et al.*, 2004). In reducing CD44 activation, the inflammatory response can be modulated and chronic inflammation would be less destructive. Our results showed no significant difference across our treatment groups, but again there was a trend towards an anti-inflammatory effect in both exercise groups. However, our results do not show a consistent effect of LPS in Sed_LPS and Ex_LPS groups.

3.4.1.8 Analysis of ERK1/2 protein expression

ERK1 and 2 are known to be vitally important in mediating the effects of BDNF upon its binding to the TrkB receptor (Han and Holtzman, 2000, Ying *et al.*, 2002), and exercise has been shown to upregulate ERK1/2 activity (Creer *et al.*, 2005, Goodyear *et al.*, 1996). At the protein level, our results show a significant difference in total ERK 1 in both Ex_Sal and Ex_LPS groups when compared to sedentary animals, showing an upregulation in response to exercise. However, this did not translate to the expression of ERK 2. Also noted was a lack of significant difference in levels of phosphorylation in both ERK 1 and 2 relative to total ERK 1 and 2, showing us that there was no change in activation of this protein at this time. Again, we saw no effect of LPS across our treatment groups.

3.4.1.9 Analysis of Akt protein expression

The PI3K/Akt pathway is known to be important in modulating the cellular changes triggered by the binding of BDNF to its TrkB receptor. Exercise has been shown to be effective in increasing activation of this pathway, but this increase comes very quickly after exercise has been completed (Camera *et al.*, 2010). Known to modulate cell survival, among other functions (Kandel and Hay, 1999), the activation of Akt is also modulated by increases in BDNF concentration, and so it is not expected that we would see a change in concentration of this signalling factor at this time point, 48hrs after exercise protocol ended, and also in the absence of an up-regulation of BDNF. We report no difference in total or activated Akt expression at the protein level across all treatment groups, and again, no effect of LPS administration.

3.4.1.10 Analysis of CREB protein expression

CREB is known to play an important role in cell survival and proliferation, but in agreement with unchanged levels of mRNA expression, we saw no change in either total CREB protein expression across all treatment groups at this time point. As discussed, CREB expression was found to peak after ten days of exercise (Vaynman *et al.*, 2004a), so it is perhaps unexpected that we found no change with this particular exercise regime. CREB activation is dependent on the activity of BDNF, ERK and Akt, so it is expected that without an increase in these effectors, an increase in phosphorylated CREB would not been seen in these particular treatment groups.

3.4.1.11 Analysis of dentate gyrus volume and cell survival

The process of labelling cells with BrdU was initiated on the first day of the exercise programme, and continued for seven days after with a daily i.p. injection of the compound. This meant that the system was flooded with this substitute for the thymidine base of DNA, and cells undergoing proliferation in the presence of this analogue could incorporate BrdU in place of thymidine. Three to four weeks later, when exercise had ended and tissue was collected, we were able to tag slices of brain tissue with an antibody to BrdU and detect the presence of BrdU in cells of the dentate gyrus, where proliferation had been occurring at the time of injection, and where we would now hope to see surviving BrdU tagged cells. A single dose of LPS was not expected to have an effect on this process so long after it had taken place, so we compare only sedentary and exercised groups in this instance. We found no difference in volume of the dentate gyrus when measured in both

groups, but we did see an insignificant tendency toward a higher number of BrdU positive cells in the dentate gyrus of exercised animals. With further analysis and an increase in n number, this difference may be more pronounced, and would agree with studies that have repeatedly demonstrated an increase in BrdU positive cells following exposure to exercise (Van Praag *et al.*, 1999a, Van Praag *et al.*, 1999b, Van Praag *et al.*, 2005).

3.4.1.12 Summary

In Experiment I, our efforts to definitively account for the exercise-induced cognitive enhancement we observed in the OD task have been unsuccessful to date. Despite much evidence in the literature reporting the benefits of exercise – whether in an exercise-induced increase in BDNF (Griffin *et al.*, 2011), or an exercise-induced decrease in cytokine secretion (Sloan *et al.*, 2007) we are thus far unsuccessful in elucidating an instrumental physiological factor in this process. We still wanted to investigate the protective effect of exercise in experimentally-induced inflammation, and so decided to alter the timeline of testing while maintaining the timing of the exercise programme and BrdU administration, as an increase in BrdU positive cells was very likely, implicating neurogenesis as a contributing factor in cognitive enhancement. The time delay between our LPS injection and OD testing meant that we did not see any conclusive acute effect of LPS in these animals, and the aversion of our control animals to exploration further restricted the perspective of our findings. For this reason, we repeated the experiment, but assessed learning and memory, and collected tissue samples on the same day as LPS administration (Experiment II).

3.4.2 Experiment II – Can exercise protect against experimentally-induced neuroinflammation in the C57/BL6 mouse: assessment at 1 hour post-learning?

3.4.2.1 Behavioural analysis

During the training phase of the OD task there was no significant difference across treatment groups, but unpaired t-test analysis showed that Sed_Sal and Ex_LPS animals explored for similar amounts of time. During the testing phase, there was significantly lower exploration in the Sed_LPS group when compared to both Sed_Sal and DHF_LPS animals, despite any decline in overall locomotor activity. These findings could suggest that there was an LPS-induced decrease in exploration that was rescued by one month of exercise. In fact, Ex_LPS animals were observed to explore at levels similar to Sed_Sal. We can report that a subseptic dose of LPS administration had a negative effect on object exploration, but was rescued in animals pre-treated with a programme of exercise.

During the training session, two-way ANOVA showed that all treatment groups explored all three objects equally, as is expected upon initial exposure to three novel objects. Object location was rotated after each animal underwent training, so we could eliminate the combined effect of a fixed object in a fixed location and the possibility that this might not be attractive for all animals.

During testing, Sed_LPS animals showed no difference in object exploration, and Sed_Sal, Ex_Sal and Ex_LPS animals showed a preference for the displaced object; object C. From this, we can conclude that the timing of the task was sufficient to permit acquisition and recall, as our healthy controls were capable of successfully completing the task. Concurrently, the detrimental effects of LPS were diminished in animals who had been exercised. This was in agreement with existing reports (Griffin *et al.*, 2009, Uysal *et al.*, 2005). Here we show that one month of moderate treadmill exercise had a beneficial effect on performance in a test of spatial memory, and we infer that exercise rescues an LPS-induced inability to perform this task.

3.4.2.2 Analysis of BDNF and its receptor; TrkB

PCR analysis of mRNA expression showed no difference in BDNF concentration in cortical tissue across all groups. With regard to BDNF mRNA levels in hippocampal tissue, we also found no difference across all treatments. Previous studies in our laboratory observed an increase in BDNF concentration immediately after the cessation of exercise (Bechara and Kelly, 2013, Griffin *et al.*, 2009, O'Callaghan *et al.*, 2007). In the current study, exercise was completed <24hrs before sacrifice to allow for behavioural testing, so there is a possibility that an acute increase in mRNA expression was not being stimulated at this time. The lack of significant difference in expression means we conclude there was no disruptive effect of LPS administration on levels of BDNF in the cortex or the hippocampus.

Similarly, there was no difference observed in multiple comparisons of all groups in the expression of TrkB receptor mRNA in the cortex. Analysis of hippocampal tissue found a discrete difference in TrkB expression when Sed_LPS animals were compared to Ex_LPS at this time point. It is possible that TrkB receptor phosphorylation as assessed by protein analysis, as opposed to mRNA analysis carried out here, might show some significant differences in activation of the receptor. However, we would likely need to see a difference in BDNF protein expression also, as it is unlikely that TrkB receptors would be activated at varying levels throughout our treatment groups without an elevation in BDNF itself. From this, we cannot conclude that LPS administration had any effect on TrkB mRNA expression, as we found no significant difference from saline treated control animals.

3.4.2.3 Analysis of Ki67 expression

In analysing expression of Ki67 mRNA, we can deduce whether or not cells are actively proliferating at the time of tissue collection. Ki67 is an endogenous marker of neuronal and glial proliferation, and is thought to be required for a cell's progression through the cell cycle (Scholzen and Gerdes, 2000). Therefore, expression of Ki67 is a direct indicator of proliferation. As was observed with expression of BDNF and TrkB mRNA, Ki67 mRNA concentration was not significantly different when measured across all treatment groups, in either cortical or hippocampal tissue. It is possible that cell proliferation was not stimulated at the time of tissue collection. Again, we did not see any effect of LPS treatment on expression of this marker.

3.4.2.4 Analysis of CREB expression

PCR analysis showed no significant difference in expression of CREB mRNA in the cortex or the hippocampus of either sedentary or exercising mice. This would agree with evidence that shows CREB expression to rise and fall parallel to BDNF expression in animals that have been subject to exercise (Vaynman *et al.*, 2004b, Gómez-Pinilla *et al.*, 2002). Both of these studies found an increase in BDNF and CREB expression after exercise programmes lasting between three and ten days, so perhaps this increase was not sustained for the duration of our intervention. We did not see any effect of LPS treatment on expression of this transcription factor, so again this does not conclude that exercise was protective against the effects of LPS.

3.4.2.5 Analysis of proinflammatory cytokines; IL1 β and TNF α

As expected, analysis of cortical and hippocampal tissue revealed a significant difference in mRNA expression of IL1 β in both Sed_LPS and Ex_LPS treatment groups when compared to their saline-treated counterparts. IL1 β is a proinflammatory cytokine released from microglia as a response to infection, and the administration of LPS triggered a significant difference in its release and subsequent upregulation in mRNA expression. The difference between Sed_LPS and Ex_LPS groups is not significant, so we cannot credit exercise with demonstrating a protective effect on LPS-induced inflammation. There are studies that have shown a reduction in IL1 β concentration attributed to exercise, in the periphery in the rat (Lira *et al.*, 2009, Smith *et al.*, 2000) and in the brain in mice (Souza *et al.*, 2013), but we have not replicated these findings here.

Similar to PCR analysis of IL1 β , mRNA expression of TNF α in the cortex was higher in both Sed_LPS and Ex_LPS groups compared to their control groups, and in the hippocampus Sed_LPS expression was higher than Sed_Sal. TNF α expression has also been shown to be lower in exercising animals in the periphery in the rat (Lira *et al.*, 2009, Smith *et al.*, 2000) and in the brain in mice (Souza *et al.*, 2013), but from our results we cannot conclude that exercise has a tendency to protect against an LPS-induced increase in TNF α expression.

3.4.2.6 Analysis of Iba1 expression

Our results show no change in mRNA expression of Iba1 across all treatment groups. Iba1 expression is upregulated during infection-induced microglial activation and was found to be increased along with IL1 β expression (Autieri *et al.*, 2000, Fukui *et al.*, 2012), so it would be expected

that its expression would be upregulated at some point in the inflammatory response, but that does not necessarily translate to an increase in its mRNA expression at this particular time point. Studies have shown that exercise decreases the expression of Iba1 in both the cortex and hippocampus of exercised mice (Gerecke *et al.*, 2013) and in the rat hippocampus (Yoo *et al.*, 2015). We cannot conclude that Iba1 expression in the cortex or the hippocampus is altered by either exercise or LPS treatment.

3.4.2.7 Analysis of CD44 expression

CD44 is an adhesion molecule expressed on the surface of memory T cells. CD44 assists in mediating protection from re-infection, and so its expression is usually upregulated and persists long after the infection has been cleared (Baaten *et al.*, 2010). Exercise has been shown to reduce the expression of CD44 in aged mice (Kohut *et al.*, 2004). In reducing CD44 activation, the inflammatory response can be modulated and chronic inflammation would be less destructive. Our results showed no significant difference across our treatment groups when analysis of cortical tissue was carried out, but there was a significant difference in CD44 mRNA in hippocampal tissue when Ex_LPS subjects were compared to Ex_Sal. Our results do not show a consistent effect of LPS in Sed_LPS and Ex_LPS groups, and we cannot infer a protective effect of exercise against the inflammatory effects of LPS.

3.4.2.8 Analysis of dentate gyrus volume and cell survival

The process of labelling cells with BrdU was initiated on the first day of the exercise programme, and continued for seven days after with a daily i.p. injection of the compound. This meant that the system was flooded with this substitute for the thymidine base of DNA, and cells undergoing proliferation in the presence of this analogue could incorporate BrdU in place of thymidine. Three to four weeks later, when exercise had ended and tissue was collected, we were able to analyse slices of brain tissue for the presence of BrdU positive cells in the dentate gyrus, where proliferation occurs. A single dose of LPS was not expected to have an effect on the cells we wished to quantify so long after they may have incorporated BrdU, so we compare only sedentary and exercised groups in this instance. We found no difference in volume of the dentate gyrus when measured in both groups, but we did see an insignificant tendency toward a higher number of BrdU positive cells in the dentate gyrus of exercised animals. With further analysis and an increase in n number, this difference may be more pronounced, and would agree with studies that have repeatedly

demonstrated an increase in BrdU positive cells following exposure to exercise (Van Praag *et al.*, 1999a, Van Praag *et al.*, 1999b, Van Praag *et al.*, 2005).

3.4.2.9 Summary

In Experiment II, we can confirm that LPS administration has a negative effect on cognitive function as tested by the OD task, and a month-long regimen of treadmill exercise was sufficient to protect the LPS-treated mouse from this manifestation.

An investigation into the physiological impact of LPS and the effect of exercise in its impediment has thus far produced no definitive answers. A thorough exploration of downstream signalling factors may produce a contender to explain the tendency toward an increase in neurogenesis. Reports show that exercise protects against inflammation by activating the BDNF signalling pathway (Wu *et al.*, 2011), so it would be interesting to analyse expression of phosphorylated ERK, Akt, and CREB proteins in tissue collected on the same day as LPS administration.

Despite a significant difference in the expression of proinflammatory markers, Ex_LPS were still successful in the OD task, so it is clear that exercise is attenuating the cognitive impairment induced by LPS by some mechanism not yet identified by our analyses. There is much in the literature to infer that there is a beneficial effect of exercise - whether in an exercise-induced increase in BDNF (Griffin *et al.*, 2011), or an exercise-induced decrease in cytokine secretion (Sloan *et al.*, 2007), but we remain unsuccessful in implicating a contributing physiological factor in this process.

3.4.3 Experiment III – Can 7,8-DHF protect against experimentally-induced neuroinflammation in the C57/BL6 mouse: assessment at 1 hour post-learning?

3.4.3.1 Behavioural analysis

During the training phase of the OD task, there was no significant difference in levels of object exploration when all groups were compared. During the testing phase, unpaired t-test analysis showed that Veh_LPS animals spent significantly less time exploring in the open field when compared to DHF_LPS animals, but not other groups. From this, we could infer that there was an LPS-induced disinterest in exploration, but that this effect was diminished by the effect of 7,8-DHF administration, as seen in the DHF_LPS group. Comparison of the remaining treatment groups show no difference in exploratory behaviour.

During the training session, two-way ANOVA showed that all treatment groups explored all three objects equally, as is expected upon initial exposure to three novel objects. This observation was not affected by the level of total exploration reported previously. Object location was rotated after each animal underwent training, to eliminate the combined effect of a fixed object in a fixed location and the possibility that this might not be attractive for all animals.

During testing, Veh_Sal and Veh_LPS animals showed no difference in object exploration, while DHF_Sal and DHF_LPS animals showed a preference for the displaced object; object C. From this, we can postulate that either the timing or structure of the task meant it was too difficult for our healthy controls to successfully complete. We can conclude that one month of 7,8-DHF administration was sufficient to replicate results stimulated by exercise in Experiment II, but we cannot currently credit 7,8-DHF with a protective effect against LPS-induced inflammation.

3.4.3.2 Analysis of BDNF and its receptor; TrkB

PCR analysis of mRNA expression showed a significantly higher BDNF concentration in the cortex of DHF_LPS animals when compared to Veh_LPS. Previous studies in our laboratory showed an increase in BDNF concentration immediately after the cessation of exercise (Bechara and Kelly, 2013, Griffin *et al.*, 2009, O'Callaghan *et al.*, 2007), and our study permitted access to 7,8-DHF treated drinking water up until the time of sacrifice. However, there was no significant difference when compared to Veh_Sal subjects, and no change was observed in hippocampal tissue across all groups. Despite a significant difference between Veh and DHF saline controls, we could propose

that 7,8-DHF treatment in general has a tendency to increase BDNF expression in the cortex, and 7,8-DHF treatment inhibited the effect of LPS. This may be possible with further analysis, and an increase in n number.

There was no difference across all groups in expression of TrkB receptor mRNA in either cortical or hippocampal tissue. This is interesting because 7,8-DHF exerts its effects directly through TrkB binding, as shown in numerous studies to date (Devi and Ohno, 2012, Zeng *et al.*, 2012b, Zhang *et al.*, 2014). However, it is possible that TrkB receptor phosphorylation as assessed by protein analysis, as opposed to mRNA analysis carried out here, might show some difference in activation of the receptor. However, we can conclude that LPS administration had no effect on TrkB mRNA expression, regardless of treatment received.

3.4.3.3 Analysis of Ki67 expression

In analysing expression of Ki67 mRNA, we can deduce whether or not cells are actively proliferating at the time of tissue collection. Ki67 is an endogenous marker of neuronal and glial proliferation, as the only time Ki67 is not expressed is when cells are inactive. We did not observe a change in Ki67 mRNA concentration upon analysis of all treatment groups. It is possible that cell proliferation was not stimulated at the time of tissue collection. There is nothing yet in the literature to suggest that 7,8-DHF provokes proliferation in a ubiquitous manner. At this time, we cannot credit 7,8-DHF with having a neuroprotective effect on levels of Ki67 mRNA.

3.4.3.4 Analysis of CREB expression

mRNA analysis showed no significant difference in expression of CREB mRNA in the hippocampus of either Vehicle or 7,8-DHF treated mice. This would agree with evidence that shows CREB expression to rise and fall parallel to BDNF expression in animals that have been subject to exercise (Vaynman *et al.*, 2004b, Gómez-Pinilla *et al.*, 2002), as we also observed no change in BDNF expression in the hippocampus. Interestingly, we observe a tendency toward an increase in CREB expression in the cortex of both DHF_Sal and DHF_LPS animals, coinciding with a tendency toward the same in cortical BDNF expression. Studies with 7,8-DHF treatment have also shown an increase in phosphorylation of CREB (Agrawal *et al.*, 2015, Zeng *et al.*, 2012a, Zeng *et al.*, 2012b), so there is the possibility that western blot analysis of activated CREB would provide some more clarification.

We did not see any effect of LPS treatment on expression of this transcription factor, so again we cannot conclude that 7,8-DHF treatment was protective against the effects of LPS.

3.4.3.5 Analysis of proinflammatory cytokines; IL1 β and TNF α

We observed no statistically significant difference in cortical mRNA expression of IL1 β in either Veh_LPS or DHF_LPS treatment groups when compared to their saline-treated counterparts. However, there is a tendency toward a significant difference that may be drawn out by further analysis and an increase in n number. In the hippocampus, these changes were significantly different in both LPS groups when compared to their control groups. IL1 β is a proinflammatory cytokine released from microglia as a response to infection, and the administration of LPS triggers an increase in its release and subsequent upregulation in mRNA expression. In both the cortex and the hippocampus, the difference between Veh_LPS and DHF_LPS groups is insignificant, but there is a trend towards a decrease with 7,8-DHF treatment, as has been shown in the literature (Park *et al.*, 2012, Park *et al.*, 2014). This is of course purely speculative, and as of yet we cannot credit 7,8-DHF with displaying a protective effect against LPS.

In the cortex, mRNA expression of TNF α was significantly different in Veh_LPS when compared to Veh_Sal, but this change was not significant when DHF_LPS was compared to DHF_Sal. In the hippocampus, both Veh_LPS and DHF_LPS displayed a significant difference in levels of TNF α when each was compared to both Veh_Sal and DHF_Sal, respectively. Again, DHF_LPS animals showed a trend toward a lower expression of TNF α in both cortex and hippocampus when compared to Veh_LPS, but this also cannot be confirmed at this time. However, this change in TNF α has been reported (Park *et al.*, 2014). From our results at this time, we cannot conclude that 7,8-DHF has the ability to protect against an LPS-induced increase in TNF α expression, but further analysis and an increase in n number may clarify this.

3.4.3.6 Analysis of Iba1 expression

Our results show higher levels of cortical Iba1 mRNA expression in DHF_LPS animals when compared to Veh_LPS. Iba1 expression is upregulated during infection-induced microglial activation and was found to be increased along with IL1 β expression (Autieri *et al.*, 2000, Fukui *et al.*, 2012), so it would be expected that its expression would be upregulated at some point in the inflammatory

response. However, levels of Iba1 mRNA did not interfere with cognitive function. Studies have shown that treatment with 7,8-DHF can decrease Iba1 expression (Sanz García *et al.*, 2014, Wurzelmann, 2016), but we have not replicated that finding in our saline treated controls, so we cannot conclude that Iba1 expression is altered by 7,8-DHF alone.

3.4.3.7 Analysis of CD44 expression

There was no significant difference found in CD44 mRNA expression in cortical tissue, but analysis of the hippocampus showed that CD44 expression was significantly different in both Veh_LPS and DHF_LPS when each was compared to Veh_Sal and DHF_Sal, respectively. CD44 is an adhesion molecule expressed on the surface of memory T cells. CD44 assists in mediating protection from re-infection, and so its expression is usually upregulated and persists long after the infection has been cleared (Baaten *et al.*, 2010). So far, there is nothing in the literature to show an effect of 7,8-DHF on CD44 expression but exercise has been shown to reduce its expression in aged mice (Kohut *et al.*, 2004). In reducing CD44 activation, the inflammatory response can be modulated and chronic inflammation would be less destructive. Our results showed no significant difference between Veh_LPS and DHF_LPS, but again there was a trend towards lower hippocampal expression of CD44 in DHF treated animals. However, we cannot currently comment on the ability of 7,8-DHF to protect against the effects of LPS.

3.4.3.8 Analysis of dentate gyrus volume and cell survival

The process of labelling cells with BrdU was initiated on the same day that 7,8-DHF was first added to drinking water, and continued for seven days after, with a daily i.p. injection of BrdU. This meant that the system was flooded with this substitute for the thymidine base of DNA, and cells undergoing proliferation in the presence of this analogue could incorporate BrdU in place of thymidine. Three to four weeks later, when tissue was collected, we were able to analyse coronal sections of the brain to detect the presence of BrdU in cells of the dentate gyrus, where proliferation was occurring at the time of injection. A single dose of LPS was not expected to have an effect on this process so long after it had taken place, so we compare only Vehicle and 7,8-DHF groups in this instance. We found a significant difference in volume of the dentate gyrus in 7,8-DHF treated animals when compared to Vehicle controls, but we also saw an insignificant tendency toward lower numbers of BrdU positive cells in the dentate gyrus of 7,8-DHF treated subjects. With further analysis and an increase in n number, we may see if treatment with 7,8-DHF really does

lower numbers of new cells, or if what we are actually seeing here is an increase in volume of the dentate gyrus skewing the average numbers of cells per mm³. There are reports of an increase in neurogenesis caused by 7,8-DHF (Hsiao *et al.*, 2014, Liu *et al.*, 2010, Zhao *et al.*, 2015), and it would be interesting to see if our results contradict this, or if 7,8-DHF does mimic BDNF.

3.4.3.9 Summary

In Experiment III, we successfully replicated the effects of one month of exercise on behaviour in the OD task, via the administration of the BDNF mimetic; 7,8-DHF. Our efforts to definitively account for the 7,8-DHF-induced cognitive enhancement we observed in this task have been unsuccessful to date, but further analysis and an increase in n number may explain this protective effect on cognition. There is evidence in the literature reporting various physiological modifications – among them inhibition of hippocampal synapse loss, restoration of synapse number, and synaptic plasticity (Zhang *et al.*, 2014), as well as increased BDNF expression, and upregulated activation of Akt and CREB (Wu *et al.*, 2014). Despite these reports, we are thus far unsuccessful in elucidating a key factor in this process. It is interesting that this lack of a finding in Experiment III agrees with every test we have carried out in Experiment II, and shows that there is some element we are yet to discover. This could lie with the analyses of downstream signalling factors activated by BDNF protein expression, as discussed. Thus, we will start with the detection of ERK, Akt, and CREB expression and phosphorylation, due to their importance in mediating the effects of BDNF on synaptic plasticity, LTP, neurogenesis, and cognitive enhancement. The potential that this factor, or a combination of factors, may be common to both, would further cement the evidence that supports 7,8-DHF as a pharmacomimetic of exercise.

Chapter 4

An investigation into the effect of long-term
7,8-DHF administration on cognition and
related neuronal physiology in a model of aging.

4.1. Introduction

Various human and rodent studies have outlined the detrimental effects of aging on the brain as a whole. The process is characterised by a decline in cognitive function, primarily linked to a reduction in neurogenesis, reduced blood-flow to the brain, reduced stimulation of neurotrophic activity, increased oxidative damage, and an overall decrease in hippocampal volume (Small *et al.*, 2002, Navarro *et al.*, 2004, Van Praag *et al.*, 2005, Kim *et al.*, 2010). However, these studies also reported on the effect of exercise at varying intensities and in multiple age-groups.

Previous study in the laboratory has shown that eight months of treadmill exercise can enhance cognitive function in the rat when compared to sedentary controls of the same age (O'Callaghan *et al.*, 2009). In human studies, long-term exercise protocols were shown to improve psychological well-being in elderly subjects (Blumenthal *et al.*, 1991), and enhanced attention, concentration, short-term memory, and overall higher cognitive function (Stevenson and Topp, 1990).

As has been discussed, many studies have shown that exercise upregulates concentration of BDNF mRNA (Oliff *et al.*, 1998), BDNF protein (Griesbach *et al.*, 2004), and both BDNF mRNA and protein in the rat hippocampus (Huang *et al.*, 2006), and in human serum (Griffin *et al.*, 2011). Long-term exercise was also shown to counteract age-related decline in NGF and BDNF mRNA expression in the rat hippocampus (O'Callaghan *et al.*, 2009). A long-term comparison of daily or intermittent running exercise also showed an increase in rat hippocampal BDNF protein concentration (Berchtold *et al.*, 2005), and also observed an acute increase in BDNF with re-exposure to a single bout of exercise even after a long delay between sessions of exercise – a change that was not seen in control animals that had not exercised before this time.

Targeting BDNF and its activity at the TrkB receptor in enhancing cognition has been well documented (Pardridge *et al.*, 1994, Pardridge, 2003). However, delivery of BDNF to the brain is difficult, and requires vectors for transport of recombinant BDNF across the BBB. It is possible that there would be more success with a TrkB agonist, to mimic the effects of BDNF, and thus mimic the effect of exercise. A number of alternatives have been identified, among them and a flavonoid; 7,8-DHF (Jang *et al.*, 2010). This compound is a phytochemical – a plant antioxidant found abundantly in various fruit and vegetables, as well as black tea, red wine, and dark chocolate (Spencer, 2009). Flavonoids are consumed ubiquitously in a healthy diet, they are absorbed well in the intestine, and have a high bioavailability, but the level of intake from food sources is deemed too low to have a therapeutic effect (Cermak and Wolfram, 2006). 7,8-DHF has been reported to successfully cross the blood-brain barrier and exert its effects at the TrkB receptor, and thus induces intracellular

signals that go on to enhance neuronal proliferation, reduce neuronal death, and improve general function of existing neurons. (Jang *et al.*, 2010).

7,8-DHF has been shown to counteract age-related spatial memory decline in aged rats (Zeng *et al.*, 2012b) when administered to 24 month old animals for one month, but there is not yet anything in the literature investigating the influence of long-term administration of this agonist on cognitive function, and more importantly how it will take effect during the aging process from adolescence, all the way through the old age. This study will perhaps provide more translational evidence for the success of long-term TrkB stimulation and how it affects cognition, and whether or not a pharmacomimetic can sustain its activation throughout the life-span.

This study attempts to replicate the prevention of age-related decline observed in studies of long-term exercise, with long-term administration of the TrkB agonist, 7,8-DHF instead.

Tracking cognitive performance throughout the study using episodic and spatial memory tasks every six weeks allows us to compare the effect of this compound at various time-points during the course of aging.

Measurement of inflammatory markers and signalling proteins allows us to compare the cellular components of aging between aged vehicle and compound-treated animals, and again in comparison to healthy younger subjects.

BrdU administration one month before sacrifice allows us to determine whether or not 7,8-DHF administration coincides with an increase in numbers of cells in the dentate gyrus when compared to controls.

4.2 Methods

4.2.1 Animals

A total of 40 male C57BL/6 Jax mice were obtained from the Comparative Medicine unit, TCD, from a line bred from Charles River Laboratories (UK) and maintained in the Comparative Medicine unit, Trinity Biomedical Sciences Institute, TCD. Animals were 12wks old, with a body mass ranging from 20 – 25g, at the beginning of the experimental protocol.

4.2.2 Animal housing

Animals were group housed, with between three and five animals per cage. Tecniplast 1285LN individually ventilated cages (W x D x H - 396 x 215 x 172 mm) were used to house subjects, and they were maintained in a facility offering a constant temperature of 22°C ($\pm 1^\circ\text{C}$), humidity of 55% ($\pm 5\%$), and on a 12hr light/dark cycle (lights on a.m., lights off p.m.). Cages and cage bedding were changed every two weeks and there was no environmental enrichment provided. Food and water were available *ad libitum*. While drug was being administered in drinking water, water-bottle levels were monitored and animals were weighed weekly to ensure that subject hydration was unaffected.

Mice were allowed at least one week of acclimatization to extended surroundings upon arrival into the research unit before experimental proceedings began.

4.2.3 Experimental design

Animals aged 3 months old were randomly assigned to either vehicle or 7,8-DHF groups (n=20). In their home cages, they received either vehicle or 7,8-DHF in drinking water for a period of 9 months, after which time they were 12 months old.

Animals were subject to cognitive testing every six weeks throughout the experimental timeline, starting at 3 months of age, just before 7,8-DHF administration began. This provided baseline comparisons of cognitive ability, and involved both NOR and OD tasks.

Young healthy controls were used to provide information on baseline levels of cellular proliferation and survival, and protein and mRNA expression. These animals were 12 – 14wks old. They received seven days of i.p. BrdU administration starting one month before sacrifice.

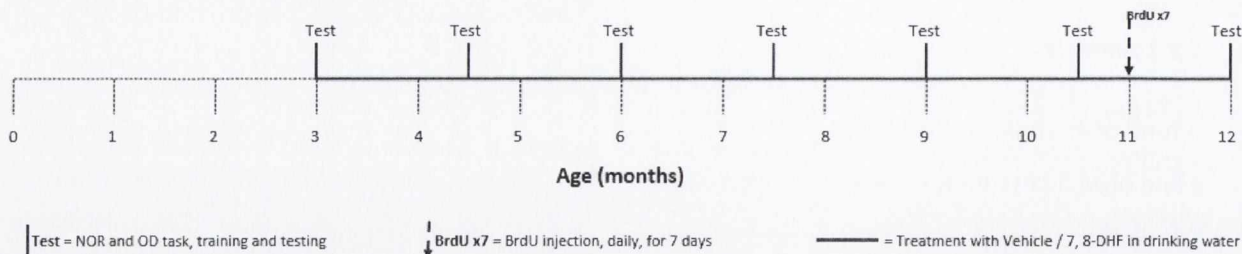


Fig. 4.2.1 - Timeline of experimental protocol

4.2.4 7,8-DHF administration

The small molecule TrkB receptor agonist 7,8-DHF (100mg) was dissolved in DMSO (100µl). This stock solution was added to dH₂O (100ml) containing sucrose (1g) (0.1% DMSO, 1% sucrose) and mixed until the solution was uniform. This brought the final concentration to approximately 23mg/kg of 7,8-DHF, per animal, per day. Calculations for this concentration were revised as animals aged and gained weight, so that dosage remained unchanged. Control animals received a vehicle solution (0.1% DMSO, 1% sucrose, dH₂O). Animals were randomly assigned to Veh and 7, 8-DHF groups, but allocation was on a cage by cage basis. Water bottles were changed every two to three days, before 7,8-DHF started to come out of solution. Levels of water consumption were monitored to ensure there was no aversion to taste, which could result in dehydration in the animals. Subjects were also weighed individually on a weekly basis to ensure that there was no significant fluctuation in body mass of any one specific animal as a result of dehydration, but which may have been masked by water consumption of the cage as a whole.

4.2.5 BrdU administration

Starting four weeks prior to sacrifice, subjects received a daily intraperitoneal injection of BrdU (50mg/kg BrdU made up in .89% w/v Saline solution) for seven consecutive days. Each day, approximately 200µl of solution per animal was administered via i.p injection. Injections were carried out by licensed individuals, in accordance with procedural guidelines.

4.2.6a Behavioural assessment – Novel Object Recognition

The NOR task is based on the idea that animals will naturally explore a novel object. The hippocampus becomes involved in this form of memory when a time delay of ten minutes – 24hrs is imposed between training and testing sessions. To perform testing, a cylindrical open field 50cm in diameter was positioned in a dimly-lit room. For two days prior to the task all animals were habituated to the open field – the first day in groups on a cage-by-cage basis for ten minutes, the second day individually for five minutes.

On day three of exposure to the open field each mouse was placed by the experimenter in the centre of the open field and allowed to explore alone for five minutes. On this occasion, two identical objects (constructed from standard Lego blocks) were placed into the open field and a spatial cue was placed in a fixed position on the wall of the open field. Time spent exploring each object was recorded. All equipment was cleaned between subjects to remove any olfactory cues. 24hrs following training, one object was replaced with a new, different object (of similar size and height). The familiar object, along with the spatial cue, remained in the same position. Upon completion of the five minute task, animals were returned to their home cage.

4.2.6b Behavioural assessment - Object Displacement

The purpose of the OD task is to assess spatial learning and memory in all animals, using a task designed to test this hippocampal-dependent memory. To perform testing, a cylindrical open field, 50cm in diameter, was positioned in a dimly-lit room. For two days prior to the task all animals were habituated to the open field – the first day in groups on a cage-by-cage basis for ten minutes, the second day individually for five minutes.

On day three of exposure to the open field each mouse was placed by the experimenter in the centre of the open field and allowed to explore alone for five minutes. On this occasion, three objects of similar size and colour (constructed from standard Lego blocks) were placed into the open field and a spatial cue was placed in a fixed position on the wall of the open field. Time spent exploring each object was recorded. All equipment was cleaned between subjects to remove any olfactory cues. 24hrs following training, one object was moved to the previously empty quadrant of the circle while the other two objects, as well as the spatial cue, remained in the same position. Upon completion of the five minute task, animals were removed from the arena and returned to their home cage.

A wide selection of objects were assembled at the beginning of all testing, and records were made of which objects were used when, so as to avoid object recycling and prevent animals from developing a familiarity with objects if they were accidentally chosen for use again.

4.2.7 Tissue collection and preparation

24hrs after the final session of behavioural testing, animals were sacrificed via either cervical dislocation or transcardial perfusion, as described in section 2.9. The former was used for collection of fresh hippocampal and cortical tissue, and the latter for *in situ* whole-brain fixation for immunohistochemical analysis. All samples were stored at -80°C until required for further analysis.

Fresh tissue samples were homogenised by hand using a manual homogeniser in 500µl of freshly prepared lysis buffer (for western blot), or Krebs buffer (for ELISA). Once suspended in buffer these samples were kept in standard freezing conditions of between -14 and -18°C. Concentration of total protein in fresh tissue homogenates was measured using the BCA assay, as described in section 2.10.1.

Additional fresh tissue samples were mechanically homogenized in lysis buffer. RNA was extracted, cDNA was synthesized from the purified mRNA, and cDNA was amplified and detected during PCR.

Whole-brain fixation was carried out via transcardial perfusion, and these brains were stored in a cryoprotective sucrose solution at 4°C until required for further analysis.

4.2.8 Analysis of mRNA expression by Polymerase Chain Reaction

Expression of various neurotrophic factors, receptors and endogenous markers of proliferation; such as BDNF, TrkB, and Ki67, and inflammatory cytokines, such as IL1β and TNFα, in both the hippocampus and cortex were assessed using PCR, as described in section 2.11. β-Actin was used as an endogenous control in calculation of Rq values.

4.2.9 Quantification of BrdU⁺ cells in dentate gyrus

Fixed, whole brains were sliced, and stained for BrdU⁺ cells using fluorescent doubling labelling with NeuN, as described in section 2.13.

4.2.10 Statistical analysis

All data was analysed and grouped using Microsoft Excel (2013), and then imported into Graphpad Prism (version 5.01) where column statistics were used to examine the data for outliers, and these were omitted in the case of values lying two standard deviations above or below the mean value. All data were represented as mean $\pm$ SEM.

Analysis was carried out using unpaired student t-test - when one factor is compared in two groups, one-way ANOVA - when one factor is compared three or more groups, and two-way ANOVA - when two or more factors are compared in two or more groups, as well as performing Bonferroni post hoc tests. Statistical significance was determined at $p < 0.05$ based upon these results.

4.3 Results

4.3.1 Trends in NOR task behaviour during 9 months of 7,8-DHF treatment

The ratio of exploration of the novel object : exploration of the familiar object is shown (Fig 4.3.2) and broken down over 9 months of treatment with 7,8-DHF. At 7.5 months ($p < 0.001$) of treatment, this ratio is significantly higher in 7,8-DHF treated subjects when compared to vehicle treated controls at the same time-point.

Baseline (*Vehicle* = 2.612839 ± 0.4681257 , $n = 20$; *7,8-DHF* = 1.646867 ± 0.1965832 , $n = 20$)

1.5 months (*Vehicle* = 1.422998 ± 0.1969507 , $n = 20$; *7,8-DHF* = 1.718741 ± 0.3070247 , $n = 20$)

3 months (*Vehicle* = 1.644186 ± 0.4069702 , $n = 18$; *7,8-DHF* = 2.314838 ± 0.4813989 , $n = 19$)

4.5 months (*Vehicle* = 1.128277 ± 0.180347 , $n = 17$; *7,8-DHF* = 1.58692 ± 0.2467149 , $n = 19$)

6 months (*Vehicle* = 0.9405016 ± 0.03715914 , $n = 16$; *7,8-DHF* = 2.03492 ± 0.1674576 , $n = 18$)

7.5 months (*Vehicle* = 1.033504 ± 0.04137287 , $n = 16$; *7,8-DHF* = 2.436717 ± 0.3105304 , $n = 19$)

9 months (*Vehicle* = 0.9902216 ± 0.04580453 , $n = 14$; *7,8-DHF* = 1.956863 ± 0.219873 , $n = 19$)

Assessed by two-way ANOVA and Bonferroni post-hoc test. Expressed as mean $\pm$ SEM.

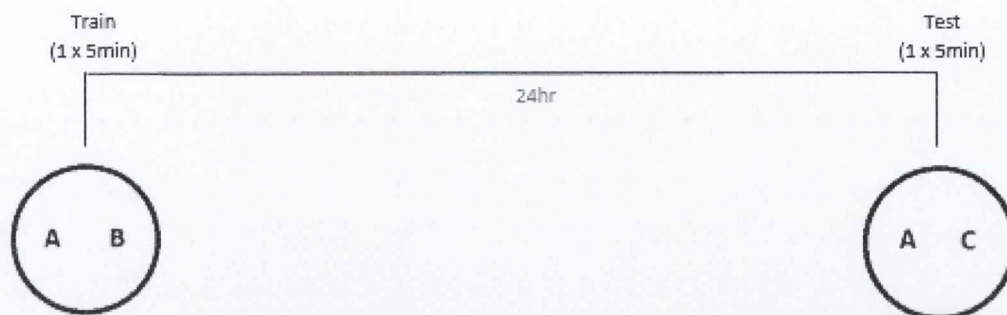


Fig. 4.3.1 – Timeline of Novel Object Recognition testing

A schematic of the timeline utilized every six weeks to test subjects in the NOR task. This involved one 5min training trial, and a 5min testing trial 24 hrs later.

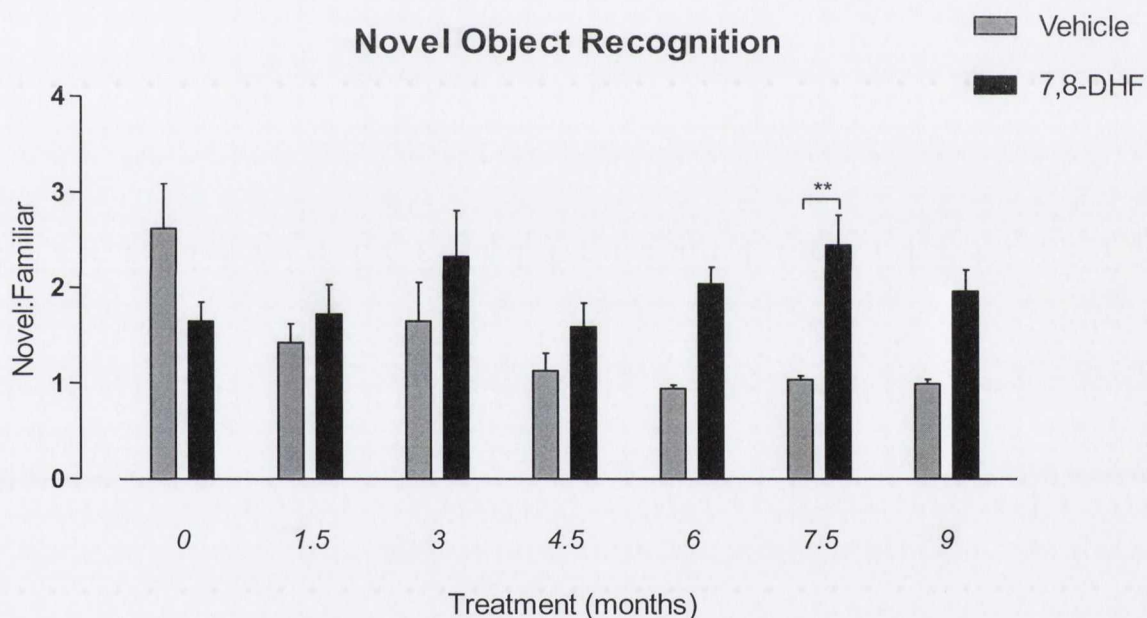


Fig. 4.3.2 - Analysis of Novel Object Recognition task over time

There was a significant difference in the ratio of novel object exploration to familiar object exploration when 7,8-DHF subjects were compared to vehicle subjects 7.5 months of 7,8-DHF administration.

Assessed by two-way ANOVA and Bonferroni post-hoc analysis. ** $p < 0.01$.

All data expressed as mean $\pm$ SEM. (n = 14 - 20).

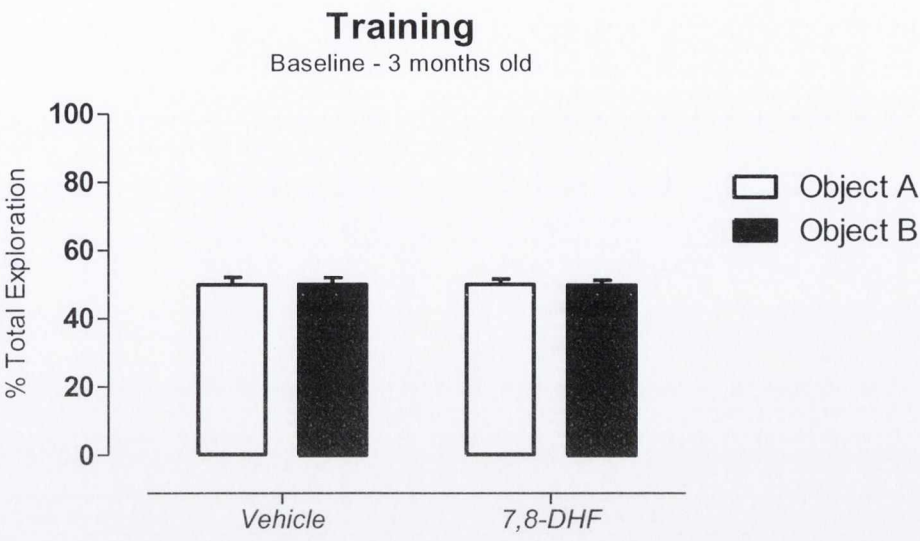
4.3.2a Baseline abilities of Vehicle and 7,8-DHF treated animals in the NOR task.

During the acquisition stage of the NOR task (Fig 4.3.3a), there was no significant difference in object exploration in either group (*Vehicle*, Object A % = 50.08218 ± 2.077107 , Object B % = 49.91782 ± 2.077107 ; *7,8-DHF*, Object A % = 50.15416 ± 1.541361 , Object B % = 49.84584 ± 1.541361). ($p > 0.05$; $n = 20$).

During the memory recall phase of the NOR task (Fig 4.3.3b), object B was replaced with a previously unseen object; C. There was a significant difference in object exploration in both treatment groups (*Vehicle*, Object A % = 28.8548 ± 3.597659 , Object C % = 71.1452 ± 3.597659 ; *7,8-DHF*, Object A % = 35.95509 ± 3.628829 , Object C % = 64.04491 ± 3.628829). ($p < 0.001$; $n = 20$).

Assessed by two-way ANOVA and Bonferroni post-hoc test. Expressed as mean $\pm$ SEM.

a)



b)

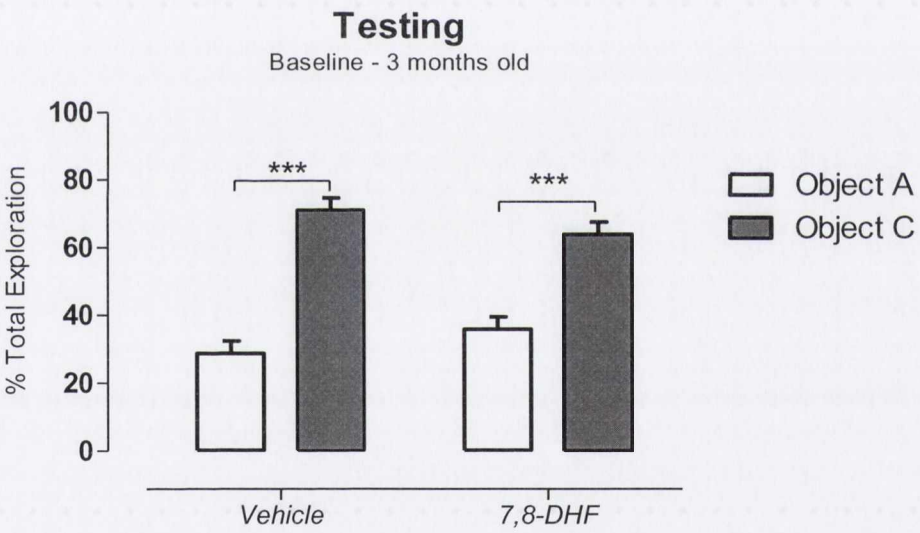


Fig. 4.3.3 – Baseline performance in NOR task prior to 7,8-DHF administration.

(a) shows exploration during a single 5min training trial. There was no significant difference in object exploration.

(b) shows exploration during a single 5min test. All animals displayed a significant preference for the novel object; C.

Assessed by two-way ANOVA and Bonferroni post-hoc analysis. *** $p < 0.001$.

All data expressed as mean $\pm$ SEM. (n = 20).

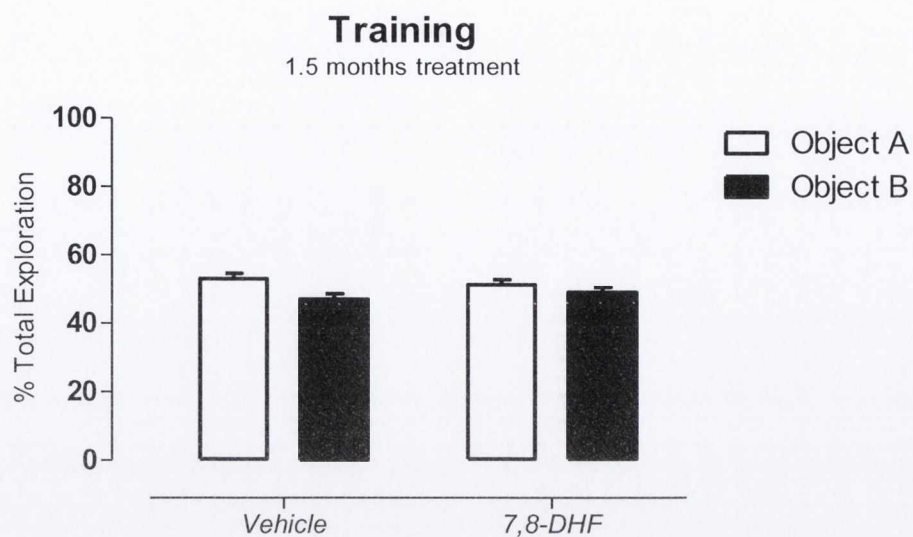
4.3.2b The effect of 1.5 months of 7,8-DHF administration on behaviour in NOR task.

During the acquisition stage of the NOR task (Fig 4.3.4a), there was no significant difference in object exploration in either group (*Vehicle*, Object A % = 53.00383 ± 1.538136 , Object B % = 46.99617 ± 1.538136 ; *7,8-DHF*, Object A % = 51.23605 ± 1.4599 , Object B % = 48.7639 ± 1.4599). ($p > 0.05$; $n = 20$).

During the memory recall phase of the NOR task (Fig 4.3.4b), object B was replaced with a previously unseen object; C. There was a significant difference in object exploration in both treatment groups (*Vehicle*, Object A % = 44.6300 ± 2.410615 , Object C % = 55.36995 ± 2.410615 ; *7,8-DHF*, Object A % = 42.8627 ± 3.327064 , Object C % = 57.1373 ± 3.327064). ($p < 0.05$; $n = 20$).

Assessed by two-way ANOVA and Bonferroni post-hoc test. Expressed as mean $\pm$ SEM.

a)



b)

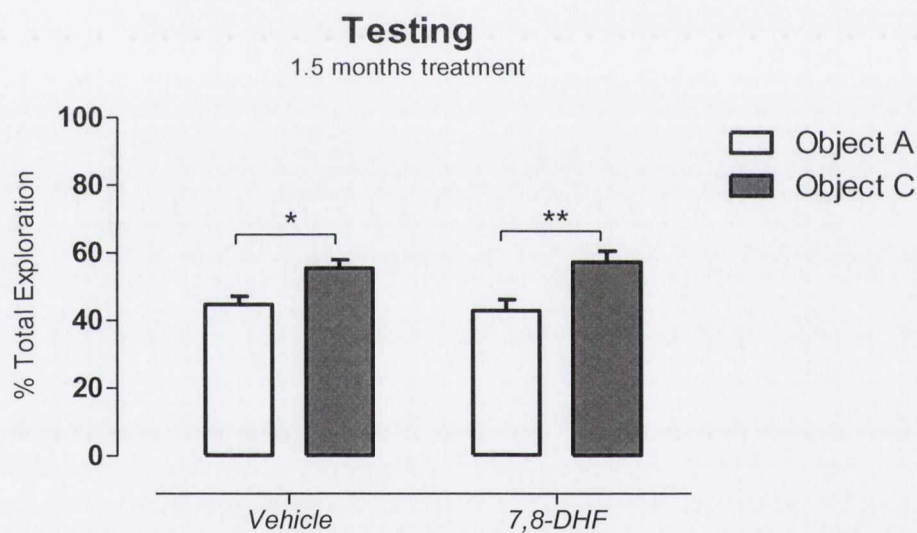


Fig. 4.3.4 – Performance in NOR task after 1.5 months 7,8-DHF administration.

(a) shows exploration during a single 5min training trial. There was no significant difference in object exploration.

(b) shows exploration during a single 5min test. All animals displayed a significant preference for the novel object; C.

Assessed by two-way ANOVA and Bonferroni post-hoc analysis. * $p < 0.05$, ** $p < 0.01$.

All data expressed as mean $\pm$ SEM. (n = 20).

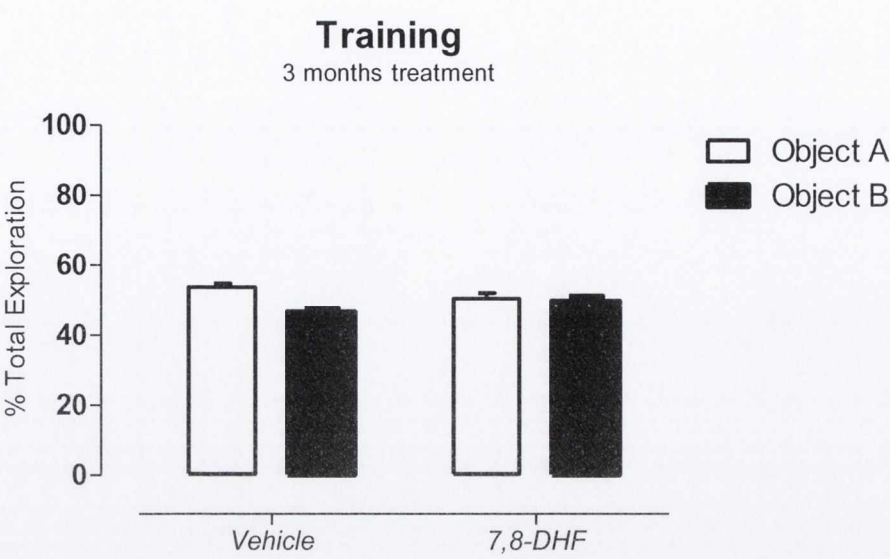
4.3.2c The effect of 3 months of 7,8-DHF administration on behaviour in NOR task.

During the acquisition stage of the NOR task (Fig 4.3.5a), there was no significant difference in object exploration in either group (*Vehicle*, Object A % = 53.69652 ± 1.033503 , Object B % = 46.30348 ± 1.033503 ; *7,8-DHF*, Object A % = 50.45828 ± 1.544976 , Object B % = 49.54172 ± 1.544976). ($p > 0.05$; Veh, $n = 18$; 7,8-DHF, $n = 19$).

During the memory recall phase of the NOR task (Fig 4.3.5b), object B was replaced with a previously unseen object; C. There was a significant difference in object exploration in both treatment groups (*Vehicle*, Object A % = 44.14364 ± 2.741919 , Object C % = 55.85636 ± 2.741919 ; *7,8-DHF*, Object A % = 37.01533 ± 3.177875 , Object C % = 62.98467 ± 3.177875). ($p < 0.05$; Veh, $n = 18$; 7,8-DHF, $n = 19$).

Assessed by two-way ANOVA and Bonferroni post-hoc test. Expressed as mean $\pm$ SEM.

a)



b)

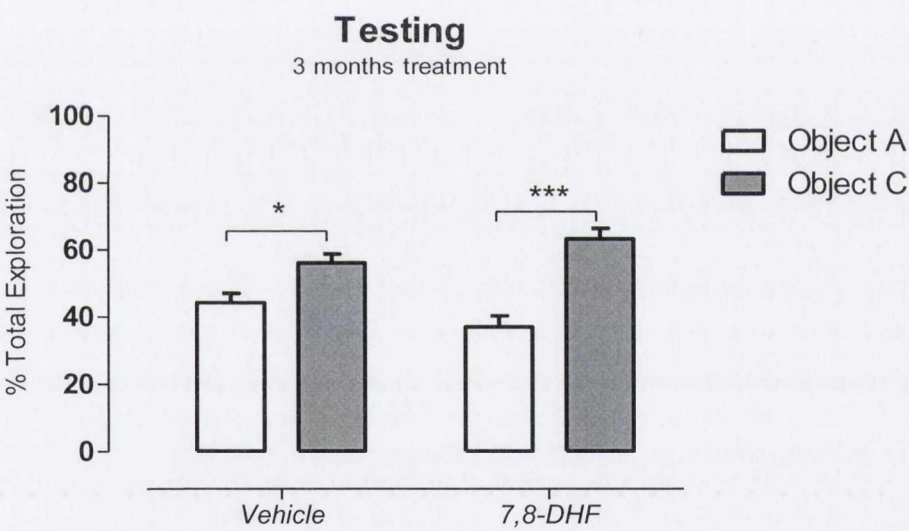


Fig. 4.3.5 – Performance in NOR task after 3 months 7,8-DHF administration.

(a) shows exploration during a single 5min training trial. There was no significant difference in object exploration.

(b) shows exploration during a single 5min test. All animals displayed a significant preference for the novel object; C.

Assessed by two-way ANOVA and Bonferroni post-hoc analysis. * $p < 0.05$, *** $p < 0.001$.

All data expressed as mean $\pm$ SEM. (Veh, $n = 18$; 7,8-DHF, $n = 19$).

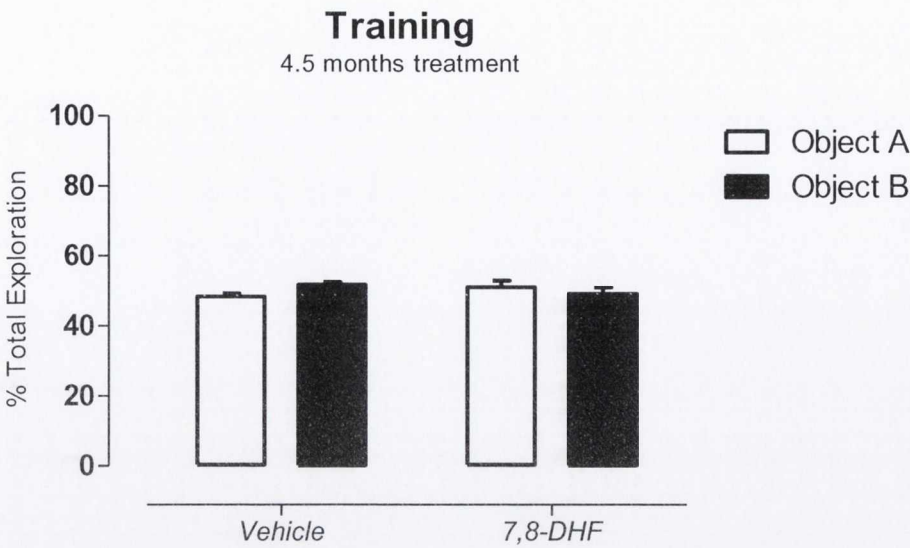
4.3.2d The effect of 4.5 months of 7,8-DHF administration on behaviour in NOR task.

During the acquisition stage of the NOR task (Fig 4.3.6a), there was no significant difference in object exploration in either group (*Vehicle*, Object A % = 48.42129 ± 0.9184198 , Object B % = 51.57871 ± 0.9184198 ; *7,8-DHF*, Object A % = 51.07698 ± 1.858384 , Object B % = 48.92302 ± 1.858384). ($p > 0.05$; Veh, $n = 17$; 7,8-DHF, $n = 19$).

During the memory recall phase of the NOR task (Fig 4.3.6b), object B was replaced with a previously unseen object; C. There was a significant difference in object exploration in 7,8-DHF treated animals, but not in Vehicle subjects (*Vehicle*, Object A % = 50.01833 ± 2.386959 , Object C % = 49.98167 ± 2.386959 ; *7,8-DHF*, Object A % = 42.78839 ± 2.67128 , Object C % = 57.21161 ± 2.67128). ($p < 0.05$; Veh, $n = 17$; 7,8-DHF, $n = 19$).

Assessed by two-way ANOVA and Bonferroni post-hoc test. Expressed as mean $\pm$ SEM.

a)



b)

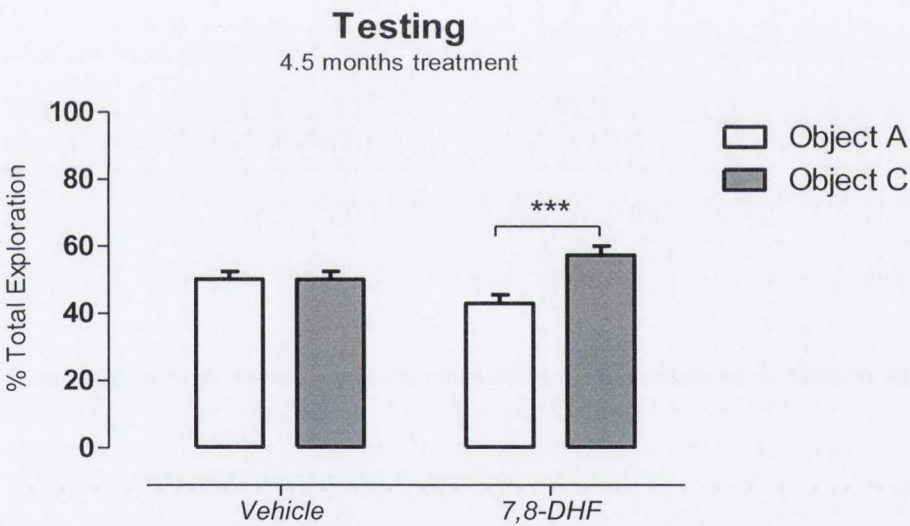


Fig. 4.3.6 – Performance in NOR task after 4.5 months 7,8-DHF administration.
(a) shows exploration during a single 5min training trial. There was no significant difference in object exploration.
(b) shows exploration during a single 5min test. 7,8-DHF treated animals displayed a significant preference for the novel object; C.
Assessed by two-way ANOVA and Bonferroni post-hoc analysis. ***p<0.001.
All data expressed as mean ± SEM. (Veh, n = 17; 7,8-DHF, n = 19).

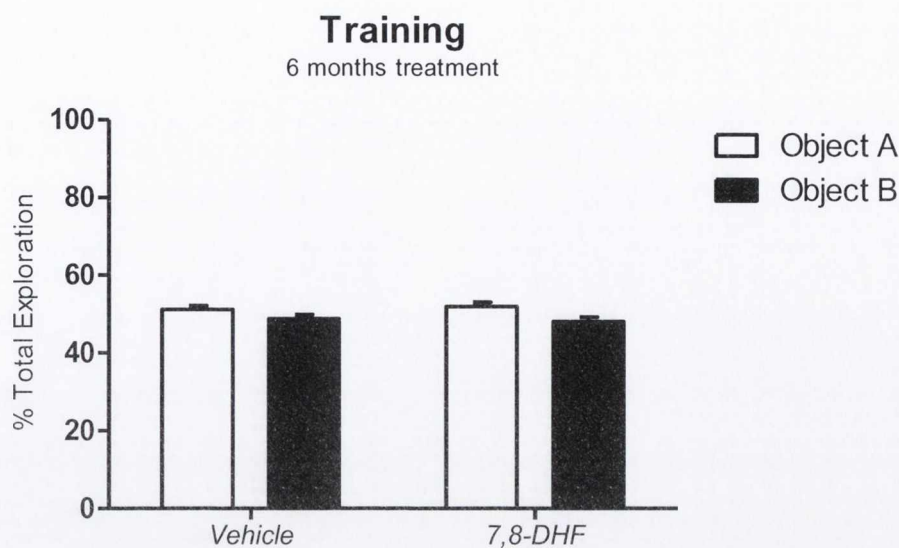
4.3.2e The effect of 6 months of 7,8-DHF administration on behaviour in NOR task.

During the acquisition stage of the NOR task (Fig 4.3.7a), there was no significant difference in object exploration in either group (*Vehicle*, Object A % = 51.18832 ± 1.057923 , Object B % = 48.81168 ± 1.057923 ; *7,8-DHF*, Object A % = 51.94111 ± 1.135859 , Object B % = 48.05889 ± 1.135859). ($p > 0.05$; Veh, $n = 16$; 7,8-DHF, $n = 18$).

During the memory recall phase of the NOR task (Fig 4.3.7b), object B was replaced with a previously unseen object; C. There was a significant difference in object exploration in 7,8-DHF treated animals, but not in Vehicle subjects (*Vehicle*, Object A % = 51.82668 ± 1.024874 , Object C % = 48.17332 ± 1.024874 ; *7,8-DHF*, Object A % = 34.59702 ± 1.810533 , Object C % = 65.40298 ± 1.810533). ($p < 0.05$; Veh, $n = 16$; 7,8-DHF, $n = 18$).

Assessed by two-way ANOVA and Bonferroni post-hoc test. Expressed as mean $\pm$ SEM.

a)



b)

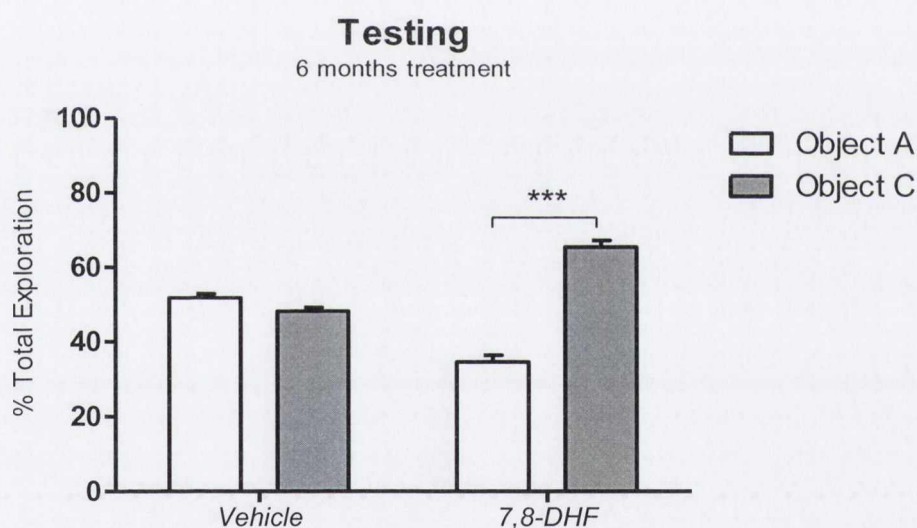


Fig. 4.3.7 – Performance in NOR task after 6 months 7,8-DHF administration.

(a) shows exploration during a single 5min training trial. There was no significant difference in object exploration.

(b) shows exploration during a single 5min test. 7,8-DHF treated animals displayed a significant preference for the novel object; C.

Assessed by two-way ANOVA and Bonferroni post-hoc analysis. ***p<0.001.

All data expressed as mean $\pm$ SEM. (Veh, n =16; 7,8-DHF, n = 18).

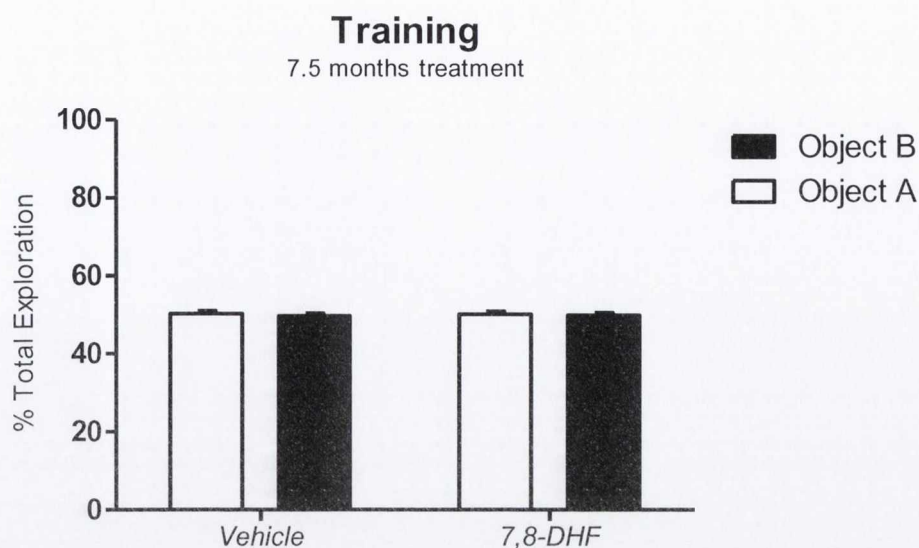
4.3.2f The effect of 7.5 months of 7,8-DHF administration on behaviour in NOR task.

During the acquisition stage of the NOR task (Fig 4.3.8a), there was no significant difference in object exploration in either group (*Vehicle*, Object A % = 50.31519 ± 0.6891351 , Object B % = 49.68481 ± 0.6891351 ; *7,8-DHF*, Object A % = 50.1656 ± 0.7279119 , Object B % = 49.8344 ± 0.7279119). ($p > 0.05$; Veh, $n = 16$; 7,8-DHF, $n = 19$).

During the memory recall phase of the NOR task (Fig 4.3.8b), object B was replaced with a previously unseen object; C. There was a significant difference in object exploration in 7,8-DHF treated animals, but not in Vehicle subjects (*Vehicle*, Object A % = 49.49455 ± 1.052621 , Object C % = 50.50545 ± 1.052621 ; *7,8-DHF*, Object A % = 32.26958 ± 2.073825 , Object C % = 67.73042 ± 2.073825). ($p < 0.05$; Veh, $n = 16$; 7,8-DHF, $n = 19$).

Assessed by two-way ANOVA and Bonferroni post-hoc test. Expressed as mean $\pm$ SEM.

a)



b)

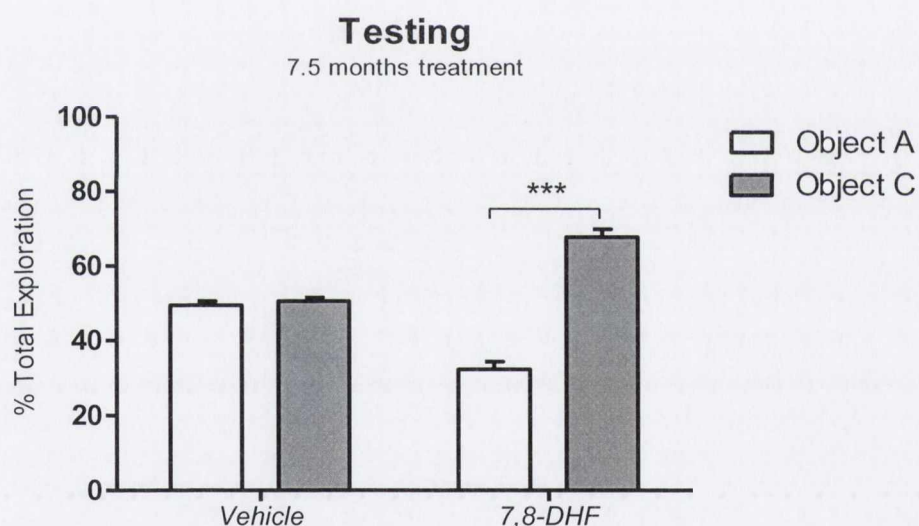


Fig. 4.3.8 – Performance in NOR task after 7.5 months 7,8-DHF administration.

(a) shows exploration during a single 5min training trial. There was no significant difference in object exploration.

(b) shows exploration during a single 5min test. 7,8-DHF treated animals displayed a significant preference for the novel object; C.

Assessed by two-way ANOVA and Bonferroni post-hoc analysis. *** $p < 0.001$.

All data expressed as mean $\pm$ SEM. (Veh, $n = 16$; 7,8-DHF, $n = 19$).

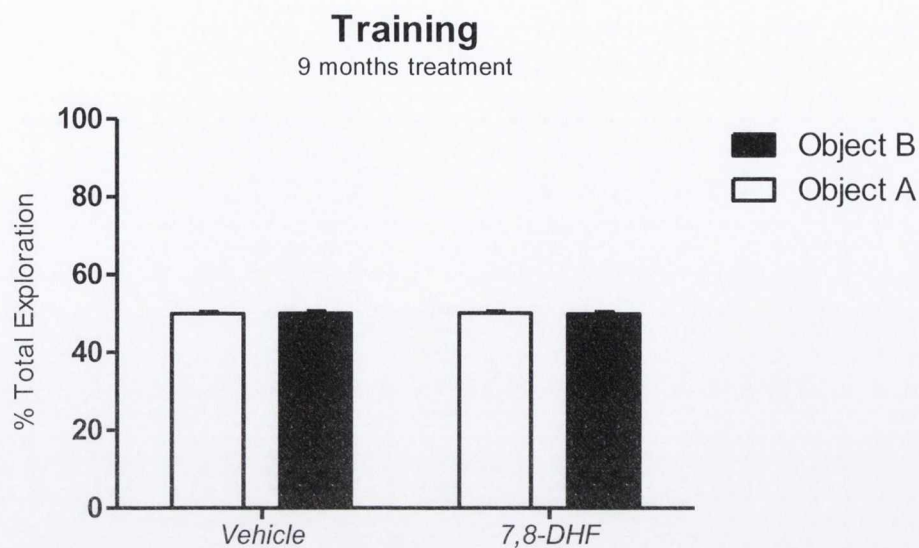
4.3.2g The effect of 9 months of 7,8-DHF administration on behaviour in NOR task.

During the acquisition stage of the NOR task (Fig 4.3.9a), there was no significant difference in object exploration in either group (*Vehicle*, Object A % = 49.94482 ± 0.5466567 , Object B % = 50.05518 ± 0.5466567 ; *7,8-DHF*, Object A % = 50.11554 ± 0.539325 , Object B % = 49.88446 ± 0.539325). ($p > 0.05$; Veh, $n = 14$; 7,8-DHF, $n = 19$).

During the memory recall phase of the NOR task (Fig 4.3.9b), object B was replaced with a previously unseen object; C. There was a significant difference in object exploration in 7,8-DHF treated animals, but not in Vehicle subjects (*Vehicle*, Object A % = 48.89228 ± 0.6774089 , Object C % = 51.10772 ± 0.6774089 ; *7,8-DHF*, Object A % = 43.64895 ± 1.792276 , Object C % = 56.35105 ± 1.792276). ($p < 0.05$; Veh, $n = 14$; 7,8-DHF, $n = 19$).

Assessed by two-way ANOVA and Bonferroni post-hoc test. Expressed as mean $\pm$ SEM.

a)



b)

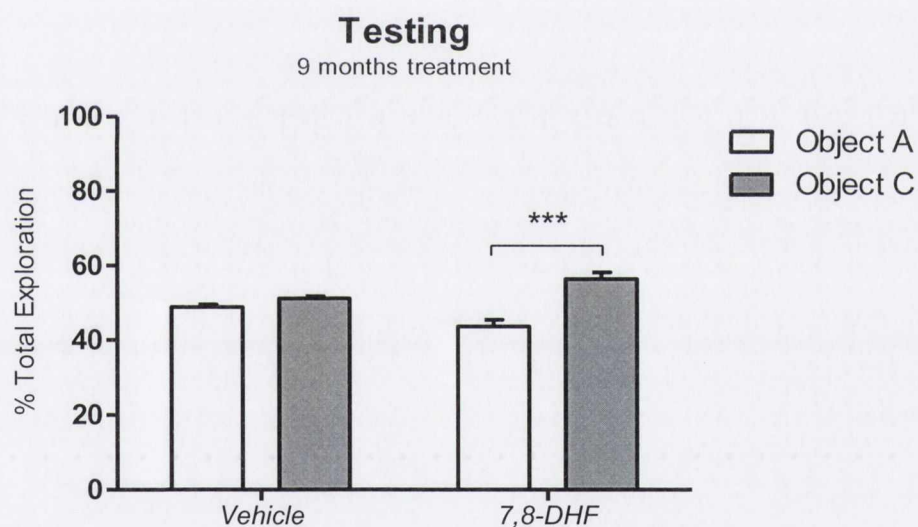


Fig. 4.3.9 – Performance in NOR task after 9 months 7,8-DHF administration.

(a) shows exploration during a single 5min training trial. There was no significant difference in object exploration.

(b) shows exploration during a single 5min test. 7,8-DHF treated animals displayed a significant preference for the novel object; C.

Assessed by two-way ANOVA and Bonferroni post-hoc analysis. ***p<0.001.

All data expressed as mean $\pm$ SEM. (Veh, n = 14; 7,8-DHF, n = 19).

4.3.3 Trends in OD task behaviour during 9 months of 7,8-DHF treatment

The ratio of exploration of displaced object : average exploration of the stationary objects is shown (Fig 4.3.11) and broken down over 9 months of treatment with 7,8-DHF. At 6 months ($p < 0.05$), 7.5 months ($p < 0.001$), and 9 months ($p < 0.001$) of treatment, this ratio is significantly higher in 7,8-DHF treated subjects when compared to vehicle treated controls.

Baseline (*Vehicle* = 2.022379 ± 0.2832689 , $n = 20$; *7,8-DHF* = 1.54167 ± 0.1693521 , $n = 20$)

1.5 months (*Vehicle* = 1.457476 ± 0.1353721 , $n = 20$; *7,8-DHF* = 1.522987 ± 0.2019058 , $n = 20$)

3 months (*Vehicle* = 1.559763 ± 0.1806088 , $n = 18$; *7,8-DHF* = 2.132339 ± 0.3269499 , $n = 19$)

4.5 months (*Vehicle* = 1.014865 ± 0.05819153 , $n = 17$; *7,8-DHF* = 1.663556 ± 0.119333 , $n = 19$)

6 months (*Vehicle* = 1.216073 ± 0.1432787 , $n = 16$; *7,8-DHF* = 2.119209 ± 0.2316632 , $n = 18$)

7.5 months (*Vehicle* = 1.020974 ± 0.03009925 , $n = 16$; *7,8-DHF* = 2.252474 ± 0.1762185 , $n = 19$)

9 months (*Vehicle* = 1.115387 ± 0.05198109 , $n = 14$; *7,8-DHF* = 2.393762 ± 0.223158 , $n = 19$)

Assessed by two-way ANOVA and Bonferroni post-hoc test. Expressed as mean $\pm$ SEM.

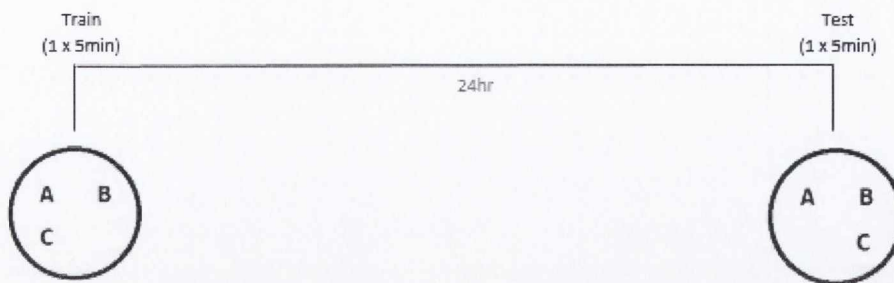


Fig. 4.3.10 – Timeline of Object Displacement testing

A schematic of the timeline utilized every 6 weeks to test subjects in the OD task. This involved one 5min training trial, and a 5min testing trial 24hrs later.

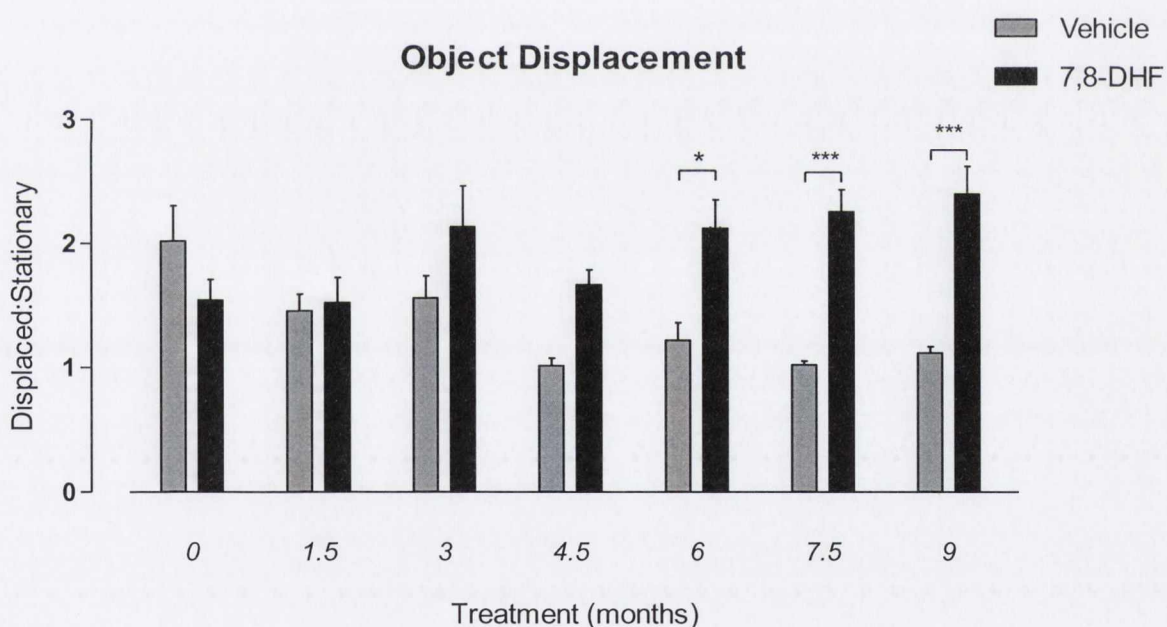


Fig. 4.3.11 - Analysis of Object Displacement task over time

There was a significant difference in the ratio of displaced object exploration to stationary object exploration when 7,8-DHF subjects were compared to vehicle subjects after 6 months, 7.5 months, and 9 months of 7,8-DHF administration.

Assessed by two-way ANOVA and Bonferroni post-hoc analysis. *p < 0.05, ***p < 0.001.

All data expressed as mean $\pm$ SEM. (n = 14 - 20).

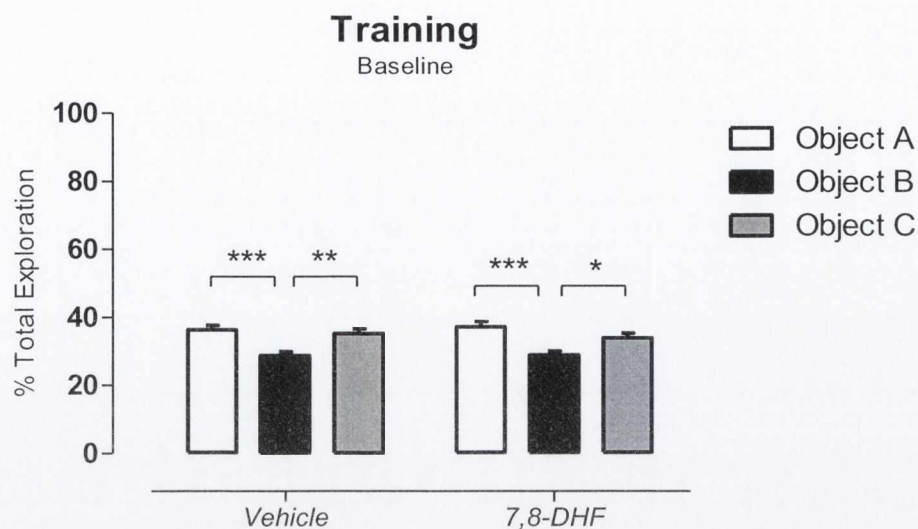
4.3.4a Baseline abilities of Vehicle and 7,8-DHF treated animals in the OD task.

During the acquisition stage of the OD task (Fig 4.3.12a), there was a significant difference in the exploration of object B compared to objects A and C, in both groups (*Vehicle*, Object A % = 36.3583 ± 1.267735, Object B % = 28.63744 ± 1.139882, Object C % = 35.00426 ± 1.421037; *7,8-DHF*, Object A % = 37.18472 ± 1.592169, Object B % = 28.89618 ± 1.1271, Object C % = 33.9191 ± 1.376307) ($p < 0.05$; $n = 20$).

During the memory recall phase of the OD task (Fig 4.3.12b), object C was moved to the previously empty quadrant of the arena. There was a significant difference in exploration of the displaced object relative to the stationary objects in both treatment groups (*Vehicle*, Object A % = 33.48835 ± 2.988195, Object B % = 20.50568 ± 2.225314, Object C % = 46.00597 ± 3.394371; *7,8-DHF*, Object A % = 34.97573 ± 2.823379, Object B % = 24.17405 ± 1.684209, Object C % = 40.85023 ± 3.049008). ($p < 0.01$; $n = 20$).

Assessed by two-way ANOVA and Bonferroni post-hoc test. Expressed as mean ± SEM.

a)



b)

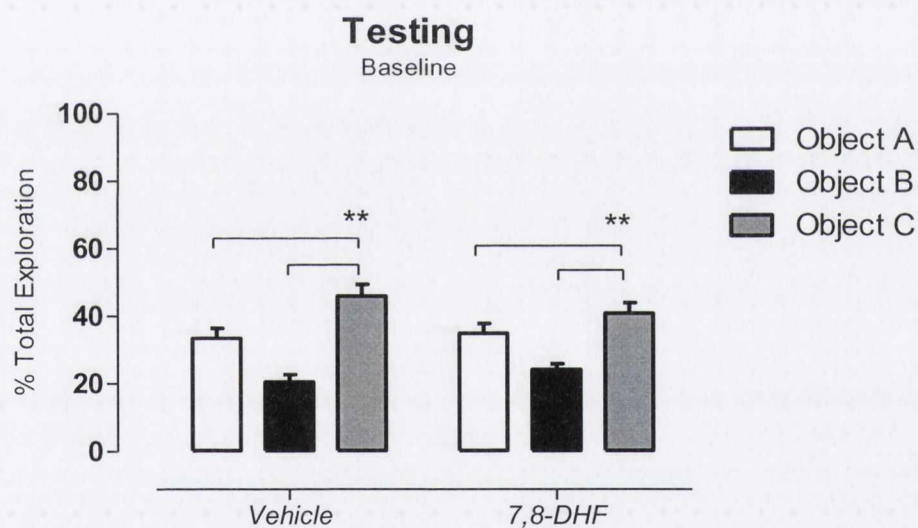


Fig. 4.3.12 – Baseline performance in OD task prior to 7,8-DHF administration.

(a) shows exploration during a single 5min training trial. There was no significant difference in object exploration.

(b) shows exploration during a single 5min test. All animals displayed a significant preference for the displaced object; C.

Assessed by two-way ANOVA and Bonferroni post-hoc analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All data expressed as mean $\pm$ SEM. (n = 20).

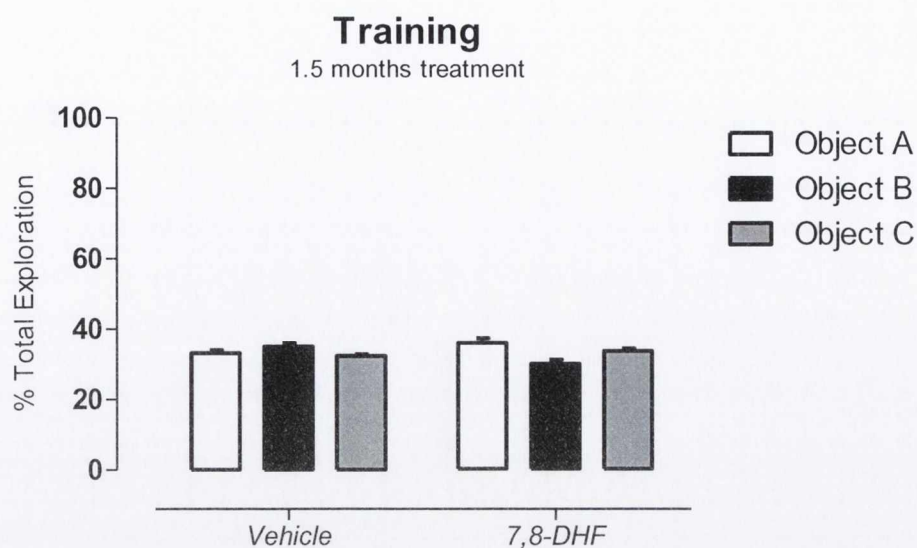
4.3.4b The effect of 1.5 months of 7,8-DHF administration on behaviour in OD task.

During the acquisition stage of the OD task (Fig 4.3.13a), there was no difference in object exploration in either group (*Vehicle*, Object A % = 32.97461 ± 0.8597929 , Object B % = 34.876 ± 1.042439 , Object C % = 32.14939 ± 0.4855938 ; 7,8-DHF, Object A % = 36.07301 ± 1.311764 , Object B % = 30.14225 ± 1.195935 , Object C % = 33.78474 ± 0.6699762) ($p > 0.05$; $n = 20$).

During the memory recall phase of the OD task (Fig 4.3.13b), object C was moved to the previously empty quadrant of the arena. There was a significant difference in exploration of the displaced object relative to the stationary objects in both treatment groups (*Vehicle*, Object A % = 33.56313 ± 1.639085 , Object B % = 25.86339 ± 1.605709 , Object C % = 40.57347 ± 2.148021 ; 7,8-DHF, Object A % = 31.65285 ± 2.622174 , Object B % = 28.33028 ± 2.762651 , Object C % = 40.01687 ± 3.117556). ($p < 0.01$; $n = 20$).

Assessed by two-way ANOVA and Bonferroni post-hoc test. Expressed as mean $\pm$ SEM.

a)



b)

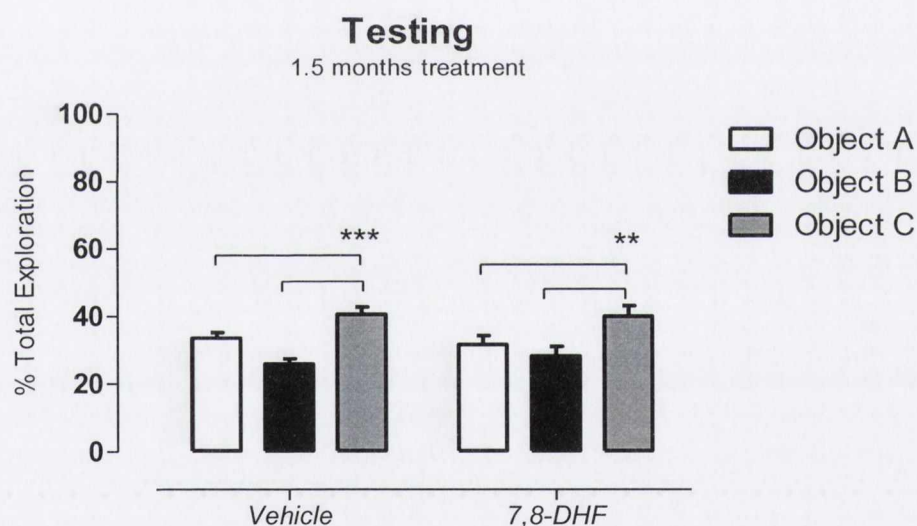


Fig. 4.3.13 – Performance in OD task after 1.5 months 7,8-DHF administration.

(a) shows exploration during a single 5min training trial. There was no significant difference in object exploration.

(b) shows exploration during a single 5min test. All animals displayed a significant preference for the displaced object; C.

Assessed by two-way ANOVA and Bonferroni post-hoc analysis. ** $p < 0.01$, *** $p < 0.001$.

All data expressed as mean $\pm$ SEM. (n = 20).

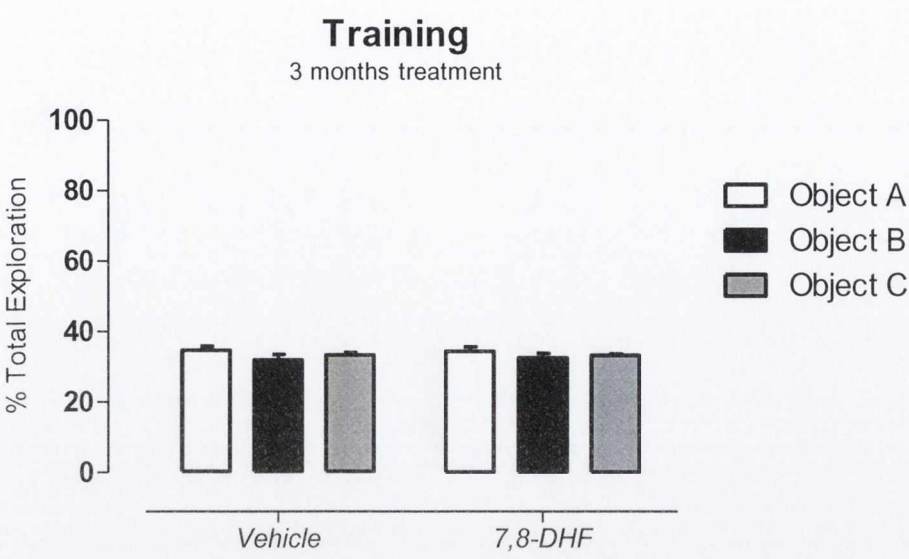
4.3.4c The effect of 3 months of 7,8-DHF administration on behaviour in OD task.

During the acquisition stage of the OD task (Fig 4.3.14a), there was no difference in object exploration in either group (*Vehicle*, Object A % = 34.71976 ± 1.130223 , Object B % = 31.95684 ± 1.526952 , Object C % = 33.3234 ± 0.6669471 ; *7,8-DHF*, Object A % = 34.34535 ± 1.216553 , Object B % = 32.4639 ± 1.263981 , Object C % = 33.19075 ± 0.400235) ($p > 0.05$; Veh, $n = 18$; 7,8-DHF, $n = 19$).

During the memory recall phase of the OD task (Fig 4.3.14b), object C was moved to the previously empty quadrant of the arena. There was a significant difference in exploration of the displaced object relative to the stationary objects in both treatment groups (*Vehicle*, Object A % = 26.51659 ± 2.01655 , Object B % = 31.76969 ± 2.09286 , Object C % = 41.71372 ± 2.476982 ; *7,8-DHF*, Object A % = 28.99665 ± 2.313183 , Object B % = 23.30474 ± 2.44889 , Object C % = 47.69861 ± 2.885788). ($p < 0.01$; Veh, $n = 18$; 7,8-DHF, $n = 19$).

Assessed by two-way ANOVA and Bonferroni post-hoc test. Expressed as mean $\pm$ SEM.

a)



b)

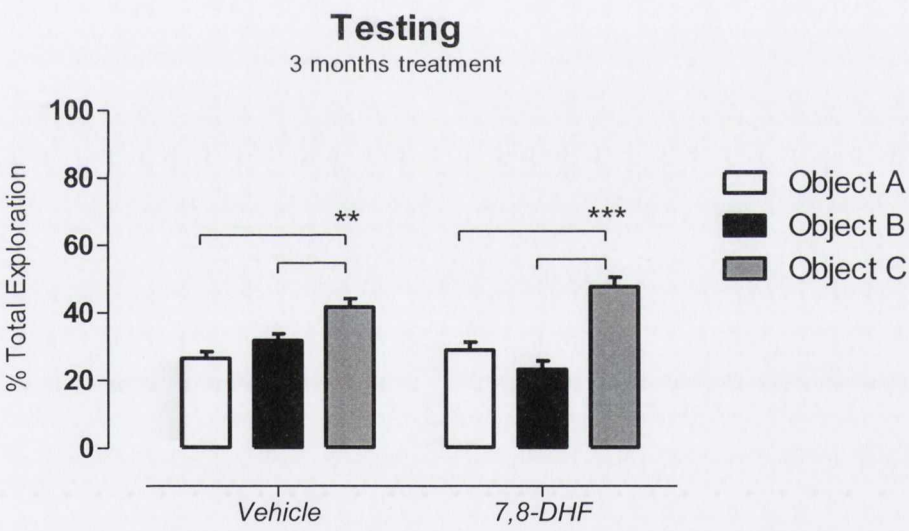


Fig. 4.3.14 – Performance in OD task after 3 months 7,8-DHF administration.

(a) shows exploration during a single 5min training trial. There was no significant difference in object exploration.

(b) shows exploration during a single 5min test. All animals displayed a significant preference for the displaced object; C.

Assessed by two-way ANOVA and Bonferroni post-hoc analysis. **p<0.01, ***p<0.001.

All data expressed as mean ± SEM. (Veh, n = 18; 7,8-DHF, n = 19).

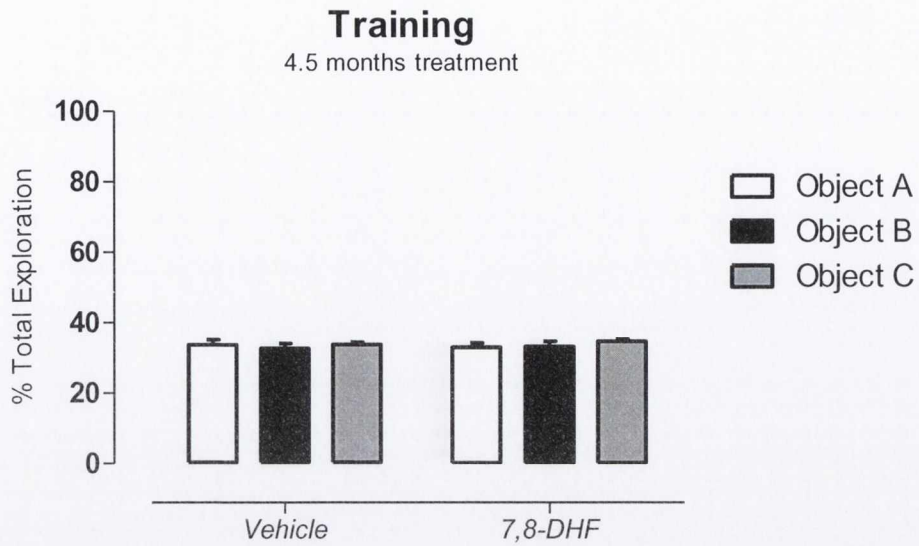
4.3.4d The effect of 4.5 months of 7,8-DHF administration on behaviour in OD task.

During the acquisition stage of the OD task (Fig 4.3.15a), there was no difference in object exploration in either group (*Vehicle*, Object A % = 33.68225 ± 1.467963 , Object B % = 32.60056 ± 1.390152 , Object C % = 33.71719 ± 0.671625 ; 7,8-DHF, Object A % = 32.7015 ± 1.246591 , Object B % = 32.88946 ± 1.478113 , Object C % = 34.40904 ± 0.6557004) ($p > 0.05$; Veh, $n = 17$; 7,8-DHF, $n = 19$).

During the memory recall phase of the OD task (Fig 4.3.15b), object C was moved to the previously empty quadrant of the arena. There was a significant difference in exploration of the displaced object relative to the stationary objects in the 7,8-DHF treatment group, but not in Vehicle treated subjects (*Vehicle*, Object A % = 34.45866 ± 1.314227 , Object B % = 32.27976 ± 1.481588 , Object C % = 33.26158 ± 1.305619 ; 7,8-DHF, Object A % = 27.26967 ± 2.399644 , Object B % = 28.39597 ± 1.4691 , Object C % = 44.33435 ± 1.87703). ($p < 0.001$; Veh, $n = 17$; 7,8-DHF, $n = 19$).

Assessed by two-way ANOVA and Bonferroni post-hoc test. Expressed as mean $\pm$ SEM.

a)



b)

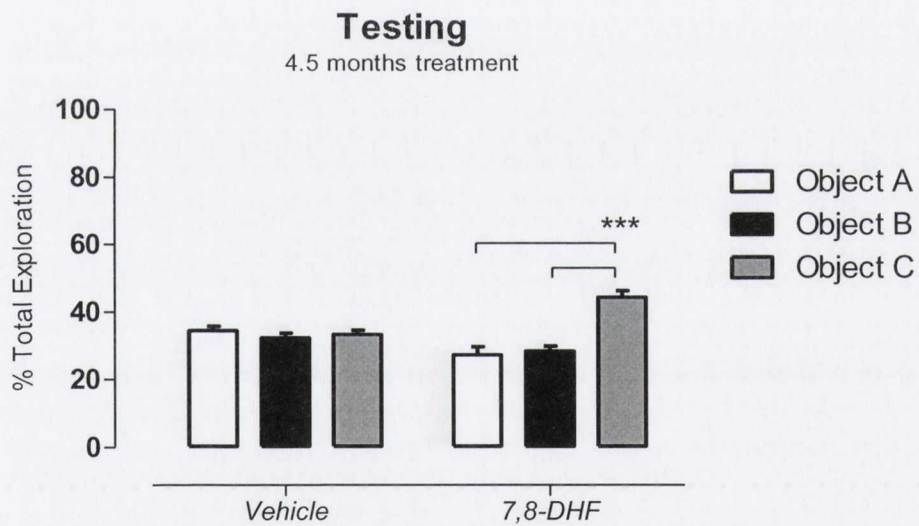


Fig. 4.3.15 – Performance in OD task after 4.5 months 7,8-DHF administration.

(a) shows exploration during a single 5min training trial. There was no significant difference in object exploration.

(b) shows exploration during a single 5min test. 7,8-DHF treated animals displayed a significant preference for the displaced object; C.

Assessed by two-way ANOVA and Bonferroni post-hoc analysis. *** $p < 0.001$.

All data expressed as mean $\pm$ SEM. (Veh, $n = 17$; 7,8-DHF, $n = 19$).

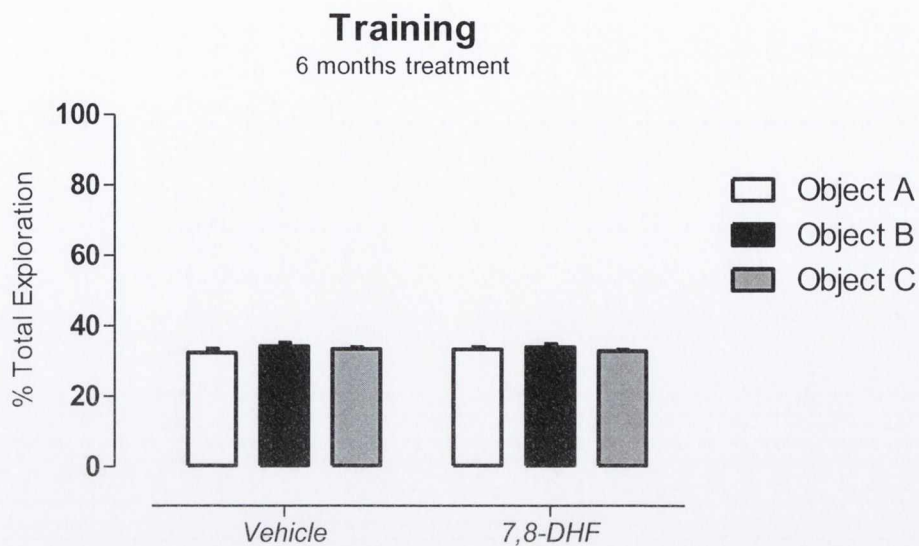
4.3.4e The effect of 6 months of 7,8-DHF administration on behaviour in OD task.

During the acquisition stage of the OD task (Fig 4.3.16a), there was no difference in object exploration in either group (*Vehicle*, Object A % = 32.37756 ± 1.134783 , Object B % = 34.2013 ± 1.030348 , Object C % = 33.42115 ± 0.5157093 ; 7,8-DHF, Object A % = 33.24212 ± 0.7087175 , Object B % = 33.9525 ± 0.9365887 , Object C % = 32.80538 ± 0.4108717) ($p > 0.05$; Veh, $n = 16$; 7,8-DHF, $n = 18$).

During the memory recall phase of the OD task (Fig 4.3.16b), object C was moved to the previously empty quadrant of the arena. There was a significant difference in exploration of the displaced object relative to the stationary objects in the 7,8-DHF treatment group, but not in Vehicle treated subjects (*Vehicle*, Object A % = 31.06354 ± 2.532636 , Object B % = 32.43783 ± 2.349079 , Object C % = 36.49863 ± 2.011759 ; 7,8-DHF, Object A % = 24.96792 ± 1.683192 , Object B % = 25.97749 ± 1.502415 , Object C % = 49.05459 ± 2.597987). ($p < 0.001$; Veh, $n = 16$; 7,8-DHF, $n = 18$).

Assessed by two-way ANOVA and Bonferroni post-hoc test. Expressed as mean $\pm$ SEM.

a)



b)

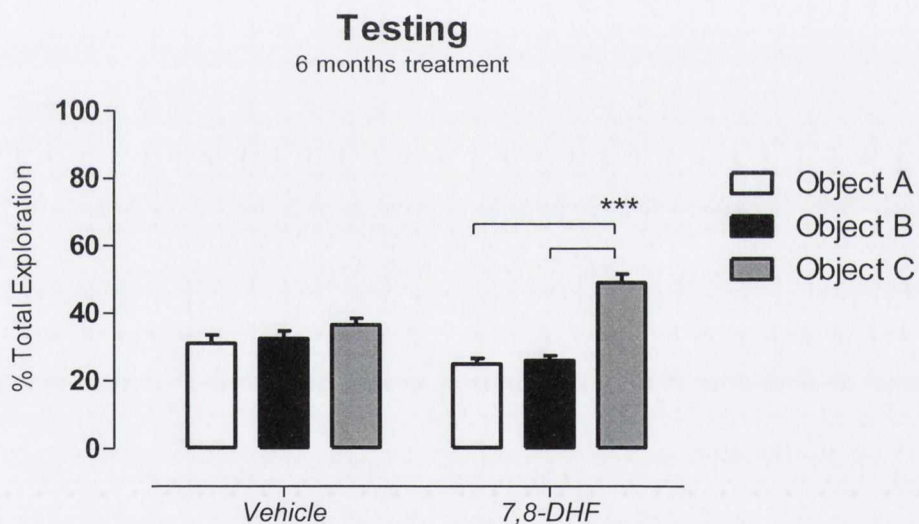


Fig. 4.3.16 – Performance in OD task after 6 months 7,8-DHF administration.

(a) shows exploration during a single 5min training trial. There was no significant difference in object exploration.

(b) shows exploration during a single 5min test. 7,8-DHF treated animals displayed a significant preference for the displaced object; C.

Assessed by two-way ANOVA and Bonferroni post-hoc analysis. ***p<0.001.

All data expressed as mean $\pm$ SEM. (Veh, n = 16; 7,8-DHF, n = 18).

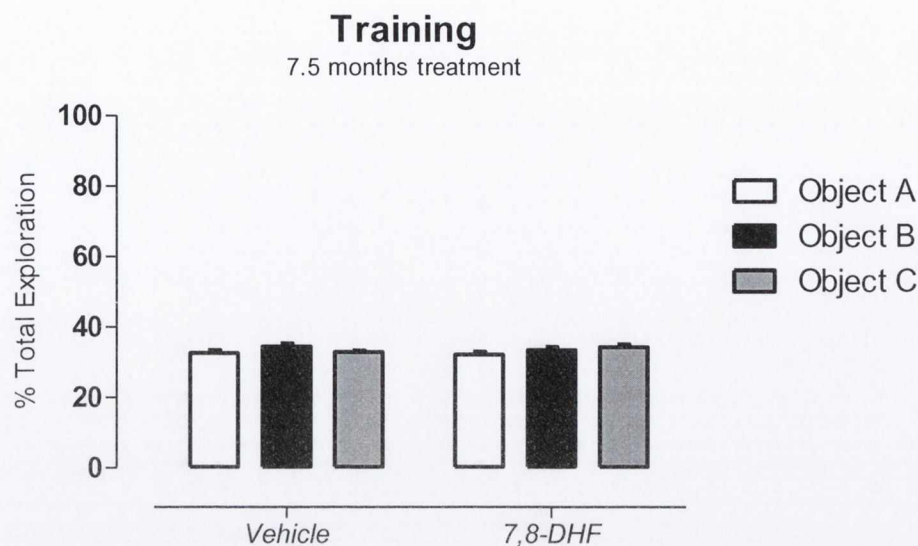
4.3.4f The effect of 7.5 months of 7,8-DHF administration on behaviour in OD task.

During the acquisition stage of the OD task (Fig 4.3.17a), there was no difference in object exploration in either group (*Vehicle*, Object A % = 32.6595 ± 0.7978504 , Object B % = 34.46989 ± 0.3732497 , Object C % = 34.46989 ± 0.4735607 ; 7,8-DHF, Object A % = 32.1787 ± 0.8244245 , Object B % = 33.48996 ± 0.9162058 , Object C % = 34.33134 ± 0.796431) ($p > 0.05$; Veh, $n = 16$; 7,8-DHF, $n = 19$).

During the memory recall phase of the OD task (Fig 4.3.17b), object C was moved to the previously empty quadrant of the arena. There was a significant difference in exploration of the displaced object relative to the stationary objects in the 7,8-DHF treatment group, but not in Vehicle treated subjects (*Vehicle*, Object A % = 33.18266 ± 0.9820835 , Object B % = 33.12431 ± 1.138072 , Object C % = 33.69303 ± 0.6912384 ; 7,8-DHF, Object A % = 24.58894 ± 0.991803 , Object B % = 23.81773 ± 1.105907 , Object C % = 51.59332 ± 1.870746). ($p < 0.001$; Veh, $n = 16$; 7,8-DHF, $n = 19$).

Assessed by two-way ANOVA and Bonferroni post-hoc test. Expressed as mean $\pm$ SEM.

a)



b)

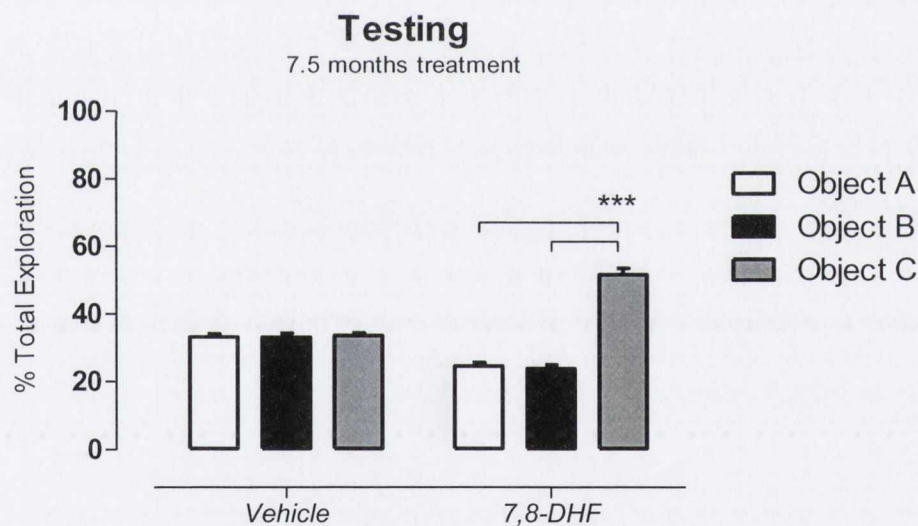


Fig. 4.3.17 – Performance in OD task after 7.5 months 7,8-DHF administration.
(a) shows exploration during a single 5min training trial. There was no significant difference in object exploration.
(b) shows exploration during a single 5min test. 7,8-DHF treated animals displayed a significant preference for the displaced object; C.
Assessed by two-way ANOVA and Bonferroni post-hoc analysis. ***p<0.001.
All data expressed as mean ± SEM. (Veh, n = 16; 7,8-DHF, n = 19).

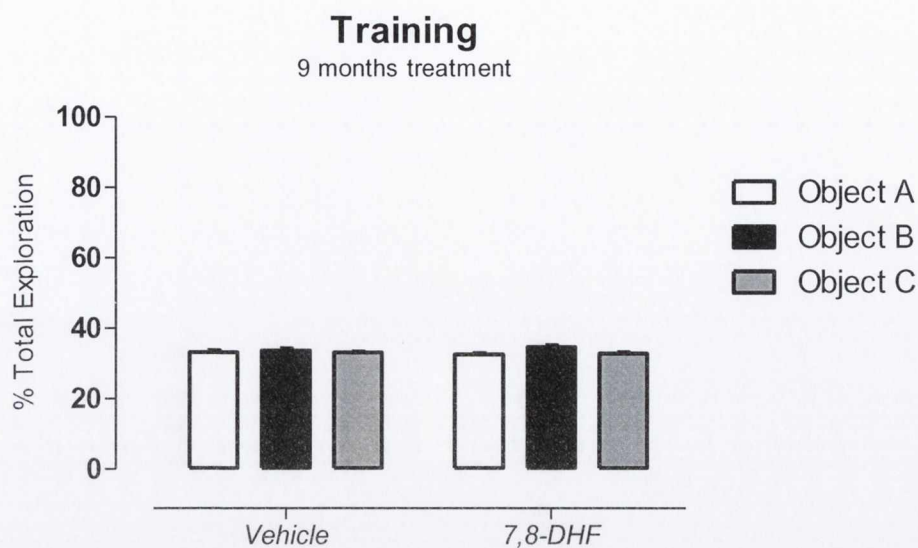
4.3.4g The effect of 9 months of 7,8-DHF administration on behaviour in OD task.

During the acquisition stage of the OD task (Fig 4.3.18a), there was no difference in object exploration in either group (*Vehicle*, Object A % = 33.18726 ± 0.5696031 , Object B % = 33.68671 ± 0.6166632 , Object C % = 33.12603 ± 0.2732497 ; *7,8-DHF*, Object A % = 32.57275 ± 0.3769464 , Object B % = 34.64257 ± 0.6215976 , Object C % = 32.78467 ± 0.4118469) ($p > 0.05$; Veh, n = 14; 7,8-DHF, n = 19).

During the memory recall phase of the OD task (Fig 4.3.18b), object C was moved to the previously empty quadrant of the arena. There was a significant difference in exploration of the displaced object relative to the stationary objects in the 7,8-DHF treatment group, but not in Vehicle treated subjects (*Vehicle*, Object A % = 32.94384 ± 1.159723 , Object B % = 31.48439 ± 1.47853 , Object C % = 35.57178 ± 1.065293 ; *7,8-DHF*, Object A % = 23.20428 ± 1.551481 , Object B % = 24.14889 ± 1.586791 , Object C % = 52.64684 ± 2.077586). ($p < 0.001$; Veh, n = 14; 7,8-DHF, n = 19).

Assessed by two-way ANOVA and Bonferroni post-hoc test. Expressed as mean $\pm$ SEM.

a)



b)

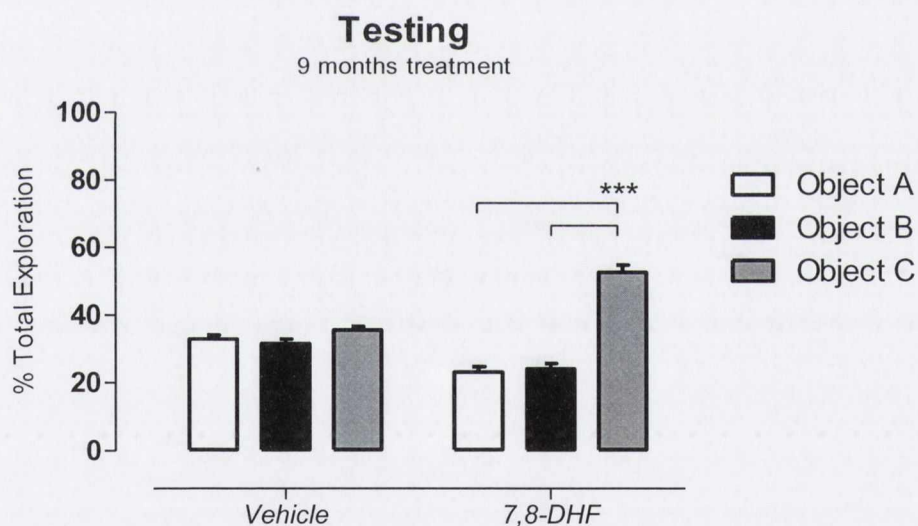


Fig. 4.3.18 – Performance in OD task after 9 months 7,8-DHF administration.

(a) shows exploration during a single 5min training trial. There was no significant difference in object exploration.

(b) shows exploration during a single 5min test. 7,8-DHF treated animals displayed a significant preference for the displaced object; C.

Assessed by two-way ANOVA and Bonferroni post-hoc analysis. ***p<0.001.

All data expressed as mean ± SEM. (Veh, n = 14; 7,8-DHF, n = 19).

4.3.5a The effect of 9 months of 7,8-DHF administration on mRNA expression of BDNF and TrkB in cortex and hippocampus.

PCR analysis showed there was a significant difference in BDNF mRNA expression in the cortex when Rq measurement in 3m/o controls was compared to Vehicle ($p = 0.0001$) and 7,8-DHF treated subjects ($p < 0.0001$), but not when Vehicle was compared to 7,8-DHF ($p = 0.8305$). (*Vehicle* = 0.9221 ± 0.08232 ; *7,8-DHF* = 0.9023 ± 0.04488 ; *3m/o control* = 1.659 ± 0.09). ($p < 0.0001$; Veh, $n = 8$; 7,8-DHF, $n = 9$; 3m/o control, $n = 5$). (Fig 4.3.19a)

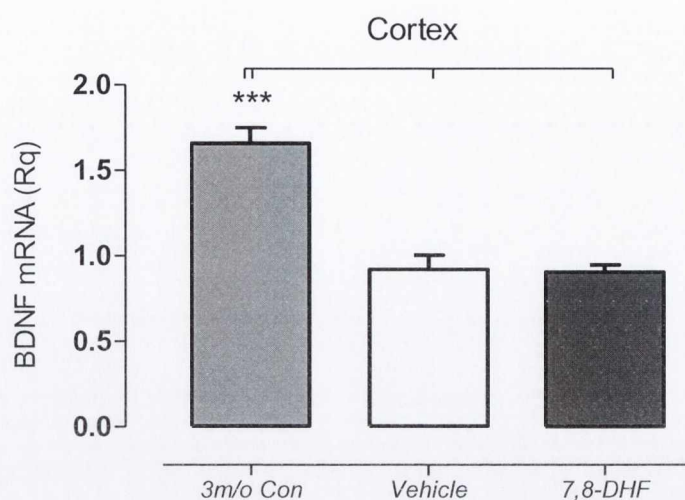
PCR analysis showed there was no significant difference in BDNF mRNA expression in the hippocampus when Rq measurement was compared across all groups (*Vehicle* = 0.9732 ± 0.06025 ; *7,8-DHF* = 1.061 ± 0.07882 ; *3m/o control* = 1.264 ± 0.1521). ($p = 0.1336$; Veh, $n = 8$; 7,8-DHF, $n = 9$; 3m/o control, $n = 5$). (Fig 4.3.19b)

PCR analysis showed there was a significant difference in TrkB mRNA expression in the cortex when Rq measurement in 3m/o controls was compared to Vehicle ($p = 0.0019$) and 7,8-DHF treated subjects ($p = 0.0004$), but not when Vehicle was compared to 7,8-DHF ($p = 0.27$). (*Vehicle* = 1.078 ± 0.04802 ; *7,8-DHF* = 1.160 ± 0.0515 ; *3m/o control* = 0.7281 ± 0.07821). ($p = 0.0002$; Veh, $n = 8$; 7,8-DHF, $n = 9$; 3m/o control, $n = 5$). (Fig 4.3.20a)

PCR analysis showed there was a significant difference in TrkB mRNA expression in the hippocampus when Rq measurement in 3m/o controls was compared to Vehicle ($p < 0.0001$) and 7,8-DHF treated subjects ($p < 0.0001$), but not when Vehicle was compared to 7,8-DHF ($p = 0.0547$). (*Vehicle* = 1.171 ± 0.03351 ; *7,8-DHF* = 1.271 ± 0.03409 ; *3m/o control* = 0.7160 ± 0.04964). ($p < 0.0001$; Veh, $n = 8$; 7,8-DHF, $n = 9$; 3m/o control, $n = 5$). (Fig 4.3.20b)

Data analysed by unpaired t-test and/or one-way ANOVA and Bonferroni post-hoc test, expressed as mean $\pm$ SEM.

a)



b)

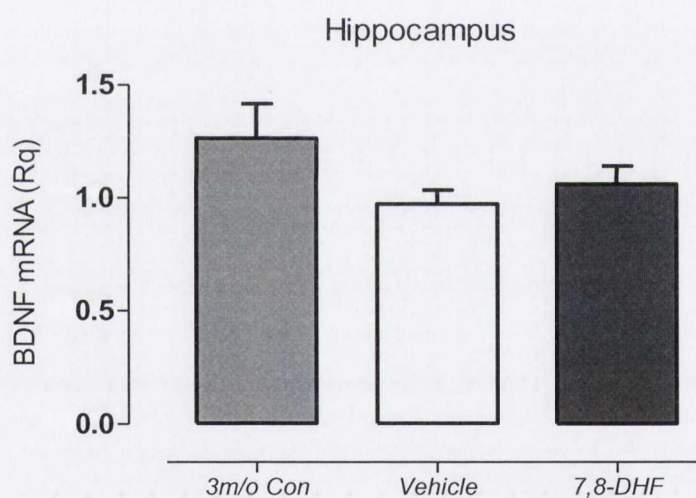


Fig. 4.3.19 – Effect of 9months of 7,8-DHF administration on BDNF mRNA expression

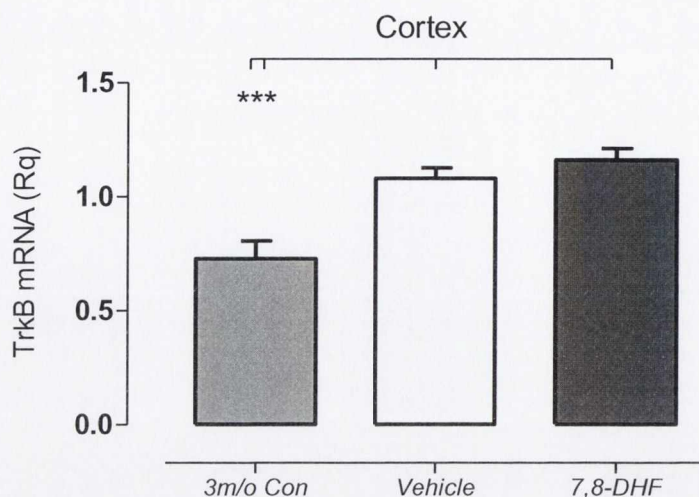
(a) shows Rq of BDNF mRNA expression in cortical tissue. There was a significant difference in BDNF expression in both Vehicle ($p = 0.0001$) and 7,8-DHF ($p < 0.0001$) treated subjects when compared to 3 month old controls.

(b) shows Rq of BDNF mRNA expression in hippocampal tissue. There was no significant difference in BDNF expression between subject groups.

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. *** $p < 0.001$.

Data expressed as mean $\pm$ SEM (Vehicle, $n = 8$; 7,8-DHF, $n = 9$; 3m/o Con, $n = 5$).

a)



b)

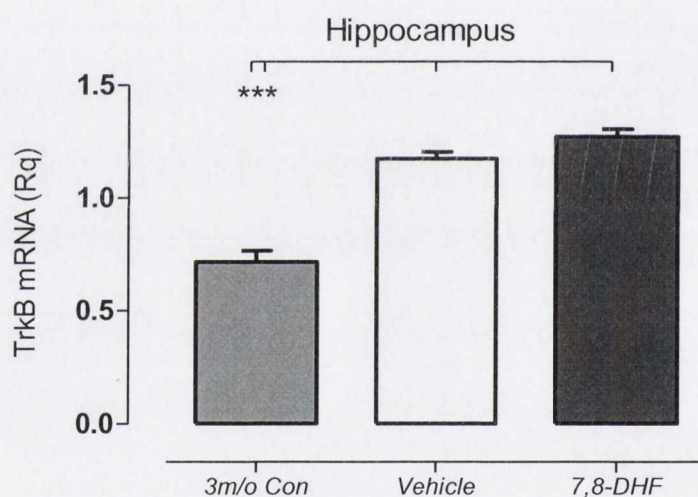


Fig. 4.3.20 – Effect of 9months of 7,8-DHF administration on TrkB mRNA expression

(a) shows Rq of TrkB mRNA expression in cortical tissue. There was a significant difference in TrkB expression in 3 month old controls when compared to both Vehicle ($p = 0.0019$) and 7,8-DHF treated subjects ($p=0.0004$).

(b) shows Rq of TrkB mRNA expression in hippocampal tissue. There was a significant difference in TrkB expression in 3 month old controls when compared to both Vehicle ($p < 0.0001$) and 7,8-DHF ($p < 0.0001$) treated subjects.

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. *** $p < 0.001$.

Data expressed as mean $\pm$ SEM (Vehicle, $n = 8$; 7,8-DHF, $n = 9$; 3m/o Con, $n = 5$).

4.3.5b The effect of 9 months of 7,8-DHF administration on mRNA expression of Ki67 and CREB in cortex and hippocampus.

PCR analysis showed there was a significant difference in Ki67 mRNA expression in the cortex when Rq measurement in 3m/o controls was compared to Vehicle ($p < 0.0001$) and 7,8-DHF treated subjects ($p < 0.0001$), but not when Vehicle was compared to 7,8-DHF ($p = 0.8435$). (Vehicle = 1.043 ± 0.05974 ; 7,8-DHF = 1.060 ± 0.05923 ; 3m/o control = 12.34 ± 1.928). ($p < 0.0001$; Veh, $n = 8$; 7,8-DHF, $n = 9$; 3m/o control, $n = 5$). (Fig 4.3.21a)

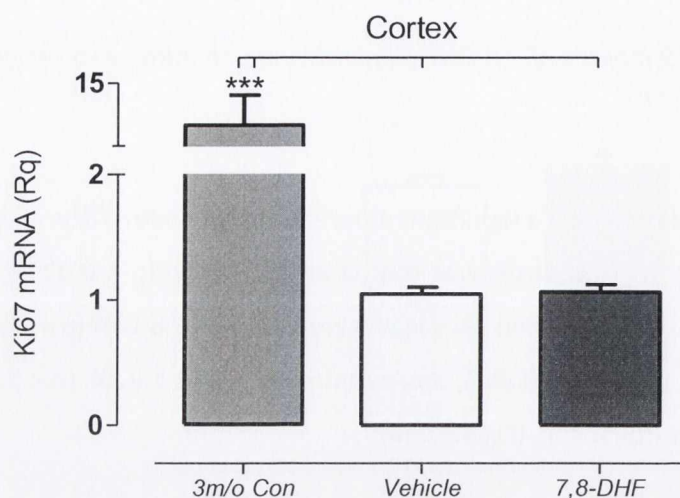
PCR analysis showed there was a significant difference in Ki67 mRNA expression in the hippocampus when Rq measurement in 3m/o controls was compared to Vehicle ($p < 0.0001$) and 7,8-DHF treated subjects ($p < 0.0001$), but not when Vehicle was compared to 7,8-DHF ($p = 0.5253$). (Vehicle = 1.186 ± 0.08585 ; 7,8-DHF = 1.289 ± 0.1281 ; 3m/o control = 17.79 ± 3.311). ($p < 0.0001$; Veh, $n = 8$; 7,8-DHF, $n = 9$; 3m/o control, $n = 5$). (Fig 4.3.21b)

PCR analysis showed there was a significant difference in CREB mRNA expression in the cortex when Rq measurement in 3m/o controls was compared to Vehicle ($p = 0.0381$) and 7,8-DHF treated subjects ($p = 0.0341$), but not when Vehicle was compared to 7,8-DHF ($p = 0.6724$). (Vehicle = 1.399 ± 0.1123 ; 7,8-DHF = 1.459 ± 0.08451 ; 3m/o control = 1.790 ± 0.1069). ($p = 0.0555$; Veh, $n = 8$; 7,8-DHF, $n = 9$; 3m/o control, $n = 5$). (Fig 4.3.22a)

PCR analysis showed there was a significant difference in CREB mRNA expression in the hippocampus when Rq measurement in 3m/o controls was compared to 7,8-DHF treated subjects ($p = 0.0263$), but not when compared to Vehicle ($p = 0.0731$), and not when Vehicle was compared to 7,8-DHF ($p = 0.3440$). (Vehicle = 1.961 ± 0.2027 ; 7,8-DHF = 2.274 ± 0.2416 ; 3m/o control = 1.370 ± 0.1897). ($p = 0.0523$; Veh, $n = 8$; 7,8-DHF, $n = 9$; 3m/o control, $n = 5$). (Fig 4.3.22b)

Data analysed by unpaired t-test and/or one-way ANOVA and Bonferroni post-hoc test, expressed as mean $\pm$ SEM.

a)



b)

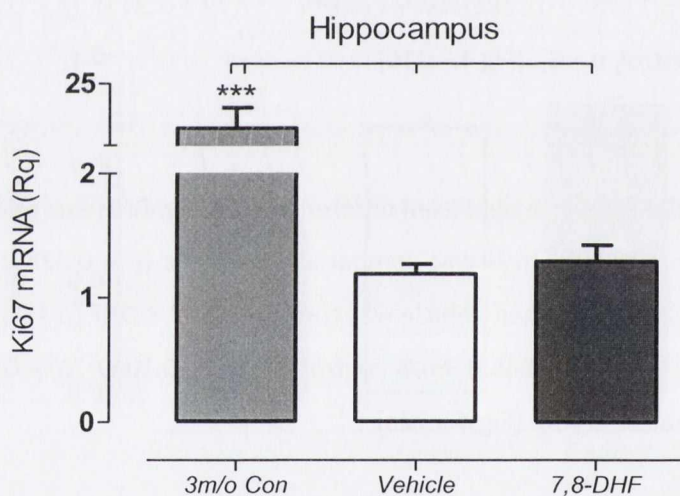


Fig. 4.3.21 – Effect of 9months of 7,8-DHF administration on Ki67 mRNA expression

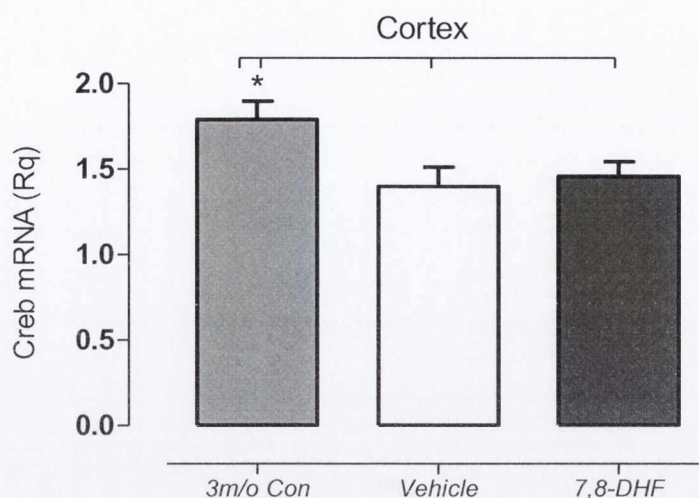
(a) shows Rq of Ki67 mRNA expression in cortical tissue. There was a significant difference in Ki67 expression in both Vehicle ($p < 0.0001$) and 7,8-DHF ($p < 0.0001$) treated subjects when compared to 3 month old controls.

(b) shows Rq of Ki67 mRNA expression in hippocampal tissue. There was a significant difference in Ki67 expression in both Vehicle ($p < 0.0001$) and 7,8-DHF ($p < 0.0001$) treated subjects when compared to 3 month old controls.

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. *** $p < 0.001$.

Data expressed as mean $\pm$ SEM (Vehicle, $n = 8$; 7,8-DHF, $n = 9$; 3m/o Con, $n = 5$).

a)



b)

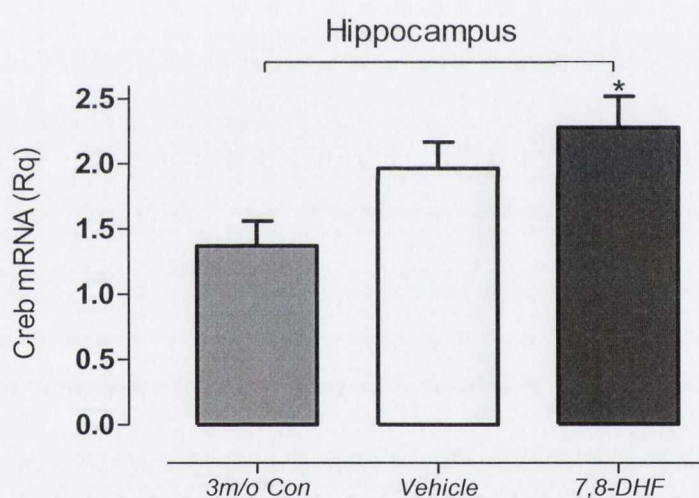


Fig. 4.3.22 – Effect of 9months of 7,8-DHF administration on CREB mRNA expression

(a) shows Rq of CREB mRNA expression in cortical tissue. There was a significant difference in CREB expression when 3 month old controls were compared to Vehicle ($p = 0.0381$) and 7,8-DHF ($p = 0.0341$) subject groups.

(b) shows Rq of CREB mRNA expression in hippocampal tissue. There was a significant difference in CREB expression in 7,8-DHF treated subjects when compared to 3 month old controls ($p = 0.0263$). Assessed by one-way ANOVA and Bonferroni post-hoc analysis. $*p < 0.05$.

Data expressed as mean $\pm$ SEM (Vehicle, $n = 8$; 7,8-DHF, $n = 9$; 3m/o Con, $n = 5$).

4.3.5c The effect of 9 months of 7,8-DHF administration on mRNA expression of IL1 β and TNF α in cortex and hippocampus.

PCR analysis showed there was a significant difference in IL1 β mRNA expression in the cortex when Rq measurement in 3m/o controls was compared to Vehicle ($p < 0.0001$) and 7,8-DHF treated subjects ($p < 0.0001$), but not when Vehicle was compared to 7,8-DHF ($p = 0.0831$). (Vehicle = 1.897 ± 0.1625 ; 7,8-DHF = 1.545 ± 0.09566 ; 3m/o control = 0.3502 ± 0.1777). ($p < 0.0001$; Veh, $n = 8$; 7,8-DHF, $n = 9$; 3m/o control, $n = 5$). (Fig 4.3.23a)

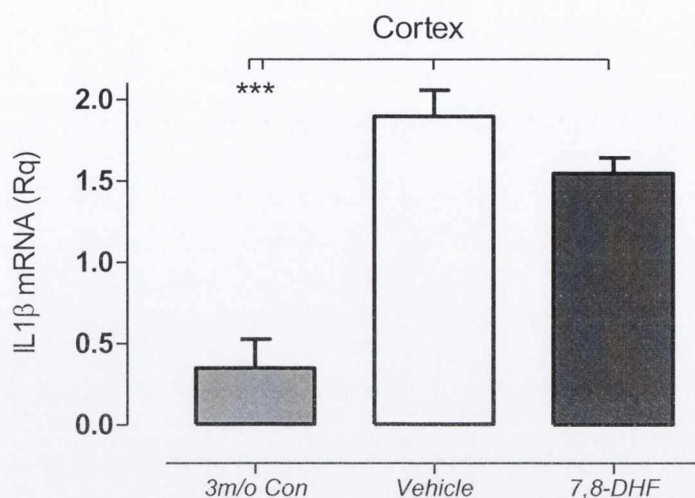
PCR analysis showed there was a significant difference in IL1 β mRNA expression in the hippocampus when Rq measurement in 3m/o controls was compared to Vehicle ($p = 0.0045$) and 7,8-DHF treated subjects ($p = 0.0007$), but not when Vehicle was compared to 7,8-DHF ($p = 0.1115$). (Vehicle = 1.851 ± 0.3828 ; 7,8-DHF = 1.139 ± 0.1714 ; 3m/o control = 0.1023 ± 0.01386). ($p = 0.0024$; Veh, $n = 8$; 7,8-DHF, $n = 9$; 3m/o control, $n = 5$). (Fig 4.3.23b)

PCR analysis showed there was no significant difference in TNF α mRNA expression in the cortex when Rq measurement was compared across all groups (Vehicle = 0.8314 ± 0.1054 ; 7,8-DHF = 0.8061 ± 0.07363 ; 3m/o control = 1.021 ± 0.1530). ($p = 0.4065$; Veh, $n = 8$; 7,8-DHF, $n = 9$; 3m/o control, $n = 5$). (Fig 4.3.24a)

PCR analysis showed there was no significant difference in TNF α mRNA expression in the hippocampus when Rq measurement was compared across all groups (Vehicle = 0.8295 ± 0.1399 ; 7,8-DHF = 0.6996 ± 0.1060 ; 3m/o control = 0.6567 ± 0.06199). ($p = 0.5896$; Veh, $n = 8$; 7,8-DHF, $n = 9$; 3m/o control, $n = 5$). (Fig 4.3.24b)

Data analysed by unpaired t-test and/or one-way ANOVA and Bonferroni post-hoc test, expressed as mean $\pm$ SEM.

a)



b)

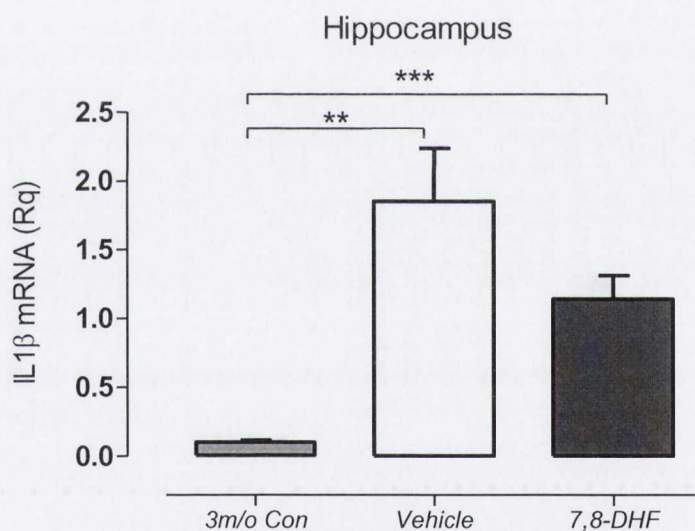


Fig. 4.3.23 – Effect of 9months of 7,8-DHF administration on IL1β mRNA expression

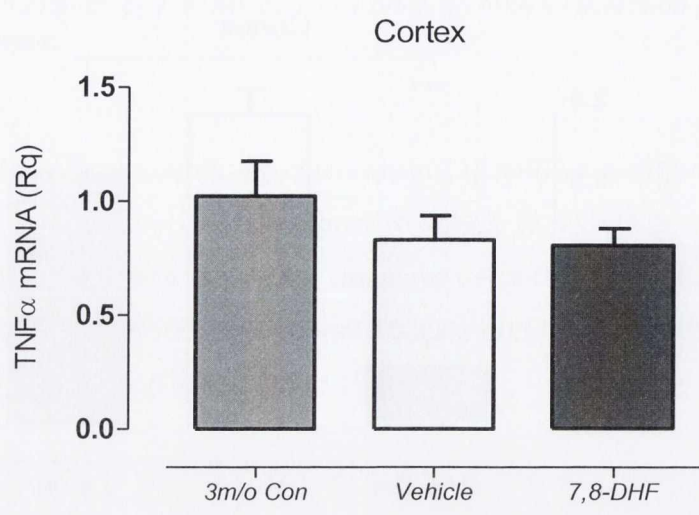
(a) shows Rq of IL1β mRNA expression in cortical tissue. There was a significant difference in IL1β expression in both Vehicle ($p < 0.0001$) and 7,8-DHF ($p < 0.0001$) treated subjects when compared to 3 month old controls.

(b) shows Rq of IL1β mRNA expression in hippocampal tissue. There was a significant difference in IL1β expression in Vehicle ($p = 0.0045$) and 7,8-DHF ($p = 0.0007$) treated subjects when compared to 3 month old controls.

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. ** $p < 0.01$, *** $p < 0.001$.

Data expressed as mean $\pm$ SEM (Vehicle, $n = 8$; 7,8-DHF, $n = 9$; 3m/o Con, $n = 5$).

a)



b)

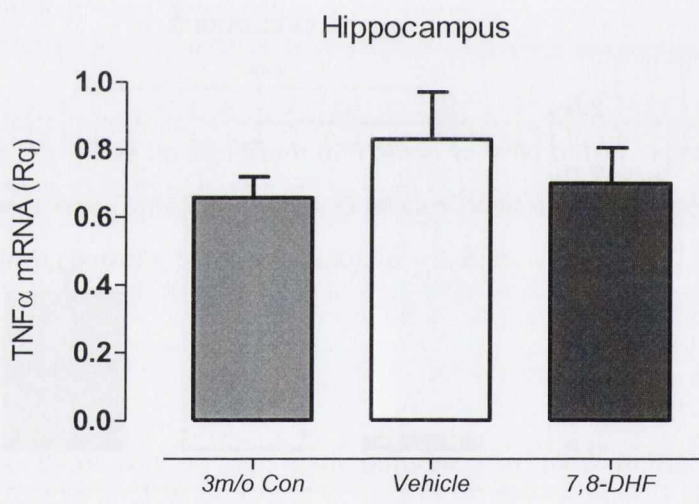


Fig. 4.3.24 – Effect of 9months of 7,8-DHF administration on TNF α mRNA expression
(a) shows Rq of TNF α mRNA expression in cortical tissue. There was no significant difference in TNF α expression between subject groups.
(b) shows Rq of TNF α mRNA expression in hippocampal tissue. There was no significant difference in TNF α expression between subject groups.
Assessed by one-way ANOVA and Bonferroni post-hoc analysis. $p>0.05$.
Data expressed as mean $\pm$ SEM (Vehicle, $n = 8$; 7,8-DHF, $n = 9$; 3m/o Con, $n = 5$).

4.3.5d The effect of 9 months of 7,8-DHF administration on mRNA expression of Iba1 and CD44 in cortex and hippocampus.

PCR analysis showed there was a significant difference in Iba1 mRNA expression in the cortex when Rq measurement in 3m/o controls was compared to 7,8-DHF treated subjects ($p = 0.0006$), but not when Vehicle was compared to 7,8-DHF ($p = 0.0996$). (*Vehicle* = 1.151 ± 0.06770 ; *7,8-DHF* = 1.276 ± 0.02940 ; *3m/o control* = 1.059 ± 0.03527). ($p = 0.0252$; Veh, $n = 8$; 7,8-DHF, $n = 9$; 3m/o control, $n = 5$). (Fig 4.3.25a)

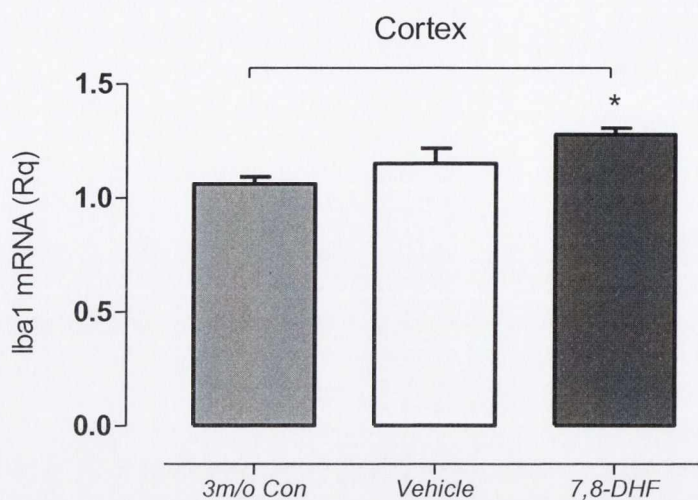
PCR analysis showed there was a significant difference in Iba1 mRNA expression in the hippocampus when Rq measurement in 3m/o controls was compared to Vehicle ($p = 0.0001$) and 7,8-DHF treated subjects ($p = 0.0005$), but not when Vehicle was compared to 7,8-DHF ($p = 0.4792$). (*Vehicle* = 1.216 ± 0.04365 ; *7,8-DHF* = 1.169 ± 0.04699 ; *3m/o control* = 0.8249 ± 0.04545). ($p < 0.0001$; Veh, $n = 8$; 7,8-DHF, $n = 9$; 3m/o control, $n = 5$). (Fig 4.3.25b)

PCR analysis showed there was a significant difference in CD44 mRNA expression in the cortex when Rq measurement in 3m/o controls was compared to Vehicle ($p = 0.0229$) and 7,8-DHF treated subjects ($p = 0.0057$), but not when Vehicle was compared to 7,8-DHF ($p = 0.0684$). (*Vehicle* = 1.372 ± 0.06511 ; *7,8-DHF* = 1.644 ± 0.117 ; *3m/o control* = 1.004 ± 0.145). ($p = 0.0036$; Veh, $n = 8$; 7,8-DHF, $n = 9$; 3m/o control, $n = 5$). (Fig 4.3.26a)

PCR analysis showed there was no significant difference in CD44 mRNA expression in the hippocampus when Rq measurement was compared across all groups (*Vehicle* = 0.6776 ± 0.1034 ; *7,8-DHF* = 0.6218 ± 0.05525 ; *3m/o control* = 0.6547 ± 0.07988). ($p = 0.8754$; Veh, $n = 8$; 7,8-DHF, $n = 9$; 3m/o control, $n = 5$). (Fig 4.3.26b)

Data analysed by unpaired t-test and/or one-way ANOVA and Bonferroni post-hoc test, expressed as mean $\pm$ SEM.

a)



b)

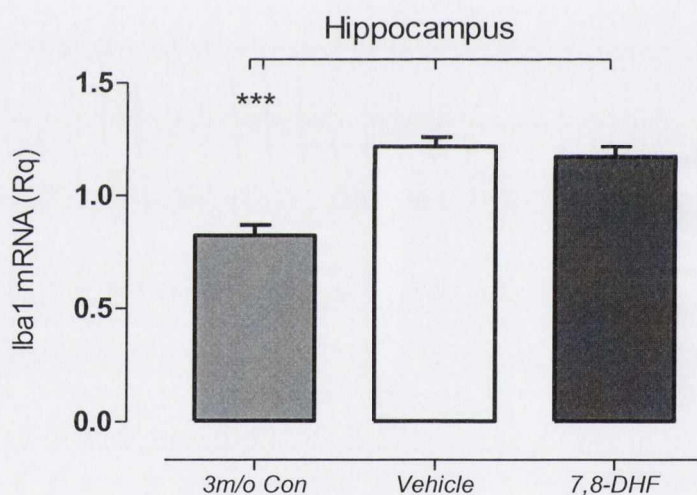


Fig. 4.3.25 – Effect of 9months of 7,8-DHF administration on Iba1 mRNA expression

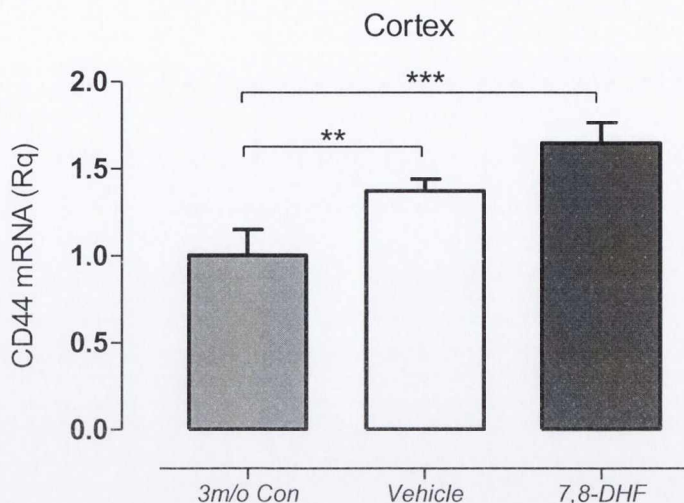
(a) shows Rq of Iba1 mRNA expression in cortical tissue. There was a significant difference in Iba1 expression in 7,8-DHF treated subjects when compared to 3 month old controls ($p = 0.0006$).

(b) shows Rq of Iba1 mRNA expression in hippocampal tissue. There was a significant difference in Iba1 expression in both Vehicle ($p = 0.0001$) and 7,8-DHF ($p = 0.0005$) treated subjects when compared to 3 month old controls.

Assessed by one-way ANOVA and Bonferroni post-hoc analysis. * $p < 0.05$, *** $p < 0.001$.

Data expressed as mean $\pm$ SEM (Vehicle, $n = 8$; 7,8-DHF, $n = 9$; 3m/o Con, $n = 5$).

a)



b)

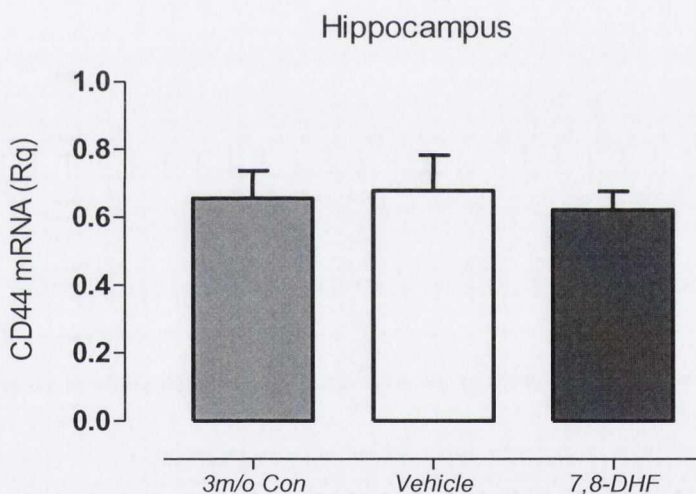


Fig. 4.3.26 – Effect of 9months of 7,8-DHF administration on CD44 mRNA expression

(a) shows Rq of CD44 mRNA expression in cortical tissue. There was a significant difference in CD44 expression in Vehicle ($p = 0.0229$) and 7,8-DHF ($p = 0.0057$) treated subjects when compared to 3 month old controls.

(b) shows Rq of CD44 mRNA expression in hippocampal tissue. There was no significant difference in CD44 expression between subject groups. Assessed by one-way ANOVA and Bonferroni post-hoc analysis. $**p < 0.01$, $***p < 0.001$.

Data expressed as mean $\pm$ SEM (Vehicle, $n = 8$; 7,8-DHF, $n = 9$; 3m/o Con, $n = 5$).

4.3.6 The effect of 9 months of 7,8-DHF administration on dentate gyrus volume, and number of BrdU⁺ cells.

Immunohistochemical staining and stereological estimation techniques show that there was no significant difference in dentate gyrus volume (Fig 4.3.27) when Vehicle animals were compared to 7,8-DHF treated animals, or when either was compared to 3 months old controls. (*Vehicle* = 0.5255 ± 0.01208 ; *7,8-DHF* = 0.5398 ± 0.02148 , *3m/o control* = 0.4928 ± 0.03089) ($p = 0.3688$, $n = 4$).

There was a significant difference in BrdU⁺ cell numbers (Fig 4.3.28) in animals that were administered Vehicle for 9 months when compared to 3m/o controls ($p = 0.0056$), but not when compared to 7,8-DHF treated animals (*Vehicle* = 18.21 ± 0.7595 ; *7,8-DHF* = 19.73 ± 1.755 , *3m/o controls* = 24.27 ± 1.219) ($p = 0.0240$, $n = 4$).

Data analysed using unpaired t-test and/or one-way ANOVA, with Bonferroni post-hoc test. Expressed as mean $\pm$ SEM.

Figure 4.3.29 shows an example of images obtained from Vehicle (a, c, e) and 7,8-DHF (b, d, f) treated animals.

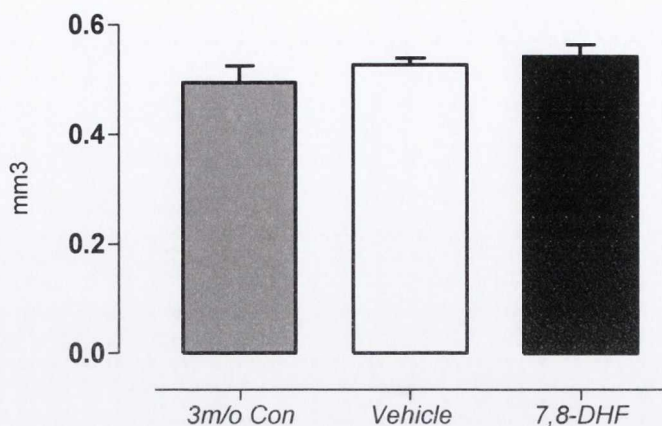


Fig. 4.3.27 – The effect of 9 months of 7,8-DHF administration on dentate gyrus volume
 There was no significant difference in overall volume of dentate gyrus as measured using the Cavalieri method of volume estimation.
 Assessed by one-way ANOVA and Bonferroni post-hoc analysis ($p > 0.05$).
 Data expressed as mean $\pm$ SEM ($n = 4$).

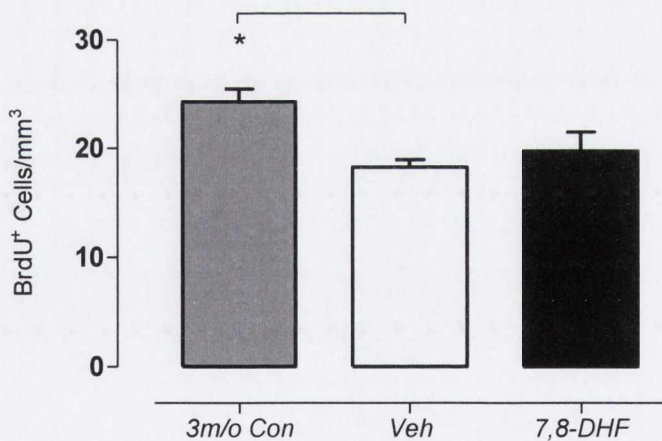


Fig. 4.3.28 - The effect of 9 months of 7,8-DHF administration on BrdU⁺ cells in the dentate gyrus
 There was a significant difference in the number of BrdU⁺ cells in the dentate gyrus of Vehicle treated animals when compared to 3 month old controls ($p = 0.0056$).
 Assessed by one-way ANOVA and Bonferroni post-hoc analysis ($*p < 0.05$).
 Data expressed as mean $\pm$ SEM ($n = 4$).

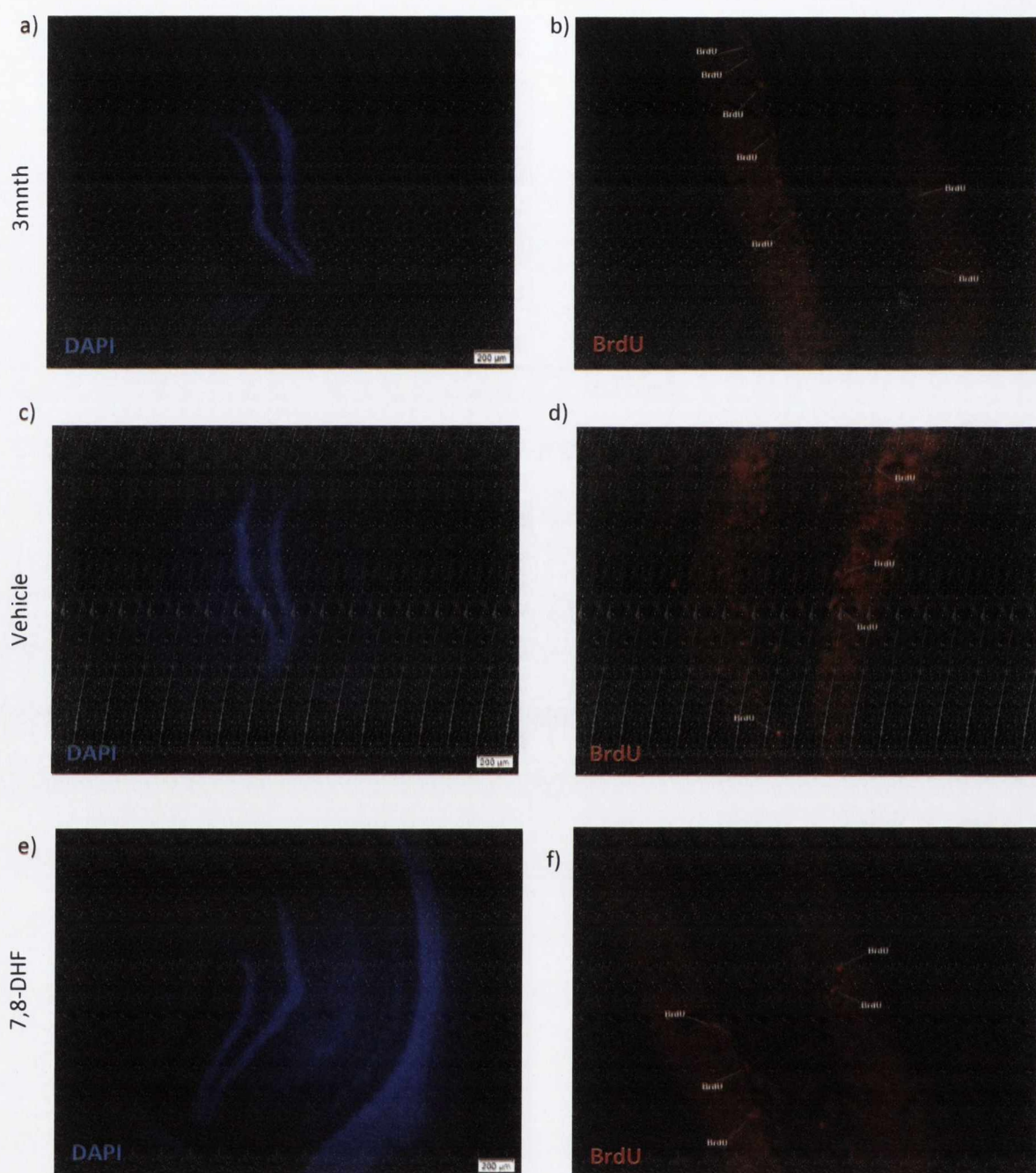


Fig. 4.3.29 - BrdU⁺ cells in the dentate gyrus

Images obtained at x4 (DAPI) and x20 (BrdU) magnification of 3m/o control (a, b), Veh (c, d), and 7,8-DHF (e, f) treated subjects.

BrdU⁺ cells are indicated with white marker.

4.4 Discussion

4.4.1 Behavioural analysis – episodic memory

Baseline performance in the NOR task was determined immediately preceding the commencement of Vehicle and 7,8-DHF administration as a measure of task difficulty and overall ability of animals to complete the test. We observed that all subjects recognised the object they had seen 24hrs beforehand, and chose to explore the novel object presented to them. From this we can conclude that there was no evidence of cognitive deficit in these 3 month old mice.

After 1.5 months and 3 months of treatment with either Vehicle or 7,8-DHF, animals were tested in the NOR task again, but we observed a preference in all animals for the novel object; C during the testing phase. At 4.5 and 6 months of age, we conclude that there was no evidence of cognitive deficit in these animals.

With 4.5 months of treatment, we observed that Vehicle treated mice showed a deficit in episodic memory and 7,8-DHF treated were successful in the task. This effect persisted at 9 months of age, when animals had received either Vehicle or 7,8-DHF in their drinking water for 6 months. We report that Vehicle treated animals show a decline in cognitive function at 9 months of age, and treatment with 7,8-DHF protects against this.

After 7.5 months of treatment, 7,8-DHF animals spent significantly more time exploring the novel object. This shows a persistence in the effect of 7,8-DHF on task performance, while we observe that Vehicle treated animals were unable to complete the task. With the final session of NOR testing at 12 months of age, we can conclude that 9 months of treatment with 7,8-DHF protects against an age-related decline in cognition, as was shown in Vehicle treated subjects from 7.5 months of age onwards.

4.4.2 Behavioural analysis – spatial memory

Baseline performance in the OD task was determined immediately preceding the commencement of Vehicle and 7,8-DHF administration as a measure of task difficulty and overall ability of animals to complete the test. We observed that all subjects spent significantly less time exploring object B during the training session, but there was no difference between objects A and C. This was despite the use of a variety of objects and combination of locations, and changing them on rotation after

each animal. Every effort was made to eliminate a spatial or object-specific aversion. Regardless of this result in training, it was observed that all animals spent significantly more time exploring the displaced object; C, during the testing phase of the task. From this observation, we can conclude that the animals were receptive to the novelty of location change in the arena. This suggests that there was no evidence of cognitive deficit in these 3 month old mice.

After 1.5 and 3 months of treatment with either Vehicle or 7,8-DHF, all animals showed a preference for the displaced object during testing, and from this we conclude that there is no change in cognitive function in animals at 4.5 or 6 months of age.

With 4.5 months of treatment, 7,8-DHF treated animals spent significantly more time exploring Object C, but Vehicle treated animals did not. We conclude that at 7.5 months of age, Vehicle treated mice showed a deficit in spatial memory, and this persisted when tested at 9 months of age, following 6 months of treatment. 7,8-DHF treated animals spent significantly more time exploring the displaced object, but again Vehicle treated animals did not recall the earlier location of object C. We conclude that treatment with 7,8-DHF protects against the cognitive disturbance observed in Vehicle treated subjects.

After 7.5 months of treatment, 7,8-DHF animals spent significantly more time exploring object C. This was not the case with Vehicle treated mice, and shows a persistence in the effect of 7,8-DHF on task performance. After testing at 12 months of age, we can conclude that 9 months of treatment with 7,8-DHF protects 12 month old mice from age-related decline in spatial cognition, as was observed in Vehicle treated subjects.

The findings from both behavioural tasks are novel because to date there is no report of the effects of long-term 7,8-DHF administration on cognitive function in the literature. Studies have shown a beneficial cognitive effect of two weeks of 7,8-DHF administration in 22month old mice (Zeng *et al.*, 2012b), and an improvement in the NOR and OD task performance in rats following a single dose of 7,8-DHF (Bollen *et al.*, 2013). However, our findings here mimic as yet unpublished work in our laboratory (Mota, BC and Kelly, ÁM) that has shown a persistent effect of eight months of treadmill running on cognition in the mouse.

4.4.3 Analysis of BDNF and its receptor; TrkB

PCR analysis of mRNA expression showed a significant difference in BDNF concentration in the cortex of Vehicle and 7,8-DHF treated animals when compared to 3 month old controls, but no

significant difference was observed in the hippocampus. We do know however that BDNF expression in general is decreased with age (Croll *et al.*, 1998, Lommatzsch *et al.*, 2005), and our comparison to young healthy controls confirms this. Evidence in the literature for a 7,8-DHF mediated upregulation in BDNF expression is unclear, but it could be argued that access to a constant supply of 7,8-DHF in drinking water makes an increase in BDNF expression redundant. Our study permitted access to 7,8-DHF treated drinking water up until the time of sacrifice, meaning there was no withdrawal from ambient 7,8-DHF availability in the system. In the sudden absence of 7,8-DHF, the demand for TrkB activation may induce an upregulation in BDNF expression. Maintaining access to 7,8-DHF could eliminate the requirement of an intense spike in BDNF concentration in its place. We infer that 7,8-DHF administration has no effect on the age-related decline in BDNF expression.

TrkB receptor mRNA in the cortex and hippocampus was significantly different in both aged Vehicle and 7,8-DHF treated animals when compared to 3 month old controls. In hippocampal tissue, we observed a tendency toward higher TrkB expression in 7,8-DHF treated animals when compared to Vehicle ($p=0.0547$). This is interesting, because 7,8-DHF exerts its effects directly through TrkB binding, as shown in numerous studies to date (Devi and Ohno, 2012, Zeng *et al.*, 2012b, Zhang *et al.*, 2014). It would also be of value to determine if TrkB receptor phosphorylation was increased in 7,8-DHF treated animals, via protein analysis, as opposed to mRNA analysis carried out here. We can infer aged animals express higher levels of TrkB mRNA, and 7,8-DHF has the potential to increase this expression in the hippocampus.

4.4.4 Analysis of Ki67 expression

In analysing expression of Ki67 mRNA, we can deduce whether or not cells are actively proliferating at the time of tissue collection. Ki67 is an endogenous marker of neuronal and glial proliferation, and is thought to be required for a cell's progression through the cell cycle (Scholzen and Gerdes, 2000). Ki67 is present during all active stages of the cell cycle. The only time Ki67 is not expressed is when cells are inactive, or resting. So its expression is a direct measure of the level of cellular proliferation. We report a significant difference in Ki67 mRNA concentration in the cortex and hippocampus of aged mice when compared to young healthy controls, confirming existing observations in the literature (Ben Abdallah *et al.*, 2010, Rao *et al.*, 2006). Upon analysis of all Vehicle and 7,8-DHF treated mice, we report no change in Ki67 expression. It is possible that cell proliferation was not stimulated at the time of tissue collection. There is nothing yet in the literature

to suggest that 7,8-DHF provokes proliferation in a ubiquitous manner. At this time, we cannot credit 7,8-DHF with having a protective effect on the age-related decline in levels of Ki67 mRNA.

4.4.5 Analysis of CREB expression

mRNA analysis showed a significant difference in expression of CREB mRNA in the cortex in both Vehicle and 7,8-DHF treated mice when compared to 3 month old controls. In the hippocampus, we observed significantly higher CREB expression in 7,8-DHF animals when compared to 3 month old controls. The literature reports CREB expression to rise and fall parallel to BDNF expression in animals that have been subject to exercise (Vaynman *et al.*, 2004b, Gómez-Pinilla *et al.*, 2002). As 7,8-DHF activates the same signalling pathways as BDNF, we would expect to see an increase in CREB expression. So far, the majority of promise in effect of 7,8-DHF has been observed in hippocampal tissue. It could be argued that 7,8-DHF is exerting some effect specifically in the hippocampus and would explain the lower CREB expression reported in the cortex. Studies have shown an increase in phosphorylation of CREB (Agrawal *et al.*, 2015, Zeng *et al.*, 2012a, Zeng *et al.*, 2012b), so there is the possibility that western blot analysis of activated CREB would provide some more clarification. For now, we can conclude that CREB mRNA expression in the aged hippocampus is increased by 7,8-DHF administration.

4.4.6 Analysis of proinflammatory cytokines; IL1 β and TNF α

We observed a statistically significant difference in cortical and hippocampal mRNA expression of IL1 β in 3 month old controls when compared to both Vehicle and 7,8-DHF. However, there is also a tendency toward lower IL1 β expression in 7,8-DHF animals when compared to Vehicle, and this may be drawn out by further analysis and an increase in n number. IL1 β is a proinflammatory cytokine released from microglia as a response to insult, and its expression is reported to increase with age (Chung *et al.*, 2009, Licastro *et al.*, 2005, O'Mahony *et al.*, 1998). We report a possible trend toward lower IL1 β expression with 7,8-DHF treatment, as has been shown in the literature (Park *et al.*, 2012, Park *et al.*, 2014). As of yet, we cannot credit 7,8-DHF with displaying a protective effect against an age-induced inflammatory response.

We report no significant differences in cortical mRNA expression of TNF α when levels were compared across all groups. However, in the hippocampus we again see a trend toward lower concentration of TNF α in 7,8-DHF treated animals compared to Vehicle treated, but this also cannot

be confirmed at this time. However, this decrease in TNF α has been reported (Park *et al.*, 2014). From our current results, we cannot conclude that 7,8-DHF has the ability to protect against an age-related increase in TNF α expression, but further analysis and an increase in n number may clarify this.

4.4.7 Analysis of Iba1 expression

Analysis of cortical tissue shows lower mRNA expression of Iba1 in 3 month old controls when compared to 7,8-DHF treated subjects, and in the hippocampus this lower expression is seen when compared to both Vehicle and 7,8-DHF treated subjects. Iba1 expression is upregulated while microglia are activated (Autieri *et al.*, 2000, Fukui *et al.*, 2012), so it would be expected that its expression would be elevated in parallel with an age-related inflammatory response. Studies have shown that treatment with 7,8-DHF can decrease Iba1 expression (Sanz García *et al.*, 2014, Wurzelmann, 2016), but we have not replicated that finding here, so we cannot conclude that Iba1 expression is altered by 7,8-DHF treatment.

4.4.8 Analysis of CD44 expression

There was no significant difference found in CD44 mRNA expression in hippocampal tissue when comparisons were made across all groups, but analysis of the cortex showed that CD44 expression was significantly higher in both Vehicle and 7,8-DHF treated subjects when each was compared to 3 month old controls. We also observed a trend toward higher CD44 expression in 7,8-DHF subjects when compared to Vehicle ($p = 0.0684$). CD44 is an adhesion molecule expressed on the surface of memory T cells. CD44 assists in mediating protection from re-infection, and so its expression is usually upregulated and persists long after the infection has been cleared (Baaten *et al.*, 2010). So far, there is nothing in the literature to show an effect of 7,8-DHF on CD44 expression. It could be argued that an increase in CD44 expression could benefit the organism, providing more widespread surveillance and thus greater protection from recurrent damage from an age-related chronic inflammatory response. This however is purely speculative and we cannot conclude from our current findings that 7,8-DHF has an effect on the increase or decrease CD44 expression.

4.4.9 Analysis of dentate gyrus volume and cell survival

The process of labelling cells with BrdU was initiated one month before sacrifice and continued for seven daily i.p. injections of BrdU. This meant that the system was flooded with this substitute for the thymidine base of DNA, and cells undergoing proliferation in the presence of this analogue could incorporate BrdU in place of thymidine. Three to four weeks later, when tissue was collected, we were able to analyse coronal sections of the brain to detect the presence of BrdU in surviving cells in the dentate gyrus, suggesting that these cells had been proliferating in the presence of BrdU. We found no difference in volume of the dentate gyrus when comparisons were made across all groups. However, we saw a significant difference in the numbers of BrdU positive cells in the dentate gyrus of Vehicle treated animals when compared to 3 month old controls, and a tendency toward higher numbers of BrdU positive cells in 7,8-DHF treated animals when compared to Vehicle treated subjects. With further analysis of the co-labelling of NeuN and BrdU⁺ cells and an increase in n number, we may see if there truly is an increase in BrdU⁺ cells induced by 7,8-DHF treatment. The literature reports an increase in neurogenesis caused by 7,8-DHF administration (Hsiao *et al.*, 2014, Liu *et al.*, 2010, Zhao *et al.*, 2015), and it would be interesting to see if our results agree with this. For now, we conclude that numbers of BrdU positive cells in the dentate gyrus are lower in aged animals, and with further analysis we may attribute a reduction in this decline to the effects of long term administration with 7,8-DHF.

4.4.10 Summary

In this long term investigation into the effects of 7,8-DHF in the aging brain, we successfully demonstrated the protective effects of this compound against age-related cognitive decline. We did this using both the NOR and the OD task, and found that Vehicle treated controls began to show signs of cognitive decline, in both episodic and spatial memory, as early as 7.5 months of age. Generally, this time-point would be classified as young adulthood, but the decline observed at this early stage was present throughout the remainder of experimentation, so we do not doubt that 7,8-DHF was exerting a positive effect in animals of the same age.

Our efforts to elucidate cellular mechanisms underlying the 7,8-DHF-induced cognitive enhancement we observed in this study have been somewhat promising to date, but additional analysis and an increase in n number may further explain this protective effect on cognition. Despite much evidence in the literature reporting various physiological modifications, we have been thus far unsuccessful in exposing a central contributing factor in this process. It is possible

that a reduction in the expression of proinflammatory cytokines coinciding with an increase in CD44 concentration in 7,8-DHF treated animals could collectively modulate an age-related inflammatory response. The combination of a number of elements influenced by 7,8-DHF may present a novel channel that we have not yet seen reported in the literature. Additional investigation may further strengthen the evidence that supports 7,8-DHF as a pharmacomimetic of exercise.

Chapter 5

General Discussion

5.1 Discussion

The importance of the hippocampus in higher cognitive function is undisputed, and it has been recognised as being highly susceptible to the destructive effects of stress (Li *et al.*, 2009, Smith *et al.*, 1995), depression (Sapolsky, 2000, Sheline *et al.*, 1996, Stockmeier *et al.*, 2004), infection (Bilbo *et al.*, 2008, Sharma *et al.*, 2002), neurodegenerative disease (Guan *et al.*, 2000, Phillips *et al.*, 1991, Sze *et al.*, 1997, Tang *et al.*, 2013), and aging (Barrientos *et al.*, 2015, Lindsey *et al.*, 1979, Luellen *et al.*, 2007, Montagne *et al.*, 2015). Advances in research over the last few decades have exposed us to an abundance of opportunity through manipulating the mechanisms involved in these processes and exerting a protective effect on the hippocampus itself, thereby impeding its structural and functional decline. Exercise - and the physiological changes it induces, has long been accepted as a front-runner in providing this protection for the hippocampus (Cotman *et al.*, 2007, Dey *et al.*, 1992, Erickson *et al.*, 2011).

The work carried out here was an investigation of the possibility that a pharmacomimetic intervention could reproduce the same effects as a programme of physical exercise; the outcome of which has been extensively reported by this laboratory in recent years (O'Callaghan *et al.*, 2007, Griffin *et al.*, 2009, O'Callaghan *et al.*, 2009, Griffin *et al.*, 2011, Bechara and Kelly, 2013, Callaghan and Kelly, 2013, Bechara *et al.*, 2014). Elucidating the intricacy of cognitive enhancement, and manipulating the methods of doing so is an area of research that has long been encouraged by the pharmaceutical industry. The advantages of pharmacological intervention have the opportunity to reach a much wider community of beneficiaries, when we consider the constraints of a person with limited physical ability. An individual in this instance may not necessarily have the capacity to exercise, and is thereby deemed incapable of reaping the reward of its physiological enhancement.

With the recognition of BDNF as a profound contributor to cognitive enhancement, much research has focused around the means of increasing its expression (Lee *et al.*, 2000, Chen *et al.*, 2001, Obrietan *et al.*, 2002), with exercise being a popular contender in the field (Hillman *et al.*, 2008). The direct administration of BDNF has shown some promise (Terenghi, 1999, Xu *et al.*, 2004), but with an inability to cross the blood-brain barrier, these effects have only been seen centrally with intra-hippocampal infusion, meaning that oral or intravenous administration will not stimulate the desired effect in the hippocampus (Nagahara and Tuszynski, 2011). As has been discussed, the search for a mimetic of the activity of BDNF has produced many possible candidates, and the small synthetic flavonoid 7,8-DHF is among them (Jang *et al.*, 2010).

With the current study, we focused mainly on the behavioural effects of exercise and 7,8-DHF administration in the context of insult or injury in the central nervous system. At the same time, we wished to investigate the potential involvement of neurogenesis in this process. Having previously looked at the acute effects of exercise in short-term studies, our protocol was adapted to observe the effects of exercise at a time-point that coincides with the maturation of new neurons and their recruitment into the circuitry. It is known to take approximately three to four weeks for neurons to be classed as mature; with the activation of dormant granule cells in the subgranular zone of the dentate gyrus, their subsequent proliferation and production of nerve fibres, and eventual connection with the entorhinal cortex preceding this mature state.

At the beginning of a one-month exercise programme, animals received 7 daily i.p. injections of BrdU. To observe mature neurons, this covered a time-line of three to four weeks before sacrifice. Once this programme was complete, animals received an i.p. injection of either saline or LPS in a bid to induce acute neuroinflammation in the subject, while we hypothesised that exercise would protect against this insult. Our first study (Experiment 3.1) showed that LPS induced an increase in expression of proinflammatory cytokines $IL1\beta$ and $TNF\alpha$ at the mRNA level, but we could not report an effect of LPS on cognitive function in these animals, although we did observe enhanced spatial-memory-dependent performance in all exercised subjects, as determined by increased exploration of a displaced object. It was concluded that this timeframe for testing was not within an ideal window. A 24hr delay between training and testing was possibly too long for recall, and the task was deemed too difficult for completion by healthy subjects. Synaptic plasticity in the hippocampus is heavily involved in short-term memory consolidation, a process that generally lasts up to 60, or possibly 90 minutes (Davis and Agranoff, 1966) after which time LTP-dependent long-term consolidation determines the fate of the memory. With the knowledge that the hippocampus can be so highly plastic in response to exercise (Cotman and Berchtold, 2002), the potential for enhanced short-term memory consolidation could be harnessed by using a different inter-trial interval following LPS administration.

With Experiment 3.2, the decision to alter our timeline meant that subjects would receive an i.p. injection of LPS, and would be trained and tested in the Object Displacement task within five hours of its administration, with a one hour delay for memory recall. On this occasion, our healthy sedentary subjects were successful in the task, and again we saw this with both exercised groups. The task was simple enough to complete, but difficult enough that any degree of cognitive dysfunction would be observed in impaired spatial memory. Animals that had been sedentary for the duration of the experiment succumbed to the effect of LPS, and animals that had been exposed

to treadmill running for that same month were able to complete the task in spite of an inflammatory response. This was confirmed through mRNA analysis, where we observed an increase in IL1 β and TNF α , combined with an increase in CD44 expression, as upregulated by T cell activation. In addition to the cognitive enhancement reported, immunohistochemical detection of BrdU showed some promise in the inclination toward an increase in cell count in exercised subjects. From these findings, we can conclude that one month of exercise preserves spatial memory capacity and protects from neuroinflammation.

The findings of Experiment 3.2 provided a template for a direct comparison of the effects of 7,8-DHF as a mimetic of this exercise-induced enhancement. Utilizing the same timeline, animals in Experiment 3.3 received access to this synthetically-derived TrkB-specific agonist and were found to display the same improvement in spatial memory when tested in the OD task, despite LPS administration and the induction of an inflammatory response as shown by increases in IL1 β , TNF α , and CD44 mRNA expression. With our vehicle treated control subjects, we saw an inability to successfully navigate the OD task, despite an ability in sedentary controls tested in this same timeline. On reflection, we did not account for the effects of a novel environment on our sedentary animals, who had been removed from their home cage on a daily basis and exposed to the surroundings of the stationary treadmill. Our vehicle treated controls remained in their home cage for the majority of this experimental period and did not benefit from the possibility of an inadvertent stimulation. Having carried out the Exercise/LPS study on this same time scale, we can conclude that the timing was sufficient for animals to learn, but that the animals in this control group did not. There could be any number of factors at play here that we failed to foresee. Aside from the daily change of environment when placed on stationary treadmills, these sedentary mice also benefitted from more frequent handling, whereas vehicle-treated animals were periodically removed from their home cage to be weighed, and have their tail markings refreshed for identification, but rarely handled aside from these occasions, while sedentary animals were handled in addition to this. The effect of incidental tactile enrichment is one that should be taken under consideration in future studies, and can easily be resolved by including a home-cage-bound group that receives standard drinking water for the duration of the study, or handling all animals on a daily basis.

Throughout each of these experiments, we observed no change in levels of TrkB or BDNF mRNA expression, or BDNF's downstream target; CREB. We also report no upregulation in Ki67 mRNA expression, showing no increase in active neuronal or glial proliferation at this time. This does not confirm a lack of physiological effect of exercise or 7,8-DHF administration, but rather a lack of

change in messenger RNA at the time of tissue collection. Perhaps protein concentration is increased, or further still, these proteins are activated at this time and this is how they exert a change in cognitive function. Our results from immunohistochemical analysis so far do not show a consistent increase or decrease in numbers of BrdU-expressing cells, so we cannot currently credit cytotogenesis for the protective effect on spatial memory. There are a number of unexplored factors, such as increases in expression of BDNF and TrkB at the protein level, along with phosphorylation of TrkB itself and subsequent activation of its downstream signalling factors; ERK, Akt, and CREB. As has been shown in the literature, any combination of these factors hold the potential to clarify mechanisms at a physiological level when we are seeing this behavioural effect.

To our knowledge, there is no existing investigation in the literature that monitors the effects of long-term TrkB activation with the flavonoid; 7,8-DHF. Subsequently, there is also no report of periodic testing of hippocampal-dependent memory during the aging process as this compound is being administered. Our findings in Chapter 4 are thus novel in many respects. We tested both episodic and spatial memory in all subjects, every 6 weeks, over a 9 month period, and report that we observe a decline in hippocampal-dependent cognitive function from 7.5 months of age onwards. This is relatively early in the aging process; at a time of transitioning into middle-age. However, this trend continued for the remainder of our study, without any discerning change in general levels of exploration across all animals. Again, these animals experienced considerably less handling than if they had been exercised on a daily basis for 9 months, but showed no cognitive dysfunction in any test of hippocampal-dependent memory up until this age. We conclude that 7,8-DHF administration coincides with the maintenance of cognitive function through middle age, when subjects were sacrificed.

One unexplored factor is the potential effect of DMSO administration – both here in the long-term study and in our earlier investigation of the neuroprotective role of 7,8-DHF in inflammation. There is a possibility that DMSO-treated water could cause some degree of cognitive dysfunction, and may explain the inability of Veh_Sal animals to perform the OD task in the LPS study, or the onset of cognitive decline at 7.5 months of age in our long-term investigation. Controlling for this effect would be simple enough and requires an additional treatment group that receives standard drinking water only.

Recognition memory is known to depend on the function of both the hippocampus (Clark *et al.*, 2000) and the adjacent perirhinal cortex (Aggleton *et al.*, 2010), while spatial memory relies heavily upon the dorsal hippocampus (Broadbent *et al.*, 2004) and its interactions with the prefrontal cortex (Spellman *et al.*, 2015). For this reason, when analysing mRNA expression we used tissue samples from both the hippocampus and prefrontal cortex. In addition to this, when carrying out tissue analysis on Vehicle and 7,8-DHF treated animals, we made comparisons with 3 month old untreated animals to account for any change in expression observed in middle-age. We observed lower BDNF expression with age, but no effect of treatment in modulating this decline. With 7,8-DHF administration providing presumably consistent TrkB activation we may not expect to see an increase here, so conclude that 7,8-DHF does not exert its effects on cognition through an upregulation of BDNF at this age. TrkB and CREB mRNA expression were significantly different in our 7,8-DHF treated subjects when compared to younger controls, but vehicle treated mice did not display this. From this we can infer that there is a 7,8-DHF mediated increase in synthesis of these proteins, permitting 7,8-DHF to further activate TrkB receptors and exert downstream effects on CREB, which is known to be intimately involved in neuronal plasticity and the late stages of long-term potentiation – both crucial processes in hippocampal-dependent memory.

Unsurprisingly, we reported a significantly lower Ki67 expression in groups at middle-age. Cellular proliferation is known to be higher during adolescence, and at 3 months of age these animals had just reached early adulthood. At 12 months old, we would expect to see a decline, but we report no difference in expression between aged groups, concluding that active upregulation in proliferation-related factors is not involved in enhanced cognitive function at this time-point. This is not to state that 7,8-DHF does not exert an effect on cellular proliferation, but it could be argued that production of new cells is in an on-demand basis, and at the time of sacrifice, there was no cognitive testing taking place.

We expected to observe higher concentration of inflammatory factors in middle-aged animals when compared to 3 month old controls, and this is what we report with IL1 β and TNF α . Younger animals show significantly lower expression of these cytokines, but 7,8-DHF animals also show a decline when compared to Vehicle subjects, indicating that 7,8-DHF is protective against an age-related modulation of the inflammatory response. From this, we can infer that the cognitive decline observed in vehicle treated animals was related to circulating proinflammatory factors, and 7,8-DHF was protective against this, perhaps through the expression of CD44, which provides surveillance to protect from re-infection, and was increased in 7,8-DHF treated animals.

With regard to the quantity of BrdU expressing cells, we cannot credit 7,8-DHF with an increase here, but observed significantly more cells in younger animals. Our investigation into the involvement of neurogenesis in modulating a protective effect on cognitive function in the injured or aging brain is inconclusive to this end. There are many reports of 7,8-DHF affecting neurogenesis in the rodent model, but to our knowledge there is nothing in the literature at this time point, and nothing that reports on a study of this length. Our results suggest that 7,8 DHF has therapeutic potential, as observed in its effect on hippocampal-dependent memory, but also that 7,8 DHF may have different mechanistic routes of implementing change when compared to BDNF, and this warrants further investigation.

5.2 Alternative considerations

A comprehensive analysis of neurogenesis via the quantification of surviving NeuN and BrdU co-labelled cells, with an increase in n number, would contribute greatly to this study, as this work has not yet been completed. Although BrdU administration was three to four weeks prior to sacrifice, it may be worthwhile to analyse additional tissue for co-labelling of BrdU and DCX (doublecortin), with the possibility of quantifying immature neurons that have not yet begun to express NeuN. To supplement this investigation, staining for GFAP (glial fibrillary acidic protein) or Ki67 could clarify if there was active proliferation of neurons or glia at the time of sacrifice, even without the upregulation of Ki67 mRNA expression.

If after this, there is still no change in cell number to account for this improvement in cognitive function, it could be worthwhile to investigate a possible change in synaptogenesis. In measuring the levels of synapsin1 (a presynaptic membrane protein) and PSD 95 (postsynaptic density protein), and their active phosphorylated counterparts, we may explain an upregulation in connectivity of the circuitry without observing a significant difference in cell numbers. 7,8-DHF administration rescued a decrease in the expression of these synaptic proteins in a model of AD at 6 months of age (Zhang *et al.*, 2014).

As discussed, further investigation into the activation of key signalling molecules at the protein level may elucidate an effect in relation to BDNF, TrkB, and CREB. An unclear effect of 7,8-DHF on their mRNA expression does not necessarily translate at the protein level. TrkB activation in particular will be a crucial factor, since 7,8-DHF specifically exerts its effect at this receptor.

In addition to CREB, the activation and expression of proteins in the MAPK/ERK, the PI3K/Akt, and the PLC γ pathways as downstream effectors of change have been shown to be modulated by exercise through BDNF/TrkB binding, and reports have shown that 7,8-DHF mediates these signalling factors.

5.3 Limitations of this research

As with all research, the most dominant constraint of this study was time. Within the field of exercise and cognitive enhancement, behavioural findings can be difficult to account for due to the abundance of potential pathways that can be taken when investigating physiological alterations. Unfortunately with this particular study we are still to pinpoint specific physiological or morphological changes that can account for the benefit to hippocampal-dependent memory that we have reported here. However, with time we have the potential to discover characteristics of the processes of learning and memory that have not yet been reported.

In addition to this, our laboratory has recently transitioned from studies using the rat to work with mice, and this meant that a lot of the protocols that had been used for many years and that I had been trained in had to be adapted for work with mice and mouse tissue. This required a lot of additional time to troubleshoot and optimise various procedures, as well as stocking the laboratory with solutions and tools that worked with mouse tissue. Also, Project Authorisations had to be updated, modified and resubmitted to include work on the mouse, and we were not immune to administrative errors. These issues, or a combination thereof, caused unforeseen delays in commencing behavioural work and generating data.

On the subject of venturing into the unknown, working with a novel agonist of TrkB was both enticing and worrying. Having only been discovered in the last decade, there is not a lot in the literature as a basis for experimentation with 7,8-DHF. That being said, there are no reports of any other long-term studies of the effects of this compound, meaning there is great potential for novelty with our findings – when we eventually pinpoint exact mechanisms of the activity of 7,8-DHF in this particular application. Hopefully we can add to its promise as a pharmacomimetic.

5.4 Future directions

Despite uncertainty around the underlying physiological mechanisms implementing the effect of 7,8-DHF in the cognitively compromised brain, this study strongly supports a role for 7,8-DHF in the enhancement of hippocampal-dependent memory. With this in mind, there are a number of proposals for further research to answer remaining questions.

Would there be any benefit from a programme of exercise while simultaneously administering 7,8-DHF in drinking water?

This could provide answers in a more translational sense. There are many people who are physically active and benefit from the advantages of this, while also taking vitamins and supplements for general health and wellbeing. Would there be an additive, or a ceiling effect of both exercise and 7,8-DHF administration together? Is there the potential for a neuro-toxic level of TrkB activation that would in fact be detrimental?

Is there a case for environmental enrichment while administering 7,8-DHF?

This would be interesting, mainly because our vehicle treated animals in Experiment 3.3 did not successfully complete the OD task, and the sedentary animals from Experiment 3.2 did. Were they benefitting from the enrichment of a new environment outside of the home cage every day?

Would 7,8-DHF continue to exert its effects into old age?

To answer this, we need to either carry out a longer period of testing, to bring subjects into old age, or else start treatment with 7,8-DHF in middle age and continue from there. Both would answer valuable questions. Does synthetically-supplementing endogenous BDNF eventually lose its therapeutic effect? Does the body become intolerant to its prolonged presence? Or is middle-age an early enough time point to exert the same cognitive effect?

Would 7,8-DHF have effect in a model of AD?

Replicating a naturally occurring physiological function is one issue. Replicating this in a model of enhanced cognitive decline and neuro-toxicity presents further challenges. There is so much to learn about how 7,8-DHF exerts its effects that there is every possibility of it therapeutically rectifying aspects of conditions associated with hippocampal impairment and cognitive decline, such as AD, that are yet to be pinpointed.

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