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Interactions between endocannabinoids and neurotrophins in adult neurogenesis: implications for spatial memory.

Jack Prenderville B.A. (Mod.)

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

Submitted January 2014

Department of Physiology

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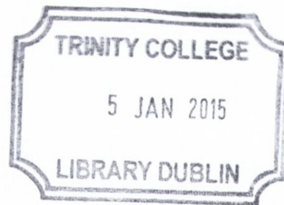

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Jack Prenderville B.A. (Mod)

Thesis 10733

Summary

The cellular mechanisms underlying learning and memory have yet to be fully elucidated. Over the past fifteen years there has been a dramatic increase in the study of adult neurogenesis; a process in which neurons are generated from stem cells in the adult brain and which has been suggested to be relevant to learning and memory. This process has been confirmed in two areas of the adult brain, one being the dentate gyrus of the hippocampus, a brain region known to be important for learning and memory. The fact that neurogenesis occurs in this brain region throughout life has prompted many researchers to hypothesise that it may be a form of brain plasticity that is important for learning and memory. Exercise has been shown to be a potent enhancer of hippocampal-dependent memory and hippocampal plasticity, including neurogenesis. However, the signalling mechanisms that underlie exercise-induced enhancement of hippocampal neurogenesis and the contribution of this form of plasticity to the cognitive enhancing ability of exercise are not yet identified. In contrast to exercise, ageing is associated with a decrease in adult hippocampal neurogenesis and a decline in hippocampal-dependent memory; again the contribution of neurogenesis to the cognitive decline observed in ageing is not known. The main objectives of this thesis were to further current understanding of some of the signalling mechanisms underlying neurogenesis and identify how changes in the rate of hippocampal neurogenesis induced by exercise and ageing may impact on hippocampal-dependent spatial memory.

Using both an *in vitro* and *in vivo* approach the present study identified a novel link between two signalling systems known to regulate neurogenesis and known to be enhanced in the brain during exercise, the endocannabinoid system and the neurotrophin signalling system. Specifically, we report for the first time that endocannabinoids can increase the expression of the neurotrophins brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in cultured neurons and in the adult dentate gyrus and hippocampus proper of the Wistar rat. Having established this link we proceeded to assess whether the increase in hippocampal BDNF and neurogenesis usually observed following exercise is mediated by endocannabinoid signalling. The study focused on the functional consequences of increasing the rate of hippocampal neurogenesis, namely how it affects spatial memory. Exercise was found to increase cell proliferation and early stage survival in the dentate gyrus and this was completely blocked by administering the cannabinoid receptor type 1 (CB_1) inverse agonist AM 251 during exercise. Interestingly, exercise alone was found to have no effect on short-term spatial memory but exercise in combination with CB_1 receptor antagonism enhanced short-term spatial memory, suggesting that newly born cells in the dentate gyrus (<10 days old) do not have a role to play in short-term spatial memory formation. The cellular

mechanisms responsible for the memory enhancement observed have yet to be identified but appear to be independent of synaptic growth in the dentate gyrus and hippocampus proper. Exercise was found to enhance long-term spatial memory, something that was not affected by CB_1 receptor antagonism during exercise. Results from this study suggest that neurogenesis does not have a role to play in long-term spatial memory. Considering all of these results, we show that endocannabinoids can regulate neurotrophin expression and that exercise can increase hippocampal neurogenesis in a CB_1 receptor-dependent mechanism, a process that appears to have no implications for standard short-term and long-term spatial memory. These data suggest that exercise can induce long-term changes in the brain that do not affect short-term memory but enhance the ability to encode a long-term memory.

Finally, the contribution of hippocampal neurogenesis to the age-related decline in spatial memory was assessed. The possibility of an age-related dysfunction of neurotrophin and endocannabinoid signalling contributing to this was evaluated. Short-term spatial memory was found to be impaired by middle age and this impairment persisted to old age. Interestingly, cell proliferation and early survival was found to be decreased only in aged rats, suggesting spatial memory impairments at middle age are not due to a decline in the rate of neurogenesis. Ageing is associated with changes in BDNF expression in the dentate gyrus; however, these changes do not appear to cause the impairments in memory and neurogenesis, and age-related changes in the hippocampal function do not appear to result from altered CB_1 receptor expression.

Taken together, these results suggest that some of the neurophysiological processes regulated by the endocannabinoid system may be mediated, at least in part, by neurotrophin signalling. The results presented here also indicate that adult hippocampal neurogenesis may not be important for short-term or long-term spatial memory. These results also suggest that exercise can enhance long-term spatial memory but not short-term spatial memory through an as yet unidentified mechanism

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List of abbreviations

AC	Adenylate cyclase
AEA	Anandamide
Akt	Protein kinase B
AM 251	1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3-carboxamide
ANOVA	Analysis of variance
ATP	Adenosine-5'-triphosphate
BDNF	Brain derived neurotrophic factor
BrdU	5'-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
CA 1	Cornu Ammonis 1
CA 2	Cornu Ammonis 2
CA 3	Cornu Ammonis 3
CA 4	Cornu Ammonis 4
CBD	Cannabidiol
CB_1	Cannabinoid type 1 receptor
CB_2	Cannabinoid type 2 receptor
CNS	Central nervous system
CREB	c-AMP response element-binding protein
CT	Cycle number
DAG	Diacylglycerol
DAGL	Diacylglycerol lipase
DCX	Doublecortin

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extacellular signal-related kinase
FAAH	Fatty acid amide hydrolase
FLAT	Fatty acid amide hydrolase-like anadamide transporter
GCL	Granular cell layer
GDP	Guanosine diphosphate
GFAP	Glial fibrillary acidic protein
GTP	Guanosine-5'-triphosphate
HRP	Horseradish peroxidase conjugate
IgG	Immunoglobulin G
IP	Intraperitoneal
IP_3	Inositol trisphosphate
JNK	c-Jun N-terminal kinases
KCl	Potassium chloride
K_i	Dissociation constant
KO	Knockout
LTP	Long-term potentiation
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
mRNA	Messenger ribonucleic acid
MW	Molecular weight
MWM	Morris water maze
NAPE	*N*-Acylphosphatidylethanolamine
NAT	N-acetyltransferase
NPC	Neural progenitor cell
NSC	Neural stem cell

OCT	Optical cutting temperature
OD	Opitical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-Tween®20
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
pERK	Phosphorylated ERK
PI	Phosphatidylinositol
PI3K	Phosphotidylinositol-3 kinase
PKC	Protein kinase C
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
RT-PCR	Real-time polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SGZ	Subgranular zone
SVZ	Subventricular zone
TBS	Tris buffered saline
TBS-T	Tris buffered saline-Tween®20
TMB	3,3',5,5'-Tetramethyl-benzidine
Trk A	Tropomyosin receptor kinase A
Trk B	Tropomyosin receptor kinase B
2-AG	2-Arachidonylglycerol

Δ^9-THC	Δ^9-Tetrahydrocannabinol

Chapter 1

General introduction

Chapter 1

1.1 Cannabis; a brief history

"when pure and administered carefully, it [cannabis] *is one of the most valuable medicines we possess." Sir John Russell Reynolds, Physician-in-Ordinary to HM Queen Victoria, 1890*

The cannabis plant has been utilised by humans for thousands of years as a raw material for manufacturing and as a drug both recreationally and medicinally. The first recorded use of cannabis was approximately 4000 B.C. in China where fibres from the plant were used for the manufacturing of rope and textiles. It has been suggested that it was used medicinally in ancient China for pain, constipation and malaria; however, it was in India where its medicinal use really began. The use of cannabis in India dates back to approximately 1000 B.C. where it was used for treatment of various different disorders including anxiety, epilepsy, rabies, tetanus and as a general painkiller/anti-inflammatory.

The introduction of cannabis to Western medicine is accredited to the Irish physician William O'Shaughnessy who served for the British Army in India. Cannabis was often used in 19th Century Europe to treat pain and was even prescribed to Queen Victoria for menstrual pain. In an article in the Lancet in 1890 the Queen's physician Sir John Russell Reynolds described cannabis as one of the most valuable medicines available. Despite its popularity the introduction of medications that could be more efficiently administered such as aspirin and intravenous morphine brought about a decline in the use of cannabis as a medicine. In the 1920s some countries, perhaps in error, began to consider cannabis to be an extremely harmful drug; its recreational use in the UK was banned in the late 1920s, its medicinal use continued but was eventually banned in 1971.

Fast-forward to 2005 and cannabis is back in medicinal use. Sativex®, a drug containing Δ^9-tetrahydrocannabinol (Δ^9-THC); the main psychoactive component of the cannabis plant and cannabidiol (CBD); a non-psychoactive substance (possibly with anti-psychotic properties) found in the cannabis plant is being prescribed for treatment of neuropathic pain associated with multiple sclerosis. In fact, it is possible that Sir John Russell Reynolds's claim is still relevant today; there is massive therapeutic potential for cannabinoid based medicines including the treatment of neuropathic pain, loss of appetite in terminal diseases such as cancer, epilepsy, psychosis and addiction (Robson, 2013).

1.2 Cannabinoid receptors

Although cannabis use in the Western World dates back to the 19th century it was only in the 1990s that the receptors in which cannabis exerts its effects on the brain (and the rest of the body) were discovered. To date there are two known cannabinoid-specific receptors; cannabinoid type 1 (CB_1) receptor and cannabinoid type 2 (CB_2) receptor, although there is speculation as to the existence of others. CB_1 and CB_2 receptors are metabotropic receptors and their cellular effects are mediated by a G-protein linked to the intracellular domain of the receptor. CB_1 and CB_2 receptors therefore belong to a super family of receptors known as G protein-coupled receptors (GPCRs).

The majority of investigation into the physiological effects of cannabinoids focuses on signal-transduction via CB_1 and CB_2 receptors, however, it should be noted that cannabinoids can act on/interact with many different receptor types which could also account for cannabinoid-induced physiological changes. The two non-CB_1/CB_2 cannabinoid receptors that have gained the most attention are the ion channel transient receptor potential vanilloid type 1 ion channel ($TRPV_1$) and the orphan GPCR GPR 55. In addition, there is evidence to suggest that cannabinoids may activate the family of nuclear receptors known as peroxisome proliferator-activated receptors (PPARs), that regulate gene expression.

1.2.1 Cannabinoid type 1 receptor

The CB_1 receptor was first cloned from rat cerebral cortex complimentary deoxy-ribonucleic acid (cDNA) in 1990 (Matsuda *et al.*, 1990). Matsuda and colleagues concluded that it was a 473-amino-acid protein and like all GPCRs has seven hydrophobic trans-membrane domains. Soon after this a 472-amino-acid GPCR that was activated by both phytocannabinoids and synthetic cannabinoids was cloned from human brain stem and testis cDNA. This human CB_1 receptor was found to have 97.3% sequence homology to the rat CB_1 receptor (Gerard *et al.*, 1991). The molecular weight of the mature CB_1 receptor is 64 kDa, however it is also reported to exist at 59 kDa and 53 kDa depending on its glycosylation status (Song *et al.*, 1995).

The CB_1 receptor is thought to be one of the most abundant GPCRs in the brain and there is mounting evidence in the literature confirming its dense distribution throughout the central nervous system (CNS). In an experiment using the radiolabeled synthetic cannabinoid [^{3}H]CP

55,940 (a high affinity CB_1 agonist) it was found that the receptor was abundantly expressed in brain areas associated with movement, including the cerebellum and the basal ganglia as well as areas important in learning and memory like the hippocampus and the forebrain (Herkenham *et al.*, 1990). Another approach used to evaluate CB_1 receptor distribution was to quantify CB_1 receptor messenger ribonucleic acid (mRNA) expression in tissues, which indicates the level of CB_1 gene expression. Using this method it was found that CB_1 receptor was expressed mainly in the brain but also found at low levels in areas of the periphery such as the adrenal gland, the heart, the testis and some immune cells (e.g. B type white blood cells) (Galiegue *et al.*, 1995). Tsou and colleagues reported CB_1 immunoreactivity in nearly all brain areas, including the hippocampal formation, the cerebral cortex, the olfactory bulb and the midbrain. The study also reported that dendrites, axons and cell soma expressed CB_1 receptors. Intensely stained and moderately stained neurons were observed throughout the brain and interestingly the hippocampus contained only intensely stained and not moderately stained neurons suggesting that hippocampal neurons have a high level of CB_1 expression (Tsou *et al.*, 1998). These findings suggest that cannabinoid signalling via CB_1 is likely to have a substantial effect on hippocampal function including learning and memory.

There is a wide variety of cell phenotypes within the CNS expressing the CB_1 receptors. Studies have suggested that GABAergic (Hoffman *et al.*, 2000), glutamatergic (Gerdeman *et al.*, 2001) serotonergic and dopaminergic (Lau *et al.*, 2008) neurons express the receptor, where it is predominantly found on axons and synaptic terminals, however its intracellular expression on mitochondrial membranes has been recently discovered (Benard *et al.*, 2012). In addition to mature neuronal expression of the CB_1 receptor it has been shown that neural stem cells (NSCs)/neural progenitor cells (NPCs) isolated from the adult brain are immunoreactive for the receptor (Jiang *et al.*, 2005; Molina-Holgado *et al.*, 2007). Finally, glia have also been shown to express the CB_1 receptor; it was found to be present on both astrocytes (Han *et al.*, 2012) and microglia (Walter *et al.*, 2003). These data suggest that CB_1 signalling has the potential to modulate multiple transmitter systems, adult neurogenesis and glial function.

1.2.2 Cannabinoid type 2 receptor

The CB_2 receptor, perhaps incorrectly coined “the peripheral cannabinoid receptor” at the time, was first cloned in 1993 from cDNA prepared from the human promyelocytic leukaemic line HL60 (Munro *et al.*, 1993). The CB_2 receptor is composed of approximately 360-amino acids and, like the CB_1 receptor, is a seven trans-membrane domain GPCR but shares only 44% homology with CB_1. The CB_2 receptor was not preserved as well through

evolution as the CB_1 receptor; there is only 81% sequence homology between human and rat CB_2 that has been linked to the different pharmacological profiles observed between human and rat CB_2 (Mukherjee *et al.*, 2004). It was found to be activated by phytocannabinoids, synthetic cannabinoids and endogenous ligands. The CB_2 receptor has a reported molecular weight of 41 kDa and 46 kDa in its glycosylated form (Filppula *et al.*, 2004).

The mammalian distribution of the CB_2 receptor differs considerably from the CB_1 receptor. Analysis of expression of CB_2 at mRNA level revealed that the CB_2 receptor was not expressed in the brain but was primarily expressed in areas of the body associated with the immune system such as tonsils, spleen and bone marrow (Galiegue *et al.*, 1995). Using human hematopoietic cell lines Galiégue and colleagues discovered the rank order of CB_2 gene expression in blood cells; B-cells > natural killer cells > monocytes > polymorphonuclear neutrophil cells > T8 cells > T4 cells. The expression of the CB_2 receptor at both mRNA and protein level has also been confirmed in peripheral mast cells (Facci *et al.*, 1995). However, in the past decade the idea that the CB_2 receptor is "the peripheral cannabinoid receptor" has been challenged. Brain structures such as the hippocampus, the cortex and the cerebellum were found to be immunoreactive for the CB_2 receptor where it appears to be expressed on both neuronal and glial cells (Gong *et al.*, 2006). In fact there are several studies claiming neuronal expression of CB_2 however, there are as many studies arguing against this notion (Atwood *et al.*, 2010). Like the CB_1 receptor the CB_2 receptor is expressed on NSC/NPCs grown *in vitro*, suggesting a role for cannabinoid signalling in neurogenesis (Molina-Holgado *et al.*, 2007). Finally, CB_2 receptors have been shown to be expressed in both astrocytes and microglia, however it appears that its expression in these cells is only induced under certain pathological conditions such as Alzheimer's disease (Benito *et al.*, 2003) and multiple sclerosis (Benito *et al.*, 2007).

1.2.3 Signal transduction through cannabinoid receptors

Cannabinoid receptors, like all GPCRs, are ligand-activated receptors and their activation requires binding of an agonist (i.e. a cannabinoid). The three main types of cannabinoids are: plant-derived cannabinoids known as phytocannabinoids, synthetic cannabinoids and endogenous cannabinoids known as endocannabinoids. Cannabinoid receptors are classical GPCRs linked to $G_{i/o}$ proteins. Binding of an agonist activates the receptor and brings about a conformational change causing the receptor to activate the $G_{i/o}$ -protein that it is linked to (the G-protein is activated when the receptor removes the guanine diphosphate [GDP] molecule bound to the G-protein and replaces it with a molecule of guanine triphosphate [GTP]). Activation of the G-protein liberates the $\beta\gamma$ and α subunits.

The βγ subunits of G-proteins primarily act on plasma membrane ion channels. In the case of the CB_1 receptor the βγ subunit acts to inhibit voltage gated N- and P/Q type Ca^{2+} channels, which inhibits intracellular Ca^{2+} currents (see Figure 1.1). This cannabinoid inhibition of Ca^{2+} influx was found in neuroblastoma cells (Caulfield *et al.*, 1992) and cultured hippocampal neurons (Twitchell *et al.*, 1997). The inhibition of intracellular Ca^{2+} currents was not seen when cannabinoids were co-administered with N- and P/Q Ca^{2+} channel blockers confirming that it is specifically this subset of Ca^{2+} channels that are affected by the CB_1 receptor.

Cannabinoid receptor activity also modulates K^+ currents (see Figure 1.1), although it should be noted that it remains elusive as to whether this is due to second messenger activity or βγ subunit activity. Mackie and colleagues found that the synthetic cannabinoid WIN 55, 21 2-2 activates inwardly rectifying K^+ channels, which are important for maintaining resting membrane potential (Mackie *et al.*, 1995). Cannabinoids have also been shown to affect K^+ efflux. WIN 55, 21 2-2 has been shown to stimulate A-type K^+ channels, which are modulated by cyclic adenosine monophosphate (cAMP) activity (suggesting it may not be a direct effect of the G-protein βγ subunit) in hippocampal cell cultures (Deadwyler *et al.*, 1995). A consequence of this cannabinoid induced increase in outward K^+ currents and decrease in inward Ca^{2+} currents is hyperpolarisation of a cell which in neurons leads to decreased neurotransmitter release (Wilson *et al.*, 2002).

The α subunit of the G-protein inhibits adenylate cyclase, a membrane bound enzyme responsible for the production of cAMP from adenosine triphosphate (ATP). This inhibition of cAMP production can affect many different signalling cascades, one of which is protein kinase A (PKA) activity (see Figure 1.1). PKA can affect the activity of other proteins/enzymes via phosphorylation (at serine or theronine sites) and it can also regulate gene transcription via phosphorylation of the transcription factor cAMP response element binding (CREB). PKA has been implicated in suppression of activation of the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinases (ERK) due to inhibition of raf-1. This is consistent in relation to cannabinoid signalling; ERK 1 and ERK 2 are both activated in a cell line expressing human CB_1 receptors following application of synthetic cannabinoids (Bouaboula *et al.*, 1995). In neurons endocannabinoids, synthetic cannabinoids and phytocannabinoids increased neuronal ERK activity *in vitro* and *in vivo* (Derkinderen *et al.*, 2003). This increase in ERK activation was reversed when cells were pretreated with a cAMP analogue illustrating that this increase is dependent on a reduction in cAMP production. Cannabinoids have also been linked to activation of another protein kinase, protein kinase B (PKB) also known as Akt. Both Δ^9-THC and the synthetic cannabinoid CP-55940 increase Akt activation via CB_1 receptor/ phosphatidylinositol 3-

kinase (PI3K) dependent mechanism (Gomez del Pulgar *et al.*, 2000). More recently it has been shown that cannabinoid activation of the PI3K/Akt pathway may not be exclusive to the CB_1 receptor as CB_2 specific agonists can also activate the pathway (Gomez *et al.*, 2011). The exact mechanism by which cannabinoid receptors activate the PI3K/Akt pathway (i.e. whether it is by direct G-protein-PI3K interaction or an indirect consequence of adenylate cyclase suppression) remains to be identified. ERK and Akt are responsible for many cellular functions and are especially important in promoting cell survival; differentiation and migration, which may account for the pivotal role cannabinoids have to play in these processes.

In addition to ERK the CB_1 receptor is involved in activating another group of MAPKs; c-Jun N-terminal kinases (JNKs). Δ^9-THC, the synthetic cannabinoid HU-210 and the endocannabinoid anandamide have been shown to increase JNK activation in Chinese hamster ovary cells transfected with rat CB_1 receptor cDNA and this increase in JNK activation was reversed by co-treatment with the CB_1 antagonist SR 141716 (Rueda *et al.*, 2000). JNK is a pro-inflammatory mediator and an increase in JNK activity has been implicated in the neurotoxicity associated with Δ^9-THC use (Downer *et al.*, 2003).

The above description of cannabinoid receptor signalling focuses mainly on signalling through the CB_1 receptor; signalling through the CB_2 receptor follows very similar patterns and the majority of signalling cascades activated by CB_1 are also activated by CB_2 receptors (Basu *et al.*, 2011).

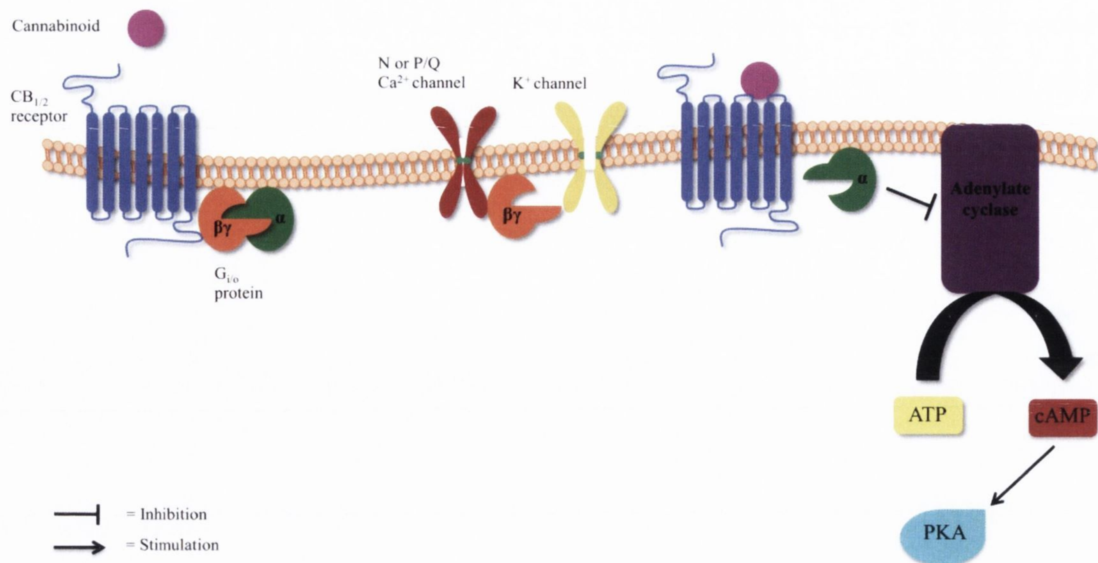

Figure 1.1 Signal transduction through the CB_1/CB_2 receptor

CB_1/CB_2 are G protein coupled receptors linked to $G_{i/o}$ G proteins. When a ligand (i.e. a cannabinoid) binds to the receptor the receptor is activated leading to liberation of the G protein subunits. The βγ subunit induces opening of plasma membrane K^+ channels and closing of Ca^{2+} channels. The α subunit acts to inhibit activity of the membrane bound enzyme adenylate cyclase, which is responsible for producing cAMP from ATP. cAMP is an important cell signalling molecule and one of its functions is to activate cAMP dependent protein kinase (PKA). By phosphorylating various other proteins/target molecules, PKA can activate other signalling pathways and have many effects on cell function.

1.3 Endogenous cannabinoids (endocannabinoids)

Following the cloning of a receptor that was activated by phytocannabinoids contained in the cannabis plant (the CB_1 receptor) researchers began to search for endogenous cannabinoid receptor ligands. This search would eventually lead to the discovery of the endocannabinoid system; a signalling system that heavily influences how we learn and remember, how we move, how we eat, how we fight disease, how we feel pain and how we sleep. The endocannabinoid system is comprised of cannabinoid receptors, their endogenous ligands and the enzymes responsible for synthesising and degrading them. The two endocannabinoids that have been studied in most detail are N-arachidonoylethanolamide also known as anandamide and 2-arachidonoylglycerol.

1.3.1 Anandamide

Anandamide (AEA) was the first endocannabinoid to be identified, it was discovered in the porcine brain in 1992 (Devane *et al.*, 1992). AEA is a phospholipid derived molecule that is an agonist at the CB_1 and CB_2 receptor, however it has a higher affinity for CB_1 (K_i=~400

nM); CB_2 (K_i=~1760 nM). AEA is present in blood plasma and throughout the mammalian brain; in particular it is found at high concentrations in the hippocampus, cerebellum and cortex (Felder *et al.*, 1998).

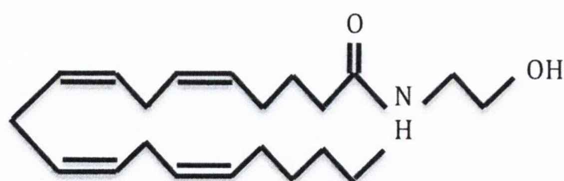

Figure 1.2 Chemical structure of anandamide

AEA is rapidly synthesised in neurons following depolarisation and subsequent Ca^{2+} influx (Di Marzo *et al.*, 1994). In neuronal signalling AEA is thought to act as a retrograde neurotransmitter; it is synthesised and released by a post-synaptic neuron, travels back across the synaptic cleft and activates receptors on the presynaptic neuron. The basic pathways involved in AEA synthesis and degradation are outlined in Figure 1.4. AEA is synthesised directly from cleavage of the phospholipid precursor present in neuronal membranes, *N*-arachidonoyl phosphatidylethanolamine (NAPE). This cleavage is catalysed by the enzyme phospholipase D (PLD), whose activity is stimulus-dependent. Alternatively NAPE can be cleaved by a phospholipase C (PLC)-catalysed reaction producing phosphorylated AEA that can then be dephosphorylated by a protein phosphatase. As AEA is a lipid-based molecule it can pass through the plasma membrane with ease and therefore acts as an efficient intercellular signalling molecule. Another important reaction in AEA synthesis that should not be overlooked is the formation of NAPE. NAPE is present in the brain at very low concentrations and it can be rapidly depleted during AEA synthesis (Piomelli, 2003). NAPE is formed from phosphatidylethanolamine (PE) in a reaction catalysed by *N*-acyltransferase (NAT).

The enzyme responsible for AEA degradation is known as fatty acid amide hydrolase (FAAH). FAAH is an intracellular membrane bound enzyme that degrades fatty-acid amides and it is responsible for inactivating AEA by catalysing its breakdown to arachidonic acid and ethanolamine (Cravatt *et al.*, 1996). The importance of FAAH in AEA degradation was re-reinforced using either of the specific FAAH inhibitors URB 597 or URB 532. Both inhibitors lead to accumulation of AEA *in vitro* and *in vivo* (Kathuria *et al.*, 2003). FAAH was found to be expressed throughout the rat brain, specifically it is expressed at high levels in pyramidal cells in the hippocampus and the cortex in close proximity to cells that express

CB_1 receptors (Tsou *et al.*, 1998). The exact mechanism by which AEA is removed from the synaptic cleft and transported to the intracellular FAAH-containing membranes was unknown for many years. The fact that AEA was efficiently broken down by intracellular FAAH suggested that the process relied on transportation of AEA across the plasma membrane rather than just relying on passive diffusion. Recently the transporter responsible for this has been identified. A membrane bound transporter that became known as FAAH-like anandamide transporter (FLAT) was found to be responsible for transport of AEA across plasma membranes (Fu *et al.*, 2012).

1.3.2 2-arachidonoylglycerol

Researchers have known of the presence of 2-arachidonoylglycerol (2-AG) in mammalian neurons since the 1980s but it was only in 1995 that it was discovered to be a cannabinoid receptor agonist. 2-AG isolated from the canine gut was found to bind both the CB_1 receptor (K_i=~472 nM) and the CB_2 (K_i=~1400 nM) receptor (Mechoulam *et al.*, 1995). Like AEA, 2-AG is found in many areas of the brain. However, the concentration of 2-AG is up to 1000 times that of AEA (Sugiura *et al.*, 1995), although other studies report a more conservative figure of 170 times that of AEA (Stella *et al.*, 1997).

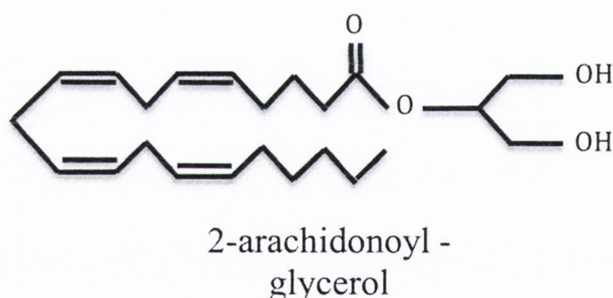

Figure 1.3 Chemical structure of 2-arachidonoyl glycerol

Similar to AEA, 2-AG is rapidly synthesised in stimulated cells via a Ca^{2+}-dependent mechanism. There are several different pathways hypothesised to be involved in 2-AG synthesis, however, the most likely pathway is shown below in Figure 1.4. Briefly, phosphatidylinositol (PI) is cleaved by phospholipase C (PLC) to form 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG is then cleaved by the enzyme diacylglycerol lipase (DAGL) to form 2-AG. The case for this pathway for 2-AG formation over other pathways is strong. Addition of exogenous PLC increases 2-AG production in mouse neuroblastoma cells (Bisogno *et al.*, 1997), and PLC and DAG lipase inhibitors respectively prevent 2-AG production in cultured cortical neurons (Stella *et al.*, 1997).

Finally, DAGLα knock out (KO) mice have an 80% reduction in 2-AG levels in the brain whereas DAGLβ KO mice have a 50% reduction in 2-AG levels (Gao *et al.*, 2010).

Deactivation of 2-AG is primarily achieved by the enzyme monoacylglycerol lipase (MAGL). MAGL was initially cloned from rat cDNA and characterised in 2002 (Dinh *et al.*, 2002). MAGL was found to be expressed at high levels in brain areas that have been identified as hotspots for endocannabinoid activity; the hippocampus, the cortex and the cerebellum. MAGL specifically catalyses 2-AG breakdown as over expression of MAGL in a cell line reduced 2-AG accumulation without affecting AEA levels. Further evidence for the role of MAGL in 2-AG inactivation was found through MAGL RNA interference. Suppressing MAGL mRNA expression in HeLa cells brought about a three-fold increase in cellular 2-AG levels (Dinh *et al.*, 2004). MAGL was found to be concentrated mainly in nerve terminals rather than cell bodies suggesting presynaptic breakdown of 2-AG (Dinh *et al.*, 2002). Unlike AEA the exact mechanism by which 2-AG is removed from the extracellular environment by cellular uptake/reuptake remains unknown. It has been suggested that this may occur by a combination of activity of a specific 2-AG transporter, simple diffusion and activity of an AEA transporter (Hermann *et al.*, 2006). Recent evidence suggests that cellular uptake of 2-AG may be coupled to AEA uptake (Chicca *et al.*, 2012).

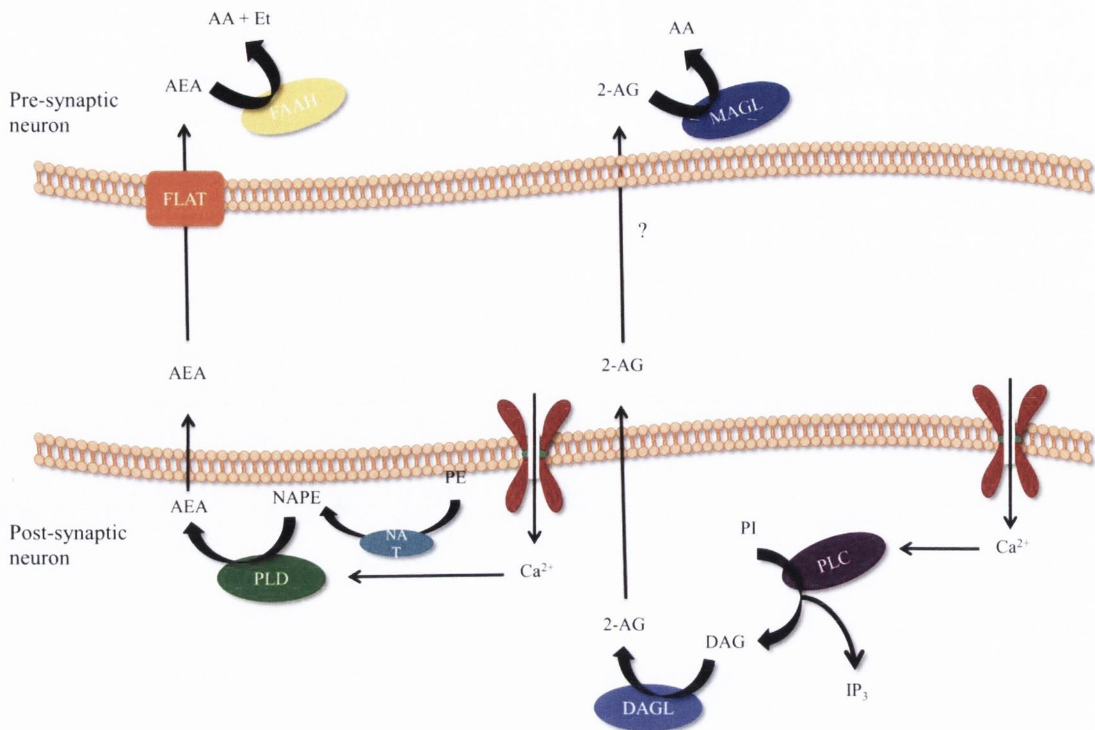

Figure 1.4 Anandamide and 2-arachidonoylglycerol synthesis and degradation in neurons

AEA and 2-AG are synthesised in a Ca^{2+}-dependent manner. Ca^{2+} enters a cell via voltage gated ion channels or is released from intracellular stores. Ca^{2+} activates phospholipase D (PLD). PLD can cleave *N*-acyl-phosphatidylethanolamine (NAPE), a product of *N*-acyltransferase (NAT) mediated phosphatidylethanolamine (PE) cleavage, to form AEA. AEA can then be released into the synapse. AEA can be taken up by a pre-synaptic cell via the AEA transporter fatty acid amide hydrolase-like anandamide transporter (FLAT) and degraded by fatty acid amide hydrolase (FAAH) to arachidonic acid (AA) and ethanolamine (Et). Ca^{2+} also activates phospholipase C (PLC) which cleaves phosphatidylinositol (PI) to form inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). DAG can then be cleaved by DAG lipase (DAGL) to form 2-AG. 2-AG can then be released into the synapse. 2-AG can be taken up by pre-synaptic cells by a mechanism that is yet to be identified and degraded by monoacylglycerol lipase (MAGL) forming AA and other products. 2-AG and AEA can also be degraded in the same cells in which they are produced. This figure uses neurons as an example; these pathways also exist in other cell types.

1.3.3 Endocannabinoid pharmacology

Common methods to manipulate an endogenous signalling system is to use agonists to activate the receptors of the system and antagonists to block activation of the receptors by endogeneous ligands. There is an abundance of drugs available to manipulate endocannabinoid signalling; including CB_1 agonists/antagonists, CB_2 agonists/antagonists and endocannabinoid 'inactivation enzyme' inhibitors (Di Marzo *et al.*, 2004). Generally researchers opt to use inhibitors of enzymes responsible for AEA or 2-AG hydrolysis rather than directly using cannabinoid receptor agonists. The reason for this is the fact that enzymatic inhibition is a more physiologically relevant method of manipulating cannabinoid signalling as it increases endocannabinoid tone in specific areas where endocannabinoids are already present whereas administering agonists leads to global cannabinoid receptor activation.

URB 597 is one of the most common FAAH inhibitors used by researchers. URB 597 inhibits the hydrolytic activity of FAAH and leads to accumulation of AEA in the rat and mouse brain (Fegley *et al.*, 2005). It was found to have no effect on AEA concentration in the brain of FAAH knockout mice. It should be noted that FAAH is not only responsible for AEA degradation but also degrades palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) and URB 597 also increases concentration of PEA and OEA in the rat and mouse brain. Both PEA and OEA are endogenous PPARα agonists (Melis *et al.*, 2013).

URB 602 is a widely used MAGL inhibitor. URB 602 specifically inhibits rat brain MAGL activity without affecting FAAH activity (Hohmann *et al.*, 2005). In rat forebrain slice cultures URB 602 increased 2-AG concentration without altering AEA concentration (Makara *et al.*, 2005).

There are several cannabinoid receptor antagonists available; the CB_1 antagonist used in this thesis is AM 251. AM 251 was found to bind CB_1 receptors in the brain following systemic administration (Gatley *et al.*, 1996). The cardiovascular effects of cannabinoids, including mesenteric vasoconstriction and hindquarter vasodilation, were found to be blocked by pre-treatment with AM 251 suggesting that the antagonist efficiently blocks binding of agonists to CB_1 receptors (Gardiner *et al.*, 2002).

1.4 Neurotrophic factors

Neurotrophic factors or neurotrophins are a family of polypeptide growth factors primarily affecting development, growth, survival and functioning of both developing

and mature neurons. Neurotrophins therefore have a pivotal role to play in brain functioning, particularly brain plasticity (e.g. synaptic plasticity and adult neurogenesis) and as a consequence have an important role to play in learning and memory. The first neurotrophin identified, nerve growth factor (NGF) was initially discovered in 1951 in ground-breaking research carried out by Rita Levi-Montalcini (Levi-Montalcini *et al.*, 1951). To date a total of four mammalian neurotrophins have been identified: NGF, brain-derived neurotrohic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5). All four neurotrophins have a low-affinity for the pan neurotrophin receptor ($p75^{NTR}$) and a high-affinity for a specific tropomyosin-receptor-kinase (Trk) receptor subtype; NGF has a high affinity for TrkA, BDNF and NT-4/5 have a high affinity for TrkB and NT-3 has a high affinity for TrkC (Lu *et al.*, 2005).

1.4.1 NGF-TrkA signalling

As mentioned above, identification and characterisation of NGF began in the early 1950s; pioneering work from Rita Levi-Montalcini showed that without the release of NGF from target cells, sensory and sympathetic neurons innervating these cells die. The NGF protein exists as a complex of α, β and γ subunits. The β subunit is the biologically active subunit that, under physiological conditions, exists as a protein dimer with a molecular weight of 26.5 kDa (Bothwell *et al.*, 1977). Like most proteins NGF is initially synthesised as a precursor protein known as proNGF (approximate molecular weight 35 kDa), it is then cleaved in the Golgi apparatus by the enzyme furin producing mature NGF (mNGF) and released constitutively from the cell (Mowla *et al.*, 1999).

mNGF was found to have a high affinity for Trk (later renamed TrkA) receptor (Kaplan *et al.*, 1991). Many different cell types have been found to be responsive to NGF, presumably through TrkA receptor activity. TrkA mRNA has been shown to be expressed in the adult hippocampus (Cellerino, 1996) and also on cultured adult NSCs (Keung *et al.*, 2013).

TrkA, like all mammalian Trk receptors, is a single transmembrane domain receptor. It has an extracellular component that contains a ligand binding site and an intracellular tyrosine kinase containing-region. Activation of a Trk receptor by a neurotrophin leads to dimerisation of the receptor with another activated Trk receptor. Following dimerisation, the receptor undergoes autophosphorylation requiring the liberation of phosphate (P) from several ATP molecules. The now phosphorylated intracellular domains of the receptor can interact with and activate several different enzymes and adaptor proteins. Once activated Trk receptors lead to induction of three major signalling cascades

(signalling cascades activated by Trk receptors can be seen in Figure 1.5); the MAPK/ERK pathway, the PI3K/Akt pathway and the phospholipase C (PLC)γ pathway. NGF induces ERK, Akt and PLCγ activation in neuronal PC12 cells in a TrkA-dependent manner (Lecht *et al.*, 2010). Another study found NGF promotes proliferation of epithelial cells via activation of ERK and Akt (Hong *et al.*, 2012).

1.4.2 BDNF-TrkB signalling

BDNF was initially discovered and purified from the porcine brain in the early 1980s. It was found to support the survival of neurons both *in vitro* (Johnson *et al.*, 1986) and *in vivo* (Hofer et al., 1988). It was fully characterised in 1989 (Leibrock *et al.*, 1989) when it was first cloned and found to be expressed mainly in the CNS. Like NGF, BDNF can exist as both proBDNF (approximate molecular weight 32 kDa) and mature BDNF (mBDNF) [approximate molecular weight 14.5 kDa] (Seidah *et al.*, 1996). In neurons BDNF protein is synthesised in the classical manner as proBDNF and packaged in secretory vesicles in the Golgi apparatus. proBDNF can be converted to mBDNF by the intracellular enzymes furin or protein convertase 1 before the vesicles are subjected to constitutive release from the cell soma or trafficked to synaptic terminals to undergo regulated (usually Ca^{2+}-dependent) release. BDNF is not as efficently processed in the Golgi apparatus as NGF and therefore is also released as proBDNF. proBDNF can then be converted to mBDNF in the extracellular environment by plasmin or matrix metalloproteinase (Lu *et al.*, 2005). BDNF is expressed throughout the brain and at particularly high levels in the cerebral cortex and the hippocampus where BDNF mRNA expression is 50 times that of NGF (Hofer *et al.*, 1990).

As mentioned above BDNF has a high affinity for the TrkB receptor. The TrkB receptor is widely distributed throughout the mammalian brain and is expressed at particularly high levels in areas associated with learning and memory (e.g. the hippocampus). In the adult hippocampus, TrkB is expressed on mature (Chakravarthy *et al.*, 2006) and developing neurons (Donovan *et al.*, 2008).

Binding of BDNF to TrkB leads to activation of the receptor in a similar fashion to NGF activation of TrkA described above. Activation of the three general Trk receptor-signalling pathways; the MAPK/ERK pathway, the PI3K/Akt pathway and the PLCγ pathway (shown in Figure 1.5) is consistent with BDNF-TrkB signalling. Overexpression of BDNF in mesenchymal stem cells (MSCs) is associated with phosphorylation (activation) of TrkB, which is coupled to phosphorylation of ERK and, as a consequence, neuronal differentiation (Lim *et al.*, 2011). The differentiation of the cells in this case is

dependent on TrkB activation of the ERK pathway. Another study found that treatment of MSCs with BDNF also induced activation of the PI3K/Akt pathway (as well as the MAPK/ERK pathway) and that blocking BDNF-induced Akt activation impaired the survival of newly generated neurons (Barnabe-Heider *et al.*, 2003). Finally, BDNF-TrkB interaction activates the PLCγ pathway resulting in both Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) activation. BDNF, via a PLCγ-dependent rise in intracellular Ca^{2+}, has been shown to increase CaMKII phosphorylation in the hippocampus (Blanquet *et al.*, 1997). CaMKII is an enzyme that is required for induction of some forms of synaptic plasticity and will be discussed in more detail in Section 1.6.2.1. PKC activation by BDNF can have many effects on cell function as PKC can induce transcription of a wide variety of genes.

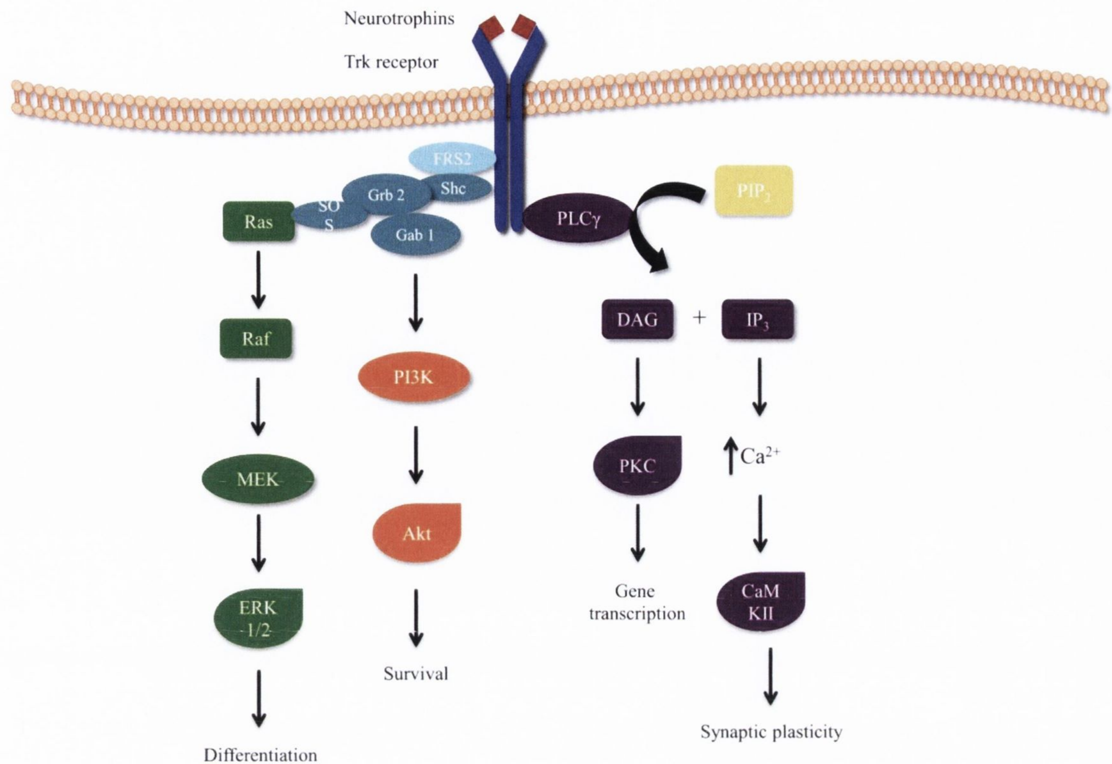

Figure 1.5 Signal transduction through Trk receptors

Binding of neurotrophin molecules to Trk receptors leads to dimerisation of the receptor and subsequent autophosphorylation of its intracellular domains. Shc acts as a linker protein that binds to a specific phosphorylated site on the Trk receptor and recruits the Grb-SOS complex. SOS activates Ras by replacing the GDP molecule bound to it with a GTP molecule. Ras activates the kinase Raf that phosphorylates MEK; MEK then acts to phosphorylate ERK 1 and ERK 2. The Grb-SOS complex also acts to directly activate PI3K, which phosphorylates Akt. Trk receptor activation also activates the phospholipase PLCγ that cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) producing DAG and inositol trisphosphate (IP_3). DAG interacts with and activates PKC whereas IP_3 induces release of Ca^{2+} from intracellular stores leading to activation of Ca^{2+}-dependent enzymes such as CaMKII.

1.5 Endocannabinoids and neurotrophins

To date there has been almost no investigation into the possibility of a link between the endocannabinoid system and neurotrophin signalling. The electrophysiological effects of BDNF in the neocortex (i.e. the spontaneous suppression of IPSCs) is blocked by CB_1 receptor antagonists and by inhibiting the synthesis of 2-AG, suggesting that BDNF induces release of endocannabinoids from neocortical cells (Lemtiri-Chlieh *et al.*, 2010). In addition to this, AEA has been shown to increase TrkB receptor phosphorylation in developing interneurons in a CB_1 receptor-dependent manner (Berghuis *et al.*, 2005).

However no study has examined the effect of cannabinoids on expression of BDNF or the TrkB receptor.

There has been very little investigation into an interaction between cannabinoids and NGF signalling but has been shown recently that NGF can increase 2-AG concentration and CB_1 receptor expression in cultured neurons (Keimpema *et al.*, 2013).

1.6 Adult hippocampal neurogenesis

In 1965, a study was published showing that cells in the adult rat dentate gyrus had the ability to undergo mitosis, and this prompted the authors to introduce the phenomenon of adult hippocampal neurogenesis (Altman *et al.*, 1965). This discovery was met with widespread scepticism as the brain was considered to be incapable of neurogenesis beyond early development. Scepticism continued well into the 1990s until it was shown that the mammalian brain contained NSCs that were capable, under the right conditions, to produce neurons, astrocytes and oligodendrocytes (Gage, 2000). Since Fred Gage's review was published in 2000 our understanding of the process of adult neurogenesis has come a long way. Actively dividing NSCs have been discovered in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus. As they begin differentiation NSCs from the SVZ migrate to the olfactory bulb where they mature into interneurons and NSCs in the SGZ migrate to the granular cell layer of the dentate gyrus where they become mature granular neurons. Once mature, these newly born neurons can integrate into neuronal networks and form synaptic connections with interneurons, mossy cells and CA3 pyramidal cells where they release glutamate as their neurotransmitter (Toni *et al.*, 2008). A brief summary of the process of adult neurogenesis in the dentate gyrus including the precursor cell subtypes involved can be seen in Figure 1.6.

In the adult rat there is thought to be approximately 9,000 new cells generated each day and 5-12 days following the birth of these cells approximately 50% express neuronal markers. It is estimated that each month the number of new granular cells generated equates to about 6% of the total granular cell number (Cameron *et al.*, 2001). It is because of this regular dramatic remodelling of the hippocampal circuitry that adult neurogenesis is thought to have a profound effect on hippocampal function, particularly learning and memory. The rate of neurogenesis is particularly sensitive to environmental cues and some of the factors that influence this rate of neurogenesis such as age; physical activity and learning will be discussed in more detail later.

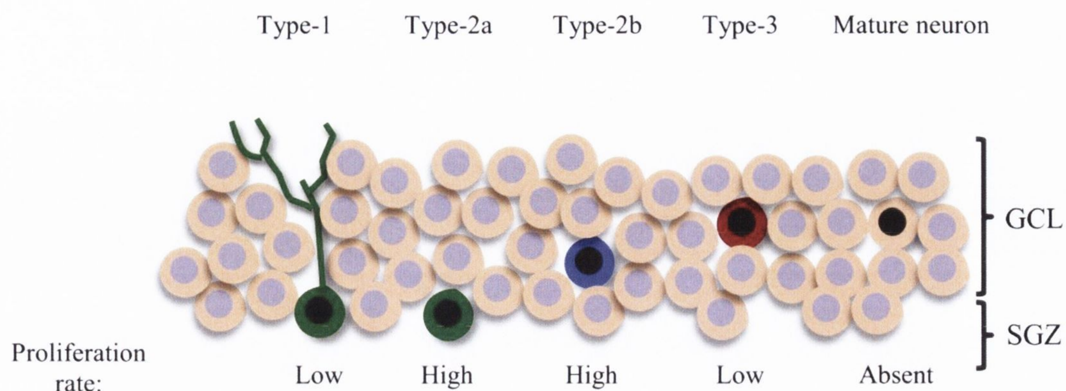

Figure 1.6 Adult neurogenesis in the dentate gyrus

There are four types of NPC in the dentate gyrus. The different cell types can be distinguished from each other based on proliferation rate, protein expression and morphology. Type-1 NPC cells are radial glial-like cells; they express the astrocytic protein glial fibrillary acidic protein (GFAP) and the intermediate filament protein nestin which is only expressed in NPCs or developing neurons. Type-1 NPCs have a low proliferation rate and can give rise to Type-2a NPCs. Type-2a NPCs do not express GFAP or have a radial process however they do maintain nestin expression. Type-2a NPCs have a high proliferation rate and under the right conditions can give rise to type-2b cells. Type-2b cells are neuronal determined NPCs; they express the immature-neuron specific protein doublecortin (DCX) and maintain nestin expression. They have a high proliferation rate and as they begin neuronal differentiation they migrate upwards from the SGZ to the granular cell layer (GCL). The fourth type of NPC is the type-3 cell; it maintains DCX expression but no longer expresses nestin. It has a low proliferation rate and it continues to migrate into the GCL as it is transformed from an immature neuron to a fully mature granular neuron. Once the neuron is mature it can integrate into synaptic networks; it will receive inputs from the entorhinal cortex/interneurons and project mossy fibre axons to the CA3 region of the hippocampus. It can take several months for a newly born neuron to fully mature but after approximately three weeks it will begin to resemble a mature granular cell with regard to morphological and electrophysiological properties (Suh *et al.*, 2009).

1.6.1 Signalling in adult hippocampal neurogenesis

The fate of NSCs in the adult brain is influenced by a wide variety of signalling molecules present in their microenvironment including neurotransmitters (Song *et al.*, 2012), growth factors (Fournier *et al.*, 2012) and cytokines (Vallieres *et al.*, 2002). In

addition to these the endocannabinoid system and neurotrophin signalling have an important role to play in regulating proliferation, differentiation and survival of NSCs in the adult brain.

1.6.1.1 Cannabinoids and adult hippocampal neurogenesis

There are several lines of evidence pointing to a role for endocannabinoid signalling in adult hippocampal neurogenesis. Firstly, one of two specific neurogenic regions in the adult brain is the hippocampus, which is a hotspot for endocannabinoid activity. Enzymes responsible for the synthesis and degradation of endocannabinoids, as well as endocannabinoids themselves, are highly concentrated throughout the hippocampus (see sections 1.3.1 and 1.3.2). Secondly, NSCs/NPCs in the adult brain express both CB_1 and CB_2 receptors and therefore are directly responsive to cannabinoids (see sections 1.2.1 and 1.2.2). Finally, cannabinoids activate intracellular signalling cascades associated with cell proliferation, differentiation and survival (e.g. the ERK pathway and the PI3K pathway) [see section 1.2.3]. It is likely that for these reasons recent neurogenesis research has focused on the endocannabinoid system.

The effect of administration of exogenous cannabinoids on adult neurogenesis varies depending on the drug and duration of treatment. Chronic treatment with the synthetic cannabinoid HU-210 enhances NSC proliferation in the rat brain (Jiang *et al.*, 2005) whereas chronic Δ^9-THC administration decreases cell proliferation in the mouse brain without affecting net neurogenesis (Wolf *et al.*, 2010). The same study found that chronic oral CBD administration also decreased cell proliferation, however, interestingly it induced an increase in net neurogenesis suggesting a strong role for cannabinoids in promoting survival and/or differentiation of neural precursor cells. There appears to be no effect of acute administration of exogenous cannabinoids on NSC/NPC proliferation whether they are synthetic (Mackowiak *et al.*, 2007) or plant derived (Kochman *et al.*, 2006).

As mentioned above, NSCs/NPCs are immunoreactive for cannabinoid receptors and one of two specific regions in the adult brain where they have the potential to proliferate and differentiate, the hippocampus, is an endocannabinoid-rich area. Furthermore, NPCs obtained from adult mice have been shown to express FAAH while NPCs obtained from rat embryos produce both AEA and 2-AG in a Ca^{2+}-dependent manner (Aguado *et al.*, 2005). Whether or not adult NPCs produce endocannabinoids remains to be tested.

Taken together these findings suggest a regulatory role for the endocannabinoid system in neurogenesis. In fact knockdown of the enzyme responsible for AEA hydrolysis, FAAH, increases cell proliferation in the dentate gyrus of adult mice (Aguado *et al.*, 2005). Complete knockdown of the α subtype of the DAGL enzyme (DAGLα) reduces brain 2-AG and AEA levels by approximately 80% and 40% respectively and leads to a decrease in cell proliferation rate and a 50% reduction in immature doublecortin (DCX) positive neurons in the hippocampus (Gao *et al.*, 2010). The same study shows that a reduction in central 2-AG alone can also interfere with neurogenesis; knockdown of the DAGL β subtype reduces 2-AG levels in the brain without significantly affecting AEA and results in a decrease in cell proliferation in the hippocampus. Further evidence supporting a role for endocannabinoid signalling in adult hippocampal neurogenesis can be found in studies involving cannabinoid receptor knock-out animals; a $CB_1^{-/-}$ genotype is accompanied by a 50% decrease in proliferating cells in the dentate gyrus (Jin *et al.*, 2004; Kim *et al.*, 2006) whereas $CB_2^{-/-}$ animals also exhibit a decreased proliferation rate (Palazuelos *et al.*, 2006).

The effects of cannabinoids on the process of adult neurogenesis appear to be mediated by both the CB_1 and the CB_2 receptor and in recent years the signalling pathways downstream of receptor activation responsible for these effects have been investigated. In order to carry out these investigations many studies have opted to use cultured NSCs. The CB_1 specific agonist arachidonyl-2'-chloroethylamide (ACEA) was found to induce neuronal differentiation and maturation of NSCs isolated from mouse embryos (Compagnucci *et al.*, 2013). Specifically, ACEA enhanced neurite outgrowth and branching complexity, which could potentially be due to the temporal effects it has on cellular ERK activity. However, caution is urged as this may or may not be applicable to adult NSCs; another study found that CB_1 activity had no effect on ERK phosphorylation in a NSC cell line (Sutterlin *et al.*, 2013), suggesting that the results may be specific to the age/type of NSC used. CB_2 receptor agonists were found to enhance proliferation rather than differentiation in both embryonic NSCs (Molina-Holgado *et al.*, 2007) and a NPC cell line (Palazuelos *et al.*, 2012). The latter study attributed CB_2 mediated activation of the PI3K/Akt/mammalian target of rapamycin C1 (mTORC1) signalling pathway as a mechanism for this, something that was also replicated in an *in vivo* model.

As alluded to earlier, certain factors such as age and physical activity can affect rate of neurogenesis and there is a case for the endocannabinoid system mediating these activity-dependent rate alterations. This will be discussed in more detail later under the headings of ageing and exercise.

In summary, cannabinoids have a role to play in adult neurogenesis, likely through activation of both CB_1 and CB_2 receptors. In recent years some of the signalling pathways involved in cannabinoid-mediated regulation of neurogenesis have been identified, however further investigation is required to characterise the specific roles for cannabinoids in the process.

1.6.1.2 Neurotrophins and adult hippocampal neurogenesis

The signalling pathways activated by the Trk family of neurotrophin receptors are pathways that in general are thought to promote proliferation, differentiation and survival. BDNF and NGF have been shown to affect cell fate in many different cell types including NSCs/NPCs and immature neurons.

There is considerable evidence to suggest that BDNF is one of the key regulators of neurogenesis in the adult hippocampus. A common method of labelling proliferating cells in the dentate gyrus is to administer the thymidine analogue 5-bromo-2'deoxyuridine (BrdU), which incorporates into the DNA of cells during the S-phase of the cell cycle. This method allows post-mortem quantification of BrdU positive cells using immunohistochemistry. Intrahippocampal infusion of BDNF was found to increase the number of cells positive for BrdU and the mature neuron specific protein Neuronal Nuclei (NeuN) suggesting that BDNF increases neurogenesis (Scharfman *et al.*, 2005). Furthermore, knockdown of BDNF expression in the dentate gyrus by region- specific RNA interference reduces neurogenesis (Taliaz *et al.*, 2010). Specifically, BDNF induces differentiation and survival of immature neurons however, whether it affects proliferation remains controversial. Expression of TrkB in adult NSCs while they are in the cell cycle is low but expression in immature neurons that have exited the cell cycle is high (Donovan *et al.*, 2008), suggesting that BDNF-TrkB signalling may be more important in the latter stages (differentiation and survival) of the neurogenesis process. This notion is further supported by the fact that deletion of TrkB *in vivo* has no effect on the number of cells exiting the cell cycle, however dendritic spine growth and the survival of immature neurons was compromised (Bergami *et al.*, 2008). Knockdown of BDNF in the dentate gyrus was also found to have no effect on proliferation rate although there was a reduction in net neurogenesis (Taliaz *et al.*, 2010). Although BDNF knockdown does not affect baseline cell proliferation it is required for some forms of activity-dependent increases in cell proliferation (Choi *et al.*, 2009). This will be discussed in more detail in Section 1.7.2.

NGF, although not as extensively studied as BDNF in relation to adult neurogenesis can affect cell fate in the adult brain. Chronic intracerebroventricular infusion of NGF was found to increase cell proliferation in the dentate gyrus (Birch *et al.*, 2013a). This method of NGF administration was also shown to enhance survival of DCX-expressing immature neurons in the dentate gyrus (Frielingsdorf *et al.*, 2007).

Like cannabinoids, neurotrophins have been implicated in altering neurogenic rate following physical activity or during ageing and this will be discussed in detail below.

1.7 Learning and memory

The capabilities of an individual to learn and remember are two actions that are of paramount importance to everyday life. Due to the importance of these processes they have been extensively investigated for many years, however, despite this it could be argued that the mechanisms underlying learning and memory remain poorly understood.

Learning at its simplest can be defined as the ability to acquire knowledge from the external environment. Memory could be seen as the ability to retain and retrieve learned information; it has been defined as "the process by which knowledge of the world is encoded, stored and later retrieved." (Kandel *et al.*, 2000). Memory can be divided into two distinct sub-types; declarative or explicit memory and non-declarative or implicit memory. Explicit memory refers to factual memory (i.e. memory relating to places or people), whereas implicit memory is usually unconscious memory that refers to information on how to perform a task (i.e. motor skills). These two distinct forms of memory are thought to be formed by similar physiological mechanisms involving functional changes in the neural circuitry of the brain; these mechanisms will be described later. What appears to be the difference between these forms of memory is the brain regions involved. For example implicit memory is thought to involve areas such as the cerebellum, the striatum and the neocortex whereas explicit memory is dependent on areas of the medial temporal lobe such as the perirhinal and entorhinal cortices, the dentate gyrus and the hippocampus proper (Squire, 2004); Figure 1.7 is a flow chart of some of the brain regions involved in explicit memory.

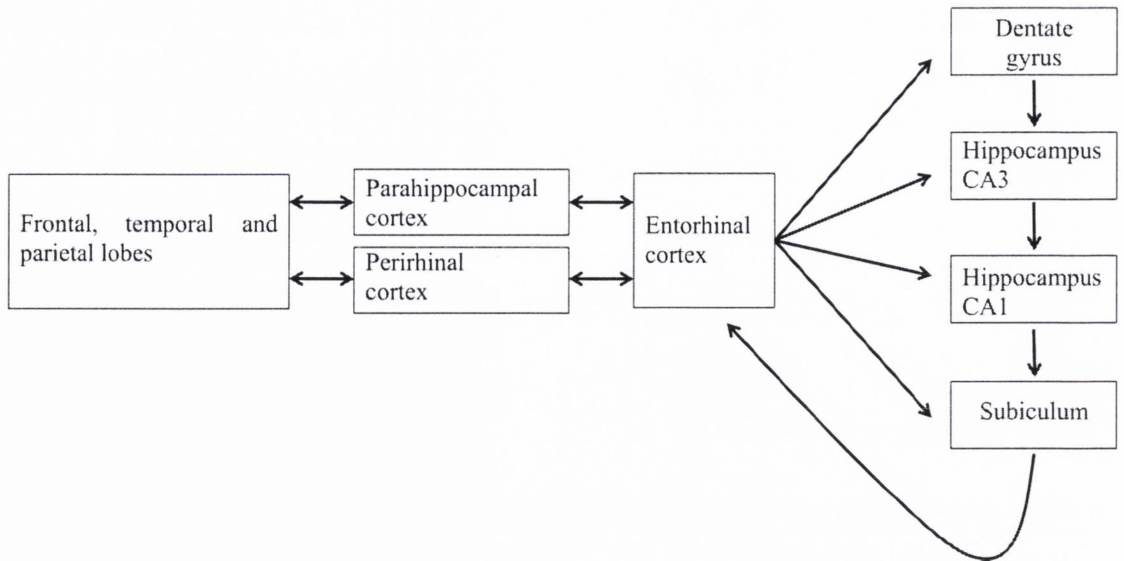

Figure 1.7 Brain areas involved in explicit memory (adapted from Kandel, 2000)

Information from the external environment is initially acquired through the various sensory areas of the brain located in the frontal, temporal and parietal lobes. After interpretation this information is transferred to the parahippocampal and perirhinal cortices. It is then transferred to the entorhinal cortex that sends information to various areas of the hippocampus including the dentate gyrus, cornu ammonis (CA) 3, CA1 and the subiculum. The information is then sent back to the entorhinal, perirhinal and parahippocampal cortices.

1.7.1 The hippocampus

Of the brain areas involved in explicit memory the hippocampus has possibly gained the most attention. The hippocampus is a bilateral brain region located in the medial temporal lobe in humans and can be found positioned between the thalamus and the cerebral cortex in rodents. The hippocampus is usually referred to as either the hippocampus proper that consists of the cornu ammonis (CA) areas; CA1, CA2, CA3 and CA4 or the hippocampal formation that consists of the dentate gyrus, the hippocampus proper and the subiculum. Areas of the hippocampal formation, namely the dentate gyrus and CA3 regions, receive input from the entorhinal cortex via the perforant pathway. The dentate gyrus projects axons known as mossy fibers to the CA3 region where they synapse with pyramidal neurons. The CA3 region projects Schaffer collateral axons to the CA1 region. The CA1 region then projects axons to the subiculum and to cortical areas including the entorhinal cortex (Deng *et al.*, 2010); a diagram of the basic hippocampal circuitry can be seen in Figure 1.8.

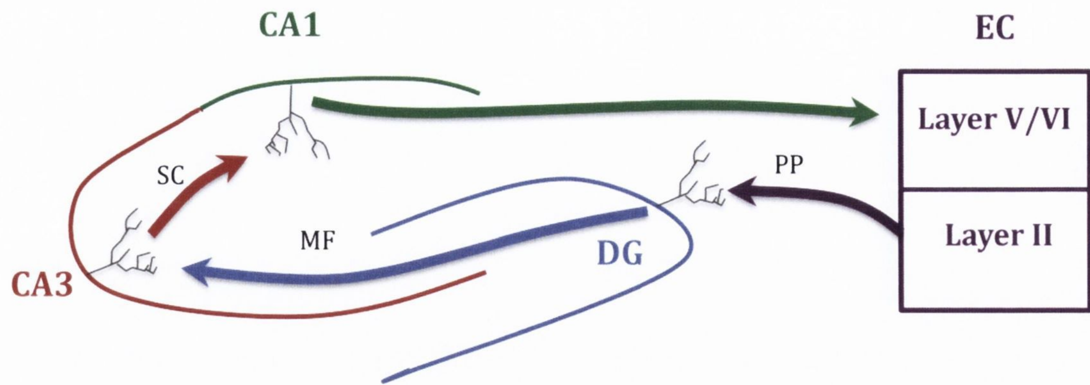

Figure 1.8 Basic hippocampal formation circuitry

The dentate gyrus (DG) receives input from the entorhinal cortex (EC) via the perforant pathway (PP). The DG granular cells project mossy fiber (MF) connections to the pyramidal neurons located in CA3 region that can also receive input directly from the EC via the PP. The CA3 region projects Schaffer collateral (SC) axons that synapse with neurons in the CA1 region. The CA1 projects axons back to cortical areas including the EC.

Much of the early knowledge on the role the hippocampus has to play in memory came from patient H.M. that the world first learned about in 1957. H.M. underwent experimental surgery to treat epilepsy; the surgery involved removing a large area of the medial temporal lobe. Following this surgery it was reported that H.M. had severe short-term memory loss, scoring well below average on the Wechsler Memory Scale (Scoville *et al.*, 1957). Interestingly, it was reported that only H.M.'s short-term memory was affected, he could recall events that occurred in his childhood up until three years previous to the surgery but was unable to form new memories, a disorder that became known as anterograde amnesia. In the decades following this H.M. was extensively studied under many different memory paradigms (Corkin, 2002). Much of the evidence suggested that with regard to explicit memory H.M. had severe learning impairments and could not retain new memories, however, maintained the ability to learn various motor tasks.

In addition to studies of H.M., various hippocampal lesion experiments in rodents have helped to delineate the role of the hippocampus in memory formation and retention. In one of the first studies to ever use the well-known Morris water maze it was demonstrated that rats with total hippocampal lesions had impaired spatial learning (Morris *et al.*, 1982). Morris and colleagues showed that rats with hippocampal lesions had a significantly higher latency to escape (i.e. reach the hidden platform) from the Morris water maze across 28 trials compared to control animals and animals with

superficial cortical lesions. This showed that brain damage alone, in the form of a cortical lesion, was not sufficient to impair spatial memory but that damage to the hippocampus was. In a similar experiment it was shown that there is a significant negative correlation between performance in the Morris water maze and hippocampal lesion size (Broadbent *et al.*, 2004). Rats with a lesion equivalent to the loss of 30-50% of total hippocampal volume had impaired performance in the task compared to the sham surgery control group, a lesion equal to 50-75% loss of hippocampal volume further decreased performance but interestingly the group with a 75-100% loss of hippocampal volume did not differ in performance capacity compared to the 50-75% lesion group. Studies using different spatial memory tasks have obtained complimentary results. Barker and Warburton assessed spatial memory using the object location (displacement) task and the object-in-place task. Both tasks assess spatial memory by capitalising on rats' innate preference for novelty. Briefly, rats are familiarised to two objects (object location task) or four objects (object-in-place task) and following an interval of twenty-four hours an object was displaced to a different quadrant of the testing arena (object location task) or the position of two objects was swapped (object-in-place task). The ability of the rats to discriminate between the stationary objects and the objects that had changed location was used as an index of their ability to successfully retain/recall the memory. It was found that rats with a cytotoxic lesion equivalent to an approximate 60% loss of total hippocampal volume had severely impaired performance in both tasks compared to sham surgery controls (Barker *et al.*, 2011). These results suggest that loss of half of overall hippocampal volume is sufficient to block spatial learning and memory.

With regards to spatial memory it seems to be undisputed that the hippocampus has a large role to play. However, the role that the hippocampus has to play in other types of memory is not as clear. For example the role of the hippocampus in recognition memory appears to be slightly controversial. A common way of assessing recognition memory is the novel object recognition task. This tasks involves allowing an animal to explore objects (commonly two objects) for a certain period of time, following an interval the animal is again allowed to explore two objects, one being a familiar object and one being a novel object. The ability of the animal to discriminate between the novel object and the familiar object suggests that the animal has successfully learned the task. One study found that animals with hippocampal lesions equal to approximately 90% total hippocampal loss had significantly lower percentage exploration of the novel object compared to sham surgery animals (Broadbent *et al.*, 2010). Another study suggested that loss of approximately 60% of the hippocampus had no effect on performance in the novel object recognition task (Barker *et al.*, 2011), whereas other investigations suggest that

there may be limited roles for the hippocampus in object recognition memory (Piterkin *et al.*, 2008). Taken together, these results suggest that the hippocampus may not play as big a role in recognition memory as it does in spatial memory, however there is insufficient evidence to prove that the hippocampus does not have a role to play in recognition learning and memory.

1.7.2 The cellular analogues of memory

The various brain regions thought to be involved in explicit memory have been discussed and it seems that the hippocampal formation, with regard to spatial memory at least, has an important role to play, but what are the physiological changes that take place in the hippocampus during memory formation? One possibility is changes in the strength of synaptic connections between cells in the form of synaptic plasticity. Another possibility is changes in the number of cells in the form of adult neurogenesis. It should be noted that synaptic plasticity and adult neurogenesis are hypothesised to be the cellular mechanisms of memory formation but definitive proof of this hypothesis remains to be identified.

1.7.2.1 Synaptic plasticity and memory

Long-term potentiation (LTP), initially discovered in 1973 (Bliss *et al.*, 1973), is the form of synaptic plasticity that has gained most attention. LTP is a long-lasting activity-dependent increase in synaptic strength that has been demonstrated in many brain areas both *in vitro* and *in vivo*. Several different forms of LTP have been discovered, the most common form of LTP is N-methyl D-aspartate (NMDA) receptor-dependent LTP (Collingridge *et al.*, 1983). A summary of the possible mechanisms of this process is briefly outlined in Figure 1.9.

The argument for LTP being a cellular basis of learning and memory is strong for several reasons. Firstly the fact that LTP is easily induced in the hippocampus by electrical stimulation that is similar to theta rhythm activity that has been recorded from the hippocampus during normal exploratory behaviour. The second argument is the fact that inhibiting protein synthesis blocks long-lasting LTP but not early-phase LTP and interestingly inhibiting protein synthesis blocks long-term memory but not short-term memory (Lynch, 2004). Further evidence linking LTP to memory can be found by blocking LTP *in vivo*. Morris and colleagues as far back as 1986 reported that blockade of NMDA receptors using the antagonist D, L- AP5 blocks hippocampal LTP and spatial memory (Morris *et al.*, 1986). More recently it has been shown that transgenic mice that

exhibit impairments in hippocampal LTP also exhibit impairments in spatial memory and object recognition memory (Molinaro *et al.*, 2011). It has also been demonstrated that enhancing hippocampal LTP, through aerobic exercise in this case, can lead to memory improvements in the form of enhanced object recognition memory (O'Callaghan *et al.*, 2007).

Long-term depression (LTD) is another form of synaptic plasticity that has been studied in detail, although possibly not in as much detail as LTP. LTD can be defined as an activity-dependent decrease in synaptic strength. Like LTP, it can be induced by several different mechanisms but the one that will be discussed here is NMDA receptor-dependent LTD. In contrast to LTP (as described in Figure 1.9), LTD involves a decrease in AMPA receptor conductance (Banke *et al.*, 2000) and a decrease in AMPA receptor surface expression (Lee *et al.*, 2002). This decrease in synaptic strength, that may also involve a decrease in postsynaptic density size, is brought about by dephosphorylation of AMPA receptors and possibly of endocytotic machinery that mediate AMPA receptor internalisation by protein phosphatases such as calcineurin and protein phosphatase 1 (Malenka *et al.*, 2004).

The link between LTD and memory is not as explicit as the proposed link between LTP and memory, however, there is evidence emerging that LTD may be important for hippocampal-dependent learning. For example, transgenic mice that lack β-arrestin-2 (a protein involved in NMDA receptor mediated cytoskeletal remodelling) exhibit normal hippocampal LTP but have impaired expression of hippocampal LTD, show deficits in spatial learning when tested in the radial arm water maze (Pontrello *et al.*, 2012). Although these mice exhibit only minor deficits in learning, which could be attributed to the knock down of a protein that can mediate LTP-independent synapse remodelling, the study does suggest that LTD could possibly be important for hippocampal-dependent learning.

Another form of plasticity that involves the alteration of synaptic connections between neurons and may be important in learning and memory is synaptogenesis (i.e. the growth of new synapses). The functional role of this form of synaptic plasticity has gained little attention, which is probably due, at least in part, to the difficulty in analysing its role in learning and memory. It has been suggested that synaptogenesis is enhanced by certain forms of learning (Bruel-Jungerman *et al.*, 2007) and therefore may be important in the process of learning and memory storage

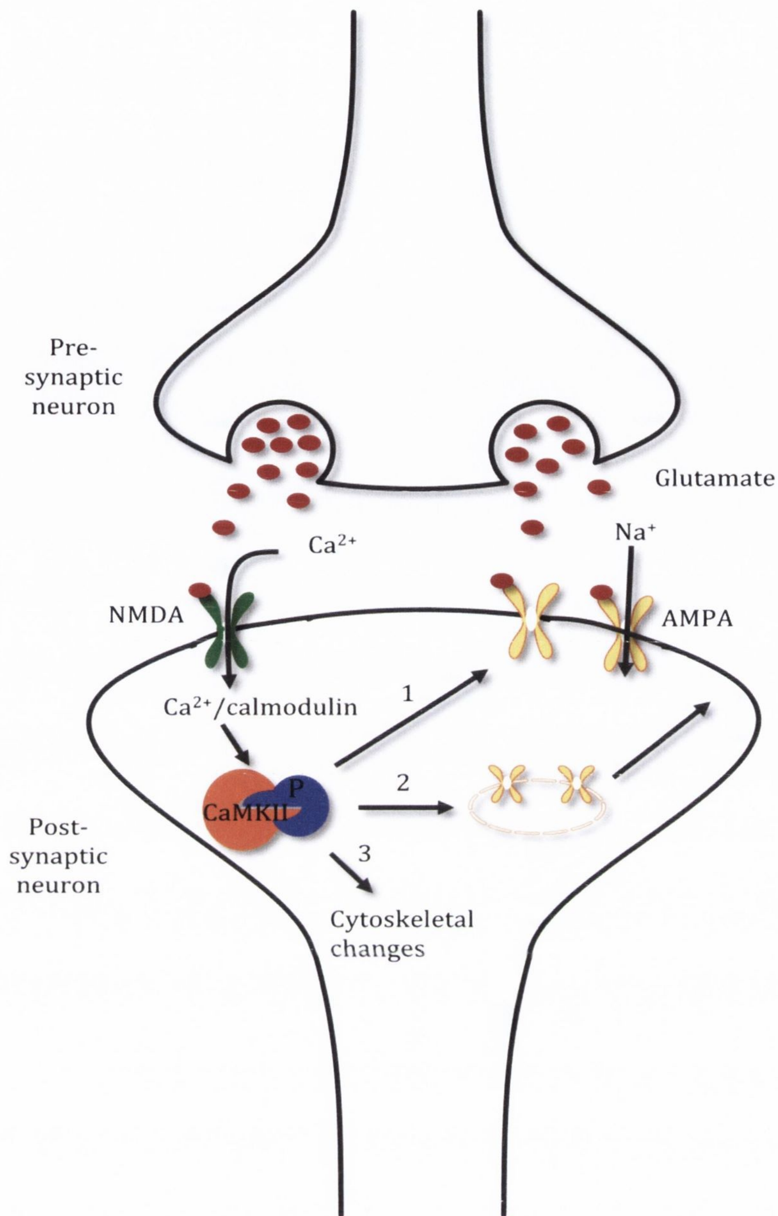

Figure 1.9 Possible mechanisms of induction of NMDA receptor-dependent LTP

AMPA receptor activation leads to depolarisation of a cell via influx of Na^{+} ions. If the cell becomes sufficiently depolarised Ca^{2+} permeable NMDA receptors become activated. Once in the cell Ca^{2+} binds the calcium binding protein calmodulin and this Ca^{2+}/calmodulin complex activates the protein kinase CaMKII. CaMKII can induce an increase in synaptic strength in three possible ways. (1) CaMKII can phosphorylate AMPA receptors expressed at the plasma membrane and increase their conductance; (2) CaMKII can phosphorylate AMPA receptor subunits and/or other substrates and induce insertion of the AMPA subunits into the plasma membrane or relocation of extra synaptic AMPA receptors to the post-synaptic density; (3) CaMKII can phosphorylate cytoskeletal proteins that allow cytoskeletal reorganisation required for synapse enlargement and synaptogenesis. Mechanism (2) appears to be the mechanism that is most widely accepted (Kerchner *et al.*, 2008).

1.7.2.2 Adult hippocampal neurogenesis and memory

Despite the considerable research carried out on the regulation of adult neurogenesis described earlier, its role in learning and memory still remains unclear. Many studies have found that certain environmental factors such as exercise and environmental enrichment can improve learning and memory as well as hippocampal neurogenesis (Kempermann *et al.*, 1997; van Praag *et al.*, 1999b). However, both exercise (O'Callaghan *et al.*, 2007) and environmental enrichment (Malik & Chattarji, 2012) have been shown to enhance LTP in the hippocampus and therefore it is impossible to attribute these cognitive improvements to neurogenesis alone.

A common way to assess the role of a cellular function on cognition is to block the cellular function. Complete knockdown of neurogenesis without damage to other brain areas has not yet been achieved but it has been possible to reduce the rate of neurogenesis. Transgenic mouse models that show reduced neurogenesis have impaired learning in the Morris water maze task (Zhao *et al.*, 2003) but these mice also show deficits in LTP expression. Similarly rats with inhibited Wnt signalling show reduced neurogenesis and impaired spatial memory (Jessberger *et al.*, 2009) but again Wnt signalling is important for LTP expression (Chen *et al.*, 2006). Recently, conditional knockdown of ERK 5, a protein that is not expressed in mature neurons and therefore its knockdown is likely not to affect mature neuronal function, was found to reduce the rate of adult neurogenesis in the dentate gyrus (Pan *et al.*, 2012). Interestingly it was found that this phenotype displayed normal spatial learning in the Morris water maze, however reversal learning (learning to locate the platform after it had been moved to a different quadrant of the maze) was impaired.

The case for adult neurogenesis being involved in spatial memory is further substantiated by the discovery that spatial learning can affect neurogenesis in the dentate gyrus. Hippocampal-dependent learning in the Morris water maze task increased the number of BrdU positive cells in the dentate gyrus (Gould *et al.*, 1999) suggesting that spatial learning can increase cell proliferation/survival. This effect was only observed in rats subjected to hippocampal-dependent learning as rats trained to perform hippocampal-independent conditioning tasks did not exhibit-altered neurogenesis. Following this discovery some studies emerged contradicting this finding and suggested that learning decreased the survival of newly born neurons in the dentate gyrus (Ambrogini *et al.*, 2004). In 2007, Dupret and colleagues discovered that spatial learning did in fact increase and decrease the survival of newly born cells and that the fate of the cell depended on the

age of the cell (Dupret *et al.*, 2007b) Specifically, training in the Morris water maze enhanced the survival of cells 7-12 days old while it induced apoptosis in cells 3 days old. This suggests that using newly generated neurons, learning induces remodelling of the circuitry of the hippocampus, which heavily implicates neurogenesis as a key physiological mediator of learning.

Although current research is yet to identify a definitive link between adult hippocampal neurogenesis and hippocampal-dependent memory there is a strong case for a relationship between both processes.

1.8 Exercise

Physical exercise has been well documented to be of benefit to overall health and well-being. In particular aerobic exercise (e.g. running, cycling, swimming) promotes efficient functioning of the cardiovascular system, the immune system and metabolism. It has also been shown to be particularly beneficial in combating type II diabetes, obesity and heart disease. Over the past couple of decades some of the beneficial effects of exercise on the brain have been identified. Exercise has been suggested to have a positive impact on mental health and importantly has been shown to enhance learning and memory.

1.8.1 Exercise and learning and memory

Exercise has been widely accepted as a potent enhancer of hippocampal-dependent memory, particularly in relation to, although not limited to, spatial memory. Voluntary exercise (i.e. unlimited access to a running wheel for one month) was shown to improve spatial memory in the Morris water maze in mice (van Praag *et al.*, 1999a). This memory improvement coincided with an enhancement in LTP and neurogenesis in the hippocampus. Shorter periods of forced exercise (one week) were found to enhance both recognition and spatial memory (Griffin *et al.*, 2009). This study found that exercise increased expression of BDNF in the dentate gyrus, hippocampus and perirhinal cortex (a brain region associated with recognition memory). Interestingly, infusion of BDNF directly into the brain boosted performance in the spatial memory task in a similar capacity to that of exercise, suggesting the cognitive improvements following periods of exercise may be mediated by BDNF. Additionally, eight weeks of treadmill running also improves spatial memory, increases BDNF expression and expression of the TrkB receptor in the hippocampus (Cassilhas *et al.*, 2012). Another study also found a strong positive correlation between BDNF expression and performance in the Morris water maze (O'Callaghan *et al.*, 2009). However, probably the most definitive proof of

exercise-induced improvements in memory being mediated by BDNF can be seen in a study by Vaynman and colleagues; blocking BDNF activity in the hippocampus of exercising animals, by administering a TrkB-IgG that selectively binds BDNF molecules, blocks the enhancement of spatial learning and memory observed following a period of exercise (Vaynman *et al.*, 2004).

1.8.2 Exercise and adult hippocampal neurogenesis

There is an abundance of evidence in the literature suggesting that physical exercise increases the rate of neurogenesis in the dentate gyrus and this may account for the positive effects that exercise has been shown to have on hippocampal-dependent memory. Exercise has been shown to enhance neurogenesis in young (van Praag *et al.*, 1999a), middle aged (Wu *et al.*, 2008) and aged animals (van Praag *et al.*, 2005). Exercise appears to have an effect at many different phases of the neurogenic process. Specifically, exercise has been shown to enhance NSC proliferation (van Praag *et al.*, 1999b), in addition to dendrite growth and neuronal differentiation of NPCs (Wu *et al.*, 2008). Recently, it has been observed that running can also induce cell cycle exit and enhance the survival of NPCs (Brandt *et al.*, 2010).

There are many proposed signalling mechanisms thought to be responsible for the exercise-induced enhancement of neurogenesis. One of the strongest candidates is BDNF. As mentioned above exercise increases BDNF expression as well as the amount of activated TrkB receptor in the hippocampus. Exercise protocols that have induced an increase in neurogenesis have also observed an increase in hippocampal expression of both BDNF (Marlatt *et al.*, 2012) and total TrkB (Wu *et al.*, 2008). In addition to this, mice engineered to specifically lack TrkB receptor expression in NPC do not exhibit enhanced NPC proliferation or differentiation following a period of exercise (Li *et al.*, 2008).

Another signalling system that contributes to exercise-induced enhancement of neurogenesis is the endocannabinoid system. Voluntary exercise was found to enhance cannabinoid signalling specifically in the hippocampus and co-administration of the CB_1 inverse agonist AM 251 blocked the exercise related increase in cell proliferation (Hill *et al.*, 2010). It has also been observed in another study that signalling through the CB_1 receptor is essential for the exercise-induced stimulation of cell proliferation; CB_1 knockout mice do not exhibit increased cell proliferation after bouts of exercise (Wolf *et al.*, 2010).

In addition to BDNF and endocannabinoids there are many other signalling molecules that have been implicated in exercise-induced neurogenesis. Exercise stimulates the uptake of insulin-like growth factor I (IGF-I) from the blood into the hippocampus and blocking this process inhibits the exercise induced enhancement of neurogenesis (Trejo *et al.*, 2001). Similarly, buffering circulating levels of vascular endothelial growth factor (VEGF) during a seven-day exercise period prevents an increase in neurogenesis (Fabel *et al.*, 2003). Finally, a transgenic mouse that is deficient in serotonin does not exhibit exercise-induced enhancement of neurogenesis (Klempin *et al.*, 2013).

The proposed link between adult neurogenesis and memory has been described earlier, and memory improvements following periods of exercise have often been correlated to increased hippocampal neurogenesis (Bechara *et al.*, 2013a; Li *et al.*, 2013; van Praag *et al.*, 1999a). However, caution is urged when interpreting this data as exercise can also enhance shorter-term plasticity (see Section 1.7.1). Additionally the age of newly-born cells is often not considered; it takes approximately three weeks for a newborn cell to begin to reach functional maturity (Suh *et al.*, 2009) and often studies link increased cell proliferation to enhanced cognitive function, however, in the absence of data to suggest that proliferating cells survive beyond proliferation to functional maturity it is difficult to link cell proliferation to memory improvements.

Taken together current evidence undoubtedly proves that exercise can enhance adult hippocampal neurogenesis. It looks likely that exercise does so by modulating three of the major events in the process of neurogenesis; proliferation, differentiation and survival. It appears that BDNF and endocannabinoid signalling have a pivotal role in exercise-induced enhancement of neurogenesis; however, there are other signalling systems involved.

1.9 Ageing

Ageing is usually accompanied by an overall deterioration in physiological state, a difficulty in maintaining homeostasis after encountering a stressor and an increased susceptibility to disease. As with all organs the ageing process affects the brain; it has an increased baseline inflammatory state, is more susceptible to neurodegeneration and loses plasticity. All of this can lead to impairments in brain function especially cognitive function.

1.9.1 Learning and memory in ageing

Normal ageing is generally accompanied by impairment in hippocampal-dependent memory, which can be attributed to alterations in brain plasticity and/or neurodegeneration. Aged animals have impaired spatial learning and memory as assessed by performance in the Morris water maze task (Frick *et al.*, 1995; Gage *et al.*, 1984) as well as the radial-arm maze (Gallagher *et al.*, 1985). Interestingly, it has been shown that from the onset of middle-age (8 months) rats begin to show deficits in performance in the hippocampus-dependent object recognition and the spatial variant of the task; the object location task (Wiescholleck *et al.*, 2013). Bach and colleagues identified a negative correlation between the age of mice and performance in the Morris water maze suggesting that memory progressively deteriorates with age and the authors attribute this age-related impairment in spatial memory to a deficit in LTP expression in the hippocampus (Bach *et al.*, 1999b). This proposed link between a decline in spatial memory that progresses with age and impairment in hippocampal LTP is something that has been put forward by many others (Foster, 2012). In particular one study found that only aged animals that show a deficit in LTP have impaired spatial memory (Diana *et al.*, 1995). This hypothesis that memory decline is associated with decreased plasticity is further strengthened by the fact that although normal ageing (i.e. ageing that is independent of a specific pathology) is associated with a decreased capacity for memory retention it is not accompanied by a loss of hippocampal neurons (Rapp *et al.*, 1996). Therefore it seems that under normal ageing conditions existing neurons remain viable but become dysfunctional.

Some groups who have hypothesised that impairment in brain plasticity is responsible for the age-related memory decline have attempted to identify the neurophysiological changes that may account for this. It has been suggested that alterations in the biochemical composition of NMDA receptors may be responsible (Clayton *et al.*, 2002), others implicate a decrease in hippocampal neurotrophin expression, specifically NGF and BDNF (O'Callaghan *et al.*, 2009) while another possibility is an increase in reactive oxygen species coupled to an increase in stress-activated protein kinase activity (O'donnell *et al.*, 2000). Ageing is also associated with an increased inflammatory environment in the brain, which is likely to be as a consequence of hyperactivity of the immune system (Norden *et al.*, 2013). An increased expression of pro-inflammatory cytokines in the hippocampus contribute to the inability of aged animals to sustain LTP (Griffin *et al.*, 2006), and, possibly as a consequence of this, exhibit impaired learning

and memory. Interestingly, the increase in inflammatory markers and attenuation of LTP observed in the aged hippocampus was partially rescued by inhibiting hydrolysis of the endocannabinoid AEA (Murphy *et al.*, 2012). This suggests that age-related deterioration in hippocampal function could potentially be a consequence of dysregulation of the endocannabinoid system.

1.9.2 Ageing and adult hippocampal neurogenesis

Ageing has been shown by multiple studies to decrease the rate of neurogenesis in the dentate gyrus that may contribute to the age-related decline in memory. Ageing was found to have a negative effect on all aspects of the neurogenesis process; it has been consistently shown that ageing decreases proliferation (Kuhn *et al.*, 1996), differentiation (Heine *et al.*, 2004) and survival (Speisman *et al.*, 2013) of NSCs/NPCs. The decline in neurogenesis appears to begin at an early age. It was found that the number of newly-born neurons in the hippocampus progressively decreases from the beginning of adulthood (2 months) to the onset of middle-age (10 months) where the number of newborn cells is 40-fold lower (Morgenstern *et al.*, 2008). By the onset of old-age (18 months) the rate of neurogenesis is extremely low and persists to 24 months of age (Bondolfi *et al.*, 2004).

Like exercise it appears that the alteration in neurogenic rate observed with age is caused by an aggregation of different factors rather than one specific signalling molecule/mechanism. As described above, ageing of the brain is accompanied by a decreased concentration of cerebral growth factors and an increased pro-inflammatory phenotype. A consequence of this is a dramatic alteration of the microenvironment of NSCs/NPCs and any alteration of a stem cell's microenvironment can have a profound effect on the fate of the cell. Growth factors particularly neurotrophins are important in maintaining the rate of adult neurogenesis and it has been shown that the concentration of the neurotrophins BDNF and NGF in the hippocampus is reduced in aged animals (Karege *et al.*, 2002; O'Callaghan *et al.*, 2009). In addition to a decrease in the concentration of neurotrophins, IGF-1, FGF-2 and VEGF all decrease at the onset of middle age and persist at this reduced level during ageing something that is also linked to decreased adult neurogenesis (Shetty *et al.*, 2005). The aged hippocampus is considered an inflammatory environment; there is an increased expression of the pro-inflammatory cytokines interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) in the aged hippocampus compared to young animals (Murphy *et al.*, 2012). NPCs in the hippocampus express the IL-1β receptor and IL-1β suppress NPC proliferation (Koo *et*

al., 2008). Similarly, hippocampal NPCs were shown to express the TNF-α receptors TNF-R1 and TNF-R2; complete knockdown of these receptors in mice lead to an increase in the number of new neurons in the hippocampus (Iosif *et al.*, 2006). Other age-related factors that may contribute to a decline in neurogenesis include hormones, disease and altered neurotransmission (Klempin *et al.*, 2007).

Interestingly, administration of the synthetic cannabinoid WIN-55, 212-2 can partially rescue the decline in neurogenesis in the aged hippocampus (Marchalant *et al.*, 2009). This again prompts the suggestion that the loss of brain plasticity observed with age could potentially be related to dysregulation of the endocannabinoid system. However, much more extensive investigation would be required to substantiate this claim.

An age-related decrease in hippocampal neurogenesis has been linked to a decline in hippocampal-dependent memory (van Praag *et al.*, 2005). However, as ageing is associated with many different changes in the hippocampus it is difficult to identify a direct link between one specific age-related physiological change and an age-related memory decline. It appears more likely that the age-related memory decline observed is due to an aggregation of several physiological mechanisms.

1.10 Aim and objectives

The aim of this thesis was to investigate if there was a link between the endocannabinoid system and neurotrophin signalling and if a link between these signalling systems could impact on adult hippocampal neurogenesis during periods of aerobic exercise and during ageing. Ultimately, the contribution of these alterations of neurogenesis to spatial memory was examined.

The specific objectives of this thesis were:

To examine if blocking endocannabinoid hydrolysis could affect the neuronal expression of neurotrophins or their receptors *in vitro.*

To assess if *in vitro* findings could be replicated in the hippocampus of the adult Wistar rat.

To examine if blocking endogenous cannabinoid signalling during exercise could affect:

i. Neurotrophin signalling

ii. Hippocampal neurogenesis (proliferation and short/long-term survival)
iii. Short-term and long-term spatial memory

To assess how endogenous cannabinoid signalling during ageing could affect:

i. Neurotrophin signalling
ii. Hippocampal neurogenesis (proliferation and short-term survival)
iii. Short-term spatial memory

To identify if changes in expression of cannabinoid receptors may be correlated with age-related effects on hippocampal function.

Chapter 2

Materials and methods

Chapter 2

2.1 *In vitro* experiments

2.1.1 Aseptic technique/sterilisation of work environment

Aseptic techniques were practiced throughout all stages of cell culture protocol. All cell culture techniques were carried out in a laminar flow hood (Astec-Microflow laminar flow workstation, Florida, USA). The laminar flow hood prevents the entry of contaminated external air. In the laminar flow hood air passes through high efficiency particle air filters at the top of the hood and flows downwards creating a barrier at the open section of the hood. The laminar flow hood was sterilised with 70% ethanol prior to use. Latex gloves were worn at all times and were sprayed with 70% ethanol before entering the hood.

2.1.2 Sterilisation of materials and reagents

Dissection instruments, eppendorfs (Starstedt, Ireland), glassware, pipette tips and double de-ionised water (ddH_2O) were wrapped in aluminium foil, sealed with autoclave tape (Sigma) and autoclaved for 30 min at 145°C. Dissection instruments were cleaned using a sonic cleaner (VWR International) and placed in an oven (Sanyo-Gallenkamp Hotbox Oven, Loughborough, UK) at 200°C overnight prior to use. All solutions were stored in autoclaved glassware or sterile 15 ml or 50 ml falcon tubes (BD). Solutions were filtered using a 0.2 μm cellulose acetate membrane filter (Pall Corporation, USA) and a sterile syringe (B.Braun Medical Ltd., Germany) prior to use.

2.1.3 Waste disposal

All hazardous material was packaged and disposed of via the Hazardous Material Facility, Trinity College Dublin. All waste was disposed of in accordance with EU legislation.

2.1.4 Primary culture of cerebral cortical neurons

2.1.4.1 Preparation of glass coverslips

Glass coverslips (13 mm diameter) were sterilised and coated with poly-L-lysine directly prior to use. Coverslips were soaked in 70% ethanol for 24 h. Coverslips were individually placed in the laminar flow hood under UV light for 12 h. The sterile coverslips were incubated for 1 h at 37°C in a 60 $\mu g.ml^{-1}$ poly-L-lysine/ddH_2O solution.

The coated coverslips were dried in the laminar flow hood and then placed individually into sterile 24-well plates (Greiner Bio One Gmbh, Austria).

2.1.4.2 Animals

Postnatal day 1 Wistar rats were obtained from BRU, Trinity College Dublin. The laboratory in which animals were housed in was maintained at a constant ambient temperature (21.5 ± 1.5°C) and ambient humidity (55 ± 5%) under a 12-12 hr light-dark cycle (lights on at 09:00, lights off at 21:00). Food and water was available to mothers *ad libitum*. 1-day-old rat pups were removed from their home cage and were transferred to the designated cell culture laboratory in a warm ventilated box.

2.1.4.3 Dissection

Rats were decapitated using a large scissors and the brain was exposed. The cortices were removed using a curved forceps and placed in a Petri-dish containing two drops of neurobasal medium (NBM; Invitrogen, UK) supplemented with heat inactivated horse serum (10%; Gibco BRL, USA), penicillin (100 U/ml; Gibco BRL, USA), streptomycin (100 U/ml; Gibco BRL, USA) and glutamine (2 mM; Glutamax, Gibco BRL, USA) and were cross chopped with a scalpel.

2.1.4.4 Dissociation

The cross chopped tissue was transferred to a falcon tube containing a 6 ml 0.3% (w/v) trypsin in phosphate buffered saline (PBS, 100 mM NaCl, 80 mM Na_2PHO_4, 20 mM Na_2HPO_4; pH 7.4) solution and incubated at 37°C in a humidified incubator (Binder CO_2 incubator, series CB, Binder GmbH, Germany) containing 5% CO_2 and 95% air for 2 min. 6 ml Dulbecco's modified Eagle's medium (DMEM) was added to the solution. The solution was centrifuged at 2000 g for 3 min, the supernatant was removed and the pellet re-suspended in 5 ml DMEM. The cell solution was triturated using a Pasteur pipette and passed through a sterile 40-μm-nylon cell strainer (Becton Dickinson Labware Europe, France). The filtered solution was centrifuged at 2000 g for 3 min and re-suspended in 5 ml NBM.

2.1.4.5 Plating cells

Re-suspended cells were plated at a density of 0.25 x 10^6 on poly-L-lysine coated coverslips. The plates were incubated at 37°C with 5% CO_2 and 95% air for 2 h to allow

cells to adhere to the coverslips. The wells were flooded with 0.4 ml with warm NBM containing B-27 and were incubated at 37°C with 5% CO_2 and 95% air, after 48 h the media was removed from the wells and replaced with 0.4 ml NBM without B-27. The cells were inspected daily by light microscopy (Nikon TMS Instech Co. Ltd., Japan) to assess their healthiness. The cells were ready for treatment on day 6.

2.1.5 Cell treatments

The cells were ready for treatment on day 6 of culture.

2.2.5.1 URB 597

The FAAH inhibitor 3-(3-carbamoylphenyl) phenyl] N-cyclohexylcarbamate (URB 597; Enzo Life Sciences, UK) was dissolved in DMSO at a concentration of 1 mM and stored at -20°C. For cell treatments it was diluted to a concentration of 1 μM in warm NBM. During cell treatments the media was removed from the wells and replaced with 0.2 ml of drug solution. Depending on the experiment cells were incubated for a specific period of time in the drug solution at 37°C with 5% CO_2 and 95% air.

2.2.5.2 URB 602

The MAGL inhibitor [1,1'-biphenyl]-3-yl-carbamic acid, cyclohexyl ester (URB 602; Enzo Life Sciences, UK) was dissolved in DMSO at a concentration of 1 mM and stored at -20°C. For cell treatments it was diluted to a concentration of 100 μM in warm NBM. During cell treatments the media was removed from the wells and replaced with 0.2 ml of drug solution. Depending on the experiment cells were incubated for a specific period of time in the drug solution at 37°C with 5% CO_2 and 95% air.

2.2 *In vivo* experiments

2.2.1 Animals

Three, fourteen or twenty-two month old male Wistar rats acquired from Bio-resources Unit (BRU), Trinity College Dublin were used in all experiments. All animals were experimentally naïve. Three-month-old animals weighed between 300g and 450g, fifteen-month-old animals weighed between 450 g and 620 g and twenty-two month old animals weighed between 600g and 800g at the beginning of experimental protocols. Animals were given at least one week to acclimatize to the laboratory environment following their transfer from the BRU breeding facility. During the acclimatization period all animals were handled daily for a minimum of 10 minutes. All experiments were performed in

accordance with the National and European directives on animal experiments (European Communities [Amendment to Cruelty to Animals Act 1876] Regulations 2002 and 2005) and were approved by the Animal Ethics Committee, Trinity College Dublin.

2.2.2 Housing conditions

Animals were group housed (two or three per cage) in standard housing cages obtained from BRU, Trinity College Dublin (40 L x 24 W x 20 H cm). The laboratory in which animals were housed in was maintained at a constant ambient temperature (21.5 ± 1.5°C) and ambient humidity (55 ± 5%) under a 12-12 hr light-dark cycle (lights on at 09:00, lights off at 21:00). Food and water was available *ad libitum* except in the case of the voluntary running protocol (see section 2.2.5).

2.2.3 Drug delivery

The CB_1 receptor antagonist (inverse agonist) AM 251 (1 $mg.kg^{-1}$), the FAAH inhibitor URB 597 (0.3 $mg.kg^{-1}$) and the MAGL inhibitor URB 602 (3 $mg.kg^{-1}$) were dissolved in a vehicle solution (50-55% DMSO in saline) and administered intraperitoneally based on animal total body weight (approximately 0.5 ml per animal). Control animals received the corresponding vehicle solution.

2.2.4 Forced exercise protocol

Animals were randomly assigned to a sedentary group or an exercising group. Animals were habituated to the rodent treadmills (Exer 3/6 treadmill, Colombus Instruments, Figure 2.1) for two consecutive days prior to commencement of the exercise protocol. Habituation consisted of animals walking on the treadmill for a period of 10 min at a belt speed of approximately 6 $m.min^{-1}$. Animals assigned to the sedentary group were placed on a stationary treadmill for the same period of time. The exercise protocol consisted of seven consecutive days of one hour of treadmill running at a belt speed of 16.7 $m.min^{-1}$ (approximately 1 $km.day^{-1}$). Again sedentary controls were placed on a stationary treadmill for the corresponding time period. One end of the treadmill belt was equipped with wire loops that administered a mild electric shock that encouraged animals to maintain the running pace dictated by the belt. The electrical shock system was activated at a low intensity throughout all exercise sessions (current of 0.7 mA with inter-pulse interval of 2 s).

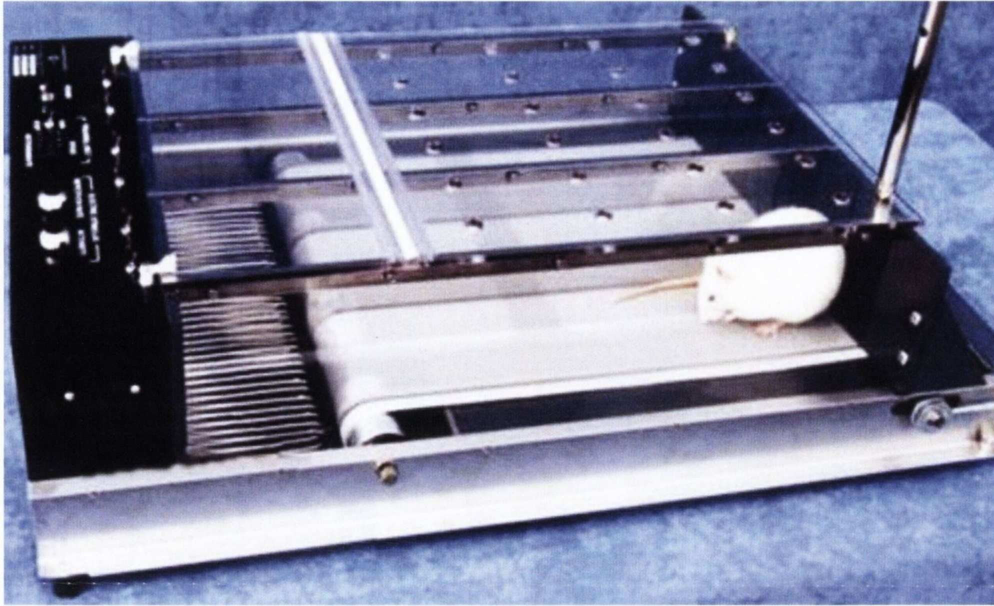

Figure 2.1 Exer 3/6 rodent treadmill, Columbus Instruments

An image of the rodent treadmill used in the forced exercise protocol. Each treadmill contains three lanes separated by Perspex walls facilitating the exercising of three animals.

2.2.5 Behavioural procedures

Behavioural tests were designed to assess hippocampal-dependent memory. Specifically spatial memory was assessed using an object displacement (OD) task.

The OD was performed in a dimly lit room of constant ambient temperature (21.5 ± 1.5°C) and humidity (55 ± 5%). The apparatus used for the OD task consisted of a circular arena constructed from black plywood (125 cm diameter; 50 cm height). The objects used in the task were constructed from Duplo Lego Blocks (Lego®). All objects were similar dimensions and colour but varied in configuration. The objects and the arena were cleaned thoroughly between animals to eliminate olfactory cues

2.2.5.1 Object displacement task

Habituation

Animals were habituated to the open field arena in the absence of objects for two consecutive days prior to the beginning of the OD task. On the first day animals were introduced to the arena in the company of their littermates for a 10 min period. On the second day of habituation rats were introduced into the open field arena individually for a period of 5 min.

Acquisition

Twenty-four hours following the habituation period three objects constructed from Lego® were placed in the open field and were fixed to the floor. Two spatial cues were placed on the walls of the arena (Figure 2.2). Animals were placed in the arena for one, two or three periods of five min (with an inter-trial interval of five min) and allowed to explore the three objects. The amount of time animals spent exploring each of the objects was timed and this was expressed as a percentage of total object exploration time. This acquisition period allowed the animals to learn the position of the objects (i.e. training phase of the task). The area of the arena that animals were initially placed in was randomised. In some experiments animals received an injection of drug or vehicle solution 30 min before the acquisition period. In the case of experiments testing long-term spatial memory the acquisition period was repeated daily for 7 days (e.g. 2 x 5 min x 7 d).

Testing

Animals were subjected to the testing phase of the task either 24 h (short-term testing experiments) or 14 day (long-term testing experiments) after the acquisition phase of the task. During testing one of the three objects was displaced to a different quadrant of the arena. Animals were placed into the arena for a five-minute period and allowed to explore the objects. The amount of time animals spent exploring the displaced object and the stationary objects was expressed as a percentage of total object exploration time.

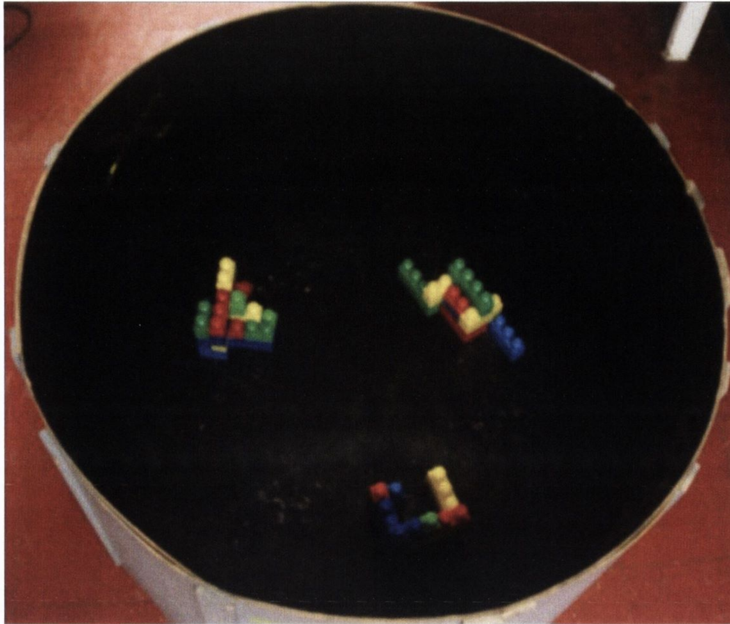

Figure 2.2 Object displacement task apparatus

An image of the objects and open field used in the object displacement task.

2.3 Tissue collection and processing

2.3.1 Transcardial perfusion

Animals were administered an overdose of the anaesthetic urethane (1500 $mg.kg^{-1}$, i.p.). Animals were monitored regularly post anaesthetic and absence of a pedal reflex indicated deep anaesthesia. Following onset of deep anaesthesia and cessation of breathing a median sternotomy was performed and the heart was exposed. All surrounding connective tissue was removed from the heart and a needle (21 G x 1 ½ in.) connected to a peristaltic pump (MINIPULS® 3, Gilson Inc., USA) was inserted into the left ventricle and fixed in place using a clamp. The right atrium was perforated to allow drainage of blood from the vascular system. The pump was activated and animals were perfused for 10 min with ice-cold saline (0.9% NaCl) containing heparin (heparin sodium, 25 $units.L^{-1}$) to prevent clotting.

2.3.2 Tissue collection

Following transcardial perfusion animals were ethuanised by decapitation; brains were removed and dissected rapidly over ice. In the case of studies where half brains were removed for immunohistochemical analysis the left hemisphere was flash-frozen in isopentane and stored at -80°C. The right hemisphere was sub-dissected to expose the hippocampus and the dentate gyrus. The tissue extracted was flash-frozen with liquid

nitrogen and stored at -80° C. In studies where blood was collected trunk bloods were removed following decapitation. Blood was stored at room temperature for 1.5 hours and centrifuged at 5°C for 20 min at 11,000 g. The supernatant consisting of the blood serum was removed and stored at -20°C.

For *in vitro* experiments cells were lysed in RA1 lysis buffer (Machery-Nagel, Germany) and stored at -20°C.

2.3.3 Homogenate preparation

Flash frozen tissue was defrosted over ice for approximately 30 min. Tissue was homogenized using a manual glass homogeniser in either 500 μl ice cold Krebs buffer (NaCl, 136 mM; KCl, 2.54 mM; KH_2PO_4, 1.18 mM; $MgSO_4.7H_2O$, 1.18 mM; $NaHCO_3$, 16 mM; glucose, 10 mM) containing 2mM $CaCl_2$ or in 500 μl ice cold lysis buffer (NP-40, 1% (v/v); Tris-base (pH 8.0), 20 mM; NaCl, 137 mM; glycerol, 10% (v/v), ethylenediaminetetraacetic acid (EDTA), 2 mM; active Na_3VO_4, 1mM; aprotinin, 10 $\mu g.ml^{-1}$; leupeptin, 10 $\mu g.ml^{-1}$).

2.3.4 Protein quantification using BCA method

Samples and protein standards were analysed in triplicate (25 μl per well) on a 96-well plate (microtest plate; Starstedt, Ireland). A standard curve was prepared from a serial dilution of a 2.0 $mg.ml^{-1}$ stock solution of BSA using lysis buffer. The standard curve consisted of nine dilutions ranging from 2000 $\mu g.ml^{-1}$ to 0 $\mu g.ml^{-1}$. The BCA working reagent was prepared from BCA reagent A and BCA reagent B (50:1, reagent A: reagent B) and 200 μl of the working reagent was added to the standards and samples on the 96-well plate. The plate was incubated for 30 min at 37°C. The plate was allowed to cool to room temperature and the absorbance of the standards and samples was read using a 96-well plate reader (Synergy HT Multi-Mode Microplate Reader, BioTek®) at 562 nm. The regression equation of the standard curve was calculated and from this the protein content of the samples was calculated in $mg.ml^{-1}$. The samples were then diluted accordingly to ensure that each sample had equal protein content.

2.4 Enzyme linked immunosorbent assay

Enzyme linked immunosorbent assays (ELISA) were used to measure the concentration of BDNF and NGFβ in serum, hippocampus and dentate gyrus tissue homogenate. A commercially available Human BDNF DuoSet ELISA Development system and Rat NGFβ DuoSet ELISA Development system (R&D Systems Europe, Oxon, United

Kingdom) were used. Manufacturer's guidelines state that the Human BDNF kit has 100% cross-reactivity with rat BDNF. In the case of the measurement of BDNF in the serum all samples were diluted 1 in 2 in distilled water prior to carrying out the ELISA.

2.4.1 Analysis of BDNF and NGFβ concentration

For the BDNF ELISA capture antibody solution (2 $\mu g.ml^{-1}$ mouse anti-human BDNF in phosphate buffered saline [PBS]) was incubated over night at 22°C in a 96-well NUNC-immuno™ plate (MaxiSorp™ plates, Denmark; 50 μl per well). The plate was washed three times with wash buffer (PBS-T; 0.05% Tween ® 20 in PBS; 400 μl per well) and incubated with reagent diluent (1% BSA in PBS; 150 μl per well) for 1 hour at 22°C to block non-specific binding. The plate was washed three times with wash buffer. Standards (diluted with reagent diluent according to the manufacturer's guidelines) and samples were added in duplicate (50 μl per well) to the plate and the plate was incubated for 2 hours at 22°C. The plate was washed three times with wash buffer. The plate was incubated for 2 hours at 22°C with detection antibody (25 $ng.ml^{-1}$ biotinylated mouse anti-human BDNF in reagent diluents; 50 μl per well). The plate was washed three times with wash buffer and incubated for 20 min at 22°C with Streptavidin-HRP solution (1 in 200 dilution in reagent diluent; 50 μl per well). The plate was washed three times with wash buffer and was incubated in the dark with 3, 3', 5, 5'- Tetramethylebenzidine (TMB, Sigma; 50 μl per well) for 20 min at 22°C. The colour reaction was stopped using stop solution (1 M H_2SO_4; 50 μl per well) and the optical density was read at 450 nm with a 96-well plate reader (Synergy HT Multi-Mode Microplate Reader, BioTek®). Similarly for the NGFβ ELISA capture antibody solution (2 $\mu g.ml^{-1}$ mouse anti-human NGFβ in phosphate buffered saline [PBS]) was incubated over night at 22°C in a 96-well NUNC-immuno™ plate (MaxiSorp™ plates, Denmark; 50 μl per well). The plate was washed three times with wash buffer (PBS-T; 0.05% Tween ® 20 in PBS; 400 μl per well) and incubated with reagent diluent (1% BSA in PBS; 150 μl per well) for 1 hour at 22°C to block non-specific binding. The plate was washed three times with wash buffer. Standards (diluted with reagent diluent according to the manufacturer's guidelines) and samples were added in duplicate (50 μl per well) to the plate and the plate was incubated overnight at 4°C. The plate was washed three times with wash buffer. The plate was incubated for 2 hours at 22°C with detection antibody (25 $ng.ml^{-1}$ biotinylated mouse anti-human NGFβ in reagent diluents; 50 μl per well). The plate was washed three times with wash buffer and incubated for 20 min at 22°C with Streptavidin-HRP solution (1 in 200 dilution in reagent diluent; 50 μl per well). The plate was washed three times with wash buffer and was incubated in the dark with 3, 3', 5, 5'- Tetramethylebenzidine

(TMB, Sigma; 50 µl per well) for 20 min at 22°C. The colour reaction was stopped using stop solution (1 M H_2SO_4; 50 µl per well) and the optical density was read at 450 nm with a 96-well plate reader (Synergy HT Multi-Mode Microplate Reader, BioTek®). The regression equation of the standard curve for each ELISA was calculated and from this the concentration of BDNF or NGFβ in each of the samples were calculated in $pg.ml^{-1}$ in the case of blood serum and in pg.mg $protein^{-1}$ in the case of brain tissue.

2.5 Immunohistochemical analysis

For experiments in which cell proliferation and/or survival was measured; 5-bromo-2'-deoxyuridine (BrdU), a thymidine analogue that incorporates into replicating DNA was administered to rats via i.p. injection at a dose of 50 $mg.kg^{-1}$ (see chapters 4 and 5). Identification of proliferation cells in the dentate gyrus was performed by immunohistochemical analysis for BrdU. In the case of experiments assessing cell proliferation and early stage survival (<10 days post first BrdU injection; short-term study Chapter 4 and Chapter 5) a percentage of BrdU positive ($BrdU^+$) cells in a particular area of the dentate gyrus was calculated. In the case of experiments assessing cell survival (>20 days post first BrdU injection; long-term study Chapter 4) the total $BrdU^+$ cell number in the whole dentate gyrus was estimated.

2.5.1 Preparation of tissue for immunostaining

Percentage $BrdU^+$ cells

Flash frozen left hemispheres were allowed to acclimatise to -20˚C, coated in OCT ™ compound (Tissue Tek, USA) and were then sectioned using a cryostat (Lecia CM1900). Saggittal sections (150 x 50 µm) were cut until the hippocampus was clearly visible. One 10 µm Saggittal section was taken, transferred onto a slide and stained with haematoxylin to confirm that the dentate gyrus was visible. Eighteen sections of 10 µm thickness were taken for each hemisphere. The sections were stored at -20˚C.

Total number $BrdU^+$ cells

Flash frozen left hemispheres were allowed to acclimatise to -20˚C, coated in OCT ™ compound (Tissue Tek, USA) and were then sectioned using a cryostat (Lecia CM1900). Saggittal sections (100 x 50 µm) were cut until the hippocampus was clearly visible. One 10 µm saggittal section was taken, transferred onto a slide and stained with haematoxylin to confirm that the dentate gyrus was visible. A one in six series of 20 µm saggittal

sections were taken until the hippocampus was no longer visible. The sections were stored at -20°C.

2.5.2 Staining of BrdU$^+$nuclei in the dentate gyrus

BrdU$^+$cells were visualised using the Aividin Biotin Complex (ABC) peroxidase method with rabbit anti-chicken conjugated IgG and 3,3 diaminobenzidine (DAB) chromogen. 10μm sections were fixed to the glass slide with 100% methanol for 10 min and washed with PBS (3 x 3 min). DNA must be denatured to allow binding of the primary antibody to BrdU, thus the sections were incubated with 2N HCl for 30 min at 37°C, sections were then neutralised with 0.1M borate buffer (2 x 5 min washes). After washing with PBS (3 x 3 min) endogenous peroxidases were blocked with 0.3% H_2O_2 (Sigma-Aldrich, USA) in dH_2O for 20 min (75μl per section). Sections were washed with PBS (3 x 3 min) and non-specific secondary antibody binding was blocked by incubating the sections in blocking buffer (1:5 normal rabbit serum) in 1% BSA in PBS; 75μl per section) for 60 min. The sections were incubated with primary antibody (1:1000 chicken anti-BrdU in 1% BSA in PBS; 75μl per section) overnight at 4°C. The sections were washed with PBS (3 x 3 min) and incubated for 30 min with secondary antibody (1:2000 rabbit anti-chicken in 1% BSA in PBS; 75μl per sections), followed by washing with PBS (6 x 3 min). Colour reaction was enhanced using Vectastain ® ABC, sections were incubated in ABC for 30 min at 4°C (75μl per section), followed by washing with PBS (3 x 3 min). The colour reagent DAB chromogen (Dako, Denmark) in PBS, at a concentration of 1mg/mL, was activated with 0.3% H_2O_2 . The sections were incubated in DAB (75μl per section) until the colour reaction developed (approximately 2 min). The colour reaction was stopped by immersing the sections in PBS. Following washing with dH_2O (2 x 3 min) the sections were then counterstained with Mayer's haematoxylin solution (2 x 5 min) and again washed with dH_2O (1 x 5 min). The sections were dehydrated by washing with 70%, 90% and 100% ethanol, immersed in xylene (Sigma-Aldrich, USA), mounted with DPX mountant and coverslipped.

As a negative control the above protocol was performed in the absence of primary antibody incubation. No BrdU$^+$nuclei were identified.

2.5.3 Quantification of BrdU$^+$nuclei in the dentate gyrus

Percentage BrdU$^+$ cells

Three sections of the dentate gyrus were stained per animal and the percentage of proliferating cells (BrdU$^+$ cells) was calculated per animal. This was achieved by counting the total number of cells and the number of proliferating cells in six views per dentate gyrus section using a light microscope at 40x objective (Olympus CH-2, Olympus Optical Company, Japan). The number of proliferating cells was divided by the total number of cells (Bechara *et al.*, 2013a). Only cells in the granular cell layer and subgranular zone were counted.

Total number BrdU$^+$ cells

Twelve sections of the dentate gyrus were stained per animal. This equates to 5% of the total dentate gyrus, a portion that has been previously determined to accurately estimate cell survival in the dentate gyrus (Thomas *et al.*, 2007). The total volume of the dentate gyrus was estimated using the point-counting Cavalieri principle (Manaye *et al.*, 2007). BrdU$^+$ cells were counted at 60x objective in three regions of interest in each of the twelve sections using the Optical Fractionator method. Applying the cell counts in each of the sections to the estimated total dentate gyrus volume the BrdU$^+$ cell numerical density (i.e. the total cell number) was calculated.

2.6 Polymerase chain reaction analysis

Polymerase chain reaction analysis was used to measure mRNA expression as previously described (Birch *et al.*, 2013a).

2.6.1 Total RNA extraction

RNA was extracted from brain tissue samples or cell lysates using the commercially available Nucleospin® RNA II isolation kit (Macherey-Nagel, Germany). The area and all apparatus used were cleaned with RNaseZap® wipes (Ambion) prior to the RNA extraction to ensure that the extraction was carried out in an RNase free environment. The extraction was carried out according to the manufacturer's guidelines. Tissue samples were mechanically homogenised in 350 µl lysis buffer solution (β-mercaptoethanol in RA1 buffer 1 in 100 dilution) using a polytron tissue disrupter (Kinetatica, Switzerland). The sample homogenates were added to Nucleospin® filters and were centrifuged for 1 min at 11,000 g. The filtrates were collected and 350 µl

molecular biology grade ethanol (70% [v/v]) was added to each sample filtrate. The filtrates were mixed with the ethanol by pipetting up and down five times. The samples were then pipetted up and down a further two times and added to Nucleospin® RNA II columns. To bind the RNA to the silica membranes of the Nucleospin® RNA II column the columns were centrifuged for 30 s at 11,000 g. Membrane desalting buffer (MDB; 350 μl) was added to the columns to dry the membrane and the columns were centrifuged for 1 min at 11,000 g. 95 μl of DNase reaction mixture (1 in 10 dilution of reconstitute rDNase in rDNase reaction buffer) was added to each of the columns and the columns were incubated at room temperature for 15 min to digest DNA. To stop the digestion reaction 200 μl RA2 buffer was added to each of the columns. The columns were centrifuged for 30 s at 11,000 g. 600 μl RA3 buffer was added to the column and it was centrifuged for 30 s at 11,000 g. A further 250 μl RA3 was added to the column and it was centrifuged for 2 min at 11,000 g. The column was placed in a nuclease-free collection tube and the RNA was collected by adding 60 μl RNase-free H_2O and centrifuging for 1 min at 11,000 g. The RNA was stored at -80°C.

2.6.2 RNA quantification and reverse transcription

The RNA concentration of each of the samples was quantified using a Nanodrop™ ND-1000 Spectrophotometer (Thermo Scientific). The optical density of each of the samples was measured at 260 nm and from this the concentration can be calculated. An optical density of 1.0 at 260 nm represents an RNA concentration of 40 $\mu g.ml^{-1}$, therefore the RNA concentration in each sample can be calculated using the following equation:

$$RNA = OD_{260\ nm} \text{ X dilution factor X } 40\ \mu g.ml^{-1}$$

In addition to this the absorbance of the samples is measured at a wavelength of 280 nm to assess the purity of the RNA. A ration of $OD_{260\ nm}$: $OD_{280\ nm}$ of approximately 1:8-2.0 indicates that the RNA is pure. Each of the samples was then appropriately diluted using RNase free H_2O to ensure that they all had an equal concentration of RNA.

The ABI High Capacity cDNA archive kit (Applied Biosystems) was used to reverse transcribe the RNA samples. 20 μl of equalised RNA was added to 20 μl 2x master mix containing: 1 in 5 dilution of 10x reverse transcription buffer; 1 in 12.5 dilution of 25x dNTPs; 1 in 5 dilution of random primers; 1 in 10 dilution of multiscribe reverse transcriptase and 1 in 2.38 dilution of H_2O). Samples were then incubated at 25°C for 10 min and then at 37°C for 120 min using a PTC-200 Peltier Thermal Cycler, Biosciences,

Dublin, Ireland). The cDNA produced was stored at -20°C until real-time polymerase chain reaction (RT-PCR) analysis.

2.6.3 RT-PCR analysis

RT-PCR was carried out using a Taqman gene expression assay kit (Applied Biosystems). Specific target primers and FAM-labeled target probes (Table 2.1) were used.

Gene expression was normalised to β-actin expression. β-actin expression was quantified using a specific target primer and a VIC-labeled rat β-actin probe.

Gene	Assay number	NCBI gene reference
β-actin	Rn.94978	NM_009608
BDNF	Rn00560868_m1	NC_000011.9
CB_1 receptor	Rn.228588	NC_000006.11
ki67	Rn01451446_m1	NC_000010.10
NGFβ	Rn01533872_m1	XM_227525.3
TrkA	Rn00572130_m1	NM_021589.1
TrkB	Rn00820626_m1	NM_012731.1

Table 2.1: PCR gene assays

2.6.4 RT-PCR

Prior to RT-PCR cDNA was diluted 1 in 4 using RNase free H_2O. 10 μl of diluted cDNA was pippetted on to a PCR plate. A mixture of target primer/probe (1.25 μl), β-actin primer/probe (1.25 μl) and Taqman® master mix (12.5 μl) was prepared and 25 μl was placed in each well of the PCR plate.

RT-PCR was performed using a real-time PCR thermocycler, ABI Prism 7300 instrument (Applied Biosystems). Samples were incubated at 95°C for 10 min then for 95°C for 15 s and 60°C for 1 min. Incubation at 95°C for 15 s and for 60°C for 1 min corresponded to one cycle. Cycles were repeated 40 times for BDNF and ki67 assays and 60 times for

CB1 receptor assay. Fluorescence was read during the annealing and extension phase (60° stage) for the duration of the programme.

2.6.5 RT-PCR analysis

Applied Biosystems 7300 RQ software was used to measure gene expression by the $\Delta\Delta$CT method. Using this method gene expression in the experimental samples was assessed relative to the mean of gene expression in the control samples. Therefore, gene expression within samples was calculated as fold change (either increase or decrease) of control. Briefly, this fold difference was assessed using the cycle number difference (CT) between samples. Threshold fluorescence is set and the CT is measured against this. The threshold was set when the PCR reaction was in the exponential phase.

2.7 Western immunoblotting

2.7.1 Preparation of samples for SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Equalised tissue homogenate (in lysis buffer) was mixed with sample buffer (Tris-HCL pH 6.8, 0.5 M; sodium dodecyl sulphate (SDS), 10% (w/v); glycerol, 10% (v/v); 2-β-mercaptoethanol, 5% (v/v); bromophenol blue, 0.05% (w/v)) at a ratio of 1:1 and boiled for 5 min.

2.7.2 SDS-PAGE

Polyacrylamide separating gels (10%; see appendix) were cast between 2 glass plates (BioRad Spacer plates, BioRad Laboratories Ltd., Hertfordshire, England) and allowed to set. Polyacrylamide stacking gel (4%; see appendix) was cast on top of the separating gel; a comb was placed into the stacking gel to induce well formation and the gel was allowed to set. The gel cassette was placed into an electrophoresis unit (BioRad Ltd.) and the unit was flooded with electrode running buffer (Tris base, 25 mM; glycine, 200 mM; SDS, 17 mM, pH: 8.5) ensuring that the inner reservoir between the gel cassettes was completely full. The first well of each gel was loaded with a colorimetric molecular weight marker (5 μl per well; Precision Plus Protein™ Standards, BioRad Ltd.) and a fluorometric molecular weight marker (2 μl per well; MagicMark™ XP Western Protein Standard, Invitrogen, Bio-sciences, Dublin, Ireland). The remaining wells were loaded with samples previously prepared in sample buffer (10 μl per well). Gels were run at 30 mA per gel for 30-40 min or until the samples had reached the bottom of the gel.

2.7.3 Western immunoblotting

The separating gel was removed from the cassette and immersed in transfer buffer (Tris base, 25 mM; glycine, 192 mM; methanol, 20% (v/v); SDS, 0.5% (w/v)). Hybond extra nitrocellulose membrane paper (Amersham Bioscience) and no. 3 grande filter paper (Whatman) were soaked in transfer buffer. The gel was placed in a sandwich of filter paper and nitrocellulose paper (Figure 2.3), the sandwich was placed in a semi-dry blotter unit (Apollo Instruments, Alpha Technologies, Dublin, Ireland) and immersed in transfer buffer.

Figure 2.3 Filter paper, gel and nitrocellulose membrane sandwich (cross section).

The upper lid of the unit was tightly fitted on top of the sandwich and the unit was run for 75 min at 225 mA to induce transfer of protein from the gel to the nitrocellulose membrane.

Nitrocellulose paper was removed from the transfer apparatus and was incubated for 2 hours at room temperature in a 5% BSA in Tris-buffered saline-Tween® 20 (TBS-T, 10 ml; 0.05% Tween® 20 in TBS) solution to block non-specific binding sites. The nitrocellulose membrane was incubated overnight at 4°C with a primary antibody (10 ml; in a 2% BSA in TBS-T solution) raised against a specific protein (see Table 2.2). The nitrocellulose paper membrane was washed with TBS-T (10 ml; 4 x 10 min) to remove excess primary antibody and the nitrocellulose membrane was incubated for 1 hour at room temperature with a secondary HRP-conjugated antibody (10 ml; in a 2% BSA in TBS-T solution) corresponding to the animal in which the primary antibody was raised (see Table 2.2). The membrane was washed with TBST-T (10 ml; 6 x 10 min) to remove excess secondary antibody. To identify immunoreactive bands HRP-conjugated secondary antibody was detected on the nitrocellulose membrane using Supersignal® West Dura chemiluminesence reagent (Pierce, Rockford, USA). Following 5 min incubation with Supersignal® West Dura chemiluminesence reagent (Pierce) the membrane was exposed to photographic film (Hyperfilm, Amersham, UK) using a Fuji Processor. Immediately following development the nitrocellulose membrane was washed

with TBS-T (10 ml; 3 x 10 min), stripped with ReBlot-Plus strong stripping solution (10 ml; 1 in 10 dilution in dH_2O, Millipore, Chemicon) to remove primary and secondary antibodies. The membrane was then ready to be re-probed for a different protein.

2.7.4 Densitometric analysis

Protein bands were imaged using Image reader LAS-3000 software (FujiFilm, USA) and a FujiFilm LAS-300 intelligent dark box (FujiFilm, USA). Protein bands were quantified by densitometric analysis using ImageJ (NIH, USA).

2.8 Statistical analysis

Prior to statistical analysis data were examined for outliers, which were identified as being more than two standard deviations removed from the mean. Outliers were excluded from the data set before statistical analysis.

All data are represented as mean ± standard error of the mean (SEM). Behavioural data were analysed using a two-way analysis of variance (ANOVA) to compare exploration of objects and the effect of group. Where a significant main effect was identified a Bonferroni *post-hoc* test was applied to detect the significant differences.

Tissue and cell data were analysed using a Student's *t*-test, a one-way ANOVA or a two-way ANOVA.

All statistical analysis was performed using Microsoft Office Excel 2007 and GraphPad Prism 5.0 (GraphPad Software Inc., USA).

Primary Antibody	Dilution	Incubation	Secondary Antibody	Dilution	Incubation
Mouse Anti-β-Actin (Sigma)	1:2000 in BSA (2% w/v) in TBS-T	Overnight @ 4˚C	Goat anti-mouse IgG (Sigma)	1:2000 in BSA (2% w/v) in TBS-T	1 h @ 22°C
Rabbit Anti-CB_1 Polyclonal IgG (Abcam)	1:1000 in BSA (2% w/v) in TBS-T	Overnight @ 4˚C	Goat anti-rabbit IgG HRP (Sigma)	1:2000 in BSA (2% w/v) in TBS-T	1 h @ 22°C
Rabbit Anti-GAPDH Polyclonal IgG (Abcam)	1:1000 in BSA (2% w/v) in TBS-T	Overnight @ 4˚C	Goat anti-rabbit IgG HRP (Sigma)	1:1000 in BSA (2% w/v) in TBS-T	1 h @ 22°C
Rabbit Anti-PSD-95 Polyclonal IgG (Cell Signaling Technology)	1:1000 in BSA (2% w/v) in TBS-T	Overnight @ 4˚C	Goat anti-rabbit IgG HRP (Sigma)	1:2000 in BSA (2% w/v) in TBS-T	1 h @ 22°C
Rabbit Anti-Synapsin Polyclonal IgG (Cell Signaling Technology)	1:1000 in BSA (2% w/v) in TBS-T	Overnight @ 4˚C	Goat anti-rabbit IgG HRP (Sigma)	1:2000 in BSA (2% w/v) in TBS-T	1 h @ 22°C
Rabbit Anti-Synaptophysin Polyclonal IgG (Abcam)	1:1000 in BSA (2% w/v) in TBS-T	Overnight @ 4˚C	Goat anti-rabbit IgG HRP (Sigma)	1:1000 in BSA (2% w/v) in TBS-T	1 h @ 22°C

Table 2.2: Antibodies, dilutions and incubations for Western immunoblotting.

Chapter 3

Evaluation of the interaction between cannabinoid signalling and neurotrophin expression in the brain

Chapter 3

3.1 Introduction

It is well known that cannabinoids can influence the activity of many cell types in the CNS and PNS. Cannabinoids can affect neuronal function by regulating neurotransmitter release (Wilson *et al.*, 2002) and expression of certain forms of synaptic plasticity (Abush *et al.*, 2010). In addition to modulating the function of mature neurons cannabinoids have also been shown to affect the process of neurogenesis. CBD enhances adult neurogenesis in the dentate gyrus by promoting the differentiation and survival of NSCs (Wolf *et al.*, 2010). Endocanninoids are also important in modulating adult neurogenesis; blockade of 2-AG synthesis *in vivo* was shown to reduce NSC proliferation and differentiation in the dentate gyrus (Gao *et al.*, 2010) and AEA enhances the survival and differentiation of NSCs isolated from the mouse dentate gyrus (Compagnucci *et al.*, 2013). Finally, endocannabinoids have been shown to be important in certain pathological states, such as cerebral ischemia, traumatic brain injury and amyloid-β induced toxicity as they exhibit neuroprotective effects (Zogopoulos *et al.*, 2013).

Neurotrophins can regulate many similar processes in the nervous system, for example neurotrophins have a role to play in basal neurotransmission as well as modification of long-term synaptic strength. The BDNF mimetic 7,8-dihydroxyflavone, acting viva the BDNF receptor TrkB, has been shown to affect neurotransmission (Marongiu *et al.*, 2013), while decreased expression of BDNF and NGFβ and their respective receptors TrkB and TrkA has been linked to a reduction in LTP expression in the dentate gyrus (Hennigan *et al.*, 2009). Like cannabinoids, neurotrophins have a pivotal role to play in neurogenesis. Blocking BDNF-TrkB receptor signalling results in a decrease in the rate of adult hippocampal neurogenesis (Quadrato *et al.*, 2012), and reducing cerebral BDNF concentration by half has been found to reduce the survival of newly born neurons (Sairanen *et al.*, 2005). NGFβ has been shown to have survival enhancing effects on newly generated neurons in the dentate gyrus (Frielingsdorf *et al.*, 2007). Finally, the neuroprotective properties of neurotrophins in the CNS have been well documented (Tabakman *et al.*, 2004).

Although cannabinoids and neurotrophins have similar actions at the cellular level within the nervous system there has been limited investigation into the interactions been these two signalling systems. Acute BDNF application reduces GABA mediated IPSPs in the neocortex and the striatum and this effect is dependent initially on activation of TrkB

followed by CB_1 receptor activation (De Chiara *et al.*, 2010; Lemtiri-Chlieh *et al.*, 2010), suggesting that BDNF may mediate endocannabinoid production/release. NGF can also interact with endocannabinoid signalling and recently has been shown to increase 2-AG and CB_1 receptor expression in cultured neurons (Keimpema *et al.*, 2013). However, there has been little investigation into the possibility of cannabinoid signalling regulating neurotrophin expression, though it has been reported that chronic administration of Δ^9-THC can increase BDNF expression in certain brain regions in rats (Butovsky *et al.*, 2005) and acute intravenous administration of Δ^9-THC in humans results in an increase in serum BDNF (D'Souza *et al.*, 2009). To date no study has focused on the acute effects of endocannabinoids nor have the effects of endocannabinoids on neurotrophin expression been investigated.

The aim of the present study was to investigate if cannabinoids, with particular focus on endocannabinoids, regulate expression of neurotrophins in the brain. The initial aim of the present study was to evaluate how neuronal expression of BDNF, NGFβ and their respective receptors TrkB and TrkA may be affected by the phytocannabinoid CBD or the endocannabinoid degradation enzyme inhibitors URB 597 and URB 602. URB 597 inhibits the enzyme FAAH that is primarily responsible for AEA hydrolysis while URB 602 inhibits MAGL that is primarily responsible for 2-AG degradation. A time profile of the effects of the three drugs was first evaluated using previously verified concentrations of CBD (1 μM), URB 597 (1 μM) and URB 602 (100 μM) (Noonan *et al.*, 2010; Ryan *et al.*, 2007; Tanveer *et al.*, 2012). Based on findings from the *in vitro* component of the study, two *in vivo* experiments were designed. The aim of these experiments was to attempt to translate *in vitro* findings to an animal model. First, the effect of a systemic injection of URB 597 (0.3 $mg.kg^{-1}$), which has been shown at this concentration to induce maximal FAAH inhibition in the brain (Fegley *et al.*, 2005), on BDNF concentration in the dentate gyrus, hippocampus and serum was assessed. Finally, the effect of a systemic injection of URB 602 (3 $mg.kg^{-1}$), a sub-psychoactive dose previously shown at this concentration to induce central effects (Comelli *et al.*, 2007), on BDNF and NGFβ concentration in the dentate gyrus, hippocampus and serum was assessed.

3.2 Methods

In vitro experiments

3.2.1 Primary culture of cerebral cortical neurons

Primary cortical neurons were cultured under sterile conditions (see section 2.2.1) from postnatal day one Wistar rats as outlined in section 2.3.4.

3.2.2 Experimental design and drug treatments

Neurons were cultured for six days prior to drug treatment. For the timecourse experiment neurons were incubated with either the phytocannabinoid cannabidiol (1 μM), the FAAH inhibitor URB 597 (1 μM) or the MAGL inhibitor URB 602 (100 μM) for 1, 2, 4, 8 or 24 h. Following specific treatments neurons were lysed in RA1 lysis buffer and stored at -20°C.

3.2.3 Analysis of mRNA expression by RT-PCR

Cell lysates were defrosted, RNA was isolated and RT-PCR was carried out (see section 2.6) to assess expression of BDNF, TrkB, NGFβ and TrkA mRNA.

In vivo experiments

3.2.4 Animals

A total of 28 three-month old male Wistar rats (Bio Resources, Trinity College Dublin) were used in these experiments. Animals weighed between 300g and 400g and were experimentally naïve at experiment onset. Following transfer from the Bio Resources breeding facility animals were given seven days to acclimatise to the housing facility before the experimental procedure, during this acclimatisation period animals were handled every day by the experimenter. Animals were housed as described in section 2.1.2.

3.2.5 Experimental design

Animals were assigned to one of two experiments 1) to assess how inhibition of AEA hydrolysis by the enzyme FAAH would effect neurotrophin expression in the brain (n=16) or 2) to assess how inhibition of 2-AG hydrolysis by the enzyme MAGL would effect neurotrophin expression in the brain (n=12). Within each study animals were

randomly divided into a vehicle treated group (n=8 or n=6 respectively) and a group injected with a specific enzyme inhibitor URB 597 (n=8) or URB 602 (n=6) respectively. In experiment 1) animals were euthanised 3 h following URB 597 or vehicle injection, in experiment 2) animals were euthanised 5 h following URB 602 or vehicle injection. Duration of drug treatment was decided based on results obtained from *in vitro* experiments. The hippocampus and dentate gyrus were removed for RNA and protein analysis. Trunk blood was collected and serum isolated as outlined in section 2.4.2 and stored at -20°C.

3.2.6 Drug treatments

Animals were injected i.p. with either URB 597 (0.3 $mg.kg^{-1}$) dissolved in vehicle solution (50% DMSO in 0.9% saline) or URB 602 (3 $mg.kg^{-1}$) dissolved in vehicle solution (50% DMSO in 0.9% saline), while control animals received corresponding vehicle solution (approximately 1 ml per animal).

3.2.7 Analysis of mRNA expression by RT-PCR

RNA was isolated from hippocampal and dentate gyrus tissue and RT-PCR was carried out (see section 2.7) to assess expression of BDNF and NGFβ mRNA.

3.2.8 Analysis of protein expression by ELISA

BDNF and NGFβ protein was quantified in serum and tissue samples using a Human BDNF ELISA kit (R&D Systems Europe, Oxon, United Kingdom) and a Rat NGFβ ELISA kit (R&D Systems Europe, Oxon, United Kingdom) respectively (see section 2.4). Serum was diluted 1 in 2 with distilled water before the ELISA was carried out. Hippocampus and dentate gyrus samples were homogenised in Krebs calcium buffer (see section 2.3) prior to carrying out the ELISA.

3.2.9 Statistical analysis

Prior to statistical analysis data were examined for outliers, which were identified as being more than two standard deviations outside the mean. Outliers were excluded from the data set before statistical analysis.

In vitro timecourse data were analysed using a one-way ANOVA. Data from *in vivo* experiments were analysed using a Student's *t*-test. All RNA data was calculated as fold-change of control.

Data are represented as mean ± standard error of the mean (SEM). All statistical analysis was performed using Microsoft Office Excel 2007 and GraphPad Prism 5.0 (GraphPad Software Inc., USA). Statistical significance was inferred at $p<0.05$.

3.3 Results

In vitro experiments

3.3.1 Effect of cannabidiol treatment on neurotrophin expression in neurons

CBD increased the expression of BDNF mRNA in cultured cortical neurons ($F_{5,30}$=5.90, $p<0.001$). Specifically, CBD treatment was found to induce a six-fold increase in BDNF mRNA at 2 h compared to control (2 h: 6.1±2.4, control: 1.0±0.6, ***$p<0.001$, Figure 3.1 A). CBD had no effect on expression of TrkB ($F_{5,12}$=1.340, $p>0.05$, Figure 3.1 B), NGFβ ($F_{5,30}$=0.8245, $p>0.05$, Figure 3.1 C) or TrkA ($F_{5,29}$=1.294,$p>0.05$, Figure 3.1 D) mRNA at any of the timepoints assessed.

3.3.2 Effect of URB 597 treatment on neurotrophin expression in neurons

URB 597 increased the expression of BDNF mRNA in cultured cortical neurons ($F_{5,18}$=7.838, $p<0.001$). Specifically, URB 597 treatment was found to induce a six-fold increase in BDNF mRNA at 2 h compared to control (2 h: 6.4±2.6, control: 1.0±0.1, ***$p<0.001$, Figure 3.2 A). URB 597 had no effect on expression of TrkB ($F_{5,12}$=1.340, $p>0.05$, Figure 3.2 B), NGFβ ($F_{5,30}$=0.8245, $p>0.05$, Figure 3.2 C) or TrkA ($F_{5,29}$=1.294, $p>0.05$, Figure 3.2 D) mRNA at any of the timepoints assessed.

3.3.3 Effect of URB 602 treatment on neurotrophin expression in neurons

URB 602 increased the expression of BDNF mRNA in cultured cortical neurons ($F_{5,10}$=1.111, $p<0.01$).. Specifically, URB 602 treatments were found to induce an approximate two-fold increase in BDNF mRNA at 4 h and 8 h compared to control (4 h: 2.0±0.3, 8 h: 2.0±0.1 control: 1.0±0.2, *$p<0.05$, Figure 3.3 A). URB 602 had no effect on expression of TrkB ($F_{5,16}$=0.6708, $p>0.05$, Figure 3.3 B) or NGFβ ($F_{5,11}$=5.556, $p>0.05$, Figure 3.3 C) mRNA at any of the timepoints assessed. URB 602 had a significant effect on TrkA mRNA expression ($F_{5,11}$=0.3014, $p<0.05$, Figure 3.3 D), however *post-hoc* analysis revealed no difference at any of the timepoints assessed.

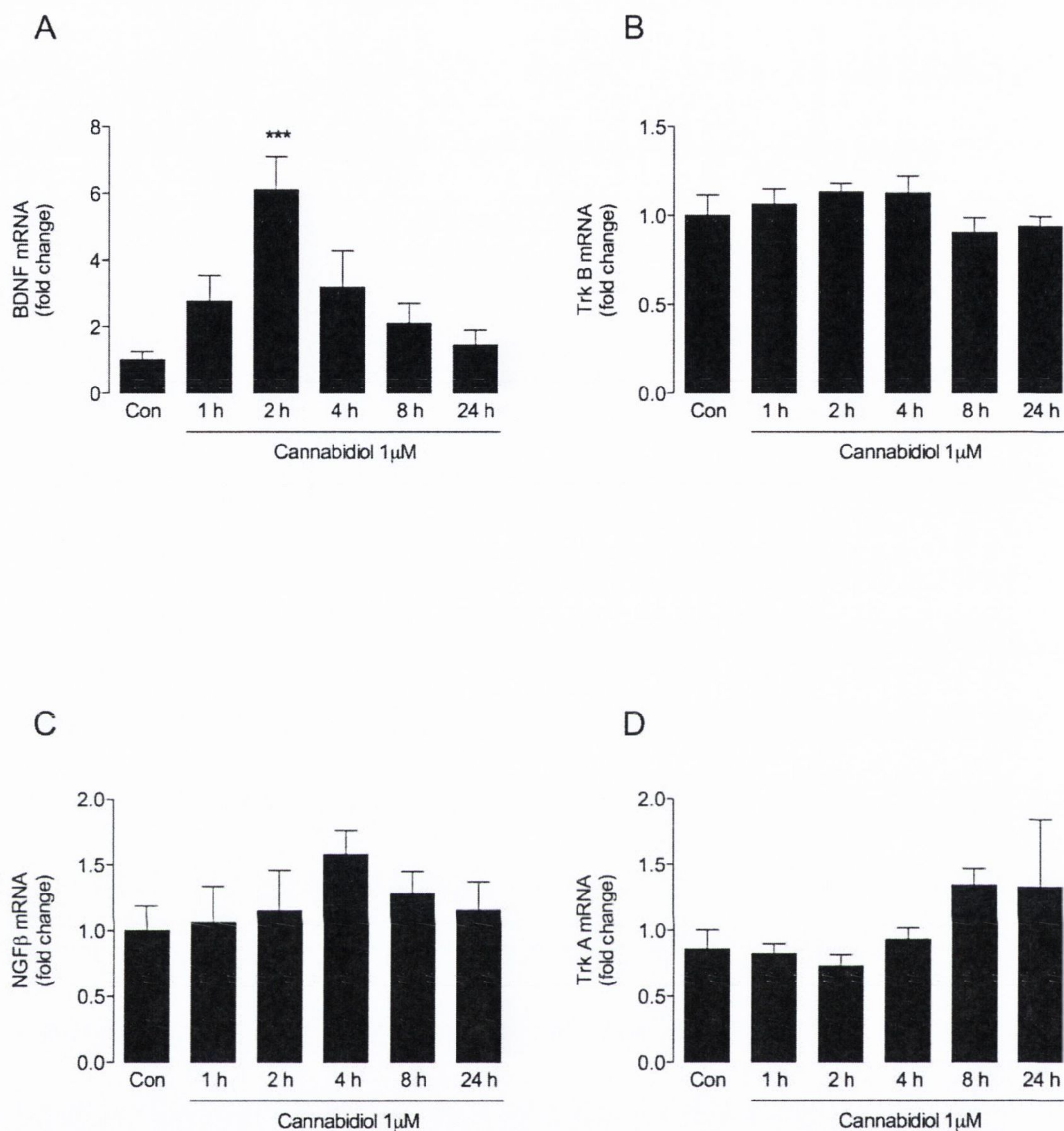

Figure 3.1 The effect of 1, 2, 4, 8 or 24 h CBD (1 μM) treatment on expression of BDNF, TrkB, NGFβ and TrkA mRNA in cultured neurons.

(A) There was a significant effect of CBD treatment on BDNF mRNA expression with *post-hoc* a significant increase in BDNF mRNA expression at the 2 h timepoint compared to control (***p<0.001). (B) There was no effect of CBD treatment on TrkB mRNA expression. (C) There was no effect of CBD treatment on NGFβ mRNA expression. (D) There was no effect of CBD treatment on TrkA mRNA expression. Bars represent mean±SEM, fold change of control, one-way ANOVA, n=6.

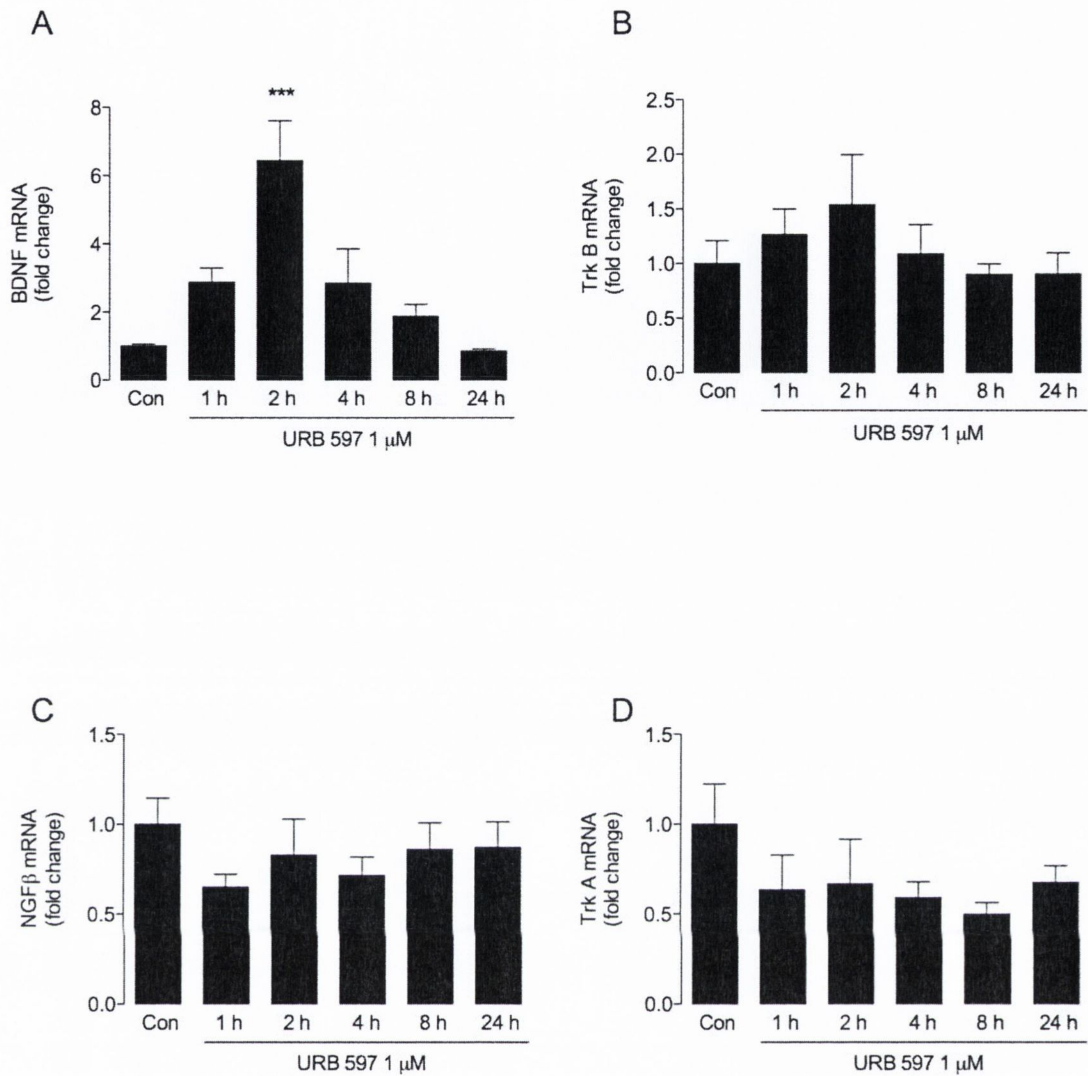

Figure 3.2 The of effect 1, 2, 4, 8 or 24 h URB 597 (1 μM) treatment on expression of BDNF, TrkB, NGFβ and TrkA mRNA in cultured neurons.

(A) There was a significant effect of URB 597 treatment on BDNF mRNA expression with *post-hoc* a significant increase in BDNF mRNA expression at the 2 h timepoint compared to control (***p<0.001). (B) There was no effect of URB 597 treatment on TrkB mRNA expression. (C) There was no effect of URB 597 treatment on NGFβ mRNA expression. (D) There was no effect of URB 597 treatment on TrkA mRNA expression. Bars represent mean±SEM, fold change of control, one-way ANOVA, n=6.

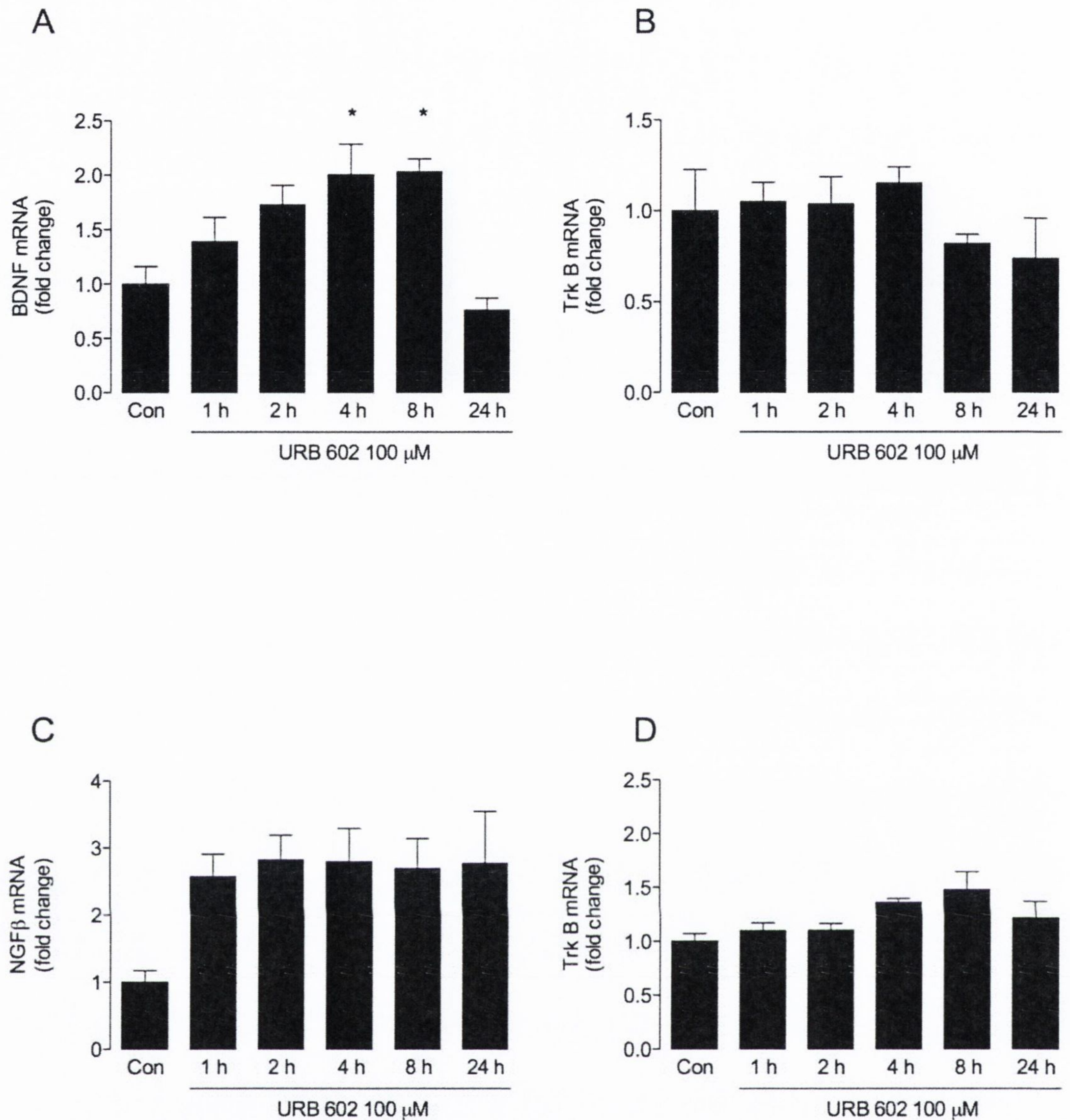

Figure 3.3 The effect of 1, 2, 4, 8 or 24 h URB 602 (100 μM) treatment on expression of BDNF, TrkB, NGFβ and TrkA mRNA in cultured neurons.

(A) There was a significant effect of URB 602 treatment on BDNF mRNA expression with a significant increase in BDNF mRNA expression at the 4 h and 8 h timepoints compared to control (*$p<0.05$). (B) There was no effect of URB 602 treatment on TrkB mRNA expression. (C) There was no effect of URB 602 treatment on NGFβ mRNA expression. (D) There was a significant effect of URB 602 treatment on TrkA mRNA expression, however *post-hoc* analysis revealed no difference between the groups. Bars represent mean±SEM, fold change of control, one-way ANOVA, n=3.

In vivo **experiments**

3.3.4 Effect of URB 597 on BDNF expression in the dentate gyrus, hippocampus and serum

There was no effect of systemic administration of URB 597 on BDNF protein concentration in the dentate gyrus (Vehicle: 1451±203.0 pg.mg protein^{-1}, URB 597: 1706±485.5 pg.mg protein^{-1}, t_7=0.6914, p>0.05, Figure 3.4).

There was no effect of URB 597 on BDNF mRNA expression (t_{13}=0.0007, p>0.05, Figure 3.5 A) or protein expression (Vehicle: 625.4±82.48 pg.mg protein^{-1}, URB 597: 817±54.91 pg.mg protein^{-1}, t_{14}=1.936, p>0.05, Figure 3.5 B) in the hippocampus, although the effect on BDNF protein expression approached significance (p=0.07).

There was no effect of URB 597 on BDNF protein concentration in the serum (Vehicle: 4136.0±287.0 pg.ml^{-1}, URB 597: 3962.0±318.6 pg.ml^{-1}, t_{14}=0.4046, p>0.05, Figure 3.6).

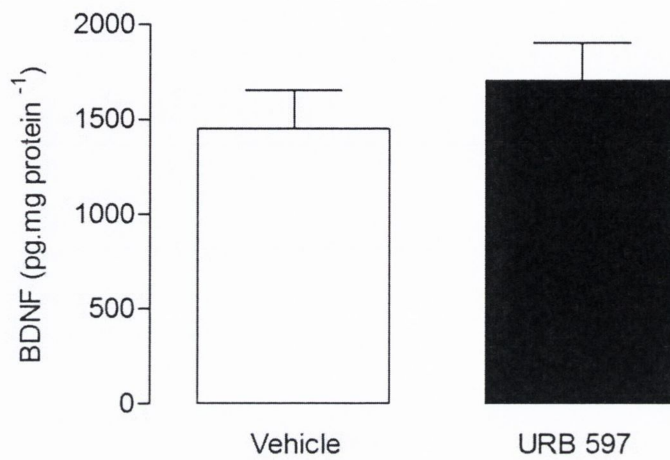

Figure 3.4 The effect of a single injection of URB 597 on BDNF protein concentration in the dentate gyrus

There was no effect of URB 597 on BDNF protein expression. Bars represent mean±SEM, Student's *t*-test, n=8 per group.

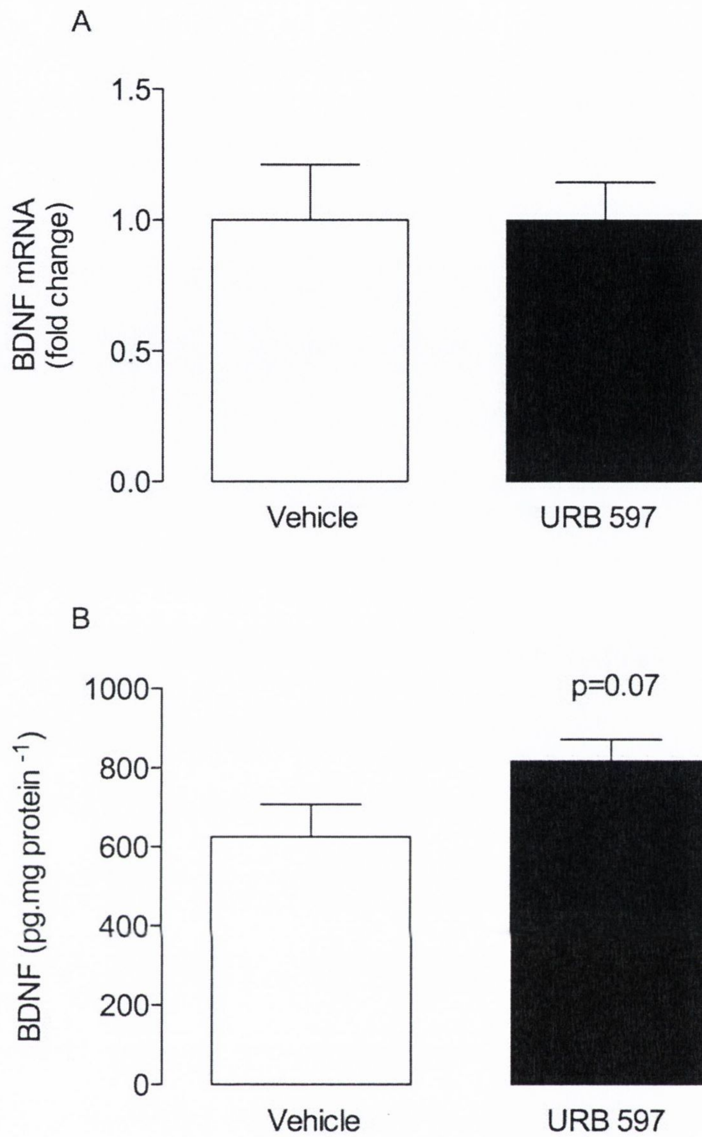

Figure 3.5 The effect of a single injection of URB 597 on BDNF mRNA and protein expression in the hippocampus

(A) There was no effect of URB 597 on BDNF mRNA expression. (B) There was no effect of URB 597 on BDNF protein concentration. Bars represent mean±SEM, Student's *t*-test, n=8 per group.

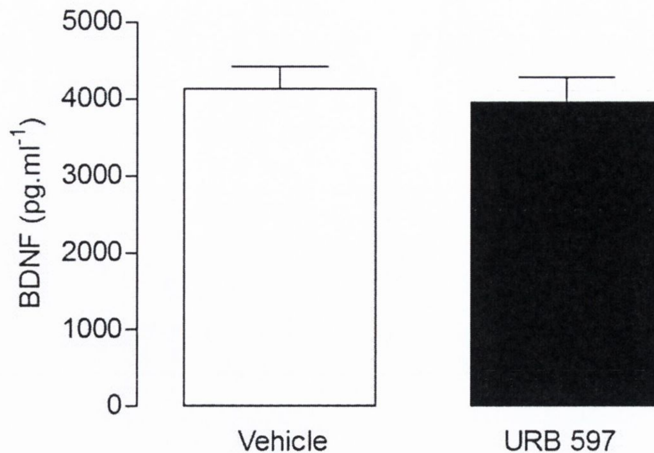

Figure 3.6 The effect of a single injection of URB 597 on BDNF protein concentration in the serum

There was no effect of URB 597 on BDNF protein concentration. Bars represent mean±SEM, Student's *t*-test, n=8 per group.

3.3.5 Effect of URB 602 on BDNF expression in the dentate gyrus, hippocampus and serum

There was no effect of URB 602 on BDNF mRNA expression in the dentate gyrus (t_{10}=0.3422, $p>0.05$, Figure 3.7 A). There was a significant increase in BDNF protein concentration in the URB 602 group compared to control (Vehicle: 217.7±26.17 pg.mg protein^{-1}, URB 602: 368.0±30.38 pg.mg protein^{-1}, t_{10}=3.749, **$p<0.01$, Figure 3.7 B).

There was no effect of URB 602 on BDNF mRNA (t_7=0.4770, $p>0.05$, Figure 3.8 A) or protein concentration (Vehicle: 209.1±13.44 pg.mg protein^{-1}, URB 602: 241.9±17.31 pg.mg protein^{-1}, t_{10}=1.497, $p>0.05$, Figure 3.8 B) in the hippocampus.

There was no effect of URB 602 BDNF protein concentration (Vehicle: 1757.0±140.4 pg.ml^{-1}, URB 602: 1769.0±210.8 pg.ml^{-1}, t_{10}=0.0447, $p>0.05$, Figure 3.9) in the serum.

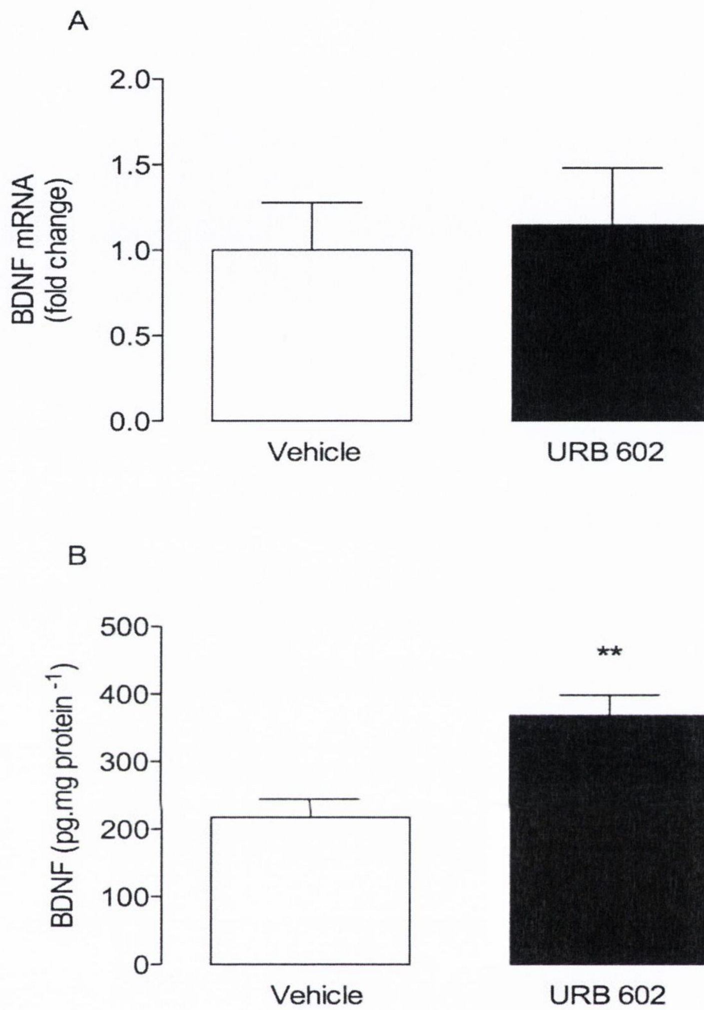

Figure 3.7 The effect of a single injection of URB 602 on BDNF mRNA and protein expression in the dentate gyrus

(A) There was no effect of URB 602 on BDNF mRNA expression. (B) There was a significant increase BDNF protein concentration in the URB 602 group compared to vehicle group (**$p<0.01$). Bars represent mean±SEM, Student's t-test, n=6 per group.

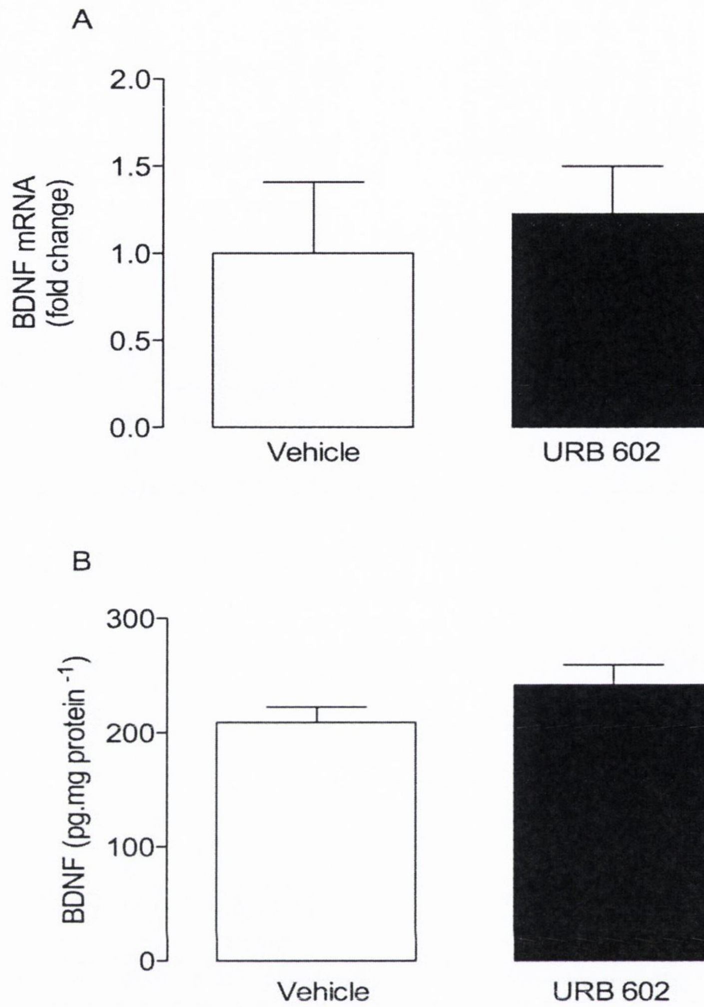

Figure 3.8 The effect of a single injection of URB 602 on BDNF mRNA and protein expression in the hippocampus

(A) There was no effect of URB 602 on BDNF mRNA expression. (B) There was no effect of URB 602 on BDNF protein expression. Bars represent mean±SEM, Student's *t*-test, n=6 per group.

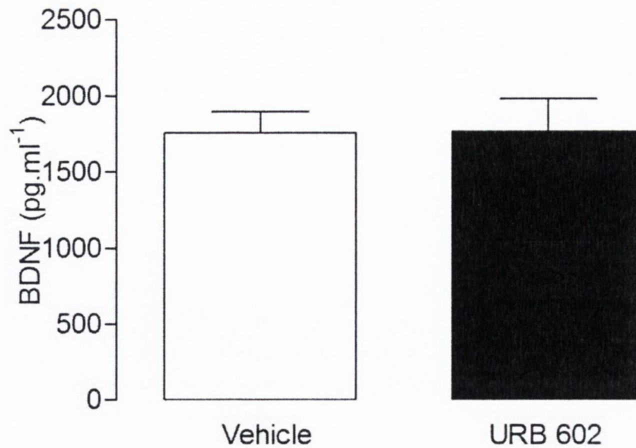

Figure 3.9 The effect of a single injection of URB 602 on BDNF protein concentration in the serum

There was no effect of URB 602 on BDNF protein concentration. Bars represent mean±SEM, Student's *t*-test, n=6 per group.

3.3.6 Effect of URB 602 on NGFβ expression in the dentate gyrus, hippocampus and serum

There was no effect of URB 602 on NGFβ mRNA expression in the dentate gyrus (t_{10}=1.907, p>0.05, Figure 3.10 A). There was a significant increase in NGFβ protein concentration in the URB 602 group compared to vehicle group (Vehicle: 13.4±4.4 pg.mg protein^{-1}, URB 602: 42.1±11.8 pg.mg protein^{-1}, t_6=2.768, *p<0.05, Figure 3.10 B).

There was no effect of URB 602 on NGFβ mRNA (t_8=1.020, p>0.05, Figure 3.11 A) in the hippocampus. There was a significant increase in NGFβ protein concentration in the URB 602 group compared to vehicle group (Vehicle: 35.7±7.7 pg.mg protein^{-1}, URB 602: 63.3±4.5 pg.mg protein^{-1}, t_{10}=3.090, *p<0.05, Figure 3.11 B).

There was no effect of URB 602 NGFβ protein concentration (Vehicle: 192.9±87.15 pg.ml^{-1}, URB 602: 75.93±26.70 pg.ml^{-1}, t_9=1.177, p>0.05, Figure 3.12) in the serum.

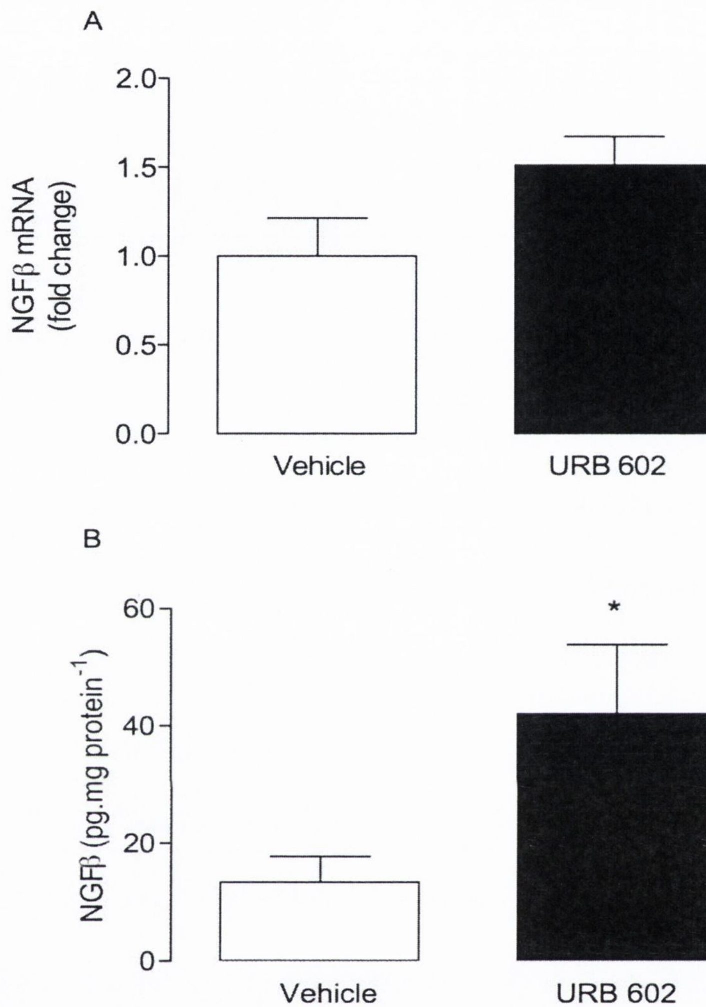

Figure 3.10 The effect of a single injection of URB 602 on NGFβ mRNA and protein expression in the dentate gyrus

(A) There was no effect of URB 602 on NGFβ mRNA expression. (B) There was a significant increase in NGFβ concentration in the URB 602 group compared to vehicle group (*$p<0.05$). Bars represent mean±SEM, Student's t-test, n=6 per group.

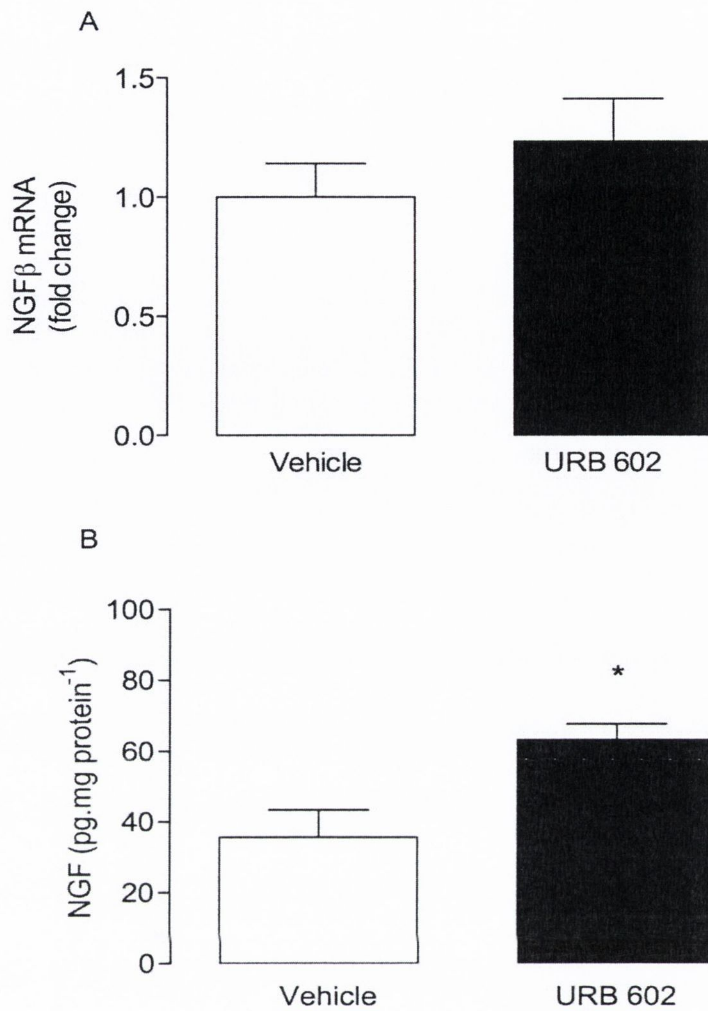

Figure 3.11 The effect of a single injection of URB 602 on NGFβ mRNA and protein expression in the hippocampus

(A) There was no effect of URB 602 on NGFβ mRNA expression. (B) There was a significant increase in NGFβ concentration in the URB 602 group compared to vehicle group (*$p<0.05$). Bars represent mean±SEM, Student's *t*-test, n=6 per group.

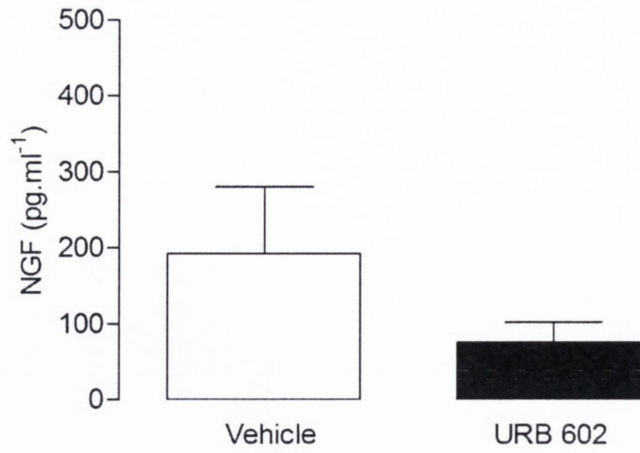

Figure 3.12 The effect of a single injection of URB 602 on NGFβ protein concentration in the serum

There was no effect of URB 602 on NGFβ protein concentration. Bars represent mean±SEM, Student's *t*-test, n=6 per group.

3.4 Discussion

CBD was found to time-dependently increase neuronal expression of BDNF mRNA, peaking at 2 h post-treatment and gradually returning to control levels by 24 h. CBD had no effect on expression of TrkB, NGFβ or TrkA mRNA at any of the five timepoints ranging from 1 h – 24 h. The finding that CBD can induce transcription of BDNF mRNA is a novel finding as CBD remains a relatively unexplored cannabinoid and this finding may help to explain some of the previously reported cellular effects of CBD. For example CBD was found to increase neurogenesis in mice by enhancing the survival of NPCs and newly-born neurons (Wolf *et al.*, 2010), this increase in net-neurogenesis by the promotion of cell survival is a property also attributed to BDNF signalling (Choi *et al.*, 2009). This suggests that the pro-neurogenic effects of CBD could at least partially or perhaps solely, be due to up regulation of BDNF expression. Another process regulated by CBD that could be explained by CBD-induced expression of BDNF is neuroprotection. Alzheimer's disease is associated with an accumulation of Aβ-plaques, which are thought to induce neuronal apoptosis. CBD has been shown to reduce neuronal apoptosis *in vitro* following treatment of a neuronal cell line with Aβ (Iuvone *et al.*, 2004) and a drug which enhances BDNF signalling in the brains of mutant mice that accumulate Aβ was shown to reduce apoptosis (Li *et al.*, 2010). These findings again show that CBD and BDNF regulate very similar mechanisms and it is possible that the effects of CBD on these mechanisms may potentially be mediated by CBD-induced up regulation of BDNF expression.

Interestingly, the effects observed in CBD treated neurons were almost identical to those observed in neurons treated with URB 597. URB 597 increased the expression of BDNF mRNA in a time-dependent manner; peaking with an approximate six-fold increase after a 2 h treatment which gradually returned to control level by 24 h. URB 597 had no effect on expression of TrkB, NGFβ or TrkA mRNA in any of the treatment durations measured over a 24 h period. Although there is pharmacological differences between the mechanism of action of CBD and URB 597 their ability to induce the same end result may account for the similarities in the neurotrophin expression profile observed following treatment with the two drugs. URB 597 can inhibit FAAH activity and therefore lead to the accumulation of AEA without interacting with AEA reuptake or cannabinoid receptors (Piomelli *et al.*, 2006). CBD has a far more complex pharmacological profile interacting with a wide variety of receptors and modulating the

activity of many different enzymes (Pertwee, 2008). There is evidence to suggest that CBD can inhibit the cellular uptake of AEA at concentrations similar to that used in this study (Rakhshan *et al.*, 2000). As previously mentioned FAAH is predominantly an intra-cellular enzyme therefore inhibition of cellular AEA uptake could lead to an accumulation of extracellular AEA. The fact that, albeit by different mechanisms, CBD and URB 597 both lead to the extracellular accumulation of AEA may explain the similarities between the results of CBD and URB 597 treatments observed in the present study. Due to the fact that URB 597 induced a robust increase in BDNF expression in cultured neurons the study moved to assess if this result could be replicated in an *in vivo* model. With particular overall attention of this thesis being given to the interactions between cannabinoids and neurotrophins in the process of adult neurogenesis and spatial memory the effect of URB 597 on BDNF expression was assessed in an adult neurogenic zone responsible for spatial memory formation; the dentate gyrus and its surrounding area; the hippocampus proper. Also, due to the fact that URB 597 was administered systemically circulating BDNF levels were measured. URB 597 had no effect on BDNF mRNA expression in the hippocampus nor did it have a significant effect on BDNF concentration in the dentate gyrus, hippocampus or serum. A limitation of this *in vivo* experiment was that, unlike the *in vitro* experiment, a timecourse analysis of expression was not carried out and only one timepoint post URB 597 administration was analysed (3 h post injection). This timepoint was based on estimation from the *in vitro* results obtained (i.e. 2 h treatment with URB 597 induced a six-fold increase in BDNF mRNA in neurons) and attempting to estimate *in vivo* treatment times from results obtained in cultured cells has limitations. Also, previous unpublished data from our lab has suggested that changes in BDNF concentration (in response to an intervention) in the rat brain are particularly transient, something that has also been observed in cultured astrocytes (Zhu *et al.*, 2012) as well as human serum (Griffin *et al.*, 2011), emphasising the importance of the timepoint on the results. However, interestingly it was found that URB 597 induced an increase in hippocampal BDNF concentration that closely approached statistical significance (p=0.07). This suggests that it is likely that URB 597 does effect BDNF expression in the hippocampus and that altering the timeline of the experiment may identify a timepoint where hippocampal BDNF concentration may be elevated.

An interesting point to note is that although URB 597 induces an increase (that is approaching statistical significance) in BDNF concentration in the hippocampus no increase is observed in the dentate gyrus. The concept that cannabinoids can induce region-specific changes in BDNF has been observed before; Butovsky *et al.* (2005)

reported that chronic Δ^9-THC administration caused increases in BDNF mRNA in certain brain regions but not others.

The fact that inhibition of AEA hydrolysis by URB 597 causes an increase in neuronal BDNF expression and appears to increase BDNF expression in the adult hippocampus may aid to explain some of the neurogenic and neuroprotective effects of AEA. For instance, AEA is important for dictating NSC/NPC cell fate and has been shown to induce neuronal differentiation of NSCs isolated from the mouse brain (Compagnucci *et al.*, 2013; Soltys *et al.*, 2010) while the induction of neuronal differentiation of NSCs by BDNF has been well documented (Ahmed *et al.*, 1995; Chen *et al.*, 2013). Therefore, it is possible that the AEA induced promotion of neuronal differentiation may be mediated or at least enhanced by the fact that AEA increases BDNF expression. It is possible that the neuroprotective effects elicited by AEA may be mediated in some way by its effects on BDNF expression. Chronic URB 597 treatment elevates AEA concentration (Murphy *et al.*, 2012) and a chronic exercise protocol elevates BDNF concentration (O'Callaghan *et al.*, 2009) in the aged hippocampus both of which prove to be neuroprotective as these interventions have been shown to rescue an age-related decline in LTP. However, further investigation would be required to confirm such links.

URB 602 was also found to affect BDNF expression, however the temporal profile differed to that of CBD and URB 597. An approximate two-fold increase in neuronal BDNF mRNA was observed after a URB 602 treatment of 4 h or 8 h with the increase returning to control level by the 24 h timepoint. After acquiring these results the study attempted to replicate the URB 602 associated BDNF increase *in vivo* in the adult brain. Again with overall attention being focused on the interaction between cannabinoid signalling and neurotrophin signalling in adult neurogenesis and spatial memory the effect of URB 602 on BDNF expression in the dentate gyrus and the hippocampus was examined. As URB 602 was administered systemically its effect on serum BDNF concentration was also measured. It was found initially that URB 602 had no effect on BDNF mRNA expression in the dentate gyrus or the hippocampus; however, it was found that the drug induced an arguably more important increase at the functional protein level in the dentate gyrus, but not the hippocampus. Again, the region specificity of the drug is observed here, as the increase in BDNF protein concentration is only present in the dentate gyrus and not the hippocampus, which interestingly is in contrast to URB 597 where the almost significant increase in BDNF protein was found in the hippocampus and not the dentate gyrus. The fact that inhibition of 2-AG degradation by URB 602 increases BDNF concentration in the dentate gyrus specifically, one of the two distinct

neurogenic zones of the adult brain, may have direct consequences for adult neurogenesis. URB 602 was found to have no effect on serum BDNF suggesting that changes may have been specific to the particular areas of the CNS.

URB 602 was also found to affect NGFβ expression. *In vitro* all five treatment durations assessed (1, 2, 4, 8, 24 h) were found to induce an approximate two and a half-fold increase in NGFβ mRNA, however this increase did not reach statistical significance which is likely due to the low sample size in this experiment (n=3). In a similar fashion to the BDNF result described above, URB 602 was found to have no effect on NGFβ expression at the mRNA level in the dentate gyrus or the hippocampus, however, interestingly there was an increase in NGFβ protein concentration in both the dentate gyrus and the hippocampus. URB 602 was found to have no effect on serum NGFβ concentration suggesting that the drug is not having an effect on circulating NGFβ.

The fact that increasing 2-AG tone leads to an increase in expression of the neurotrophins BDNF and NGFβ in the dentate gyrus and NGFβ in the hippocampus suggests that 2-AG can interact with neurotrophin signalling to affect hippocampal function. 2-AG increases BDNF and NGFβ in the dentate gyrus which means that 2-AG could be responsible for regulating the concentration of neurotrophins in the microenvironment of proliferating and differentiation NSCs and maturing neurons which is likely to have a profound effect on their fate. Although there has been little investigation into the role of 2-AG in adult neurogenesis there is evidence to suggest it has a role to play; knockdown of the 2-AG synthesising enzyme DAGLα which is associated with an 80% reduction in concentration of 2-AG in the brain reduces both the number of proliferating cells and the number of immature neurons in the dentate gyrus by approximately 50% (Gao *et al.*, 2010), however it should be noted that this effect may not be exclusively due to 2-AG depletion as DAGLα knockdown also decreases AEA concentration by approximately 40% in the brain. BDNF knockdown, which leads to an 80% reduction in cerebral BDNF concentration, has also been shown to reduce the number of immature neurons in the dentate gyrus by approximately 50% (Taliaz *et al.*, 2010). This again illustrates the fact that blocking activity of a specific endocannabinoid, 2-AG in this case, or a neurotrophin that it can regulate the expression of e.g. BDNF (as shown by the data presented in this chapter), can have the same end-effect on adult neurogenesis; suggesting the possibility that some of the effects of endocannabinoids may be related to their ability to alter neurotrophin expression. The fact that 2-AG has also been shown to regulate NGFβ expression in the dentate gyrus and hippocampus could also potentially explain the

effects of this particular endocannabinoid on neurogenesis; reducing 2-AG concentration in the brain can reduce the survival of immature neurons and NGFβ is strongly linked with the survival of immature neurons (Birch *et al.*, 2013b; Frielingsdorf *et al.*, 2007). Another process that is of great importance to the successful generation of a new functional neuron in the adult dentate gyrus is its ability to project an axon and form a synapse with cells within the CA3 region of the hippocampus. Neuriteogenesis, the formation of a projection from the cell soma, is a process that has been shown to be regulated by both 2-AG (Jung *et al.*, 2011) and NGFβ (Behar *et al.*, 1994). Interestingly the present study shows that systemically increasing 2-AG tone can increase NGFβ expression in the hippocampus, therefore there is a possibility that an interaction between 2-AG signalling and NGFβ expression may aid to regulate axonal guidance of newly generated cells in the dentate gyrus, however further investigation would be required to confirm such a possibility.

In summary, the present results show that cannabinoids can regulate neurotrophin expression in primary neuronal cultures and in the adult dentate gyrus and hippocampus. There is evidence in the literature proving that cannabinoids and neurotrophins can have similar cellular effects, especially with respect to proliferation, differentiation and survival of NPCs and immature neurons in the adult dentate gyrus. Taking into account the results from the current study and the fact that cannabinoids and neurotrophins have converging cellular effects opens up the possibility that some of the cellular effects induced by cannabinoids may be partially, or even solely, mediated by their ability to alter neurotrophin expression, however extensive future investigation is needed to investigate such a possibility. If this possibility is substantiated it could identify many future therapeutic targets of cannabinoid-based drugs and help to explain some of the mechanisms of currently available cannabinoid-based drugs.

Chapter 4

An investigation into the role of endocannabinoids into exercise-induced changes in the brain

Chapter 4

4.1 Introduction

It has been well established that exercise has a positive effect on hippocampal function. Both voluntary and forced exercise can enhance spatial learning and memory in a variety of different tasks; including the Morris water maze (van Praag *et al.*, 1999a), the object displacement task (Griffin *et al.*, 2009) and the radial arm maze (Khabour *et al.*, 2010). Several neurophysiological changes associated with exercise have been hypothesised to be the mechanisms underlying exercise-induced memory enhancement.

Cognitive improvements associated with exercise have often been attributed to hippocampal BDNF signalling. There is a positive correlation between BDNF expression in the hippocampus of exercising rats and performance in the Morris water maze (O'Callaghan *et al.*, 2009). Exercise increases both expression of BDNF and its receptor TrkB in the hippocampus (Cassilhas *et al.*, 2012), while administering an immunoglobulin that binds BDNF and blocks its interaction with the TrkB receptor prevents exercise-induced spatial learning enhancement (Vaynman *et al.*, 2004).

Neurogenesis is another neurophysiological change that is often suggested to be responsible for the memory-improving effects of exercise. Exercise is a potent stimulator of the neurogenesis process; it enhances NSC/NPC proliferation (van Praag *et al.*, 1999b) and survival (Brandt *et al.*, 2010), in addition to promoting neuronal differentiation and dendritic growth (Wu *et al.*, 2008). Increased cell proliferation and survival in the dentate gyrus resulting from periods of exercise have been linked to memory improvements (Bechara *et al.*, 2013a; Li *et al.*, 2013). However, to date in most studies memory tasks have been performed directly after exercise protocols and therefore fail to discriminate between short-term (e.g. enhanced BDNF signalling) and long-term (e.g. increased neurogenesis) effects of exercise.

Recently exercise has been shown to affect endocannabinoid signalling. It has been reported that exercise increases AEA concentration and CB_1 receptor signalling in the hippocampus (Hill *et al.*, 2010), while increased dentate gyrus cell proliferation observed in exercised mice is absent when the CB_1 receptor is knocked down (Wolf *et al.*, 2010).

The overall aim of the present study was to assess if the cognitive and cellular effects of exercise were mediated by endocannabinoid signalling through the CB_1 receptor. The study also focused on identifying if some of the effects of exercise persist over long time

periods. To achieve this, rats were either subjected to a one-week forced exercise protocol or remained sedentary with or without daily injections of the CB_1 receptor antagonist AM 251. The four groups used in this study were: sedentary-vehicle, sedentary-AM 251, exercise-vehicle, and exercise-AM 251. For the duration of the exercise/drug administration protocol rats received daily injections of BrdU to allow analysis of cell proliferation and survival in the dentate gyrus. BrdU is a thymidine analogue that incorporates into replicating DNA and therefore labels cells that have undergone division, a method that has been used in over 20,000 peer-reviewed articles (Cavanagh *et al.*, 2011). Following the seven-day exercise/drug administration protocol each of the four groups was further sub-divided into a short-term study group or a long-term study group (see Figure 4.1). The short-term group's performance in the object displacement task was assessed 24 h after the last exercise bout; the short-term variation of the task was used here (see Figure 4.2). Following testing the hippocampus and dentate gyrus were analysed to assess how exercise with or without AM 251 administration affected neurotrophin signalling and the expression of synaptic markers. Cell proliferation and early-stage survival of newly born cells was assessed in the dentate gyrus. The long-term groups' performance was assessed in a long-term variation of the object displacement task (see Figure 4.2). This variation of the task was carried out over a three-week period where retention of spatial memory was assessed two weeks after the acquisition period. This is a novel variation of the task that has not previously been reported in the literature. Following testing in the task survival of cells labelled with BrdU during the exercise/drug administration protocol was quantified.

4.2 Methods

4.2.1 Animals

A total of 64 three-month old male Wistar rats (Bio Resources, Trinity College Dublin) were used in these experiments. Animals weighed between 300g and 400g and were experimentally naïve at experiment onset. Following transfer from the Bio Resources breeding facility animals were given seven days to acclimatise to the housing facility before the experimental procedure. During this acclimatisation period animals were handled every day by the experimenter. Animals were housed three per cage and were maintained on a 12:12 hour light-dark cycle at constant ambient temperature (21.5 ± 1.5 °C) and humidity (55 ± 5%). Food and water was available *ad libitum*. Methods were performed in accordance with the national law and European Union directives on animal experiments.

4.2.2 Experimental design

Animals were assigned to the short-term study (n=32) or the long-term study (n=32) [see Figure 4.1]. Within each study animals were randomly divided into four groups (n=8 per group); a vehicle treated sedentary group (sedentary-vehicle), a sedentary group that were administered the CB_1 specific inverse agonist AM 251 (sedentary-AM 251), a vehicle treated exercise group (exercise-vehicle) and an AM 251 treated exercise group (exercise-AM 251). To assess the effects of AM 251, either alone or in combination with exercise, on short-term spatial memory animals were tested in the object displacement task. Animals assigned to the long-term study were tested in a long-term variation of the object displacement task (see section 4.2.5). Animals were euthanised following testing in the object displacement task to assess how the above treatments affect the cells of the brain. In order to examine if the above treatments had an effect on NSC/NPC proliferation and/or survival animals were i.p. injected daily for the duration of the drug treatment/exercise protocol with the thymidine analogue BrdU (50 $mg.kg^{-1}$ in 0.9% saline; ~1 ml per animal) [Sigma]. BrdU incorporates into replicating DNA and therefore allows identification of cells that have undergone mitosis during the experimental protocol.

4.2.3 Drug treatments

Animals were injected i.p. with either AM 251 (1 $mg.kg^{-1}$) dissolved in vehicle solution (55% DMSO in 0.9% saline) or with vehicle solution based on animals total body weight (~1 ml per animal) daily for seven days approximately two hours before each exercise session.

4.2.4 Forced exercise protocol

Animals were habituated to the rodent treadmills (Exer 3/6 treadmill, Colombus Instruments) for two consecutive days prior to the exercise protocol. Habituation consisted of animals walking/running on the treadmill for a period of 15 min at a belt speed of approximately 8.5 $m.min^{-1}$. Animals assigned to the sedentary group were placed on a stationary treadmill for the same period of time. The exercise protocol consisted of seven consecutive days of one hour of treadmill running at a belt speed of 16.6 $m.min^{-1}$ (approximately 1 km per day). Again sedentary controls were placed on a stationary treadmill for the corresponding time period. One end of the treadmill belt was equipped with wire loops that administered a mild electric shock that encouraged animals to maintain the running pace dictated by the belt. The electrical shock system was activated at a low intensity throughout all exercise sessions (current of 0.7 mA with inter-pulse interval of 2 s).

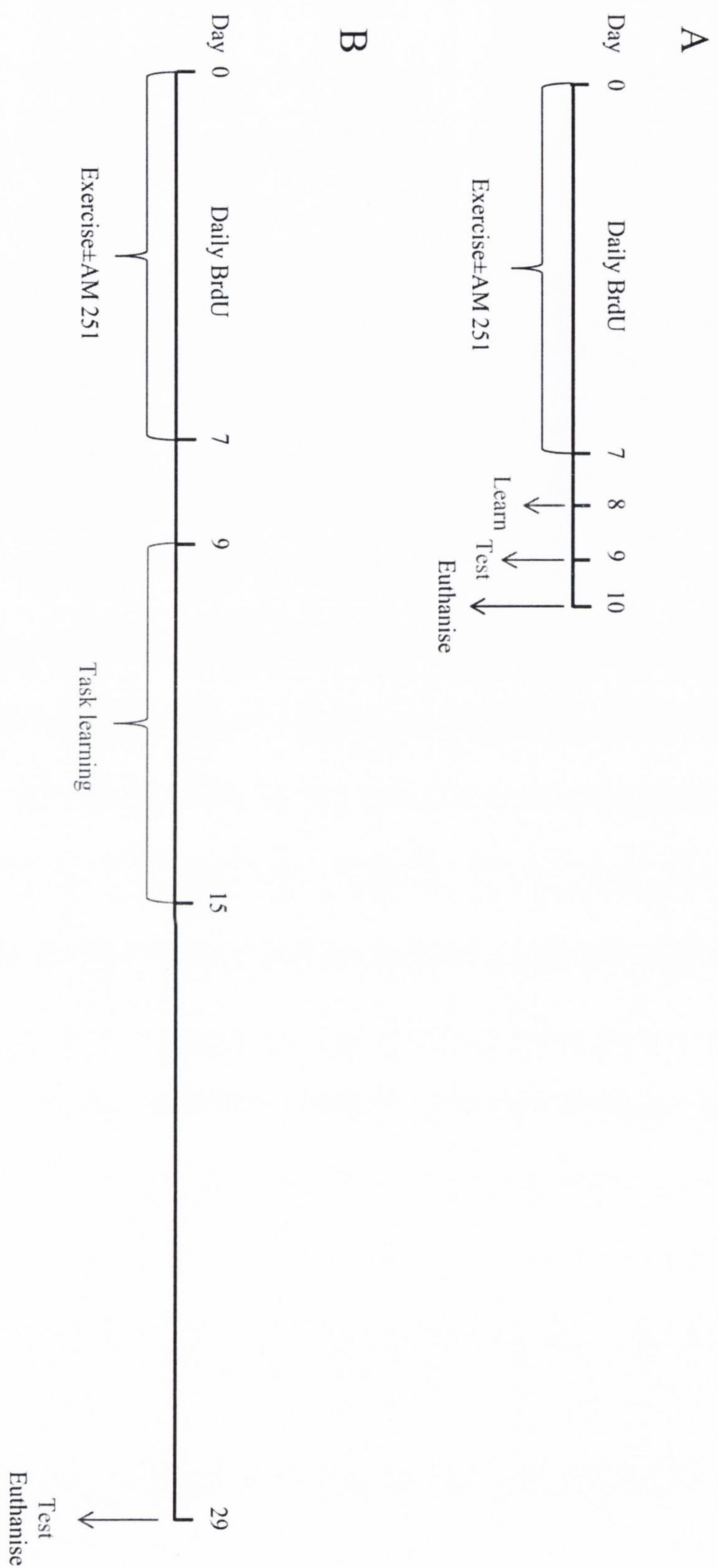

Figure 4.1 Timelines illustrating the short-term study protocol (A) and the long-term study protocol (B)

4.2.5 Object displacement task

To eliminate the short-term behavioural effects of exercise and AM 251 the short-term object displacement task was carried out 24 hours after the last exercise bout (26 hours post last drug administration). The acquisition period of the long-term object displacement task commenced 48 hours post last exercise bout.

Short-term variation of task

During the acquisition phase of the task animals were placed in the open field containing three objects and were allowed to explore freely for five minutes. The open field and the room in which the open field was placed contained several spatial cues. Twenty-four hours later, again in the presence of spatial cues, animals were placed in the open field containing the same three objects with one of the objects repositioned to another quadrant of the open field (Figure 4.2).

Long-term variation of task

The acquisition phase of the task consisted of allowing animals to explore the three objects for two five-minute periods with a five-minute inter-trial interval daily for seven days (2 x 5 min x 7 d). The open field and the room in which the open field was placed contained several spatial cues. Fourteen days after the last acquisition trial animals were placed in the open field containing the same three objects with one of the objects repositioned to another quadrant of the open field (Figure 4.2).

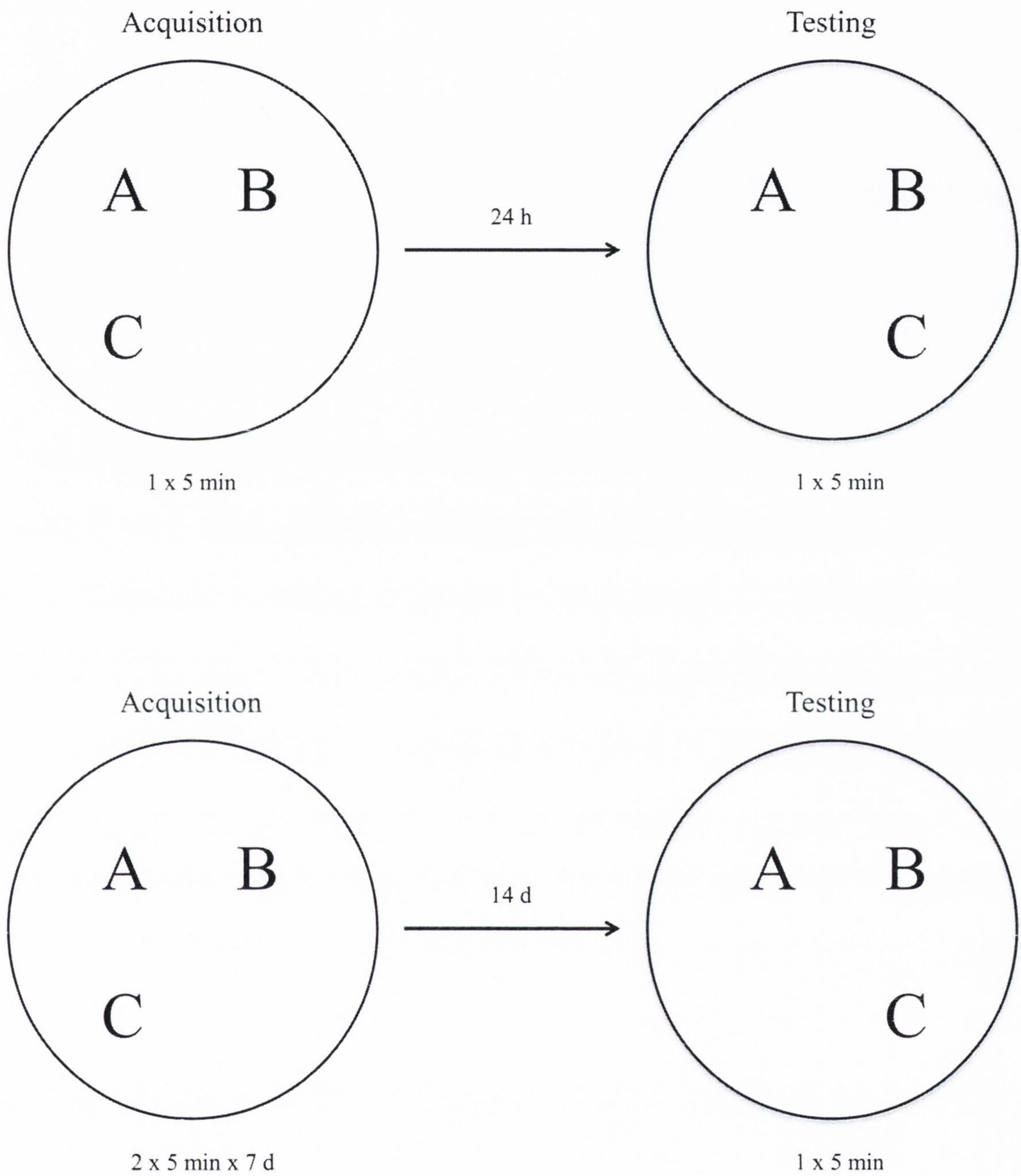

Figure 4.2 Short-term (24 h retention period) and long-term (14 d retention period) variations of the object displacement task

The short-term object displacement task (top) consisted of a single 5-minute acquisition/learning session, a 24-hour retention period and one 5-minute testing session. The long-term object displacement task (bottom) consisted of two 5-minute acquisition/learning sessions daily for 7 days, a 14-day retention period and one 5-minute testing session.

4.2.6 Analysis of mRNA expression by RT-PCR

BDNF and CB_1 mRNA expression in the hippocampus and dentate gyrus were assessed using RT-PCR (see section 2.6). Ki67 expression was assessed in the dentate gyrus.

4.2.7 Analysis of protein expression by ELISA

BDNF protein was quantified in serum and tissue samples using a Human BDNF ELISA kit (R&D Systems Europe, Oxon, United Kingdom) [see section 2.4]. Trunk blood was taken immediately after the animal was euthanised and stored at room temperature for two hours. It was then centrifuged at 11,000 g for 20 min at 4°C. The serum was removed and stored at -20°C for later analysis by ELISA. Serum was diluted 1 in 2 with distilled water before the ELISA was carried out. Hippocampus and dentate gyrus samples were homogenised in Krebs calcium buffer (see section 2.3) prior to carrying out the ELISA.

4.2.8 Analysis of $BrdU^+$nuclei by immunohistochemistry

$BrdU^+$ cells were visualised using the Aividin Biotin Complex (ABC) peroxidase method with rabbit anti-chicken conjugated IgG and 3,3 diaminobenzidine (DAB) chromogen (see section 2.5).

Percentage $BrdU^+$ cells (short-term study)

Three sections were stained per animal and the percentage of cells in the dentate gyrus that were $BrdU^+$ was calculated. Only cells that were located in the subgranular zone or the granular cell layer were included in this analysis.

Total number $BrdU^+$ cells (long-term study)

Twelve sections of the dentate gyrus were stained per animal. The total number of $BrdU^+$ cells in the dentate gyrus was estimated using a combination of the Cavalieri principle and the Optical Fractionator method (see section 2.5.3). Only cells that were located in the subgranular zone or the granular cell layer were included in this analysis.

4.2.9 Analysis of protein expression by Western immunoblotting

Synapsin, synaptophysin and post-synaptic density protein 95 (PSD-95) expression was measured by SDS-PAGE and Western immunoblotting (see section 2.7). GAPDH expression was measured as a loading control.

4.2.10 Statistical analysis

Prior to statistical analysis data were examined for outliers, which were identified as being more than two standard deviations outside the mean. Outliers were excluded from the data set before statistical analysis.

Behavioural data were analysed using a two-way ANOVA to compare exploration of objects and the effect of group. Where a significant main effect was identified a Bonferroni *post-hoc* test was applied to detect the significant differences.

Tissue analysis data were analysed using a two-way ANOVA.

Data are represented as mean ± standard error of the mean (SEM). All statistical analysis was performed using Microsoft Office Excel 2007 and GraphPad Prism 5.0 (GraphPad Software Inc., USA). Statistical significance was inferred at $p<0.05$.

4.3 Results

Short-term study

4.3.1 Object displacement task

Short-term spatial memory was assessed using the short variation of the object displacement task. On the acquisition day of the object displacement task there was a significant difference in object exploration ($F_{2,81}$=4.824, p<0.05) and there was no interaction ($F_{6,83}$=1.854, p>0.05). Bonferroni *post-hoc* tests revealed that the sedentary-vehicle group spent significantly more time exploring object B compared to object A (percentage exploration of objects: object A: 25.68±2.24, object B: 44.27±2.96, **p<0.01, Figure 4.2 A). No difference was found with respect to object exploration in any of the other groups.

On testing day there was a significant difference in object exploration ($F_{1,54}$=5.247, p<0.05). Bonferroni *post-hoc* analysis showed that the exercise-AM 251 group spent significantly more time exploring the displaced object compared to the stationary objects (percentage exploration of objects: stationary: 37.57±3.61, displaced: 62.43±3.61, *p<0.05, Figure 4.2 B). There was no significant interaction ($F_{3,54}$=2.547, p>0.05). No difference was found with respect to object exploration in any of the other groups.

4.3.2 Analysis of BDNF protein in the serum

There was no effect of exercise ($F_{1,28}$=0.4817, p>0.05) or drug ($F_{1,28}$=0.9523, p>0.05) on the concentration of BDNF protein in the serum. There was no interaction (Sedentary-vehicle: 884.5±78.2 $pg.ml^{-1}$, Sedentary-AM 251: 862.5±79.2 $pg.ml^{-1}$, Exercise-vehicle: 882.5±71.5 $pg.ml^{-1}$, Exercise-AM 251: 766.0±51.54 $pg.ml^{-1}$, $F_{1,28}$=0.4434, p>0.05, Figure 4.4).

4.3.3 Analysis of BDNF expression in the dentate gyrus and the hippocampus

There was no effect of exercise ($F_{1,25}$=0.0482, p>0.05) or drug ($F_{1,25}$=0.0335, p>0.05) on BDNF mRNA expression in the dentate gyrus and there was no interaction ($F_{1,25}$=0.0067, p>0.05, Figure 4.5 A). There was no effect of exercise ($F_{1,23}$=0.0031, p>0.05) or drug ($F_{1,23}$=0.3120, p>0.05) on BDNF protein expression in the dentate gyrus and there was no

interaction (Sedentary-vehicle: 4773.0±715.3 pg.mg protein^{-1}, Sedentary-AM 251: 4820.0±1058.0 pg.mg protein^{-1}, Exercise-vehicle: 4255.0±312.4 pg.mg protein^{-1}, Exercise-AM 251: 4551.0±412.6 pg.mg protein^{-1}, $F_{1,23}$=0.0526, p>0.05, Figure 4.5 B).

There was no effect of exercise ($F_{1,28}$=0.1541, p>0.05) or drug ($F_{1,25}$=1.0760, p>0.05) on BDNF mRNA expression in the hippocampus and there was no interaction ($F_{1,28}$=1.1460, p>0.05, Figure 4.6 A). There was no effect of exercise ($F_{1,27}$=0.7019, p>0.05) or drug ($F_{1,27}$=0.0437, p>0.05) on BDNF protein concentration in the hippocampus and there was no interaction (Sedentary-vehicle: 892.0±168.6 pg.mg protein^{-1} Sedentary-AM 251: 1082.0±160.3 pg.mg protein^{-1}, Exercise-vehicle: 1222.0±179.7 pg.mg protein^{-1}, Exercise-AM 251: 843.0±180.9 pg.mg protein^{-1} , $F_{1,27}$=0.4173, p>0.05, Figure 4.6 B).

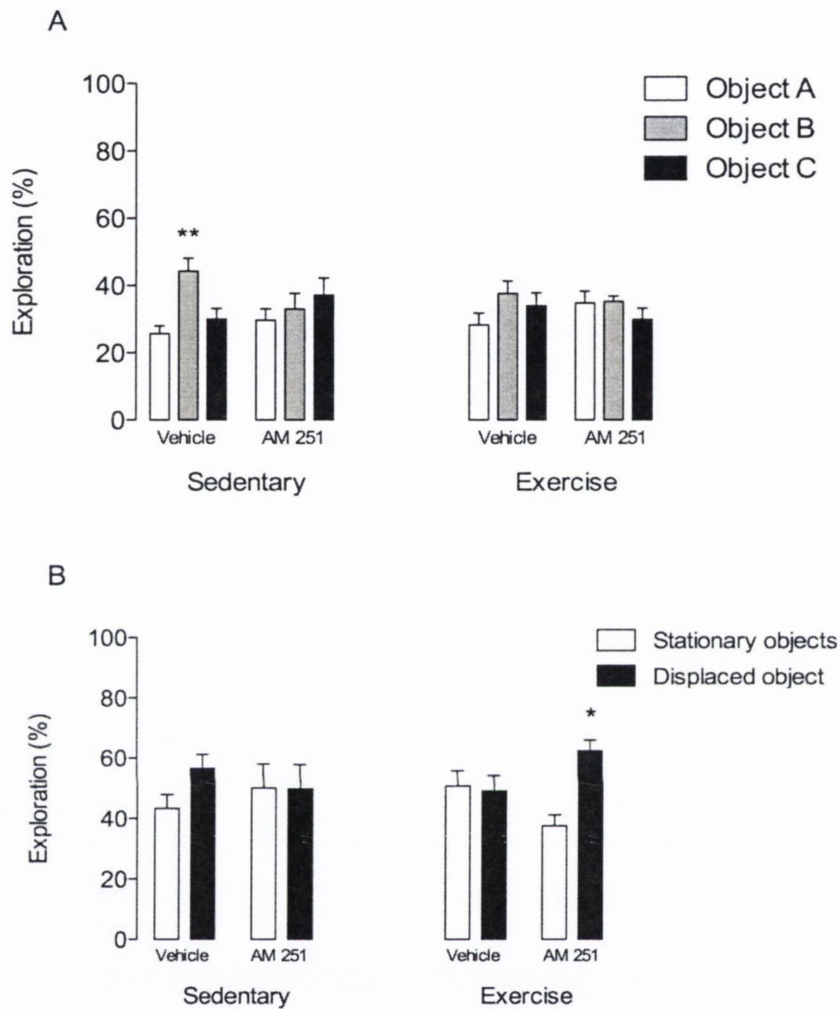

Figure 4.3 The effect of 7 days of AM 251 injections alone, or in combination with daily exercise on performance in the object displacement task

(A) There was a significant difference in object exploration during the acquisition period with sedentary-vehicle group exploring object B significantly more than object A (**p<0.01). No difference was found with respect to object exploration in any of the other groups. (B) There was a significant difference in object exploration in the testing period with the exercise-AM 251 group exploring the displaced object significantly more than the stationary objects on the testing day (*p<0.05). No difference was found with respect to object exploration in any of the other groups. Sedentary-vehicle: n=7, sedentary-AM 251: n=8, exercise-vehicle: n=8, exercise-AM 251: n=8. Bars represent mean±SEM percentage, two-way ANOVA.

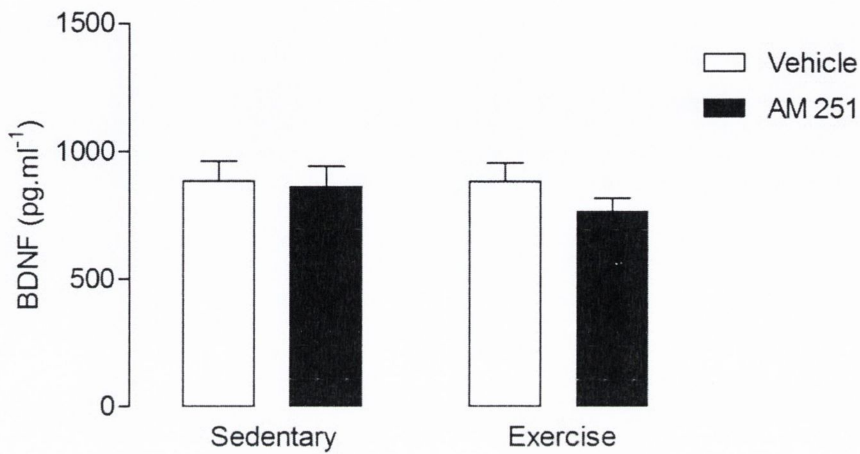

Figure 4.4 The effect of 7 days of AM 251 injections alone, or in combination with daily exercise, on BDNF protein concentration in the serum

There was no effect of exercise or drug on concentration of BDNF protein in the serum. There was no interaction. n=8 per group. Bars represent mean±SEM in $pg.ml^{-1}$, two-way ANOVA.

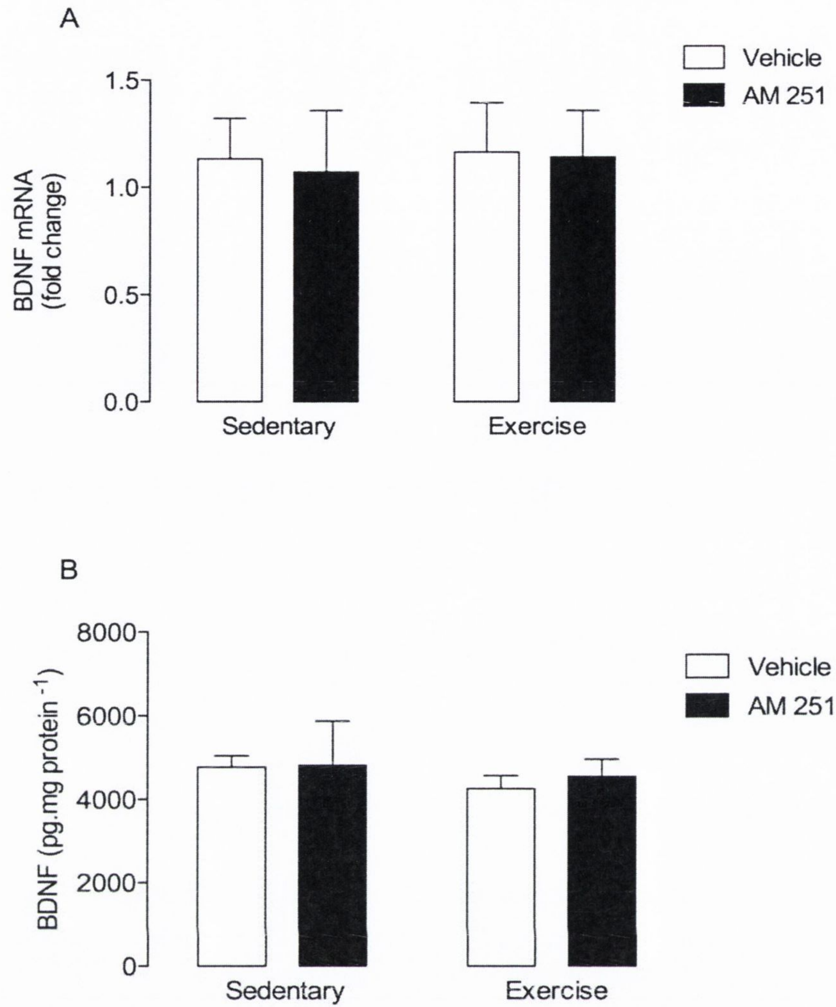

Figure 4.5 The effect of 7 days of AM 251 injections alone, or in combination with daily exercise on BDNF mRNA protein expression in the dentate gyrus

(A) There was no effect of exercise or drug on BDNF mRNA expression and there was no interaction. Sedentary-vehicle: n=7, sedentary-AM 251: n=6, exercise-vehicle: n=8, exercise-AM 251: n=8. Bars represent mean±SEM, arbitrary units, 2-way ANOVA. (B) There was no effect of exercise or drug on BDNF protein expression and there was no interaction. Sedentary-vehicle: n=8, sedentary-AM 251: n=6, exercise-vehicle: n=7, exercise-AM 251: n=6. Bars represent mean±SEM in $pg.mg^{-1}$, two-way ANOVA.

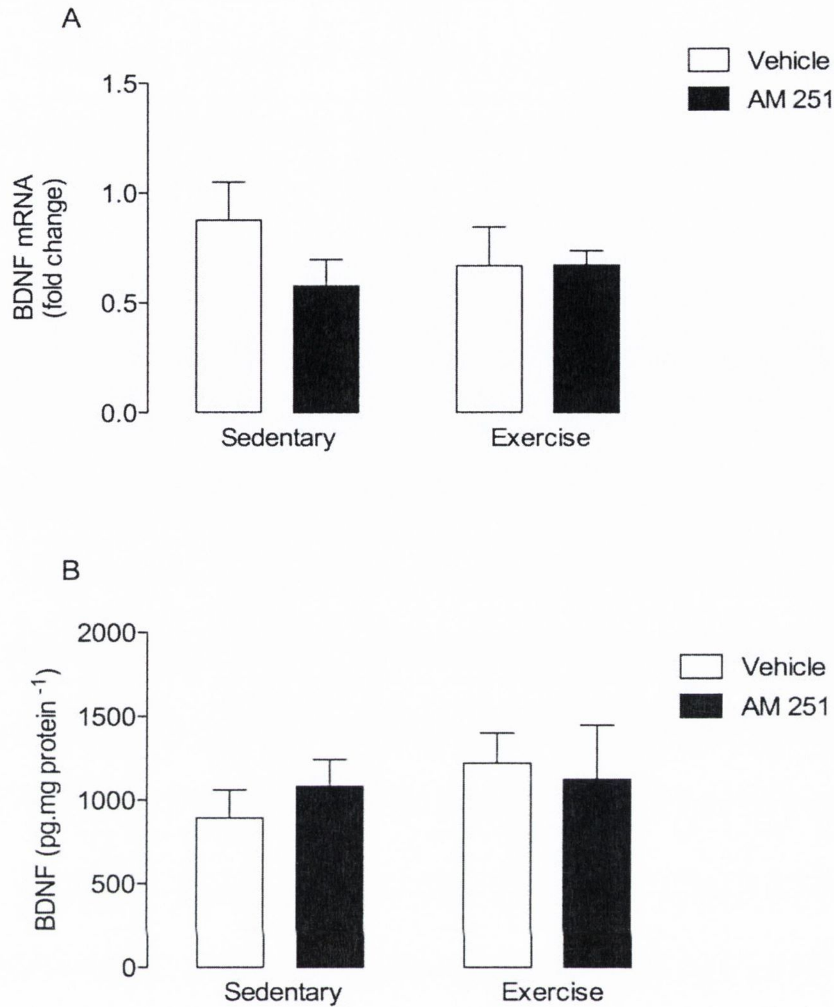

Figure 4.6 The effect of 7 days of AM 251 injections alone, or in combination with daily exercise on BDNF mRNA and protein expression in the hippocampus

(A) There was no effect of exercise or drug on BDNF mRNA expression and there was no interaction. Sedentary-vehicle: n=8, sedentary-AM 251: n=8, exercise-vehicle: n=8, exercise-AM 251: n=8. Bars represent mean±SEM, arbitrary units, 2-way ANOVA. (B) There was no effect of exercise or drug on BDNF protein expression and there was no interaction. Sedentary-vehicle: n=8, sedentary-AM 251: n=7, exercise-vehicle: n=8, exercise-AM 251: n=8. Bars represent mean±SEM in $pg.mg^{-1}$, two-way ANOVA.

4.3.4 Analysis of CB_1 receptor mRNA expression in the dentate gyrus and the hippocampus

There was no effect of exercise ($F_{1,28}=0.5581$, $p>0.05$) or drug ($F_{1,28}=1.6020$, $p>0.05$) on CB_1 receptor mRNA in the dentate gyrus and there was no interaction ($F_{1,28}=0.0444$, $p>0.05$, Figure 4.7).

There was no effect of exercise ($F_{1,26}=0.2650$, $p>0.05$) or drug ($F_{1,26}=0.3691$, $p>0.05$) on CB_1 receptor mRNA in the hippocampus and there was no interaction ($F_{1,26}=2.302$, $p>0.05$, Figure 4.8).

4.3.5 Analysis of synapsin protein expression in the dentate gyrus and the hippocampus

There was no significant effect of exercise ($F_{1,17}=1.6770$, $p>0.05$) or drug ($F_{1,17}=1.5610$, $p>0.05$) on synapsin expression in the dentate gyrus and there was no interaction ($F_{1,17}=1.5310$, $p>0.05$, Figure 4.9 B).

There was no significant effect of exercise ($F_{1,24}=1.5370$, $p>0.05$) or drug ($F_{1,24}=2.1800$, $p>0.05$) on synapsin expression in the hippocampus and there was no interaction ($F_{1,24}=2.1700$, $p>0.05$, Figure 4.10 B).

4.3.6 Analysis of synaptophysin protein expression in the dentate gyrus

There was no significant effect of exercise ($F_{1,19}=0.1836$, $p>0.05$) or drug ($F_{1,19}=0.8334$, $p>0.05$) on synapsin expression in the dentate gyrus and there was no interaction ($F_{1,19}=0.3306$, $p>0.05$, Figure 4.11 B).

4.3.7 Analysis of PSD-95 protein expression in the dentate gyrus

There was no significant effect of exercise ($F_{1,22}=1.527$, $p>0.05$) or drug ($F_{1,22}=0.4274$, $p>0.05$) on PSD-95 expression in the dentate gyrus and there was no interaction ($F_{1,22}=0.0047$, $p>0.05$, Figure 4.12 B).

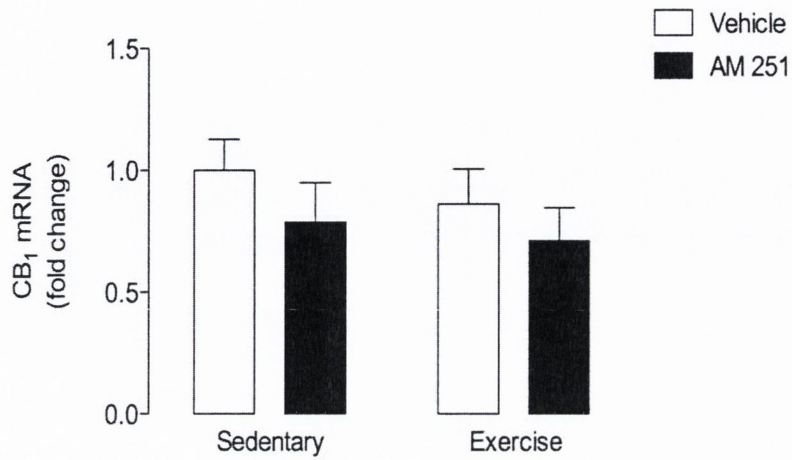

Figure 4.7 The effect of 7 days of AM 251 injections alone, or in combination with daily exercise on CB_1 receptor mRNA expression in the dentate gyrus

There was no effect of exercise or drug on CB_1 receptor mRNA expression. There was no interaction. Sedentary-vehicle: n=7, sedentary-AM 251: n=6, exercise-vehicle: n=8, exercise-AM 251: n=8. Bars represent mean±SEM, arbitrary units, two-way ANOVA.

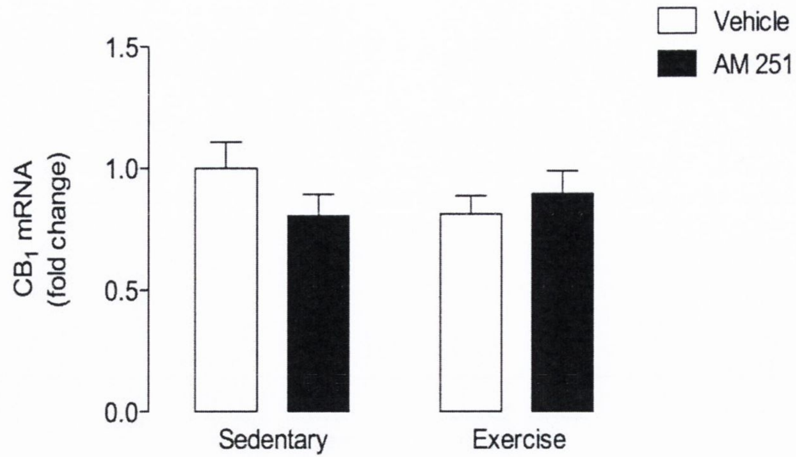

Figure 4.8 The effect of 7 days of AM 251 injections alone, or in combination with daily exercise on CB_1 receptor mRNA expression in the hippocampus

There was no effect of exercise or drug on CB_1 receptor mRNA expression. There was no interaction. Sedentary-vehicle: n=7, sedentary-AM 251: n=8, exercise-vehicle: n=8, exercise-AM 251: n=8. Bars represent mean±SEM, arbitrary units, two-way ANOVA.

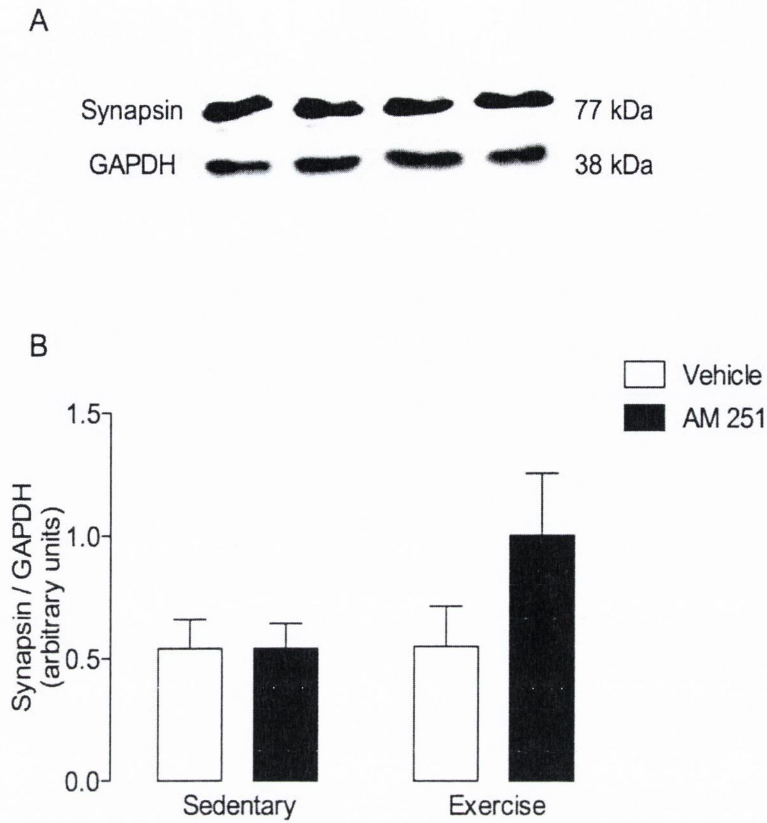

Figure 4.9 The effect of 7 days of AM 251 injections alone, or in combination with daily exercise on synapsin protein expression in the dentate gyrus

(A) Representative image from a western blot of synapsin (77 kDa) and GAPDH (38 kDa). Left to right: sedentary-vehicle, sedentary-AM 251, exercise-vehicle, exercise-AM 251. (B) There was no effect of exercise or drug on synapsin protein expression in the dentate gyrus. There was no interaction. Sedentary-vehicle: n=6, sedentary-AM 251: n=5, exercise-vehicle: n=4, exercise-AM 251: n=6. Bars represent mean±SEM, arbitrary units, 2-way ANOVA.

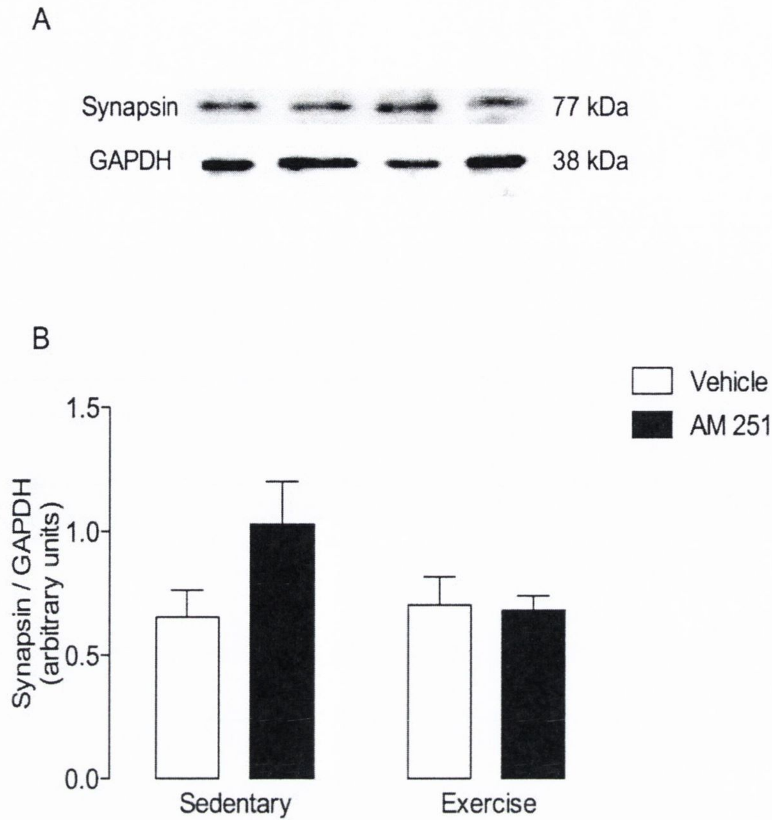

Figure 4.10 The effect of 7 days of AM 251 injections alone, or in combination with daily exercise on synapsin protein expression in the hippocampus

(A) Representative image from a western blot of synapsin (77 kDa) and GAPDH (38 kDa). Left to right: sedentary-vehicle, sedentary-AM 251, exercise-vehicle, exercise-AM 251. (B) There was no effect of exercise or drug on synapsin-I protein expression in the hippocampus. There was no interaction. Sedentary-vehicle: n=8, sedentary-AM 251: n=6, exercise-vehicle: n=8, exercise-AM 251: n=6. Bars represent mean±SEM, arbitrary units, two-way ANOVA.

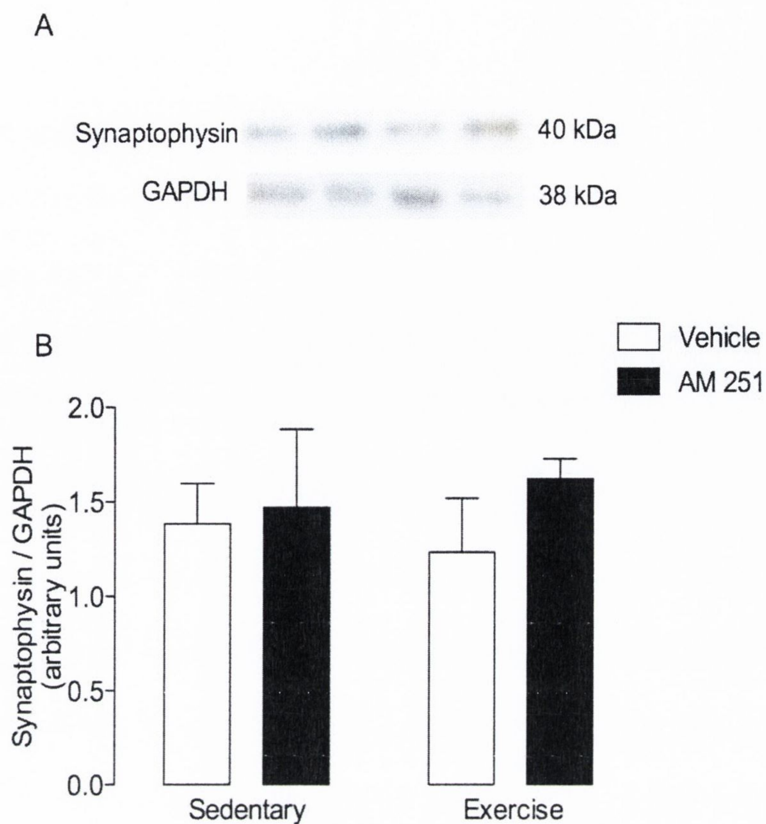

Figure 4.11 The effect of 7 days of AM 251 injections alone, or in combination with daily exercise on synaptophysin protein expression in the dentate gyrus

(A) Representative image from a western blot of synaptophysin (40 kDa) and GAPDH (38 kDa). Left to right: sedentary-vehicle, sedentary-AM 251, exercise-vehicle, exercise-AM 251. (B) There was no effect of exercise or drug on synaptophysin protein expression in the dentate gyrus. There was no interaction. Sedentary-vehicle: n=7, sedentary-AM 251: n=5, exercise-vehicle: n=5, exercise-AM 251: n=6. Bars represent mean±SEM, arbitrary units, two-way ANOVA.

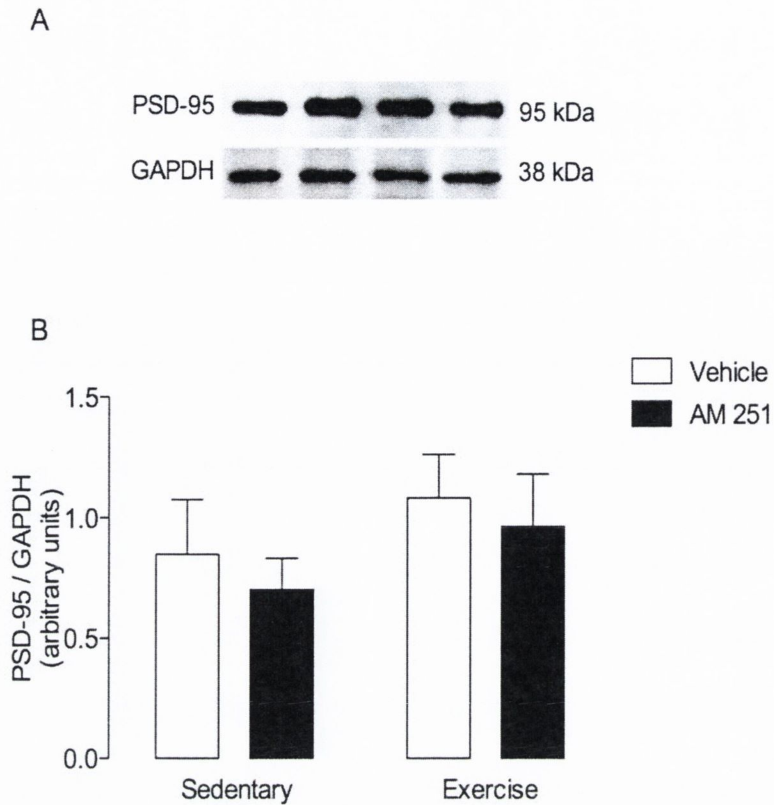

Figure 4.12 The effect of 7 days of AM 251 injections alone, or in combination with daily exercise on PSD-95 protein expression in the dentate gyrus

(A) Representative image from a western blot of PSD-95 (95 kDa) and GAPDH (38 kDa). Left to right: sedentary-vehicle, sedentary-AM 251, exercise-vehicle, exercise-AM 251. (B) There was no effect of exercise or drug on PSD-95 protein expression in the dentate gyrus. There was no interaction. Sedentary-vehicle: n=6, sedentary-AM 251: n=6, exercise-vehicle: n=6, exercise-AM 251: n=8. Bars represent mean±SEM, arbitrary units, two-way ANOVA.

4.3.8 Analysis of Ki-67 mRNA expression in the dentate gyrus

There was no significant effect of exercise ($F_{1,28}$=0.7082, p>0.05) or drug ($F_{1,28}$=0.6304, p>0.05) on Ki67 mRNA in the dentate gyrus and there was no interaction ($F_{1,28}$=1.958, p>0.05, Figure 4.13).

4.3.9 Analysis of $BrdU^+$ nuclei in the dentate gyrus

There was a no significant effect of exercise ($F_{1,22}$=3.175, p>0.05) on the percentage of $BrdU^+$ nuclei in the dentate gyrus. There was a significant effect of drug ($F_{1,22}$=6.428, p<0.05) and a significant interaction ($F_{1,22}$=7.255, p>0.05). Bonferroni *post-hoc* analysis revealed a significant increase in $BrdU^+$ nuclei in the exercise-vehicle group compared to the sedentary-vehicle group (percentage $BrdU^+$ nuclei: exercise-vehicle group: 8.14±1.21, sedentary-vehicle: 4.40±0.57, **p<0.01, Figure 4.14). This increase was attenuated by co-administration of AM 251 group (percentage $BrdU^+$ nuclei: exercise-vehicle group: 8.14±1.21, exercise-AM 251: 3.77±0.36, ++p<0.01, Figure 4.14).

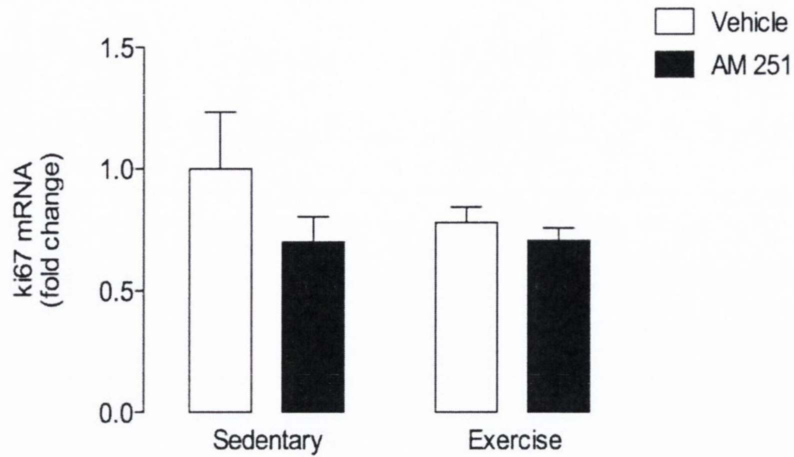

Figure 4.13 The effect of 7 days of AM 251 injections alone, or in combination with daily exercise on Ki67 mRNA expression in the dentate gyrus

There was no effect of exercise or drug on ki67 mRNA expression. There was no interaction ($F_{1,28}$=1.958, p>0.05). Sedentary-vehicle: n=8, sedentary-AM 251: n=8, exercise-vehicle: n=8, exercise-AM 251: n=8. Bars represent mean±SEM, arbitrary units, two-way ANOVA.

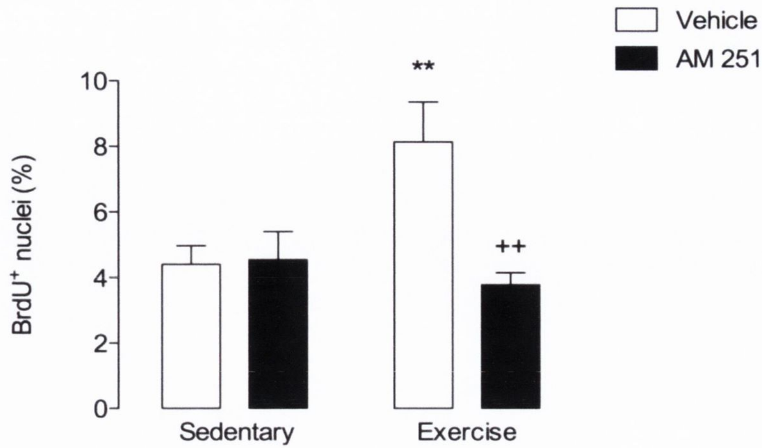

Figure 4.14 The effect of 7 days of AM 251 injections alone, or in combination with daily exercise on BrdU$^+$ nuclei in the dentate gyrus

There was no effect of exercise; there was a significant effect of drug treatment on the percentage of BrdU$^+$ nuclei. There was an increase in percentage of BrdU$^+$ nuclei in the exercise-vehicle group compared to the sedentary-vehicle group ($^{**}p<0.01$), this increase was attenuated by AM 251 treatment ($^{++}p<0.01$). There was a significant interaction. Sedentary-vehicle: n=7, sedentary-AM 251: n=8, exercise-vehicle: n=8, exercise-AM 251: n=8. Bars represent mean±SEM, data expressed as a percentage, two-way ANOVA with Bonferroni *post-hoc* tests.

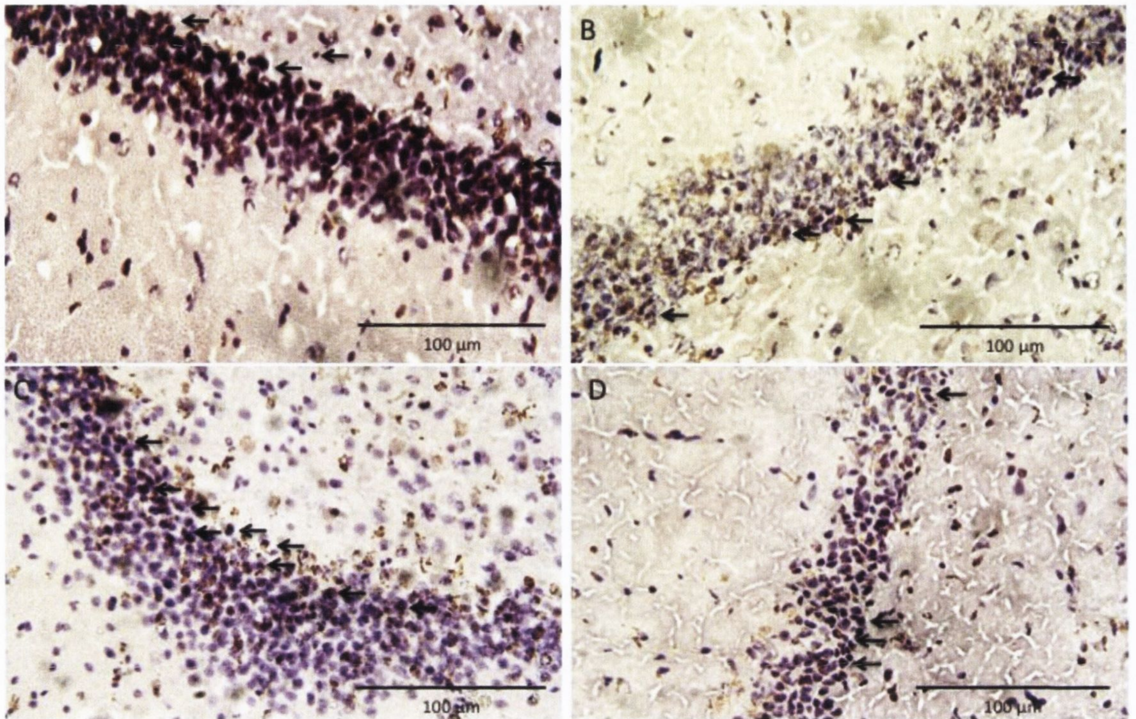

Figure 4.15 Representative pictures of BrdU$^+$ nuclei in the dentate gyrus

Cell nuclei are stained in purple and BrdU$^+$ nuclei are indicated by purple co-localised with brown staining. Arrows indicate some BrdU$^+$ nuclei; (A) sedentary-vehicle, (B) sedentary-AM 251, (C) exercise-vehicle and (D) exercise-AM 251.

Long-term study

4.3.10 Object displacement task

Long-term spatial memory was assessed using the long variation of the object displacement task. On the acquisition day of the object displacement task there was a significant difference in object exploration ($F_{2,84}$=21.210, p<0.001) and there was no interaction ($F_{6,84}$=0.800, p>0.05). Bonferroni *post-hoc* tests revealed that the exercise-vehicle group spent significantly more time exploring object C compared to object A and object B (percentage exploration of objects: object A: 31.72±1.01, object B: 29.55±1.13 object C: 38.73±1.66, *p<0.05 ***p<0.001, Figure 4.16 A) and the exercise-AM251 group spent significantly more time exploring object C compared to object B (percentage exploration of objects: object B: 28.71±1.30, object C: 38.01±1.37 ***p<0.001, Figure 4.16 A).

On testing day there was a significant interaction ($F_{3,56}$=16.28, p<0.001), and there was a significant difference in object exploration ($F_{1,56}$=37.08, p<0.001). Bonferroni *post-hoc* analysis showed that the exercise-vehicle group (percentage exploration of objects: stationary: 33.63±2.17, displaced: 66.37±2.17, ***p<0.001, Figure 4.16 B) and exercise-AM 251 group (percentage exploration of objects: stationary: 34.95±2.02, displaced: 65.05±2.02, ***p<0.001, Figure 4.16 B) spent significantly more time exploring the displaced object compared to the stationary objects.

4.3.11 Analysis of $BrdU^+$ nuclei in the dentate gyrus

There was no significant effect of exercise ($F_{1,7}$=0.5832, p>0.05) or drug ($F_{1,7}$=0.0514, p>0.05) on BrdU+ nuclei in the dentate gyrus. There was a signficant interaction ($F_{1,7}$=6.883, p<0.05, Figure 4.17).

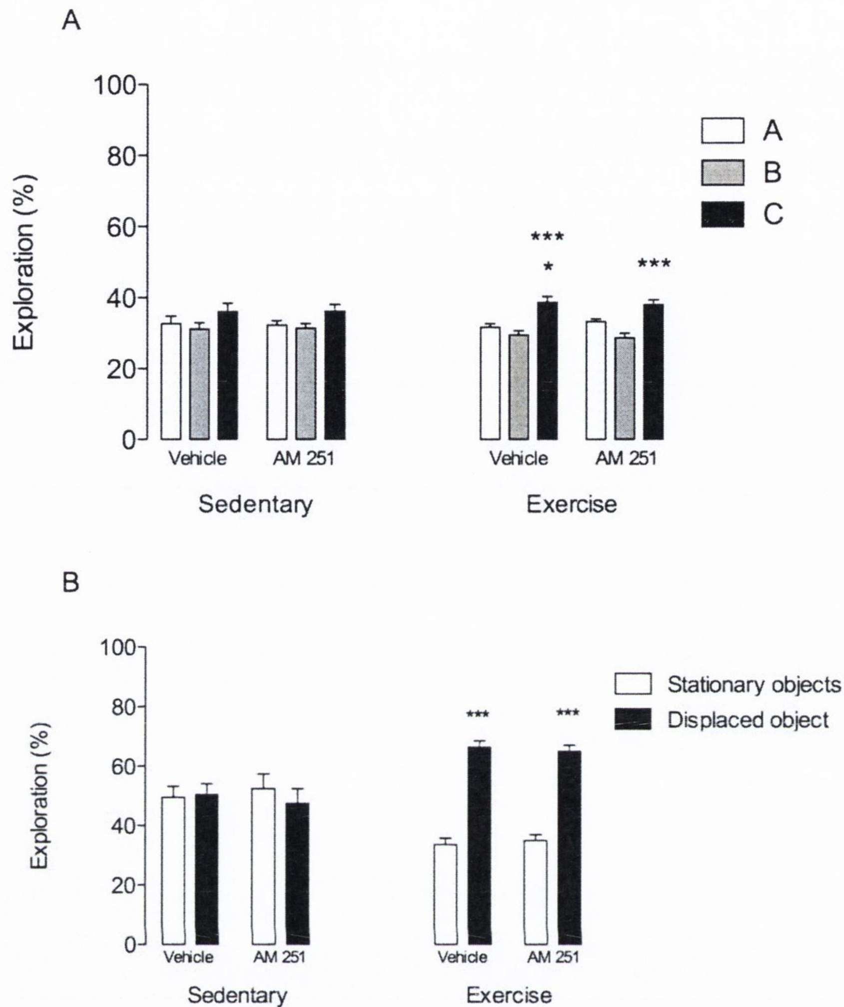

Figure 4.16 The effect of 7 days of AM 251 injections alone, or in combination with daily exercise on performance in the long-term object displacement task

(A) There was a significant difference in object exploration during the acquisition period with exercise-vehicle exploring object C significantly more than object A (*p<0.05) and object B (*p<0.001) and the exercise-AM251 group exploring object C more than object B (***p<0.001). No difference was found with respect to object exploration in any of the other groups. (B) There was a significant difference in object exploration with both the exercise-vehicle and the exercise-AM 251 group exploring the displaced object significantly more than the stationary objects on the testing day (***p<0.001). No difference was found with respect to object exploration in any of the other groups. Sedentary-vehicle: n=8, sedentary-AM 251: n=8, exercise-vehicle: n=8, exercise-AM 251: n=8. Bars represent mean±SEM percentage, two-way ANOVA.

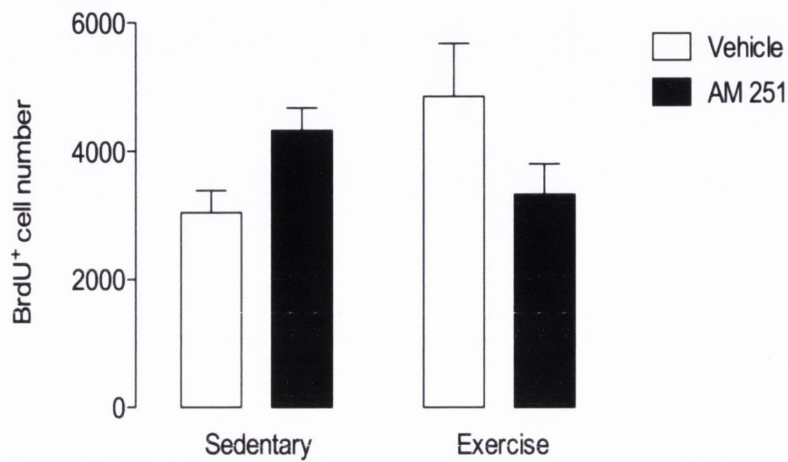

Figure 4.17 The effect of 7 days of AM 251 injections alone, or in combination with daily exercise on long-term survival of BrdU$^+$ nuclei in the dentate gyrus

There was no significant effect of exercise or drug on BrdU$^+$ nuclei in the dentate gyrus. There was a significant interaction. Sedentary-vehicle: n=3, sedentary-AM 251: n=2, exercise-vehicle: n=2, exercise-AM 251: n=4. Bars represent mean±SEM, data expressed as total cell number, two-way ANOVA.

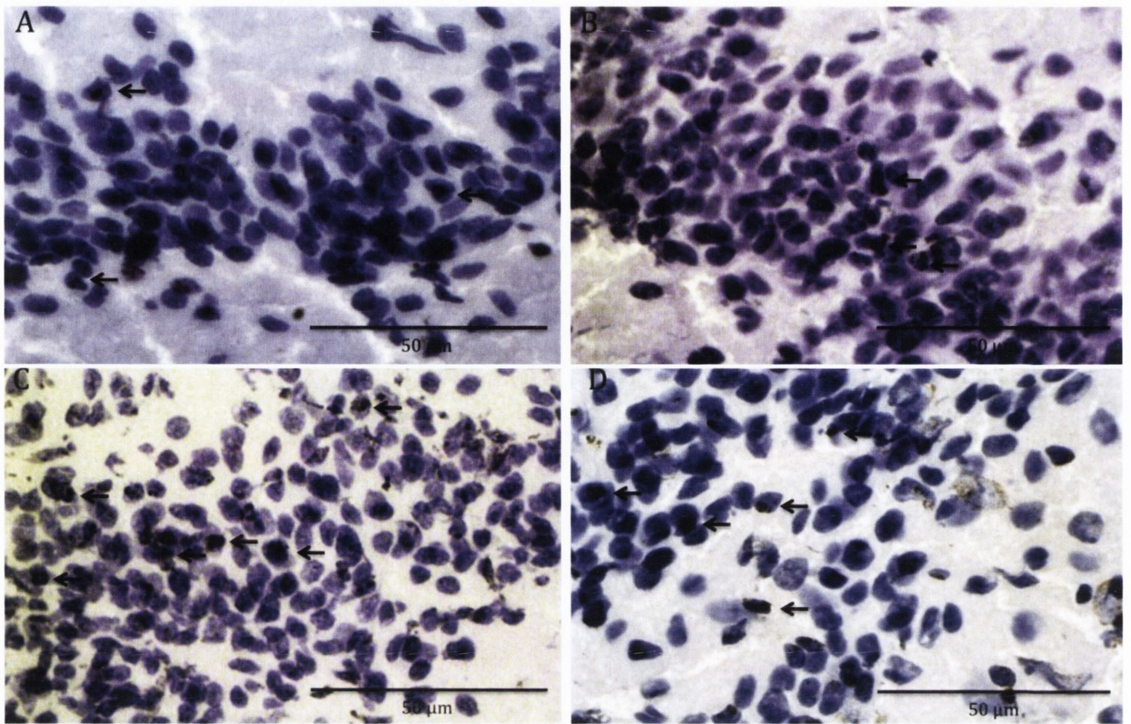

Figure 4.18 Representative pictures of BrdU$^+$ nuclei in the dentate gyrus

Cell nuclei are stained in purple and BrdU$^+$ nuclei are indicated by purple co-localised with brown staining. Arrows indicate some examples of BrdU$^+$ nuclei. (A) sedentary-vehicle, (B) sedentary-AM 251, (C) exercise-vehicle and (D) exercise-AM 251.

4.4 Discussion

Short-term study

The first aim of the short-term study was to assess how exercise with or without AM 251 administration affected spatial memory tested in the object displacement task, a task known to be dependent on the hippocampus (Mumby *et al.*, 2002). The task was performed over a short 24 h time period. During the acquisition phase the sedentary-vehicle group explored object B significantly more than object A. A spontaneous object preference can occasionally occur during acquisition and in order to prevent this from distorting the results of the task object B was not used as the displaced object. The control (i.e. sedentary-vehicle) group could not discriminate between the displaced object and the stationary objects during testing and therefore did not successfully perform the task. A week of AM 251 treatment or a week of exercise alone had no effect on performance. Interestingly, a combination of exercise and AM 251 treatment synergistically facilitated learning of the task. This result shows for the first time that one week of exercise in combination with daily systemic blockade of CB_1 activity enhances spatial memory. The fact that exercise alone did not improve spatial memory was somewhat surprising, since many studies in the literature report such an effect of exercise (Khabour *et al.*, 2010; van Praag *et al.*, 1999a). Interestingly studies using the same exercise protocol and same short-term object displacement task as the present study report that exercise enhances performance in the task (Bechara *et al.*, 2013b; Griffin *et al.*, 2009). This is very likely to be due to the differences in the latency of the rest period between the last exercise bout and the acquisition/learning period of the task. Rats in the present study were subjected to the acquisition phase of the object displacement task 24 h after the last exercise bout whereas in studies carried out by Griffin *et al.,* and Bechara *et al.,* the acquisition phase of the task was carried out on the same day as the last exercise bout. The fact that when exercised rats learn the task 24 h post exercise they are not successful in recalling the memory (Figure 4.3) but when they learn on the last day of exercise they can recall the memory (Bechara *et al.*, 2013b; Griffin *et al.*, 2009) suggests that short-term changes following exercise are responsible for these memory enhancements. Further investigation using varying lengths of rest periods between exercise and learning would need to be carried out to identify the limits of the short-term effects of exercise.

Exercise has often been linked to an increase in BDNF concentration in the hippocampus. Varying lengths of both voluntary (Wrann *et al.*, 2013) and forced (Cassilhas *et al.*, 2012) aerobic exercise is associated with increased hippocampal BDNF expression at both mRNA and protein level. Interestingly, a study comparing forced and voluntary exercise shows a similar increase in hippocampal BDNF in both exercise groups compared to control (Alomari *et al.*, 2013), showing both forms of exercise equally increase BDNF expression. As exercise has been shown to increase endocannabinoid signalling in the hippocampus (Hill *et al.*, 2010) and the previous chapter shows that endocannabinoids can regulate BDNF expression one of the aims of the study presented in this chapter was to assess if exercise-induced increases in BDNF expression are dependent on CB_1 receptor activity, a hypothesis that has not previously been explored. In order to assess this BDNF expression in the serum, dentate gyrus and hippocampus was examined in the sedentary-vehicle, sedentary-AM 251, exercise-vehicle and exercise-AM 251 groups. The results in Figures 4.4-4.6 show no changes across the four groups in BDNF expression in the serum, dentate gyrus and the hippocampus proper. The fact that exercise alone had no effect on BDNF expression is in stark contrast to previous findings from this lab which report that the same forced treadmill running protocol used by this study increases BDNF expression. It has been consistently reported that this exercise protocol can increase BDNF (mRNA and protein) in the dentate gyrus and BDNF protein in the hippocampus (Bechara *et al.*, 2013a; Bechara *et al.*, 2013b; Griffin *et al.*, 2009). However, it should be considered that rats in the studies by Bechara *et al.,* and Griffin *et al.,* were euthanised and brains removed for analysis 24 h after the last exercise bout and rats in this study were ethuanised 72 h after the last exercise bout. This suggests that exercise-induced increases in BDNF in the dentate gyrus and hippocampus return to baseline at some point between 24 h and 72 h post exercise. Further analysis of the temporal profile of BDNF expression post exercise would need to be carried out to identify the precise dynamics, however the present study does further our knowledge of the expression profile of BDNF in response to exercise. This lack of BDNF response may also explain the lack of effect of exercise on cognition discussed above.

As mentioned above exercise has been reported to selectively increase endocannabinoid signalling in the hippocampus (Hill *et al.*, 2010). Specifically, the authors report an increase in hippocampal AEA concentration and an increase in CB_1 receptor activity in the hippocampus following 8 days of voluntary running. An increase in CB_1 receptor binding site density and an increase in CB_1 receptor ^{35}S- GTPγS binding in the hippocampus is reported in the exercise group, however CB_1 receptor expression is not

directly assessed. Here we report that forced exercise does not affect CB_1 receptor mRNA in the dentate gyrus or the hippocampus. This is in contrast to a study reporting an increase in CB_1 receptor mRNA in the hippocampus following ten days of voluntary exercise (Wolf *et al.*, 2010). Considering results from the present study, the fact that Hill and colleagues report an increase in CB_1 receptor binding site density in the hippocampus and the fact that Wolf and colleagues show an increase in CB_1 receptor mRNA in the hippocampus one of two conclusions may be drawn. First it may be the case that voluntary exercise increases CB_1 receptor expression in the hippocampus and forced exercise does not. Alternatively, considering the time points post exercise that tissue was analysed (0 h in the case of Wolf *et al.,* 2010; 24 h in the case of Hill *et al.,* 2010 and 72 h in the case of this study) it could be hypothesised that exercise, both forced and voluntary, increase CB_1 expression and that CB_1 mRNA is efficiently translated to CB_1 protein within 24 h of exercise.

The fact that changes in BDNF expression were not observed in this study raises the possibility that the spatial memory enhancement observed (in the exercise-AM 251 group) could potentially be due to a more long-term structural neurophysiological change rather than a short-term signalling change. There is evidence that synaptogenesis (i.e. the formation of new synapses) may have a role to play in hippocampal-dependent memory. Meta-analysis of the available literature shows that spatial learning coincides with a large increase in total synapse density in the hippocampal formation while an increase in vesicle content at synapses was observed in the CA3 region (Marrone, 2007), there is no data on vesicle content in other sub-regions of the hippocampus. In addition to this, one week of exercise sufficient to enhance spatial memory was found to induce an increase in the synaptic vesicle protein synapsin in the dentate gyrus (Bechara *et al.*, 2013b). Taking this into account it is possible that synaptogenesis may account for the memory enhancement observed in the exercise-AM 251 group. In order to assess synaptogenesis, the expression of synapsin, synaptophysin and PSD-95 was evaluated in each of the four experimental groups. Synapsin and synaptophysin are synaptic vesicle proteins and expression of these proteins is often used to quantify synaptogenesis (Fletcher *et al.*, 1994). Increased expression of synapsin or synaptophysin indicates an increase in total presynaptic bouton number or an increase in total synaptic vesicle content in a particular brain region. PSD-95 is a protein found in the hippocampus almost exclusively in dendrites (Cho *et al.*, 1992), and therefore an increase in expression of this protein can indicate and increase in the total number of dendrites or an increase in the size of dendritic terminals in a particular brain region. Using Western immunoblotting the expression of synapsin, synaptophysin and PSD-95 were measured relative to the

expression of GAPDH in the dentate gyrus, while synapsin expression relative to GAPDH was measured in the hippocampus proper. None of the three treatment groups (sedentary-AM 251, exercise-vehicle, exercise-AM 251) exhibited altered expression of any of these proteins compared with the sedentary-vehicle control group. This implies that synaptogenesis is not likely to be responsibe for the spatial learning enhancement seen in the exercise-AM 251 group. The fact that exercise alone had no effect on synapsin expression is interesting when compared to the findings of Bechara *et al.,* 2013b where an increase in synapsin was found using the same exercise protocol, but, as discussed earlier, tissue analysis carried out in this study was 72 h post exercise and 24 h post exercise in the case of Bechara *et al.,* 2013b. This suggests that exercise increases synapsin expression in the dentate gyrus but that this increase is transient as it returns to baseline 24-72 h post exercise.

Another long-term mechanism of hippocampal plasticity that may account for spatial memory enhancements is adult hippocampal neurogenesis. Increased cell proliferation/early-stage survival does not necessarily suggest an increase in overall neurogenesis in the dentate gyrus but it has in the past been linked to memory improvements (Bechara *et al.*, 2013a; Bechara *et al.*, 2013b). In this chapter (Figure 4.14) it is reported that, like Bechara *et al.,* 2013a & 2013b, one week of forced exercise increases the percentage of $BrdU^+$ cells in the dentate gyrus approximately two-fold, illustrating that exercise can enhance the proliferation and early-stage survival of NSC/NPCs in the dentate gyrus. Interestingly, Figure 4.14 also shows that administering AM 251 daily during the exercise protocol completely blocks this increase in $BrdU^+$ cells, showing that the effects of exercise on cell proliferation/early survival are completely dependent on CB_1 receptor activity. It has been previously reported that voluntary exercise increases the number of cells in the dentate gyrus expressing the cell cycle protein Ki67 and that this was blocked by AM 251 (Hill *et al.*, 2010). We do not observe an exercise related change in Ki67 expression suggesting that the effect of exercise on inducing cells to enter the cell cycle is short lived (i.e. less than 72 hours). It has also been reported that increased cell proliferation following exercise is absent in CB_1 knockout mice (Wolf *et al.*, 2010), however the effect of a CB_1 receptor antagonism on the exercise-induced enhancement of early-stage survival of proliferating cells had not been assessed until now.

The fact that AM 251 blocks the observed increase in cell proliferation/early-stage survival in the exercise group appears counterintuitive considering that the spatial memory enhancement was only observed in the exercise-AM 251 group. However, this

can possibly be explained when the developmental timeline of adult neurogenesis is explored. The latency between proliferation of a NSC/NPC in the adult dentate gyrus and its development into a cell that exhibits morphological and electrophysiological properties of a mature neuron is approximately 28 days (Suh *et al.*, 2009) [although it should be noted that newly-generated neurons can continue to mature for several months]. $BrdU^+$ cells in the present study are 3-10 days old (see experimental timeline in Figure 4.1 A) and therefore are unlikely to exhibit mature neuronal properties, which make these cells unlikely to have the ability to contribute to memory.

Taken together, this suggests that exercise in combination with CB_1 receptor antagonism enhances short-term spatial memory through an as-yet unidentified mechanism but this cognitive enhancement appears to be independent of BDNF signalling, synaptogenesis and hippocampal neurogenesis.

Long-term study

The considerations discussed in the previous paragraph prompted the design of this long-term study (see experimental timeline in Figure 4.1 B). The aim of this study was to assess spatial memory at a more relevant timepoint in which cells generated during the exercise/AM 251 administration protocol are likely to be functionally mature neurons. To achieve this rats underwent the same exercise/AM 251/BrdU administration protocol as the short-term study and where then subjected to a spatial learning paradigm during the early maturation period of the BrdU labelled cells. Recall of the spatial learning task was tested on day 29 when $BrdU^+$ cells were 21-28 days old. The spatial memory task used was a purpose-designed variation of the object displacement task in which the acquisition period and retention periods were extended (Figure 4.2). The survival of BrdU labelled cells was assessed after testing in the task to give an indication of the effect of the interventions on net-neurogenesis (as the phenotype of these cells is not being examined assessing survival of $BrdU^+$ cells can only indicate neurogenesis).

During the acquisition phase of the task the exercise-vehicle group explored object C significantly more than object A and B and the exercise-AM 251 group explored object C significantly more than object B. To prevent this spontaneous preference from affecting the results of the task object C was not used as the displaced object. Consistent with the hypothesis of the study neither the sedentary-vehicle nor the sedentary-AM 251 groups were successful in discriminating between the displaced object and stationary objects on testing day, whereas the exercise-vehicle group (the group which exhibited increased proliferation/survival in the short-term study) successfully performed the task. This

shows that the positive effects of exercise on hippocampal-dependent memory can be detected 21 days post exercise. This is an extremely interesting finding, as the persistence of the cognitive-enhancing effects of exercise was not been examined before. It has been discussed throughout this chapter that many studies in the literature report that exercise enhances spatial memory; studies using Morris water maze assess memory retention (i.e. perform a probe trial) over a 4 h period (Li *et al.*, 2013) while studies using the object displacement task use a 24 h retention period (Bechara *et al.*, 2013b; Griffin *et al.*, 2009). To the authors' knowledge only one study has assessed the exercise-enhancing effects on spatial memory over a longer time period (Intlekofer *et al.*, 2013). Using the object displacement task this study reports that access to a running wheel enhances spatial memory when tested 24 h post acquisition of the task but has no effect 7 days post acquisition. However, this is due to the fact that, unlike the long-term study in the present chapter, Intlekofer and colleagues fail to adjust the duration of the acquisition period to coincide with the increased retention period, an acquisition period of 3 min is used for both 24 h and 7 day retention tests.

Another interesting finding, not fitting with the hypothesis of the experiment, is that the exercise-AM 251 group (the group which exhibited baseline proliferation/survival in the short-term study) performed the task to the same level as the exercise-vehicle group. This shows that exercise enhances long-term spatial memory and this enhancement is independent of an exercise-induced increase in cell proliferation/early-stage survival at the onset of learning.

The study also reports that there is no effect of exercise or AM 251 either alone or in combination on the long-term (22-29 day) survival of $BrdU^+$ cells labelled during the exercise/AM 251 protocol. However, due to a low sample size (n= 2-4 per group) resulting from an equipment failure it is not possible to fully interpret this result. Comparing absolute values, the survival of $BrdU^+$ cells appears to mirror the proliferation/early survival result (Figure 4.14), at least with respect to the effect of exercise, with or without AM 251. The number of $BrdU^+$ cells is increased in the exercise-vehicle group (4856±828 cells) compared to the sedentary-vehicle group (3046±349 cells). AM 251 administered during exercise appears to block this effect; exercise-AM 251 group: 3333±475 cells. It also appears that AM 251 alone enhances cell survival; sedentary-AM 251 group: 4327±498. From this it could be speculated that exercise enhances the long-term survival of newborn cells in the dentate gyrus and that this is blocked administering a CB_1 receptor antagonist during exercise, while the antagonist alone seems to also enhance long-term cell survival.

Taken together, this suggests that exercise can enhance spatial memory independent of the effects it has on neurogenesis. Although further work is required to investigate this notion results from this study can help to further current knowledge on the role that neurogenesis has to play in spatial learning and memory.

To summarise, a week of daily exercise in combination with daily CB_1 receptor antagonist injections enhanced short-term spatial memory, tested after a 24 h delay. This memory enhancement is independent of synaptogenesis in the dentate gyrus and possibly the hippocampus. Increases in BDNF expression in the dentate gyrus and the hippocampus often associated with exercise have been shown not to persist to the third day post exercise. One week of daily exercise increases cell proliferation and early-stage survival of these cells in the dentate gyrus and this increase requires CB_1 receptor activation during exercise. It could also be speculated that this is the case for the long-term survival of these newly born cells. Exercise enhances long-term spatial memory tested over a 14-day retention period; this memory enhancement is independent of the CB_1 receptor activity during exercise. The fact that this enhancement is not affected by CB_1 inverse agonism proves that it is also independent of an exercise-induced increase in cell proliferation/early-stage survival and possibly net neurogenesis. This result suggests that neurogenesis may not mediate the effects of exercise on spatial memory reported here.

Chapter 5

Age-related changes in spatial memory and neurogenesis; is there a role for endocannabinoid and neurotrophin signalling?

Chapter 5

5.1 Introduction

Ageing is associated with widespread physiological changes in the body, the functioning of many organs is altered with age and dramatic changes can occur within the brain. Deficits in hippocampal-dependent learning and memory are one consequence of brain ageing. Aged animals exhibit deficits in spatial learning/memory as tested by performance in the Morris water maze task (Frick *et al.*, 1995; O'Callaghan *et al.*, 2009; Rapp *et al.*, 1996) and the object displacement task (Benice *et al.*, 2006). Interestingly, some studies report that deficits in performance of the object displacement task can occur from onset of middle-age (Wiescholleck *et al.*, 2013).

Brain ageing is associated with alterations in the functioning of multiple interconnected signalling systems, therefore, it is difficult to attribute age-related cognitive decline to dysfunction of one particular system. It is possible that cognitive impairments may be a result of an increased inflammatory environment in the CNS (Lynch, 2010), an altered composition/functioning of glutamate receptors (Clayton *et al.*, 2002), while impaired expression of long-term potentiation is correlated to age-related spatial memory impairments (Bach *et al.*, 1999a). Another neurophysiological process that is altered during ageing and has been suggested to contribute to age-related memory impairments is adult hippocampal neurogenesis (van Praag *et al.*, 2005). The rate of neurogenesis is decreased in aged animals; proliferation (Kuhn *et al.*, 1996), differentiation (Heine *et al.*, 2004) and survival (Speisman *et al.*, 2013) of NSC/NPCs is reduced in the aged dentate gyrus. Both Kuhn and Heine report that age-related decline in cell proliferation in the dentate gyrus is observed from 12 months of age suggesting that dysfunction of neurogenesis begins well before the onset of old-age. In addition, middle-aged rats (17 months old) have a dramatic decrease in number of dentate gyrus cells expressing the cell cycle protein ki67 and the immature neuron protein DCX compared to 5 month-old rats (Touyarot *et al.*, 2013) suggesting that neurogenesis is severely impaired by middle-age.

Neurotrophins, particularly BDNF, have been shown to play roles many processes in the brain including memory (Griffin *et al.*, 2009) and hippocampal neurogenesis (Scharfman *et al.*, 2005). Therefore, it is possible that the age-related changes in memory and neurogenesis may be a result of altered neurotrophin signalling. Until recently there had been little work published on the effects of ageing on BDNF expression in the hippocampus however several studies published in 2013 addressed this question. One

study reports an increase in BDNF concentration in the hippocampus of middle-aged rats compared to young rats (Sallaberry *et al.*, 2013), another shows no change in middle-aged or aged rats (Perovic *et al.*, 2013) while a third finds a decrease in the ventral but not the dorsal hippocampus of aged rats (Calabrese *et al.*, 2013). The effects of age on hippocampal NGF expression appear equally unclear; aged rats have been found to have both decreased (Terry *et al.*, 2011) and unchanged (Perovic *et al.*, 2013) levels of hippocampal NGF compared to young.

The endocannabinoid system may contribute to age-related impairments in brain function. Although direct evidence of age-related endocannabinoid dysfunction has not been reported there is some indirect evidence supporting this hypothesis. The synthetic cannabinoid WIN-55212-2 improves spatial memory, reduces the number of activated microglia and partially rescues neurogenesis in the aged hippocampus (Marchalant *et al.*, 2009; Marchalant *et al.*, 2008). It has also been shown that inhibiting the hydrolysis of the endocannabinoid AEA reduces inflammation and partially rescues LTP in the aged hippocampus (Murphy *et al.*, 2012).

The first aim of the present study was to investigate how ageing affects spatial memory as assessed by performance in previously described hippocampal-dependant object displacement task. Three groups were used in the present study: young (3 month-old), middle-aged (15 month-old) and aged (22 month-old). There are no strict guidelines defining young, middle-aged and aged rats however the age of each group in this study approximately complements (± 2 months in the case of middle-aged and aged groups) other studies in the literature (Kadar *et al.*, 1990). Following behavioural testing in the object displacement task proliferation and early stage survival of cells in the dentate gyrus were assessed. Examining the expression of Ki67 mRNA in the dentate gyrus was used as an indication of proliferation rate. Ki67 is a protein expressed by cells only during the cell cycle (Scholzen *et al.*, 2000) and therefore alterations in its expression can be indicative of alterations in the rate of cell proliferation. Rats also received seven daily injections of BrdU which was used indicate how age affected proliferation and early-stage survival of cells in the dentate gyrus. In an attempt to identify underlying mechanisms responsible for any age-related cognitive or cellular changes observed, expression of the neurotrophins BDNF and NGFβ was assessed in the hippocampus and the dentate gyrus, while CB_1 expression was assessed in the dentate gyrus.

5.2 Methods

5.2.1 Animals

A total of 28 male Wistar rats (Bio Resources, Trinity College Dublin) were used in these experiments. Young animals weighed approximately 300g, middle-aged animals weighed approximately 500-600g and aged animals weighed approximately 600-800g. All animals were experimentally naïve at experiment onset. Following transfer from the Bio Resources breeding facility animals were given at least seven days to acclimatise to the housing facility before the experimental procedure, during this acclimatisation period animals were handled every day by the experimenter. Animals were group-housed three-four per cage and were maintained on a 12:12 hour light-dark cycle at constant ambient temperature (21.5 ± 1.5 °C) and humidity (55 ± 5%). Food and water was available *ad libitum.* Methods were performed in accordance with the national law and European Union directives on animal experiments.

5.2.2 Experimental design

Animals were assigned to one of three groups depending on age; young (3 months old), middle-aged (15 months) and aged (22 months). To assess the effects of age on short-term spatial memory animals were tested in the object displacement task. Animals were euthanised following testing in the object displacement task to assess how age affects cellular changes and signalling systems in the brain. In order to examine whether age had an effect on NSC/NPC proliferation and early-stage survival animals were i.p. injected with BrdU (50 $mg.kg^{-1}$ in 0.9% saline; 1-2 ml per animal) [Sigma] daily for 7 days prior to the object displacement protocol. BrdU is a thymidine analogue and incorporates into replicating DNA during the S-phase of the cell cycle prior to cell division and therefore allows identification of cells that have undergone mitosis during the experimental protocol.

5.2.3 Object displacement task

Short-term object displacement task

During the acquisition phase of the task animals were placed in the open field containing three objects and were allowed to explore freely for three five minute trials with a five minute inter-trial interval during which the animals were returned to their home cage. The open field and the room in which the open field was placed contained several spatial cues. Twenty-four hours later, again in the presence of spatial cues, animals were placed

in the open field containing the same three objects with one of the objects repositioned to another quadrant of the open field (Figure 5.1).

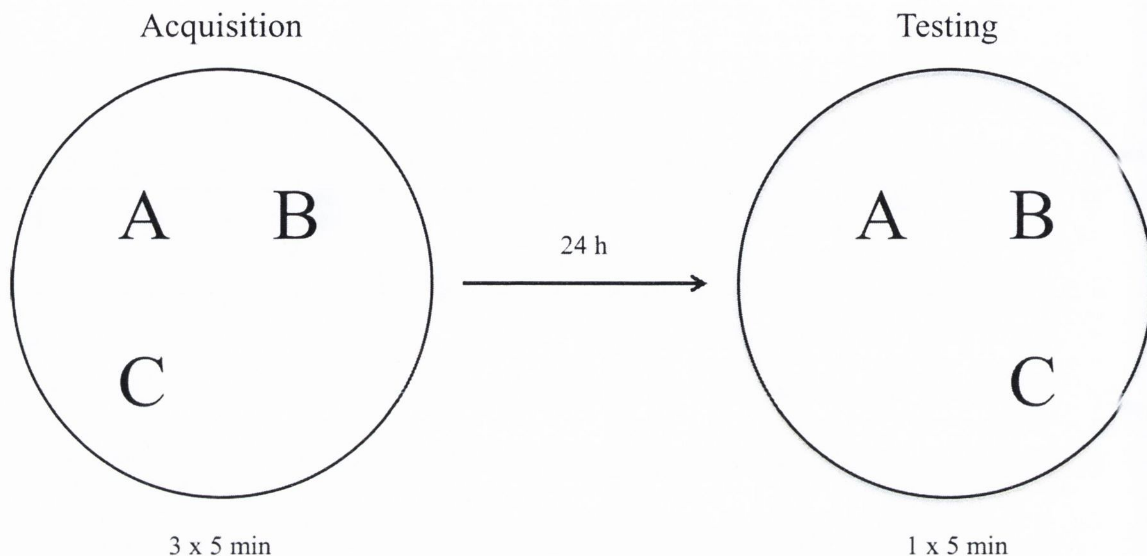

Figure 5.1 Short-term object displacement task protocol

5.2.4 Analysis of mRNA expression by RT-PCR

BDNF and NGFβ mRNA expression in the hippocampus and dentate gyrus was assessed using RT-PCR (see section 2.7). As ki67 is a cell cycle protein its expression was assessed in the dentate gyrus only.

5.2.5 Analysis of protein expression by ELISA

BDNF protein was quantified in serum and tissue samples using a Human BDNF ELISA kit (R&D Systems Europe, Oxon, United Kingdom). The active subunit of the NGF protein NGFβ was quantified in tissue samples using a Rat NGFβ DuoSet ELISA Developmental system (R&D Systems Europe, Oxon, United Kingdom) [see section 2.4]. Hippocampus and dentate gyrus samples were homogenised in Krebs calcium buffer (see section 2.3) prior to carrying out the ELISA.

5.2.6 Analysis of $BrdU^+$ nuclei by immunohistochemistry

BrdU positive ($BrdU^+$) cells were visualised using the Aividin Biotin Complex (ABC) peroxidase method with rabbit anti-chicken conjugated IgG and 3,3 diaminobenzidine (DAB) chromogen (see section 2.5). Three sections were stained per animal and the percentage of cells in the dentate gyrus that were BrdU positive was calculated. Only cells that were located in the subgranular zone or the granular cell layer were included in this analysis.

5.2.7 Analysis of protein expression by western immunoblotting

CB_1 expression was measured in the dentate gyrus by SDS-PAGE and western immunoblotting (see section 2.7). β-actin expression was measured to indicate total protein expression in the sample.

5.2.8 Statistical analysis

Prior to statistical analysis data were examined for outliers, which were identified as being more than two standard deviations outside the mean. Outliers were excluded from the data set before statistical analysis.

Behavioural data were analysed using a two-way ANOVA to compare the exploration of objects and the effect of group. Where a significant main effect was identified a Bonferroni *post-hoc* test was applied to detect the significant differences.

Tissue analysis data were analysed using a one-way ANOVA.

5.3 Results

5.3.1 Object displacement task

Spatial memory was assessed using the object displacement task. For the acquisition trial there was no significant effect of object ($F_{1,46}=0.3401$, $p>0.05$), and there was no significant interaction ($F_{4,42}=0.6654$, $p>0.05$).

During the testing trial there was no significant effect of object ($F_{1,46}=1.435$, $p>0.05$) and there was a significant interaction ($F_{2,46}=9.852$, $p<0.0001$). Bonferroni *post-hoc* analysis revealed that the young group spent significantly more time exploring the displaced object compared to the stationary objects (percentage exploration of objects: stationary: 28.67±5.1, displaced: 71.33±5.1, ***$p<0.001$, Figure 5.2 B). No difference was found with respect to object exploration in the middle-aged or aged groups.

There was no effect of age on total exploration object exploration time during the task ($F_{2,21}=2.798$, $p>0.05$, Figure 5.3).

5.3.2 Analysis of Ki67 mRNA expression in the dentate gyrus

There was no effect of age on Ki67 mRNA expression in the dentate gyrus ($F_{2,20}=3.003$, $p>0.05$, Figure 5.4), however the effect of age on Ki67 expression approached significance ($p=0.07$).

5.3.3 Analysis of the number of $BrdU^+$ nuclei in the dentate gyrus

There was a significant effect of age on the number of $BrdU^+$ nuclei in the dentate gyrus ($F_{2,15}=11.360$, $p=0.001$, Figure 5.5). Bonferroni *post-hoc* analysis revealed that there was significantly lower percentage of $BrdU^+$ nuclei in the dentate gyrus of aged animals compared with young and middle-aged groups (percentage of $BrdU^+$ cells: young: 7.32±0.92, middle-aged: 6.82±0.51, aged: 2.75±0.31, **$p<0.01$, Figure 5.5).

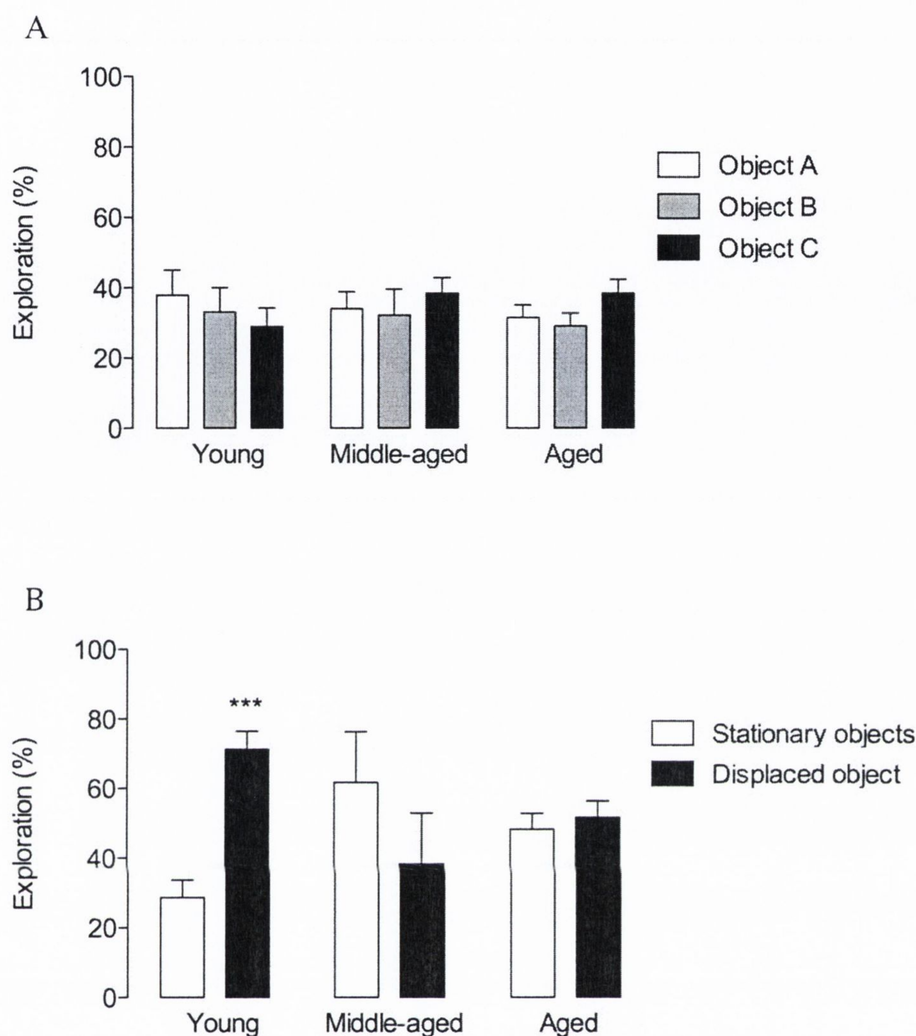

Figure 5.2 The effect of age on performance in the object displacement task

(A) There was no significant difference in object exploration during the acquisition period in any of the three groups. (B) There was a significant interaction during the testing period with the young animals exploring the displaced object significantly more than the stationary objects (***$p<0.001$). No such difference was observed in the middle-aged or aged groups. Young: n=12, Middle-aged: n=6, Aged: n=8. Bars represent mean±SEM percentage, two-way ANOVA.

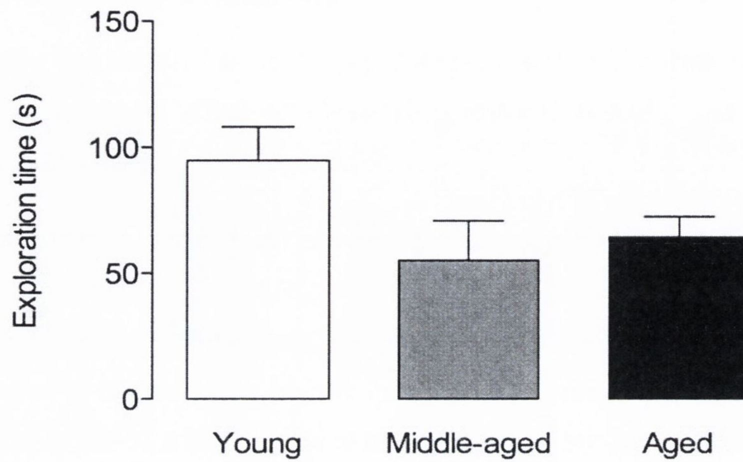

Figure 5.3 The effect of age on total object exploration time in the object displacement task

There was no significant effect of age on total exploration time. Young: n=12, Middle-aged: n=6, Aged: n=8. Bars represent mean±SEM, arbitrary units, one-way ANOVA.

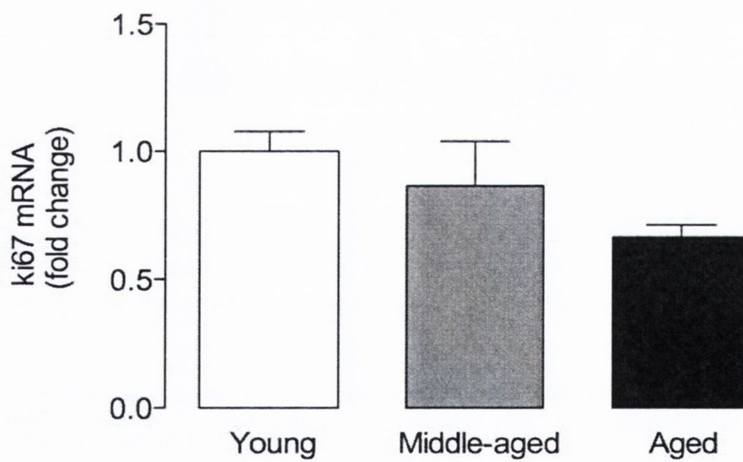

Figure 5.4 The effect of age on Ki67 mRNA expression in the dentate gyrus

There was no significant effect of age on Ki67 mRNA expression in the dentate gyrus. Young: n=12, Middle-aged: n=6, Aged: n=8. Bars represent mean±SEM, arbitrary units, one-way ANOVA.

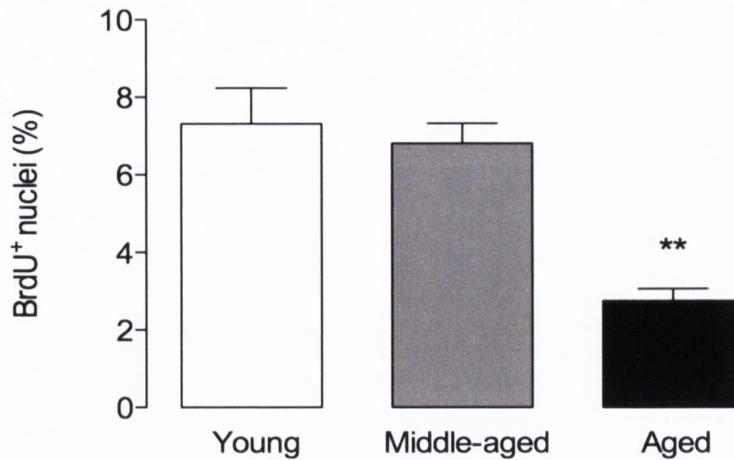

Figure 5.5 The effect of age on BrdU$^+$ nuclei in the dentate gyrus

There was a significant effect of age on the percentage of BrdU$^+$ nuclei in the dentate gyrus, *post-hoc* analysis revealed there was decrease in BrdU$^+$ nuclei in the aged group compared to young and middle-aged groups (**p<0.01). Young: n=7, Middle-aged: n=6, Aged: n=5. Bars represent mean±SEM percentage, one-way ANOVA.

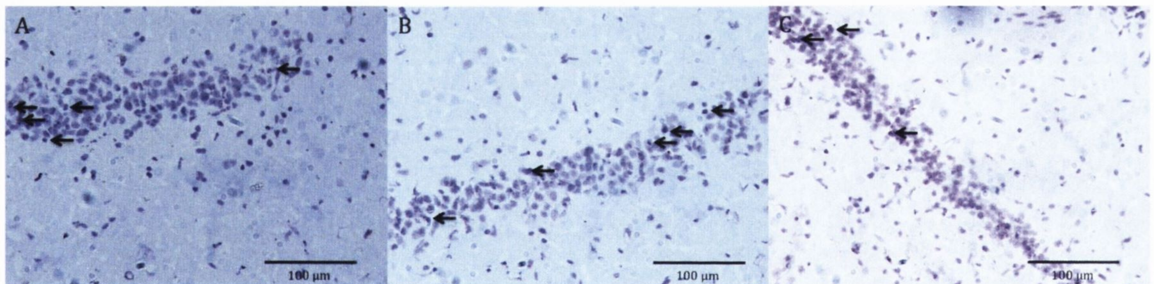

Figure 5.6 Representative images of BrdU$^+$ nuclei in the dentate gyrus

Cell nuclei are stained purple (haematoxylin) and BrdU$^+$ nuclei are indicated by purple co-localised with brown (DAB). Arrows indicate some BrdU$^+$ nuclei; (A) Young (B) Middle-aged (C) Aged.

5.3.4 Effect of age on BDNF expression in the dentate gyrus and the hippocampus

There was a significant effect of age on BDNF mRNA expression in the dentate gyrus ($F_{2,17}$=7.380, p<0.01, Figure 5.7 A). Bonferroni *post-hoc* analysis revealed the middle-aged and aged groups had an approximate three-fold decrease in BDNF mRNA compared with the young group (fold change of control: young: 1.00±0.19, middle-aged: 0.33±0.04, aged: 0.35±0.21, *p<0.05, Figure 5.7 A). There was a significant effect of age on BDNF protein expression in the dentate gyrus ($F_{2,17}$=10.790, p<0.001, Figure 5.7 B). Bonferroni *post-hoc* analysis revealed the aged group had a significantly higher concentration of BDNF compared with the young and middle-aged groups (Young: 802.10±75.56 pg.mg protein^{-1}, Middle-aged: 575.20±99.53 pg.mg protein^{-1}, Aged: 1365.00±158.90 pg.mg protein^{-1}, *p<0.05; ***p<0.001, Figure 5.7 B).

There was no effect of age on BDNF mRNA expression in the hippocampus ($F_{2,20}$=2.190, p>0.05, Figure 5.8 A). There was no significant effect of age on BDNF protein expression in the hippocampus (Young: 1280.0±131.0 pg.mg protein^{-1}, Middle-aged: 1132±230.6 pg.mg protein^{-1}, Aged: 13276.00±113.5 pg.mg protein^{-1}, $F_{2,20}$=0.2369, p>0.05, Figure 5.8 B).

5.3.5 Effect of age on NGFβ expression in the dentate gyrus and the hippocampus

There was no effect of age on NGFβ mRNA expression in the dentate gyrus ($F_{2,20}$=2.051, p>0.05, Figure 5.9 A). There was no effect of age on NGFβ protein expression in the dentate gyrus (Young: 175.9±10.6 pg.mg protein^{-1}, Middle-aged: 176.4±31.6 pg.mg protein^{-1}, Aged: 183.2±28.3 pg.mg protein^{-1}, $F_{2,19}$=0.0196, p>0.05, Figure 5.9 B).

There was a significant effect of age on NGFβ mRNA expression in the hippocampus ($F_{2,22}$=6.150, p<0.01, Figure 5.10 A). Bonferroni *post-hoc* analysis revealed the aged group had an approximate two-fold decrease in NGFβ mRNA compared with the young group (fold change of control: Young: 1.00±0.14, Aged: 0.48±0.09, **p<0.01, Figure 5.10 A). There was no effect of age on NGFβ protein expression in the hippocampus (Young: 155.1±12.7 pg.mg protein^{-1}, Middle-aged: 116.9±20.45 pg.mg protein^{-1}, Aged: 164.0±2.6 pg.mg protein^{-1}$F_{2,19}$=2.447, p>0.05, Figure 5.10 B).

5.3.6 Effect of age on CB_1 expression in the dentate gyrus

There was no effect of age on CB_1 protein expression in the dentate gyrus ($F_{2,17}=0.252$, $p>0.05$, Figure 5.11 B).

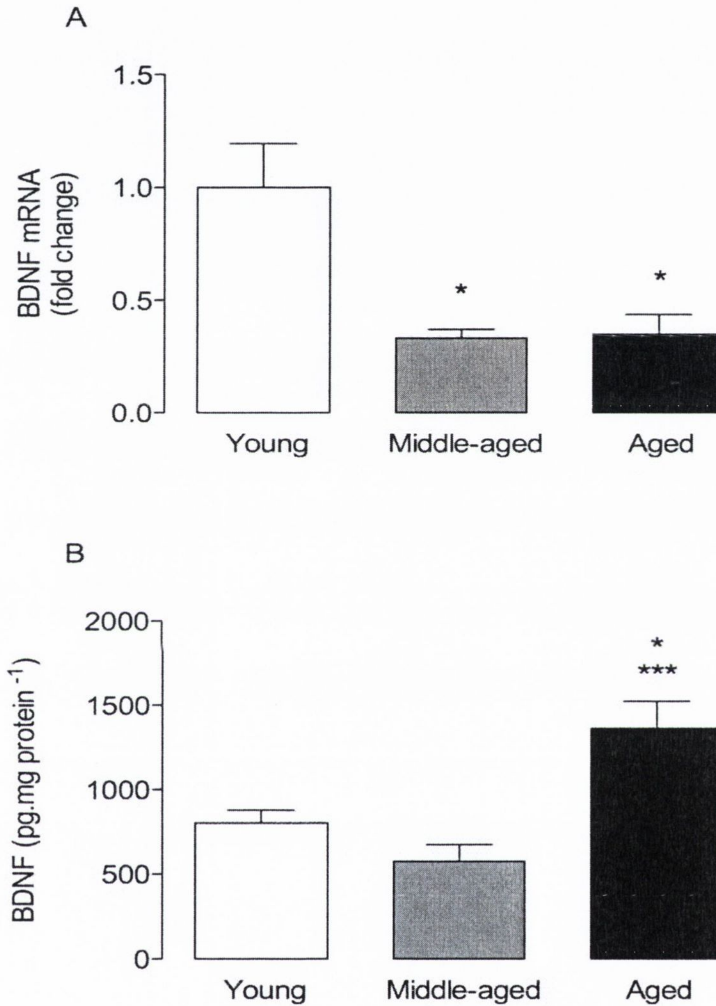

Figure 5.7 The effect of age on BDNF mRNA and protein expression in the dentate gyrus

(A) There was a significant effect of age on BDNF mRNA expression in the dentate gyrus, *post-hoc* analysis revealed there was decrease in BDNF mRNA expression in the middle-aged and aged groups compared to the young group (*$p<0.05$). Young: n=8, middle-aged: n=6, aged: n=6. (B) There was a significant effect of age on BDNF protein expression in the dentate gyrus; *post-hoc analysis* revealed there was an increase in BDNF protein in the aged group compared to the middle-aged (***$p<0.001$) and the young group (*$p<0.05$). Young: n=4, middle-aged: n=8, aged: n=8. Bars represent mean±SEM, one-way ANOVA.

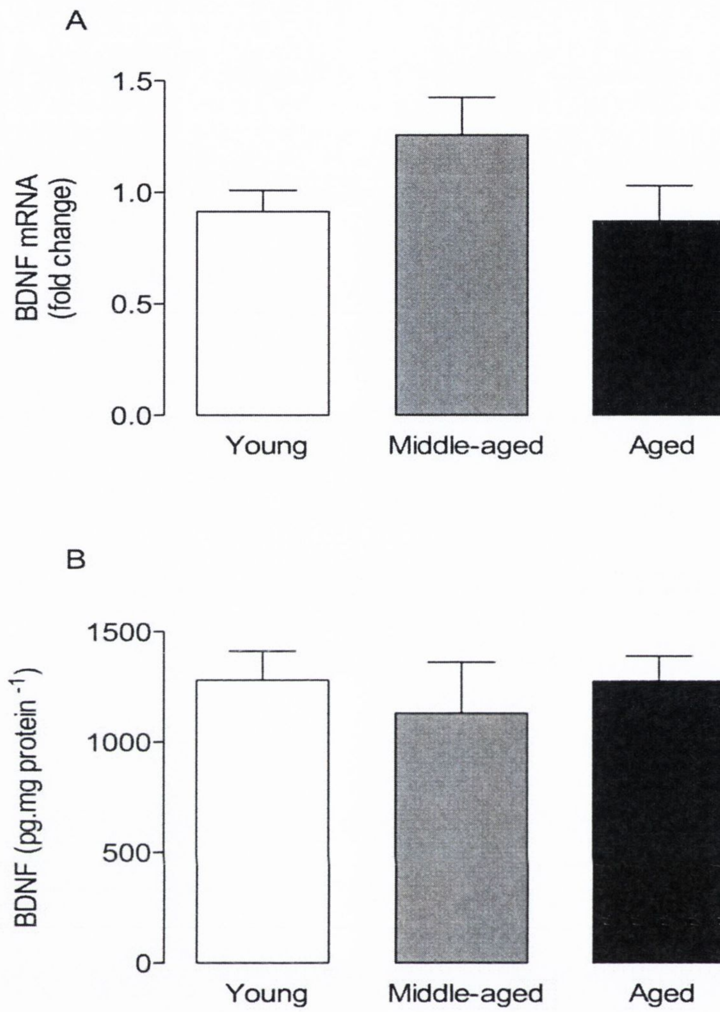

Figure 5.8 The effect of age on BDNF mRNA and protein expression in the hippocampus

(A) There was no effect of age on BDNF mRNA expression in the. Young: n=8, middle-aged: n=8, aged: n=7. (B) There was no effect of age on BDNF protein expression in the hippocampus. Young: n=10, middle-aged: n=8, aged: n=5. Bars represent mean±SEM, one-way ANOVA.

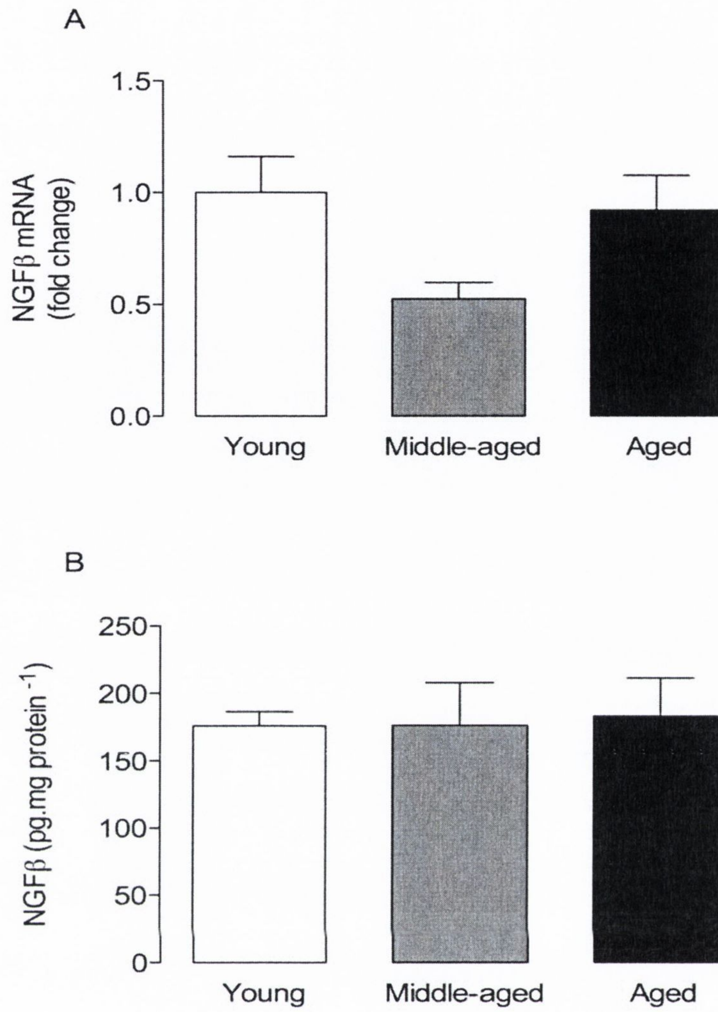

Figure 5.9 The effect of age on NGFβ mRNA and protein expression in the dentate gyrus

(A) There was no effect of age on NGFβ mRNA expression in the dentate gyrus. Young: n=10, middle-aged: n=5, aged: n=8. (B) There was no effect of age on NGFβ protein expression in the dentate gyrus. Young: n=4, middle-aged: n=8, aged: n=8. Bars represent mean±SEM, one-way ANOVA.

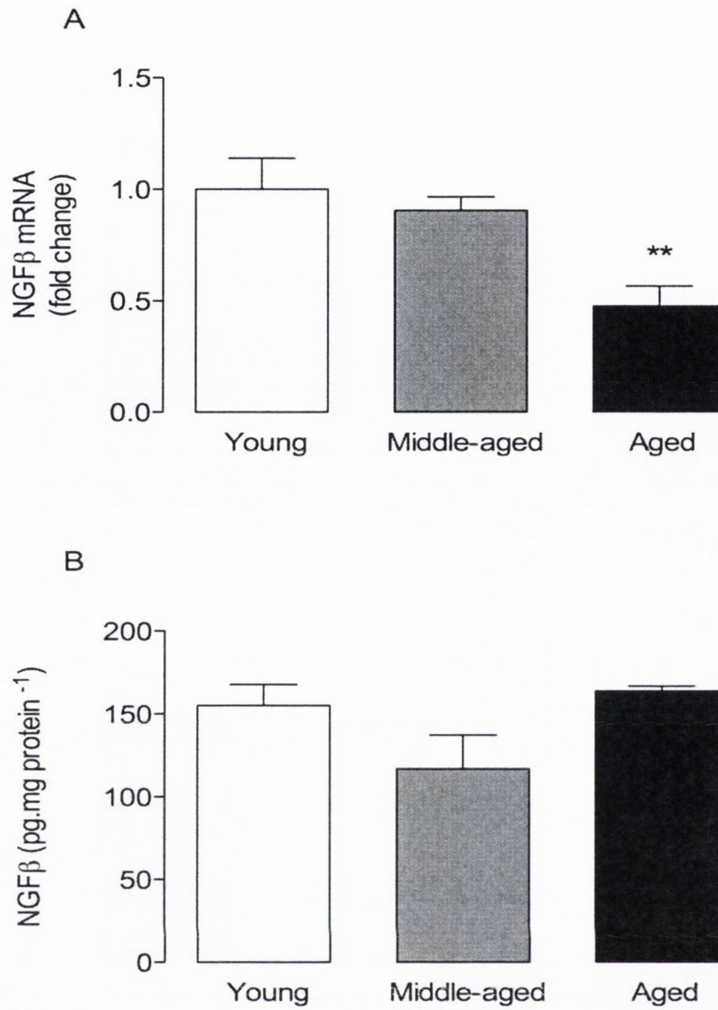

Figure 5.10 The effect of age on NGFβ mRNA and protein expression in the hippocampus

(A) There was a significant effect of age on NGFβ mRNA expression in the hippocampus, *post-hoc* analysis revealed there was decrease in NGFβ mRNA expression in the aged group compared to the young group (**p<0.01). Young: n=9, middle-aged: n=7, aged: n=7. (B) There was no effect of age on NGFβ protein expression in the hippocampus. Young: n=10, middle-aged: n=5, aged: n=5. Bars represent mean±SEM, one-way ANOVA.

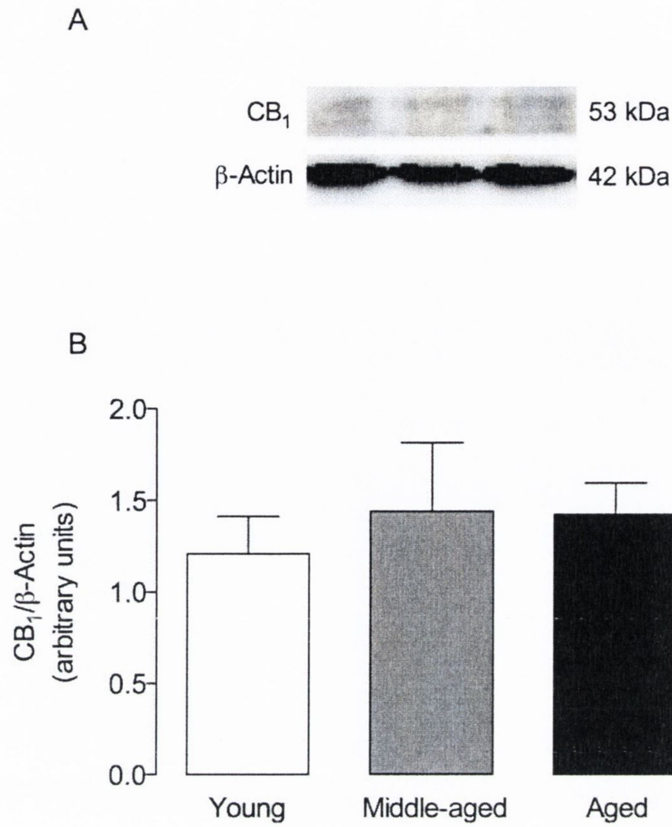

Figure 5.11 The effect of age on CB_1 protein expression in the dentate gyrus

(A) Representative image from a western blot of CB_1 (53 kDa) and β-Actin (42 kDa). Left to right: young, middle-aged and aged. (B) There was no effect of age on CB_1 protein expression in the dentate gyrus. Young: n=6, middle-aged: n=6, aged: n=8. Bars represent mean±SEM, one-way ANOVA.

5.4 Discussion

Ageing was found to impair spatial memory. Only the young group successfully performed the object displacement task. On the training day all three groups spent a similar portion of time exploring each of the objects, on the testing day young rats successfully discriminated between the displaced object and the stationary objects whereas the middle-aged and aged groups could not. This suggests that spatial learning/memory is impaired by middle age and this impairment persists during ageing. Importantly no change in total object exploration time was observed during ageing showing that learning impairments during ageing is not due to an overall decline in exploratory behaviour. As mentioned above, ageing has long been associated with impaired spatial memory. Spatial memory impairments are usually measured by performance of the Morris water maze (Frick *et al.*, 1995; O'Callaghan *et al.*, 2009; Rapp *et al.*, 1996) and there have been limited studies focusing on the object displacement task. It has been reported that old mice (20 months) have impaired performance in the object location (i.e. displacement) task compared to middle-aged (12 months) and young (4 months) mice (Benice *et al.*, 2006). This study from Benice and colleagues supports some of the findings from the present study. The results presented here show that both middle-aged and aged rats cannot perform the object location task whereas Benice and colleagues report that only aged mice and not middle-aged mice have impaired object location memory. This suggests that there is a clear impairment in object location memory in aged animals, however the effect of middle age on this form of memory is slightly less clear. The fact that the results presented here show memory deficits at 'middle-age' and Benice and colleagues do not may be due to one of two confounds when comparing both studies. One is that it is difficult to directly compare results observed in mice to rats and the other is that they have defined middle-aged as 12 months whereas middle-aged rats in this study were 15 months. Another study that examined the effects of age on performance in the object displacement task in the rat used young (2 months) and 'older' (8 months) Wistar rats (Wiescholleck *et al.*, 2013). It was found the young but not 'older' rats could discriminate between the displaced object and the stationary object 1 h post-acquisition. This suggests that impairments in short-term spatial memory can occur at 8 months of age, well in advance of the 15-month age used in our study. Interestingly it was found that young and 'older' rats can both discriminate between the displaced and stationary objects when tested 5 min post-acquisition, therefore the memory impairment appears to be due to a deficit in consolidation rather than a deficit in acquisition. Taken together the results from the present study and the literature suggest that spatial memory tested by the object location task is impaired by

late-adulthood (8 months) and persists through middle age (15 months) to old age (22 months). It is possible that this impairment is due to consolidation deficits; however further investigation would be required to confirm this.

Ageing was found to affect cell proliferation/early survival in the dentate gyrus. There was no difference across the three age groups with respect to expression of the cell cycle protein Ki67 at the mRNA level in the dentate gyrus, however the effect of age on Ki67 mRNA expression closely approached significance (p=0.07). It was found that aged rats had an approximate two-fold decrease in the number of BrdU$^+$ cells compared to young and middle-aged rats. This shows that by middle age (15 months) cell proliferation and early-stage survival of these proliferating cells is not impaired. By old age (22 months) there appears to be no alteration in the rate of cell proliferation as indicated by ki67 mRNA expression however there is a decrease in the number of BrdU$^+$ cells in the dentate gyrus. In this particular experiment rats received daily BrdU injections for seven days prior to euthanasia, therefore BrdU$^+$ cells are 1-7 days old and a decrease in BrdU$^+$ cells suggests impairment in NSC/NPC cell proliferation (1 day-old cells) or early survival (cells 2-7 days old). These data could be interpreted in a number of ways. It could be suggested that cell proliferation is not decreased with age, as there is no change in Ki67 mRNA but that survival of these proliferating cells is impaired as there is a decrease in the number of BrdU$^+$ cells. However, it should be noted that Ki67 mRNA was quantified here and not ki67 protein therefore it is not possible to definitively say that there is no change in cell proliferation rate in the dentate gyrus. Another confound that should be considered here is the fact that the percentage of BrdU$^+$ cells presented in this study is achieved by counting the total number of cells in the granular cell layer and the number of BrdU$^+$ cells in the granular cell layer and subgranular zone of the dentate gyrus and expressing BrdU$^+$ cells as a percentage of total cell number. Due to the fact that only BrdU$^+$ cells within the subgranular zone and the granular cell layer were quantified it is likely that the majority of these cells are of neuronal linage. In contrast to this, Ki67 PCR analysis in this study was performed on mRNA isolated from whole dentate gyrus homogenate, therefore, it is not possible to differentiate between proliferating cells in the granular cell layer/subgranular zone and those outside of these regions.

Ageing is associated with an increase in microglia density in the hilus, molecular and granular cell layers of the dentate gyrus (Gebara *et al.*, 2013). It is therefore possible that a decrease in NSC/NPC cell proliferation in aged rats (measured by Ki67 mRNA expression) in the granular cell layer/subgranular zone may be masked by an increase in microglial cell proliferation in the hilus and molecular layer. Another line of evidence in

support of this notion is the fact that other studies have shown a decrease in Ki67$^+$ cells in the granular cell layer and subgranular zone of aged rats (Cowen *et al.*, 2008). Considering all of this the results from the present study identify a decrease in cell proliferation and/or early stage survival in the dentate gyrus of aged but not middle-aged rats when compared to young rats.

This decrease in cell proliferation/survival found in aged animals is in agreement with other studies in the literature. Although it is difficult to directly compare studies due to differences in BrdU administration protocol it has been shown that aged rats (24 months) when compared to young rats (6 weeks) have a decrease in cell proliferation as measured 1 day post BrdU injection and decreased early stage survival as measured 7 days post BrdU injection (Heine *et al.*, 2004), a result that complements the findings in the present study. Interestingly, Heine and colleagues find that this decrease in proliferation and early survival is observed from 12 months of age, a finding that is contradictory to the results presented here. It is likely that this contradiction is due to the different housing conditions used; this study uses group housing (three-four per cage) whereas Heine and colleague use singly housing (one per cage). Housing condition is a variable that makes comparison between theses two studies difficult as social isolation has been shown to decrease cell proliferation and survival in the dentate gyrus of Wistar rats (Evans *et al.*, 2012). Taking this into account, it is possible that Heine and colleagues have obtained a false negative result and the decrease in proliferation/early survival they observed in middle-aged rats (12 months) is in fact due to social isolation and not as a result of ageing. To summarise, the present study shows that non-socially isolated aged rats exhibit reduced cell proliferation and/or early stage survival in the dentate gyrus when compared to middle-aged and young rats, further investigation would be required to identify at what stage during the proliferation/survival processes the deficits occur.

Interestingly the spatial memory deficits in aged rats are accompanied by a decrease in cell proliferation/early survival in the dentate gyrus, however the memory deficit observed in middle-aged rats is not accompanied by a change in neurogenic activity in the dentate gyrus suggesting that another neurophysiological change within the hippocampus may be responsible. In an attempt to explain the cognitive and cellular effects of ageing observed neurotrophin expression in the hippocampus and dentate gyrus was examined. BDNF has been shown to modulate memory (Griffin *et al.*, 2009) and hippocampal neurogenesis (Scharfman *et al.*, 2005), therefore changes in hippocampal and dentate gyrus BDNF expression may explain the deficits in spatial memory observed in middle-aged and aged rats and the decrease in cell proliferation/survival seen in aged

rats. It has been shown here that BDNF expression is unchanged in the hippocampus proper during ageing. The finding that hippocampal BDNF expression is unchanged during ageing conflicts the findings from previous studies which report decreases in hippocampal BDNF during ageing (Calabrese *et al.*, 2013). However, as with the cell proliferation/survival result above, a confound with comparing the present study to that of Calabrese and colleagues is that they have opted to singly house rats whereas this study uses group housing. Hippocampal BDNF has been shown to be decreased as a result of (single housing) social isolation (Evans *et al.*, 2012; Pisu *et al.*, 2011) and therefore Calabrese *et al.,* could potentially be observing a false negative result as the decrease in hippocampal BDNF may be due to social isolation and not ageing. This notion is further substantiated by the fact that a study that group housed rats (two-four per cage) found no age-related change in hippocampal BDNF protein expression (Perovic *et al.*, 2013).

The data presented here show that in the dentate gyrus BDNF mRNA expression is decreased in middle-aged and aged rats, whereas aged rats exhibit a dramatic increase in BDNF protein compared to young and middle-aged. The finding that middle-aged rats have decreased BDNF mRNA specifically in the dentate gyrus is a novel finding that has not been previously reported. There has been almost no investigation into the expression of BDNF mRNA in the dentate gyrus during middle age, however, it has been reported that BDNF protein expression is not altered in middle-aged rats (Li *et al.*, 2009), something that has been found in the present study. These results suggest that although middle age is accompanied by decrease in BDNF mRNA transcription it appears that a compensatory increase in translation and/or an increase in the rate of processing of proBDNF to mature BDNF maintains a level of (mature) BDNF protein similar to that of young animals. This lack of effect of middle age on functional BDNF protein expression in the hippocampus and the dentate gyrus fails to account for the spatial memory deficit observed in middle-aged rats suggesting other mechanisms may be responsible. Aged rats were found to have decreased BDNF mRNA in the dentate gyrus, a result observed by others (O'Callaghan *et al.*, 2009), and increased BDNF protein a result that conflicts a study that reports decreases in aged rats (Hattiangady *et al.*, 2005). Again, the confound of single housing is presented in the latter study. The results from the present study with respect to aged dentate gyrus again, in a similar fashion to the middle-aged results, suggest hyperactivity of BDNF mRNA translation. It appears that the proposed increase in BDNF mRNA translation observed in middle age that is acting to maintain a normal BDNF protein concentration may be reaching a non-homeostatic level of hyperactivity inducing an increase in BDNF protein compared to young. This is an interesting finding

that has not been reported before and warrants further investigation to fully decipher the dynamics of this process. The fact that BDNF is increased in the aged dentate gyrus is counter-intuitive to the fact that spatial memory and hippocampal neurogenesis are compromised in these animals. Perhaps it is possible that such a large increase in BDNF is detrimental to dentate gyrus function, however this hypothesis requires further investigation to be substantiated.

NGFβ expression was also examined in the hippocampus and the dentate gyrus of these animals. Like BDNF, NGFβ has been shown to affect memory and cell proliferation/survival in the dentate gyrus (Birch *et al.*, 2013a; Frielingsdorf *et al.*, 2007) and changes in NGFβ expression may help to identify mechanisms responsible for the changes in hippocampal function observed with age. The results present here show that NGFβ mRNA expression is unchanged in dentate gyrus of middle-aged or aged rats. This is in contrast to a previous finding from our group that reports a decrease in NGFβ mRNA in the dentate gyrus of aged (23 months) rats (O'Callaghan *et al.*, 2009). This suggests the possibility of variability within cohorts of rats and/or a progressive decrease in NGFβ mRNA expression from the age of 22 months (rats used in the present study) and 23 months (rats used by O'Callaghan *et al.*). The results presented here found no difference in NGFβ expression at the protein level in the dentate gyrus, a result that has not previously been reported. The fact that NGFβ expression is not altered in the aged dentate gyrus suggests that the age –related decrease in cell proliferation/survival is not a consequence of altered NGFβ expression.

NGFβ expression in the hippocampus proper was found to be unchanged in middle-aged rats when compared to young rats, at both mRNA and protein level. This has been recently shown at two stages of 'middle-age'; 12 months and 18 months (Perovic *et al.*, 2013). Perovic and colleagues also report that aged (24 months) rats have no change in NGFβ mRNA or protein expression in the hippocampus, the mRNA result contrasts whereas the protein result supports the findings presented here in Figure 5.10. Again it is possible that slight variations in experimental conditions (e.g. cohort of rats used) could account for the variation in NGFβ mRNA transcription efficiency when comparing the two studies. Importantly no difference was found by the present study or by Perovic *et al.,*at the functional protein level. The lack of an ageing effect on NGFβ protein expression in the hippocampus suggests that spatial memory deficits observed in middle-aged and aged rats is not due to lack of NGFβ availability.

It should be noted that neurotrophin analysis in the present study was carried out on whole dentate gyrus or hippocampus proper homogenate and therefore it is not possible to attribute changes in neurotrophin expression to one particular cell type. This could be an important point to consider as there is evidence that the number of astrocytes in the hippocampus increases during ageing (Lynch *et al.*, 2010), therefore changes in neurotrophin expression could be a consequence of an increased number of astrocytes rather than an increase in activity-dependent neuronal release.

Another signalling system involved in adult hippocampal neurogensis is the endocannabinoid system and, interestingly administering a synthetic cannabinoid partially rescues the age-related decline in neurogenesis (Marchalant *et al.*, 2009). No study has investigated the effect of ageing on endocannabinoid concentration in the hippocampus proper or the dentate gyrus. It has been reported that ageing has no effect on concentrations of AEA and 2-AG in the cerebellum (Murphy *et al.*, 2012). It may be hypothesised from this finding that age has no effect on AEA or 2-AG expression in other regions of the brain, although further investigation would be required to confirm this. Taking this hypothesis and the fact that synthetic cannabinoids partially rescues neurogenesis in ageing into account it could be speculated that the dysfunction in age-related dentate gyrus endocannabinoid signalling may lie with cannabinoid receptor expression and/or function. In other to examine this and to explain the deficit in cell proliferation/survival in the dentate gyrus of aged animals the expression of the CB_1 receptor in the dentate gyrus was evaluated. Age was found to have no effect on CB_1 receptor in the dentate gyrus (Figure 5.11). This result suggests that impaired proliferation/survival observed in aged rats is not due to altered CB_1 receptor expression. However caution is urged with this interpretation as CB_1 receptor expression was examined in whole dentate gyrus homogenate and therefore it is not possible to speculate expression level on a particular cell phenotype, namely NSC/NPCs. CB_2 receptor was not examined due to limited availability and reliability of anti-CB_2 antibodies (Atwood *et al.*, 2010).

In summary, ageing is accompanied by a deficit in spatial memory, this deficit is observed during middle age and persists to old age. The short-term spatial memory deficit appears to be independent of cell proliferation/early-stage survival in the dentate gyrus as only aged and not middle-aged rats exhibit a decrease in this measure of neurogenic capacity. Middle-aged and aged rats have a decreased level of BDNF mRNA in the dentate gyrus compared to young rats. This change does not translate to a decrease in BDNF protein expression in middle-aged rats, and, somewhat surprisingly leads to an

increase in BDNF protein expression in aged rats. This suggests the possibility of a compensatory increase in the rate of BDNF mRNA-protein translation that maintains protein expression in middle age but that possibly becomes hyperactive during ageing and leads to non-homeostatic increases in BDNF protein expression in old age. Ageing does not affect NGFβ expression with the exception of a decrease in mRNA in the hippocampus. These age-related changes in dentate gyrus function appear to be independent of CB_1 receptor signalling in the dentate gyrus, as its expression is not affected by age.

Chapter 6

General discussion

Chapter 6

6.1 General discussion

Learning and memory can be simply defined as the ability to acquire information from the environment, store this information and recall it at a later time. As the study of learning and memory has progressed it has become evident that although these processes can be easily defined the mechanisms underlying learning and memory are not easily understood. Several cellular mechanisms have been suggested to be responsible for memory formation; however, proof of a definitive link between these mechanisms and learning and memory does not yet exist. Since the phenomenon of adult hippocampal neurogenesis was accepted by modern neuroscience in the 1990s it has gained widespread attention, particularly into the possibility of the process playing a major role in learning and memory (Deng *et al.*, 2010). Despite extensive research into adult neurogenesis and the identification of a potential relationship between it and memory formation a conclusive link is yet to be established. The primary aim of this thesis was to aid the progression of our knowledge of adult neurogenesis and ultimately evaluate its role in spatial memory. To achieve this we attempted to identify a novel link between two signalling systems known to regulate neurogenesis and assess what this link may mean for regulation of neurogenesis by aerobic exercise and the effects that ageing has on neurogenesis. The thesis maintained a focus on how hippocampal neurogenesis may influence spatial memory.

Identifying the signalling mechanisms responsible for regulating adult neurogenesis at the cellular level is of great importance. Determining the precise signalling pathways that induce NSCs in the adult hippocampus to divide, differentiate and mature into functional neurons will allow manipulation of neurogenesis at many different stages of the process and the ability to manipulate adult neurogenesis offers massive therapeutic potential. Neurodegenerative diseases are usually accompanied by altered, mainly reduced, adult hippocampal neurogenesis. Animal models of Alzheimer's disease, Huntington's disease and Parkinson's disease usually exhibit a decreased rate of neural precursor cell survival and a decrease in the number of immature neurons in the dentate gyrus of the hippocampus (Winner *et al.*, 2011). These neurodegenerative diseases are complex and the contribution of decreased neurogenesis in the dentate gyrus to the progression of the disease is not clear, however, restoring neurogenesis to its homeostatic level may aid to combat these pathologies. For example, environmental enrichment reverses deficits in

spatial learning in a mouse model of Alzheimer's disease and the authors attribute this to the fact that enrichment reverses deficits in adult neurogenesis exhibited in this mouse model (Valero *et al.*, 2011). Therefore, a full understanding of the signalling mechanisms regulating neurogenesis can lead to development of therapies that can correct dysfunctional neurogenesis in certain disease states.

Results from the present study have helped with the progression of our understanding of the signalling pathways regulating neurogenesis in the dentate gyrus. Both endocannabinoid signalling and neurotrophin signalling have been shown to regulate adult neurogenesis. Endocannabinoids modulate proliferation, differentiation and survival of NSC/NPCs (Compagnucci *et al.*, 2013; Gao *et al.*, 2010), similarly neurotrophins have also been shown to be important in regulating these cellular processes (Frielingsdorf *et al.*, 2007; Quadrato *et al.*, 2012; Sairanen *et al.*, 2005). This thesis shows for the first time that endocannabinoids can regulate the expression of neurotrophins. Specifically, our results suggest that 2-AG regulates NGF expression in the dentate gyrus and the hippocampus proper, expression of BDNF in the dentate gyrus is also regulated by 2-AG, while AEA appears to induce BDNF expression in the hippocampus proper. Results from *in vitro* experiments show that this increase in neurotrophin expression is due to cannabinoid-stimulated neuronal expression of BDNF and NGF, however, the involvement of other cell types has not been ruled out. For example, astrocytes express the CB_1 receptor (Han *et al.*, 2012) and can release neurotrophins (Kajitani *et al.*, 2012). This discovery could have huge implications for how we interpret the physiological effects of the endocannabinoid system, not only in the context of neurogenesis but also in many other phenomena including development, inflammation, behaviour and neurotransmission. For example, BDNF has been shown to induce endocannabinoid release in the neocortex (Lemtiri-Chlieh *et al.*, 2010). When the results from this thesis and those from the study by Lemtiri-Chlieh and colleagues are considered it is possible that a novel positive feedback loop has been identified; BDNF induces cells to release endocannabinoids and these released endocannabinoids can act to increase BDNF expression, which leads to further endocannabinoid release (Figure 6.1). There is also the potential of a NGF-endocannabinoid positive feedback loop as NGF has been shown to increase 2-AG levels in culture (Keimpema *et al.*, 2013). Although further investigation is required to decipher the precise dynamics of these hypothesised positive feedback loops, data from this thesis have potentially identified their existence.

The identification of this link between the endocannabinoid system and neurotrophin expression may also help to explain and further our understanding of how exercise affects

the body. Aerobic exercise is hugely beneficial to many physiological systems, including the cardiovascular system, the immune system and general metabolic function. Exercise also has profoundly positive effects on brain function; these effects are thought to be mediated, at least in part, by the fact that exercise increases growth factor expression in the brain. The beneficial effects exercise has on hippocampal function have been attributed to increased BDNF expression (Griffin *et al.*, 2009; O'Callaghan *et al.*, 2009; Vaynman *et al.*, 2004), however, the source of this BDNF and the mechanism by which exercise increases BDNF expression in the hippocampus remains contentious. Recently, the release of the protein irisin from skeletal muscle during exercise has been linked to increased hippocampal BDNF gene expression (Wrann *et al.*, 2013), however, a definitive mechanism remains to be identified. Exercise has been shown to induce CB_1 gene expression in the hippocampus, increase hippocampal AEA concentration as well as CB_1 binding site density and activity (Hill *et al.*, 2010; Wolf *et al.*, 2010). Considering findings from these two studies and the fact that the data in Chapter 3 of this thesis establishes a link between hippocampal endocannabinoids and BDNF expression it is a strong possibility that increased hippocampal BDNF following exercise is mediated by enhanced hippocampal endocannabinoid signalling. One of the aims of this thesis was to investigate this link by administering a CB_1 antagonist during exercise. Unfortunately the present study failed to comprehensively test this hypothesis, due to the fact that in order to incorporate other experiments BDNF expression analysis was carried out outside of a time window appropriate to observe exercise-induced increase of BDNF expression. However, this does not rule out the possibility of the endocannabinoid system mediating exercise and BDNF expression. Although the study failed to test this hypothesis it did produce an interesting result concerning the persistence of the effect of exercise on BDNF expression. When considered with findings from other studies using the same exercise protocol (Bechara *et al.*, 2013b; Griffin *et al.*, 2009) it shows that the BDNF increase is transient and returns to baseline 24-72 h post exercise. This is something that should be considered in the planning of future studies, particularly studies using exercise as an intervention to manipulate brain function in a neurotrophin-related mechanism.

Due to the timepoint confound described above, this thesis failed to show an interaction between exercise and endocannabinoid signalling with respect to BDNF expression, but it did show interactions at other levels, namely at the cognitive level. Data from the short-term study in Chapter 4 suggest that a week of CB_1 antagonist treatment or a week of exercise alone has no effect on short-term spatial memory; however, a combination of these interventions enhances spatial memory. This is an interesting result on two levels. Firstly, the fact that exercise alone has no effect on spatial memory is interesting when

others report that the same exercise protocol enhances spatial memory using the same task (Bechara *et al.*, 2013b; Griffin *et al.*, 2009). This discrepancy is likely due to the fact these studies subjected rats to the learning portion of the task on the same day as the last bout of exercise and the present study performs the learning period of the task 24 h post exercise. This suggests that the cognitive enhancing effects of exercise, with respect to short-term memory, are short-lived and that in order to utilise exercise as a cognitive enhancing intervention regular daily exercise is likely to be required. The second interesting finding from this experiment was that blocking CB_1 receptor activity during exercise enhanced spatial memory. This seems to suggest that a CB_1 receptor inverse agonist prolongs the cognitive enhancing ability of exercise on short-term memory. This is an extremely novel finding and further work would need to be carried out to delineate the neurophysiological mechanism responsible for this, although results from this thesis suggest it is independent of synaptogenesis in the dentate gyrus and the hippocampus proper.

In addition to identifying a link between exercise and endocannabinoid signalling at the cognitive level we also report an interaction at the cellular level. Exercise enhances cell proliferation and early-stage survival in the dentate gyrus and Chapter 4 shows that this enhancement is dependent on endocannabinoid signalling through the CB_1 receptor. Other studies report that increased cell proliferation in the dentate gyrus during exercise requires the activation of the CB_1 receptor (Hill *et al.*, 2010; Wolf *et al.*, 2010). However, importantly this thesis shows for the first time that this proliferation effect translates to early-stage survival, here defined as 3-7 days post-birth. The significance of assessing early survival is the fact that 3-7 days of age is a critical period in the life of a NPC. Spatial learning promotes the survival and integration of cells >3 days old at the onset of long-term spatial learning paradigm whereas apoptosis is initiated in cells <3 days old (Dupret *et al.*, 2007a). Therefore, although Hill *et al.,* and Wolf *et al.,* show an effect of CB_1 antagonism on exercise-induced cell proliferation this thesis has taken this result one step further and has shown that this interaction is observed beyond proliferation to survival and may have implications for learning. Taken together, this prompted the formulation of the hypothesis that the exercise protocol used in the present study would enhance long-term spatial memory when the learning/memory encoding phase of the task took place over a long period of time post-exercise when the cells born during exercise were >3 days old. As the CB_1 antagonist prevented exercise from increasing the number of cells at this timepoint it was hypothesised that the CB_1 antagonist would prevent exercise from enhancing long-term memory. This hypothesis was tested in the long-term study in Chapter 4. This study produced very interesting findings that could have

significance for the field of neurogenesis. Exercise enhanced long-term spatial memory and although a CB_1 receptor antagonist prevented exercise from increasing cell proliferation/early survival in the dentate gyrus it did not alter the exercise enhancement of long-term memory. As discussed in Chapter 4 it was not possible to quantify the long-term survival of the cells generated during exercise using a full data-set, however, the survival data appears to mirror the proliferation/early survival data, at least with respect to the effects of exercise with or without CB_1 receptor antagonist. Taking all of this together, what we show here is that the mechanism responsible for exercise enhancing long-term spatial memory is likely not to be adult hippocampal neurogenesis, suggesting that neurogenesis may not important for spatial learning. This is a controversial statement that conflicts much of the literature, however, recently published studies also present evidence in support of this statement. Attenuation of neurogenesis using conditional knockdown of ERK5 had no effect on spatial learning in the Morris water maze over an 8-day period (Pan *et al.*, 2012). Similarly, almost complete ablation (a 98% reduction) of neurogenesis using a novel pharmacogenetic technique in which the proliferation of viral enzyme expressing neural stem cells in transgenic rats is specifically knocked down by antiviral drugs had no effect on Morris water maze learning over a 3 day period (Groves *et al.*, 2013). Pan and colleagues report an impairment in spatial reversal learning in the water maze whereas Groves and colleagues do not, however this is possibly due to the fact that the former study uses a 7-day reversal-learning period while the latter opts for a 1-day period. Taking these studies and the results from this thesis into consideration it appears that adult hippocampal neurogenesis is not important for standard long-term spatial learning but has a role to play in long-term spatial reversal learning. Therefore, although slightly controversial, findings from this thesis along with two recently published studies have brought us closer to answering the long unanswered question; 'What is the function of adult hippocampal neurogenesis?'

Results from the ageing study in this thesis could potentially be interpreted as further evidence of neurogenesis having no role to play in standard spatial learning. The aged group in this study have a decreased rate of proliferation and decrease in cell survival in the dentate gyrus. This is something that is not observed in the middle-aged group, however, both middle-aged and aged groups have impaired spatial memory. One could conclude from this that decreased neurogenesis is not responsible for age-related decline in spatial memory. Although it should be noted that short-term spatial memory was assessed in this experiment, with learning occurring on 1 day and recall being tested the following day. As mentioned above learning promotes cell survival/integration over a period of days and therefore it is unlikely that encoding of a memory over a short period

of time, such as in this experiment, would directly involve neurogenesis. Independent of the role of neurogenesis in the type of memory assessed in this study some significant results were observed. It is well known that aged animals have impaired spatial memory, however, there are some discrepancies in the literature with regard to the age of onset of this impairment. Although there has been limited study into the effects of middle-age on memory, one study reports no effect (Benice *et al.*, 2006) whereas another study reports an impairment at middle-age (Wiescholleck *et al.*, 2013). The present study may help to resolve this discrepancy as it shows that spatial memory is impaired from middle age and persists to old age. This suggests that the detrimental effects that ageing has on hippocampal function begins at middle age. This is something that should be considered when treatments for age-related cognitive decline are being developed.

An interesting finding that resulted from this study is the novel finding that although BDNF mRNA is decreased in the middle-aged and aged dentate gyrus this does not translate to a decrease in protein expression, in fact it is actually accompanied by an increase in protein expression in the aged dentate gyrus. A possible mechanism responsible for this is an age-related change in the rate of BDNF mRNA-protein translation and is outlined in section 5.4. This result is also interesting on another level; a reoccurring theme referred to throughout this thesis is the cognitive-enhancing effects of neurotrophins, particularly BDNF, and in this study although aged rats exhibit a dramatically increased level of BDNF in the dentate gyrus they also exhibit impaired spatial memory and reduced cell proliferation/early survival. This suggests that irreversible damage to the dentate gyrus occurs during age and that even a dramatic increase in BDNF is incapable of combating this. This would also fit in with current theory that astrogliosis (i.e. increase in the number of astrocytes induced by neuronal damage) can occur in the aged hippocampus due to hippocampal damage resulting from a persistent inflammatory state (Lynch *et al.*, 2010) and, it is possible that increased BDNF observed in age animals is due to an increase in the density of astrocytes actively secreting the protein.

Another finding of note from this thesis is the inter-study variation of BDNF concentration in brain (dentate gyrus and hippocampus) and serum. In particular the BDNF concentration in all tissues analysed in the URB 602 experiment is lower than the URB 597 study, while the BDNF concentration in the dentate gyrus in the Exercise-AM 251 experiment is considerably higher than URB 597, URB 602 and Young, Middle-aged, Aged experiments (Table 6.1). Although it is difficult to identify the precise explanation for these variations it is likely that the different experimental protocols

employed, particularly in relation to drug administration, handling, treadmill exposure and behavioural testing are responsible. For example, all animals in the Exercise-AM 251 were subjected to extensive handling due to twice daily i.p. injections and daily treadmill exposure for one week, in addition to two days of behavioural testing. It has been shown that treadmill exposure (O'Callaghan *et al.*, 2009) and learning in hippocampal-dependent behavioural tasks (Hall *et al.*, 2000) increase BDNF expression in the brain while handling neonatal rats (Garoflos *et al.*, 2005) has also been shown to increase hippocampal BDNF, therefore, increased dentate gyrus BDNF concentration in this experiment may be due to one, or a combination of these factors. The lower BDNF concentration in all tissues analysed in the URB 602 experiment in comparison to the URB 597 experiment could potentially be due to the temporal effect of the potentially stressful injection procedure on BDNF expression. Tissue was collected 5 h post URB 602 (or vehicle) injection while tissue was collected 3 h post URB 597 (or vehicle) injection raising the possibility that the injection procedure and/or the vehicle solution may have temporal effects on BDNF expression. Further investigation would need to be carried out to test this hypothesis, however the fact that acute stress has been shown to increase hippocampal BDNF expression (Uysal *et al.*, 2012) shows that acute stressful situations can interact with BDNF expression.

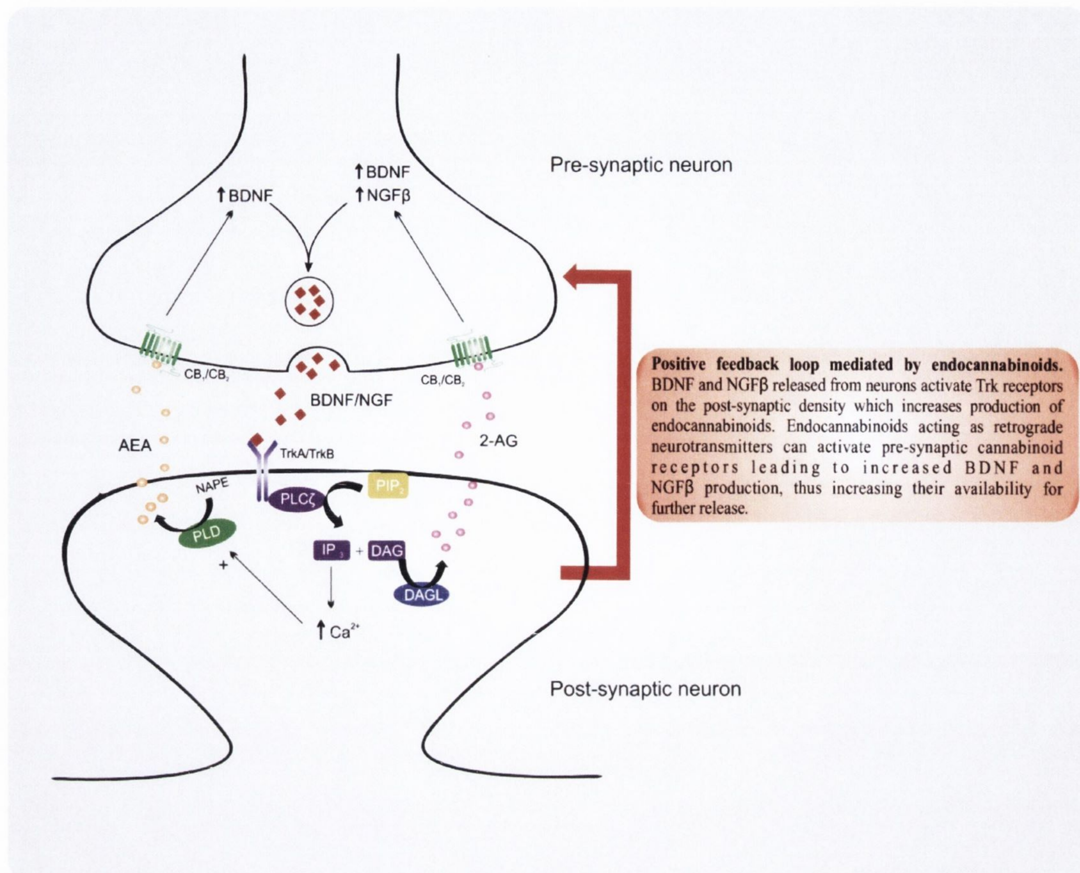

Figure 6.1 Possible interactions between endocannabinoid signalling and neurotrophin signalling in the neuron

This figure illustrates possible interactions between the endocannbinoid system and neurotrophin signalling using a neuronal synapse as an example. The figure also describes a positive feedback loop linking neurotrophin release to increased neurotrophin expression mediated by the endocannabinoid system. This positive feedback loop is hypothesised to exist due to findings from this thesis identifying the endocannabinoids AEA and 2-AG as potential regulators of BDNF and NGFβ expression.

Tissue	Group	BDNF (pg.mg protein^{-1})
URB 597 experiment		
Dentate gyrus	Vehicle	1451.0±203.0
	URB 597	1706.0±485.5
Hippocampus	Vehicle	625.4±82.48
	URB 597	817.0±54.91
Serum	Vehicle	4136.0±287.0
	URB 597	3962.0±318.6
URB 602 experiment		
Dentate gyrus	Vehicle	217.7±26.2
	URB 602	368.0±30.4
Hippocampus	Vehicle	209.1±13.4
	URB 602	241.9±17.3
Serum	Vehicle	1757.0±140.4
	URB 602	1769.0±210.8
Exercise-AM 251 experiment		
Dentate gyrus	Sedentary-vehicle	4773.0±715.3
	Sedentary-AM 251	4820.0±1058.0
	Exercise-vehicle	4255.0±312.4
	Execise-AM 251	4820.0±1058.0
Hippocampus	Sedentary-vehicle	892.0±168.6
	Sedentary-AM 251	1082.0±160.3
	Exercise-vehicle	1222.0±179.7
	Execise-AM 251	843.0±180.9
Serum	Sedentary-vehicle	884.5±78.2
	Sedentary-AM 251	862.5±79.2
	Exercise-vehicle	882.5±71.5
	Execise-AM 251	766.0±51.5
Young, Middle-aged, Aged experiment		
Dentate gyrus	Young	802.1±75.56
	Middle-aged	575.2±99.5
	Aged	1365.0±158.9
Hippocampus	Young	1280.0±131.0
	Middle-aged	1132.0±230.6
	Aged	1276.0±113.5

Table 6.1 Comparison of BDNF concentrations across experiments

The endocannabinoid system is a complex signalling system that has widespread physiological effects. The exact mechanisms by which endocannabinoids exert their effects are not fully understood and this thesis identifies a novel interaction between the endocannabinoid system and neurotrophin expression. The identification of this novel interaction could help further our knowledge of the functioning of the endocannabinoid system and potentially identify novel cannabinoid-based therapies. Exercise is well known to modulate neurotrophin expression and the endocannabinoid system appears to have a role to play in the neurophysiological effects of exercise. Endocannabinoid activation of the CB_1 receptor is required for activity-dependent enhancement of neurogenesis. Recently some research groups have suggested that adult neurogenesis may not be important for spatial learning, a hypothesis that is supported by this thesis. Ageing is associated with impaired spatial memory, something that is observed here from the onset of middle age. The contribution of neurogenesis, neurotrophin signalling and the endocannabinoid system to this age-related cognitive decline is unclear and warrants further investigation.

6.2 Future directions

Chapter 3

A novel link between the endocannabinoid system and neurotrophin signalling has been identified here. Specifically, we have shown that endocannabinoids can induce expression of BDNF and NGF in the hippocampus proper and the dentate gyrus. Results from *in vitro* experiments suggest the source of these neurotrophins is likely to be neuronal, however, other cell types have not been examined. Further experiments would need to be carried out to assess the glial contribution to this. In addition to this, the receptor(s) and intracellular signalling cascade responsible activated by endocannabinoids that lead to BDNF and NGF gene expression have not been explored. A study using antagonists specific to cannabinoid receptor subtypes is required to determine the precise signalling mechanisms linking cannabinoids to neurotrophin gene expression.

Chapter 4

This chapter shows that the endocannabinoid system can interact with exercise on both a cognitive and cellular level. Exercise in combination with CB_1 antagonism enhances spatial memory. The mechanism underlying this has yet to be identified. The data suggests BDNF signalling and synaptogenesis are not responsible for this enhancement but further experimentation would be required to test other possible mechanisms, for example synaptic plasticity.

Data from the short-term and long-term studies in this chapter show that exercise enhances long-term spatial memory irrespective of its effects on cell proliferation/early survival, and possibly net neurogenesis. Exercise, therefore, must induce another long-term neurophysiological change that facilities spatial learning over a long period of time but does not effect short-term spatial learning, one possibility is changes in the neuronal expression of proteins involved in learning such as ERK and CaMKII could occur post-exercise. Future experiments should focus on identifying this neurophysiological change, again the effects of these exercise/drug interventions have on synaptic plasticity should be explored.

Short-term spatial memory is impaired with age and this impairment is observed from middle age. This deterioration in spatial memory is independent of a decrease in cell proliferation/early survival and neurotrophin expression. Future studies should focus on identifying the potential neurophysiological mechanism. Investigating the effects of ageing on synaptogenesis and shorter forms of synaptic plasticity may help to explain the observed effects of middle-age and age on memory. Further investigation should also be carried out to identify the source and the physiological significance of the age-related increase in BDNF expression.

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Appendix

Materials

Animals

Wistar rats (1 day old, male or female)	Bio Resources Unit, TCD
Wistar rats (3, 15 or 24 month old, males)	Bio Resources Unit, TCD
Irradiated laboratory animal diet	Harlan™, Madison, WI, USA

Animal and cell treatments

AM 251 [*N*-(Piperdin-1-yl)-5-(4-idophenyl)-1-(2,4 dichlorophenyl)-4-methyl-1 H-pyrazole-3-carboxamide	Tocris Cookson Ltd., Bristol, UK
URB 597 [3'-(aminocarbonyl)[1,1'-biphenyl]-3-yl)-cyclohexylcarbamate]	Enzo Life Sciences, UK
URB 602 [1,1'-(biphenyl)-3-yl-carbamic acid, cyclohexly ester]	Sigma, Wicklow, Ireland

Cell culture solutions

Dulbecco's modified Eagle's medium (DMEM)	Invitrogen, UK
Glutamax	Gibco, BRL, UK
Heat inactivated horse serum	Gibco, BRL, UK
Neurobasal medium (NBM)	Invitrogen, UK
Penicillin/streptomycin	Gibco, BRL, UK
Trypsin	Gibco, BRL, UK

ELISA kits

Human BDNF Duoset® ELISA kit	R&D Systems Europe, Oxon, UK

Rat NGFβ Duoset® ELISA kit	R&D Systems Europe, Oxon, UK
Substrate Solution	R&D Systems Europe, Oxon, UK

General laboratory chemicals

Acryl amide electrophoresis reagent	Sigma, Wicklow, Ireland
Ammonium persulphate	Sigma, Wicklow, Ireland
Aprotinin	Sigma, Wicklow, Ireland
Bio-Rad dye reagent concentrate	Bio-Rad, California, USA
Bovine serum albumin (BSA)	Sigma, Wicklow, Ireland
Bromopehnol blue sodium salt	Sigma, Wicklow, Ireland
Calcium chloride ($CaCl_2$)	Lennox, Dublin, Ireland
Dimethyl sulphoxide (DMSO)	Sigma, Wicklow, Ireland
Di-Sodium hydrogen orthophosphate (Na_2HPO_4)	Sigma, Wicklow, Ireland
Ethanol	Sigma, Wicklow, Ireland
Glucose	Lennox, Dublin, Ireland
Glycerol	Sigma, Wicklow, Ireland
Glycine	Sigma, Wicklow, Ireland
Hydrochloric acid (HCl)	Lennox, Dublin, Ireland
Hydrogen peroxide (H_2O_2)	Sigma, Wicklow, Ireland
Leupeptin	Sigma, Wicklow, Ireland
Magnesium sulphate ($MgSO_4$)	Sigma, Wicklow, Ireland
Magnesium Chloride ($MgCl_2$)	Sigma, Wicklow, Ireland
β-Mercaptoethanol	Sigma, Wicklow, Ireland
Methanol (MeOH)	Sigma, Wicklow, Ireland

N, N' – Methylenebisacrylamide	Sigma, Wicklow, Ireland
Nitrocellulose membrane	Amersham Bioscience, Stockholm, Sweden
NP-40	Sigma, Wicklow, Ireland
Potassium chloride (KCl)	Sigma, Wicklow, Ireland
Potassium dihydrogen orthophosphate (KH_2PO_4)	Sigma, Wicklow, Ireland
Potassium hydroxide (KOH)	Sigma, Wicklow, Ireland
Potassium phosphate (KH_2PO_4)	Sigma, Wicklow, Ireland
2-Propanol	Sigma, Wicklow, Ireland
Sodium carbonate (Na_2CO_3)	Sigma, Wicklow, Ireland
Sodium bicarbonate ($NaHCO_3$)	Sigma, Wicklow, Ireland
Sodium chloride (NaCl)	Sigma, Wicklow, Ireland
Sodium dodecylsulphate (SDS)	Sigma, Wicklow, Ireland
Sodium hydrogen carbonate ($NaHCO_3$)	Sigma, Wicklow, Ireland
Sodium hydroxide (NaOH)	Lennox, Dublin, Ireland
Sodium orthovanadate (Na_3VO_4)	Sigma, Wicklow, Ireland
Sodium phosphate, monobasic (NaH_2PO_4)	Sigma, Wicklow, Ireland
Sodium phosphate, dibasic (Na_2HPO_4)	Sigma, Wicklow, Ireland
3,3', 5,5'-Tetramethylbenzidine (TMB)	Sigma, Wicklow, Ireland
N, N, N', N'-Tetramethylethylene-diamine (TEMED)	Sigma, Wicklow, Ireland
Tris-base	Sigma, Wicklow, Ireland
Tris-HCl	Sigma, Wicklow, Ireland
Tween®-20	Sigma, Wicklow, Ireland

General laboratory products and plastics

Biosphere filter pipette tips	Sarstedt, Nümbrecht, Germany
Cork discs	R.A. Lamb Ltd., Sussex, UK
Falcon tubes (15ml, 50ml)	Sarstedt, Nümbrecht, Germany
Microtest 96-well flat bottomed plates	Sarstedt, Nümbrecht, Germany
Microtubes (0.5ml, 1.5ml)	Sarstedt, Nümbrecht, Germany
96 Microwell Nunc ELISA plates	Nunc, Roskilde, Denmark
Needles 26G/21G	BD Microlance, Oxford, UK
Optical adhesive covers	Applied Biosystems, Warrington, UK
96-well optical reaction plates	Applied Biosystems, Warrington, UK
Parafilm	Lennox, Dublin, Ireland
PCR tubes	Sarstedt, Nümbrecht, Germany
Pipette tips	Sarstedt, Nümbrecht, Germany
Plastic transfer pipettes	Sarstedt, Nümbrecht, Germany
Scalpels (disposable)	Swann-Morton, Sheffield, UK
Standard grade No.1 filter paper	Whatman Ltd., UK
Standard grade No.3 filter paper	Whatman Ltd., UK
Sterile syringes (1ml, 2ml, 10ml, 50ml)	BD Plastpak, Oxford, UK
Syringe-drive filter unit	Millipore, Chemicon, Cork, Ireland

Immunohistochemistry

5-bromo-2'-deoxyuridine (BrdU)	Sigma, Wicklow, Ireland

Chicken anti-BrdU monoclonal IgG	Abcam, Cambridge, UK
Rabbit anti-chicken IgG	Abcam, Cambridge, UK
DAB Chromagen tablets	Dako Diagnostics Ltd., Ireland
DPX mountant	Sigma, Wicklow, Ireland
Normal rabbit serum	Vector Laboratories Ltd., Peterborough, UK
VECTASTAIN ABC kit standard	Vector Laboratories Ltd., Peterborough, UK

Western Immunoblotting reagents and antibodies and reagents

Anti-mouse (goat) IgG peroxidase conjugate	Sigma, Wicklow, Ireland
Anti-rabbit (goat) IgG peroxidase conjugate	Sigma, Wicklow, Ireland
β-Actin mouse monoclonal IgG	Sigma, Wicklow, Ireland
Rabbit anti- Synapsin IgG	Cell Signaling Technology®, Massachusetts, USA
Mouse anti- GAPDH	Abcam, Cambridge, UK
Rabbit Anti-CB_1 Polyclonal IgG	Abcam, Cambridge, UK
Rabbit Anti- PSD-95, Polyclonal IgG	Cell Signaling Technology®, Massachusetts, USA
Hybond-C extra nitrocellulose membrane	Amersham Biosciences, UK
Precision Plus Protein Standards (Dual Colour)	Bio-Rad Laboratories, California, USA

ReBlot Plus strong antibody stripping solution	Millipore, Cork, Ireland
SuperSignal® chemiluminescence	Pierce, Rockford, USA
MagicMark™ XP Western Protein Standard	Invitrogen, Bio-sciences, Dublin, Ireland

Molecular reagents

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

Solutions Used

Electrode Running Buffer

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

Krebs Solution

NaCl	136mM
$NaHCO_3$	16mM
KCl	2.54mM
KH_2PO_4	1.18mM
$MgSO_4.7H_2O$	1.18mM
Glucose	10mM
Containing $CaCl_2$	2mM
Distilled water	

Lysis buffer (pH 7.4)

NP-40	1% (v/v)
Tris base	20mM
NaCl	137mM
Glycerol	10% (v/v)
EDTA	2mM

Activated Sodium Orthovanadate (Na_3VO_4)	1mM
Aprotinin	
Leupeptin	
Distilled water	

Phosphate Buffered Saline (PBS, pH 7.4)

NaCl	100mM
Na_2HPO_4	80mM
NaH_2PO_4	20mM
Distilled water	

PBS-Tween (PBS-T)

0.1% Tween-20 solution in PBS

Sample Buffer

Tris-HCl	(pH 6.8)	0.5mM
Glycerol		10% (v/v)
SDS		0.05% (w/v)
2-β-mercaptoethanol		5% (v/v)
Bromophenol blue		0.05% (w/v)

Separating Gel 10%

Bisacrylamide		33% (w/v)
Tris-HCl	(pH 8.8)	1.5M
SDS		1% (w/v)
Ammonium persulphate		0.5% (w/v)
TEMED		0.1% (v/v)
Distilled water		

Stacking Gel 4%

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

Transfer Buffer (pH 8.3)

Tris-base	25mM
Glycine	192mM
Methanol	20% (v/v)
SDS	0.05% (w/v)

Distilled water

TBS-Tween wash buffer

Tris-HCl (pH 7.5)	20mM
NaCl	150mM
Tween-20	0.05% (v/v)
Distilled water	