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The role of exercise and NMNAT1 on functional and behavioural aspects of brain ageing in the mouse.

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

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Declaration

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically stated in the text. The work presented here is not substantially the same as any I have submitted for a degree or diploma or other qualification at any other University.

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List of abbreviations used

AD	Alzheimer's disease
BDNF	Brain-derived neurotrophic factor
BrdU	Bromodeoxyuridine
DAB	3,3'-diaminobenzidine
Dcx	Doublecortin
DG	Dentate Gyrus
DMNT	DNA methyltransferase
dNTP	deoxyribose nucleoside triphosphates
DPX	Distyrene, plasticizer and xylene mix
EDTA	Ethylenediaminetetraacetic acid
EE	Environmental enrichment
EPM	Elevated plus maze
HAT	Histone acetyltransferase enzyme
Het	Heterozygous knockout NMNAT1
IP	Intraperitoneal
miRNA	micro-RNA
mRNA	messenger RNA
NAD	Nicotinamide adenine dinucleotide
NaMN	Nicotinic acid mononucleotide
NAMPT	Nicotinamide phosphoribosyltransferase
ncRNA	non-coding RNA
NMNAT	nicotinic mononucleotide adenylyltransferase
NOR	Novel object recognition

NPC	Neural progenitor/stem cell
OF	Open field
PARP1	Poly [ADP-ribose] polymerase 1
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
qPCR	Quantitative polymerase chain reaction
SA	Spontaneous alternation/Y maze
SAP	Stretch attenuated postures
siRNA	short-interfering RNA
SIRT1	Sirtuin 1
SVZ	Sub-ventricular zone
TET	Ten-eleven translocation enzyme
TG	Transgenic overexpressing NMNAT1
WT	Wildtype

Abstract

Ageing is a complex process that occurs in every living organism and is the result of interactions between genetics and the environment. As we age, the risk of developing a number of diseases like Alzheimer's disease, cardiovascular disease and cancer increases significantly. However, not everyone will develop any of these illnesses, and may instead undergo 'healthy ageing', a process broadly defined in humans as "ageing in the absence of illness". Healthy ageing can be promoted by, amongst other things, regular exercise and caloric restriction. Previous research has shown that caloric restriction can delay the onset of the negative impacts of ageing, and also extend maximum lifespan. These effects are regulated via the activity of the NAD^+ dependent deacetylase SIRT1, and overexpression of SIRT1 can have the same effect as caloric restriction. Exercise also promotes improved health with ageing, thought the underlying mechanisms are not fully understood.

Here, we aimed to investigate the impact of long-term exercise in an ageing mouse model, and how changing the expression of the NAD^+ biosynthetic enzyme NMNAT1 could impact on ageing in both sedentary and voluntary exercise conditions. For this we compared two colonies of mice: transgenic overexpression of NMNAT1 (TG) and heterozygous knockout of NMNAT1 (Het). We also included the wildtype (WT) littermates from both colonies. We focused on four key areas that are known to be affected in ageing: behaviour, adult neurogenesis, miRNAs as blood biomarkers of healthy ageing and hippocampal gene expression.

In order to ensure that our behavioural results were ecologically relevant, we first determined how the phase of the light cycle under which behavioural tests are performed can alter the behaviour outcomes in mice

that had access to a running wheel. Within the literature there is conflicting data on how exercise affects behaviour in a mouse, mainly focused on the effect on anxiety. Half of the mice were given access to a running wheel for one month before the behavioural tests. At the end they all underwent a series of behavioural tests, either under white light or in the dark. When the tests were performed in the light it appears as though the mice that exercise go into the open areas of the elevated plus maze less which can be interpreted as the mice being more anxious. However, when the same test was performed in the dark this did not happen. We also found that all mice showed increased activity in all of the tests when under dark conditions, as mice are nocturnal and are more active at night. From these results we decided to perform all other behavioural tests in the dark phase of the light/dark cycle.

Afterwards, we focused on the impact of exercise and NMNAT1 genotype on ageing. Male mice of all three genotypes (WT, Het and TG) grew to 18 months of age under group housed conditions. At both 11 and 18 months of age, all of the mice underwent a series of behavioural tests for cognition, anxiety and locomotor activity. From 12 to 18 months of age, the mice either had access to a running wheel or remained sedentary but with a broken wheel to control for environmental enrichment. All mice received an injection of BrdU at 17 months of age. We also had a group of young (2-month old) male and female mice from all three genotypes, and we compared the levels of adult neurogenesis in the young and old mice.

In WT mice, exercise tended to increase/maintain cognitive ability with age. In the brains of these mice, we found an increase in the number of new cells in the brain becoming neurons and surviving long enough to mature and potentially integrate into the neuronal network. Exercise also increased the expression of miR-21-5p in the blood plasma of WT mice, a

miRNA that has previously been shown to have a role in the regulation of neural stem/progenitor cell (NPC) proliferation in the adult brain.

When we compared the levels of adult neurogenesis in the young and aged mice of all three genotypes, we saw a significant decrease with age in all groups. This comparison has not been done in Het and TG mice before, and we saw some slight variations between the different genotypes with age.

When comparing adult neurogenesis in the WT and Het mice, we saw a significant increase in the NPC proliferation and immature neuron numbers in the Het mice. However, exercise reduced the number of proliferating cells in the Het mice.

In the brains of TG mice, we saw an increase in the number of new cells becoming immature neurons and the number of cells surviving long enough to mature. Exercise had no significant effect on adult neurogenesis in TG mice. We saw a significant increase in miR-21-5p in the TG mice in comparison to the WT mice, similar to the increase seen with exercise in the WT mice. The expression of miR-92a-5p and miR-21a-3p was significantly decreased in TG mice that had exercised. Both of these miRNAs have been previously linked with regulating adult neurogenesis. Finally, we saw a significant increase in the expression of BDNF in the hippocampus of TG mice that had exercised.

We saw no significant changes with exercise or genotype in the hippocampal expression of NMNAT2, SIRT1, NAMPT, Ezh2 or Utx.

Overall, we concluded that prolonged voluntary exercise has a significant impact on adult neurogenesis and circulating miRNA expression in aged mice but does not significantly affect the behaviour of the mice or gene expression in the hippocampus. Changing the expression of NMNAT1 also

impacts adult neurogenesis and circulating miRNA expression in aged mice and can impact the effect that exercise has in aged mice.

Further research is required to fully understand the underlying mechanisms behind the changes we have found in this study, both with exercise and the change in NMNAT1 expression.

1. Chapter 1:

Introduction

1.1 The ageing process

Ageing is a process that occurs in every living organism and is the result of a complex set of interactions between genetics and the environment. The complete underlying mechanisms of ageing are not yet fully understood. In the brain, many structural and functional changes are known to occur with age, including reduced cognition, a reduction in brain volume and a significant decrease in levels of neurogenesis. There is an increased risk with age of developing diseases such as Alzheimer's disease (AD), Parkinson's disease, cancers, cardiovascular disease and osteoarthritis. It is not a guarantee though that an ageing subject will encounter any of these problems, indeed many people age successfully without significant health problems.

Healthy or successful ageing has been broadly defined in humans as ageing with an absence of illness or health related problems (Bowling and Dieppe, 2005). More detailed definitions vary depending on the point of view they are looking from, for example biomedical theories focus on the optimisation of life expectancy with minimal physical and mental deterioration (Depp, Glatt and Jeste, 2007). More lay approaches focus on social engagement and personal resources (Stubbs *et al.*, 2017). For the purposes of this study we have focused more on the biomedical definition of healthy ageing.

Healthy ageing can be promoted by a calorie restricted diet and regular exercise across the life span (Martin, Mattson and Maudsley, 2006; Fontana and Klein, 2007). Both have been shown to significantly improve quality of life and reduce the risk and severity of age-related neuropathies. It is important to understand the underlying molecular processes of healthy ageing and how exercise and caloric restriction affect the ageing process.

With a better understanding, improved treatments and care plans can be used on an ageing population, therefore improving quality of life for many.

1.2 The impact of ageing and exercise on behaviour

1.2.1 Ageing and cognitive decline

The levels of cognitive decline vary from person to person (Wilson *et al.*, 2002), some showing severe impairment whilst others show little to no cognitive decline at the same age or older (Stott, 2006). The underlying causes of this decline or the reasons for the variations in severity from person to person are still not fully understood.

Cognitive decline is known to occur gradually from early adulthood onwards, with a higher rate of decline in advanced ageing (Salthouse, 2009, 2010). It affects a range of functions in the brain, including memory, processing speed, executive function and reasoning. Age-related cognitive decline is different from age-related neuropathies like AD. Whilst they can have similar symptoms on occasion, the severity is significantly higher in the neuropathies compared to normal age-related cognitive decline, and the underlying causes are not the same.

In normal ageing, episodic and working memory seem to be affected, along with some types of short term memory. Both cross-sectional and longitudinal studies on cognitive decline have found a strong decline in the ability to form new episodic memories and process information quickly (Nilsson, 2003; Hedden and Gabrieli, 2004; Spaan, 2015). A decline in processing speed has been connected with a decline in episodic and working memory with age (Bopp and Verhaeghen, 2009). Slow processing

speed could be a significant contributor to the apparent decline in working and episodic memory (Salami *et al.*, 2012). Many of the symptoms of cognitive decline are associated with structural changes in various regions of the brain (Raz *et al.*, 2005; Kennedy and Raz, 2009). Processing speed seems to be affected by changes in white matter structure with ageing (Salami, 2012; Kuznetsova *et al.*, 2016).

Other cognitive abilities seem to be more resilient to the effects of normal ageing. Most people seem to retain their full vocabulary and verbal skills with age (Harada, Love and Triebel, 2013), though there is some reduction in visual confrontation naming (naming a common object when placed in front of you) beyond the age of 70 (Zec *et al.*, 2005). General knowledge levels also seem to remain relatively consistent with age.

1.2.2 The effect of exercise on cognition

There is a growing body of evidence suggesting that both acute and long-term cardiovascular exercise have a significantly positive affect on cognitive ability, anxiety and depressive symptoms in humans (Hillman, Erickson and Kramer, 2008) and rodents (Binder *et al.*, 2004; Duman *et al.*, 2008; Mul, 2018).

In children, a strong correlation between physical activity levels and cognitive performance in schools has been shown (Sibley and Etnier, 2003; Hillman, 2008). Trials in rodents have also shown significantly better results in cognitive tests such as the Morris water maze (van Praag, 2009; Mustroph *et al.*, 2012) and novel object recognition (O'Callaghan, Ohle and Kelly, 2007; Bechara and Kelly, 2013) after a period of exercise, usually 1-4 weeks of training or running wheel access, when compared to sedentary controls. A virtual Morris water task completed by humans with different levels of aerobic fitness, using a computer and a joystick to navigate,

showed similar results of improved memory with increased fitness (Herting and Nagel, 2012).

Environmental enrichment (EE) has been shown to have a strong impact on cognitive performance, especially in rodents, and exercise is a component of this. EE involves having a stimulating environment, and often for rodents this includes things like chew toys and tunnels placed in the cages for them to use (see Figure 1.1). In exercise studies, the inclusion of a running wheel counts as environmental enrichment, and not including the equivalent enrichment in the sedentary cages can have a significant effect on the experimental outcomes. This includes changes in neurogenesis (Olson *et al.*, 2006; Clemenson, Deng and Gage, 2015) and behaviour (Fox, Merali and Harrison, 2006; Simpson and Kelly, 2011). Exercise has been shown to have a stronger effect on cognition and neurogenesis than some forms of EE however (Olson, 2006).

Figure 1.1: Example of mouse cage with environmental enrichment factors

Photograph of a mouse cage with labelled environmental enrichment factors. These include free access to food and water, chew toys, a running wheel, multiple cardboard tubes and separate nesting materials for nesting behaviours.

Exercise is also known to have a significant effect on anxiety and depression levels in both humans and rodents. Jayakody et al (2014) performed a systematic review of eight randomised controlled trials on humans suffering with anxiety disorders and treated with exercise,

placebo, anxiety medication, cognitive behavioural therapy, or a combination of treatments. They found that exercise was an effective addition to treatment, but alone was less effective than medical treatment. Stubbs et al (2017) performed a similar systematic review on the benefits of exercise in anxiety related disorders, and also found exercise to be an effective treatment alongside medical treatment.

In rodents, there is some controversy over the effect exercise has on anxiety behaviours when studied using tests such as the elevated plus maze and open field. Some studies show similar findings to those in humans, where exercise decreases the anxiety behaviour (Binder, 2004; Duman, 2008; Santos-Soto *et al.*, 2013). Other studies have found the opposite to be true, with no change or increases in anxiety behaviour (Fuss, Ben Abdallah, Vogt, *et al.*, 2010; Nguyen, Killcross and Jenkins, 2013). There is evidence to suggest that housing conditions may be a contributing factor to these differences (Pan-Vazquez *et al.*, 2015), and part of our study looks into the effect of light cycle conditions on behavioural outcomes in mice.

Research has shown that one of the likely mechanisms by which the positive effects of exercise on the brain are mediated is by a brain-derived neurotrophic factor (BDNF) pathway (Cotman and Berchtold, 2002; Cotman and Engesser-Cesar, 2002). BDNF is a neurotrophic growth factor found in the brain and peripheral nervous system, contributing to cell survival, neuronal growth and differentiation. BDNF plays a significant role in neuronal survival and growth, as well as neuronal plasticity which is essential for learning and forming new memories (Bathina and Das, 2015). In young adults, the levels of peripheral BDNF have been shown to increase with bouts of acute (Griffin *et al.*, 2011; Roig *et al.*, 2013) and long term exercise (Huang *et al.*, 2014), which has correlated with

improvements in cognitive performance during testing and improved memory.

1.2.3 Impact of exercise on behaviour with ageing

Having seen that cognitive decline is significantly related with ageing, and that exercise has a significant positive effect on cognition and anxiety, we now need to see the effect of exercise on cognition in ageing.

In human studies on exercise and ageing, the most consistent finding is a rescue of executive functions with exercise (Colcombe and Kramer, 2003; Erickson and Kramer, 2009), though there is evidence for rescue of other cognitive functions affected by age-related decline with exercise (Smith *et al.*, 2011).

There are often a number of differences in the methods used by studies on exercise and ageing. This includes the type of exercise, what age the participants started exercising, how long they were exercising for, general lifestyle differences, gender splits and the types of analysis performed. Currently there is some debate over which type of exercise has the most benefits for rescuing cognitive decline. Many people support the hypothesis that a combination of mental stimulation and cardiovascular based exercise, such as dancing, is the most efficient at rescuing cognitive decline (Merom *et al.*, 2016).

Van Praag (2005) demonstrated that after 1 month of voluntary exercise aged mice showed faster learning and better retention of water maze tasks than their sedentary age matched controls. Similar improvements have been seen in other behaviour tests in rodents, such as the Y-maze, novel object recognition, learned helplessness and elevated plus maze

(Greenwood *et al.*, 2003; O'Callaghan, 2007; Van Praag, 2008; van Praag, 2009). Most of the rodent studies of ageing and exercise only use 1-3 months of exercise time, usually at the end of the experiment. The rest of the life of the rodent is spent sedentary. Longer term exercise experiments in rodents may show different results.

Our understanding of the underlying processes by which exercise influences cognition and behaviour is steadily advancing, but it is still not fully understood. A consistent finding within the literature is improved cognition correlating with increased levels of neurogenesis in the hippocampus of subjects that have exercised for a period of time.

1.3 The ageing brain

Across the lifespan, the brain undergoes a number of physical changes. The overall volume of the brain is known to slowly decrease over time, with brain atrophy rates markedly increasing around 70 years of age (Good and Johnsrude, 2002; Scahill *et al.*, 2003; Raz, 2005). A significant shrinkage in the volume of the grey matter has been commonly reported (Anderton, 2002; Taki *et al.*, 2004; Toescu, 2014), but it is unclear whether this loss is the primary cause of the overall volume reduction (Peters, 2006). Unlike in neurodegenerative disorders, this age-related loss of volume does not appear to be caused by increased widespread cell apoptosis in the brain; no evidence has been found for increased apoptosis with age in the brain (Pollack, Phaneuf and Dirks, 2002). Instead, it is believed that neurons in the brain undergo significant structural changes. These include reductions in dendritic branching and a reduction in the myelin sheaths, leading to an overall decrease in brain volume (Pannese, 2011). A decrease in hippocampal volume over time has been correlated with a loss in both verbal and non-verbal memory, suggesting a strong relationship between

episodic memory and hippocampal volume (Bonner-Jackson *et al.*, 2015). Schuff et al (2009) used MRI scans to measure the hippocampal volume of cognitively normal, mild cognitive impairment and AD patients over 12 months. Both the mild cognitive impairment and AD groups showed hippocampal volume loss at 6 months, with accelerated loss by 12 months. Monitoring hippocampal volume with age could be a strong early indicator of AD risk.

Exercise in aged subjects has been linked to a reduced risk or delay in the onset of age related neuropathies such as AD (Larson *et al.*, 2006; Lautenschlager *et al.*, 2008). The underlying molecular causes behind this are not yet known, though rodent models of AD, such as those with double mutant APP genes such as the TgCRND8 mice, have shown reductions in the deposition of extracellular amyloid-β plaques and improvements in behavioural learning with exercise (Paul. A. Adlard, Perreau and Cotman, 2005; Yuede *et al.*, 2009). These studies saw increases in the hippocampal volume, suggesting a potential increase in neurogenesis in the area.

Adult neurogenesis in the human hippocampus was first shown in a paper by Eriksson et al (1998). They treated human brain tissue with bromodeoxyuridine (BrdU) that labels DNA in S phase, and neuronal markers such as NeuN, and found clear evidence for cell division and new neurons forming.

The formation of new neurons in the brain, neurogenesis, decreases over time. The rate of neurogenesis is higher during the developmental stages and early life, before it declines throughout adulthood. It was once thought that adult neurogenesis did not exist, though nowadays it is widely accepted that neurogenesis occurs in the adult brain and persists throughout the lifespan, and there have been many studies showing evidence for this. However, recently the debate over adult neurogenesis re-

emerged with the study by Sorrells et al (2018) suggesting that neurogenesis drops to undetectable levels in the adult brain, and that "neurogenesis in the dentate gyrus does not continue, or is extremely rare, in adult humans". At the same time, a second study by Boldrini et al (2018) found similar numbers of intermediate neural progenitor cells in young and old humans, as well as a high number of immature neuronal cells in the hippocampus. They agree that there is a decline in neurogenesis in the adult brain but suggest that healthy older subjects with no age-related neuropathies display preserved neurogenesis. The reason for the significantly different outcomes of these two papers is not clear, though it is likely due to differences in staining techniques and analysis.

1.3.1 Neurogenesis

Neurogenesis describes the production and integration of new neurons into the brain. The neural tube is formed early in embryonic development and becomes the basis for the entire central nervous system. Some of the neural stem cells that make up this neural tube become radial glial cells during embryonic neurogenesis (Anthony *et al.*, 2004), and these produce the majority of the neurons that make up the networks of the brain. These neuronal cells must migrate long distances before they settle, mature and integrate into the new networks. The process of embryonic neurogenesis in the developing brain is significantly different to that in the adult brain (Urbán and Guillemot, 2014).

1.3.1.1 Adult neurogenesis

Adult neurogenesis is the formation of new neuronal cells in the fully developed brain from neural progenitor/stem cells (NPCs). In adult neurogenesis, these cells are restricted to three specific regions of the brain: the hypothalamus (not analysed in this study), the subgranular zone of the dentate gyrus (DG) within the hippocampus, and the subventricular zone (SVZ) of the striatum and hypothalamus (See Figure 1.2).

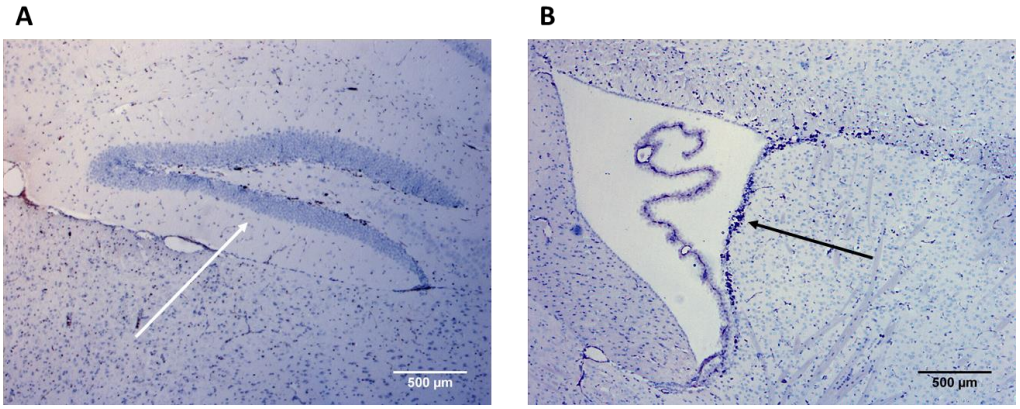

Figure 1.2: Example sagittal images of the DG and SVZ regions in the adult mouse brain

Images showing the location of adult neurogenesis within the mouse brain. Darker stained cells represent those which were actively proliferating. Blue colour is background staining from counterstaining with Harris Haematoxylin. A) The subgranular zone of the DG within the hippocampus. B) The SVZ of the striatum.

Images taken by author at the University of Nottingham

Neurogenesis in the SVZ primarily produces new neuronal cells which then migrate to the olfactory bulb (Alvarez-Buylla and García-Verdugo, 2002; Lim and Alvarez-buylla, 2016), which is especially important in rodents and other animals that rely strongly on their sense of smell. Neurogenesis that occurs within the DG of the hippocampus is associated with learning and integration of memories (Jessberger *et al.*, 2009).

Spalding et al (2013) measured the levels of ^{14}C (Carbon 14) in the genomic DNA of adult human hippocampal cells, and found that around 700 new cells are added to each hippocampus per day. This is comparable

to middle-aged mice, though the exchange rates of cells within the dentate gyrus is different between humans and mice.

Neurogenesis was believed to be limited to embryonic development until the early 1960s when pioneering research by Joseph Altman discovered the formation of new neurons in the brains of adult rats (Altman, 1962). He used intracranial injection of [H^3] thymidine to mark new cells appearing in damaged brain regions. Since that time, adult neurogenesis has been shown via immunostaining in a number of animals, including rodents (Gage *et al.*, 1998; Alvarez-Buylla, 2002; Clarke and Van Der Kooy, 2011), non-human primates (Gould, 2014) and humans (Spalding, 2013; Seib and Martin-Villalba, 2015). Neurogenesis levels in the adult brain decline with ageing (Gage, 1998; van Praag, Kempermann and Gage, 1999; Deary *et al.*, 2009) but it is widely agreed that it never fully stops (Seib, 2015). This decline has been attributed to the decrease in the number of actively proliferating NPCs in the aged brain.

The majority of NPCs within the adult brain are in a quiescent state, with a prolonged cell cycle and self-renewal phase. There is also a pool of active NPCs within the same regions of the brain. These NPCs are highly proliferative cells which retain their multipotency (Wang *et al.*, 2011). Active NPCs do not yet have a defined cell fate. Once NPCs differentiate into neuroblast cells they become unipotent and have a determined cell fate. This means they will either become a neuronal cell, or they will become one of two types of glial cells: oligodendrocytes or astrocytes.

Neuronal fated cells become immature neurons, and only those that survive longer than 28 days after differentiation are likely to become mature, integrated cells. Many cells die during the proliferation stages (Dayer *et al.*, 2003), and more die as immature neurons that fail to integrate (Kuhn, 2015). Only a small number succeed in becoming mature

cells. See Figure 1.3 for the progression of adult neurogenesis resulting in a mature neuron.

Adult neurogenesis is widely believed to be partially regulated via the Wnt/β-catenin signalling pathway, also known as the canonical Wnt pathway (Varela-Nallar and Inestrosa, 2013). This pathway causes the protein β-catenin to accumulate in the cytoplasm, and eventually localise in the nucleus where it has a role in regulating gene expression. Lie et al (2005) demonstrated that hippocampal NPCs express receptors for Wnt proteins, which are known to be critical in embryonic development. They also demonstrated how an overexpression of Wnt3 can significantly increase neurogenesis and blocking Wnt signalling significantly reduces it. Wnt signalling has also been shown to affect neuronal shape and synaptic assembly (Ortiz-Matamoros *et al.*, 2013).

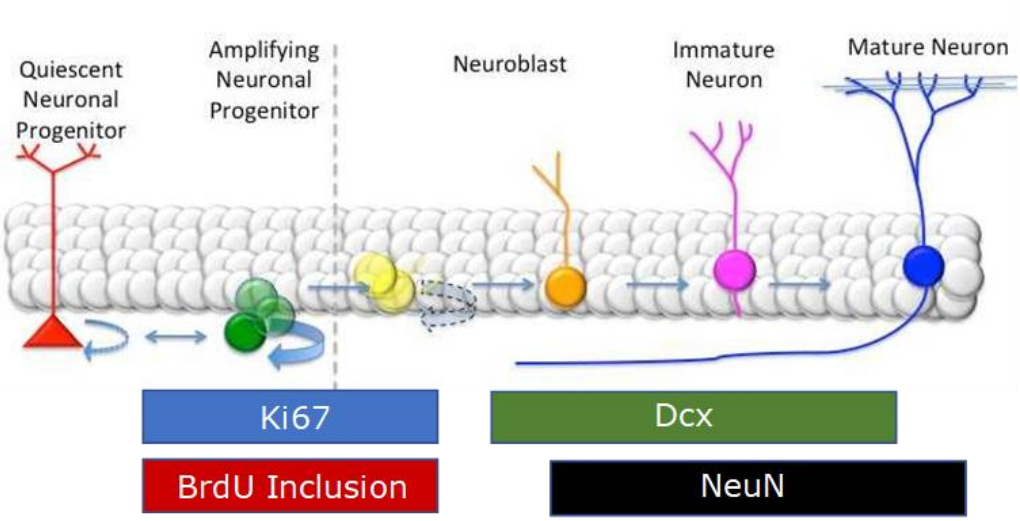

Figure 1.3: Diagram showing the stages of neurogenesis and markers used for analysis

Diagram representation of neurogenesis, showing the different cellular stages in the production of a new mature neuron. The stages where the neurogenic markers Ki67, Dcx and NeuN are expressed is shown, along with the stages during which BrdU is taken up into the nucleus. Other markers are not shown.

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1.3.1.2 Stages of adult neurogenesis

There are three main stages of adult neurogenesis based on what phase the cell is in: Active proliferation, differentiation and mature neuronal cells. We can use some of the markers expressed at these different stages to label the cells (Von Bohlen Und Halbach, 2007). In this study, we used the marker Ki67 for cell proliferation, Doublecortin (Dcx) for cells recently differentiated into neuronal type cells, and BrdU for 28-day cell survival. See Figure 1.3 for the stages each protein is expressed at. These markers were selected over other markers, such as BrdU for proliferation or NeuN for new neuronal cells, as the protocols for these were previously set up in the lab.

Ki67 is a nuclear based protein expressed in all mammalian cells that is believed to be necessary for cell proliferation. It is a reliable endogenous marker of cell proliferation as it is expressed in all phases of the cell cycle, albeit at different concentrations, except for the resting phase and it has a short half-life.

Dcx is an endogenous microtubule-associated protein expressed by cells with a determined neuronal fate. It is expressed in the cells for around 3 weeks after the initial differentiation. As the protein is exclusively expressed in immature neurons it is a useful marker for these cells (Couillard-Despres *et al.*, 2005). Dcx was used in this study instead of the other neuronal marker NeuN due to the protocol previously being set up in the lab.

Bromodeoxyuridine (BrdU) is an exogenous thymidine analogue often used to detect cell proliferation and cell survival (Eriksson, 1998). BrdU inserts itself into the DNA of cells in the S-phase of mitosis and remains there until cell death. This can then be used as a marker for one of two things: cell

proliferation or cell survival, depending on the length of time between injection and analysis or BrdU uptake. BrdU was commonly used as a marker for cell proliferation before the discovery that Ki67 could also be reliably used as a marker of proliferation (Kee *et al.*, 2002). Ki67 is now thought to be a more useful marker for cell proliferation than the previously more commonly used BrdU, as BrdU is an exogenous marker which can only enter the cells at the S-phase of mitosis, whilst Ki67 is a nuclear based protein expressed throughout the cell cycle. In rodent studies, BrdU is usually injected via the peritoneum, and detected in the brain cells using specific antibodies that recognise the presence of BrdU. A study by Cifuentes et al (2011) showed that, whilst IP administration of BrdU reliably labels cells in the SVZ, hippocampus and other large myelinated tracks, intracerebroventricular administration of BrdU labels significantly more cells. This is believed to be due to the higher availability of BrdU when it is injected directly into ventricular cerebrospinal fluid. In our study, we used IP injection, as it is a reliable method for marking cell survival in the DG of the hippocampus and SVZ and does not require surgery on the animals. We used the dosage 20mg/kg per mouse as it is below toxic levels, but high enough for detection 28 days later.

1.3.1.3 Ageing and adult neurogenesis

Neurogenesis continues within the adult brain until death (Boldrini, 2018). However, there are some significant changes that occur to the process of adult neurogenesis with age. There is evidence of a reduction in proliferation and new neuronal cells occurring with age in various animal models, including rodents and canines (Kuhn, Dickinson-Anson and Gage, 1996; Rao *et al.*, 2005; Pekcec *et al.*, 2008). In human brain tissue, significant decreases in the levels of mRNAs for Ki67 and Dcx have been seen in the hippocampus over the lifespan by Mathews et al (2017). They

also found markers showing the consistent presence of a hippocampal stem cell population over the same span. This suggests that the proliferation and differentiation stages of neurogenesis are affected by ageing, whilst the overall pool of NPC/stem cells remains constant. Daynac et al (2016) have shown the proliferative status of active NPCs is altered as early as 6 months of age in mice, whilst both the active and quiescent NPC/stem cell pools themselves remained stable beyond 12 months. This significant reduction in proliferation is likely the cause of the decrease in neurogenesis seen with ageing.

The decrease in neurogenesis with age has been hypothesised to correlate with a decline in memory and cognitive function. In rats, the levels of cell proliferation in the DG were quantified with BrdU and correlated to the special memory task results in the water maze by Drapeau et al (2003). The study showed a clear relationship between the learning capabilities of the rats and the levels of hippocampal neurogenesis. Similarly, other studies have shown that an intervention leading to increased neurogenesis, such as decreased expression of the Wnt antagonist Dkk1, causes improvements in spatial working memory and memory consolidation (Seib *et al.*, 2013; Seib, 2015).

However, other studies such as the one by Bizon et al (2004) have not found such a strong relationship between increased neurogenesis and improved cognition. The study by Bizon et al in rats found no correlation between new hippocampal cell survival and performance in the Morris water maze task as a measure of cognitive ability. These results suggest that the relationship between adult neurogenesis levels and cognitive ability may not be as simple as cause and effect.

1.3.1.4 Neurogenesis and exercise

There is a large amount of evidence showing that exercise can have a significant effect on neurogenesis in the adult brain, especially within the DG. Comparisons of different experimental conditions in mice, such as environmental enrichment, voluntary running and groups which are taught to run mazes, have shown that exercise alone is enough to nearly double the number of proliferating cells in the adult mouse dentate gyrus (van Praag, 1999; Olson, 2006). Whilst both exercise and environmental enrichment have been shown to improve cell survival after proliferation (Kempermann, Kuhn and Gage, 1997), a combination of the two shows the largest significant increase in neurogenesis in the adult brain (Fabel *et al.*, 2009).

As has already been stated, increases in neurogenesis, such as the increase caused by exercise, have been linked to improvements in cognitive abilities such as learning and memory. Numerous rodent models of both forced and voluntary exercise have shown improvements in spatial learning in a variety of maze tasks which have been correlated with increases in neurogenesis (van Praag, Christie, *et al.*, 1999; van Praag, 2005; Cotman and Berchtold, 2007; Van der Borght *et al.*, 2007; Van Praag, 2008).

The amount of time that rodents are exercised for during a study varies from group to group, with the average time seeming to be around 28-30 days, usually performed at the end of the study. Significant changes in the levels of immature neurons are not seen until after 14 days of voluntary wheel running, whilst changes in the level of cell proliferation are seen much earlier (Patten *et al.*, 2013). Longer term exercise studies are fairly rare. Marlatt et al (2012) have shown increased levels of long-term cell survival and increased numbers of new neurons in the brains of rats who

had voluntary access to running wheels for 6 months from 9 months of age.

1.3.1.5 Neurogenesis, exercise and the ageing brain

Exercise has been shown to rescue some of the loss in neurogenesis levels with age, especially in the DG. Aged rodents that have undergone voluntary exercise show significant increases in cell proliferation and cell survival, analysed with BrdU-positive cell numbers, in the DG in comparison to rodents who remained sedentary (van Praag, 2005; Pereira *et al.*, 2007; Saraulli *et al.*, 2017).

When comparing aged and young rodents that had either had voluntary access to a running wheel or remained sedentary, van Praag (2005) found that both running groups showed a significant increase in the number of new cells in comparison to their age matched sedentary groups. Aged runners showed similar numbers of new cells to the sedentary young mice, though the new cells in the old runners were more likely to have an astrocytic cell fate than they were in the young sedentary mice.

1.4 Epigenetics

Adult neurogenesis is a tightly regulated and complex process which can be influenced by many external factors, including epigenetic changes.

Epigenetics is the study of changes in gene expression not due to changes in the DNA sequence. These changes are heritable and can be affected by the environment, disease and behaviours throughout life. The term epigenetics was first used back in the 1940s by Conrad Waddington (Waddington, 1942), who first proposed the idea that "between the genotype and the phenotype…lies a whole complex of developmental process". At this time the understanding of genes and genetic expression

was small. Today however, epigenetics is a major field of interest. Three significant epigenetic mechanisms are DNA methylation, histone modification and non-coding RNA associated gene silencing.

1.4.1 DNA methylation

DNA methylation is the addition of a methyl (CH_3) group to the DNA sequence. This addition usually modifies the expression of the affected gene. The most common form of DNA methylation is the addition of the methyl group to the 5-carbon of the cytosine ring (Singal and Ginder, 1999; Klose and Bird, 2006). This then projects into the major grove of the DNA and prevents transcription of the modified gene. In somatic cells, DNA methylation occurs almost exclusively within areas of DNA known as CpG sites. These sites consist of a cytosine nucleotide next to a guanidine nucleotide. CpGs often cluster into CpG islands found near the promotors of genes (Bird, 1986). Methylation of these islands causes repression of the gene expression, whilst a lack of methylation allows for gene expression (Varriale and Bernardi, 2010). Most instances of DNA methylation lead to gene suppression (see Figure 1.4), though there are some exceptions to this rule.

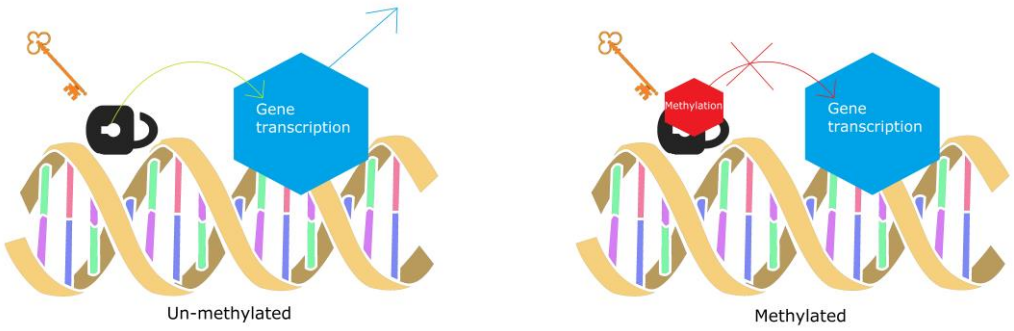


Figure 1.4: DNA methylation

Diagram showing the effect of DNA methylation on gene transcription

The addition of these methyl groups is carried out by a series of enzymes known as DNA methyltransferases (DMNTs). DMNT1 maintains and regulates the copying of DNA methylation during mitosis, whilst DMNT3a and DMNT3b create *de novo* methylation patterns and regulate methylation in non-dividing cells (Li, 2002; Kareta *et al.*, 2006; Probst, Dunleavy and Almouzni, 2009).

Certain patterns of increased DNA methylation have been associated with various disease states, such as cancers, neurological disorders and autoimmune diseases. Rett syndrome is caused by mutations in the gene which encodes the methyl CpG binding protein 2, leading to neurodevelopmental defects in patients (Kriaucionis and Bird, 2003). In Alzheimer's disease, CpG island hypermethylation has been linked to transcription repression of various proteins, including neprilysin which is a major Amyloid-β degrading enzyme in the brain (Urdinguio, Sanchez-mut and Esteller, 2009). Cancer cells have been shown to have significantly reduced global DNA methylation, with specific patterns of hypermethylation at CpG islands. These hypermethylated areas cause suppression of genes involved in DNA repair and apoptosis amongst others (Esteller, 2007; Portela and Esteller, 2010). It has been shown that a combination of DMNT1 and DMNT3a are responsible for the hypermethylation that cause significant gene silencing in these cells (Rhee *et al.*, 2002).

Demethylation of the DNA also occurs as part of the epigenetic changes in gene expression. It can be either a passive or active process. The passive demethylation process occurs on new DNA strands via DMNT1. The active demethylation occurs via the ten-eleven translocation (TET) enzymes. These enzymes modify the cytosine bases by an oxidation reaction, which results in the removal of the methyl group (Kohli and Zhang, 2013; Wu

and Zhang, 2017). They also bind to the CpG rich regions of the DNA and prevent any methyl groups from being added by the DNMT enzymes.

1.4.2 Histone modification

Histones are the proteins around which DNA strands wrap themselves to package it into chromosomes. Groups of DNA wrapped histone proteins group together to form chromatin within the nucleosome. Functionally, chromatin structures are divided into two: euchromatin and heterochromatin (see Figure 1.5). Euchromatin contains regions of actively or potentially actively transcribed genes, and usually has unmethylated CpG islands and hyperacetylated histones H3 and H4. Heterochromatin is the opposite, highly condensed, containing inactive gene sequences with methylated CpG islands and hypoacetylated histone proteins, and it is inaccessible to nucleases (Quina, Buschbeck and Di Croce, 2006). The modification of chromatin structure is essential for the regulation of gene expression as it changes the accessibility of the underlying genes (Li,

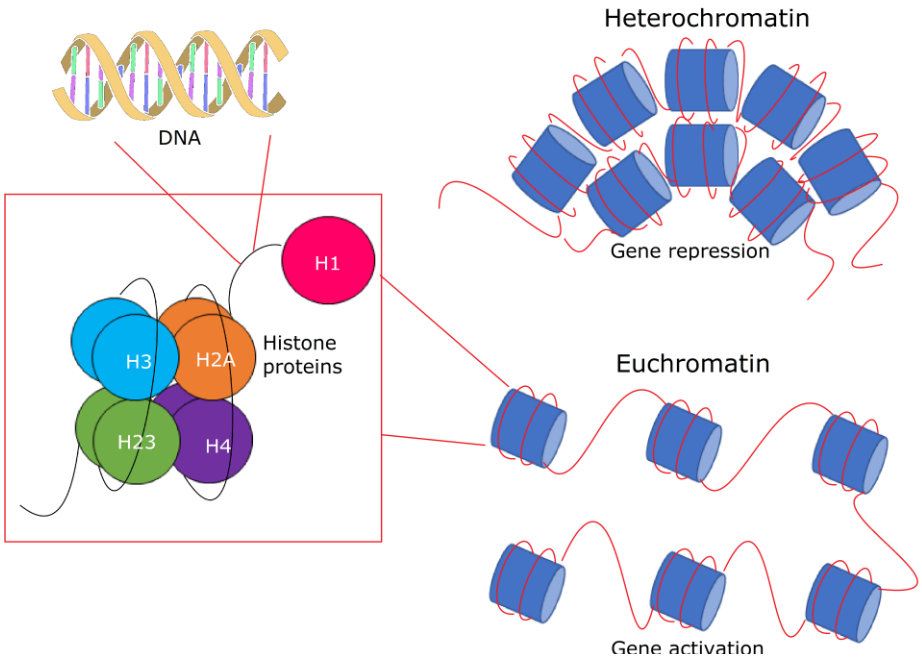


Figure 1.5: Histones and chromatin

Diagram showing how DNA wraps around histone proteins, and how these bundles form heterochromatin and euchromatin.

2002; Berger, 2007; Li, Carey and Workman, 2007). Epigenetic histone modifications are covalent post-translational modifications, and include methylation, phosphorylation and acetylation. These changes can affect many processes, including transcription, chromosome packaging and DNA repair.

1.4.2.1 Histone Acetylation

The addition of an acetyl group from acetyl coenzyme A to the histone proteins H3 and H4 occurs via histone acetyltransferase enzymes (HATs). HATs can be divided into different families based on their catalytic domains (Lee and Workman, 2007), including the GNAT and MYST families. Histone acetylation is involved in regulating a number of important cellular processes such as DNA repair, DNA replication, cell cycle, transcription and gene silencing (Tamburini and Tyler, 2005). Generally, acetylation of a gene causes that gene to be expressed.

The removal of acetyl groups from the histone proteins is catalysed by histone deacetylase enzymes. SIRT1 of the sirtuins family of proteins is a class of histone deacetylases strongly connected with regulation of the lifespan and ageing (Fraga and Esteller, 2007). The balance between histone acetylation and deacetylation has been linked to cancer and tumour growth (Ropero and Esteller, 2007).

1.4.2.2 Histone methylation

Histone methylation is the process by which 1-3 methyl groups are transferred onto a lysine or arginine residue on a histone protein. This reaction is carried out by histone methyltransferases. There are a number of different histone methyltransferases, and each one is specific to one or a set of lysine or arginine residues that they affect.

Methylation of histone 3 lysine 4 (H3K4) almost always leads to active gene expression, as it is strongly associated with numerous gene promotors in multiple cell types (Barski *et al.*, 2007), including some which are vital for embryonic development (Shilatifard, 2012). Methylation of Histone 3 lysine 27 (H3K27) is almost the opposite of H3K4, as it almost always prevents translation from occurring. It has only one known methyltransferase currently, Ezh2 (Kuzmichev *et al.*, 2002), which is known to be responsible for repressing genes associated with development and cell differentiation (Bracken *et al.*, 2006). Both are known to be involved in neurogenesis, though with very different roles. Methylation of H3K4 catalysed by TrxG promotes neuronal cell fate specification and neuronal maturation, whilst methylation of H3K27 catalysed by the PcH complex silences genes required for neural differentiation (Ma *et al.*, 2010).

Histone demethylases remove the methyl groups from the lysine and arginine groups of the histone proteins. There are two major demethylase families currently known, Lysine specific demethylase 1 (LSD1), also known as KDM1 (Shi *et al.*, 2004) and Jumonji domain containing histone demethylases (Tsukada *et al.*, 2006). The amino acid residue and level of methylation determines which demethylase works at which site. KDM1 has been shown to have roles in development in certain species (Lan *et al.*, 2007) and roles in tumour recurrence during therapy (Kahl *et al.*, 2006).

1.4.2.3 Histone phosphorylation

Phosphorylation of histones occurs at the accessible histone tails on serine, threonine and tyrosine residues. Phosphorylation occurs via a number of protein kinase enzymes such as KNK1, PRK1 and AMPK (Baek, 2011). Dephosphorylation occurs via phosphatase enzymes.

Phosphorylation of certain histone sites has been found to be important in the response to DNA sequence damage (Rossetto, Avvakumov and Côté, 2012). For example, phosphorylation of Serine 139 on histone H2 in mammals is involved in numerous DNA damage response pathways such as homologous recombination.

1.4.3 Non-coding RNAs

Non-coding RNAs (ncRNAs) are RNA molecules that are not translated into proteins but have recently been shown to have crucial roles in the regulation of gene expression. There are two main groups of ncRNAs: short ncRNAs (<30 nucleotides long) and long ncRNAs (>200 nucleotides long). The majority of ncRNAs are long ncRNAs and have roles in regulation pre and post transcription and chromatin remodelling. There are three major types of short ncRNAs: piwi-interacting RNAs, short interfering RNAs and microRNAs (miRNAs). Piwi-interacting ncRNAs interact with the piwi family of proteins for gene regulation. Short interfering RNAs (siRNA) work in a similar way to miRNAs, though there are a few subtle differences between the two. siRNAs are double stranded exogenous strands of RNA that bind perfectly to targets, whilst miRNAs are single stranded endogenous strands that often work via imperfect binding.

1.4.3.1 miRNAs

miRNAs were first identified in *C.elegans* in 1993 (Lee, Feinbaum and Ambros, 1993) and have since been found in a large range of organisms. They are usually around 20 nucleotides in length and regulate gene expression by binding to the messenger RNA (mRNA), usually at the 3'UTR end. Each miRNA can bind multiple different mRNAs, so the base pairing of the miRNA and mRNA strands is not always exact. After binding, miRNAs either cleave the mRNA strand, destabilise the polyA tail, or simply prevent

ribosomal translation from occurring (Cannell, Kong and Bushell, 2008; Gu and Kay, 2010) (Figure 1.6).

The genes encoding mammalian miRNAs are initially transcribed in the nucleus via the action of RNA polymerase II, producing pri-miRNAs (Lee *et al.*, 2004). These structures are then further processed within the nucleus via the RNase III enzyme Drosha into pre-miRNAs. The pre-miRNAs are usually hairpin loop structures of 60-70 nucleotides (Lee *et al.*, 2003; Han *et al.*, 2004). After nuclear processing, the pre-miRNAs are transported into the cytoplasm through nuclear pores via exportin-5 (Lund *et al.*, 2004). The final step is the cleavage of the pre-miRNA into a mature miRNA by the RNase III enzyme Dicer. The final mature miRNA that is left

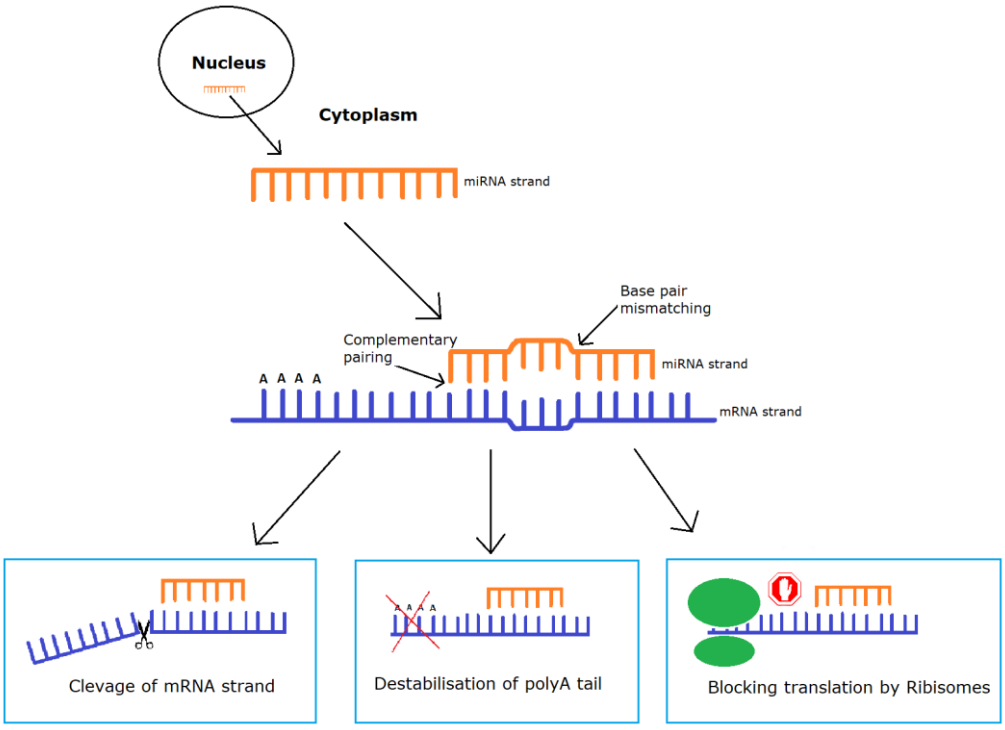


Figure 1.6: Diagram of miRNA gene silencing

Diagram representation of miRNA function within a somatic cell. miRNA strands are transported out of the nucleus and bind to the mRNA strands. From this, the mRNA strand is either cleaved, destabilised at the polyA tail, or is prevented from being translated by the ribosome being blocked.

is a double stranded length of RNA that is around 22 nucleotides long (Knight and Bass, 2001; Wahid *et al.*, 2010).

The majority of identified miRNAs are located and function intracellularly and were originally believed to be too unstable to be able to leave the intracellular environment. Recently though, several highly stable miRNAs have been found in the extracellular environment (Sohel, 2016) in body fluids such as blood plasma (Hunter *et al.*, 2008; Arroyo *et al.*, 2011). Mature miRNAs are released from the cells in exosomes into circulating body fluids and can be delivered to other locations and cells (Valadi *et al.*, 2007; Hunter, 2008; Turchinovich *et al.*, 2013).

Circulating miRNAs are now being investigated for their usefulness as biomarkers for various diseases. Most significantly, a number of different stable miRNAs have been found in circulating serum, which can be used as markers of different cancers, such as small cell lung cancer and colorectal cancers (Chen *et al.*, 2008; Mitchell *et al.*, 2008). They can also be markers of various inflammatory diseases such as asthma, arthritis and inflammatory bowel disease (Mi S., Zhang J., Zhang W., 2013) and other diseases including Alzheimer's (De Guire *et al.*, 2013; Kumar *et al.*, 2013). This field of research could lead to much earlier detection of diseases, allowing for earlier and more successful treatments. The detection of miRNAs in blood plasma is minimally invasive and could reduce the requirement for more invasive and often painful diagnostic measures.

In our study we look at miRNA expression in blood plasma, and the changes associated with ageing, exercise and varied NMNAT1 expression. As such, these will be discussed in more detail than other epigenetic mechanisms. The miRNAs discussed here were selected via previous systemic reviews to find a group associated with ageing that were also often affected by exercise.

1.4.4 Epigenetics in ageing

Recently, the significant impact that epigenetic changes have in ageing has become clear. Ageing changes the epigenome, which in turn changes the expression levels of affected genes which in turn changes the functionality of the cell. These changes in gene expression are one of the main drivers behind the ageing process (Pal and Tyler, 2016; Sen *et al.*, 2016). These age-related changes in the epigenome can also cause an increase in the likelihood of developing certain diseases.

1.4.4.1 Epigenetics in age related illness

A number of tumour suppressor genes are known to become hypermethylated with ageing, preventing their activity and increasing the risk of developing cancers such as colon cancer (Issa *et al.*, 1994; Esteller, 2007; Maegawa *et al.*, 2010). Age-related epigenetic changes have also been connected with the onset of Alzheimer's disease (AD). Bradley-Whitman et al (2013) found increases in the levels of TET1, 5mc and 5hmc in the brains of humans with early and late stage AD compared to control subjects. As the TET enzymes are key for DNA demethylation, it suggests an increase in demethylation in the brains of AD patients. Mastroeni et al (2010) show that a number of epigenetic markers and regulators are significantly decreased in the neurons of AD patients, including DNMT1 and MBD2.

Along with disease states, age related epigenetic changes can have other significant effects in the body which could lead to age related illnesses. De Magalhães et al (2009) found significant increases in the expression of inflammation and immune response genes, and significant decreases in the expression of genes associated with energy metabolism in the mitochondria and apoptosis with age.

1.4.4.2 Epigenetics, ageing and the environment

In addition to the biological process of ageing, the epigenome is affected by changes in the environment that occur throughout the lifespan, such as nutritional accessibility, exercise and circadian rhythms. The lifespan extending affects of caloric restriction are known to work via the activity of the NAD^+ dependent deacetylase SIRT1. A publication by Li et al (2011) reviews how caloric restriction regulates epigenetic process via DNA methylation and histone modifications. DNA methylation leads to an increase in DNMT activity which increases silencing of target genes involved in ageing like p16 and Ras. Histone modifications include deacetylation via the activity of SIRT1 which leads to changes in the expression of genes such as p53 and Foxo. Recently, a paper by Maegawa et al (2017) demonstrated that caloric restriction in mice, rhesus monkeys and humans delays age-related methylation drift, changes in methylation patterns that are associated with age, and could potentially be used as a biomarker to determine biological age (Bell *et al.*, 2012). Caloric restriction in mammals has also been shown to reduce the progressive destabilisation of the genome commonly seem with ageing (Heydari *et al.*, 2007), and ameliorate detrimental ageing phenotypes such as cognitive decline via chromatin modification (Benayoun, Pollina and Brunet, 2015).

Changes in the levels of miRNAs also occurs with ageing, and many miRNAs have been found to play an important role in ageing regulation. The first miRNA to be discovered in the *C.elegans* model, *Lin-4*, was found to regulate the nematode lifespan via the insulin signalling pathway (Kenyon *et al.*, 1993; Boehm and Slack, 2005; Kenyon, 2010). A number of miRNAs have since been shown to be upregulated with ageing, some involved in cell senescence such as miR-71 in C*.elegans* (Lencastre *et al.*, 2011). Change in the expression of other miRNAs has been associated with

age related diseases and neuropathies (Hébert and De Strooper, 2009), such as a decrease in miR-133b found in patients with Parkinson's disease (Kim *et al.*, 2009). An increase in miR-29a and miR-92b can cause a decrease in the expression of β-amyloid cleavage enzyme 1 which could play a significant role in AD (Hébert *et al.*, 2008; Junn and Mouradian, 2012). Many other miRNAs have altered expression levels with ageing that could affect these types of disease (Jung and Suh, 2014; Saeidimehr *et al.*, 2016; Williams *et al.*, 2017)

However, the field of miRNA research is still relatively new and so the cause and effect relationship between changes in miRNA expression and ageing are still not fully understood.

1.4.4.3 miRNAs, ageing and exercise

The affect of ageing and exercise on the expression of different miRNAs varies. In our study we looked at the expression of seven different miRNAs in blood plasma known to be affected by exercise and/or ageing. They were selected from the literature and previous work done in our lab.

Baggish et al (2011) have done a study on the effects of exercise on some miRNAs in human blood plasma. They found that MiR-222, miR-21 and miR-221 significantly increased after acute exercise, with miR-21 and miR-221 reducing after one hour's rest, whilst miR-20a showed no change. MiR-222, miR-21 and miR-221 showed significant increase in blood plasma after 90 days of exercise training, with miR-20a showing a trend towards increase. The review by Silva et al (2017) found an increase in miR-92a after 10 weeks of regular endurance exercise and an increase in miR-148b after 8 weeks voluntary wheel running. The majority of the seven miRNAs studied in our experiments are downregulated with ageing (Table 1.1), so

there may be some differences in our results with the combination of exercise and ageing.

	Ageing	*Exercise*	*Other*
miR-20a-5p	Downregulated with age	Plasma levels: No difference with acute exercise. Upregulated after prolonged exercise	Involved in neurogenesis regulation, upregulated in AD
miR-21-5p	Deregulated with age	Plasma levels: Upregulated with acute and prolonged exercise	Involved in neurogenesis and cancer development
miR-30e-5p	Downregulated with age	-	Upregulated in AD, involved in radial glial proliferation
miR-92a-3p	Downregulated with age	Plasma levels: Upregulated with prolonged exercise	Involved in neural progenitor cell development
miR-148b-3p	-	Upregulated in hippocampal brain tissue with prolonged exercise	Involved in proliferation and differentiation of neuronal cells after stroke
miR-221-3p	-	Plasma levels: Upregulated with acute and prolonged exercise	Involved in stem cell differentiation determination
miR-222-3p	Downregulated with age	Plasma levels: Upregulated with acute exercise, less affected by prolonged exercise	Involved in stem cell differentiation determination

Table 1.1: The role of miRNAs in ageing, exercise and other significant mechanisms

List of miRNAs investigated during this study, and how exercise, ageing or age-related processes affect their expression. Other column includes miRNA involvement with neurogenesis, cancer, cell development and Alzheimer's disease (AD).

(Gangaraju and Lin, 2009; Baggish, 2011; Zhang *et al.*, 2011; Olivieri *et al.*, 2013; Satoh, 2012; Nigro *et al.*, 2012; Olivieri *et al.*, 2012; Bian *et al.*, 2013; Ghosh *et al.*, 2014; Hatse *et al.*, 2014; Jung, 2014; Cheng *et al.*, 2015; Jin *et al.*, 2016; Silva, 2017; Wang, Chen and Shan, 2017).

Many of the miRNAs of interest in this study are also involved in different stages of neurogenesis and also Alzheimer's disease. However, miRNAs often regulate more than one gene and often affect multiple cellular processes. Likewise, multiple cellular processes can be affected by one miRNA. Because of this, it can be difficult to connect changes in the expression of one miRNA with changes to a specific cellular process. Studying the expression of multiple miRNAs can give a better understanding of the connections between miRNA expression and changes in the cell.

1.5 The NAD$^+$ biosynthetic pathway

Nicotinamide adenine dinucleotide (NAD) is a small molecule that has an essential role in metabolism and is a coenzyme for multiple reactions,

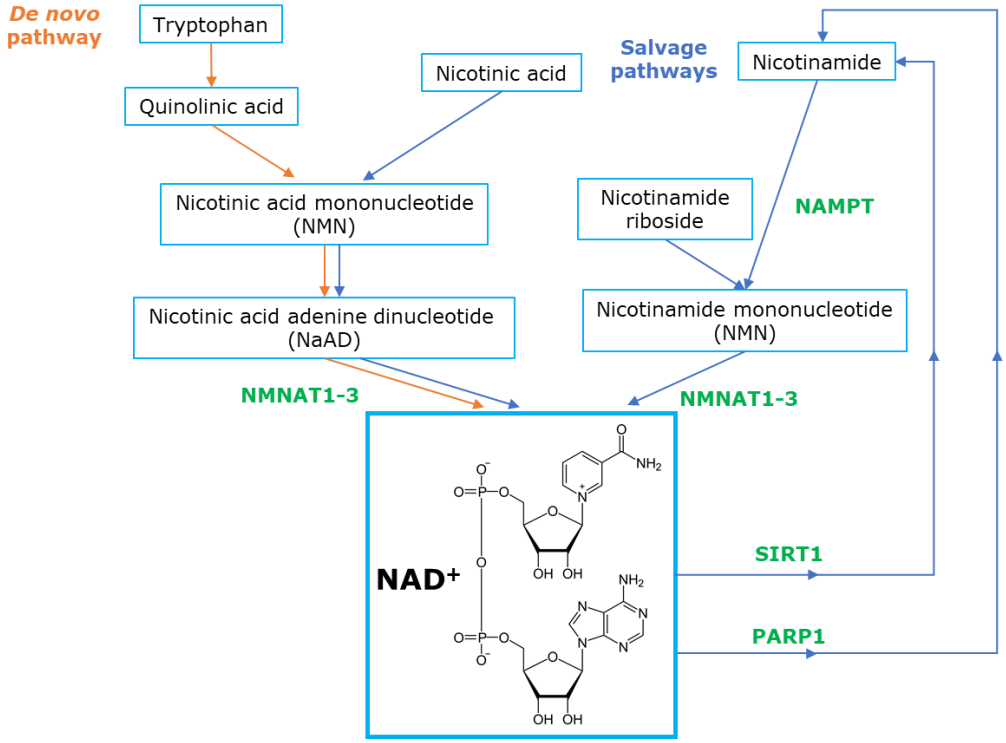


Figure 1.7: NAD biosynthetic pathways

Diagram representation of the NAD biosynthetic pathways, showing both the *de novo* (orange arrows) and salvage (blue arrows) pathways and key enzymes found in mammals.

including the redox reactions in all stages of cellular respiration. NAD is synthesised through one of two major pathways (Figure 1.7): a *de novo* pathway, or salvage pathways.

The *de novo* pathway synthesises NAD from the amino acid L-tryptophan in mammals. It produces the molecule nicotinic acid mononucleotide (NaMN) and then is converted via the nicotinic mononucleotide adenylyltransferase (NMNAT) enzymes into NAD^+ (see Figure 1.7).

There are two main salvage pathways used to make NAD^+ (Figure 1.7). The first is from Nicotinic acid taken from Niacin and then processed in the same way as the *de novo* pathway once it is converted into NaMN. The second uses Nicotinamide salvaged from reactions that have used NAD^+ as a catalyst, usually by the enzymes SIRT1 and PARP1. Nicotinamide is converted to nicotinamide mononucleotide (NMN) by the enzyme NAMPT. The NMN is then converted back to NAD^+ via the NMNAT enzymes (Kim, Zhang and Kraus, 2005).

1.5.1 NAD^+ in ageing and exercise

NAD^+ is required for the activity of the deacetylase enzyme SIRT1, which we have already shown is key in the regulation of ageing via caloric restriction (Section 1.4.4.2 Epigenetics, ageing and the environment).

Nicotinamide phosphoribosyltransferase (NAMPT), part of the NAD^+ biosynthetic pathway (See section 1.5.2 NMNAT, Figure 1.7), has previously been shown to be a rate limiting factor in the production of NAD^+ (Revollo, Grimm and Imai, 2004). NAMPT converts nicotinamide into NMN, the precursor molecule for NAD^+. There is a global decline in NAMPT and NAD^+ levels and SIRT1 activity found with ageing, which correlates

with DNA damage accrued with age (Braidy *et al.*, 2011). This suggests that NAD^+ availability may be important in healthy ageing.

A study by Koltai et al (2009) looked at the levels of NAD^+ availability in the skeletal muscle of young and aged rats that underwent exercise training. They saw no decrease in SIRT1 activity with age, but they did see age related decreases in NAMPT and NAD^+ levels. With exercise, the activity of SIRT1 significantly increased, along with NAMPT and NAD^+ levels. This suggests that regular exercise can rescue age related NAD^+ loss, potentially in a similar way to caloric restriction.

NAMPT is not the only enzyme in the NAD^+ biosynthetic pathway that is thought to be rate limiting. In our study, we looked at the enzyme which controls the conversion of NMN to NAD^+: NMNAT.

1.5.2 NMNAT

Nicotinamide mononucleotide adenylyltransferase (NMNAT) is the enzyme which catalyses the final steps of NAD^+ biosynthesis (Figure 1.7). To date there are three known isoforms of the enzyme, known as NMNAT1-3.

NMNAT3 is localised to the mitochondria, and is believed to be responsible for NAD production for and transport into the mitochondria, which are thought to be impermeable to NAD, thus maintaining the mitochondrial NAD pool (Zhang *et al.*, 2003; Hikosaka *et al.*, 2014). However, it is difficult to detect in a number of different tissues, and there are some conflicting views on the exact nature of NMNAT3's function (Felici *et al.*, 2013; Yamamoto *et al.*, 2016).

NMNAT2 is cytoplasmic and is located primarily within neuronal cells. Homozygous knock out of the NMNAT2 gene causes severe peripheral nerve/axon defects and significant axonal truncation throughout the CNS, causing perinatal death (Gilley *et al.*, 2013). This suggests that NMNAT2 is

essential for correct axonal formation during development. In mature neurons, NMNAT2 is essential for the maintenance of the axon, and due to its short half-life constant anterograde transport from the cell body is required. If the axon is damaged or cut, transport is interrupted and rapid Wallerian degeneration takes place and the axon quickly dies (Gilley and Coleman, 2010; Conforti, Gilley and Coleman, 2014).

NMNAT1 is located in the cell nucleus and is expressed in most tissues throughout the body. It plays a vital role during embryonic development, as homozygous knock out of the gene leads to death before birth (Conforti *et al.*, 2011). Heterozygous knock out (Het) of the gene does not have this effect, and mice develop and mature normally. The transgenic overexpression (TG) of this gene via a construct from a β-actin promotor in mice also allows for normal birth and development (Conforti *et al.*, 2007). In our study we look at both the heterozygous knock out and the transgenic overexpression of NMNAT1 in mice, and how this changes the effect of exercise in ageing. Previous work has looked at the effect of similar variance of NMNAT1 expression in ageing mice, with a focus on behaviour, metabolic analysis, NAD levels and gene expression (Rossi, 2015). The study found that mice overexpressing the NMNAT1 gene had a delay in the body weight increase usually found with ageing, and increased oxygen consumption and locomotor activity in comparison to the wildtype (WT) and Het groups. They also saw increases in NAD levels in the brains of aged TG mice, but no difference in NAD levels between the WT and Het groups, though the Het mice showed a decrease in NAD over time that was not seen in the other two groups. They saw no differences in the levels of NMNAT2, NAMPT or SIRT1 expression in aged mice from the WT, TG and Het groups.

Our study will take a further look at the effects of varied NMNAT1 expression and exercise on the brain with ageing, focusing on behaviour, neurogenesis, miRNA expression and gene expression.

1.5.3 NAD and SIRT1

Once NAD^+ has been synthesised by the NMNAT1 genes, it is then often used as a coenzyme by Sirtuin1 (SIRT1). An NAD-dependent deacetylase enzyme, SIRT1 plays an important role in cellular stress response, energy metabolism and regulation of gene expression via protein modification. The function of SIRT1 can be regulated by the availability of NAD^+ and thus by the activity levels of some of the enzymes in the biosynthetic pathway, including potentially NMNAT1 (Zhang *et al.*, 2009).

As discussed in Section 1.4.4.2, caloric restriction is known to increase maximum life span and delay the onset of ageing phenotypes. Caloric restriction exerts it effects primarily through the activity of SIRT1 (Cohen, 2004; Cantó and Auwerx, 2009). Transgenic overexpression of the SIRT1 gene has a similar effect, resulting in increased average and maximum lifespan in rodents (Mercken, Mitchell, *et al.*, 2014; Mitchell *et al.*, 2014). Conversely, knock out of the SIRT1 gene causes a significant decrease in median life span (Chen, 2005; Mercken, Hu, *et al.*, 2014).

1.6 Models of ageing and exercise

1.6.1 Human studies

Ideally, we would conduct ageing studies in humans. There are two main study designs for ageing in humans: Longitudinal and cross-sectional. Longitudinal studies give data on changes over time in each participant,

whilst cross-sectional studies give only snapshots at a single time point in each participant.

For longitudinal human ageing studies, we gain a good insight into changes over time and can potentially see cause and effect based on lifestyle. However, apart from the ethical issues from long term data collection in humans, longitudinal studies have a number of other problems. These include the length of time it takes for a human to age from birth, inaccuracy due to self-reporting, poor population representation and participant retention, and high costs. Cross-sectional studies often have higher numbers of participants, and therefore can sometimes be better representative of a population. However, it is much harder to see cause and effect as the data may be affected by prior behaviours and/or environments where they previously lived.

Methodology used often differs between human studies, making direct comparisons of the data difficult. These factors and more make it problematic to accurately study the ageing process in humans.

1.6.2 Ageing models

Due to the many issues surrounding human studies, animal and cellular models are often used instead, and the data from these models is extrapolated to humans.

Human cell cultures, Yeast colonies (Váchová, Čáp and Palková, 2012), *Caenorhabditis elegans* (Tissenbaum, 2015)*, Drosophila* (Piper and Partridge, 2017) and rodents such as mice and rats are all common models used to study different mechanisms underlying the ageing process. These smaller models have significantly shorter life cycles than larger models, such as humans and primates, which makes ageing studies more manageable. The short life cycle allows for rapid data acquisition and

analysis of single and multiple generations in a relatively short time frame. There is also a significant amount of research into genetic manipulation on these models, allowing for greater understanding of the underlying molecular and genetic mechanisms of many processes, including ageing. In this study, we make use of a mouse model of exercise during the ageing process.

1.6.2.1 Exercise models in rodents

There are two main rodent models of exercise. The first is voluntary wheel running where the rodent has free access to a running wheel for a set number of hours/days/weeks, usually inside their home cage. The second is forced exercise, when rodents are placed onto a treadmill and made to run for a set period of time each day (minutes/hours), usually over several days, or a forced swim test where rodents are placed in a tank of water for a set time, and the test is repeated on a number of days (Gobatto *et al.*, 2001; Kuphal, Fibuch and Taylor, 2007). Both models have advantages and disadvantages.

One of the main differences often found between the two running exercise models is the speed and time spent running and how many times the rodents stop during the running period. It has been shown that mice that have voluntary access to a running wheel will run at a higher speed in short bursts, and overall are likely to spend a longer time running on the wheel when compared to forced treadmill exercised mice (Burghardt *et al.*, 2004; Leasure and Jones, 2008). A main disadvantage to voluntary wheel running models is the amount of variability between the distance run per mouse, which is easily controlled for in treadmill running experiments.

Forced exercise has been shown to increase stress and anxiety in rodents. One model of forced exercise is the forced swim test, where a rodent is

placed in a tank of water and forced to swim for a set period of time. However, a version of the forced swim test is also used as a test for stress and depression (Duman and Monteggia, 2006; Commons *et al.*, 2017). Another model of forced exercise is forced treadmill running, where a rodent is placed on a treadmill for a set period of time and is gently encouraged to continue each time they try to stop. Forced treadmill exercise has also been shown to increase stress and anxiety in rodents in a similar way (Ke *et al.*, 2011; Svensson *et al.*, 2016), though some studies have presented conflicting data on this (Burghardt, 2004).

The increase in anxiety, stress and depression that forced exercise seems to cause in rodents could have a significant impact on the healthy ageing outcomes of our study. Due to this, and despite the disadvantage of run distance variability, we have chosen to use a voluntary exercise model.

1.7 Aims and hypotheses

The purpose of this study is to gain further understanding into the impact of ageing, exercise and varied NMNAT1 expression on the brain and behaviour.

In this study, we aim to:

1. **Investigate the effect that the phase of the light/dark cycle under which behavioural tests are performed in mice has on the effect of exercise on mouse behaviour**

2. **Investigate the effect of exercise on behaviour, adult neurogenesis, circulating miRNA expression and hippocampal gene expression in aged C57BL/6J mice**

   We hypothesise that aged mice that have exercised will:

- Show decreased anxiety, and increased cognition and locomotor activity in behavioural tests

- Have increased cell proliferation, immature neuron numbers and 28-day cell survival numbers in the dentate gyrus and subventricular zone

- Have upregulation of specific miRNAs in blood plasma

- Have increased expression of epigenetic markers and genes associated with the NAD+ biosynthetic pathway

3. Investigate the effect of varied NMNAT1 expression on behaviour, adult neurogenesis, circulating miRNA expression and hippocampal gene expression in aged mice

We hypothesise that:

- Increased NMNAT1 expression will have a similar effect to exercise in WT mice on behaviour, adult neurogenesis, miRNA expression and hippocampal gene expression

- Halved NMANT1 expression will have the opposite effect of exercise in WT mice on behaviour, adult neurogenesis, miRNA expression and hippocampal gene expression

4. Investigate the effect of exercise in aged mice with varied NMNAT1 expression on behaviour, adult neurogenesis, circulating miRNA expression and hippocampal gene expression

We hypothesise that:

- Exercise will affect mice with both increased and halved NMNAT1 expression in the same way as age matched wildtype mice.

5. Investigate the effect of varied NMNAT1 expression on adult neurogenesis in young male and female mice, and how it changes with age

We hypothesise:

- Overexpression of NMNAT1 will increase cell proliferation and immature neuron numbers, and halved expression of NMNAT1 will decrease these in young mice, regardless of sex
- All markers of adult neurogenesis will significantly decrease with time, regardless of NMNAT1 expression level

2. Chapter 2:

Methods

2.1 Animal work

2.1.1 Licencing

All animal work was authorised by the University of Nottingham Animal Welfare and Ethics Board and performed by personal licence holders in accordance with the 1986 Animals (Scientific Procedures) Act. The reverse light cycle study was performed under project licence PPL 40/3601 holder Dr Marie-Christine Pardon. All other work was performed under project licences PPL 40/3482 (January 2015-July 2016) and 30/3416 (July 2016-November 2016) both held by Dr Maria Toledo-Rodriguez and carried out by personal licence holders.

2.1.2 Housing

All mice used during these studies were housed under standard conditions, with rodent chow (Teklan 2018, Harlan) and water *ad libitum*, standard bedding materials and environmental enrichment in the form of chew toys and a cardboard tube. Lights went on at 7am and off at 7pm for ageing mice up to 12 months of age and for young mice on the normal light cycle used in the light cycle experiment. All other mice and ageing mice from 12 months of age onwards were housed with lights on at 11pm and off at 11am. Ageing mice were moved onto the reverse light cycle early in the study so that the change had minimal impact. Ageing mice up to 12 months of age and all young mice prior to being housed with a running wheel (where applicable) were housed in cages with a Floor area of 501cm^2. Ageing mice from 12 months of age onwards were housed in the larger cages with a Floor area of 904cm^2 with an accessible or dismantled running wheel (see Figure 2.3). All behavioural tests were performed 2 hours after the lights changed for all mice. Young mice involved in the light

cycle experiment were single housed in rat cages with an accessible or dismantled running wheel (See chapter 3 for details).

2.2 Reverse light cycle study

2.2.1 Reverse light cycle study

All work done under normal light cycle conditions (the "light" groups) was performed by Dr. Maria Toledo-Rodriguez, and all work done under reverse light cycle conditions was carried out by the author. Raw data was provided for the normal light cycle conditions and analysed by the author.

Male C57BL/6J mice aged 6-7 weeks were obtained from Charles River Laboratories International UK, Inc into the facility and acclimatised for one week to the new facility. For mice on the reverse light cycle, this week was also used to acclimatise to the new cycle. Mice were separated into two main groups, those on the normal light cycle (lights on 7am-7pm, N=35), the "Light" group, and those on the reverse light cycle (lights on 11pm-11am, N=18), the "Dark" group (Figure 2.1). The reverse light cycle timings were selected to facilitate access to the behaviour testing equipment.

Before starting the experiment, mice were ordered based on the number of arm entries performed correlated with distance travelled over the course of a 5-minute spontaneous alternation test. They were then evenly separated into the exercise and sedentary groups (i.e. the highest scoring mouse was placed into the exercise group, the next highest into the sedentary etc.) and single housed.

Once single housed, mice assigned to the exercise group were placed in a cage with a running wheel ("light" exercise group N=20, "dark" exercise group N=10) with free access to the running wheel for the duration of the

experiment (Figure 2.1). The rest of the mice ("light" sedentary group N=15, "dark" sedentary group N=8) had a dismantled wheel placed in the cage to control for environmental enrichment and no access to voluntary exercise. Mice tested in the light were separated further into a group which performed the EPM test, and a second group which performed all other behaviour tests. Group numbers and situation were identical.

After 28 days with or without the running wheels, all mice underwent a series of up to 4 behavioural tests carried out over consecutive days (See section 2.4 for more details). Elevated plus maze (EPM) on day one, followed by open field (OF), novel object recognition (NOR) and finally SA. See Figure 2.1 for a timeline of the study. All of the tests were started

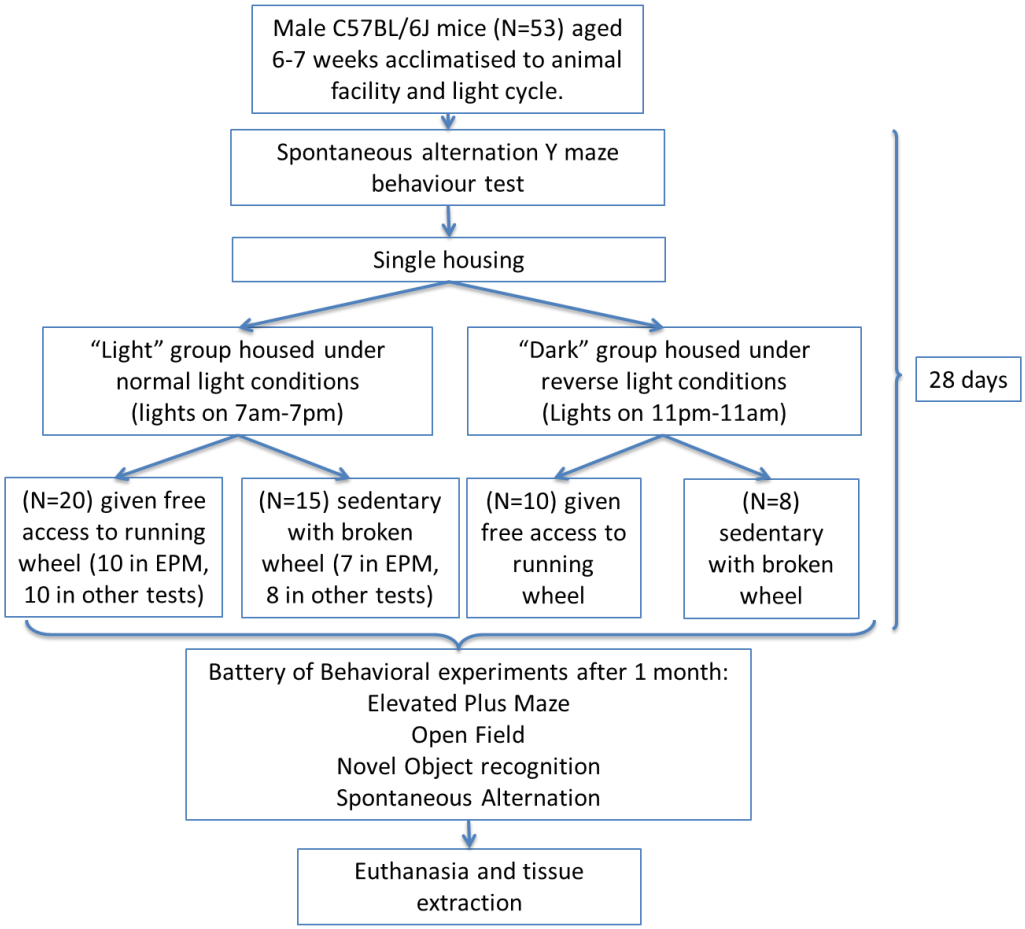


Figure 2.1: Flow diagram showing timeline of light cycle experiment

Flow diagram showing the key events for the light cycle experiments. Behaviour experiments were performed 2 hours after the lights changed.

approximately 2 hours after the change in light/dark phase for both group (11am for the light groups, 1pm for the dark groups).

For the reverse light cycle tests, light intensity did not exceed 5 lux anywhere within the arenas of the tests. Mice were transferred to the tests in a covered transporter to avoid light exposure.

2.3 NMNAT1 studies

2.3.1 NMNAT1 colonies

Two transgenic colonies of mice with altered expression of NMNAT1 were used in this study. These mice were bred in house, and both produced average litter sizes.

The first group overexpresses NMNAT1 (TG) and was originally created by the Coleman group at the Babraham Institute in Cambridge (Conforti, 2007) on a C57BL/CBA mouse background. The mice used in this study were the first 3 generations of mice back crossed with C57BL/6J mice. Female TG mice were bred with male C57BL/6J mice to create the offspring.

The second group has a heterozygous knock out (Het) NMNAT1 and was originally created by the Coleman group at the Babraham Institute in Cambridge (Conforti, 2011) in I29/J mice crossed with C57BL/6 K14 cre mice to produce the NMNAT1 HetKOs on a black background. These knock out mice were again back crossed to C57BL/6J mice, and the first three generations of this new colony made up the groups for this study. There was a noticeable reduction in the number of mice with the Het genotype in each litter compared to WT mice, leading to reduced numbers of these mice throughout the study.

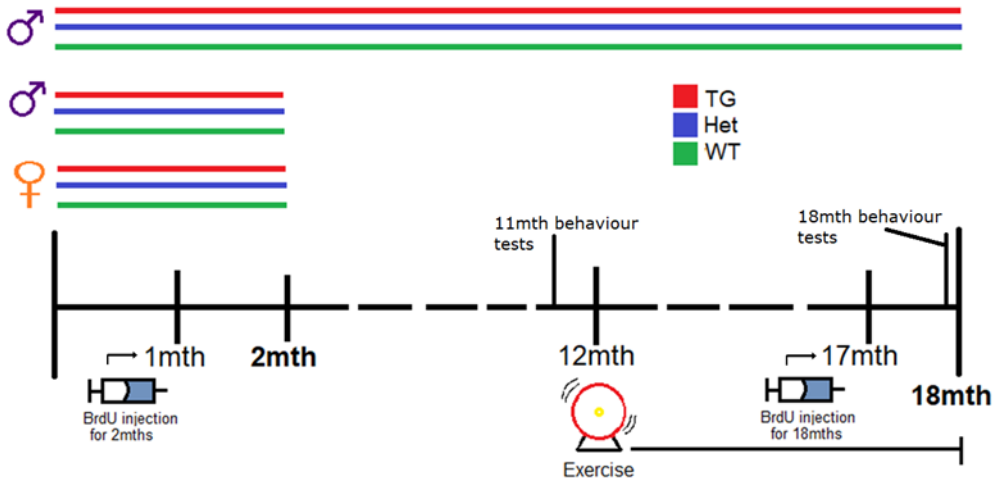


Figure 2.2: Overview of young and aged NMNAT1 experiments

Timeline of experiments with young and aged NMNAT1 mice. Colours denote the three genotypes present in each study. The top group shows the ageing experiment, and the middle and bottom groups are the young animal experiments. Along the bottom is the timeline, showing the key events and when they occurred in each experiment.

2.3.2 NMNAT1 study overview

Mice from both the overexpressing and HetKO NMNAT1 colonies were used for both the young and aged experiments in this study. Figure 2.2 shows a basic timeline for both experiments.

	Male	Female
WT	18	18
Het	7	11
TG	8	10

Table 2.1: Group numbers for the young mouse study

2.3.3 Young mouse study

Both male and female mice were genotyped and weaned at P28 into group housing (see Table 2.1 for numbers). Mice with the Het genotype were low in number in each litter, and so ended up with low overall numbers.

Each mouse was given an intraperitoneal injection of 20mg/kg BrdU dissolved in sterile saline 28 days before euthanasia. This dose has been found to be high enough for detection levels, but well beneath the level at

which it can become toxic to the animal or the developing cells. At P56 mice were euthanised via terminal anaesthesia and tissues were extracted. For details on immunostaining, see section 2.5.1 Immunohistochemistry.

2.3.4 Ageing mouse study

	Sedentary	Exercise
WT	12 (9)	11 (11)
Het	9 (6)	14 (13)
TG	11 (7)	14 (12)

Table 2.2: Group numbers for ageing study

Table showing the group numbers for each genotype and exercise category. Numbers in brackets show the group numbers at the end of the study.

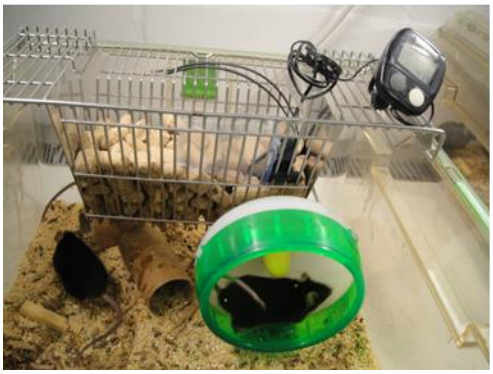
Male mice from both the overexpressing and HetKO NMNAT1 colonies were used for this experiment. See Table 2.2 for group numbers at the beginning of the study. Numbers vary due to mice dying of natural causes before the end of the study.

After genotyping, mice were group housed into the smallest number of cages. Several cages had a mix of either TG or Het mice with their respective WT littermates. This was done to avoid having single housed mice during the 17 months the experiment lasted. No cages had a mixture of TG and Het mice.

At 11 months of age, mice underwent a full series of behavioural tests (See section 2.4 for details). One month later, mice were transferred into new cages. These contained either a working running wheel (Figure 2.3A) allowing the group free access to voluntary exercise, or a dismantled wheel (Figure 2.3B) for the sedentary group. Mice were housed under these conditions for a further 6 months until they were 18 months of age. The sedentary group cages contained the dismantled wheel to control for environmental enrichment. Mice were filmed overnight on a regular basis to estimate the distance run by each individual mouse.

At 17 months of age, 1 month before the end of the experiment, all mice underwent IP injection of 20mg/kg BrdU to study survival of new neurones.

A



B

Figure 2.3: Cage set up

Photographs showing the wheel set up in the cages for the ageing experiment. A) An exercise group cage with the running wheel attached to the food hopper, and the cycle computer stored above to track running distances. B) A sedentary group cage with the dismantled running wheel placed on the floor in the corner of the cage where mice have access but cannot exercise.

1 week before the end of the experiment, mice went through the same series of behavioural experiments as they did at 11 months of age.

At the end of the experiment, mice were euthanised using terminal anaesthesia. Blood was extracted via cardiac puncture and placed into EDTA coated tubes to prevent coagulation. This was then centrifuged at low speed (1,000G) for 10 minutes to separate plasma from cells. Plasma was transferred to another tube and snap frozen on dry ice and stored at -80°C. "RNA later" was added to the remaining blood cells to prevent RNA degradation and these were stored at 4°C overnight. Afterwards "RNA later" was removed and the blood cells stored at -20°C.

The brains of the mice were immediately removed on a cool tray, and the left hemisphere was dissected into different regions: olfactory bulb, frontal cortex, cortex, striatum, cerebellum, hippocampus, amygdala, hypothalamus and pituitary gland. The body was dissected, and the eyes, various organs, gastrocnemius and adductor muscles and brown, beige and white adipose samples were taken. Organs extracted were as follows:

heart, kidneys, adrenal glands, liver, pancreas, spleen, testes, large intestine, small intestine and aorta. Tissues samples were stored for future studies and have not been investigated in this study (i.e. this study used only the brain and plasma). Half (liver, spleen, pancreas, brain minus hypothalamus) or one (kidney, adrenals, testis, heart, intestine, hypothalamus, hippocampus, PFC, striatum, cerebellum, pituitary, eye) were snap frozen on dry ice and stored at -80°C. The remaining tissues were fixed in 4% paraformaldehyde overnight and then stored in a PBS solution with 0.01% w/v sodium azide (NaN_3) at 4°C.

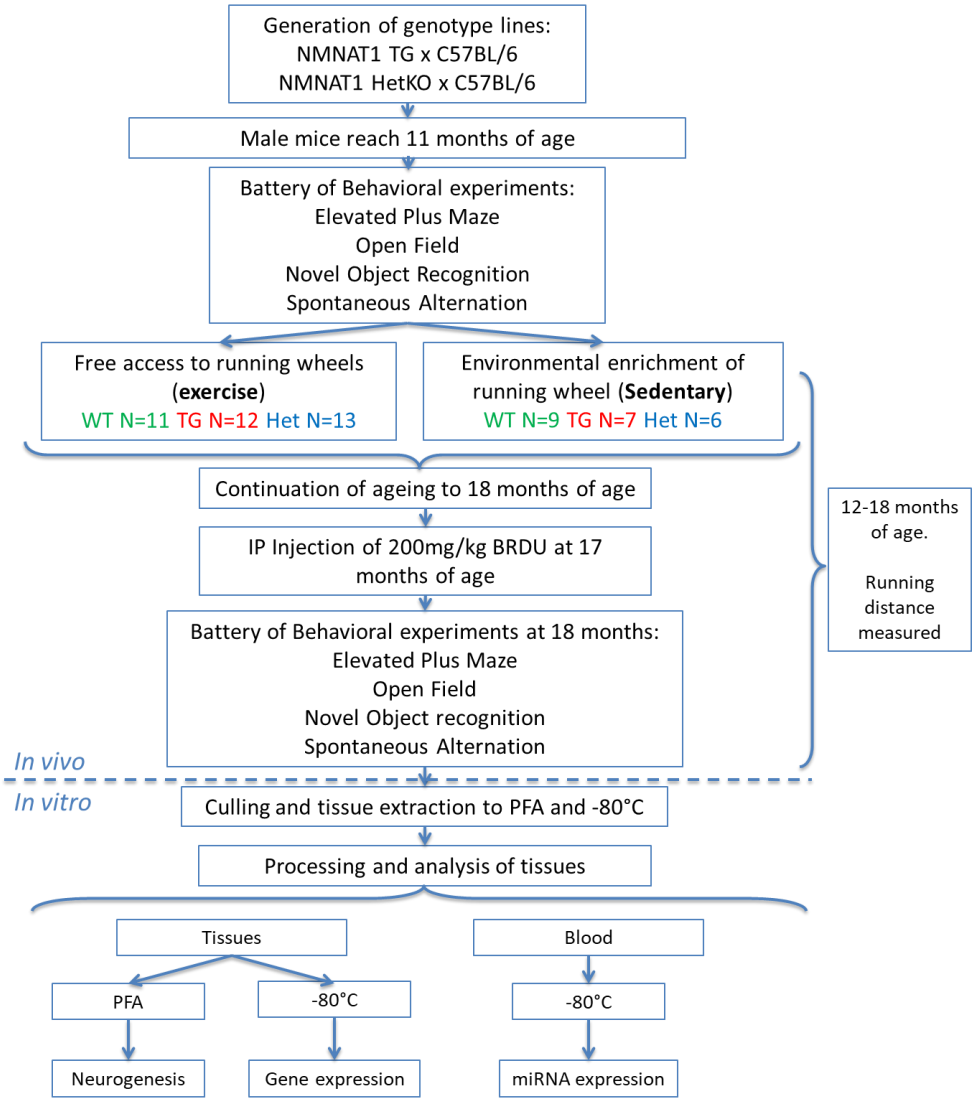


Figure 2.4: Timeline of ageing experiment

Timeline for the ageing experiment, showing the progression from genotyping, through behaviour experiments and the exercising period, to in vitro analysis experiments performed.

2.4 Behaviour tests

The behaviour tests used throughout this study were selected to investigate cognition, anxiety and locomotor activity in the mice. Other tests for these measures were considered, such as the Morris water maze for cognition. These other tests were not used, either due to lack of access to the correct equipment, or due to potential confounding factors i.e. the Morris water maze has been shown to increase stress and anxiety in mice during a test for cognition. All manual behaviour data scoring was performed blind to the exercise and genotype group.

2.4.1 Elevated plus maze (EPM)

The EPM test is one of the most commonly used tests for anxiety in mice (Komada, Takao and Miyakawa, 2008).

The EPM test consisted of mice being placed in the centre of a plus shaped maze (7.5cm x 35cm) raised 1 meter above the ground (Figure 2.5). The maze had 2 'closed' arms with high walls and 2 'open' arms with no walls. Foam blocks were placed underneath the open arms to protect the mice if they fell during the test. Any mouse that fell during the test was excluded from the data (only 2 mice fell during the ageing study).

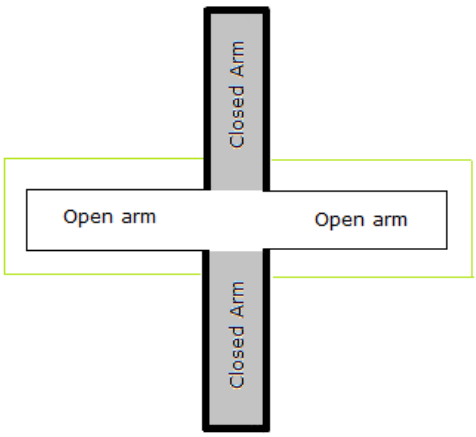


Figure 2.5: Diagram of EPM

Diagram of the EPM layout, marking the location of open and closed arms. Green lines mark the location of foam blocks.

The mice explored the maze for 5 minutes. The distance travelled over the course of the test was measured, along with the amount of time spent in the open and the closed arms. This was done via the video analysis software Ethovision (XT V10 Copyright © 2013 Noldus Information Technology), which tracked the position of the mouse throughout the trials. Exploratory behaviours were manually scored. This included stretch attenuated postures (SAPs) and dipping the head over the side of the open arms of the maze. An increase in the number of SAPs performed by a mouse suggests an increase in risk analysis and potentially increased anxiety. An increase in the number of head dips suggests an increase in risk taking behaviour, as it can lead to the mouse falling from the maze, and therefore a potential decrease in anxiety.

2.4.2 Open field (OF)

The open field (OF) test is commonly used to test for anxiety and locomotor activity in mice (Seibenhener and Wooten, 2015).

The OF behaviour test consisted of the mice being placed in the centre of a large arena (50.5cm x 30.3cm) and left to explore for 30 minutes (Figure 2.6).

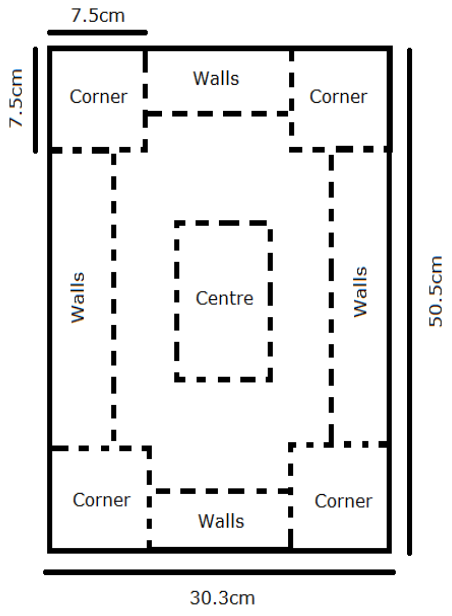


Figure 2.6: Diagram of OF arena

Diagram of the OF arena. Dotted lines denote areas marked out for Ethovision analysis.

The walls, corners and centre of the arena were marked out in EthoVision software. The time the mice spent in each section of the arena the distance travelled during the experiment was calculated with EthoVision

2.4.3 Novel object recognition (NOR)

The novel object recognition test (NOR) is commonly used to test for recognition memory in mice (Leger *et al.*, 2013).

The same arenas used for the OF tests were used for the NOR. Mice were habituated to the arena during the OF test and a second 30-minute trial 24 hours later. 24 hours after the second trial, mice were placed back into the arena with two identical objects placed in opposite corners of the arenas (Figure 2.7 upper half) for the familiarisation stage of the NOR. The objects used in this study were a small deodorant bottle made of white plastic with a blue plastic lid, and a small glass bottle full of red liquid with a green stopper lid. Mouse location and movement was tracked using Ethovision video analysis software.

The mice were then placed in the centre of the arena and allowed to explore for five minutes before being returned to their home cage for a further 5 minutes. The arena and objects were then thoroughly cleaned with a 20% EtOH solution ready for the next test. New objects were then placed into the same positions in the arena, one identical to the recognition test objects and one novel object with a different shape, colour and texture (Figure 2.7 lower half). Mice were then placed back into the arena and allowed to explore for a further 5 minutes. Investigation of each object was manually scored for both frequency and duration. A recognition index was calculated for each mouse as: $\frac{Time\ exploring\ novel\ object}{Total\ time\ exploring\ both\ objects}$ during the second, recognition, trial.

Investigation of an object was classified as direct contact with an object with the nose or front paws, or clear directed investigation of the object with the nose and whiskers. Analysis for side bias was performed during recognition trials to check for object preference, as each object was consistently placed on the same side of the arena for every recognition trial, and none was found.

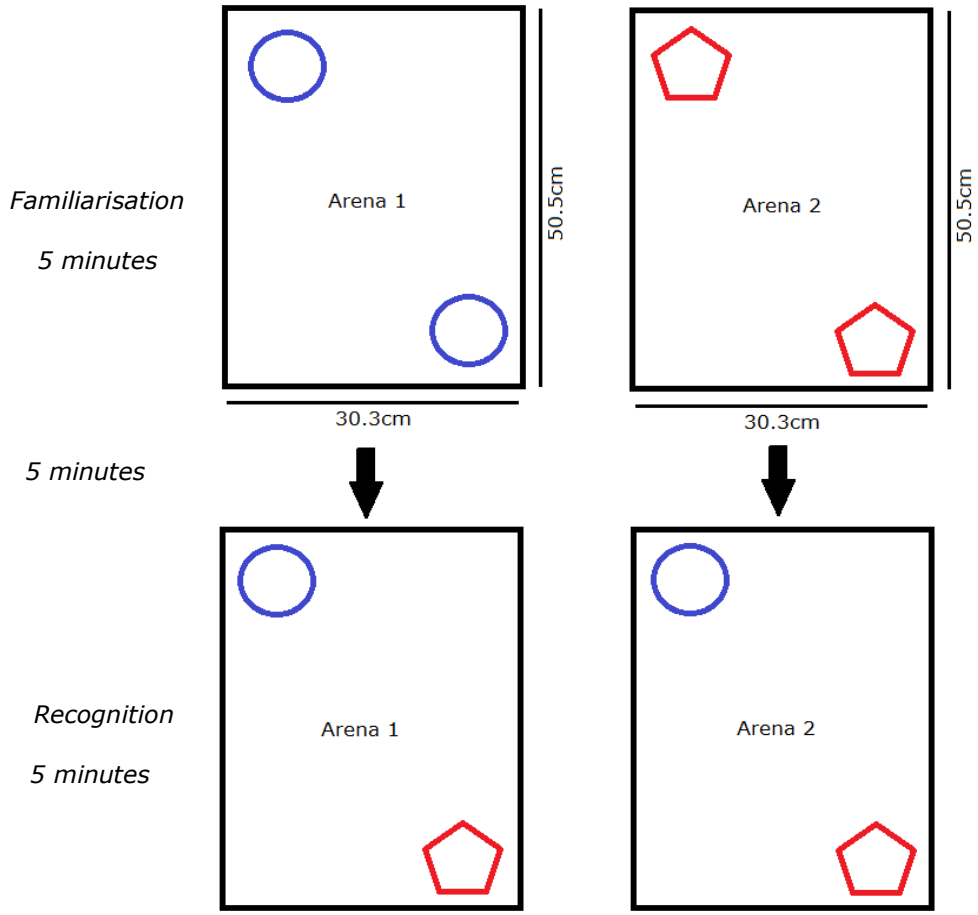


Figure 2.7: NOR arena set up

Diagram of the NOR arena set up. Top half shows arena layout for habituation phase when using 2 arenas. Bottom half shows arena layout for recognition phase. Blue circle and red pentagon represent different objects used in arenas.

2.4.4 Spontaneous alternation Y maze (SA)

The spontaneous alternation test (SA) is used to test spatial memory in mice and their willingness to explore new areas.

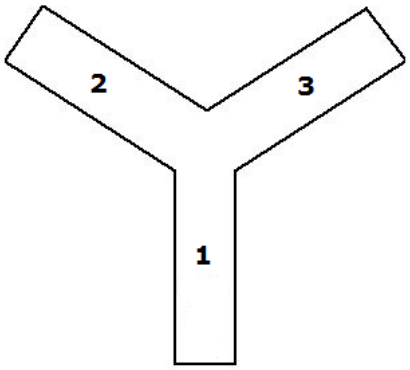


Figure 2.8: SA Y maze diagram

Diagram of the Y maze with arms labelled for entry recording.

Mice were placed into the centre of a Y shaped maze (arms 45cm x 7.5cm x 14cm) made of clear plastic and allowed to explore for 5 minutes. Arm entries were manually recorded and scored to give a spontaneous alternation index. Mouse location and movement was tracked by Ethovision video analysis software.

Each arm entry made by the mouse was given a score of 1 or 0. A 1 was awarded if the arm entered was different from the previous 2 entries. Otherwise, a 0 was awarded. This was then added up to give the alternation score (a). The total number of alternations made was also added up (n). The alternation index is then calculated as: $\frac{a}{(n-1)}$

2.4.5 Cleaning

Between each behavioural test the arena and the objects used (NOR test) were thoroughly cleaned using a 20% ethanol solution in distilled water and then dried to remove any scents left by the previous mouse.

2.5 *In vitro* tissue analysis

2.5.1 Immunohistochemistry

2.5.1.1 Tissue processing

The right hemisphere of the brain which had been fixed in 4% PFA was processed in a Leica tissue processor for paraffin embedding. During this process tissues were taken through the following steps:

Incubation in 70%, 80% and 96% alcohol for 90 minutes each, followed by three 60-minute incubations in 100% alcohol. Then two incubations of 3 hours in Chloroform before finally two incubations of 2 hours in molten paraffin wax.

Tissues were embedded into paraffin wax blocks, with each block containing up to four tissues of the same genotype and exercise group (with or without voluntary exercise). Blocks were sectioned at 7μm thickness using a Leica 2245 microtome and mounted onto APES coated glass slides (Figure 2.9).

2.5.1.2 Immunostaining

Buffers and antibodies:

Citrate buffer – 10mM sodium citrate buffer solution with a pH of 6 made up with 10mM citric acid.

Day 1 buffer - 10 ml PBS (phosphate buffered saline) + 1% BSA (bovine serum albumin) (100mg) + 0.3% triton 300ul 10% triton

Day 2 buffer – 1 in 3 dilution of day 1 buffer with PBS

	Blocking serum 2%	*Primary antibody*	*Secondary antibody*
Ki67	Donkey serum	1:100 (V.P.K #452)	1:200 Horse-anti-mouse
Dcx	Rabbit serum	1:200 (Santa Cruz #8066)	1:200 Rabbit-anti-sheep
BrdU	Rabbit serum	1:500 (Abcam #6326)	1:200 Rabbit-anti-sheep

Table 2.3: Antibodies

Table showing serum and antibody dilutions used for immunostaining.

Protocol:

Slides were first deparaffinised and rehydrated as follows. Two 5-minute incubations in Xylene were followed by two more in 100% alcohol, then 5 minutes each in 70% and 50% alcohol before two final 5-minute incubations in distilled water (dH_20).

Antigen retrieval was carried out in citrate buffer (pH6) with 0.05% triton heated to 98°C and incubated for 20 minutes. Slides were washed in dH_20 after cooling, and endogenous peroxidase activity was reduced by an incubation of 0.5% H_20_2 in PBS for 10 minutes. After washing twice for 5 minutes with PBS, tissues were blocked with a 2% serum for one hour at room temperature. Finally, they were incubated with the primary antibody diluted in day 1 buffer overnight at 4°C (Table 2.3).

The next day, samples were washed twice in day 2 buffer and then incubated with the secondary antibody for one hour at room temperature (Table 2.3). After further washing, samples were then incubated with avotin-biotin-complex (Thermofischer UK) for a further hour. Each component was diluted at 1:500. After washing once with day 2 buffer and twice with PBS, samples were developed with DAB (3,3'-diaminobenzidine) for up to 20 minutes and then washed with dH_20.

Finally, samples were counterstained as follows. After 1-minute incubation in Harris haematoxylin, samples were washed in warm tap water. Then placed for 5-7 seconds in acetic alcohol, followed by wash in warm tap

water and a further 15 seconds in Scotts water. After a last wash in tap water, samples were then dehydrated by 10 second incubations in 50%, 70% 90% and two 100% ethanol baths followed by two 2-minute incubations in xylene. Slides were the mounted with DPX (a mixture of distyrene, plasticizer and xylene) and left to dry.

2.5.1.3 Analysis of immunostaining

Slides were selected for staining and analysis using the Allen mouse brain atlas as a guide. Starting on the medial side of the tissue, the first section to have a clear and complete visible DG area was selected, and a further 3 sections were selected, spaced 70µm apart (10 slides). Each brain was coded by its position in the block (see Figure 2.9) and analysed blind to exercise and genotype group.

Slides were imaged using an axioplan upright light microscope with a QImaging micropublisher 5.0 RTV camera. The DG and SVZ areas of each brain were measured using imageJ software and a 5x lens. The cells were manually counted by eye using a 40x lens.

The number of cells/mm^2 was calculated as $\left(\frac{Cell\ count}{area\ nm2}\right) * 1{,}000{,}000$. An average was taken for each brain over up to 6 sections to get the final result for each animal.

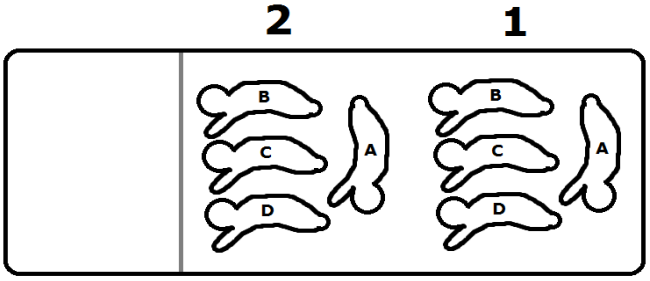


Figure 2.9: Layout of tissue samples on slides

Diagram showing the approximate layout of the tissue samples on the glass slides after sectioning. Each slide contained 2 sections of each brain e.g. tissue samples 1A and 2A are from the same brain.

2.6 Genotyping of NMNAT1 colonies

Genotyping of the mice from the NMNAT1 colonies was performed on DNA extracted from pre-weaning ear biopsies. DNA was extracted by incubating the tissue sample with DNA release additive at room temperature for 2-5 minutes. Samples were then heated to 98°C for 2 minutes before being centrifuged. For the genotyping, *Taq* DNA polymerase from Sigma Aldrich was used for the reaction enzyme and the standard protocol was followed. 20µl reactions were set up with 10µl of red TAQ, 1µl of each of the forward and reverse primers (500/500nm), 7µl dH_20 and 1µl DNA template. PCR reactions were performed (cycling 30 times at 94°C for 45 seconds, 56°C for 45 seconds and 72°C for 120 seconds). Finally, samples were run in a 3% agarose gel and bands indicated the presence or absence of the knock in/knock out.

The following primers were used to detect the TG and Het mice:

Nmnat1 transgenic (TG):
Forward 5'-ACTTCGGCTCACAGCGCG-3'
Reverse 5'-TCCTTGGCCAGCTCGAACA-3'

Nmnat1 heterozygous KO (Het):
Forward 5'-CCCAGTCACTAAGACATTCAA-3'
Reverse 5'-CCTTCTTGCTTCCCACGAGG-3'

2.7 miRNA extraction from blood plasma

2.7.1 miRNA extraction

miRNAs were extracted from blood plasma samples using the Qiagen miRNeasy serum/plasma kit and the Qiacube standard protocol for miRNeasy serum/plasma. 100µl of plasma was used from each sample, and the final RNA sample was dissolved in 12µl RNase-free water. I carried out this work in the lab of Dr Yvan Devaux in the Luxembourg institute of health department of population health. miRNA samples were then frozen at -80°C and shipped back to Nottingham until use. RNA quality was checked using a Nanodrop 3000 (Thermofischer).

2.7.2 Reverse transcription and qPCR

Reverse transcription (RT) and quantitative polymerase chain reaction (qPCR) was performed following the manufacturer's instructions as described.

Blood plasma miRNAs were reverse transcribed with the Exiqon miRCURY LNA™ Universal RT microRNA PCR kit following the manufacturer's instructions (see next paragraph). 2µl of the extracted miRNA samples were used for each reaction. We chose to use 2µl to avoid interaction from the PCR inhibitors found in samples extracted from blood plasma.

Reverse transcription. For each RT reaction we used 2µl RNA, 2µl 5x reaction buffer, 5µl nuclease free water and 1µl Enzyme mix for a total of 10µl. Samples were then incubated for 60 minutes at 42°C and then for 5 minutes at 95°C to heat inactivate the reverse transcriptase before being immediately cooled on ice. Samples were then diluted 40x in nuclease free

water in low binding tubes. The final samples from this reaction were cDNA that was used in the qPCR reactions.

qPCR reaction. For each qPCR reaction, we added 4µl PCR master mix (ExiLENT SYBR® Green master mix) and 1µl PCR primer set to 4µl of the diluted cDNA template for a total of 10µl. The reaction was done in the Rotor-Gene 6000 cycler (Corbett Research) according to the following temperature/time parameters: Enzyme activation at 95°C for 10 minutes, followed by 40 amplification cycles of: 10 seconds denaturation at 95°C followed by 1 minute annealing at 60°C with a ramp-rate of 1.6°C. Analysis of qPCR data is described in section 2.8.1.3.

2.8 RT and qPCR for gene expression

2.8.1 Gene expression in the hippocampus

RNA extraction from brain tissue was performed following the Thermofischer TRIzol protocol as follows. Frozen hippocampus tissue was weighed and the amount of TRIzol to use was calculated based on this. As all samples weighed less than 25mg, each was homogenized with 0.8ml TRIzol. They were then left at room temperature for 5 minutes. 320µl of 1-bromo-3-chloropropane was added, and samples were shaken for 15 seconds before being left at room temperature for 2-15 minutes. They were then phase separated at 4°C and 12,000g for 15 minutes.

The clear aqueous phase was transferred to a new tube, and 0.4ml room temperature isopropanol and 1µl glycogen was added to each. After mixing, these were left to stand for 10 minutes at RT before being centrifuged at 4°C and 12,000g for 15 minutes. The supernatant was removed, and the RNA pellet was washed with 0.8ml ice cold 75% ethanol and centrifuged again at 4°C and 12,000g for 10 minutes. The supernatant

was again removed, and the RNA pellet left to air dry for up to 10 minutes. 50µl of RNAse-free water was added to dissolve the pellet, and then samples were incubated at 55°C for 10 minutes. Finally, quantity and quality of the samples was measured using the nanodrop 2000 (Thermofischer) and RNA was stored at -80°C until use.

2.8.1.1 Reverse transcription

DNase treatment. For every 1ug RNA we added 1µl DNase buffer and 1µl DNase I enzyme from the Invitrogen DNase I kit and increase the mixture volume to 10µl with DPEC treated water. Samples were incubated at room temperature for 15 minutes before the enzyme was deactivated by adding 1µl EDTA and heated to 65°C for 10 minutes and then immediately cooled onto ice.

Reverse transcription. After DNAse treatment, the following was added to each sample: 1.25µl of dNTP (0.5mM), 3.75µl N15 oligonucleotides (0.52µg). Each sample then had DPEC treated water added to make it up to 17.25µl total. Samples were heated to 65°C for 5 minutes in the PCR machine before being snap cooled onto ice for at least 1 minute. Samples were then spun down and kept on ice.

Each sample then had the following added: 5µl 1x first strand buffer, 1.25µl DTT (5µM), 0.5µl RnaseIn (20U) and 1µl Superscript™ III (200U) from the Invitrogen Superscript™ III reverse transcriptase kit per sample. This left the total volume of each sample at 25µl. After mixing, samples were placed into the PCR machine with the following protocol: 25°C for 5 minutes without the lid on, 50°C for 60 minutes with the lid at 95°C and finally 70°C for 15 minutes. cDNA samples were stored at -20° until use.

2.8.1.2 qPCR

We studied the expression levels of a number of different genes in the hippocampus of our mice (see Table 2.4). qPCR was performed by reactions containing 7.5µl enzyme (either SYBR® Green JumpStart™ Taq ReadyMix™ for Quantitative PCR by Sigma-Aldrich or SensiMix™ SYBR® by Bioline) and 1.5µl cDNA and primers (see Table 2.4 for final concentrations) and mixed with RNAse-free water to a total of 15µl per reaction. SIRT1, Ezh2, NMNAT1, NAMPT and Utx expression levels were all measured using the SensiMix™ enzyme due to poor efficiency with the primers in standard curves with the JumpStart™ enzyme. NMNAT2 and housekeeping genes were run with JumpStart™ as they had good efficiency numbers. Previous tests in the lab have shown similar results in non-

Gene	Temperature	Concentration	Dilution	Primer sequences
SIRT1	56ºC	300nm	1:16	**F:**AGAACCACCAAAGCGGAAA **R:**TCCCACAGGAGACAGAAACC
NMNAT1	58ºC	300nm	1:64	**F:**GCCACCAAGAACTCACACTG **R:**TGGGCTCTTGGGGCTTCTGT
NMNAT2	58ºC	300nm	1:64	**F:**GGCTGCATCCTCTCCAACGT **R:**AAGTGAAGCGCTCCACTGGT
NAMPT	58ºC	200nm	1:64	**F:**GCCACCTTATCTTAGAGTCA **R:**CCAAGGCCATTGGTTACAAC
Ezh2	58ºC	500nm	1:16	**F:**ACTTGGATTTTCCAGCACAAGT **R:**AAGGGCGACCAAGAGTACATTA
Hprt1	63ºC	500nm	1:64	**F:**GCAGTACAGCCCCAAAATGG **R:**AACAAAGTCTGGCCTGTATCCAA
Pgk1	63ºC	400nm	1:64	**F:**GAAGGGAAGGGAAAAGATGC **R:**AAATCCACCAGCCTTCTGTG
Tatabox	63ºC	300nm	1:16	**F:**CAGCCTTCCACCTTATGCTC **R:**TGCTGCTGTCTTTGTTGCTC
BDNF	63ºC	300nm	1:16	**F:**GGTATCCAAAGGCCAACTGA **R:**CTTATGAATCGCCAGCCAAT
Utx	64ºC	500nm	1:16	**F:**ATGGAAACGTGCCTTACCTG **R:**GGACCTGCCAAATGTGAACT

Table 2.4: qPCR gene analysis

Table showing information on each gene analysed with qPCR in this study. Each row shows the gene name, the optimum temperature for cycling, the primer dilution, the cDNA dilution and finally the primer sequences used.

problematic genes with both enzymes. Reactions were prepared on ice and either used immediately or stored at 4°C in the dark for up to 2 hours.

qPCR reactions were performed using the Rotor-Gene 6000 cycler (Corbett Research) and reactions were performed in triplicate. Enzyme activation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, gene temperature (see Table 2.4) for 20 seconds and 72°C for 35 seconds. Afterwards samples underwent melt curve analysis with ramp rate of 72°C to 95°C with 1°C per step.

In order to calculate the primer's efficiency, we created a pool of all samples. This was done by taking 1µl from each sample and mixing them in one tube. This pool sample then underwent a series of 1 to 4 dilutions series (from 1:4 to 1:1024). Due to the large number of animals, we could not test all samples in a single run of the qPCR machine (capacity for 23 samples and one non-template control), so two qPCR runs were performed for each gene. In order to be able to pool the data from both runs, two reference samples were included in each run (identical samples in each run). The CT values for the reference samples was then set to be identical in both runs. The cDNA dilution used in this study for each gene can be seen in Table 2.4.

2.8.1.3 qPCR analysis

Rotor-Gene software was used to determine the CT for each sample and the efficiency of the primers. Relative expression was calculated by $CT\text{^}((Group\ average) - (Efficiency + 1))$ (Pfaffl, 2001). Normalisation factors were calculated for each sample by $\frac{Geometric\ mean\ of\ HKG\ for\ sample}{Geometric\ mean\ of\ all\ samples\ \&\ HKGs}$ (Vandesompele *et al.*, 2002). Finally, the normalised expression of each sample was calculated as $\frac{Relative\ expression}{Normalisation\ factor}$.

2.9 Statistics and software

2.9.1 Software used

All statistical analysis was performed using GraphPad Prism version 7.00 for windows, GraphPad Software, La Jolla California USA.
All behavioural tests were recorded using EthoVision XT V10 Copyright © 2013 Noldus Information Technology
Manual behavioural analysis was performed using Clicker v1.13 Copyright © 2005 Velibor Ilic
Image analysis was performed using ImageJ software (Fiji ImageJ 2.0.0, national institute of health, U.S.A) (Rueden *et al.*, 2017)

2.9.2 Statistics

For behaviour data, two-way-ANOVA with repeated measures were used to analyse the changes in the measurements with age in the exercise and sedentary groups. Student's *t*-test was used to compare the percentage changes in behaviour data.

For all other data, ordinary two-way-ANOVA was used for multiple group comparisons, and Student's *t*-test was used for single comparisons.

Normally distributed data was confirmed by D'Agostino test. Statistics reported show the mean ±SEM.

3. Chapter 3:

The influence of light-dark cycle phase on the outcome of behaviour tests in a mouse model of voluntary exercise

3.1 Background

Physical exercise is known to be beneficial to both mental and physical health (Reiner *et al.*, 2013; Knapen *et al.*, 2015). For example, in many studies patients with anxiety and/or depression have reported a significant decrease in symptoms when performing physical exercise (Hillman, 2008; Jayakody, 2014; Knapen, 2015; Stubbs, 2017). There have also been reports that physical exercise is associated with a reduction in cognitive decline in aged subjects (Bherer, Erickson and Liu-Ambrose, 2013; Gomez-Pinilla and Hillman, 2013), as well as an improvement in cognitive function in younger subjects (Hillman, 2008; van Praag, 2009).

In rodents, similar changes and improvements in both mental and physical health have been seen with exercise (O'Callaghan, 2007; van Praag, 2009). As discussed in Chapter 1, studying the underlying mechanisms of changes brought about by exercise in humans is difficult, so rodent models are commonly used instead. The two exercise models used in rodents, forced and voluntary exercise, both have their advantages and disadvantages. However, as forced exercise is more likely to increase anxiety in rodents (Leasure, 2008), this study uses a model of voluntary exercise.

In order to study levels of anxiety or cognitive ability in a rodent model we carried out a battery of behavioural tests. The elevated plus maze (EPM) and open field (OF) tests are commonly used to assess anxiety levels, whilst the novel object recognition (NOR) and spontaneous alternation or Y maze (SA) tests are used to evaluate cognition. The NOR specifically tests for recognition memory, and in our study looked at short term and working memory. Other protocols for the NOR look at more long-term memory by having a longer break between the familiarisation and recognition trials. SA

tests for spatial memory, to see whether the mouse can recognise the location it has recently visited. In humans, working memory and the ability to form new long-term memories often declines with age, and may be rescued or maintained with exercise.

In this study, we wanted to observe how exercise changed the outcome of these tests, and whether the phase of the light-dark cycle under which they are carried out can change those outcomes.

Within the literature, there are some conflicting results as to how exercise affects anxiety in rodents. Many studies present results consistent with the idea that exercise decreases anxiety levels in rodents (Duman, 2008; Salam *et al.*, 2009; Santos-Soto, 2013). Other studies however, present data which demonstrates that exercise either has no effect on anxiety (Garrett *et al.*, 2012; Nguyen, 2013), or shows an increase in anxiety in mice that have exercised (García-Capdevila *et al.*, 2009; Fuss, 2010; Pan-Vazquez, 2015).

Looking closer at the methods in these papers, it is clear that some of the tests were performed in the dark phase of the light-dark cycle, and others in the light phase. This could be a possible explanation for some of the discrepancies in the data. Rodents are nocturnal animals and are therefore most active during the dark phase of the light-dark cycle. However, the majority of behavioural tests in rodents are performed during the light phase. This is mainly because it is more convenient for the experimenters and there may be a lack of facilities for performing and recording behaviour tests in the dark. Previous experiments have already shown that certain rodent behaviours can vary with the phase of the light cycle. Roedel et al (2006) tested DBA mice in the modified hole board during the dark phase under red light or during the light phase under white light. This test uses a similar arena to open field tests but includes a series of shallow holes that

the mice can explore. Significant behavioural inhibition and cognitive disruption was seen in the DBA mice tested under white light in comparison to those tested in the dark phase. Aslani et al (2014) showed that causing stress in rats during the light phase of the diurnal cycle caused the rats to display signs of depression and anxiety related behaviours. If the same stressors were applied during the dark phase however, no change in the behaviour was seen.

The aim of this study was to determine whether the phase of the light cycle has an impact on the outcomes of behaviour tests for anxiety and cognition in rodents that have exercised. We therefore hypothesised that mice tested for anxiety during the light phase of the light cycle will show an increase in anxiety related behaviours after one month of voluntary exercise in comparison to control mice. We also hypothesised that mice tested for anxiety during the dark phase of the light cycle will show a decrease in anxiety related behaviours with one month of voluntary exercise in comparison to control mice. We compared two groups of mice, one tested in the light and one tested in the dark, with half of the mice in each group having been allowed access to running wheels for one month.

3.1.1 Aims and objectives of the chapter

The purpose of this study was to investigate the effect of light-dark cycle phase on mouse behaviour after a period of voluntary exercise.

Our objective is to discoverer whether the phase of the light/dark cycle during which mice are tested for behavioural measures will have an effect on the behaviour in young mice that have exercised.

3.2 Methods

3.2.1 Study protocol

All work on animals under a normal light cycle was performed by Dr. Maria Toledo-Rodriguez. Raw data was provided by Dr. Toledo and analysed by the author.

Male mice were acclimatised to the new conditions for one week and separated into two groups: under normal light cycle and under reverse light cycle. The reverse light cycle hours were selected so that behaviour experiments could be performed 2 hours after the lights changed whilst allowing time for care routines to be performed in the light. They were then

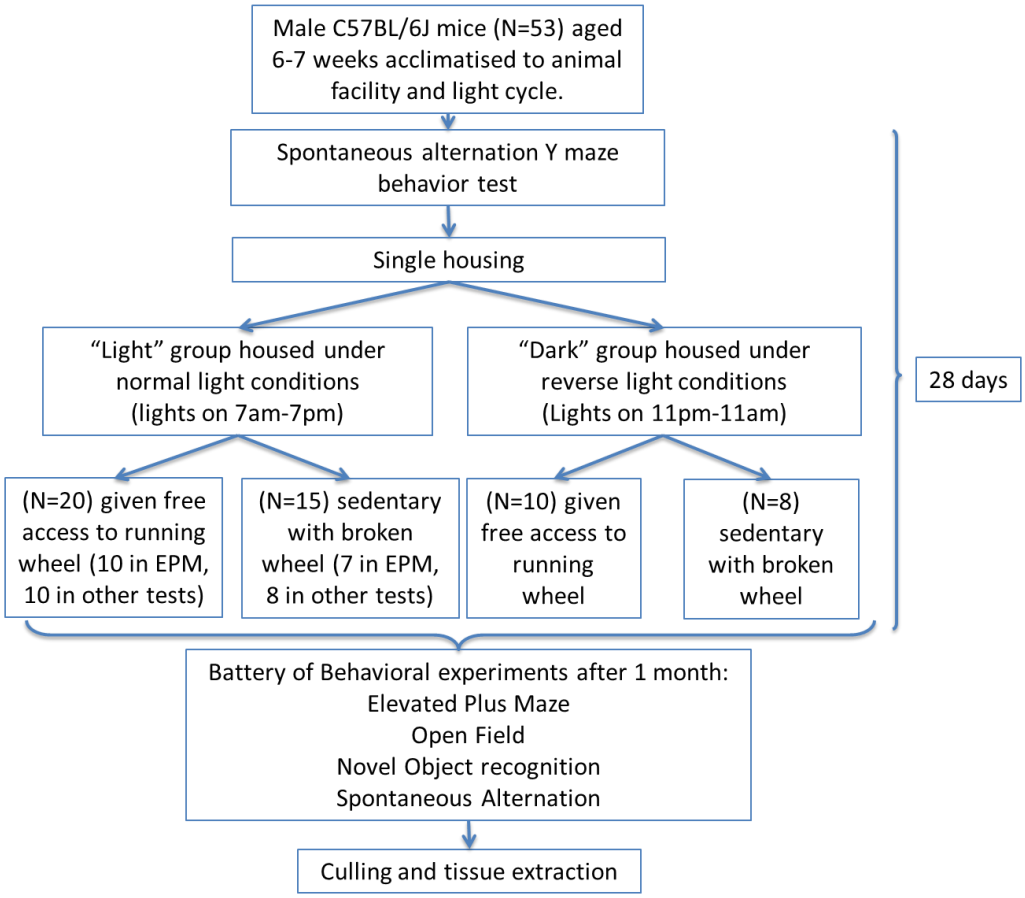


Figure 3.1: Protocol for light cycle experiment

Timeline showing key events of the experiments. Replicated from Chapter 2: Methods, section 2.2.1

distributed into single housed conditions by basal activity into the exercise and sedentary groups.

After one month, all of the mice were subjected to a series of up to 4 behavioural tests carried out over consecutive days, performed in the order of elevated plus maze, open field, novel object recognition and finally spontaneous alternation. There was alternation between the exercise and sedentary groups in the order of performing the tests to prevent timing bias. All of the tests were started approximately 2 hours after the change in light/dark phase for both groups. There was a separate group of mice who performed the EPM trials in the light, causing the increase in numbers for that group. For more details on how the behaviour tests were performed and analysed, see 'Chapter 2: Methods'.

3.2.2 Data analysis

All statistical analysis was performed using GraphPad Prism 7.00, with each data set analysed with two-way-ANOVA and Tukey's multiple comparisons where appropriate. Anomalies were calculated as data points further than two standard deviations from the mean and were removed from analysis. All graphical data is presented as mean ±SEM.

3.3 Results

3.3.1 The effect of light cycle on running distance in the mouse

Mice that had free access to the running wheels for voluntary exercise ran on average 3-12km per 24 hours (Figure 3.2). There was no difference in running distances between the two light-dark cycle groups. The mice ran on the wheels from the first day they were given access. The majority of running most likely occurred during the dark phase of the light-dark cycle. The mice showed on average a steady increase in running distance over the first 10 days of the experiment before reaching a plateau. All mice

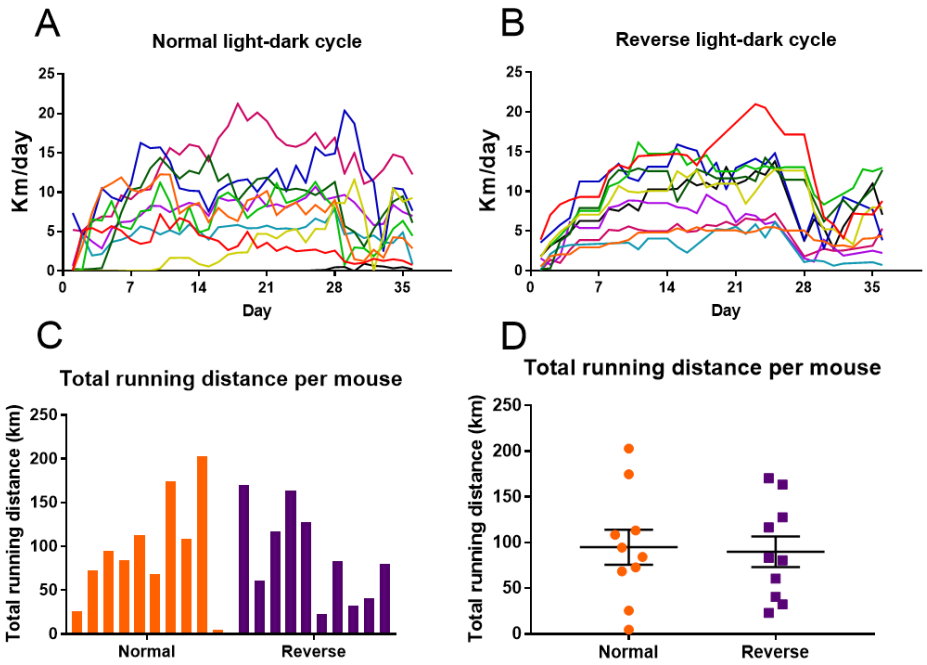


Figure 3.2: Running data for mice under normal or reverse light-dark cycle

Running distance data (km/day) for male C57BL/6 mice under reverse and normal light cycles over 36 days. Behaviour tests were performed from day 28 to 32, which is reflected in the decrease in running distance at that time. A) Running distance for mice tested under reverse light cycle conditions. B) Running distance for mice tested under normal light cycle conditions. C) Total running distance (km) per mouse. D) Comparison of mean average total running distance per light cycle group ±SEM.

showed a significant drop in running distance from day 28-32, due to disruption caused by the behaviour tests being performed.

3.3.2 The influence of light cycle on behaviour in a mouse model of voluntary exercise

3.3.2.1 Elevated plus maze

A number of differences were seen in the behaviours displayed by the mice that were tested under white light in comparison to those tested in the dark. When tests were performed in the dark, on average the mice spent significantly more time in the open arms of the arena compared to the mice tested in the light (two-way-ANOVA P=0.0010** Figure 3.3A). Tukey's multiple comparisons post hoc analysis showed that mice that were tested in the light phase and had exercised with a running wheel spent significantly less time in the open arms of the maze than the mice that remained sedentary and were also tested in the light (two-way-ANOVA Tukey's multiple comparisons P=0.0264*).

No significant difference was seen in the percentage time spent performing stretch attenuated postures (SAPs) between the groups. However, the groups tested in the light performed a significantly higher number of SAPs compared to the groups tested in the dark (two-way-ANOVA p=<0.0001**** Figure 3.3D). There was a significant increase in the percentage time spent dipping the head over the side of the maze (two-way-ANOVA p=<0.0001**** Figure 3.3E) and the number of head dips performed (two-way-ANOVA p=0.0003*** Figure 3.3F) in the group tested in the dark compared to the one tested in the light. There was no significant effect of exercise in either measure. Overall, mice that were tested in the dark phase travelled significantly further during the test than

those tested in the light phase (two-way-ANOVA p=0.0044** Figure 3.3B). There was no significant effect of exercise in either light phase group.

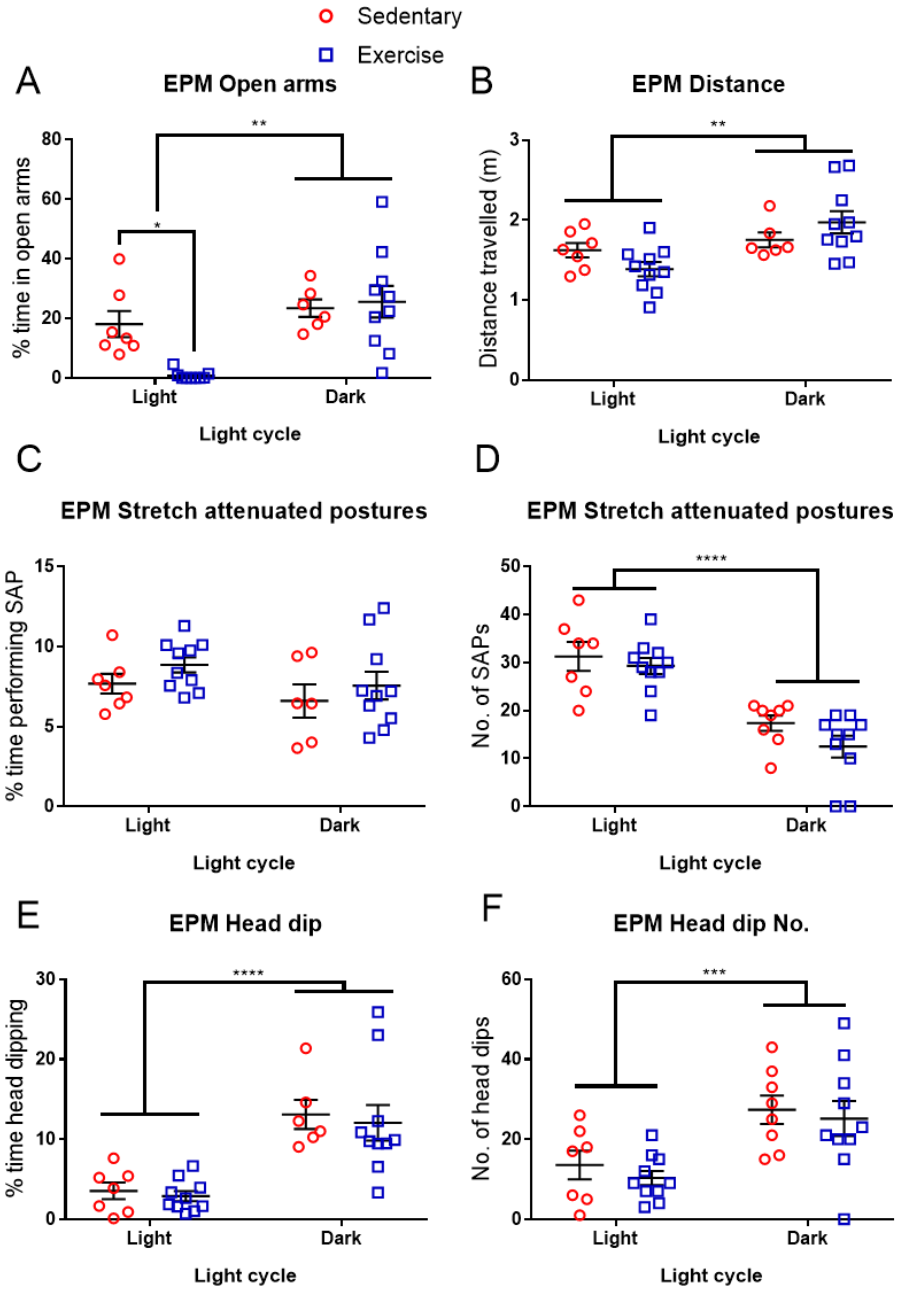


Figure 3.3: Data from EPM tests for mice tested under white light or in the dark that exercised or remained sedentary

Data from EPM tests on mice performed under white light (n=17) and in the dark (n=18). Individual values, group, mean and ±SEM bars are displayed. Mice that exercised (blue) and remained sedentary (red) are shown. A) Percentage of total test time spent in the open arms of the maze. B) Total distance travelled (m). C) Percentage of total time spent performing SAPs. D) Number of SAPs performed during the test. E) Percentage of total time spent dipping the head over the edge of the maze. F) Number of times head dipped over the edge of the maze.

3.3.2.2 Open field test

There were no significant differences seen between any of the groups for any of the parameters looked at in the OF test. Mice that had exercised and then tested in the light showed a strong tendency to spend more time in the corners of the maze than the sedentary mice tested in the light (p=0.0568). This difference is reduced in the mice that were tested in the dark (Figure 3.4A). Similar results are seen with the percentage time spent next to the walls of the arena (Figure 3.4B).

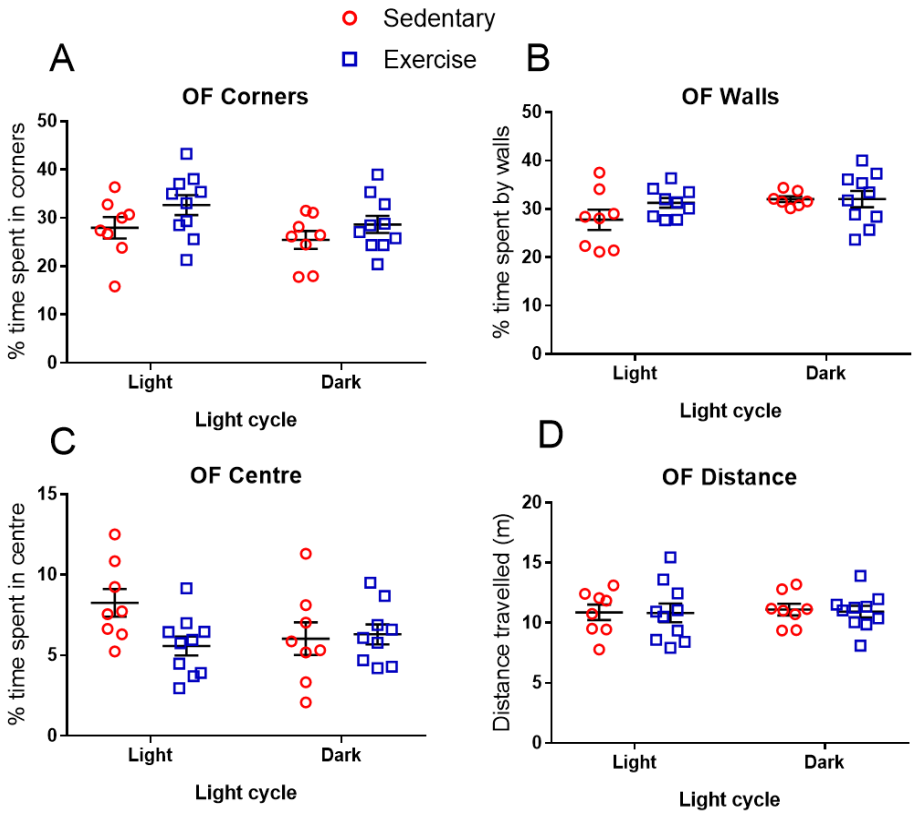


Figure 3.4: Data from OF test under white light or in the dark for mice that performed voluntary exercise or remained sedentary

Data from OF tests on mice performed under white light (n=18) and in the dark (n=18). Individual values, group, mean and SEM bars are displayed. Mice that exercised (blue) and remained sedentary (red) are shown. A) The percentage of the total test time spent in the corners of the arena. B) The percentage of the total test time mice spent beside the walls of the arena. C) The percentage of the total test time mice spent in the centre of the arena. D) Total distance travelled (m) by each mouse during the test.

In the mice tested in the light, there is a strong tendency towards the exercise group spending less time in the centre of the arena in comparison to the sedentary group (two-way-ANOVA p=0.0788 Figure 3.4C). This trend is not present in the mice tested in the dark, with both the exercised and sedentary groups spending less time in the centre of the arena overall compared to the sedentary group tested in the light.

The total distance travelled during the test was not affected by the phase of the light/dark cycle or exercise (Figure 3.4D).

3.3.2.3 Novel object recognition test

During the 5-minute familiarisation trial, mice tested in the dark spent significantly more time investigating the two objects in comparison to the mice tested in the light (two-way-ANOVA p=<0.0001**** Figure 3.5A). There was no significant preference for either of the two objects used in this trial. When looking at the time spent investigating the familiar vs novel object, only the exercise group tested in the dark show a significant increase in the time spent investigating the novel object vs the familiar one (Two-way-ANOVA object p=0.0193* multiple comparisons p=0.0488* Figure 3.5B). For the groups tested in the light, the sedentary mice spent significantly more time exploring both objects in comparison to the exercise group (Two-way-ANOVA group p=0.0124* multiple comparisons p=0.0013** Figure 3.5B). However, when calculating the ratio of investigation there was no significant difference in ratio between any of the groups (Figure 3.5C). There was also no significant difference in distance travelled between light cycle or exercise state groups (Figure 3.5D).

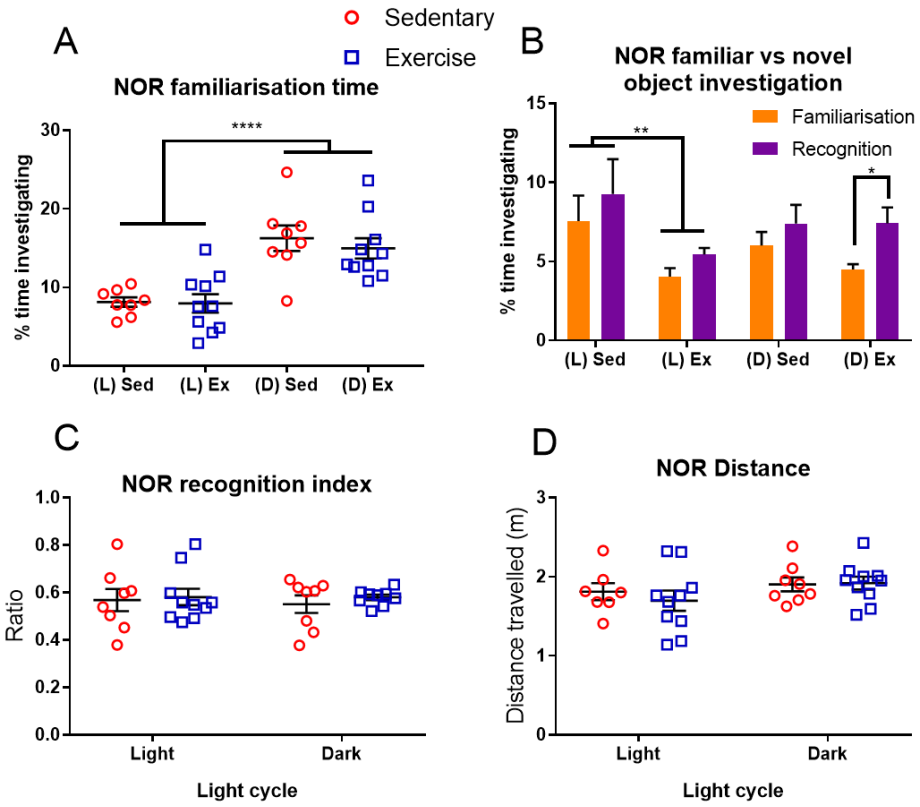


Figure 3.5: Data from NOR test under white light or in the dark for mice that performed voluntary exercise or remained sedentary

Data from NOR tests on mice performed under white light (n=18) and in the dark (n=18). Individual values, group, mean and ±SEM bars are displayed. Mice that exercised (blue) and remained sedentary (red) are shown. A) Percentage time spent exploring objects during the familiarisation trial. B) Percentage time spent exploring the familiar and novel object during the recognition trial. C) Recognition index from recognition trial. D) Distance travelled during recognition trial.

3.3.2.4 Spontaneous alternation Y maze test

The SA tests were performed both before and one month after the mice were housed with the running wheels. The first test was done to ensure that both of the exercise and sedentary groups had mice with a similar basal activity and cognitive ability.

There was no significant change in the alternation index from before being housed with the wheels and after (Figure 3.6A,B).

When focusing on the results for alternation ratio after one-month access to the wheels, there was no significant differences between the exercise and sedentary groups under either light cycle (Figure 3.6C).

However, the total number of arm entries was significantly higher in mice on the reverse light cycle compared to those on the normal light cycle (two-way-ANOVA light phase P=<0.0001****) (Figure 3.6D). This is the same for the distance travelled by the mice (Figure 3.6E), and there is a strong correlation between distance travelled and total number of entries (Figure 3.6F).

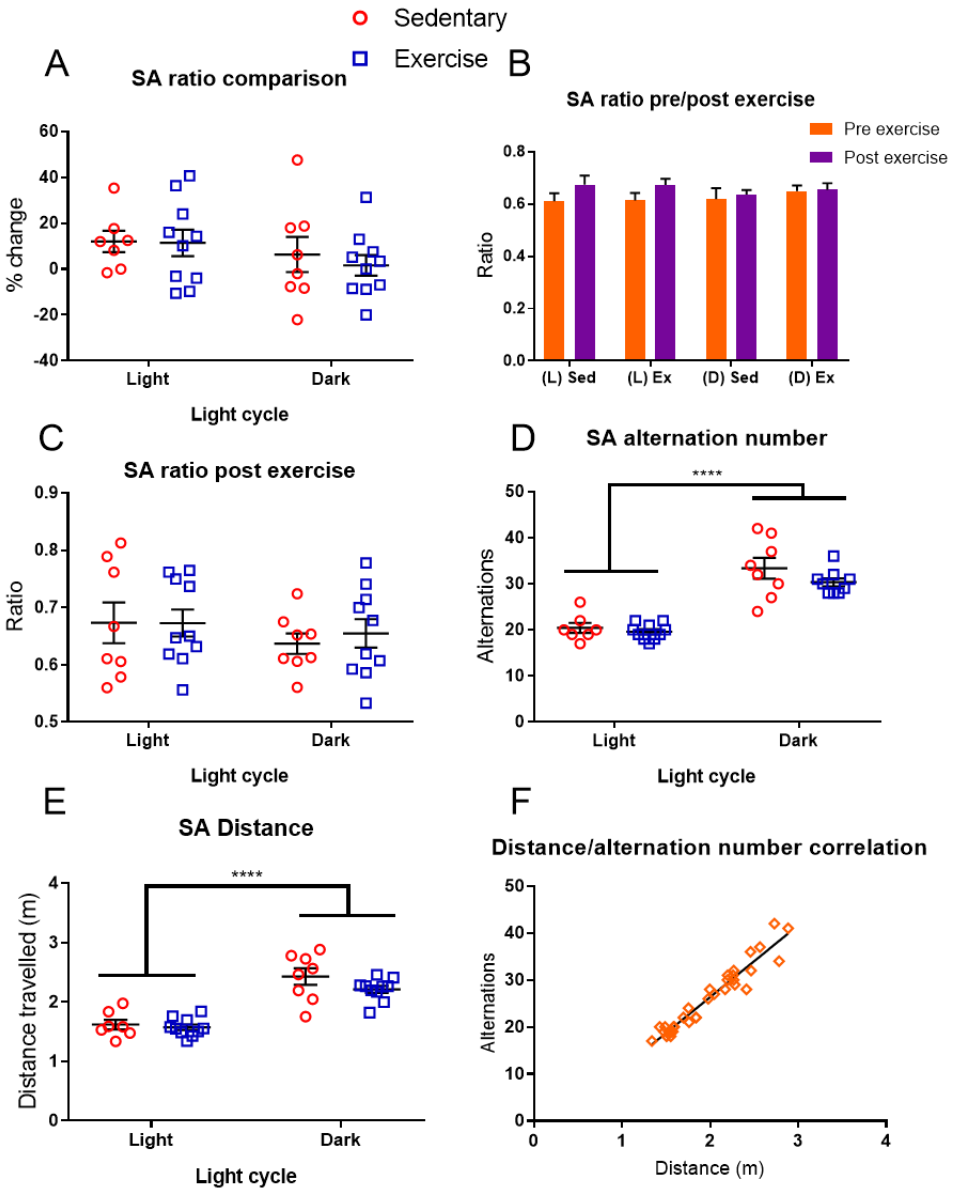


Figure 3.6: Data from SA test under white light or in the dark for mice that performed voluntary exercise or remained sedentary

Individual values, group, mean and ±SEM bars are displayed. Mice that exercised (blue) and remained sedentary (red) are shown. A) The percentage change in the SA ratio from before being housed with running wheels and the end of the experiment B) SA ratio for before and after the 28 days exercise or remaining sedentary. C) SA ratio for all groups after being housed with running wheels. D) Number of alternations after being housed with running wheels. E) Distance travelled during SA trial after being housed with running wheels. F) Correlation of distance travelled and number of alternations in the SA test post housing with running wheels.

3.4 Discussion

In this study, we have shown that there is a potential for the outcome of behaviour tests for anxiety and cognition to be affected by the phase of the light/dark cycle under which the tests are performed. The largest effects were seen in the EPM and SA tests for anxiety and cognitive ability respectively. In both of these tests, mice tested in the dark showed a significant increase in levels of locomotor activity (LMA) during the test, regardless of whether they had access to a running wheel or not.

3.4.1 Anxiety behaviour

In our study we have found that mice tested during the light phase of the light-dark cycle can appear to be more anxious after one month of voluntary exercise compared to the sedentary mice tested under the same conditions. This was shown by significantly decreased exploration of the EPM open arms, a trend towards decreased time spent in the centre of the OF arena and a tendency towards increased time spent in the corners of the OF arena (Figure 3.3 and Figure 3.4). Our results are consistent with those of Fuss et al (Fuss, 2010; Fuss, Ben Abdallah, Hensley, *et al.*, 2010) who also tested mice during the light phase of the light-dark cycle, and those of Burghardt et al (2004) who performed similar tests in rats.

When we performed the same tests during the dark phase of the light-dark cycle, we no longer saw an apparent increase in anxiety in the exercise group in comparison to the sedentary. The mice in the voluntary exercise group spent a similar length of time in the open arms of the EPM, and beside the walls and in the centre of the OF arena to the mice in the sedentary groups. In comparison to the mice tested in the light, the mice in the voluntary exercise group seem less anxious when tested in the dark.

The Santos-soto group (2013) also performed behaviour tests on mice in the dark phase of the light-dark cycle. Their results showed a significant decrease in anxiety in the EPM when the mice were tested within an hour of the lights being turned off. Their group used a similar protocol to the one used in our own study. One main difference in the protocol however was that the Santos-soto group did not have equivalent environmental enrichment in the cages of the sedentary mice. In our study, we placed a dismantled wheel on the floor of the cages of the sedentary groups to control for the increased environmental enrichment. It is known that environmental enrichment can have a significant effect on mouse behaviour, including decreasing anxiety (Benaroya-Milshtein *et al.*, 2004). Therefore, the difference in anxiety reduction seen by our study and the Santos-Soto group could be explained by the difference in environmental enrichment levels.

Mice are known to be more active during the night than during the day, and the majority of the voluntary exercised is performed during the lights off phase of the cycle (File and Day, 1972; Pan-Vazquez, 2015). It is likely that behavioural tests performed on mice during the day disturbs their resting period. This disruption to their sleep/rest is likely to cause an increase in stress levels during that time. Mice with free access to a running wheel may be have a reduced interest in exploring the arenas of the behaviour tests after a night of running on the wheels.

Studies looking into the impact of sleep deprivation in mice on tests for anxiety have shown it to increase anxiety like behaviours (Silva *et al.*, 2004). Similarly, in human studies it has been shown that the negative impacts of sleep deprivation can be increased with regular bouts of exercise during the sleep deprivation period (Scott, McNaughton and Polman, 2006). A minor form of sleep deprivation may be present in the

mice tested during the day time due to the prolonged disturbance for each day of the behaviour tests. The known negative impacts of sleep deprivation include fatigue and increased reporting of depression.

Mice with an accessible running wheel in their cage spend a significant portion of the night actively using it. Due to this, we thought it possible that these mice have a higher level of fatigue during the daylight hours than the sedentary mice, making them less likely to walk around the behavioural test arenas. A decrease in LMA leading to a lack of exploration of the open arms of the EPM test can be interpreted as an increase in anxiety, which could be a false interpretation. In our study results for the EPM, there is no significant difference in the distance travelled between the sedentary and exercise groups under either light cycle. However, there was a significant increase in distance travelled and head dipping exploratory behaviours in mice tested in the dark compared to those tested in the light, suggesting that the phase of the light cycle can have an effect on the LMA levels in mice.

However, there are problems with this hypothesis. Firstly, there is no difference in the LMA in the OF test of this study. If fatigue was a significant influence on the behaviour of the mice, then we would have expected to see a trend or significant difference in the LMA in the OF tests between the exercise and sedentary groups. Second, there is evidence within the literature to suggest that fatigue does not play a significant part in increased anxiety as a result of exercise in mice (Duman, 2008; Fuss, 2010).

Some studies have suggested that the reported increase in anxiety behaviour displayed by mice tested in the light could be interpreted as an increase in risk-analysis and a reduction in impulsiveness (Binder, 2004; Burghardt, 2004; García-Capdevila, 2009). In essence, rather than being

more anxious, the mice could instead be interpreted as taking more time to analyse the situation and be overall less inclined to explore. This could explain the significant increase in head dipping EPM behaviours displayed by the mice in our study tested in the dark in comparison to those tested in the light.

3.4.2 Memory and cognition

Both the NOR and SA studies test the memory and cognitive ability of the mice. In our study, we found that mice tested in the dark spent significantly more time investigating the objects during the familiarisation trial than those tested in the light. When we then looked at the time spent investigating both the novel and familiar objects during the recognition trial, we found that, in the light, the exercise group investigated both objects significantly less than the sedentary group. However, in the dark we saw a significant increase in the time spent investigating the novel object compared to the familiar object in the exercise group. This suggests that exercise was having an impact on the ability of the mice to recognise the new object, but that this impact was affected by the light conditions. However, when we calculated the recognition ratio, we saw no significant effect of light cycle or exercise state. This suggests that the significant increase we found in recognition time for the exercise group tested in the dark is not very robust.

A number of other studies have shown that exercise has caused a significant increase in the time spent investigating the novel object during the test (O'Callaghan, 2007; Yuede, 2009). The differences between these results and our own could be explained by differences in the protocols. Both studies had a longer familiarisation period than our own study, with the Yuede study also having a longer recognition period. Both the O'Callaghan and Yuede studies comprised two sessions habituating the

mice to the arenas on the two days prior to the recognition test in a similar way to our own study. The O'Callaghan study then performed three sets of 5-minute familiarisation to the object sessions with two identical objects, and then performed one 5-minute recognition session 24 hours later. The Yuede study had a single 10-minute familiarisation trial followed by a short 50-minute break and then finally a 10-minute recognition trial. In our study, the mice were given 5 minutes to familiarise themselves with the objects, followed by a five-minute break and then a 5-minute recognition test. Any of these differences could have contributed towards the lack of significant differences seen between the exercise and sedentary groups in our study. Some of the differences in these protocols mean that the data collected are on different types of memory, for example the 24-hour gap between the familiarisation and recognition tests in the O'Callaghan study suggest a more long-term memory test in comparison to the shorter time gaps seen in the Yuede and our own studies.

The overall percentage time spent investigating either of the objects in our NOR study was very low, ranging from 6-20% of the total test time in all groups (not shown). This is likely due to the short familiarisation trial and short break between trials. The average distance travelled by each of the mice (Figure 3.5) was comparable to that found in both the EPM and SA tests, which suggests that fatigue is not to blame for this low investigation time.

There is a possibility that the appearance of the objects in the arena caused anxiety in the mice. They were used to an empty arena and only had a relatively short time to become used to the presence of the familiar objects. If the appearance of the objects caused an anxious response in the mice, it may have caused them to avoid the objects rather than investigate them. This would then explain the lack of overall time spent investigating

the objects. Alternatively, they could have simply lacked an interest in exploring the objects. One could argue that the objects we used might have been too similar to each other and thus, mice could have problems distinguishing between them. However, our study used a clear glass bottle full of red liquid and a white plastic deodorant bottle with a blue lid as the two objects, which have different shapes, colours and textures though were of a similar size. This should have increased the likelihood that the mice could distinguish a difference between them.

In the SA tests we saw a few differences between the tests under white light and the tests in the dark. When comparing the SA ratio of pre and post exercise, there was no significant changes in the scores for any of the mice. However, when looking at the data for the SA test after the month of running wheel access, we saw a small trend towards an increased score in the mice that had exercised when they were tested in the dark. The biggest change we see in the SA data is the number of arm entries performed by each mouse. The mice tested in the dark performed significantly more arm entries during the 5-minute test than the mice who were tested under white light. As we did not see an increase in the ratio scores to correlate with this, it suggests that the mice did not show improved memory when tested in the dark, and that in this study exercise did not have an effect.

However, exercise has been shown by multiple other studies to improve performance in numerous different memory and learning tasks. The van Praag group have shown improvements in the Morris Water maze (van Praag, 1999, 2005) and the Berchtold group showed similar improvements in the radial arm water maze (Berchtold, Castello and Cotman, 2010). The differences seen between our study's results and those in the literature could potentially be due to difference methodologies; both of these groups

used water maze tasks to analyse cognition. There is a chance that the protocols used in this study were not robust enough to see the expected significant differences.

3.4.3 Locomotor activity

Animals tested in the dark showed significantly increased locomotor activity in both the EPM and SA tests in comparison to those tested in the light. Exercise did not have any significant effects on the activity levels in the mice in any of the tests. However, we did see a trend towards mice that have exercise and tested in the EPM in the light showing lower levels of locomotor activity than the sedentary group. This trend was then reversed when they are tested in the dark. We did not see this in any of the other tests.

In the SA test, there was a correlation between the number of arm entries made by the mice and the distance travelled (Figure 3.6E). As we did not see any improvement in the SA ratio scores for these mice, it can be presumed that the mice were simply more active and did not show an improvement in memory when tested in the dark when compared to those tested in the light.

The data from the EPM and SA tests suggest that the mice tested in the dark were overall more active than those tested in the light. However, because we did not see this difference in the 30-minute-long OF test for locomotor activity, this can only be said for the shorter behaviour tests. We don’t see a similar increase in locomotor activity in the NOR tests. This could be due to the mice having already been in the arena for 5 minutes during the familiar object trial. As the mice have therefore already been active for over 5 minutes, the difference in activity levels are not seen. A possible explanation for these differences is the mice on the reverse light

cycle are more likely to already be awake and alert at the time they are disturbed for the behaviour tests, so they are likely to be more active. The mice on the normal light cycle are more likely to be sleeping or resting when they are disturbed for the behaviour tests, and so are likely to take longer to become fully alert and active. After 5-10 minutes, these mice are then fully awake and alert, so the initial difference in activity levels is no longer seen in the longer tests.

In our study, we did not see any effect of the availability of a running wheel on the distances travelled in any of the behaviour tests. If there had been excess fatigue in the exercise groups, we would have expected to see lower levels of LMA in these mice compared to the sedentary groups under both light cycle conditions. As this is not the case, it suggests that excess fatigue is not present in the exercise groups.

3.5 Conclusions

We have observed that the phase of the light/dark cycle had some significant effects on the results of behaviour tests for anxiety, cognition and locomotor activity. Therefore, when performing behaviour test the phase of the light/dark cycle should be taken into consideration as they may affect the data produced. We saw the largest effect on anxiety in mice that had voluntary access to a running wheel, and in locomotor activity in short, 5-minute duration experiments. Differences in protocols for each behaviour test could explain differences seen between this study and other studies performed in the literature.

4. Chapter 4:

The effect of exercise on ageing

4.1 Background

Ageing is a natural process undergone by nearly every living organism. The progression of ageing is complex, and it is significantly influenced by the individual's genetics, actions and surrounding environment throughout the lifespan. In humans, old age is associated with illness and other health problems, both mental and physical. Healthy ageing is defined as ageing with an absence of illness or health related problems and can be promoted by a calorie restricted (CR) diet and regular exercise.

It has been demonstrated that CR can both extend the maximum lifespan of an organism and significantly delay the onset of ageing phenotypes and disease in models such as rodents and primates (Masoro, 2005; Colman *et al.*, 2009; Mattison *et al.*, 2017). Evidence suggests that CR exerts its effects on ageing via the NAD^+ dependent deacetylase SIRT1 (Cantó, 2009; Mercken, 2014; Mitchell, 2014). SIRT1 is a known histone deacetylase, and its increased activity with CR causes changes in the expression of a number of genes, including p53 (Li, 2011). Overexpressing the SIRT1 gene has been shown to extend maximum lifespan and delay ageing phenotypes in a similar way to CR (Bordone *et al.*, 2007).

SIRT1 requires the presence of NAD^+ as a coenzyme for it to function. NAD^+ is synthesised via one of two pathways, *de novo* synthesis or salvage pathways. Key enzymes in the pathway include the proven NAD^+ level limiting NAMPT (Imai, 2010), and the final enzyme in both pathways NMNAT. In our study we looked to see whether the expression of any of the key enzymes involved in NAD^+ biosynthesis, including SIRT1, was affected by exercise. We have previously shown that the expression of these genes is affected by one month of voluntary exercise in young mice (unpublished).

Regular exercise is also a part of a healthy lifestyle, and it can reduce the risk of developing age related illness such as cardiovascular disease (Manson *et al.*, 1999; Hillman, 2008; Hayes, Alosco and Forman, 2014) and have significant neuroprotective effects against dementia and other brain diseases associated with age (Ahlskog *et al.*, 2011; Paillard, Rolland and de Souto Barreto, 2015). Cognitive decline is strongly associated with ageing, and exercise has been shown to slow down its progression in older individuals (Colcombe, 2003). In both rodent and human studies, significant improvements in the results of cognitive tests have been demonstrated when comparing before and after the individual has exercised for a period of time (van Praag, 2009; Herting, 2012; Bechara, 2013). Exercise is also known to reduce anxiety and depression levels in humans with diagnosed mild anxiety and depressive disorders (Stubbs, 2017), though in more serious cases exercise is not as effective as pharmaceutical treatments (Carek, Laibstain and Carek, 2011; Jayakody, 2014).

The improvements in cognition that have been associated with exercise have also been connected with increased adult neurogenesis in the dentate gyrus (DG) of the hippocampus (Van Praag, 2008). Adult neurogenesis is the broad term covering the proliferation, differentiation and maturation of new cells within the adult brain. There is evidence of adult neurogenesis occurring in two areas of the brain: the DG and sub-ventricular zone (SVZ) of the hippocampus (Eriksson, 1998). The different stages of neurogenesis can be analysed using different cell markers. Proliferating cells in the hippocampus express the nuclear protein Ki67 in all the stages of cell division bar the resting phase. Immature neuronal cells express the microtubule associated protein doublecortin (Dcx) once they have committed to a neuronal cell fate until they become mature integrated

cells. Finally, cell survival can be studied with injection of the exogenous thymine analogue bromodeoxyuridine (BrdU), which is incorporated into the cells' DNA in the S-phase of mitosis. After proliferation, cells take around 28 days to fully mature and integrate into the brain. Injection of BrdU 28 days before euthanasia of an animal model allows us to quantify the number of cells that survived this time. In order to see whether the cell has differentiated and what cell fate has been determined, duel staining techniques are needed. This was not done in this study.

Studying aspects of ageing in humans, such as adult neurogenesis levels, can be quite challenging, and often has a number of ethical issues associated with it. A relatively new and minimally invasive method of monitoring the ageing process is looking at blood biomarkers of ageing. miRNAs are small non-coding RNA strands whose role is epigenetic modification of gene expression throughout the body. Some miRNAs are found in blood plasma, and could be used as potential blood biomarkers of ageing and disease (Olivieri *et al.*, 2017). In our study, we have looked at 7 miRNAs present in blood plasma that are known to have a role in ageing or age-related illness and are affected by exercise.

4.1.1 Aims and objectives of the chapter

The aim of this chapter is to investigate the effects of 6 months access to a running wheel on behaviour, adult neurogenesis, circulating miRNA expression and hippocampal gene expression in aged C57BL/6J mice. We also wish to investigate the extent to which adult neurogenesis is affected by age by comparing data from young and aged C57BL/6J mice that have not exercised.

We hypothesise that aged mice that have exercised will:

- Show decreased anxiety, and increased cognition and locomotor activity in behavioural tests comparing results before and after 6 months voluntary exercise

- Have increased cell proliferation, immature neuron numbers and 28-day cell survival numbers in the dentate gyrus and subventricular zone

- Have upregulation of specific miRNAs in blood plasma

- Have increased expression of epigenetic enzymes and genes associated with the NAD+ biosynthetic pathway

We hypothesise that aged mice will have significantly decreased cell proliferation, immature neuron numbers and 28-day survival rates compared to young mice.

4.2 Methods

For more detailed descriptions of the methods used, see chapter 2.

4.2.1 Study protocol

Male C57BL/6J mice were used for all experiments in this chapter.

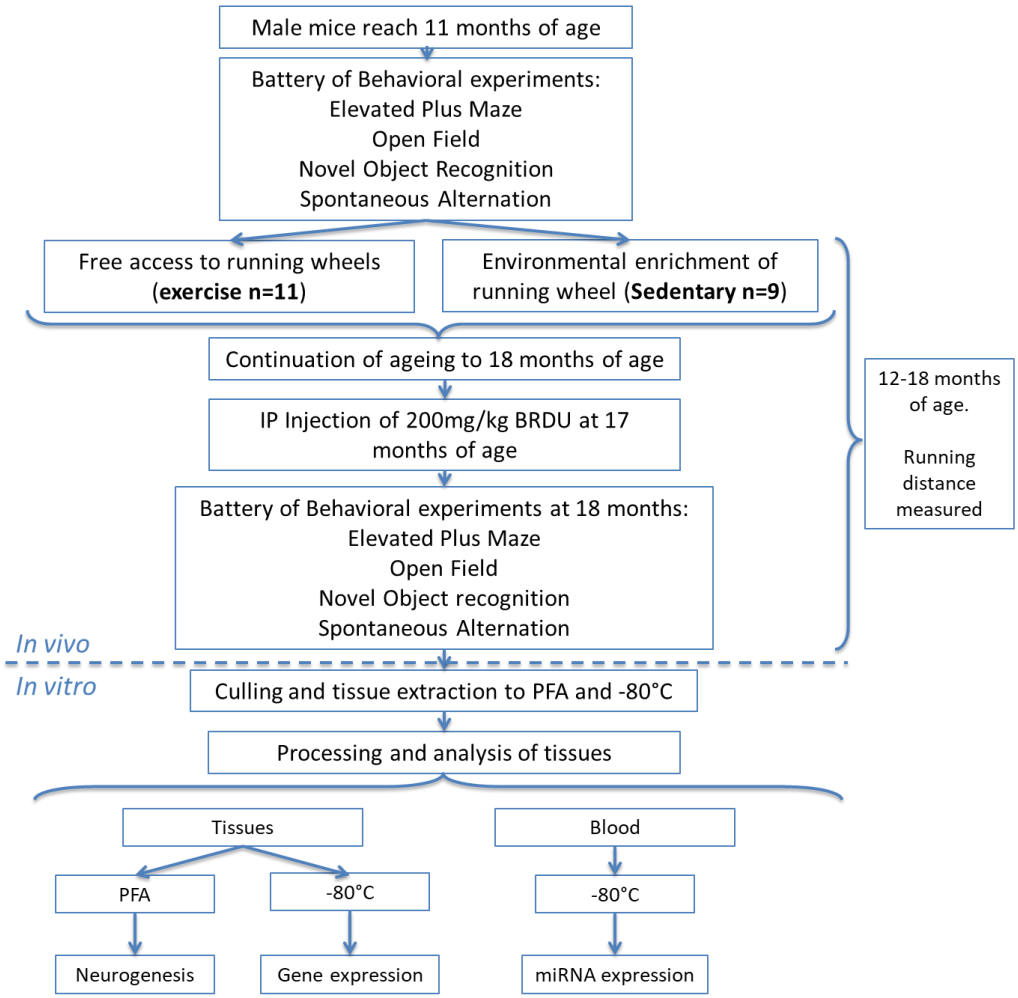


Figure 4.1: Timeline of experiments for aged WT mice with or without access to a running wheel

Flow diagram for ageing WT mice with or without free access to a running wheel, showing key events in the study.

4.2.1.1 *In vivo* work

	WT	Het	TG	Total
Exercise		4 (4)		4 (4)
	2 (2)	2 (2)		4 (4)
	2 (2)	2 (2*)		4 (4*)
	1 (1)	3 (2)		4 (3)
			4 (3)	4 (3)
			4 (3)	4 (3)
	2 (2)		2 (2)	4 (4)
	2 (2)		1 (1*)	3 (3*)
	1 (1)		3 (3*)	4 (4*)
	1 (1)		3 (2)	4 (3)
Sedentary		2 (1)		2 (1)
		4 (3*)		4 (3*)
	2 (2)	2 (1)		4 (3)
	3 (3)	1 (1)		4 (4)
			4 (2)	4 (2)
			2 (2)	2 (2)
	2 (1)		2 (2*)	4 (3*)
	3 (3)		1 (0)	4 (3)
	1 (0)		2 (2)	3 (2)

Table 4.1: Animal numbers in cages at the beginning and end of the study

Table showing cage numbers at the beginning and end of the exercise study. Numbers decrease due to natural deaths. * denotes a mouse was used for tissue analysis but not for behaviour tests due to death occurring during or just before the behaviour tests.

In the ageing experiment, mice were group housed (2-4 per group Table 4.1) from weaning until 18 months of age when they were euthanised using S1 techniques. Mice were group housed with mice of either NMNAT1 Het or NMNAT1 TG genotypes due to in house breeding and needing to ensure that no mice were single housed. Each row shows the genotypes present in each cage. Approximately half of the mice were given free access to a running wheel from 12-18 months of age, the rest had a broken wheel in the cage to control for environmental enrichment. Mice were injected IP with 20mg/kg BrdU at 17 months of age.

Mice underwent a series of 4 behavioural tests, first at 11 months of age and then again at 18 months of age after 6 months of either voluntary exercise or remaining sedentary. The behavioural tests were performed during one week in the following order: elevated plus maze (EPM), open field (OF), novel object recognition (NOR) *and spontaneous alternation Y maze* (SA). The results from before and after the 6 months running wheel access for each mouse were compared. Only data for the mice that survived until the tests at 18

months are included. EPM and OF tests were used to test for anxiety, NOR and SA tests were used to test cognitive ability.

Young male and female mice were group housed at weaning and injected IP with 20mg/kg BrdU. They were then housed for a further 28 days before being euthanised using S1 techniques. These mice were used as a control for neurogenesis in ageing.

4.2.1.2 *In vitro* work

After euthanasia, brain tissue and blood plasma were immediately extracted and processed. For analysis of neurogenesis levels in the hippocampus of both the young and aged mice, the right hemisphere from each mouse brain was embedded in paraffin and sectioned. These were then stained for Ki67, Dcx and BrdU (18-month-old mice only), and the number of stained cells/mm^2 was quantified.
The hippocampal tissue from the left brain hemisphere was immediately snap frozen following extraction and later processed using TRIzol. RNA was extracted, and the expression levels of the following genes were then analysed using Qpcr: NMNAT1, NMNAT2, NAMPT, SIRT1, Utx, Ezh2 and Bdnf.

Blood plasma was extracted via cardiac puncture from the 18-month-old mice, and miRNAs were extracted using the Qiagen miRNeasy serum/plasma kit. The levels of 7 miRNAs were analysed: miR-30e-5p, miR-148b-3p, miR-222-3p, miR-21-5p, miR-20a-5p, miR-221-3p and miR-92a-3p.

4.2.2 Data analysis

Data presented in this chapter for the behavioural tests is the percentage change in the results from the 11 months tests to the 18 months tests,

before and after the 6 months voluntary access to a running wheel. This is calculated by: $\left(\frac{18mth-11mth}{11mth}\right) * 100$

All statistical analysis was performed using GraphPad Prism 7.00, with each data set analysed with one-way-ANOVA and multiple comparisons where appropriate. Anomalies were calculated as data points over two standard deviations away from the mean and removed from the analysis. All graphical data is presented as mean ±SD.

4.3 Results

4.3.1 Body weight change

Both the sedentary and exercise groups showed an overall increase in body weight from 12 to 18 months of age (Figure 4.2). The level of weight variance within each group increases with age, and the largest individual increases in body weight were in the sedentary group (up to 30% increase in two cases).

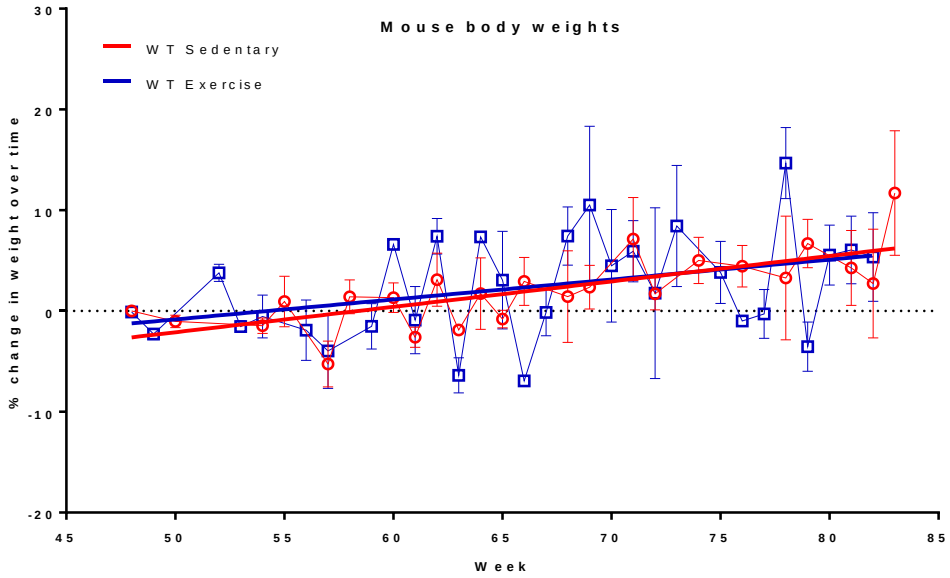


Figure 4.2: Body weight change in ageing male WT mice

Data for percentage change in body weight in ageing male WT mice from week 48 to 83 of the experiment. Mean ±SEM is shown for each time point for mice that had free access to a running wheel or remained sedentary for 6 months (12-18 months of age).

4.3.2 Behaviour tests for cognition

No significant differences were seen in cognitive ability between mice that had voluntary access to a running wheel for 6 months from 11 months of age and those that remained sedentary for the same time.

In the NOR ratio there was a significant effect of age (Two-way-ANOVA repeated measures F(1,17)=8.983 p=0.0081** Figure 4.3A), but no significant effect of exercise and no significant interaction. We also saw a similar age related effect during the first minute of the test (Two-way-ANOVA repeated measures F(1,16)=5.066 p=0.0388* Figure 4.3C). There

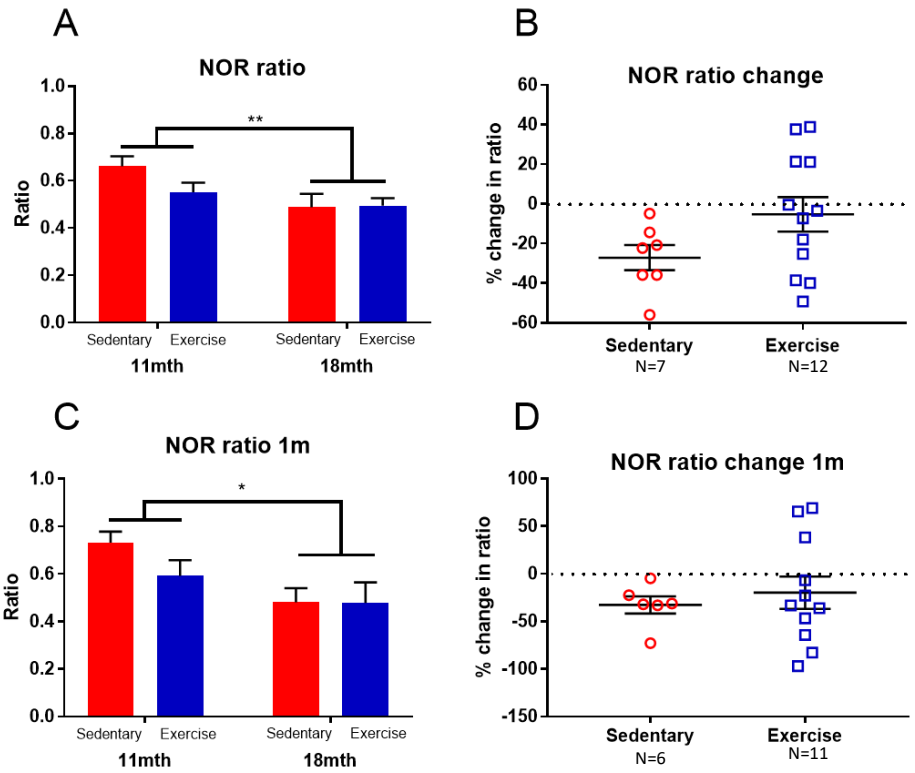


Figure 4.3: NOR cognitive behavioural data for WT mice

NOR data for WT mice at 11 and 18 months of age who remained sedentary (red) or had free access to a running wheel (blue). Mean and ±SEM bars are shown. A) NOR ratio for sedentary and exercise groups at 11 and 18 months of age. B) Percentage change with age in NOR ratio for sedentary and exercise groups. C) NOR ratio during the first 60 seconds of the test for sedentary and exercise groups at 11 and 18 months of age. D) Percentage change with age in the NOR ratio in the first minute of the test.

was a mild trend towards the exercise group maintaining a higher ratio with age in comparison to the sedentary group (t(17)=1.748 p=0.0985

Figure 4.3D), but this change was not apparent during the first minute of the test. There was a mild trend towards the exercise group maintaining a higher recognition index with age in comparison to the sedentary group (t(17)=1.748 p=0.0985 Figure 4.3B)

There was no significant change in SA ratio for either group with age Figure 4.4A,B). There was a mild trend towards the exercise group maintaining a higher ratio with age in comparison to the sedentary group (t(18)=1.786 p=0. 0910 Figure 4.4C,D). There was a significant change in the number of alternations performed by matching subjects (Two-way-ANOVA Repeated measures F(18,18)=2.482 p=0.0307*), but there was no significant effect of age or exercise group.

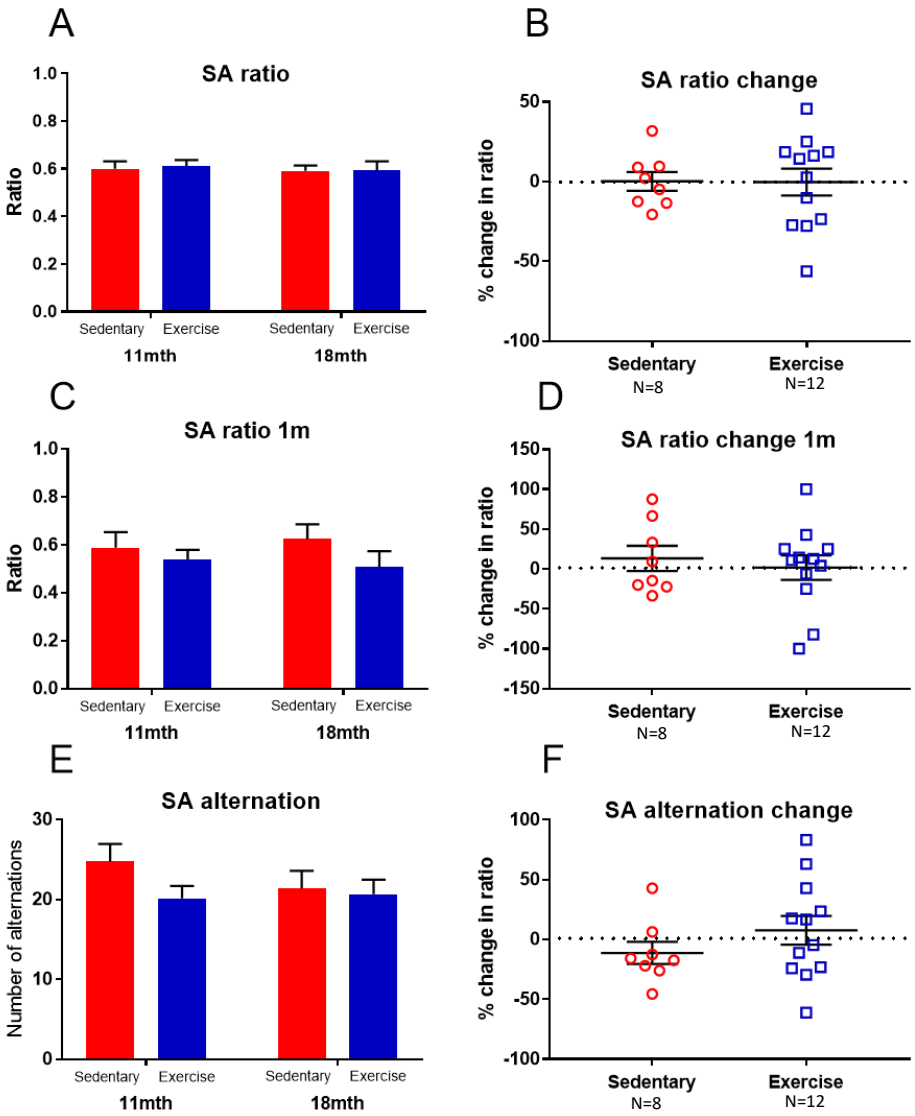


Figure 4.4: SA cognitive behavioural data for WT mice

SA data for WT mice at 11 and 18 months of age who remained sedentary (red) or had free access to a running wheel (blue). Mean and ±SEM bars are shown. A) SA ratio at 11 and 18 months of age. B) Percentage change with age in SA ratio. C) SA ratio during the first 60 seconds of the test at 11 and 18 months of age D) Percentage change with age in the SA ratio in the first minute of the test. E) Number of alternations performed at 11 and 18 months of age. F) Percentage change with age in alternations

4.3.3 Behavioural tests for anxiety

6 months of access to a running wheel had no significant effect on the results of EPM (Figure 4.5) and OF (Figure 4.6) tests for anxiety in mice.

There was a significant decrease with age in time spent in open arms (Two-way-ANOVA RM $F(1,16)=9.232$ $p=0.0078$**), SAPs (Two-way-ANOVA RM $F(1,16)=12.13$ $p=0.0031$**) and head dipping behaviour (Two-way-ANOVA RM $F(1,16)=30.82$ $p=<0.0001$****) in the EPM (Figure 4.5A,C,E). There was no effect of exercise or any significant interaction. There was no significant effect of exercise in the percentage change in any of the EPM results (Figure 4.5B,D,F).

There was a significant decrease in the amount of time spent in the centre of the OF arena with age (Two-way-ANOVA RM $F(1,17)=5.133$ $p=0.0368$* Figure 4.6A). There was no significant effect of exercise on the results of the OF test.

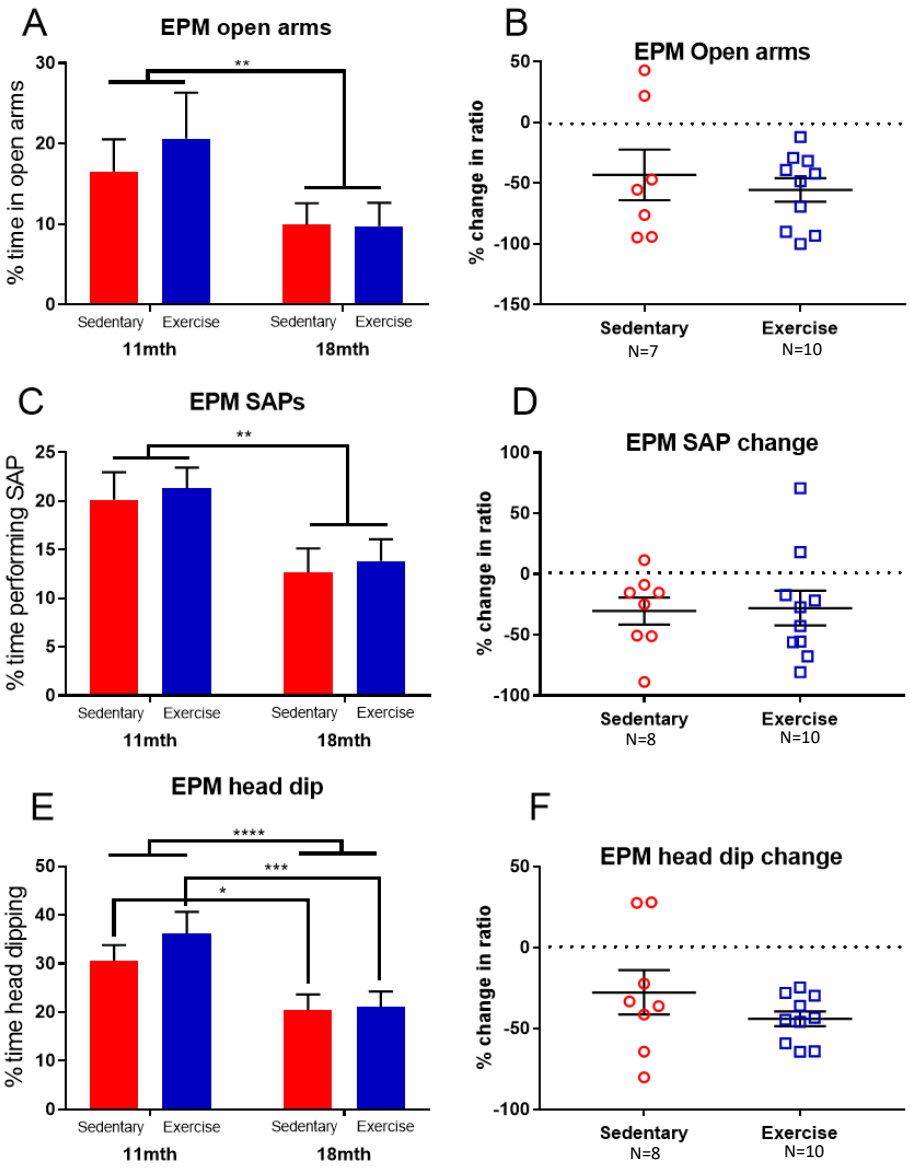


Figure 4.5: EPM behavioural tests for anxiety in WT mice

EPM behavioural data at 11 and 18 months of age for anxiety in male WT mice who remained sedentary (red) or had free access to a running wheel (blue) for 6 months. Mean and ±SEM bars are shown. A) Percentage time spent in the open arms of the EPM arena. B) Percentage change with age of time spent in the open arms of the EPM. C) Percentage time performing SAPs. D) Percentage change in time performing SAPs with age. E) Percentage time performing head dips. F) Percentage change in time spent performing head dips with age.

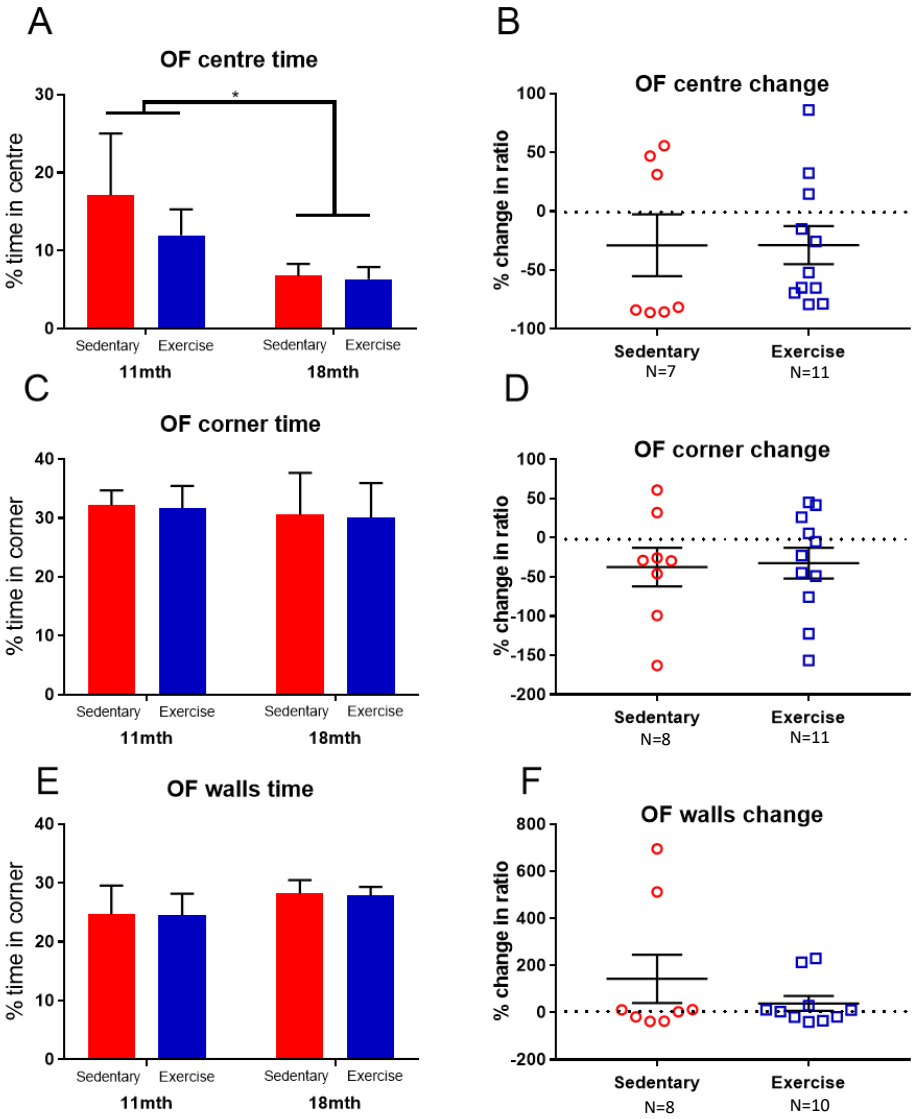


Figure 4.6: OF behavioural data for anxiety in WT mice

OF behavioural data at 11 and 18 months of age for anxiety in male WT mice who remained sedentary (red) or had free access to a running wheel (blue) for 6 months. Mean and ±SEM bars are shown. A) Percentage time spent in the centre of the OF arena. B) Percentage change with age of time spent in centre of the arena. C) Percentage time spent in the corners of the OF arena. D) Percentage change in time spent in the corners of the OF arena with age. E) Percentage time spent beside the walls of the arena. F) Percentage change in time spent beside the walls of the arena.

4.3.4 Behaviour tests for locomotor activity

Locomotor activity levels were measured by the distance travelled by the mouse in each of the behavioural experiments performed. Exercise did not have a significant effect on the locomotor activity of the mice in any of the four behavioural tests performed (Figure 4.7). In the EPM, mice had an overall decrease in locomotor activity with age, whilst in the other behavioural tests the distance travelled either stayed the same or mildly increased.

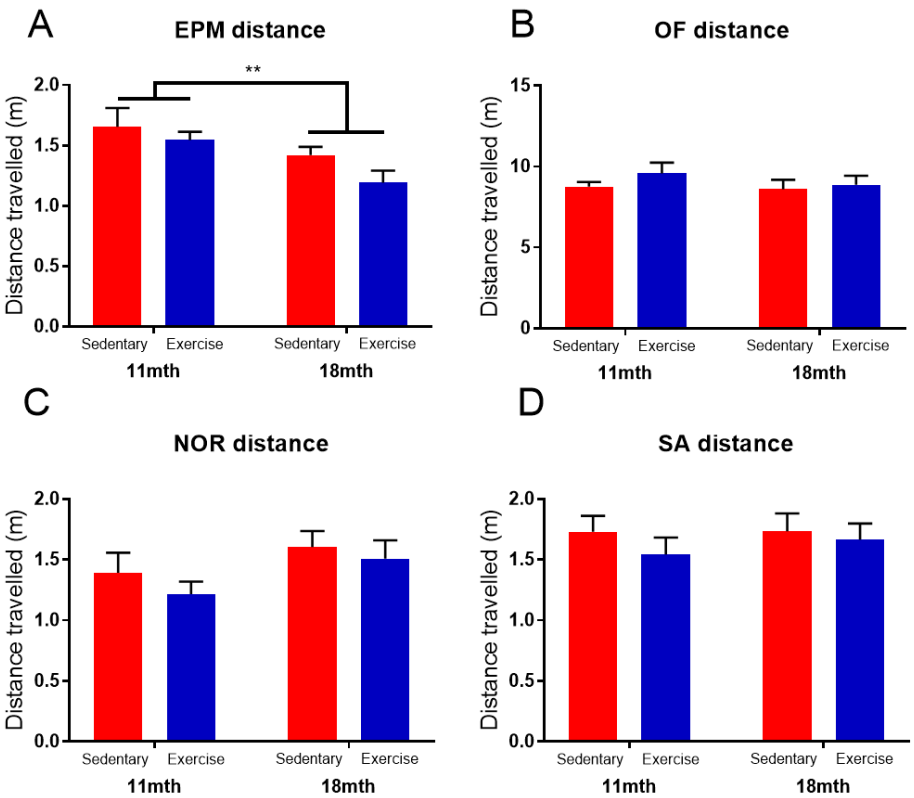


Figure 4.7: Locomotor activity from behavioural tests in WT mice

Locomotor activity data from behaviour experiments. Mean and ±SEM bars are displayed. Mice that had access to a running wheel for 6 months and their sedentary controls are shown for tests at 11 months and 18 months of age. A) Change in the locomotor activity from the EPM experiment. B) Change in the locomotor activity from the OF experiment. C) Change in the locomotor activity from the NOR experiment. D) Change in the locomotor activity from the SA experiment.

4.3.5 Results: The effect of exercise on neurogenesis in an ageing mouse model

Adult neurogenesis was measured in the DG of the hippocampus Figure 4.8A) and the SVZ (Figure 4.8B) in the brains of 18-month-old mice who had either had access to a running wheel for the last 6 months of life or remained sedentary throughout their lives.

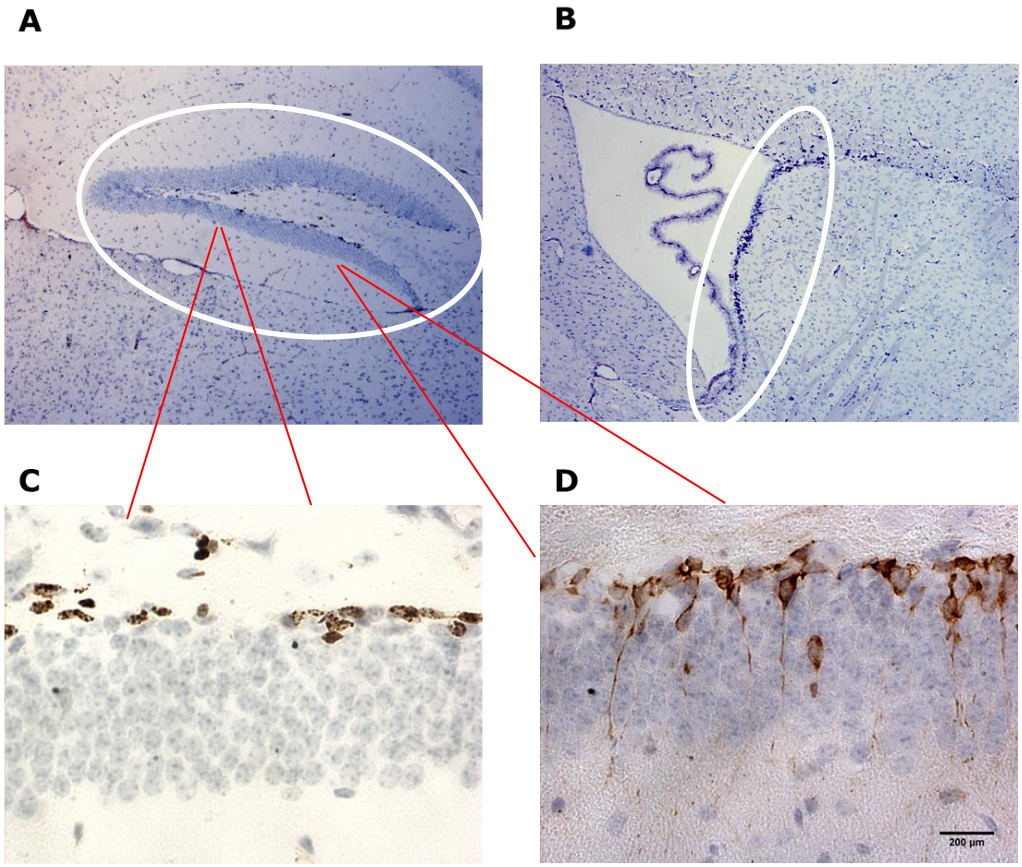


Figure 4.8: Images of neurogenesis staining in the mouse brain

Images of the mouse DG (A) and SVZ (B) with white circles indicating the area of interest. Example images of Ki67 (C) and Dcx (D) staining within the mouse DG, with brown cells showing a positive marker. Blue background colour is an artefact of H&E counterstaining and image analysis. Images taken with a 5x objective (A and B) and a 40x objective (C and D).

4.3.6 Results: Changes in adult neurogenesis with exercise

There were no significant differences in the number of proliferating (Ki67 positive) cells/mm^2 quantified in the DG (Figure 4.9A) or SVZ (Figure 4.9B) in mice with access to a running wheel for 6 months in comparison to mice that remained sedentary. Overall, more Ki67 positive cells/mm^2 were found in the SVZ than in the DG.

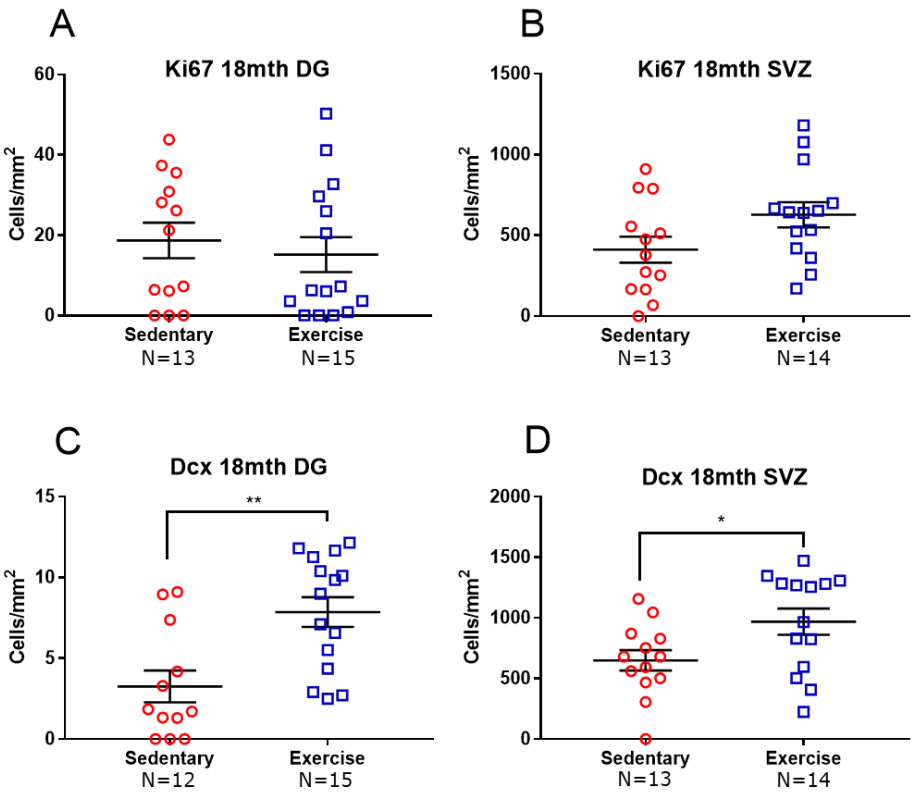


Figure 4.9: Effect of exercise on cell proliferation and immature neuron numbers in the DG and SVZ of WT mice

Data from 18 month old WT mice who had access to an exercise wheel for 6 months or remained sedentary for the same time. Mean and ±SEM bars are displayed. Data shows average number of cells/mm². A) Number of Ki67 positive stained cells/mm² in the DG. B) Number of Ki67 positive stained cells/mm² in the SVZ. C) Number of Dcx positive stained cells/mm² in the DG (t-test **P=<0.01). D) Number of Dcx positive stained cells/mm² in the SVZ (t-test *P=<0.05).

With exercise, there was a significant increase in the number of immature neuronal (Dcx positive) cells/mm^2 in both the DG and SVZ. This effect was more pronounced in the DG ($t(25)=3.399$ $p=0.0023$** Figure 4.9C) compared to the SVZ. In the SVZ, overall there were more Dcx positive cells than in the DG in both groups, and the group that had access to a running wheel had significantly more Dcx cells compared to the sedentary group ($t(25)=2.292$ $p=0.0306$* Figure 4.9D).

Mice that had access to a running wheel had a significant increase in the survival of new cells for 28 days (BrdU positive) per mm^2 compared to mice that remained sedentary in both the DG and the SVZ.

In the DG there was a significant increase in the number of BrdU positive cells in mice that had access to a running wheel compared to the sedentary group ($t(25)=2.325$ $p=0.0285$* Figure 4.10). However, only one third of the analysed brain slices from the exercise group showed any amount BrdU staining in the DG, whilst the sedentary group had only one sample with any staining (Figure 4.10B).

There was a similar significant increase in the number of BrdU positive cells in the SVZ of mice from the exercise group compared to those from the sedentary group ($t(26)=2.489$ $p=0.0195$* Figure 4.10C). Just over half (8 of 15) brain slices from the exercise group had some BrdU staining in the SVZ, in comparison to just one sample from the sedentary group (Figure 4.10D).

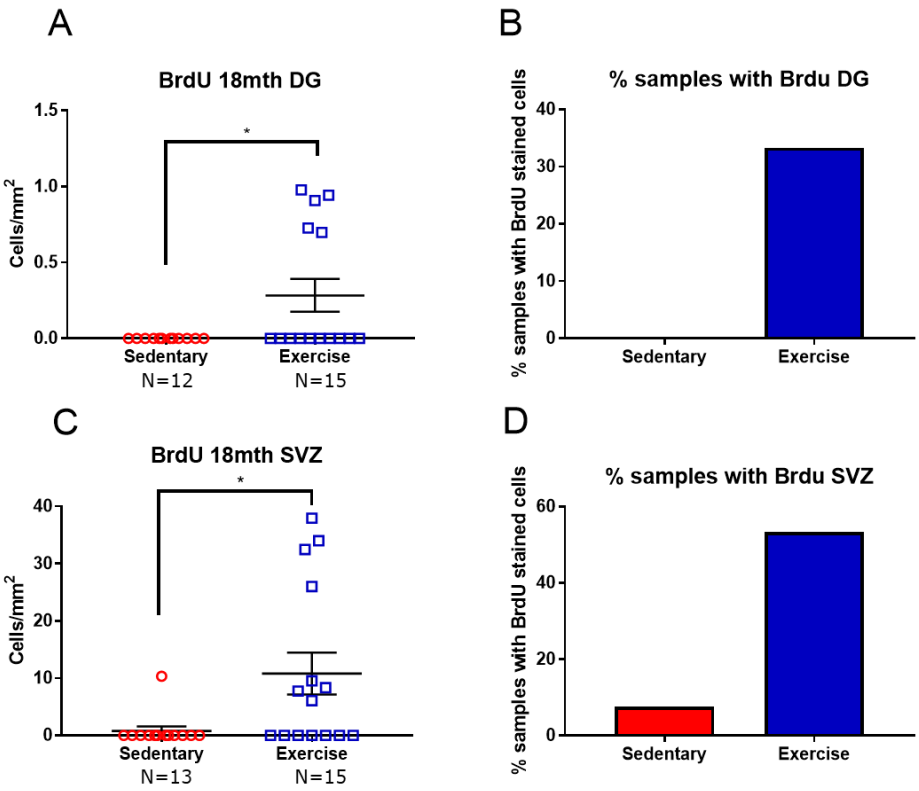


Figure 4.10: Effect of exercise on cell survival (BrdU)

Data from 18-month-old WT mice who had access to an exercise wheel for 6 months or remained sedentary for the same time. Mean and ±SEM bars are displayed. Data shows average number of cells/mm². A) Number of BrdU positive stained cells/mm² in the DG. B) Percentage of total analysed samples that had BrdU positive stained cells present in the DG. C) Number of BrdU positive stained cells/mm² in the SVZ. D) Percentage of total analysed samples that had BrdU positive stained cells present in the SVZ

4.3.7 Changes in adult neurogenesis with ageing

In order to study the change in adult neurogenesis levels in mice as they aged, cell proliferation (Ki67), immature neuron numbers (Dcx) and 28-day cell survival (BrdU) levels were analysed in the DG and SVZ of 2 month and 18 month old male mice. We saw significant decreases in these markers of adult neurogenesis in both areas in the aged mice compared to the young (t-test p=<0.0001**** Figure 4.11).

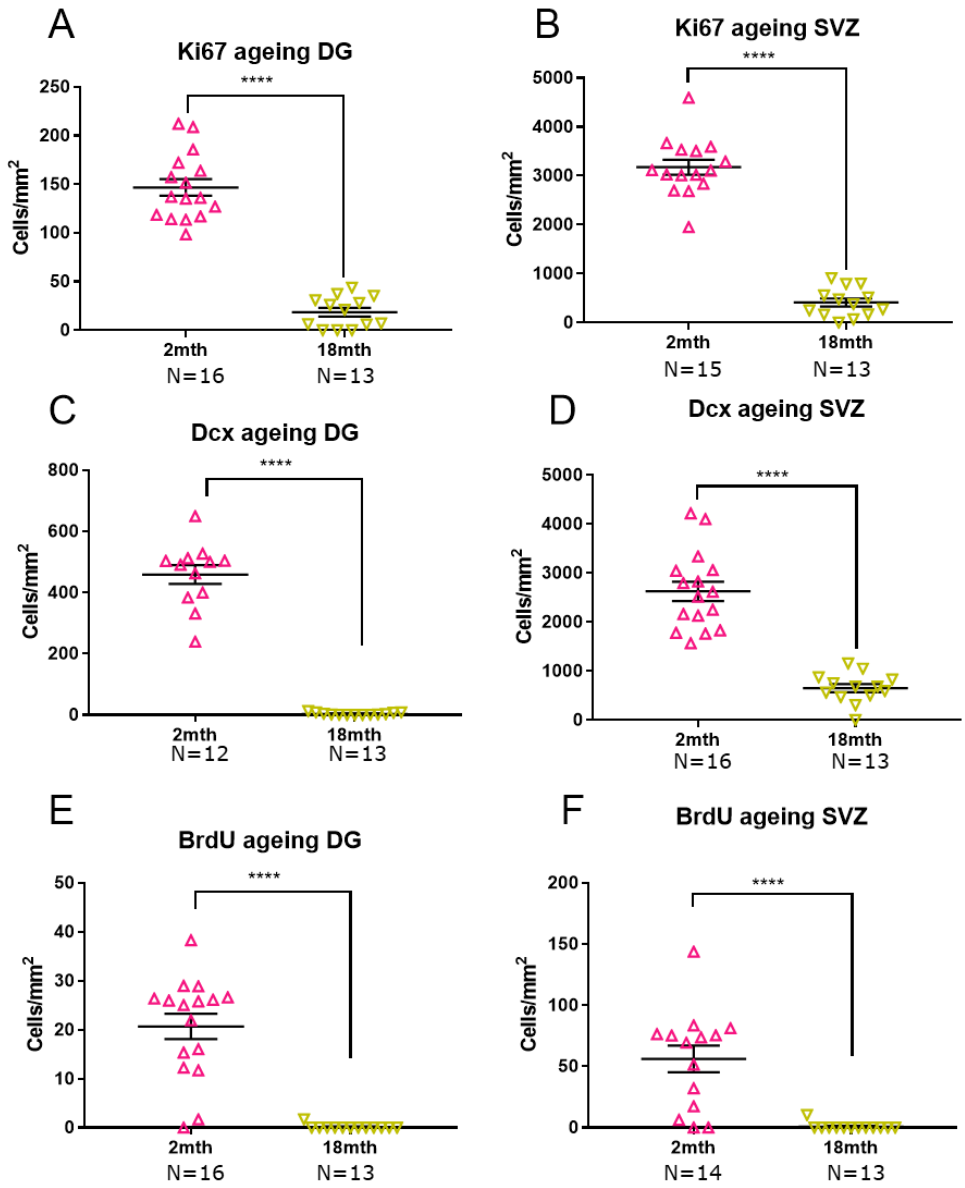


Figure 4.11: Adult neurogenesis in young (2 month) and aged (18 month) WT male mice

Data from 2 month (young) and 18 month (aged) old sedentary WT mice. Mean and ±SEM bars are displayed. Data shows average number of cells/mm². ****P=<0.0001 T-test. A) Number of Ki67 positive stained cells in the DG of young and aged mice. B) Number of Ki67 positive stained cells in the SVZ of young and aged mice. C) Number of Dcx positive stained cells in the DG of young and aged mice. D) Number of Dcx positive stained cells in the SVZ of young and aged mice. E) Number of BrdU positive stained cells in the DG of young and aged mice. F) Number of BrdU positive stained cells in the SVZ of young and aged mice.

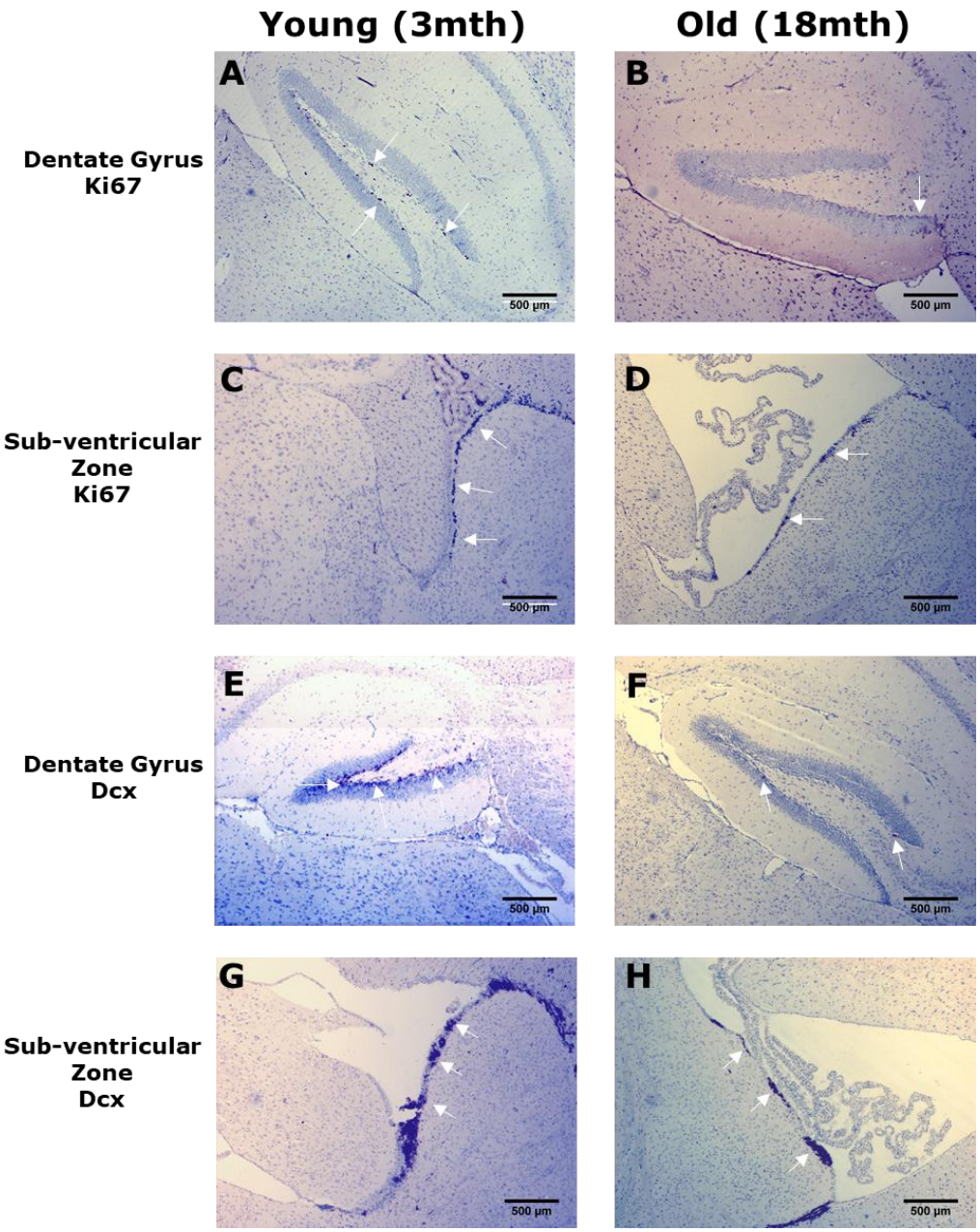


Figure 4.12: Adult neurogenesis imaging in young (L) and aged (R) WT male mice

Images of neurogenesis staining from young (2 months, left images) and aged (18 months, right images) WT male mouse brains. Background colour is an artefact of H&E counterstaining and image analysis. White arrows point to darker stained areas which are labelled with Ki67 (A-D) or Dcx (E-H). Examples in the DG (A and B, E and F) and SVZ (C and D, G and H) are shown.

4.3.8 Circulating miRNA expression levels

The levels of 7 miRNAs present in blood plasma were studied in 18-month-old male WT mice that either had access to a running wheel or remained sedentary from 12 to 18 months of age. With exercise, there was a significant increase in the levels of miR-21-5p ($t(14)=2.339$ $p=0.0347$* Figure 4.13D). There was also a strong trend towards decreased expression of miR-92a-3p ($t(13)=2.102$ $p=0.0556$ Figure 4.13G). None of the other analysed miRNAs were significantly affected by exercise.

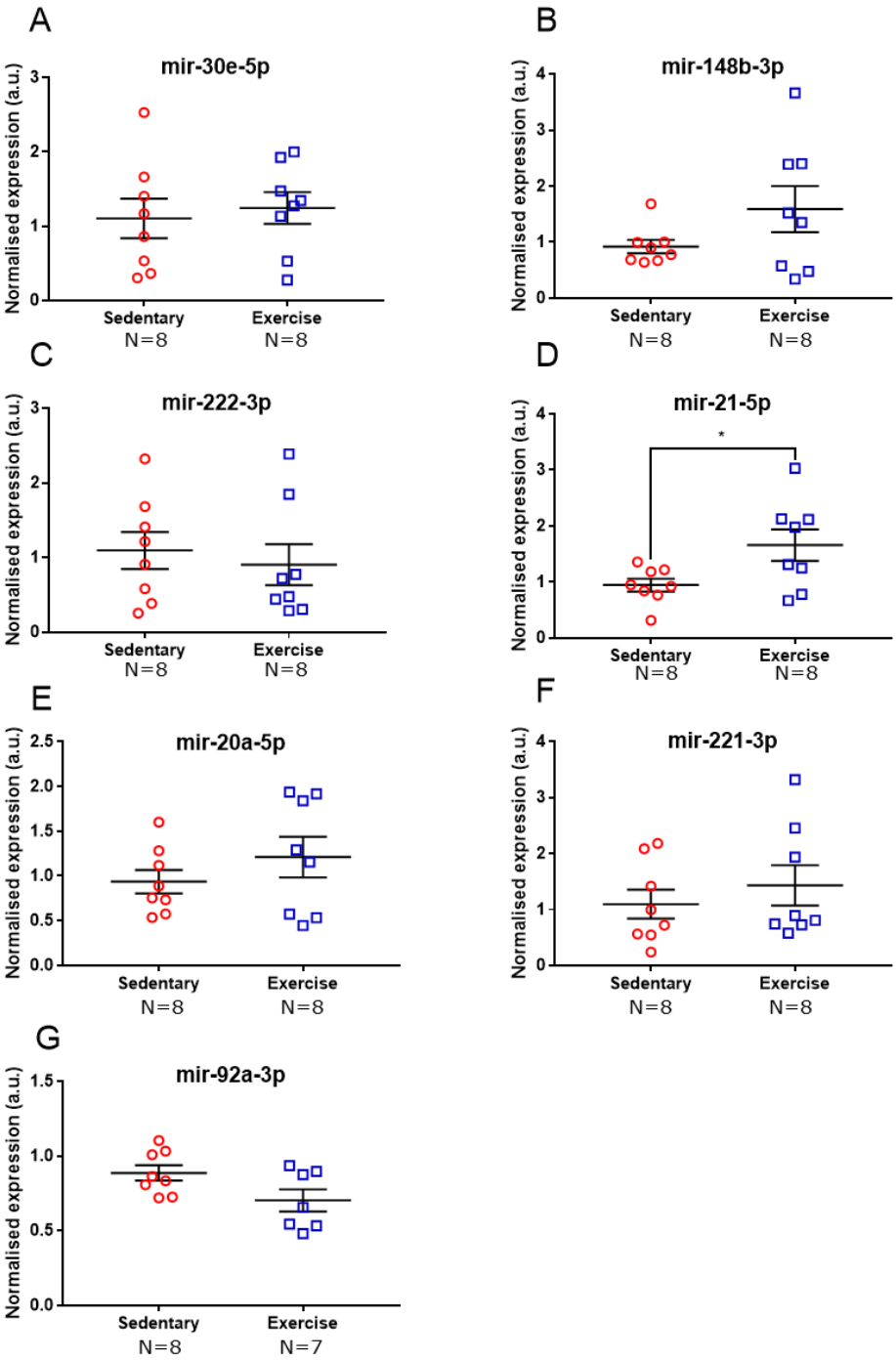


Figure 4.13: Circulating miRNA levels in blood plasma of male WT mice with and without 6 months voluntary exercise

Data shows normalised expression of miRNA's from blood plasma in 18 month old WT mice that either had access to a running wheel for 6 months or remained sedentary. Mean and ±SEM is shown. A) mir-30e-5p normalised expression B) mir-148b-3p normalised expression. C) mir-222-3p normalised expression. D) mir-21-5p normalised expression (t-test *P=<0.05). E) mir-20a-5p normalised expression. F) mir-221-3p normalised expression. G) mir-92a-3p normalised expression.

4.3.9 Effect of exercise on gene expression in the hippocampus

All genes were normalised to a set of housekeeping genes consisting of Hprt1, Pgk1 and Tatabox (ANOVA $F(2,39)=0.06837$ $p=0.5107$).

There was no significant difference in the expression of NAD^+ biosynthetic pathway related genes in the hippocampus between the exercise and sedentary groups (Figure 4.14).

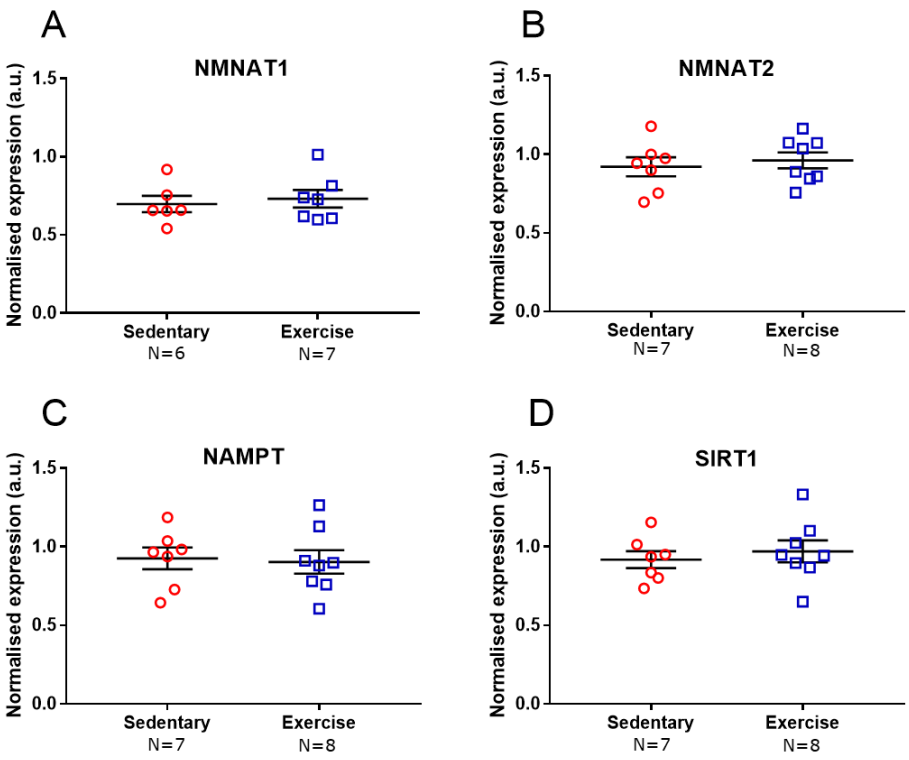


Figure 4.14: Expression levels of genes involved in the NAD+ biosynthetic pathway in aged WT male mice with and without 6 months exercise

Data shows relative expression of genes in the NAD biosynthetic pathway in mice that had access to a running wheel for 6 months or remained sedentary. Mean and ±SEM are shown. Expression normalised to HPRT1, PGK1 and Tatabox genes (see methods). A) Normalised expression of NMNAT1. B) Normalised expression of NMNAT2. C) Normalised expression of NAMPT. D) Normalised expression of SIRT1.

There was also no significant difference in the expression of the epigenetic modifiers Utx and Ezh2 (Figure 4.15A,B), nor was there any significant difference in the expression of Bdnf (Figure 4.15C).

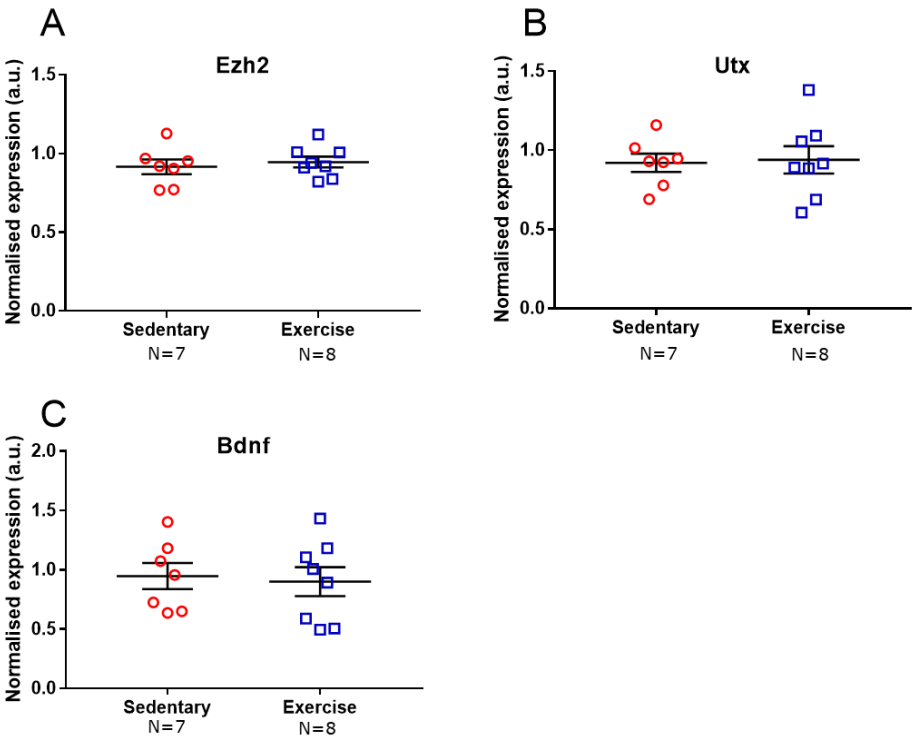


Figure 4.15: Gene expression in the hippocampus of WT male mice with and without voluntary exercise

Data shows normalised expression of genes involved in epigenetic modification and neurogenesis in the hippocampus of aged (18 months old) male WT mice that either had access to a running wheel from 12 months of age or remained sedentary for 6 months. Mean and ±SEM bars are shown. Expression normalised to HPRT1, PGK1 and Tatabox genes (see methods). A) Normalised expression of Ezh2. B) Normalised expression of Utx. C) Normalised expression of Bdnf.

4.4 Discussion

4.4.1 The effect of exercise on behaviour in an aged mouse model

4.4.1.1 Cognition

In our study, we used the NOR and SA behavioural tests for cognitive ability. The NOR test studies recognition memory by looking at the ratio of novel object investigation compared to familiar object investigation. The SA behavioural test studies spatial awareness as well as recognition memory by looking at the ratio of novel arm entries compared to recent arm entries in the Y shaped maze. We compared the results for each mouse at 11 months and 18 months and looked to see whether 6 months of voluntary exercise had a significant effect.

We saw no statistically significant improvements in the results for the cognitive tests in the exercise group in comparison to the sedentary group as the mice aged. However, within the exercise group there was a fairly large spread in the data for some of the tests. To check whether there was a correlation between running distance and the results of the behavioural tests, we analysed the time spent running by each individual for some of the mice by analysing videos of night-time running behaviour. Not all videos were of high enough quality for accurate analysis. We found no significant correlation between individual running times and the data from the cognitive behavioural experiments. This supports our previous conclusion that in our study, exercise did not maintain or improve cognition in the mice as they aged. We also compared the behavioural test results at 18 months and found no significant effect of exercise.

Despite the lack of significance, there was a trend towards exercise preventing a decline in the NOR ratio as the mice aged. On average the sedentary group showed a decrease of nearly 30% in the ratio of time spent exploring the novel object as they aged, in comparison to an average of around 5% decrease in the exercise group as they aged. Previous studies have shown that cognitive decline is strongly linked with ageing, and many have shown significant improvements in cognition in aged rodents after around 1 month of exercise (van Praag, 2009). From our data, we saw a trend towards exercise protecting against this age-related cognitive decline in our mice.

In the first minute of the NOR test, both groups showed a decrease in ratio with age. However, the decrease in the sedentary group ratio was larger than the one for the exercise group, suggesting a mild maintenance effect in the exercise group. We see similar effects for the NOR ratio for the full test, with both groups having a significant decrease in ratio with age, but no significant effect of exercise. When we looked at the percentage change in ratio for each animal, on average the exercise group maintained a higher ratio with age compared to the sedentary group. This again suggests that exercise had some protective effect against age-related cognitive decline.

There was no significant change in the SA ratio data for either group with age. When looking at the percentage change with age, both groups on average show no change in SA ratio for the full test or in the first minute. The data suggests that the exercise group may perform more novel arm entries early on in the SA test, but overall both groups perform similarly.

The lack of significant cognitive improvement in our study is in contrast to many of the findings in other studies. Bechara et al (Bechara, 2013), O'Callaghan et al (O'Callaghan, 2007) and Griffin et al (Griffin *et al.*, 2009) all found that rodents that had exercised for 7 days showed a significantly

increased ratio of exploring the novel object over familiar ones in a recognition test. However, the exercise period for these studies is significantly shorter than our own and used young adult rodents. Van Praag et al (Van Praag, 2008) looked at spatial memory in aged mice that were given access to a running wheel for 1 month at 18 months of age. They found that exercise significantly improved the spatial memory of the mice in the morris water maze test. Studies where the rodents had access to a running wheel for a much longer period of time have also shown that the exercise groups perform better than the sedentary ones. Pietrelli et al (Pietrelli *et al.*, 2012) aged rats to 18 months of age, and trained them on a treadmill 3 times a week from 2 months of age. Using the radial maze as a cognitive behavioural test, they found that the rats that had been on the treadmill entered the correct arm of the maze significantly more than the age matched sedentary controls, suggesting improved spatial memory.

Differences in the set up and methodology used by our study and those in the literature could explain the variance in the data obtained. The majority of rodent exercise studies have a short period of exercise, usually up to one month of exercise towards the end of the study. In our study, the mice had access to the running wheel for 6 months from 12 to 18 months of age. This could suggest that there is a strong effect of exercise within the first month which disappears over time with more prolonged exercise such as the 6 months used in our study. In our study we saw some trends towards improved cognition with exercise, which may have been significant with a shorter period of exercise if our hypothesis is correct.

4.4.1.2 Anxiety

Exercise has been shown to help reduce anxiety in humans (Jayakody, 2014), though mixed results are often seen in rodent studies, as we discussed in Chapter 3 (Binder, 2004; Fuss, 2010).

In our study, we used the EPM and OF behavioural tests to look at anxiety behaviour. In the EPM test, increased time spent in the open arms of the maze and increased time spent performing exploratory behaviours such as dipping the head over the edge of the maze suggest a lower level of anxiety. In the OF, more time spent in the centre of the arena suggests a lower level of anxiety, whereas more time spent by the edges and in the corners of the arena suggests a higher level of anxiety. We compared the results for each mouse at 11 months and 18 months and looked to see whether 6 months of voluntary exercise had a significant effect.

In our study we saw no significant difference in the measures of anxiety between the exercise and sedentary group. Whilst the data for the exercise group was spread out, there was no correlation between distance run and measures of anxiety. All mice showed an apparent increase in anxiety as they aged as on average both groups spent less time exploring the open arms of the EPM and less time in the centre of the OF arena. Locomotor activity in the EPM was decreased in both groups, which could explain the reduction in exploring the open arms of the arena.

In the literature, different studies have shown results of increased (Fuss, 2010), decreased (Salam, 2009) or non-significant differences (Garrett, 2012) between exercise and sedentary groups for anxiety. The protocol used in each study was different. For example, the amount of time the mice were exercising, and the age of the mice used varies between studies, and these variations may explain some of the differences in the results. Additionally, some of these discrepancies may be due to the period of the light/dark cycle the tests are performed under. From earlier work performed, as seen in Chapter 3, the behavioural tests at 18 months were performed 2 hours after lights were turned off for all mice.

The mice in our study were group housed throughout their lifespan, as ethical considerations prevented single housing for extended periods of time. Previous studies have shown that mice that are group housed and have to share a running wheel show higher levels of anxiety and potentially fight more often over use of the wheel (Pan-Vazquez, 2015). In the literature, exercise studies often single house male rodents for a short period of time with a running wheel – usually up to 1 month– or animals are on a treadmill for a similar length of time. Thus, an explanation to our findings could be that the increase in anxiety due to sharing the wheel could cancel out any beneficial effects of exercise in our study.

4.4.1.3 Locomotor activity

We measured the total distance travelled by the mice in each behavioural test using Ethovision software. We compared the results at 11 months and 18 months, with the mice either remaining sedentary or having access to a running wheel for 6 months.

There was a significant decrease in distance travelled in the EPM for both the exercise and sedentary groups with age. This could explain the apparent increase in anxiety seen in this test.

No significant differences were seen between the exercise and sedentary groups for locomotor activity in any of the behaviour tests. The lack of significant difference in locomotor activity between the exercise and sedentary groups rules out fatigue from running on the wheels being the cause for differences, or lack of differences, in behaviour between the two groups. For example, if we had seen a significant decrease in the exercise group results for any of the behaviour tests, it could not be attributed to fatigue as the locomotor activity for the exercise group was the same as the sedentary group. Likewise, if the exercise group showed a significant

increase in locomotor activity in the NOR, then this could potentially explain the increased level of exploration of the novel object during the test.

4.4.2 Adult neurogenesis

4.4.2.1 Exercise and adult neurogenesis

To analyse the levels of adult neurogenesis in the brains of our mice we utilised three different immunohistochemical stains: Ki67, Dcx and Brdu. Ki67 marks the nucleus of cells that were actively proliferating at the moment of death. Dcx marks immature neurons up to the first 28 days after differentiation. BrdU was injected into the mice 28 days before they were euthanised and marks the nucleus of cells that were actively dividing on the day of injection and were still alive at the time of euthanasia.

We saw no significant difference in the number of cells marked with Ki67 in the DG between the exercise and sedentary groups (Figure 4.9A). However, we saw a significant increase in the number of Dcx positive cells in both the DG (Figure 4.9C) and the SVZ (Figure 4.9D) in the mice that had access to running wheels. We also saw significant increases in the number of cells surviving 28 days with BrdU staining (Figure 4.10). Overall, our data shows that exercise did not affected the number of proliferating cells in the hippocampus of aged mice. However, with exercise more of the proliferating cells became immature neurons and were more likely to survive and become mature, integrated cells.

A study by Kronenberg et al (Kronenberg *et al.*, 2006) found that there was an acute upregulation spike in cell proliferation with exercise, but that with continuous exercise (up to 32 days), the difference in cell proliferation with exercise returned to non-significant. However, the number of immature neurons continued to increase after the 32-day timepoint,

suggesting that exercise drives more of the proliferating cells to become immature neurons and increases their survival rate.

Other studies have shown increases in adult hippocampal neurogenesis with exercise, usually over one month (van Praag, 1999, 1999). This upregulation of neurogenesis has been linked with the improvements in cognition also often found in exercise studies. For example, van Praag et al (van Praag, 2005) individually housed both young (3-months-old) and aged (19-months-old) mice with a running wheel for 45 days. They studied both their spatial learning abilities in the Morris water maze, and the levels of adult hippocampal neurogenesis with BrdU and NeuN – a neuronal nuclear marker. They found that the aged mice that had exercised performed much better than the age matched sedentary controls, and that the decline in neurogenesis in the aged mice was reversed by up to 50%.

4.4.2.2 Neurogenesis and ageing

Our study has compared the levels of Ki67, Dcx and BrdU in 2-month-old male mice to the 18-month sedentary male mice.

As expected from the literature we saw a significant decrease in all three measures of neurogenesis with ageing. Previous studies have shown that neurogenesis declines rapidly with age (Kuhn, 1996; Seib, 2015), reaching a very low baseline in old age but never completely stopping. A recent publication by Sorrells et al (Sorrells, 2018) disagrees and states that adult neurogenesis in human and monkeys decreases to undetectable levels in adulthood, but there are other recent publications that contradict this conclusion (Boldrini, 2018).

In our study, in the SVZ the numbers of Ki67 and Dcx cells were much higher than in the DG in both age groups. This shows that the SVZ had much higher levels of adult neurogenesis throughout life compared to the

DG. In both age groups the number of BrdU positive cells was relatively low compared to the number of Ki67 and Dcx cells. This indicates that many proliferating cells do not survive to fully differentiate, and many more die as immature cells when they fail to integrate (Dayer, 2003).

4.4.3 miRNA expression in blood plasma

Here we saw a significant increase in the expression of Mir-21-5p in the blood plasma of aged mice that had exercised compared to the age matched controls. Mir-21 has been associated with cell proliferation, different types of cancer and a reduction in apoptosis. It also has a role in recovery post traumatic brain injury by inhibiting apoptosis and promoting angiogenesis (Ge *et al.*, 2015). Overexpression of Mir-21 increases Ezh2 expression in human lung cancer cells (Xia *et al.*, 2017). Ezh2 has a regulatory role in NPC proliferation and neuronal cell fate in adult neurogenesis (Zhang *et al.*, 2014), and upregulation via the activity of Mir-21 could be linked with the increases in the number of immature neurons in the brains of our mice.

There was also a strong trend towards Mir-92a-3p being decreased with exercise in aged mice in our study. Mir-92 expression in embryonic neurogenesis has been shown to regulate the development of radial glial cells into intermediate progenitor cells by repressing the Tbr2 protein which promotes the transition (Bian, 2013). Overexpression of the miR cluster that includes Mir-92 (mir-17-92) has been shown to significantly increase axonal outgrowth in primary cortical neuron culture (Zhang *et al.*, 2013). It has also been found that antidepressant treatment can restore both cell proliferation in the adult brain and Mir-92 expression levels (Jin, 2016). All of these studies show that Mir-92 expression has a significant regulatory role in both embryonic and adult neurogenesis. A study by Taurino et al

(2010) found that Mir-92 expression in the blood was significantly increased after 10 weeks of endurance exercise in humans. In our study, we saw a tendency towards decreased expression of Mir-92a-3p with exercise and saw no increase in cell proliferation in the DG or SVZ, so we did not see a clear link between the two. More research is needed to confirm a link between Mir-92a expression and cell proliferation in aged mice who have prolonged access to a running wheel.

4.4.4 Gene expression in the hippocampus

Exercise did not have any significant effect on the expression levels of the genes measured in the hippocampus. First, we looked at the expression levels of proteins associated with the NAD^+ biosynthetic pathway. 6 months of voluntary exercise did not have an impact on the expression of any of the proteins, suggesting that the available levels of NAD^+ are also unlikely to have changed in that time. Future studies could measure the NAD^+ levels in the brain and body to see if they have changed with 6 months voluntary exercise. If they have changed, it suggests that long term voluntary exercise affects the activity levels of the NAD^+ biosynthetic pathway proteins rather than their expression levels.

We also saw no change in the expression of Utx, Ezh2 and Bdnf with siz months voluntary exercise in aged mice. The lack of increased Ezh2 expression with exercise may be linked with the lack of increase in the number of proliferating cells with exercise found in our mice.

A study by Adlard et al (Paul A. Adlard, Perreau and Cotman, 2005) looked at Bdnf protein level in the hippocampus of 2-month, 15-month and 24-month old mice that had access to a running wheel for up to 28 days. They saw significant increases in Bdnf levels in all age groups after 7 days voluntary exercise, but after 28 days exercise only the youngest group still

showed a significant increase compared to the sedentary controls. This data, along with our results, suggests that the effect of exercise on Bdnf expression is not as strong in aged mice as it is in the young mice.

4.5 Conclusions

In this chapter, we found that prolonged voluntary exercise caused a significant increase in the number of immature neurons in the DG and SVZ, and a significant increase in the number of cells surviving 28 days. However, we did not see a similar significant increase in the number of proliferating cells. This suggests that prolonged exercise causes increased survival and neuronal differentiation in the same pool of proliferating NPCs.

As the mice aged, we saw a significant decrease in all aspects of adult neurogenesis as we hypothesised.

We saw a trend towards improved/maintained cognitive ability in ageing mice after 6 months of voluntary exercise, and a possible increase in anxiety with age. However, we saw no significant changes in cognition, anxiety or locomotor activity in ageing mice after 6 months of voluntary exercise.

Prolonged voluntary exercise significantly increased the expression of Mir-21-5p, and trends towards a decrease in the expression of Mir-92a-3p in the blood plasma of an ageing mouse model.

Prolonged voluntary exercise did not have a significant effect on the expression of genes involved in the NAD^+ biosynthetic pathway, nor genes involved in epigenetic modification within the hippocampus of an ageing mouse model.

5. Chapter 5:

The influence of different NMNAT1 expression on the ageing brain and blood biomarkers, and its combined effects with exercise in an ageing mouse model

Note: Thank you to the undergraduate students who produced the Ki67 data for the young female mice used in this chapter: Nikki Drury, Desislava Nesheva, Eleanor Chapman, Jessica Hewitt, Jack Reynolds-Clark and Nathalie Badger.

5.1 Background

Nicotinamide adenine dinucleotide (NAD) has an essential role in metabolism and is required as a coenzyme for numerous reactions. It is synthesised via one of two pathways: *de novo* from the amino acid L-tryptophan, or via salvage pathways from either nicotinic acid or leftover nicotinamide from reactions using NAD^+ (Figure 5.1). In both pathways, the last reaction is catalysed by the enzyme nicotinamide mononucleotide adenylyltransferase (NMNAT).

NAD is required for the activity of the deacetylase enzyme SIRT1 which has a prominent role in the regulation of ageing (Bordone, 2007; Cantó, 2009).

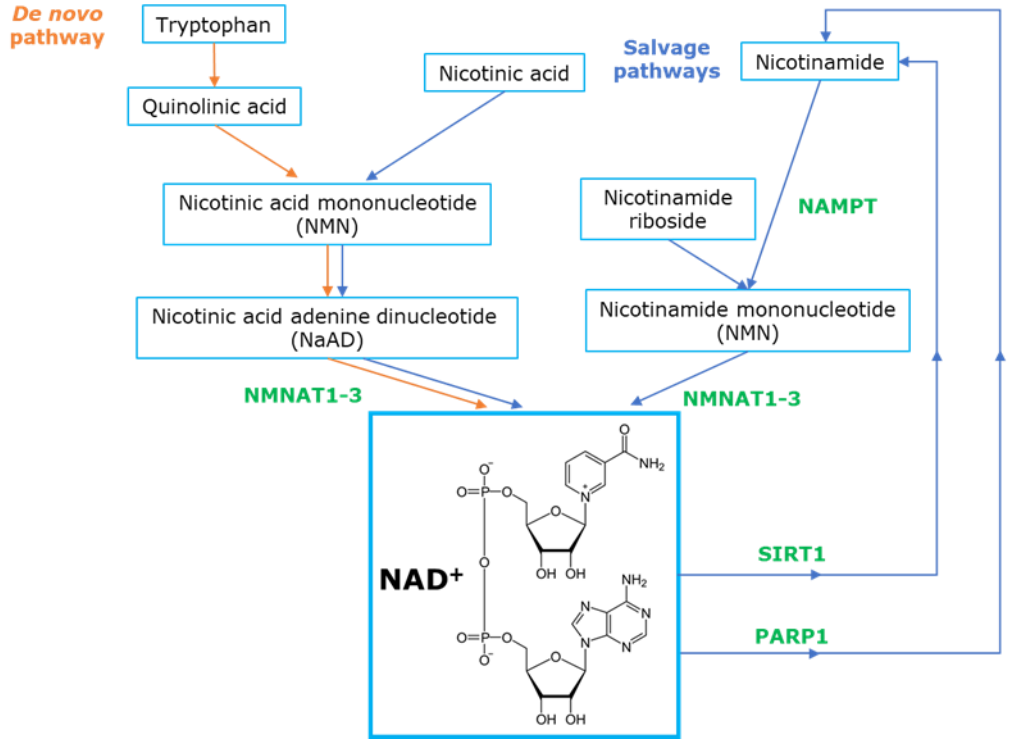


Figure 5.1: The NAD^+ biosynthetic pathways

Diagram showing the stages of the NAD^+ *de novo* and salvage pathways. Reproduced from Chapter 1 section 1.5.2

Previous studies have shown that NAD^+ levels decline with age along with the rate limiting NAD^+ biosynthetic enzyme NAMPT and the activity level of SIRT1 (Braidy, 2011; Massudi *et al.*, 2012). NMNAT1 is one of the three NMNAT isoforms, all of which perform the final reaction to convert NMN or NaAD to NAD^+ in the biosynthetic pathways. It is expressed in the nucleus of all cells and is known to be crucial during development (Conforti, 2011). More information on NMNAT1 and the NAD^+ biosynthetic pathway can be found in Chapter 1.

In our study, we investigated whether changing the expression level of the NMNAT1 gene had an effect in an ageing mouse model similar to changes in SIRT1 expression. Previous work (Rossi, 2015) has shown some significant effects of varied NMNAT1 expression, including changes in body weight, locomotor activity and changes in behavioural outcomes in a mouse model of AD. In our study, we used a similar mouse model and looked at neurogenesis in the HC, gene expression in the brain and miRNA expression in blood plasma, as well as behaviour and body weight changes. We also studied whether NMNAT1 levels influenced the effects of voluntary exercise on behaviour, neurogenesis, circulating miRNA expression and hippocampal gene expression in old age.

5.1.1 Aims and objectives

In this chapter, we aim to investigate whether heterozygous knockout or overexpression of NMNAT1 in an animal model of ageing had an effect on the following:

- Behavioural outcomes in tests for anxiety, cognition and locomotor activity

- Neurogenesis in the DG and SVZ of the hippocampus, including cellular proliferation, neuronal differentiation and 28-day cell survival
- Changes in gene expression in the hippocampus, including key genes associated with the NAD^+ biosynthetic pathway
- Levels of miRNA biomarkers present in blood plasma

Further, we aimed to see if 6 months of voluntary exercise had any impact on the list above, and whether it changes the effect of varied NMNAT1 expression in these areas.

5.2 Methods

For full methodology, see Chapter 2, Methods.

5.2.1 Animal work

Two colonies of mice were \used for the following experiments, both with altered expression of the NMNAT1 gene: Transgenic overexpression (TG) and heterozygous knock out (Het). WT littermates from both colonies were used (See chapter 4 for full WT results).

5.2.1.1 Young animal experiment

Young male and female mice from both colonies were group housed at P28 and were given an intraperitoneal injection of 20mg/kg BrdU. At P56 mice were culled and tissues extracted. Figure 5.2 shows an overview of both the young and ageing experiments.

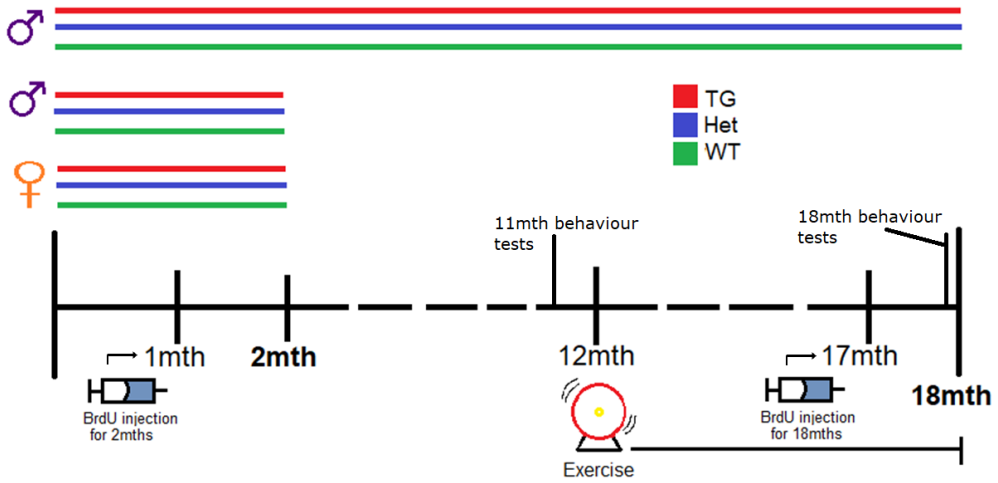


Figure 5.2: Overview of NMNAT1 mouse experiments

Diagrammatic timeline of experiments with young and aged NMNAT1 mice. Colours denote the three genotypes present in each study. The top group shows the ageing experiment, and the middle and bottom groups are the young animal experiments. Along the bottom is the timeline, showing the key events and when they occurred in each experiment. (Reproduced from Chapter 2: Methods, section 2.3.1)

5.2.1.2 Ageing and voluntary exercise

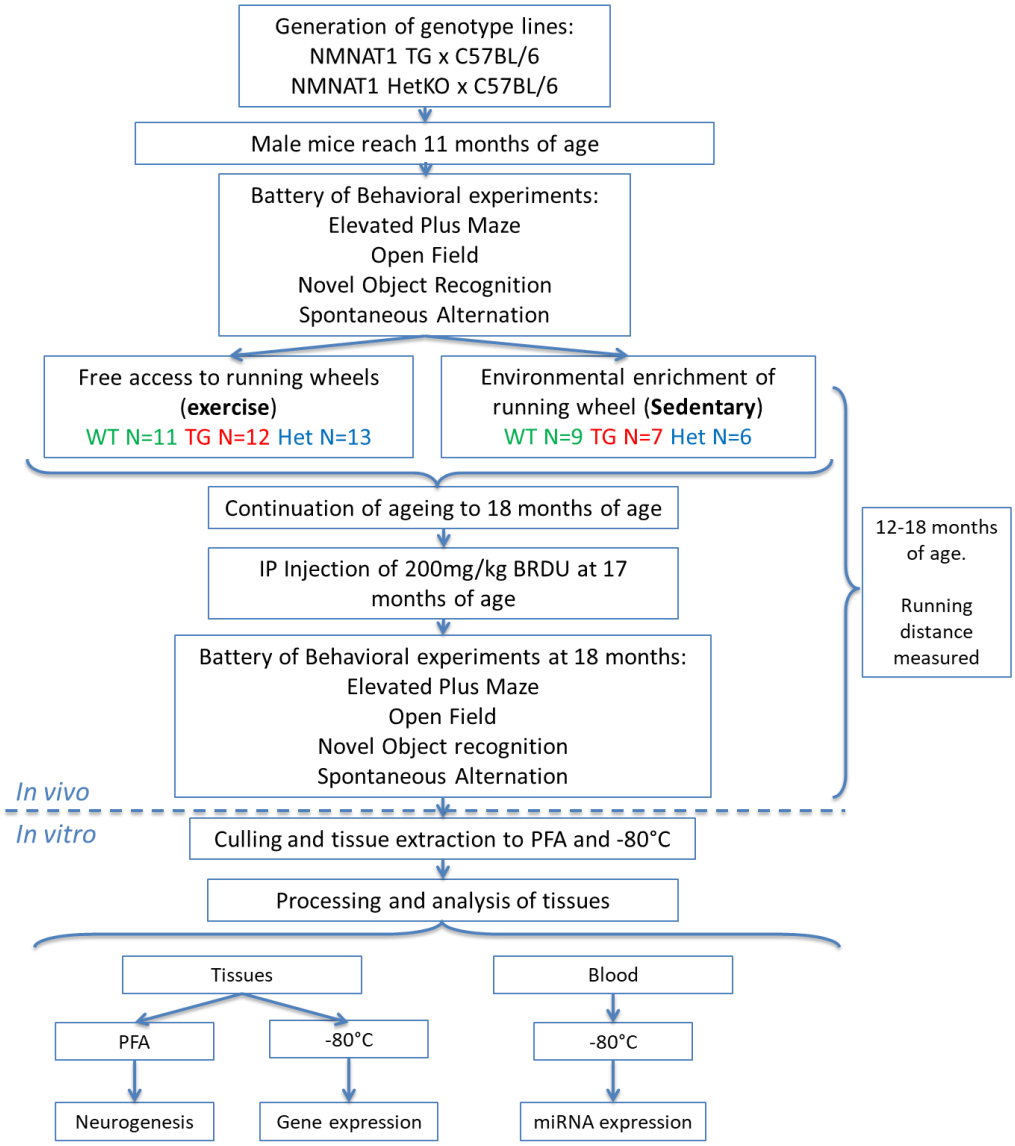


Figure 5.3: Timeline for ageing experiment

Timeline for the ageing experiment, showing the progression from genotyping, through behaviour experiments and the exercising period, to in vitro analysis experiments performed.

(Reproduced from Chapter 2: Methods, section 2.3.4

	WT	Het	TG	Total
Exercise		4 (4)		4 (4)
	2 (2)	2 (2)		4 (4)
	2 (2)	2 (2*)		4 (4*)
	1 (1)	3 (2)		4 (3)
			4 (3)	4 (3)
			4 (3)	4 (3)
	2 (2)		2 (2)	4 (4)
	2 (2)		1 (1*)	3 (3*)
	1 (1)		3 (3*)	4 (4*)
	1 (1)		3 (2)	4 (3)
Sedentary		2 (1)		2 (1)
		4 (3*)		4 (3*)
	2 (2)	2 (1)		4 (3)
	3 (3)	1 (1)		4 (4)
			4 (2)	4 (2)
			2 (2)	2 (2)
	2 (1)		2 (2*)	4 (3*)
	3 (3)		1 (0)	4 (3)
	1 (0)		2 (2)	3 (2)

Table 5.1: Animal numbers in cages at the beginning and end of the study

Table showing cage numbers at the beginning and end of the exercise study. Numbers decrease due to natural deaths. * denotes a mouse was used for tissue analysis but not for behaviour tests due to death occurring during or just before the behaviour tests.

Mice from both NMNAT1 colonies were aged to 18 months old over the duration of this experiment. See Table 5.2 for group numbers, with each row showing genotype numbers in each cage. At 11 months, all mice underwent the series of behaviour tests (EPM, OF, NOR and SA). At 12 months of age, mice were re-housed in groups, either with access to a running wheel or a dismantled wheel on the floor of the cage. They remained in these cages for the rest of the study. At 17 months of age, one month before the end of the study, mice had an intraperitoneal injection of 20mg/kg BrdU. One week before being euthanised, all mice repeated the series of behaviour experiments. Finally, at 18 months of age, mice were euthanised under terminal anaesthesia and tissues were extracted. Figure 5.3 shows a flow diagram of the key events of the experiment.

5.2.2 In vitro work

5.2.2.1 Immunohistochemistry

Fixed brain tissue was embedded into paraffin wax and sectioned at 7µm thickness onto APES coated slides. Slides were then stained with primary

antibodies for Ki67, Dcx or BrdU following the standard protocol described in Chapter 2: Methods. Slides were analysed under a light microscope, positive stained cells quantified and the areas of the DG and SVZ measured. This was then calculated as the number of cells/mm^2.

5.2.2.2 miRNA levels in blood plasma

Blood plasma was snap frozen at -80°C. miRNAs were then extracted using the Qiagen miRNeasy serum/plasma kit and the Qiacube standard protocol for miRNeasy serum/plasma.

Samples were then reverse transcribed following the Exiqon miRCURY LNA™ Universal RT microRNA PCR protocol for serum/plasma using 2µl total RNA. QPCR reactions were performed on the Rotor-Gene 6000 cycler (Corbett Research) and reactions were performed in triplicate.

5.2.2.3 Gene expression in the hippocampus

RNA extraction from hippocampal brain tissue was performed with TRIzol by the manufacturers protocol. From this, RNA samples underwent DNase treatment and were reverse transcribed using Superscript™ III from Invitrogen. QPCR reactions were performed using the Rotor-Gene 6000 cycler (Corbett Research) and reactions were performed in triplicate.

5.2.3 Data analysis

Data presented in this chapter for the behavioural tests shows the results separately at 11 months and 18 months, as well as the percentage change in the results from the 11 months tests to the 18 months tests, before and after the 6 months voluntary access to a running wheel. This is calculated by: $\left(\frac{18mth-11mth}{11mth}\right) * 100$

All statistical analysis was performed using GraphPad Prism 7.00, with each data set analysed with one-way-ANOVA and multiple comparisons where appropriate. Anomalies were calculated as data points over two standard deviations away from the mean and removed from the analysis. All graphical data is presented as mean ±SEM.

5.3 Results

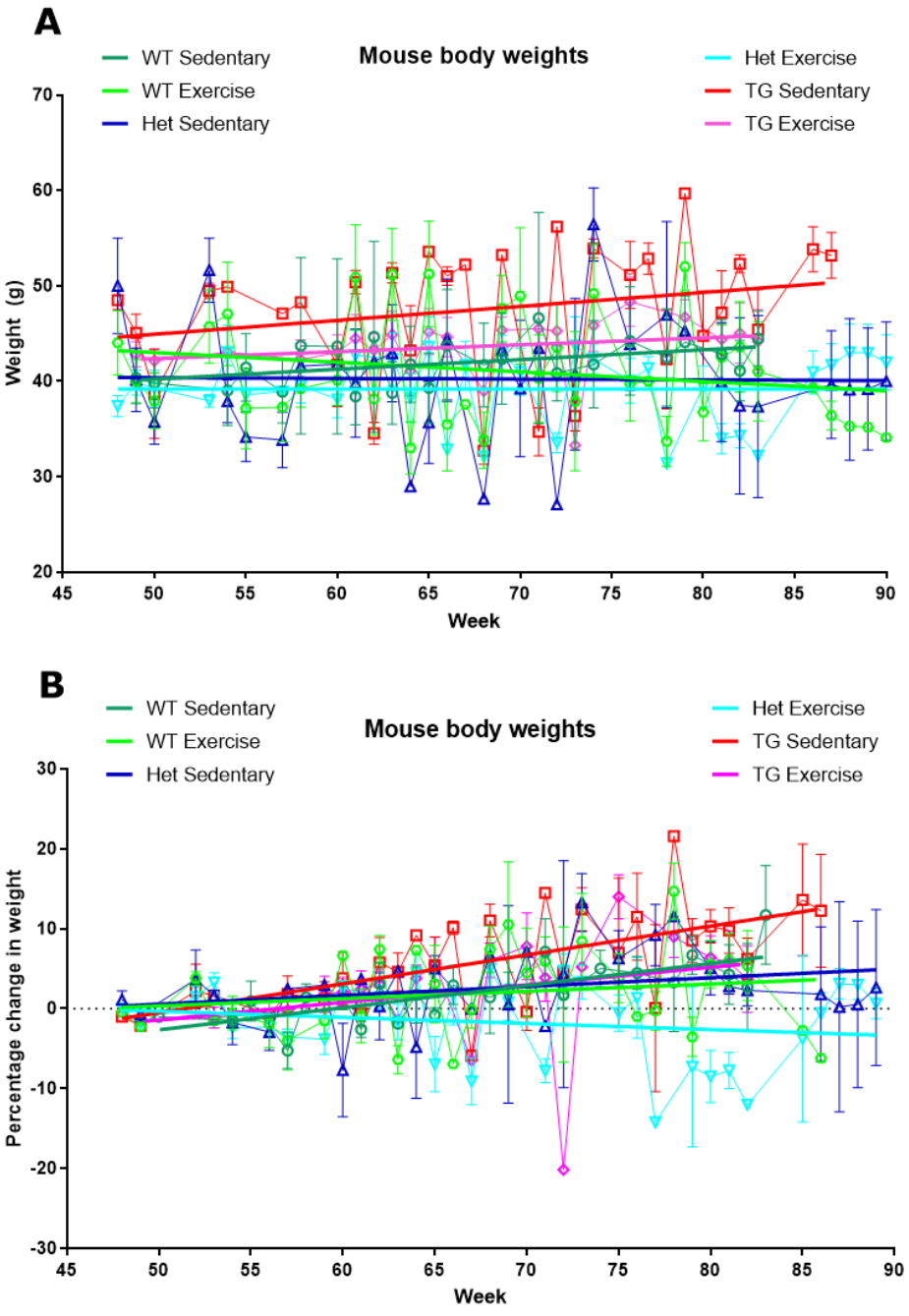
For behavioural, neurogenesis, miRNA and hippocampal gene expression results, we will first describe the comparison between the different NMNAT1 genotypes within the sedentary mice, and then how exercise impacts each parameter within the TG and Het genotypes.

5.3.1 Changes in body weight over time in aged mice with different levels of NMNAT1 expression and exercise

We monitored the body weights of the ageing mice over 6 months from 12 to 18 months of age, with half having access to a running wheel during this time. We recorded both actual weight in grams (Figure 5.4A) and the percentage change from the first weight recorded (Figure 5.4B). There was a large amount of variance within each group at most time points, as shown by the error bars.

The majority of the mice showed a trend towards increased body weight with age (Figure 5.4C,D). Exercise had a significant effect on body weight change over time (two-way-ANOVA $F(1,48)=4.67$ $p=0.0357$ Figure 5.4E). The Het mice that exercised showed an average decrease in percentage body weight change after 6 months voluntary exercise (Figure 5.4E), which

was significantly lower than the WT sedentary group (Tukey's multiple comparisons p=0.0387).



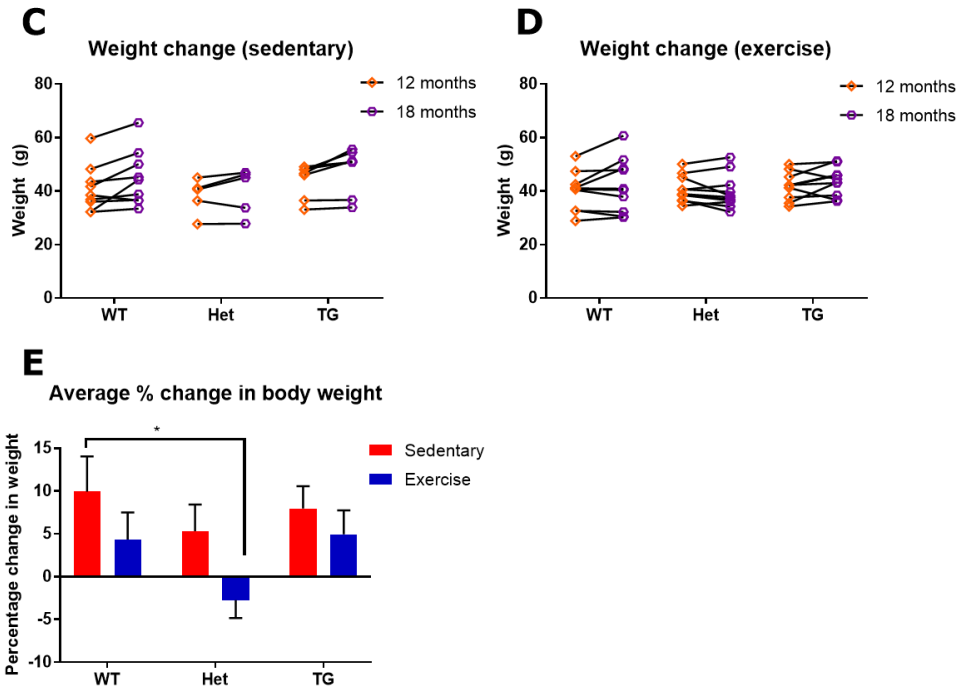


Figure 5.4: Changes in mouse body weight over 6 months with and without voluntary exercise

Data showing the changes in mouse body weights over 6 months, with or without access to a running wheel. Average data graphs C-E use first and last weights from the 6-month study. Mean and ±SEM shown. A) Actual body weight (g) change over time for all groups. B) Percentage change in body weight from 12 to 18 months of age, before and after 6 months access to a running wheel or remaining sedentary. C) Average actual weight change (g) in sedentary mice from 12 to 18 months of age. D) Average actual weight change (g) in mice with access to a running wheel from 12 to 18 months of age. E) Average percentage change in body weight for all groups from 12 to 18 months of age

5.3.2 Comparing survival rates in mice with different levels of NMNAT1 expression and exercise

Over the course of the ageing study, a number of the mice died from natural causes before the final date. When comparing the number of deaths in each group, we see a significantly higher number of deaths in the sedentary groups of all genotypes in comparison to the exercise groups ($Chi^2 = 0.030$).

Survived	Sedentary	Exercise	Started	Sedentary	Exercise
Wildtype	4	11	**Wildtype**	8	11
Het	5	11	**Het**	9	13
TG	6	11	**TG**	11	14

Table 5.2: Starting and surviving group numbers

Figure 5.5: Mouse mortality rates during the ageing study

Table: Number of mice at the beginning and end of the study per group.

Figure: Data showing the percentage of each group that survived to the end of the ageing study. There is a clear higher survival rate in the exercise groups compared to the sedentary.

5.3.3 Running distances

Mice that were given access to a running wheel were group housed with a single shared wheel for the duration of the experiment due to the ethical constraints of single housing for 6 months. This resulted in one set of running data being obtained per cage rather than individual mouse running data (Figure 5.6A). Video recordings of overnight cage activity were taken, and individual running time was assessed manually for a single time point.

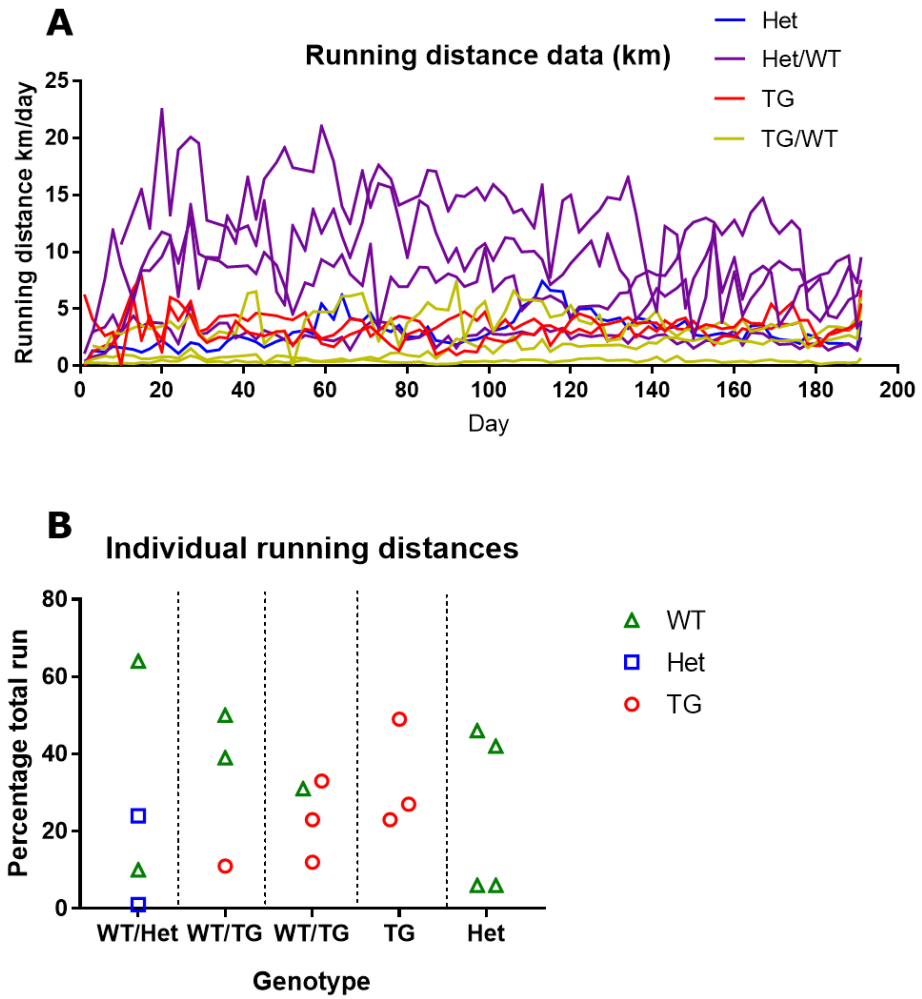


Figure 5.6: Running data

Running data from mice with free access to a running wheel for 6 months displayed as specific cage groups. A) Distance data from group cages (km/day). B) Percentage of total distance run in the first hour of activity after lights out by mice of different genotypes.

Figure 5.6A shows the group running distance data for all of the cages containing a running wheel. Data was collected 3 times a week (Monday, Wednesday, Friday) to minimise disruption to the mice. Cages with both WT and Het mice in seemed to run the furthest each night. One cage showed very low running distances. Due to in-house breeding, WT mice had to be mixed in with Het or TG littermates to avoid any being single housed.

Figure 5.6B shows the percentage of the total running for individual mice during the hour after the lights turned off for mice of each genotype. WT mice in both mixed cages seemed to spend more time running than their Het and TG cage-mates. Each cage had a clear one or two dominant mice that spent more time on the wheel than the others. The n numbers for Figure 5.6B are low due to the video quality not being high enough to identify individual mice from all of the exercise group cages.

5.3.4 The effect of different NMNAT1 expression on behaviour in mice and the changes brought about with voluntary exercise

5.3.4.1 Behavioural tests for cognition

There was a significant decrease in NOR ratio in the first minute of the test in sedentary Het mice in comparison to both WT (two-way-ANOVA RM $F(2,11)=9.407$ $p=0.0145$*) and TG ($p=0.0046$**) mice that remained sedentary, with no effect of age (Figure 5.7A). This difference is not present in the results for the full 5-minute test. However, there was a significant reduction in NOR ratio with age in the WT (two-way-ANOVA RM $F(1,14)=25.67$ $p=0.0032$**) and TG ($p=0.0133$*) sedentary mice (Figure 5.7C). There was no significant difference in the percentage change in NOR

ratio between the genotype groups. There was no significant difference in any of the SA ratio results with age or between the genotype groups.

There was no significant effect of age or exercise on the NOR ratio for Het mice. However, there was a significant decrease with age in the total NOR ratio in TG mice (Figure 5.8G). This decrease was much higher in mice that had exercised (two-way-ANOVA RM $F(1,14)=29.2$ $p= 0.0009$***) than in those that remained sedentary ($p=0.0108$*). There were no significant differences in percentage changes in NOR ratio with age.

There were no significant differences in the SA ratio with age or exercise group for Het or TG mice (Figure 5.9).

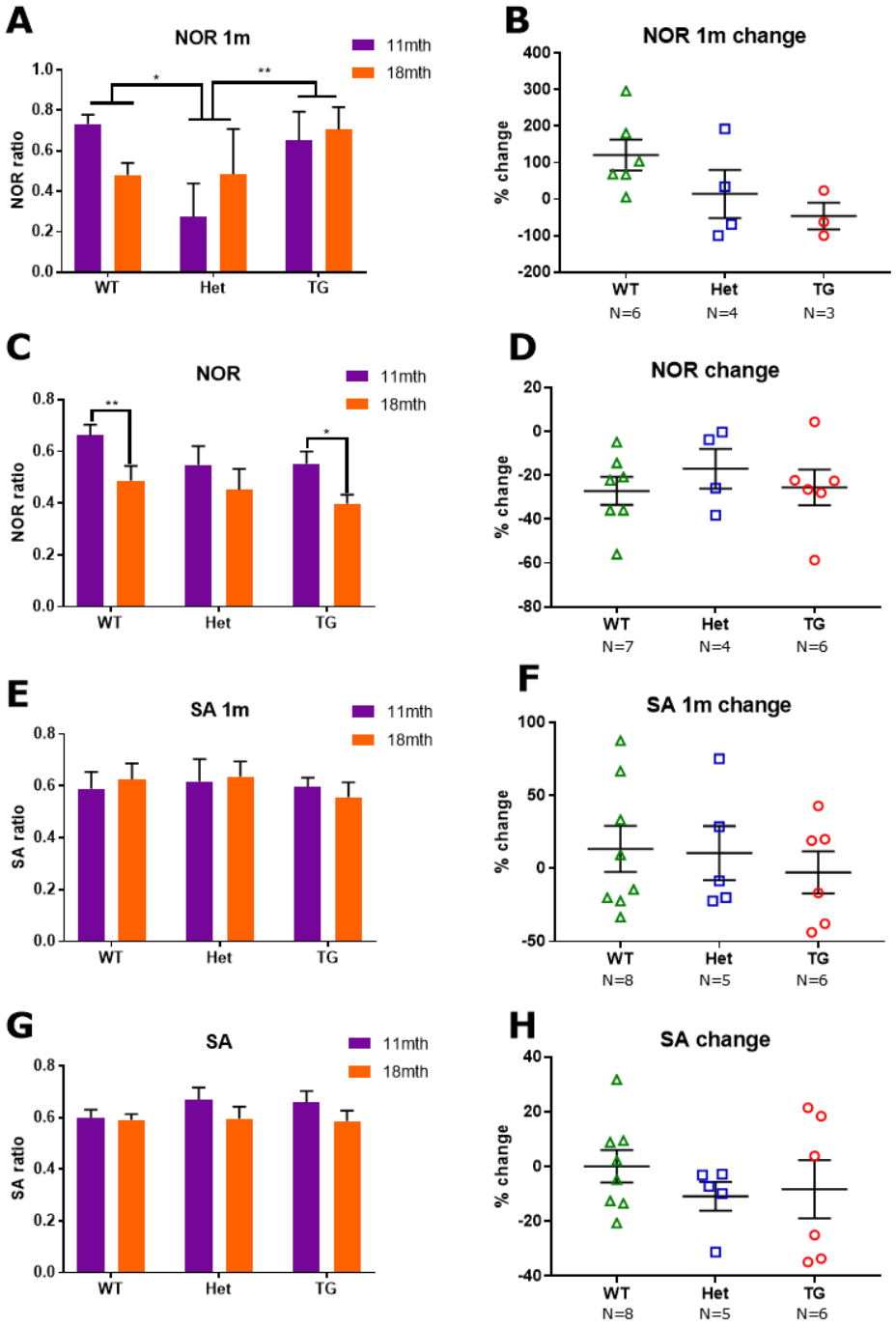


Figure 5.7: Cognitive behavioural tests in sedentary aged mice with varied NMNAT1 expression

Data from cognitive behaviour tests. Mean and ±SEM bars are displayed. WT, Het and TG mice that remained sedentary are shown. Percentage change with age is shown. Results for 11 and 18 months old. A) NOR ratio in the first 60 seconds. B) Percentage change in NOR ratio in the first 60 seconds. C) NOR ratio for the full test. D) Percentage change in NOR ratio for the full test. E) SA ratio in the first 60 seconds. F) Percentage change in SA ratio in the first 60 seconds. G) SA ratio for the full test. H) Percentage change in SA ratio for the full test.

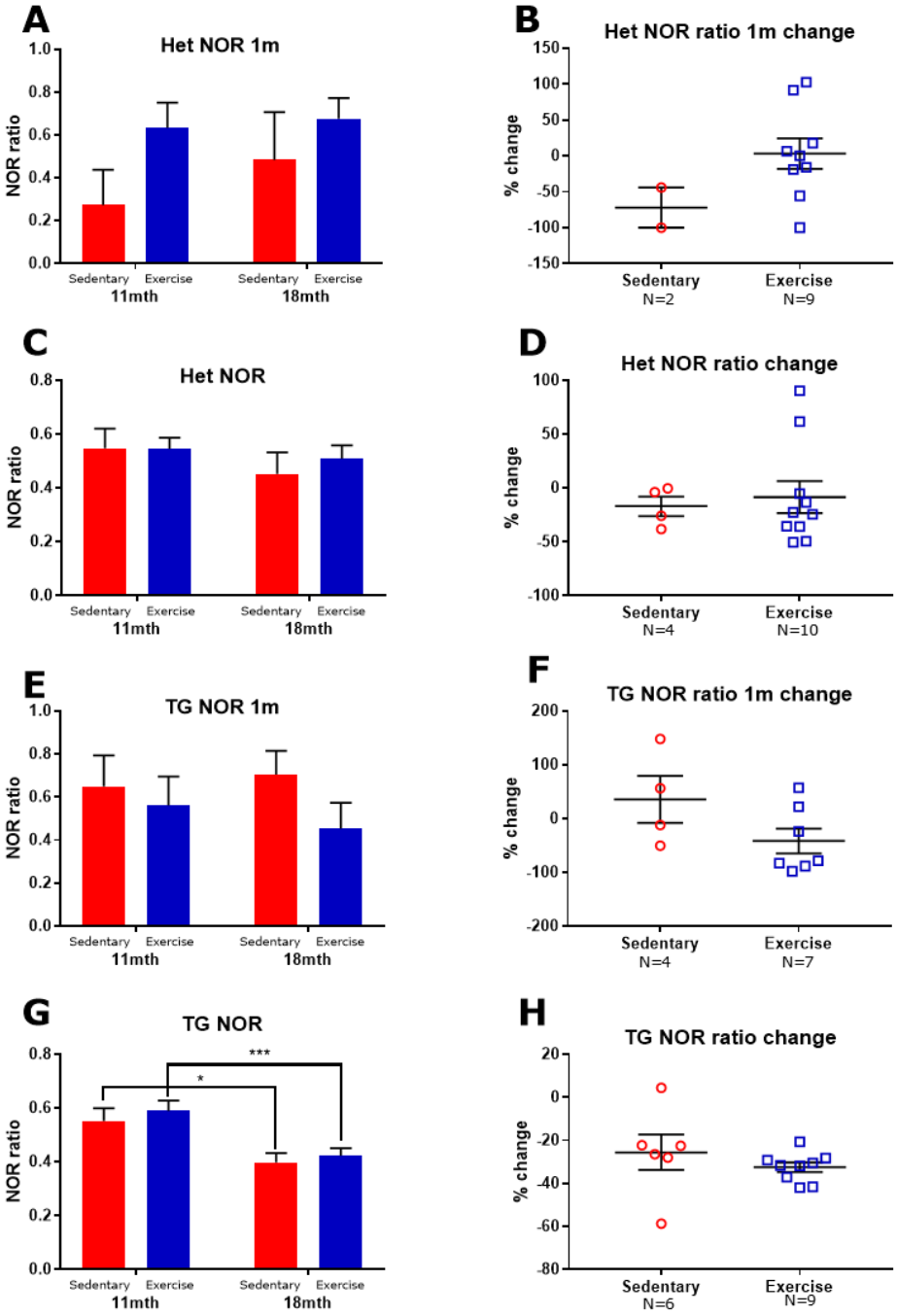


Figure 5.8: Data from NOR tests in aged TG and Het mice, comparing exercise and sedentary groups

Behaviour data for NOR cognitive tests. Mean and ±SEM bars are displayed Percentage change with age is shown. Results for exercise and sedentary groups at 11 and 18 months for the NOR test. A) NOR ratio in the first 60 seconds in Het mice. B) Percentage change in NOR ratio in the first 60 seconds in Het mice. C) NOR ratio for the full test in Het mice. D) Percentage change in NOR ratio for the full test in Het mice. E) NOR ratio in the first 60 seconds in TG mice. F) Percentage change in NOR ratio in the first 60 seconds in TG mice. G) NOR ratio for the full test in TG mice. H) Percentage change in NOR ratio for the full test in TG mice.

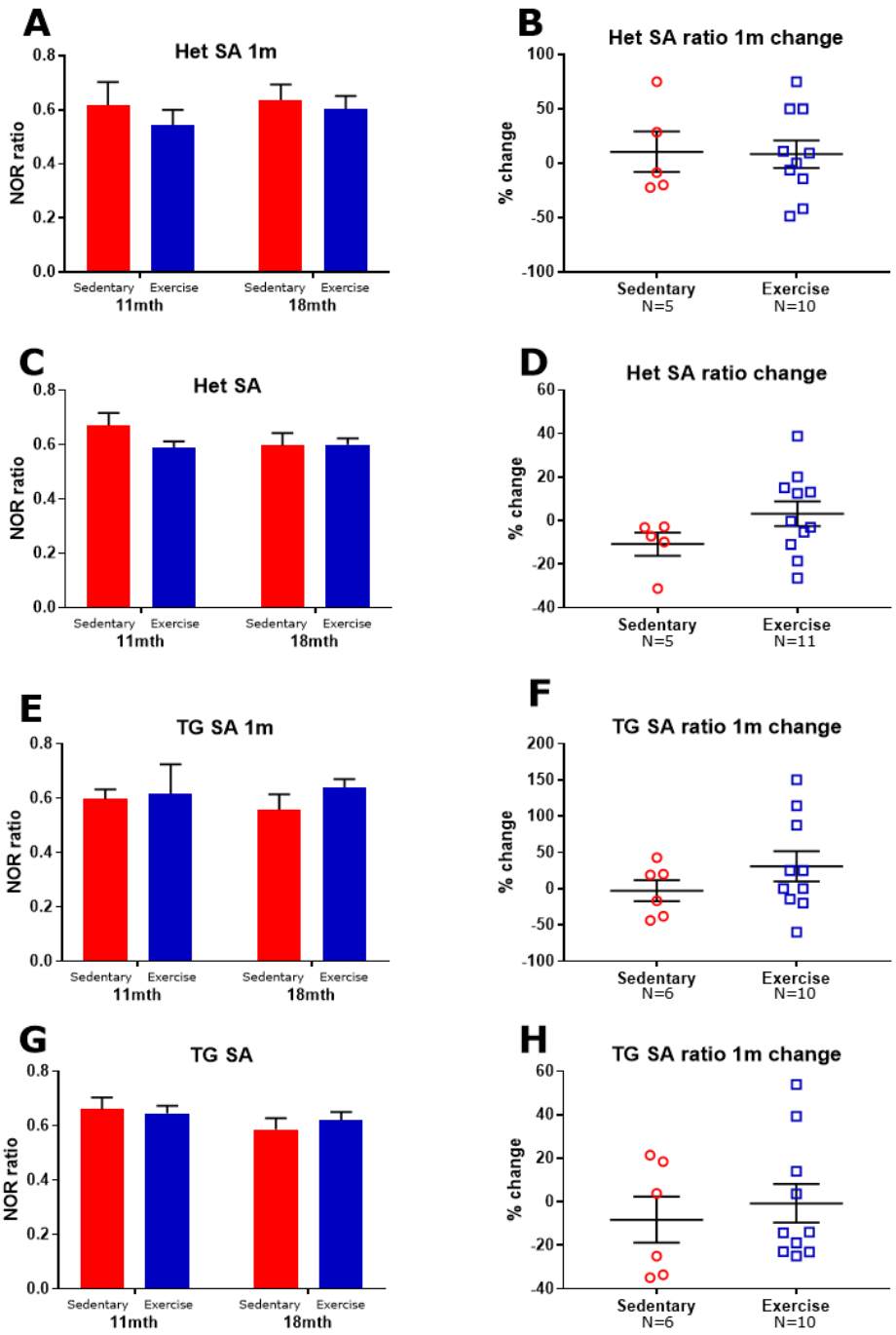


Figure 5.9: Data from SA tests in aged TG and Het mice, comparing exercise and sedentary groups

Behaviour data for SA cognitive tests. Mean and ±SEM bars are displayed Percentage change with age is shown. Results for exercise and sedentary groups at 11 and 18 months for the NOR test. A) SA ratio in the first 60 seconds in Het mice. B) Percentage change in SA ratio in the first 60 seconds in Het mice. C) SA ratio for the full test in Het mice. D) Percentage change in SA ratio for the full test in Het mice. E) SA ratio in the first 60 seconds in TG mice. F) Percentage change in SA ratio in the first 60 seconds in TG mice. G) SA ratio for the full test in TG mice. H) Percentage change in SA ratio for the full test in TG mice.

5.3.4.2 Behaviour tests for anxiety

There were some significant differences in the results from behavioural tests for anxiety between aged WT, Het and TG mice. There was a significant decrease in the percentage time spent in the open arms in the TG mice with age (two-way-ANOVA RM $F(1,18)=9.826$ $p=0.0006$***), which was not present in the WT or Het groups. There was also a significant difference in the percentage time spent in the open arms between the TG mice and the WT (two-way-ANOVA RM $F(2,18)=4.761$ $p=0.0138$*) and the TG and Het ($p=0.0004$***) groups. However, there was no significant difference in the percentage change in time spent in open arms with age between the three genotypes.

There was also a significant decrease in the SAPs (two-way-ANOVA RM $F(1,18)=5.696$ $p=0.0202$*) and head dipping (two-way-ANOVA RM $F(1,18)=5.889$) $p=0.0009$***) behaviours in the TG group from 11 to 18 months of age. We also saw a significant decrease in the percentage change in SAPs (one-way-ANOVA $F(2,18)=3.908$ $p=0.0399$*) and head dips (one-way-ANOVA $F(2,18)=3.908$ $p=0.0399$*) in the TG group in comparison to the Het group.

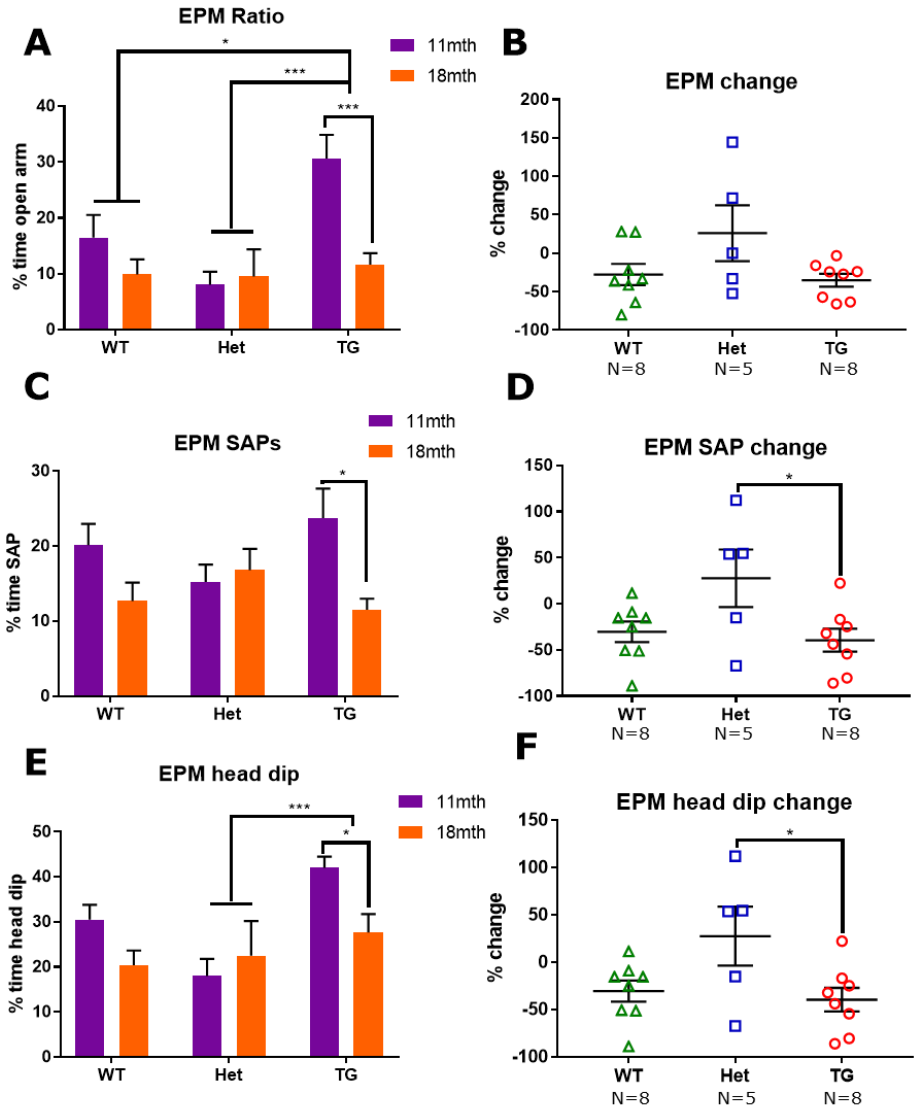


Figure 5.10: EPM data for mice with varied NMNAT1 expression

Data from EPM behaviour tests. Mean and ±SEM bars are displayed. WT, Het and TG mice that remained sedentary are shown. Percentage change with age is shown. A) Percentage time spent in open arms of EPM at different time points. B) Percentage change in time spent in open arms with age. C) Percentage time spent performing SAPs at different time points. D) Percentage change in time spent performing SAPs with age. E) Percentage time spent performing head dips at different time points. F) Percentage change in time performing head dips with age.

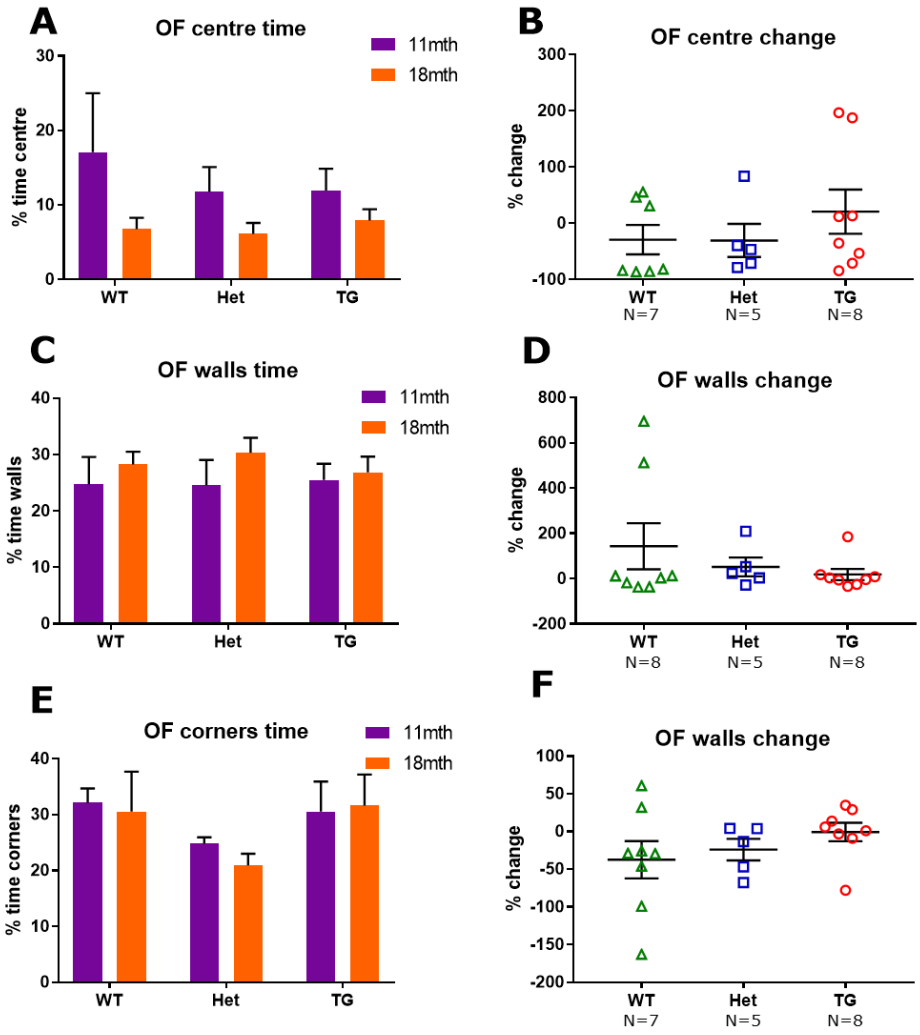


Figure 5.11: OF behavioural data for mice with varied NMNAT1 expression

Data from OF behaviour tests. Mean and ±SEM bars are displayed. WT, Het and TG mice that remained sedentary are shown. Percentage change with age is shown. A) Percentage time spent in the centre of the OF arena at different time points. B) Percentage change in time spent in the centre of the OF arena with age. C) Percentage time spent beside walls of OF arena at different time points. D) Percentage change in time spent beside walls of OF arena with age. E) Percentage time spent in corners of OF arena at different time points. F) Percentage change in time spent in corners of OF arena with age.

In the EPM, Het mice that had exercised showed a significant decrease in time spent in the open arms of the maze with age (Two-way-ANOVA RM F(1,11)=7.131 p=0.0344*) (Figure 5.12A). There was no significant difference in the Het sedentary group with age. In the TG mice, the sedentary group at 11 months of age spent significantly more time in the open arms of the EPM than the exercise group at the same age (Two-way-ANOVA RM F(1,15)=18.63 p=<0.0001****) and there was a significant decrease with age in the sedentary group (p=<0.0001****) (Figure 5.12B).

There were no significant differences between the sedentary and exercise groups with age in the number of SAPs performed in Het mice. However, for the TG mice there was a significant decrease in the number of SAPs performed in the sedentary group with age (two-way-ANOVA RM F(1,15)=10.59 p=0.0178*) (Figure 5.12D).

In the exercise group there was a significant decrease in the number of head dips performed with age in the Het mice (F(1,11)=7.22 p=0.0138*). There was a significant decrease in the number of head dips performed with age in both the exercise and sedentary groups (F(1,15)=34.53 p=<0.0001****) (Figure 5.12F).

In the OF test there was no significant effect of age or exercise on the amount of time spent in the centre or beside the walls of the arena in both the Het and TG mice (Figure 5.13). There was a significant decrease in the Het exercise group for time spent in the corners of the OF arena (Two-way-ANOVA RM F(1,13)=9.312 p=0.0139*). There were no significant effects in the TG mice.

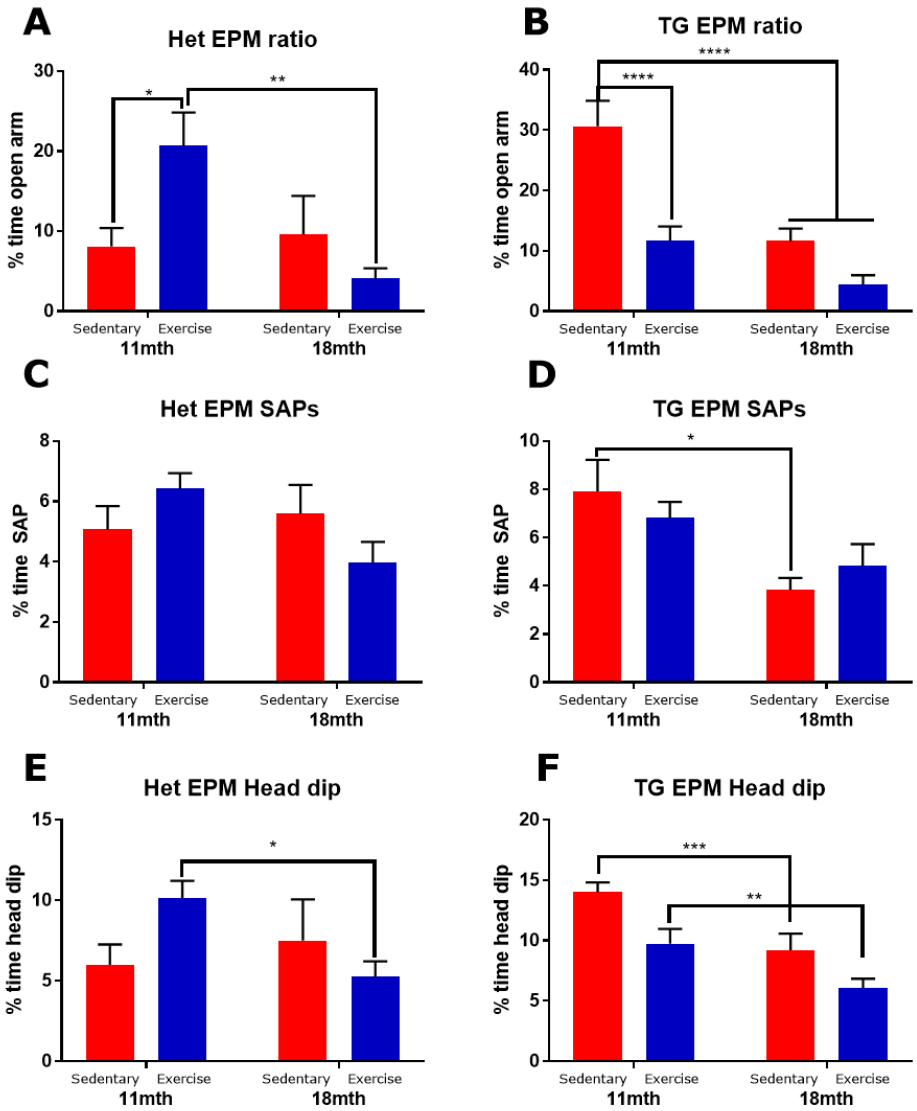


Figure 5.12: Data from EPM tests in aged TG and Het mice, comparing exercise and sedentary groups

Data from EPM behaviour tests in Het and TG mice at 11 and 18 months of age with or without 6 months of exercise. Mean and ±SEM bars are displayed. A) Percentage time spent in the open arms of the EPM arena in Het mice. B) Percentage time spent in the open arms of the EPM arena in TG mice. C) Percentage time spent performing SAPs in Het mice. D) Percentage time spent performing SAPs in TG mice. E) Percentage time spent performing head dips in Het mice. F) Percentage time spent performing head dips in TG mice.

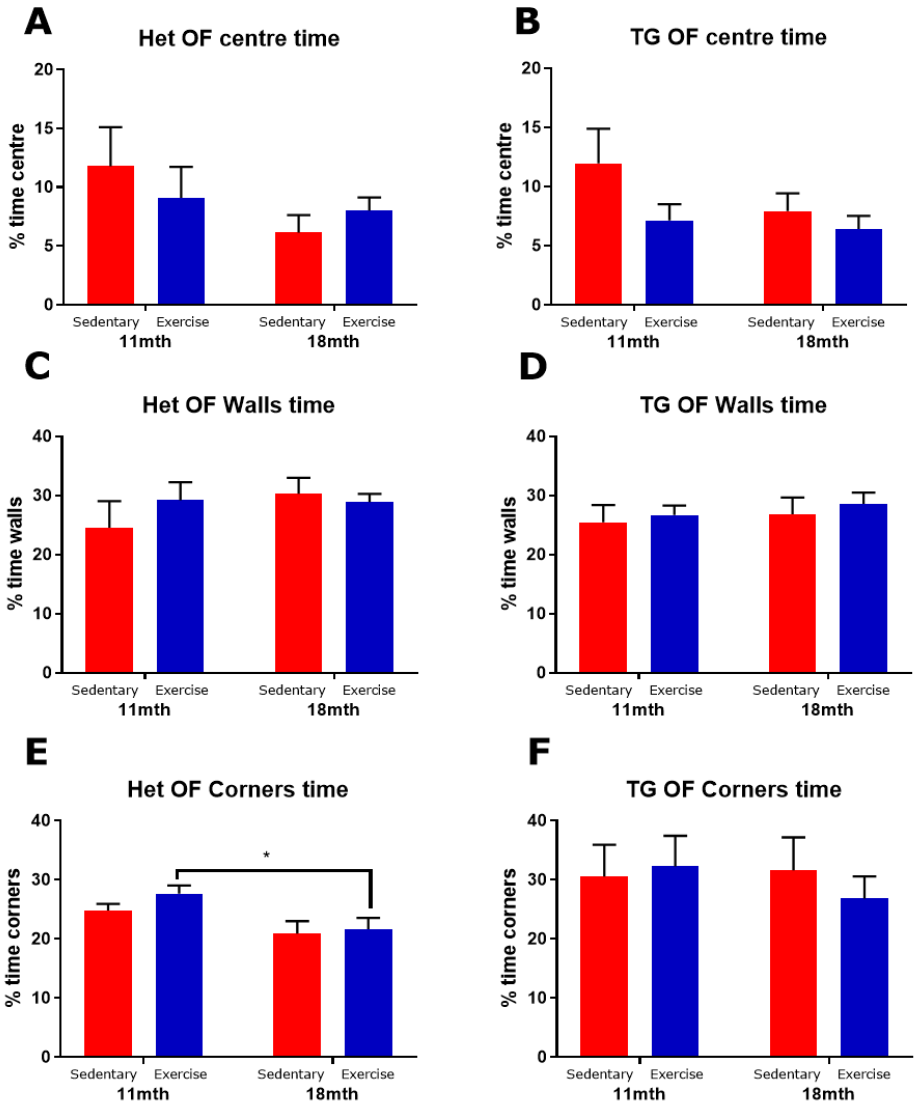


Figure 5.13: Data from OF tests in aged TG and Het mice, comparing exercise and sedentary groups

Data from OF behaviour tests in Het and TG mice at 11 and 18 months of age with or without 6 months of exercise. Mean and ±SEM bars are displayed. A) Percentage time spent in the centre of the OF arena in Het mice. B) Percentage time spent in the centre of the OF arena in TG mice. C) Percentage time spent by the walls of the OF arena in Het mice. D) Percentage time spent by the walls of the OF arena in TG mice. E) Percentage time spent in the corners of the OF arena in Het mice. F) Percentage time spent in the corners of the OF arena in TG mice.

5.3.4.3 Locomotor activity

In the EPM there was a significant effect of age on the distance travelled per mouse (Two-way-ANOVA RM F(1,18)=7.789 p=0.0121*). There was also a significant effect of age on the distance travelled in the NOR (Two-way-ANOVA RM F(1,12)=7.222 p=0.0198*). There was no significant effect of age on the distance travelled in the OF or SA. There was also no significant effect of genotype on the distance travelled in any of the behaviour tests (Figure 5.14).

There was a significant decrease in the distance travelled in the EPM by the exercise group in the Het mice (two-way-ANOVA RM F(1,11)=8.236 p=0.0220*) (Figure 5.15A) and both the sedentary (F(1,15)=18.96 p=0.0183*) and exercise (p=0.0125*) TG mice (Figure 5.15B).

There was a significant increase in the distance travelled in the NOR with age in both the sedentary (Two-way-ANOVA RM F(1,14)=38.03 p=0.0097**) and exercise (p=<0.0001****) TG mice (Figure 5.15F). There were no other significant effects of exercise or age in the behavioural tests for Het and TG mice.

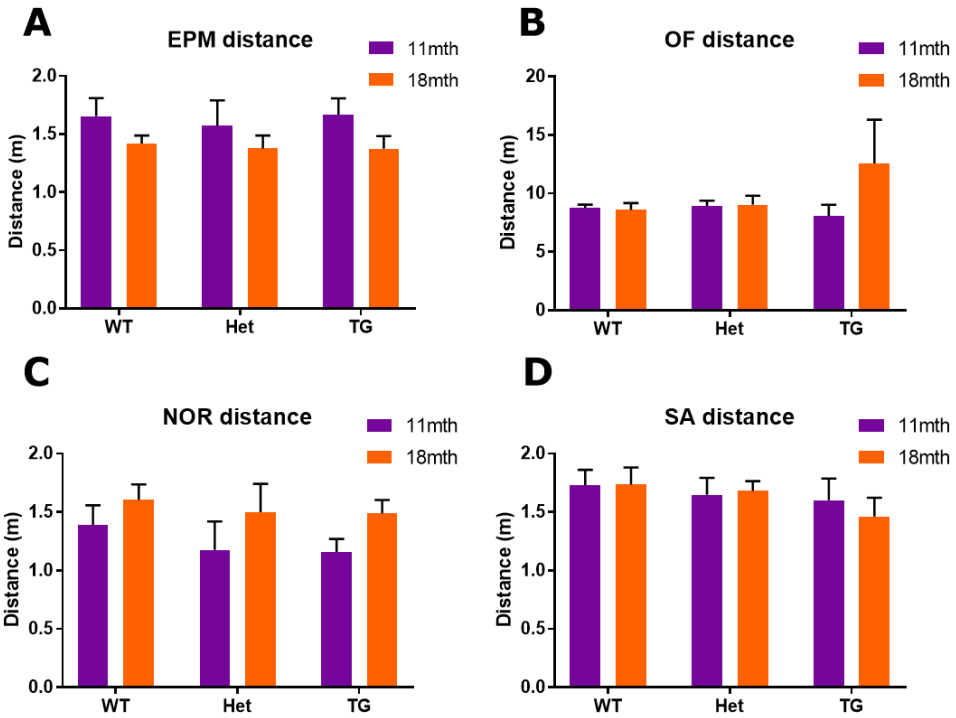


Figure 5.14: Locomotor activity data from behaviour tests performed on mice with varied NMNAT1 expression

Data from cognitive behaviour tests in mice at 11 and 18 months of age. Mean and SEM bars are displayed. WT, HetKO and TG mice that remained sedentary are shown. A) Change in the LMA in the EPM test. B) Change in the LMA in the OF test. C) Change in the LMA in the NOR test. D) Change in the LMA of the SA test.

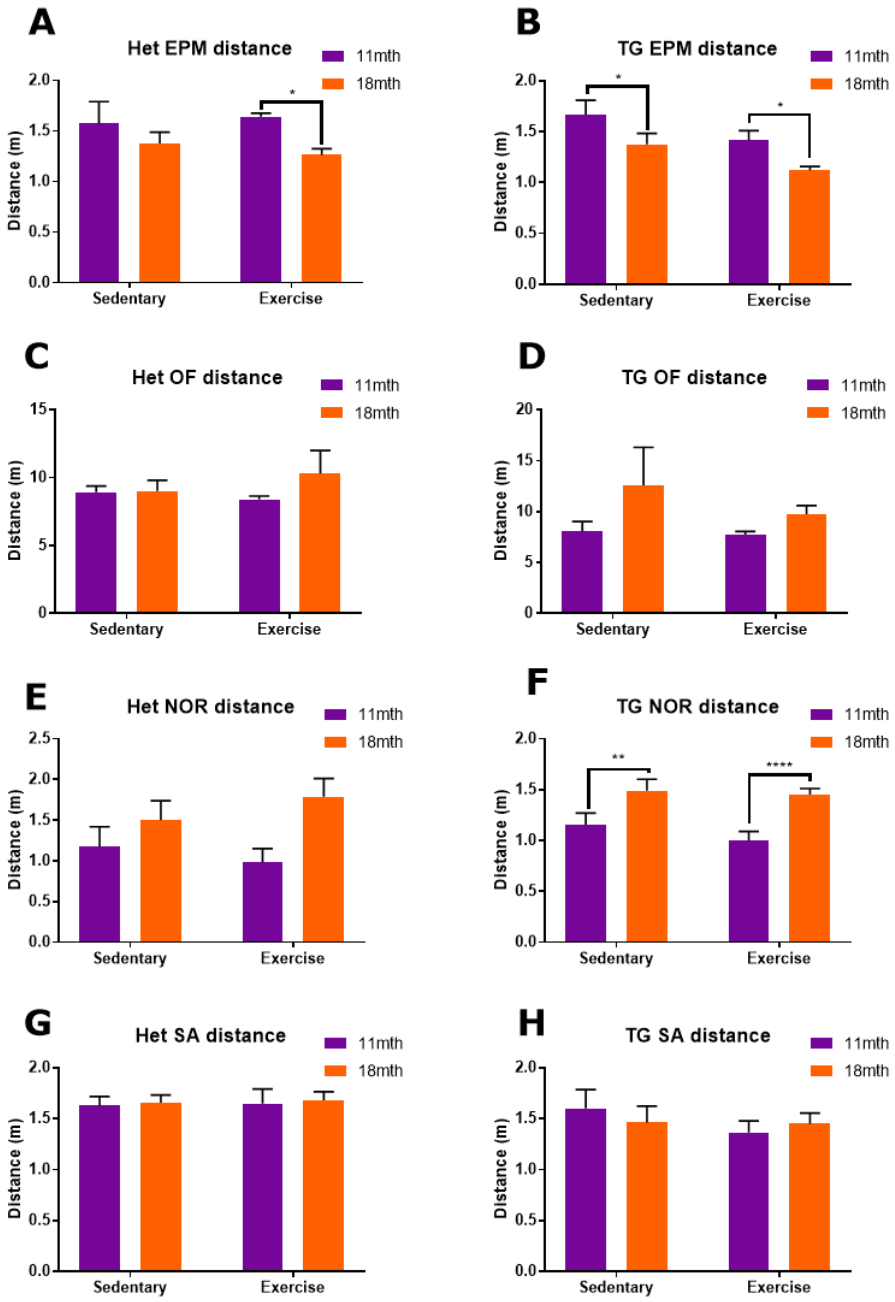


Figure 5.15: Locomotor activity data from behavioural tests in aged TG and Het mice, comparing exercise and sedentary groups

Locomotor activity data from behaviour tests in mice at 11 and 18 months of age. Mean and SEM bars are displayed. All graphs are comparing mice that had voluntary access to a running wheel for 6 months, and mice who remained sedentary for the same time. A) LMA from the EPM in Het mice. B) LMA from the EPM in TG mice. C) LMA from the OF test in Het mice. D) LMA from the OF test in TG mice. E) LMA from the NOR test in Het mice. F) LMA from the NOR test in TG mice. G) LMA from the SA test in Het mice. H) LMA from the SA test in TG mice.

5.3.5 The effects of varied NMNAT1 expression on adult neurogenesis in mice and the changes brought about with voluntary exercise

5.3.5.1 The effect of varied NMNAT1 expression and sex on Neurogenesis in the dentate gyrus of a young mouse model

There were no significant differences seen in the number of Ki67 positively stained cells between the male and female mice, or between the three genotypes (Figure 5.16A).

Two-way-ANOVA analysis showed a significant difference with genotype for the number of Dcx positive stained cells ($F(2,67)=5.458$ $p=0.0064$). With Tukey's multiple comparisons test, we found that there was no significant difference in the number of Dcx positive stained cells in the young male mice with varied expression levels of NMNAT1. However, there were significantly more Dcx positive stained cells in young female mice from the HetKO colony than the young female WT ($P=0.0203$), young female TG ($P=0.0185$) and young male WT mice ($P=0.0043$).

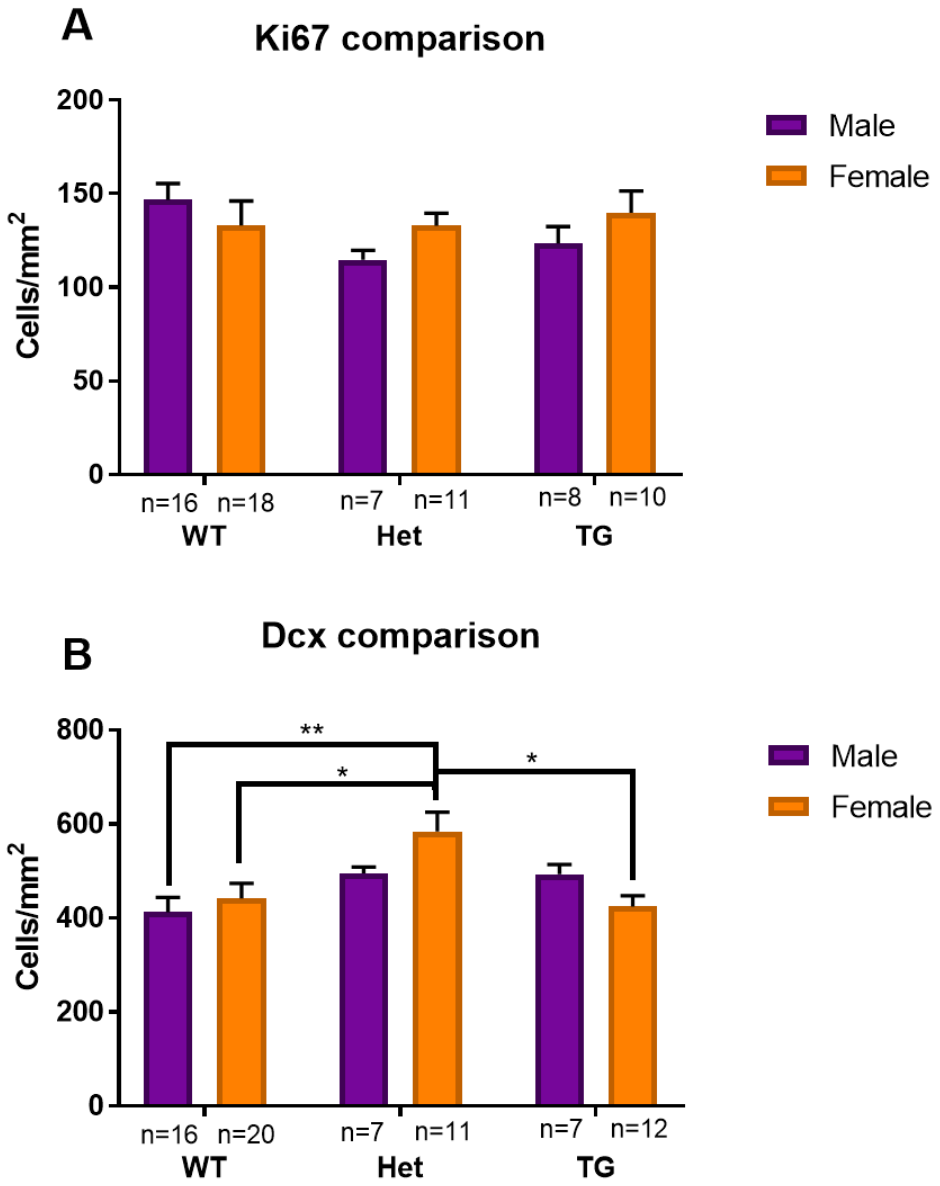


Figure 5.16: Effect of varied NMNAT1 expression and sex on neurogenesis in the dentate gyrus in 56-day old mice

Data from immunohistochemical studies for Ki67 and Dcx marked cells in the dentate gyrus of 56-day old male and female mice. Data from Mean and SEM bars are displayed. WT, HetKO and TG mice that remained sedentary are shown. (*p=≤0.05 **P=≤0.01 following ANOVA) A) Comparison of the number of Ki67 positive cells quantified in the dentate gyrus of male and female mice from all three genotypes. B) Comparison of the number of Dcx positive cells quantified in the dentate gyrus of male and female mice from all three genotypes.

5.3.5.2 Changes in adult neurogenesis in aged mice with varied NMNAT1 expression and the effect of voluntary exercise.

Changes in the expression levels of NMNAT1 had a significant effect on neurogenesis levels in the hippocampus of aged mice. There was no significant difference in the number of Ki67 cells in the DG between the three groups (Figure 5.17A). However, TG mice had significantly higher numbers of Dcx cells in the DG (ANOVA F(2,23)=7.306 p=0.0035 Figure

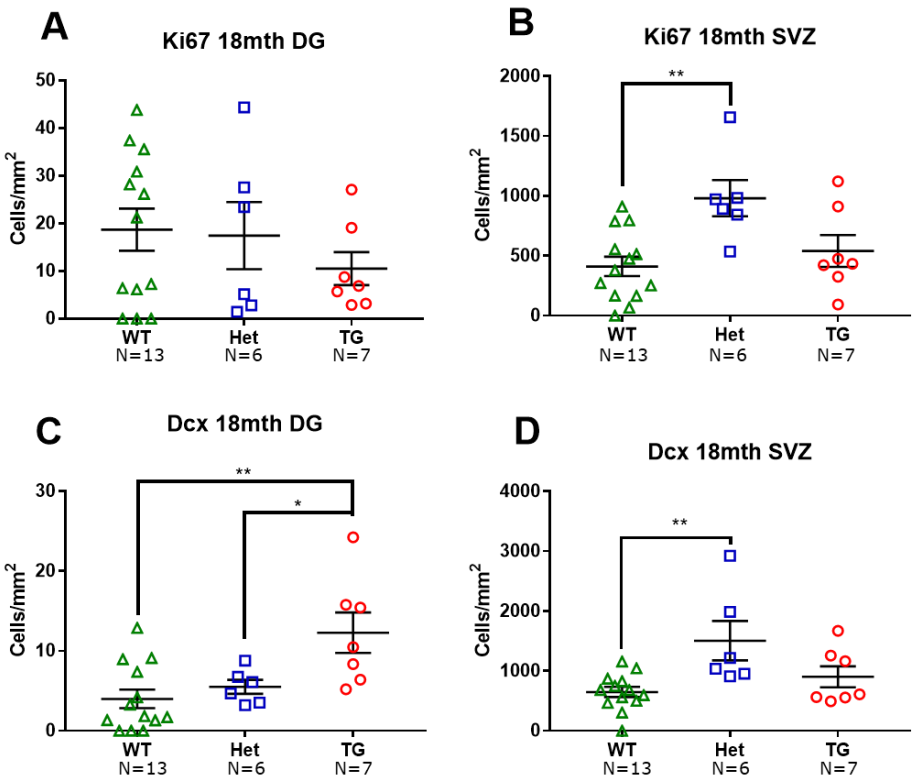


Figure 5.17: Neurogenesis data for Ki67 and Dcx in the DG and SVZ of aged sedentary mice with varied NMNAT1 expression

Data from immunohistochemical studies for Ki67 and Dcx marked cells in the DG and SVZ in aged mice. Data from Mean and SEM bars are displayed. WT, HetKO and TG mice that remained sedentary are shown. (**p=≤0.01 *p=≤0.05) A) Comparison of Ki67 positive stained cells in the DG. B) Comparison of Ji67 positive stained cells in the SVZ. C) Comparison of Dcx positive stained cells in the SVZ. D) Comparison of Dcx positive stained cells in the SVZ.

5.17C) than either Het mice (multiple comparisons p=0.0414) or WT mice (multiple comparisons p=0.0028).

Het mice had significantly higher numbers of Ki67 stained cells in the SVZ than WT mice (ANOVA F(2,23)=6.294 p=0.0066 multiple comparisons p=0.0049 Figure 5.17B) There was also a strong trend towards there being significantly higher number of Ki67 stained cells in the SVZ in Het mice than in TG mice (multiple comparisons p=0.0598). Het mice also had significantly higher numbers of Dcx cells in the SVZ than WT mice (ANOVA F(2,23)=6.189 p=0.0071 multiple comparisons p=0.0051 Figure 5.17D)

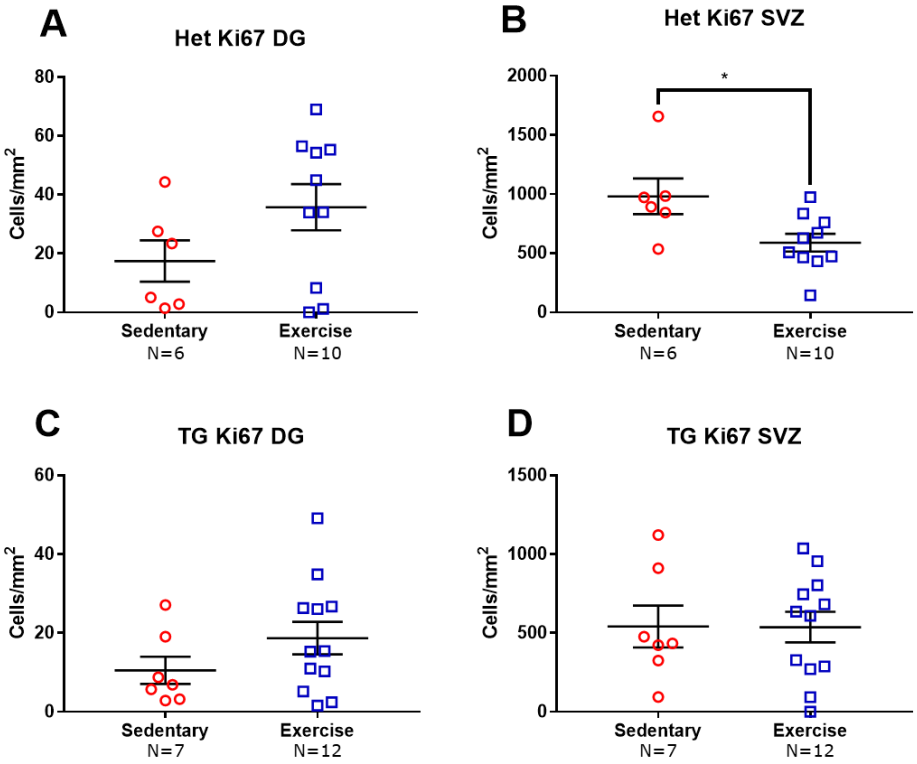


Figure 5.18: Number of cells/mm2 stained with Ki67 in aged TG and Het mice, comparing exercise and sedentary groups

Neurogenesis data for cells with positive staining for Ki67 in the hippocampus per mm^2. All graphs are comparing mice that had voluntary access to a running wheel for 6 months, and mice who remained sedentary for the same time. Mean and SEM bars are displayed. A) Number of cells in the DG of Het mice. B) Number of cells in the SVZ of Het mice (*p=≤0.05). C) Number of cells in the DG of TG mice. D) Number of cells in the SVZ of TG mice.

and tended towards having higher numbers than TG mice (multiple comparisons p=0.0931).

Exercise caused a significant decrease in the number of Ki67 stained cells in the SVZ of Het mice (t(14)=2.606 p=0.0207 Figure 5.18B). There were no other significant changes in the number of Ki67 cells in the DG or SVZ of Het and TG mice.

There were no significant changes in the number of Dcx cells in the DG or SVZ of Het and TG mice. However, there was a trend towards increased numbers of Dcx cells in the DG of Het mice with exercise (t(14)=1.854 p=0.0849). There was also a trend towards decreased numbers of Dcx cells in the SVZ of Het mice with exercise (t(14)=2.126 p=0.0518).

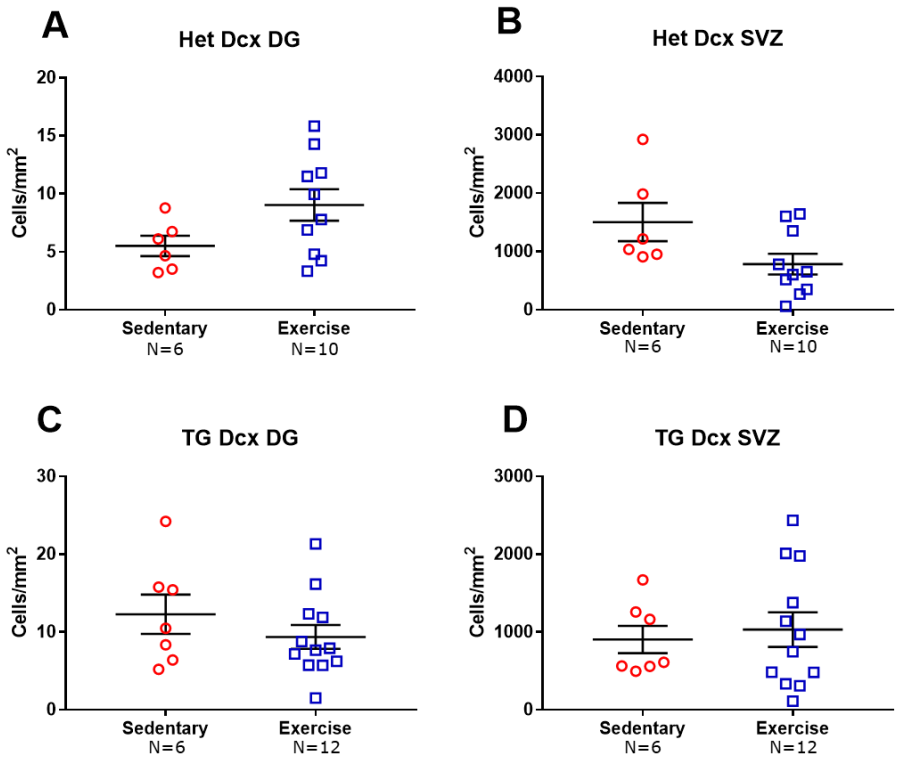


Figure 5.19: Number of cells/mm2 stained with Dcx in aged TG and Het mice, comparing exercise and sedentary groups

Neurogenesis data for cells with positive staining for Dcx in the hippocampus per mm^2. All graphs are comparing mice that had voluntary access to a running wheel for 6 months, and mice who remained sedentary for the same time. Mean and SEM bars are displayed. A) Number of cells in the DG of Het mice. B) Number of cells in the SVZ of Het mice. C) Number of cells in the DG of TG mice. D) Number of cells in the SVZ of TG mice.

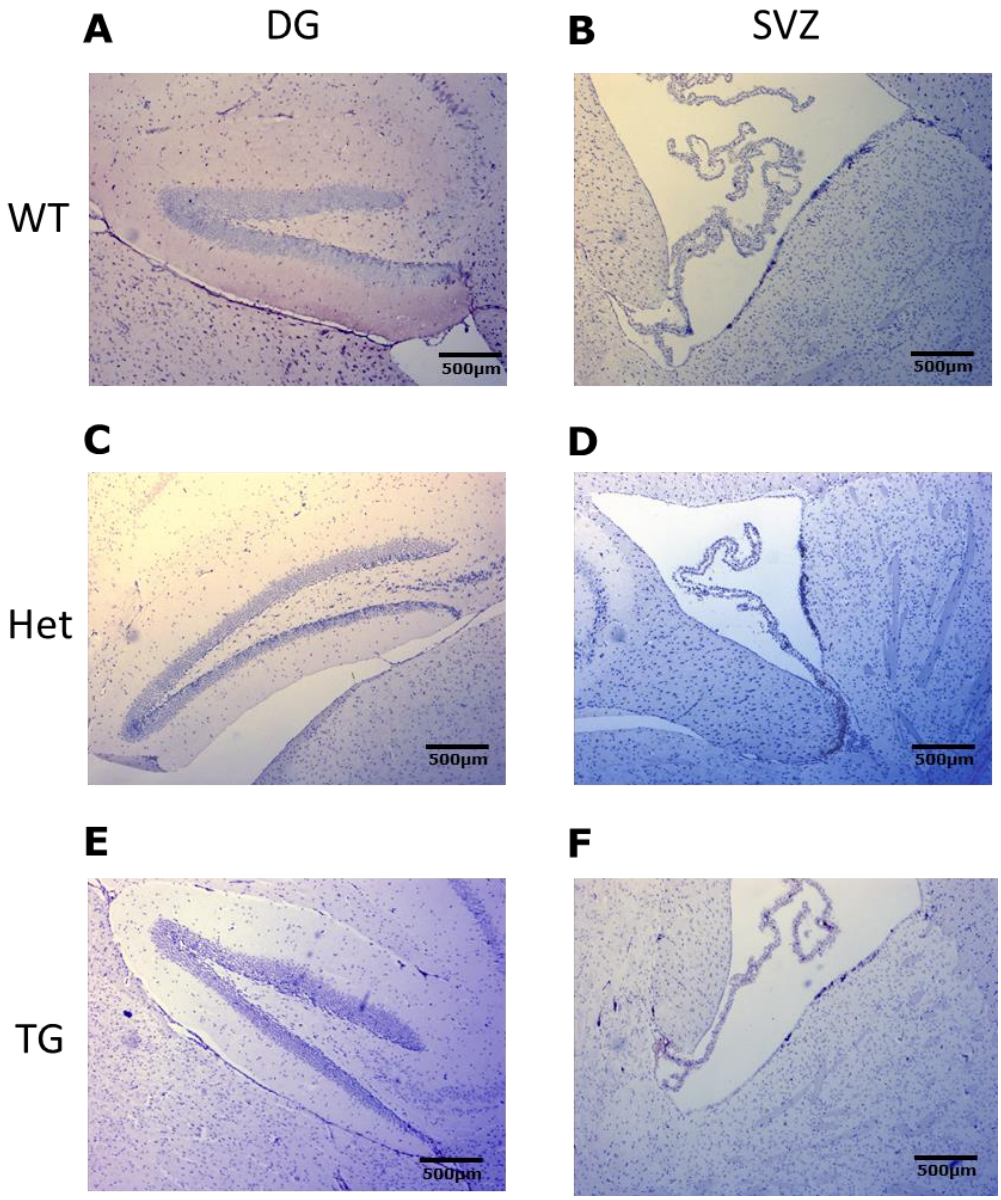


Figure 5.20: Sample images of DG and SVZ regions stained with Ki67 antibody in WT, Het and TG mice.

Sample images from analysis of Ki67 stained cells within the DG (left) and SVZ (right) in the brains of 18-month-old WT (top), Het (middle) and TG (bottom) mice that remained sedentary. Images were taken by the author at *the University of Nottingham SLIM facility*.

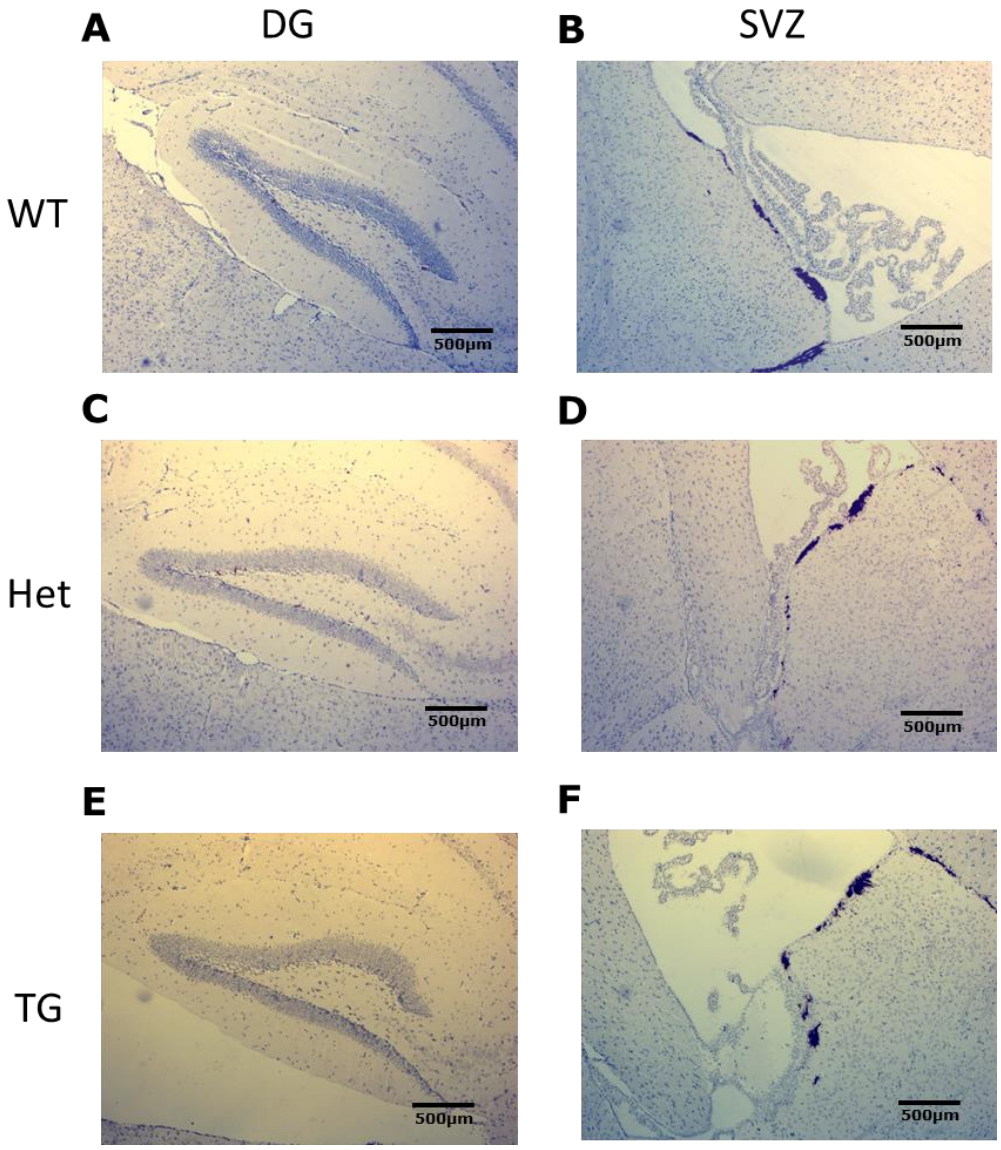


Figure 5.21: Sample images of DG and SVZ regions stained with Dcx antibody in WT, Het and TG mice.

Sample images from analysis of Dcx stained cells within the DG (left) and SVZ (right) in the brains of 18-month-old WT (top), Het (middle) and TG (bottom) mice that remained sedentary. Images were taken by the author at *the University of Nottingham SLIM facility.*

There was no significant effect of varied NMNAT1 expression on the number of BrdU positive cells in the DG of aged mice (Figure 5.22A). TG mice had significantly higher numbers of BrdU positive cells (ANOVA $F(2,23)=9.443$ $p=0.0010$ Figure 5.22B) in the SVZ than Het mice (multiple comparisons $p=0.0148$) or WT mice (multiple comparisons $p=0.0008$). In the DG, mice from the Het colony had the highest percentage of samples with BrdU staining, and in the SVZ the TG mice had the highest (Figure 5.22C,D).

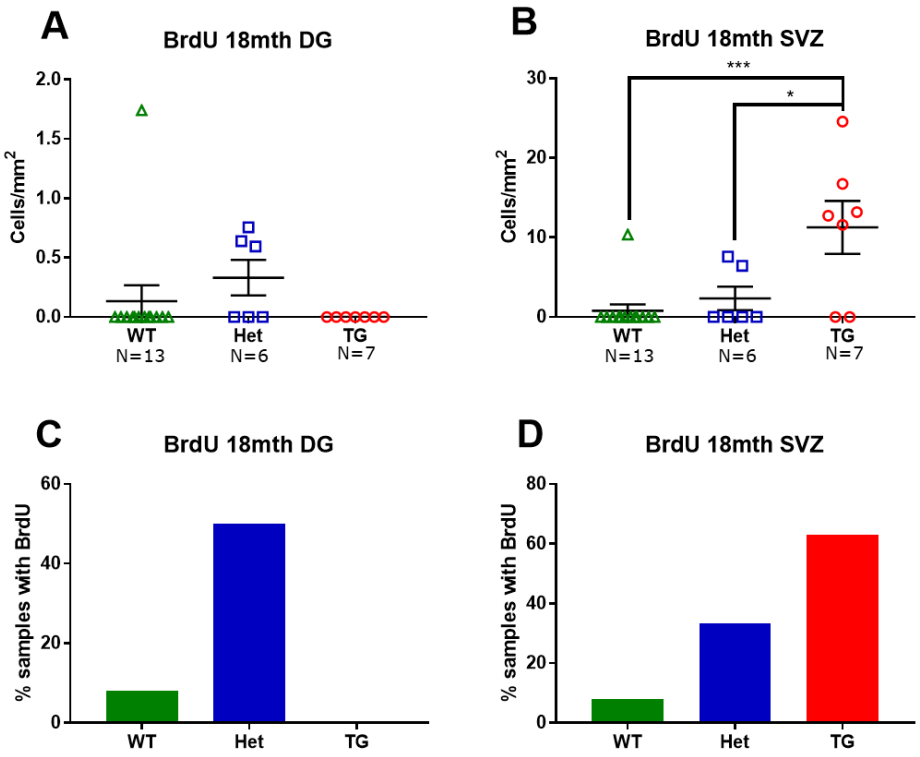


Figure 5.22: Neurogenesis data for BrdU stained cells in the DG and SVZ of aged sedentary mice with varied NMNAT1 expression

Data from immunohistochemical studies for BrdU marked cells in the dentate gyrus and subventricular zone of the hippocampus in aged mice. Data from Mean and SEM bars are displayed. WT, HetKO and TG mice that remained sedentary are shown. (*p=≤0.05 ***p=≤0.001) A) Comparison of BrdU marked cells in the DG. B) Comparison of BrdU marked cells in the SVZ. C) Comparison of the percentage of all samples with BrdU stained cells present in the DG. D) Comparison of the percentage of all samples with BrdU stained cells present in the SVZ.

Exercise had no significant effect on the number of BrdU stained cells in the DG and SVZ of mice from the TG and Het colonies (Figure 5.23). However, there was a trend towards exercise increasing the number of BrdU cells in the DG of TG mice (t(17)=2.088 p=0.0522 Figure 5.23C).

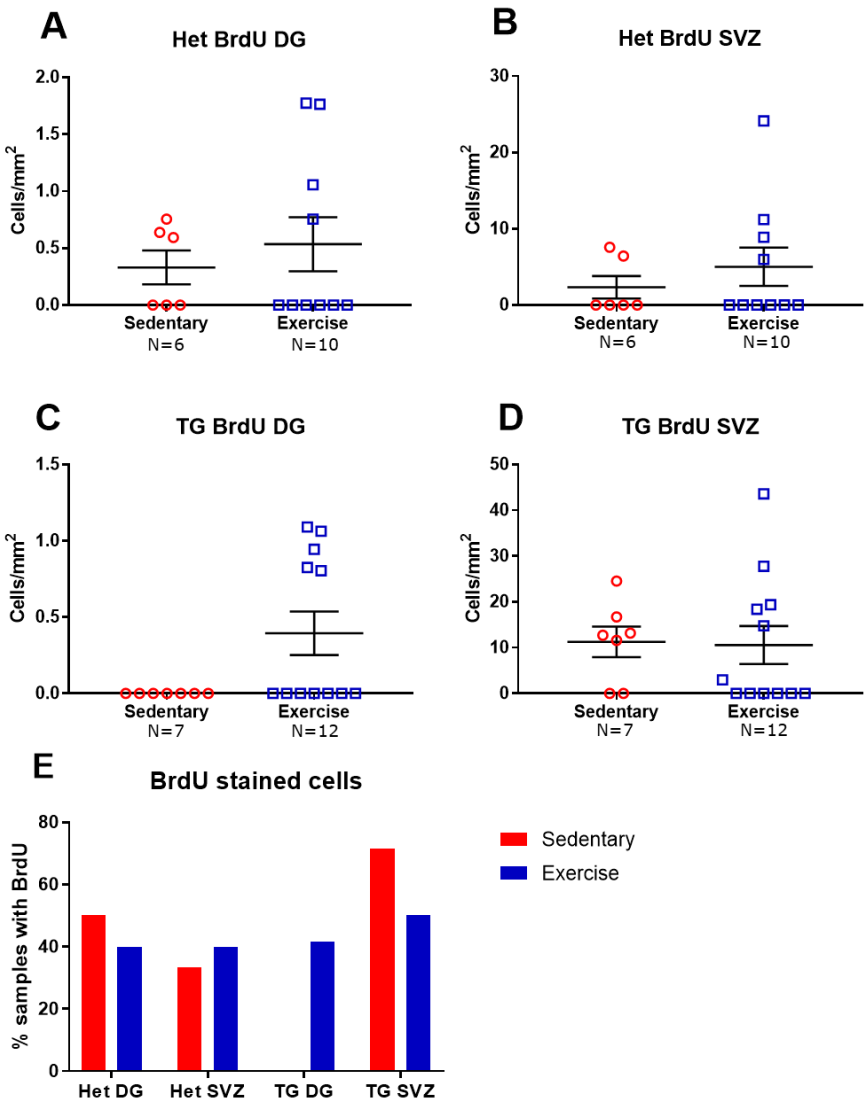


Figure 5.23: Number of cells/mm2 stained with BrdU in aged TG and Het mice, comparing exercise and sedentary groups

Neurogenesis data for cells with positive staining for BrdU in the hippocampus per mm^2. All graphs are comparing mice that had voluntary access to a running wheel for 6 months, and mice who remained sedentary for the same time. Mean and SEM bars are displayed. A) Number of cells in the DG of Het mice. B) Number of cells in the DG of TG mice. C) Number of cells in the SVZ of Het mice. D) Number of cells in the SVZ of TG mice. E) The number of brain sections with one or more cells with BrdU staining present

5.3.6 The effect of varied NMNAT1 expression on circulating miRNA expression levels in blood plasma and the changes brought about with voluntary exercise

The majority of the miRNAs tested in this study were not significantly affected by the change in NMNAT1 expression (Figure 5.24). The expression of miR-21-5p was significantly affected by the change in NMNAT1 expression (ANOVA $F(2,14)=5.765$ $p=0.0149$), and was significantly higher in TG mice compared to WT mice (Tukey's multiple comparisons $p=0.0113$ Figure 5.24D).

In Het mice, exercise had no significant effect on the expression of the miRNA's tested in this study. However, there was a trend towards exercise decreasing the expression of miR-20a-5p ($t(10)=1.969$ $p=0.0773$ Figure 5.25E).

In TG mice, exercise caused a significant decrease in the expression of miR-20a-5p ($t(13)=2.23$ $p=0.0440$ Figure 5.26E) and miR-92a-3p ($t(13)=2.47$ $p=0.0281$ Figure 5.26G). There was also a trend towards decreased expression of miR-21-5p ($t(13)=1.938$ $p=0.0746$ Figure 5.26D) and miR 221-3p ($t(13)=1.938$ $p=0.0746$ Figure 5.26F) with exercise.

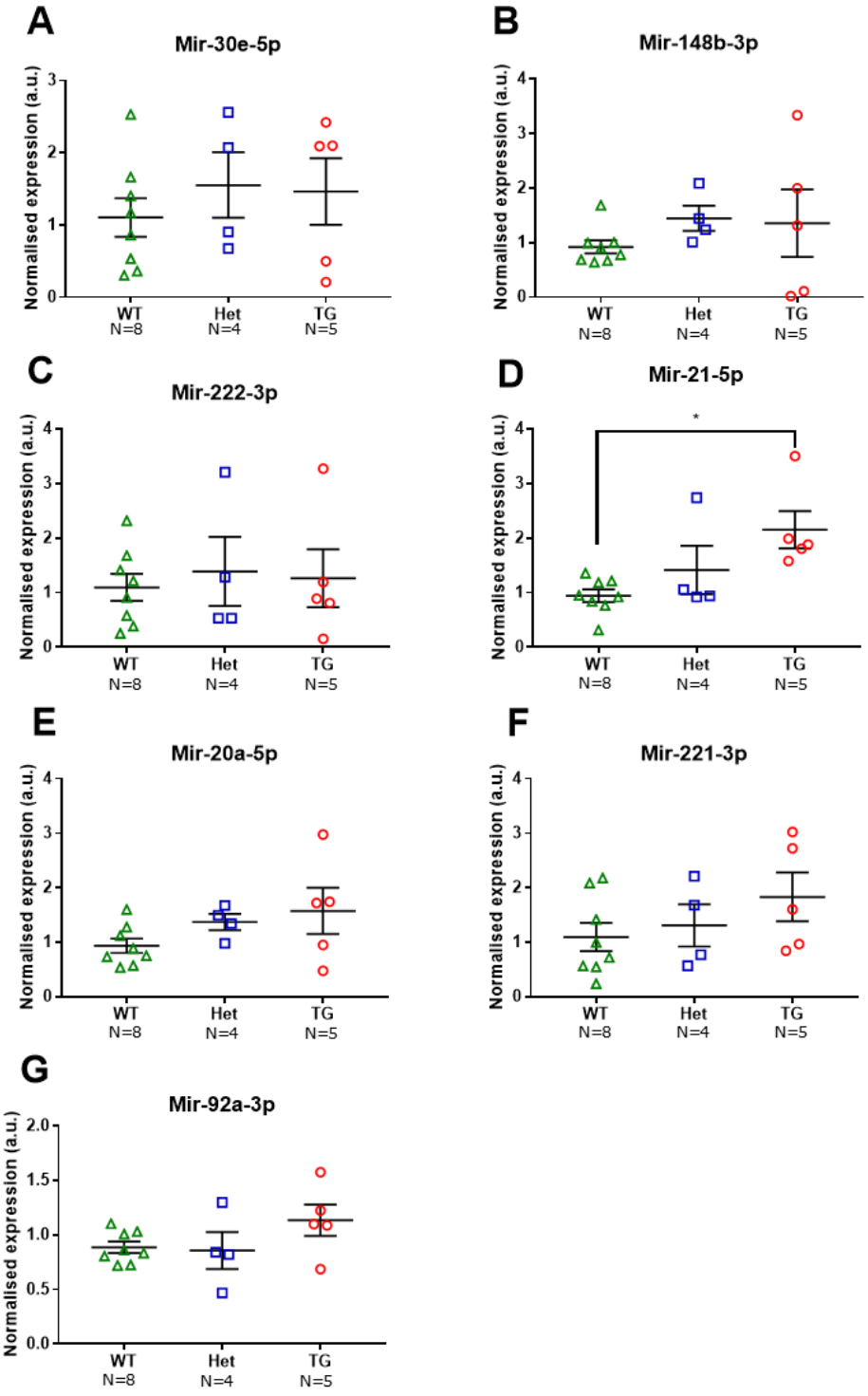


Figure 5.24: Expression levels of circulating miRNA levels in blood plasma with varied NMNAT1 expression

Data for expression levels of miRNAs from blood plasma in aged sedentary mice. Data from mean and SEM bars are shown. WT, Het and TG mouse data is shown. A) Expression levels of miR-30e-5p. B) Expression levels of miR-148b-3p. C) Expression levels of miR-222-3p. D) Expression levels of Mir 21-5p (*p=≤0.05). E) Expression levels of miR-20a-5p. F) Expression levels of Mir 221-3p. G) miR-92a-3p.

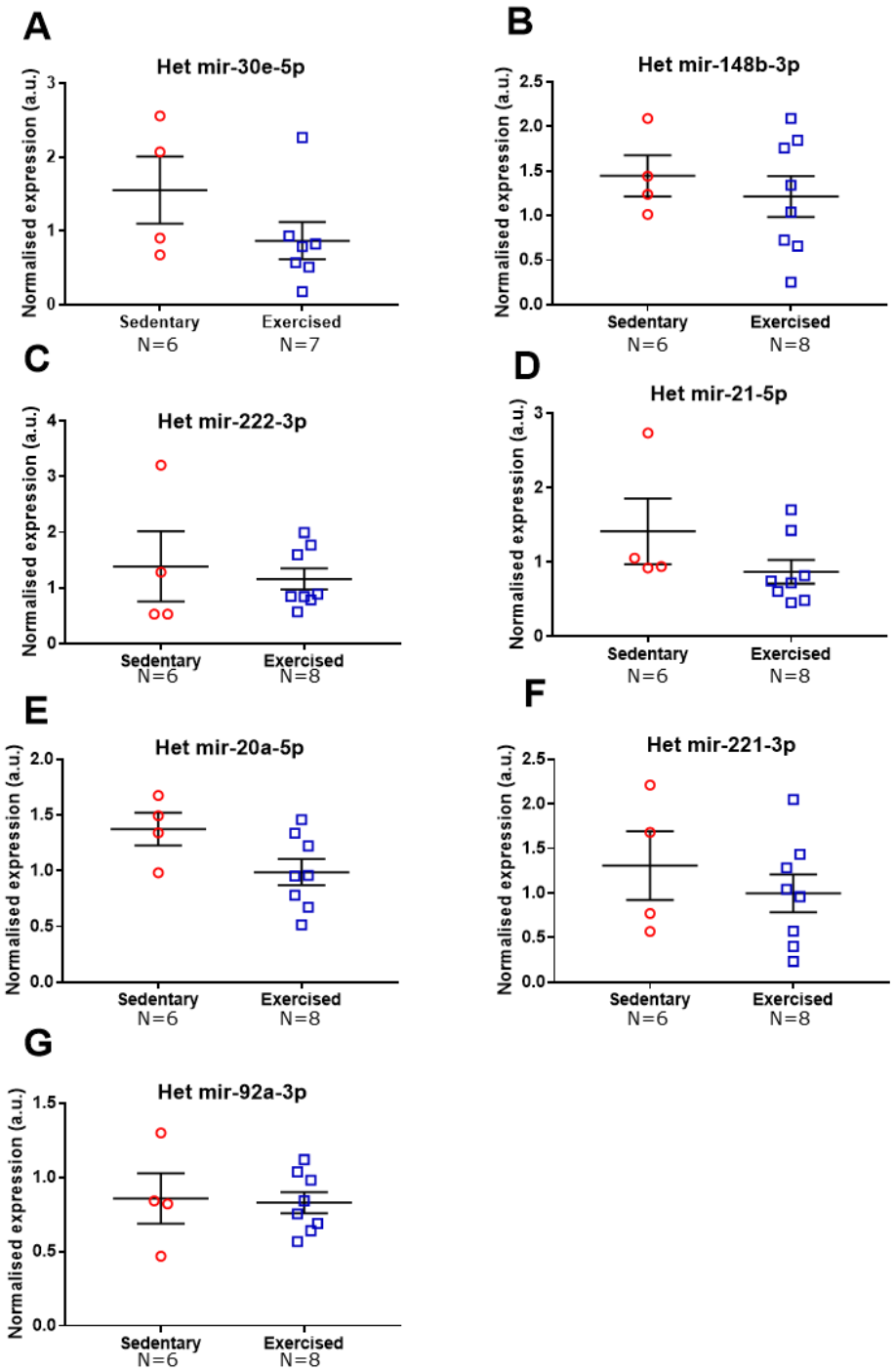


Figure 5.25: Effect of exercise on miRNA expression in blood plasma of Het mice

Normalised expression of miRNAs in blood plasma in Het mice. All graphs are comparing mice that had voluntary access to a running wheel for 6 months, and mice who remained sedentary for the same time. Mean and SEM bars are displayed. A) Normalised expression of miR-30e-5p. B) Normalised expression of miR-148b-3p. C) Normalised expression of miR-222-3p. D) Normalised expression of miR-21-5p. E) Normalised expression of miR-20a-5p. F) Normalised expression of miR-221-5p. G) Normalised expression of miR-92a-

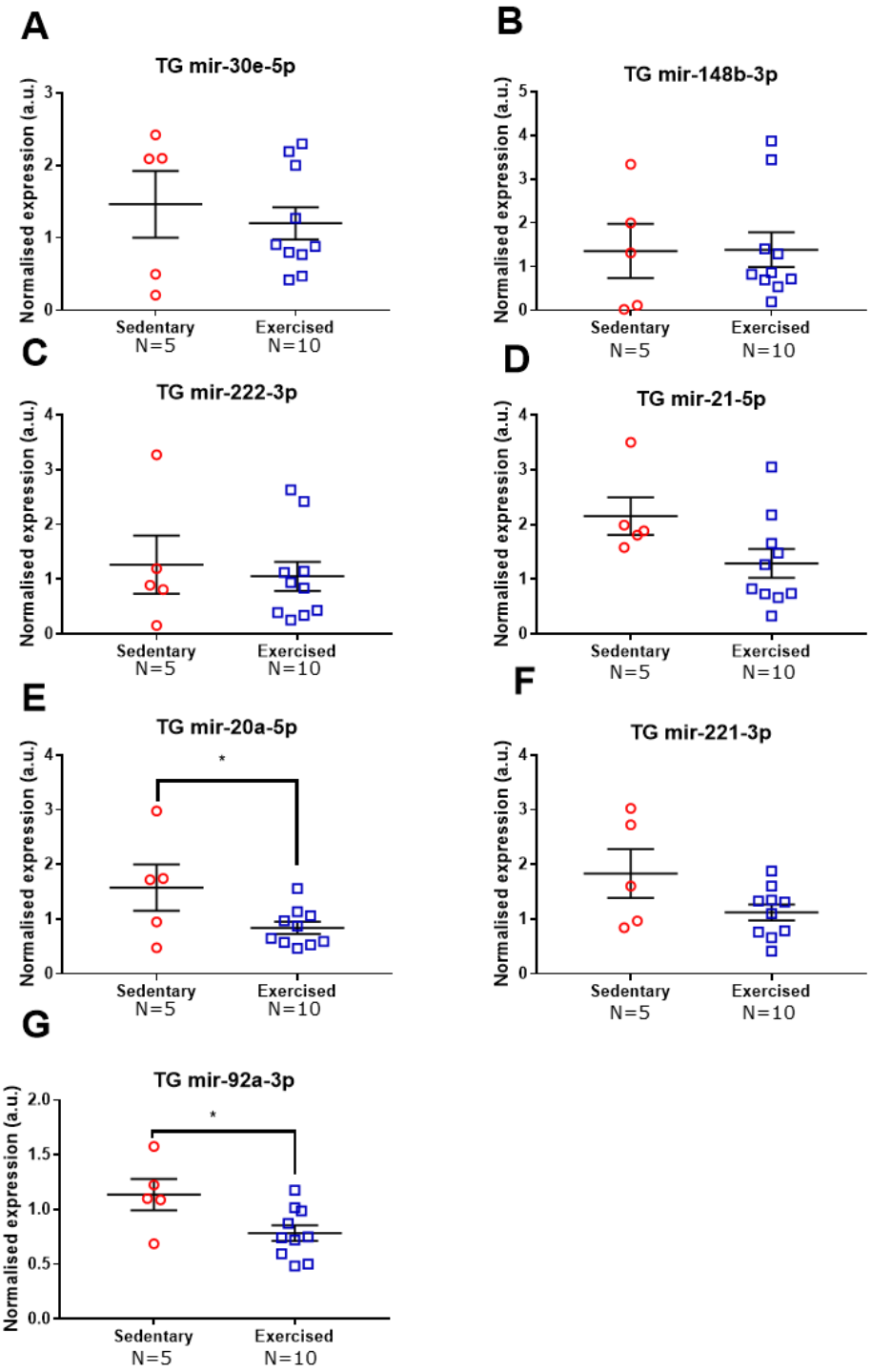


Figure 5.26: Effect of exercise on miRNA expression in blood plasma of TG mice

Normalised expression of miRNAs in blood plasma in Het mice. All graphs are comparing mice that had voluntary access to a running wheel for 6 months, and mice who remained sedentary for the same time. Mean and SEM bars are displayed. A) Normalised expression of miR-30e-5p. B) Normalised expression of miR-148b-3p. C) Normalised expression of miR-222-3p. D) Normalised expression of miR-21-5p. E) Normalised expression of miR-20a-5p. F) Normalised expression of miR-221-5p. G) Normalised expression of miR-92a-

5.3.7 The effect of varied NMNAT1 expression on gene expression in the hippocampus and the changes brought about with voluntary exercise

Apart from the expression of NMNAT1 itself, there was no significant difference in the expression of genes involved in the NAD^+ biosynthetic pathway (Figure 5.27), or genes associated with epigenetic modifications (Figure 5.28) in the hippocampus of aged mice with varied NMNAT1 expression. As expected, we saw a significant increase in the levels of NMNAT1 expression in TG mice in comparison to WT and Het mice (ANOVA $F(2,14)=17$ $P=0.0002$ Figure 5.27A). We also saw a significant decrease in NMNAT1 expression in Het mice compared to WT mice ($t(9)=3.815$ $p=0.0041$ Figure 5.27A top).

Exercise did not have a significant effect on gene expression in the hippocampus of aged Het mice. However, there was a trend towards decreased NAMPT ($t(12)=1.948$ $p=0.0752$ Figure 5.29C) and Utx ($t(13)=1.906$ $p=0.0790$ Figure 5.29F) expression with exercise.

The only significant effect of exercise in TG mice was increased expression of BDNF ($t(13)=3.362$ $p=0.0051$ Figure 5.30G).

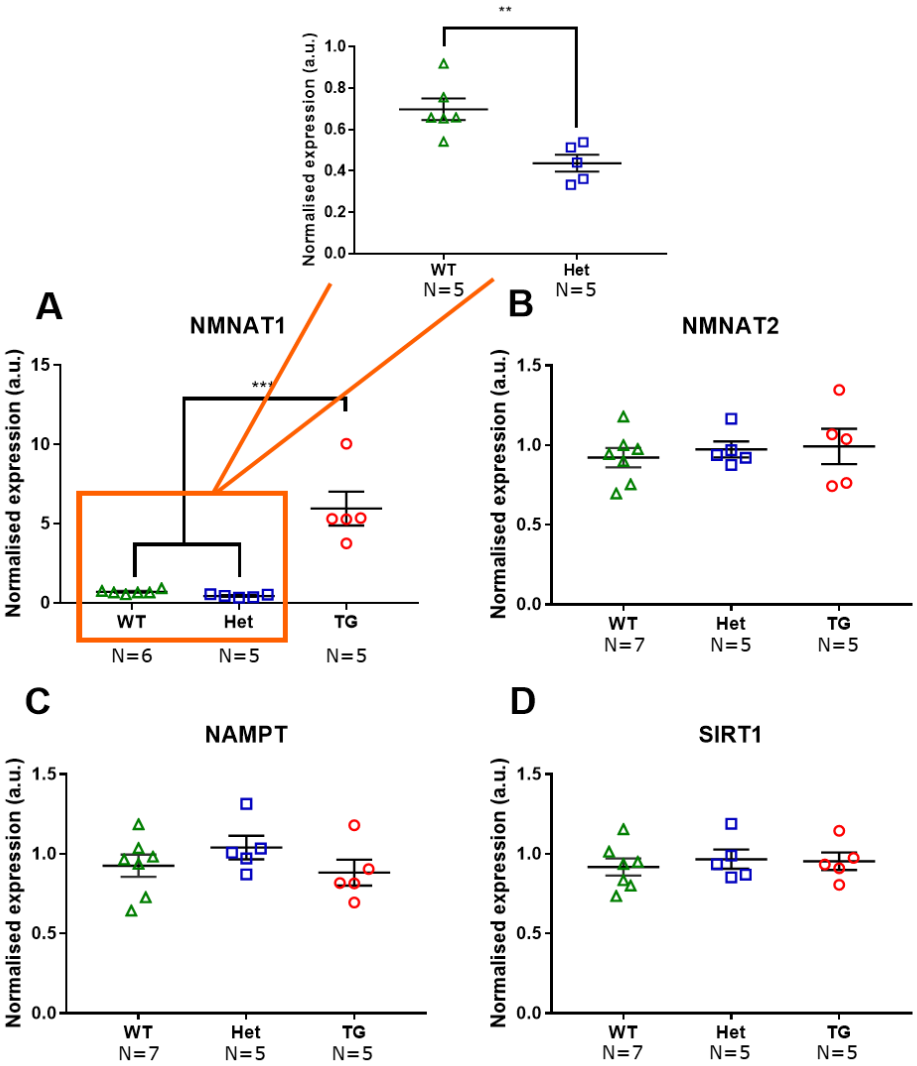


Figure 5.27: Gene expression of enzymes involved in the NAD+ biosynthetic pathway in the hippocampus of aged mice with varied NMNAT1 expression

Data for expression levels of genes in the hippocampus of aged sedentary mice. A) Expression levels of NMNAT1(***p=≤0.001, **p=≤0.01) B) Expression levels of NMNAT2. C) Expression levels of NAMPT. D) Expression levels of SIRT1.

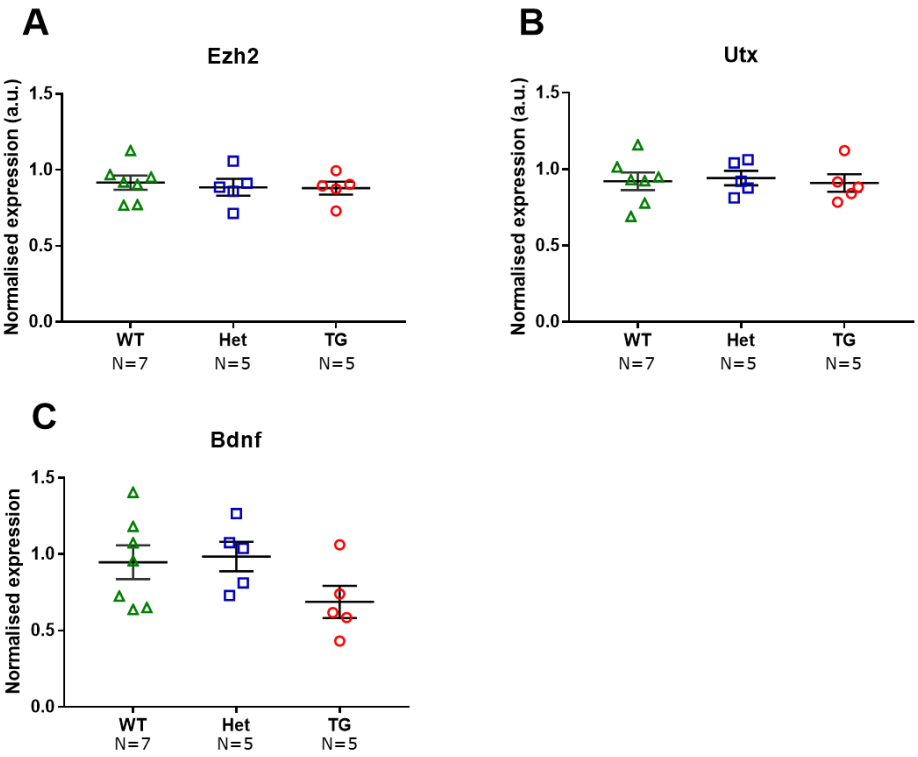


Figure 5.28: Gene expression in the hippocampus of aged mice with varied NMNAT1 expression

Data for expression levels of genes in the hippocampus of aged sedentary mice with varied NMNAT1 expression. Mean and ±SEM bars are shown. A) Expression levels of Ezh2. B) Expression levels of Utx. C) Expression levels of BDNF.

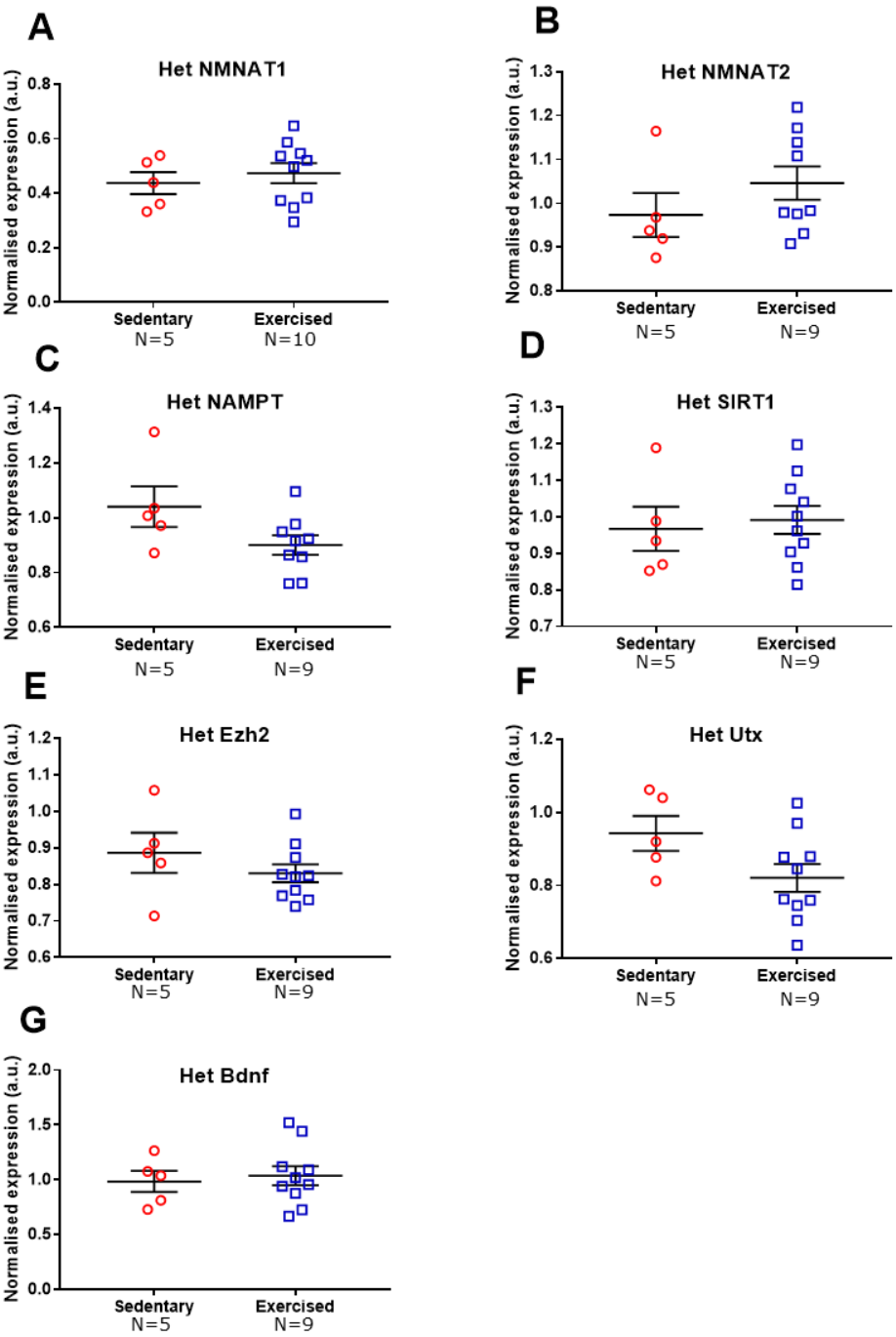


Figure 5.29: Effect of exercise on expression of NAD+ biosynthetic enzymes and epigenetic enzymes in Het mice.

Normalised expression of NAD+ biosynthetic genes in the hippocampus in Het and TG mice. All graphs are comparing mice that had voluntary access to a running wheel for 6 months, and mice who remained sedentary for the same time. Mean and ±SEM bars are displayed. A) NMNAT1 expression in Het mice. B) NMNAT2 expression in Het mice. C) NAMPT expression in Het mice. D) SIRT1 expression in Het mice. E) Ezh2 expression in Het mice. F) Utx expression in Het mice. G) Bdnf expression in Het mice.

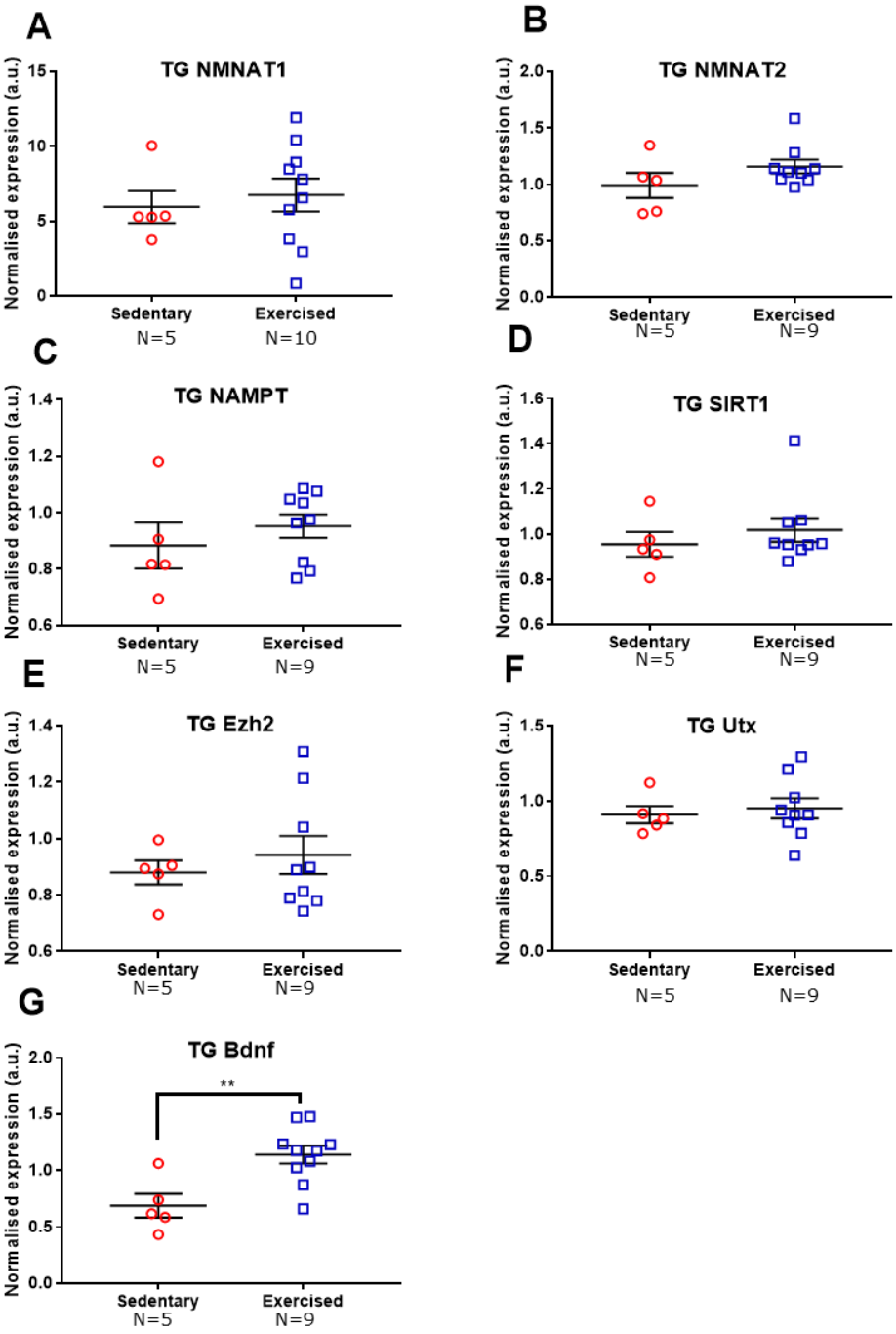


Figure 5.30: Effect of exercise on expression of NAD+ biosynthetic enzymes and epigenetic enzymes in TG mice.

Normalised expression of NAD+ biosynthetic genes in the hippocampus in Het and TG mice. All graphs are comparing mice that had voluntary access to a running wheel for 6 months, and mice who remained sedentary for the same time. Mean and ±SEM bars are displayed. A) NMNAT1 expression in TG mice. B) NMNAT2 expression in TG mice. C) NAMPT expression in TG mice. D) SIRT1 expression in TG mice. E) Ezh2 expression in TG mice. F) Utx expression in TG mice. G) Bdnf expression in TG mice.

5.4 Discussion

5.4.1 Body weight, survival and running distance

5.4.1.1 Body weight change with age and voluntary exercise

Exercise had a significant effect on the average percentage change in body weight, with Het mice that had exercised on average having a significant decrease in percentage body weight change in comparison to the WT sedentary mice. In all of the genotypes, the exercise group appears to have lower body weight at the end of the study than the sedentary controls for the corresponding genotype.

There was no significant effect of genotype on body weight. This is different to previous data in similar mice found by Dr. Francesca Rossi (Rossi, 2015). In her thesis, she reports that sedentary TG mice had significantly lower body weights at 12 and 18 months of age than either WT or Het mice that remained sedentary. She also showed that sedentary Het mice had significantly higher body weight than WT mice that remained sedentary at both 12 and 18 months. The difference in the results could be explained by the slight difference in the genetic background of the mice: in our study we used the offspring of mice from the original NMNAT1 TG and HetKO colonies used by Dr. Francesca Rossi crossed with WT C57BL/6J mice. They were backcrossed to strengthen the C57BL/6J background in all of the mice. The increase in C57BL/6J genetic background in the mice used in our study could have been sufficient to change the effect of varied NMNAT1 expression on body weight seen in the previous study.

5.4.1.2 The effect of voluntary exercise on mortality as mice age

We saw a significant increase in mortality rates in mice that remained sedentary throughout the study compared to those that had access to a running wheel for 6 months. The largest difference was in the WT group, with 50% increase in survival rates in the exercise group compared to the sedentary group. We did not investigate the maximum lifespan in our mice.

Other studies have demonstrated that, whilst exercise does not extend average lifespan, exercise increases the "health span" of mice. In a study by Garcia-Valles et al (2013), sedentary mice had a shorter median lifespan by an average of 20 days than the mice that had access to a running wheel from 3-months of age until natural death. The results of our study support the hypothesis that exercise increases the health span of the mice.

5.4.1.3 Variations in distance run

Groups of Het and WT mice housed together appeared to run much longer distances per night than any other housing group mix. When analysing the individual running distances in one of the WT/Het cages, it is clear that a WT mouse is the dominant wheel runner. WT mice also appear to be the dominant runners in WT/TG mix group housing. This suggests that WT mice may more often be the dominant mouse in a group cage or may be more aggressive in fighting over the wheels. When comparing cages with just Het or TG mice, there is very little difference in running distance.

A study by Howerton et al (2008) looked into the effect of running wheel inclusion on aggression in group housed CD-1 mice. They found that the inclusion of a wheel, both for running and for enrichment purposes, caused an increase in aggression in the mice and a disruption in dominance

hierarchy linearity. Different genetic backgrounds in laboratory mice can lead to different characteristics such as different levels of aggression. This could explain the apparent wheel running dominance of the WT mice in mixed genotype cages. More research is needed to analyse the fighting behaviour and aggression levels in the three genotypes in this study.

5.4.2 NMNAT1 expression, exercise and behaviour

5.4.2.1 Cognition

In most of the results, we saw no significant effect of different levels of NMNAT1 expression on the results of cognitive behavioural tests. There was a significant decrease in the NOR ratio in the first minute in the Het mice in comparison to both the WT and TG mice. This suggests that overexpression or reduced expression of the NMNAT1 gene does not have a significant effect on cognition in mice.

We also saw a significant reduction in the total NOR ratio with age in both the WT and TG mice. The lack of age-related difference in the Het mice suggest a possible protective effect on the cognitive abilities of the mice with age. We did not see the trend towards maintained NOR ratio with exercise that we saw in the WT mice (Figure 4.3), which suggests an effect of genotype. Rossi et al (2018) investigated the effect of different NMNAT1 expression on behaviour in a mouse model of AD. They found that overexpression of the NMNAT1 gene reduced a deficit in food burrowing behaviour normally see by 6 months of age in the model, though the exact mechanism behind the change remains unknown. So far, the effect of NMNAT1 expression on cognition has not been further explored in the literature.

We also saw no significant effect of exercise on cognition in either the Het or TG mice. This is similar to what we saw in the WT mice (Chapter 4 section 4.3.2), where exercise had no significant effect on cognition in WT mice that had access to a running wheel compared to sedentary controls. As we have already discussed (Chapter 4 section 4.4.1.1), the lack of significant change in cognition with voluntary exercise is opposed to data in the literature.

5.4.2.2 Anxiety

In the EPM test, the sedentary TG mice at 11 months of age spent a significantly higher percentage of the test time in the open arms of the arena than any other sedentary group (genotype and age). This suggests that the overexpression of NMNAT1 in younger mice may decrease the levels of anxiety, and that this effect is decreased with age. The 11-month sedentary TG mice also spent significantly more time performing head dips, a high risk behaviour which can result in the mouse falling from the maze, than the 18-month TG mice, which again suggests decreased anxiety in the young mice. However, the 11-month sedentary TG mice also spent significantly more time performing SAPs than the 18-month-old TG mice. An increase in time spent performing SAPs could suggest an increase in anxiety in the mice, as it can be interpreted as risk assessment behaviour. When we looked at the percentage change in these behaviours with age, we saw that the Het mice on average showed a significant increase in both SAPs and head dipping in comparison to the TG group. This suggests an effect of the genotype and supports the idea that the younger TG mice may have an effect of decreased anxiety with disappears with age. So far, the effect of varied NMNAT1 expression on anxiety has not been further explored in the literature.

In the Het mice, we saw no protective effect of exercise with age in the EPM test for any of the parameters. We saw a significant decrease in the ratio and head dip time in the het mice with age, which is similar to what we saw in the WT mice and suggests that the Het genotype had no significant effect on anxiety.

In the TG mice, the sedentary group at 11 months of age spent significantly more time in the open arms of the maze than the exercise group. As this was before they were housed with the running wheels, this was not an effect of exercise. This also suggests that the decrease in anxiety suggested above in the 11-month TG mice may not be as significant as it first appeared. Looking at the percentage change with age (not shown) shows no significant effect of age in the TG mice. There is no clear reason for this significant difference in the data, as the mice from the exercise and sedentary groups were alternated during the EPM test and were housed under identical conditions before and during the behavioural tests.

Whilst the data suggests some effects of genotype on anxiety in the mice, the extent of the effect is not clear, and the impact of exercise in these mice was minimal. Further investigations into the effect of NMNAT1 overexpression in younger mice would be needed to clarify our findings.

For the OF test we saw very few effects of exercise or genotype. Similar to the data in the WT mice, we saw a decrease in the time spent in the centre of the arena with age.

In our study we have shown that anxiety increases in mice with age. Shoji et al (2016) looked at a number of different behavioural tests for anxiety in mice between 2 and 12 months of age. They found on average that the older groups showed an increase in anxiety-like behaviours in comparison

to the younger groups, though they also showed some decreases in anxiety in the 12-month-old group in comparison to the younger groups. Anxiety is a complex behaviour, and there are multiple other factors that can affect it. In our study, the group housing of the animals and the dominance hierarchy may have had different effects on the mice within each cage. Parikh et al (2016) looked at the effect of caloric restriction on anxiety behaviour in young and aged mice. They found that, whilst CR had no effect in the young mice on anxiety, they saw reduced anxiety in the aged CR group in comparison to the aged mice on an *ad libitum* diet. This effect of CR could possibly be related to the reduced anxiety effects we saw in some of the TG mice in our study. We hypothesised that the changing of NMNAT1 expression in our mice could affect the activity of SIRT1, which is how CR exerts its effects. More in-depth research is required to find out more about the connection between NMNAT1 expression levels and SIRT1 activity to confirm this.

5.4.2.3 Locomotor activity

We saw no significant effect of genotype on the distance travelled in each of the behavioural tests performed. In the Het mice that exercised, we saw a significant decrease in LMA with age in the EPM test, but not in any of the other tests. This could be the cause of the decrease in EPM ratio seen in the exercise Het group. In the TG mice, we saw a significant decrease in LMA for both groups in the EPM test, and we saw an increase in LMA in both groups in the NOR test. These changes in LMA do not correlate with any changes in the behaviour results. The LMA results suggest that fatigue did not affect the results of the behaviour tests.

5.4.3 NMNAT1 expression, exercise and adult neurogenesis

5.4.3.1 The effect of varied NMNAT1 expression and sex on adult neurogenesis in young mice

There were no significant differences in the number of proliferating cells in the hippocampus of 2-month-old male and female mice. However, in female Het mice there was a significant increase in the number of immature neurons (expressing Dcx) in comparison to both male and female WT mice and to female TG mice. This suggests that heterozygous knockdown of the NMNAT1 gene can have an effect on the survival of new neurons or commitment to neurogenic lineage in young female mice.

Other studies looking at sex differences in adult hippocampal neurogenesis in C57BL/6J mice have shown no significant differences in cell proliferation or immature neuron numbers at various ages (Lagace, Fischer and Eisch, 2007; Ben Abdallah *et al.*, 2010). However, in other rodent models such as rats and voles, adult neurogenesis is affected by sex and oestrogen cycles throughout life (Galea *et al.*, 2006). The young mice in our study were 2 months old, which is the junction between late adolescence and early adulthood in mice, compared to the adult models used in the literature. Therefore, we may be seeing slightly different hormonal changes in our mice which could be responsible for the changes seen.

Unfortunately, female mice were only used in this part of the study. In the future, it would be interesting to see the effects of ageing and exercise on the female WT, Het and TG mice. In this study it was not possible due to time and space restrictions.

5.4.3.2 Adult neurogenesis in aged mice with varied NMNAT1 expression and the effect of voluntary exercise

There was no difference in the number of actively proliferating cells in the DG between mice in the WT, Het and TG colonies. In the SVZ however, there was a significant increase in the number of proliferating cells in Het mice compared to WT mice. There was also a tendency towards there being an increase in the number of proliferating cells in the Het mice SVZ in comparison to the TG mice. Changes in NMNAT1 expression also had a significant effect on the number of immature neurons found in both the DG and SVZ. In the DG, mice from the TG colony had significantly more immature neurons than either the Het or WT mice. In the SVZ, mice from the Het colony had significantly more immature neurons than the WT mice and a trend towards having more immature neurons than the TG mice.

Previous studies have shown that changes in the activity levels of NAD^+ biosynthetic enzymes such as NAMPT or the NAD^+ dependent SIRT1 can have significant effects on adult neurogenesis levels. Stein et al (Stein and Imai, 2014) found that ablation of the NAD^+ limiting enzyme NAMPT causes a significant reduction in cell proliferation and NPC pools in the hippocampus, and concluded that NAD^+ biosynthesis is a key mediator of age associated decline in adult neurogenesis. Changes in the NAD^+ dependent SIRT1 activity can also have a significant effect on neurogenesis. Ma et al (Ma *et al.*, 2014) showed that decreased SIRT1 activity can lead to increased cellular proliferation, and Rafalski et al (Rafalski and Brunet, 2011) showed decreased SIRT1 activity increasing the number of immature neurons in the hippocampus.

There was no significant difference in the number of cells that survived for 28 days in the DG of WT, Het and TG mice. However, in the SVZ there was a significant increase in the number of surviving cells in TG mice compared to the WT and Het mice.

Exercise did not significantly affect cell proliferation or immature neuron numbers in the DG and the SVZ of TG mice. Interestingly, in Het mice exercise caused a significant decrease in cell proliferation in the SVZ. The decrease brought the number of proliferating cells closer to those seen in the sedentary WT and TG mice. As mentioned in chapter 4, WT mice showed a significant increase in the number of immature neurons in both the DG and SVZ (Chapter 4 section 4.3.6). The difference between the WT and the Het and TG findings with exercise supports the hypothesis that changes to the expression of NMNAT1 influences adult neurogenesis in the hippocampus of ageing mice. However, more research is needed to understand the underlying mechanisms that are changed by the varied NMNAT1 expression.

Exercise had no significant effect on cell survival numbers in either the Het or the TG mice. From the Dcx and BrdU data in our study, it suggests that the increase in NMNAT1 expression in the TG mice affects neurogenesis in the DG and SVZ differently, as we see increases in immature neurons and cell survival only in the SVZ of these mice.

5.4.4 miRNA expression in blood plasma

Cell-free circulating miRNAs in blood plasma have recently been found to be potential biomarkers of disease and ageing, and could soon become a commonly used diagnostic marker for various cancers and other diseases (Schwarzenbach, Hoon and Pantel, 2011; Mo *et al.*, 2012). The majority of the research into the influence of cell-free circulating mRNAs has been in

connection to cancer detection and treatment targeting. Because of their potential as blood biomarkers, in our study we looked at the levels of miRNAs that have been linked with ageing, exercise and neurogenesis in the blood plasma of our mice.

In our study we found a significant increase in the expression of miR-21-5p in the plasma of TG mice in comparison to Het and WT mice. We then saw a trend of decreased expression in the TG with exercise. This is the opposite to what we saw in the WT mice (Chapter 4 section 4.3.8), where there was an increase in miR-21-5p with exercise. This suggests that for the expression of miR-21-5p, exercise in WT mice and the overexpression of NMNAT1 have a similar effect, yet exercise in the TG mice has the opposite effect. As discussed in Chapter 4, miR-21 has links to cell proliferation and reduced apoptosis in the adult hippocampus (Strickland *et al.*, 2011; Ge, 2015) in Chapter 4 (section 4.4.3). In our TG mice, we saw a significant increase in both miR-21-5p and 28-day cell survival after proliferation in the brain. The increase in cell survival could be linked with the increase in miR-21-5p due to its link with reducing apoptosis, though further research is needed to investigate this.

We saw no other significant changes in miRNA expression between the three genotypes. However, exercise significantly decreased the expression of miR-20a-5p and miR-92a-3p in TG mice.

5.4.5 Gene expression in the hippocampus

We confirmed a significant increase in NMNAT1 gene expression in the HC of TG mice and a decrease in the Het mice when both were compared with WT mice, so any differences found between the three genotypes is likely to be caused by the change in NMNAT1 expression.

We saw no significant change in the expression levels of NMNAT2, NAMPT or SIRT one. This suggests that NMNAT2 and NAMPT are not compensating for the decrease in NMNAT1 in the Het mice and are not changing in response to the increase in NMNAT1 in the TG mice. The lack of change in SIRT1 expression shows that changing NMNAT1 expression does not cause a change in SIRT1 expression due to changes in NAD^+ availability. As there is no change in SIRT1 expression, changes in neurogenesis levels in the hippocampus of the mice may be instead due to the activity level of SIRT1. Further research to investigate the activity level of SIRT1 in the Het and TG mice is needed to fully understand the link.

Exercise did not have any significant effects on the expression of NMNAT1, NMNAT2, NAMPT or SIRT1 in Het and TG mice. However, we did see a trend towards decreased NAMPT expression in the Het mice with exercise. NAMPT is known to be a key limiting factor in NAD^+ availability. Previous studies have shown that a decrease in NAMPT can lead to a decrease in cell proliferation in the hippocampus (Stein, 2014), yet in our study we saw an increase in cell proliferation in the SVZ of Het mice who had a trend towards decreased NAMPT expression.

We saw no significant effect of NMNAT1 expression on the expression levels of Ezh2, Utx or BDNF, though we saw a strong trend towards decreased expression of BDNF in TG mice. However, with exercise we saw a significant increase in BDNF in the TG mice which restored its expression to a similar level seen in the WT and Het mice. We did not see this significant change in the WT or Het mice. BDNF has a role in regulating adult neurogenesis in the DG. Waterhouse et al (2012) showed that locally synthesised BDNF in the hippocampus promoted differentiation and maturation of progenitor cells via GABA release mechanisms. As we saw a trend towards decreased expression of BDNF in the TG mice that also had

significantly increased immature neuron numbers in the DG, it is possible that the different levels of NMNAT1 expression were affecting this mechanism, leading to decreased BDNF activity. More research is needed to understand the underlying mechanisms of the TG mouse adult neurogenesis changes.

5.5 Conclusions

In this chapter, we investigated how transgenic overexpression and heterozygous knock out of the NMNAT1 gene affected behaviour, adult neurogenesis, cell-free miRNA expression in blood plasma and gene expression in the hippocampus. We also looked to see how, if at all, 6 months voluntary exercise influenced these effects.

We found that different levels of NMNAT1 expression had very little influence on behaviour, and 6 months voluntary exercise did not change this

We saw some effects of different levels of NMNAT1 expression on adult neurogenesis in both the DG and SVZ, with Het mice showing increased cell proliferation and immature neuron numbers in the SVZ, and TG mice showing increased immature neuron numbers in the DG and increased cell survival in the SVZ. Exercise had little effect in these genotypes on adult neurogenesis, apart from to reduce the levels of cell proliferation in the SVZ of Het mice.

The overexpression of NMNAT1 in TG mice caused a significant increase in the expression of miR-21 in blood plasma, and in the same mice we saw a decrease in miR-20a and miR-92a with voluntary exercise.

We also saw decreased BDNF expression in the hippocampus of TG mice that was restored with exercise.

The changes found in our study are often in contrast to those seen in the literature. Further research would be needed to understand the exact influence different levels of NMNAT1 expression have on neurogenesis and miRNA expression in blood plasma.

6. Summary and Conclusions

6.1.1 Summary of results

In our study, we aimed to gain further understanding into the impact of ageing, exercise and different levels of NMNAT1 expression on behaviour, adult neurogenesis, circulating miRNA expression and hippocampal gene expression in the mouse. The main findings of our study have been summarised below, and in Table 6.1, Table 6.3 and Table 6.2.

An important finding in our study was the significant decrease in mortality rates in the exercising groups of the ageing study in comparison to the sedentary (Table 6.3). This supports the hypothesis that exercise increases health and promotes longevity.

In our light cycle study, we found that performing behavioural tests in the dark increased the activity levels of the mice and reduced apparent increases in anxiety seen with exercise in tests in the light (Table 6.1)

In the ageing study (Table 6.2), we saw a large number of changes in the WT mice with age, and exercise in these mice boosted the neurogenesis levels in the brain.

In the Het mice there were very few changes with both ageing and exercise. This suggests that the genotype was having some effect, mainly on neurogenesis levels in the brain. Exercise however did not boost these effects, and in some ways seemed to have a negative impact in these mice.

In the TG mice, there appeared to be a decrease in anxiety in younger mice, as well as an increase in immature neuron numbers. However, these changes were not present in the TG mice that exercised, suggesting that exercise influenced the effect of the genotype. The most interesting find was the significant increase in BDNF in the TG exercise group, along with the trend towards a decrease in the sedentary group. BDNF is a key factor

in neurogenesis and long-term memory. Previous literature has shown that exercise significantly increases BDNF in the human brain (Kristin L. Szuhanya, Matteo Bugattia, 2015), though this is usually in studies of short term exercise. This suggests that the genotype combined with long term exercise may have similar effects to acute exercise.

Table 6.1: Key results from the light cycle study

	Light sedentary	Light exercise	Dark Sedentary	Dark exercise
Anxiety	-	↑ Compared to sedentary (EPM)	↓ Compared to Light groups (EPM)	
Cognition	-	↓ exploring in NOR compared to sedentary	-	↑ exploring in NOR compared to sedentary
LMA	-	-	↑ Distance in EPM and SA compared to light groups	

Table 6.3: Key results for weight, mortality and sex

Body weight	↑ WT, TG, ↓ Average in Het Ex
Mortality	↓ with exercise
Male/female neurogenesis	↑ Immature neurons in Het females than WT mice and TG females

Table 6.2: Key data from ageing study

	WT Sedentary	WT Exercise	Het Sedentary	Het Exercise	TG Sedentary	TG Exercise
Cognition	↓ NOR with age		↓ NOR compared to WT and TG	-	↓ NOR with age	-
Anxiety	↑ Anxiety with age		-	-	↓ in 11mths compared to other groups	-
LMA	↓ in EPM with age		-	↓ in EPM with age	↓ in EPM with age ↑ in NOR with age	
Cell proliferation	↓ With age in DG and SVZ	-	↑ in SVZ compared to WT	↓ with exercise	-	-
Immature neurons	↓ With age in DG and SVZ	↑ in DG and SVZ	↑ in SVZ compared to WT	-	↑ in DG compared to WT and Het	-
Cell survival	↓ With age in DG and SVZ	↑ in DG and SVZ	-	-	↑ Compared to WT and Het	-
Circulating miRNAs	-	↑ in miR-21-5p with exercise	-	-	↑ miR-21-5p compared to WT and Het	↓ in miR-20a-5p and miR-92a-3p with exercise
Hippocampal gene expression	-	-	-	-	-	↑ BDNF with exercise

6.1.2 Future work

The effect of varied NMNAT1 expression has not been extensively investigated prior to this study. Here, we have found that the varied expression does have some effects on mouse behaviour, adult neurogenesis and circulating miRNA expression. Further investigation is now needed to increase our understanding on how NMNAT1 expression exerts its effects, and what mechanisms are changed because of this.

We hypothesised that SIRT1 would be affected by the change in NMNAT1 expression, though we saw no change in the expression of SIRT1 in our mice. The next step to investigate this hypothesis would be to look at the activity levels of SIRT1 and NAD availability levels. If either are affected by the change in NMNAT1 expression, then it could explain the changes in neurogenesis and behaviour seen in this study.

Some of the results in our exercise study conflicted with those found in the literature. One significant difference between our study and the literature was the length of time the mice were given access to a running wheel for, and the group housing conditions used. These could have significantly impacted the changes seen with exercise. A further study is needed investigating the effect of the length of time spent exercise has on behaviour, neurogenesis and gene expression.

Finally, further investigation into the change in circulating miRNA expression in these genotypes is needed. This would include looking at the actions of these miRNAs in the ageing brain, and how they interact with NMNAT1 activity. These are potential future biomarkers of healthy brain ageing.

7. References

Ben Abdallah, N. M. B., Slomianka, L., Vyssotski, A. L. and Lipp, H. P. (2010) 'Early age-related changes in adult hippocampal neurogenesis in C57 mice', *Neurobiology of Aging*, 31(1).

Adlard, P. A., Perreau, V. M. and Cotman, C. W. (2005) 'The exercise-induced expression of BDNF within the hippocampus varies across life-span', *Neurobiology of Aging*, 26(4).

Adlard, P. A., Perreau, V. M. and Cotman, C. W. (2005) 'Voluntary exercise decreases amyloid load in a transgenic model of alzheimer's disease', *Journal of Neuroscience*, 25(17).

Ahlskog, J. E., Geda, Y. E., Graff-Radford, N. R. and Petersen, R. C. (2011) 'Physical exercise as a preventive or disease-modifying treatment of dementia and brain aging', *Mayo Clinic Proceedings*. Mayo Foundation for Medical Education and Research, 86(9).

Altman, J. (1962) 'Are New Neurons Formed in the brains of adult mammals', *Science*, 135(3509).

Alvarez-Buylla, A. and García-Verdugo, J. M. (2002) 'Neurogenesis in adult subventricular zone', *The Journal of Neuroscience*, 22(3).

Anderton, B. (2002) 'Ageing of the Brain', *Mechanisms of Ageing and Development*, 123(7).

Anthony, T. E., Klein, C., Fishell, G. and Heintz, N. (2004) 'Radial glia serve as neuronal progenitors in all regions of the central nervous system', *Neuron*, 41(6).

Arroyo, J. D., Chevillet, J. R., Kroh, E. M., Ruf, I. K., Pritchard, C. C., Gibson, D. F., Mitchell, P. S., Bennett, C. F., Pogosova-Agadjanyan, E. L., Stirewalt, D. L., Tait, J. F. and Tewari, M. (2011) 'Argonaute-2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma', *Proceedings of the National Academy of Sciences*, 108(12).

Aslani, S., Harb, M. R., Costa, P. S., Almeida, O. F. X., Sousa, N. and Palha, J. A. (2014) 'Day and night: diurnal phase influences the response to chronic mild stress', *Frontiers in Behavioral Neuroscience*, 8(March).

Baek, S. H. (2011) 'When Signaling Kinases Meet Histones and Histone Modifiers in the Nucleus', *Molecular Cell*, 42(3).

Baggish, A. L., Hale, A., Weiner, R. B., Lewis, G. D., Systrom, D., Wang, F., Wang, T. J. and Chan, S. Y. (2011) 'Dynamic regulation of circulating microRNA during acute exhaustive exercise and sustained aerobic exercise training', *Journal of Physiology*, 589(16).

Barski, A., Cuddapah, S., Cui, K., Roh, T. Y., Schones, D. E., Wang, Z., Wei, G., Chepelev, I. and Zhao, K. (2007) 'High-resolution profiling of histone methylations in the human genome', *Cell*, 129(4).

Bathina, S. and Das, U. N. (2015) 'Brain-derived neurotrophic factor and its clinical Implications', *Archives of Medical Science*, 11(6).

Bechara, R. G. and Kelly, á M. (2013) 'Exercise improves object recognition memory and induces BDNF expression and cell proliferation in cognitively enriched rats', *Behavioural Brain Research*. Elsevier B.V., 245.

Bell, J. T. *et al.* (2012) 'Epigenome-wide scans identify differentially methylated regions for age and age-related phenotypes in a healthy ageing population', *PLoS Genetics*, 8(4).

Benaroya-Milshtein, N., Hollander, N., Apter, A., Kukulansky, T., Raz, N., Wilf, A., Yaniv, I. and Pick, C. G. (2004) 'Environmental enrichment in mice decreases anxiety, attenuates stress responses and enhances natural killer cell activity', *European Journal of Neuroscience*, 20(5).

Benayoun, B. A., Pollina, E. A. and Brunet, A. (2015) 'Epigenetic regulation of ageing: Linking environmental inputs to genomic stability', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 16(10).

Berchtold, N. C., Castello, N. and Cotman, C. W. (2010) 'Exercise and time-dependent benefits to learning and memory', *Neuroscience*. Elsevier Inc., 167(3).

Berger, S. L. (2007) 'The complex language of chromatin regulation during transcription', 447(May).

Bherer, L., Erickson, K. I. and Liu-Ambrose, T. (2013) 'A review of the effects of physical activity and exercise on cognitive and brain functions in older adults', *Journal of Aging Research*, 2013.

Bian, S., Hong, J., Li, Q., Schebelle, L., Pollock, A., Knauss, J. L., Garg, V. and Sun, T. (2013) 'MicroRNA Cluster miR-17-92 Regulates Neural Stem Cell Expansion and Transition to Intermediate Progenitors in the Developing Mouse Neocortex', *Cell Reports*. The Authors, 3(5).

Binder, E., Droste, S. K., Ohl, F. and Reul, J. M. H. M. (2004) 'Regular voluntary exercise reduces anxiety-related behaviour and impulsiveness in mice', *Behavioural Brain Research*, 155(2).

Bird, A. P. (1986) 'CpG-Rich islands and the function of DNA methylation', *Nature*, 321(6067).

Bizon, J. L., Lee, H. J. and Gallagher, M. (2004) 'Neurogenesis in a rat model of age-related cognitive decline', *Aging Cell*, 3(4).

Boehm, M. and Slack, F. (2005) 'A developmental timing microRNA and its target regulate life span in C. elegans.', *Science (New York, N.Y.)*, 310(5756).

Von Bohlen Und Halbach, O. (2007) 'Immunohistological markers for staging neurogenesis in adult hippocampus', *Cell and Tissue Research*, 329(3).

Boldrini, M., Fulmore, C. A., Tartt, A. N., Simeon, L. R., Pavlova, I., Poposka, V., Rosoklija, G. B., Stankov, A., Arango, V., Dwork, A. J., Hen, R. and Mann, J. J. (2018) 'Human Hippocampal Neurogenesis Persists throughout Aging', *Cell Stem Cell*, 22(4).

Bonner-Jackson, A., Mahmoud, S., Miller, J. and Banks, S. J. (2015) 'Verbal and non-verbal memory and hippocampal volumes in a memory clinic population', *Alzheimer's Research and Therapy*. Alzheimer's Research & Therapy, 7(1).

Bopp, K. L. and Verhaeghen, P. (2009) 'Working Memory and Aging: Separating the Effects of Content and Context', *Psychol Aging*, 24(4).

Bordone, L., Cohen, D., Robinson, A., Motta, M. C., Van Veen, E., Czopik, A., Steele, A. D., Crowe, H., Marmor, S., Luo, J., Gu, W. and Guarente, L. (2007) 'SIRT1 transgenic mice show phenotypes resembling calorie restriction', *Aging Cell*, 6(6).

Van der Borght, K., Havekes, R., Bos, T., Eggen, B. J. L. and Van der Zee, E. A. (2007) 'Exercise Improves Memory Acquisition and Retrieval in the Y-Maze Task: Relationship With Hippocampal Neurogenesis', *Behavioral Neuroscience*, 121(2).

Bowling, A. and Dieppe, P. (2005) 'What is successful ageing and who should define it?', *BMJ : British Medical Journal*, 331(7531).

Bracken, A. P., Dietrich, N., Pasini, D., Hansen, K. H. and Helin, K. (2006) 'Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions', *Genes & Development*, 20(9).

Bradley-whitman, M. A. and Lovell, M. A. (2013) 'Epigenetic changes in the progression of Alzheimer ' s disease', *Mechanisms of Ageing and Development*, 134(10).

Braidy, N., Guillemin, G. J., Mansour, H., Chan-Ling, T., Poljak, A. and Grant, R. (2011) 'Age related changes in NAD+ metabolism oxidative stress and sirt1 activity in wistar rats', *PLoS ONE*, 6(4).

Burghardt, P. R., Fulk, L. J., Hand, G. A. and Wilson, M. A. (2004) 'The effects of chronic treadmill and wheel running on behavior in rats', *Brain Research*, 1019(1–2).

Cannell, I. G., Kong, Y. W. and Bushell, M. (2008) 'How do microRNAs regulate gene expression?', *Biochemical Society Transactions*, 36(6).

Cantó, C. and Auwerx, J. (2009) 'Caloric restriction, SIRT1 and longevity', *Trends in Endocrinology and Metabolism*, 20(7).

Carek, P. J., Laibstain, S. E. and Carek, S. M. (2011) 'Exercise for the Treatment of Depression and Anxiety', *The International Journal of Psychiatry in Medicine*, 41(1).

Chen, D. (2005) 'Increase in Activity During Calorie Restriction Requires Sirt1', *Science*, 310(5754).

Chen, X. *et al.* (2008) 'Characterization of microRNAs in serum: A novel class of biomarkers for diagnosis of cancer and other diseases', *Cell Research*, 18(10).

Cheng, L., Doecke, J. D., Sharples, R. A., Villemagne, V. L., Fowler, C. J., Rembach, A., Martins, R. N., Rowe, C. C., Macaulay, S. L., Masters, C. L. and Hill, A. F. (2015) 'Prognostic serum miRNA biomarkers associated with Alzheimer's disease shows concordance with neuropsychological and neuroimaging assessment', *Molecular Psychiatry*. Nature Publishing Group, 20(10).

Cifuentes, M., Pérez-Martín, M., Grondona, J. M., López-Ávalos, M. D., Inagaki, N., Granados-Durán, P., Rivera, P. and Fernández-Llebrez, P. (2011) 'A comparative analysis of intraperitoneal versus intracerebroventricular administration of bromodeoxyuridine for the study of cell proliferation in the adult rat brain', *Journal of Neuroscience Methods*. Elsevier B.V., 201(2).

Clarke, L. and Van Der Kooy, D. (2011) 'The adult mouse dentate gyrus contains populations of committed progenitor cells that are distinct from subependymal zone neural stem cells', *Stem Cells*, 29(9).

Clemenson, G. D., Deng, W. and Gage, F. H. (2015) 'Environmental enrichment and neurogenesis: from mice to humans', 4(July).

Cohen, H. Y. (2004) 'Calorie Restriction Promotes Mammalian Cell Survival by Inducing the SIRT1 Deacetylase', *Science*, 305(5682).

Colcombe, S. and Kramer, A. F. (2003) 'Fitness effects on the cognitive function of older adults', *Psychological Science*, 14(2).

Colman, R. J., Anderson, R. M., Johnson, S. C., Kastman, E. K., Kosmatka, K. J., Beasley, T. M., Allison, D. B., Cruzen, C., Simmons, A., Kemnitz, J. W. and Weindruch, R. (2009) 'Caloric restriction delays disease onset and mortalilty in rhesus monkeys', *Science*, 325(5937).

Commons, K. G., Cholanians, A. B., Babb, J. A. and Ehlinger, D. G. (2017) 'The Rodent Forced Swim Test Measures Stress-Coping Strategy, Not Depression-like Behavior', *ACS Chemical Neuroscience*, 8(5).

Conforti, L., Fang, G., Beirowski, B., Wang, M. S., Sorci, L., Asress, S., Adalbert, R., Silva, A., Bridge, K., Huang, X. P., Magni, G., Glass, J. D. and Coleman, M. P. (2007) 'NAD(+) and axon degeneration revisited: Nmnat1 cannot substitute for Wld(S) to delay Wallerian degeneration.', *Cell death and differentiation*, 14(1).

Conforti, L., Gilley, J. and Coleman, M. P. (2014) 'Wallerian degeneration: an emerging axon death pathway linking injury and disease.', *Nature reviews. Neuroscience*, 15(6).

Conforti, L., Janeckova, L., Wagner, D., Mazzola, F., Cialabrini, L., Di Stefano, M., Orsomando, G., Magni, G., Bendotti, C., Smyth, N. and Coleman, M. (2011) 'Reducing expression of NAD+ synthesizing enzyme NMNAT1 does not affect the rate of Wallerian degeneration', *FEBS Journal*, 278(15).

Cotman, C. W. and Berchtold, N. C. (2002) 'Exercise: A behavioral intervention to enhance brain health and plasticity', *Trends in Neurosciences*, 25(6).

Cotman, C. W. and Berchtold, N. C. (2007) 'Physical activity and the maintenance of cognition: Learning from animal models', *Alzheimer's and Dementia*, 3(2).

Cotman, C. W. and Engesser-Cesar, C. (2002) 'Exercise enhances and protects brain function.', *Exercise and Sport Sciences Reviews*, 30(2).

Couillard-Despres, S., Winner, B., Schaubeck, S., Aigner, R., Vroemen, M., Weidner, N., Bogdahn, U., Winkler, J., Kuhn, H. G. and Aigner, L. (2005) 'Doublecortin expression levels in adult brain reflect neurogenesis', *European Journal of Neuroscience*, 21(1).

Dayer, A. G., Ford, A. A., Cleaver, K. M., Yassaee, M. and Cameron, H. A. (2003) 'Short-term and long-term survival of new neurons in the rat dentate gyrus', *Journal of Comparative Neurology*, 460(4).

Daynac, M., Morizur, L., Chicheportiche, A., Mouthon, M. A. and Boussin, F. D. (2016) 'Age-related neurogenesis decline in the subventricular zone is

associated with specific cell cycle regulation changes in activated neural stem cells', *Scientific Reports*. Nature Publishing Group, 6(January).

Deary, I. J., Corley, J., Gow, A. J., Harris, S. E., Houlihan, L. M., Marioni, R. E., Penke, L., Rafnsson, S. B. and Starr, J. M. (2009) 'Age-associated cognitive decline', *British Medical Bulletin*, 92(1).

Depp, C. A., Glatt, S. J. and Jeste, D. V (2007) 'Recent advances in research on successful or healthy ageing', 9(1).

Drapeau, E., Mayo, W., Aurousseau, C., Le Moal, M., Piazza, P.-V. and Abrous, D. N. (2003) 'Spatial memory performances of aged rats in the water maze predict levels of hippocampal neurogenesis', *Proceedings of the National Academy of Sciences*, 100(24).

Duman, C. H., Schlesinger, L., Russell, D. S. and Duman, R. S. (2008) 'Voluntary exercise produces antidepressant and anxiolytic behavioral effects in mice', *Brain Research*, 1199(14).

Duman, R. S. and Monteggia, L. M. (2006) 'A Neurotrophic Model for Stress-Related Mood Disorders', *Biological Psychiatry*, 59(12).

Erickson, K. I. and Kramer, A. F. (2009) 'Aerobic exercise effects on cognitive and neural plasticity in older adults', *British Journal of Sports Medicine*, 43(1).

Eriksson, P. S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A. and Gage, F. H. (1998) 'Neurogenesis in the adult human hippocampus', *Nature medicine*, 4(11).

Esteller, M. (2007) 'Epigenetic gene silencing in cancer: The DNA hypermethylome', *Human Molecular Genetics*, 16(R1).

Fabel, K., Wolf, S. A., Ehninger, D., Babu, H., Leal-Galicia, P. and Kempermann, G. (2009) 'Additive effects of physical exercise and environmental enrichment on adult hippocampal neurogenesis in mice', *Frontiers in Neuroscience*, 3(NOV).

Felici, R., Lapucci, A., Ramazzotti, M. and Chiarugi, A. (2013) 'Insight into Molecular and Functional Properties of NMNAT3 Reveals New Hints of NAD Homeostasis within Human Mitochondria', *PLoS ONE*, 8(10).

File, S. E. and Day, S. (1972) 'Effects of time of day and food deprivation on exploratory activity in the rat', *Animal Behaviour*, 20(4).

Fontana, L. and Klein, S. (2007) 'Aging, adiposity, and calorie restriction.', *JAMA : the journal of the American Medical Association*, 297(9).

Fox, C., Merali, Z. and Harrison, C. (2006) 'Therapeutic and protective effect of environmental enrichment against psychogenic and neurogenic stress', *Behavioural Brain Research*, 175(1).

Fraga, M. F. and Esteller, M. (2007) 'Epigenetics and aging: the targets and the marks', *Trends in Genetics*, 23(8).

Fuss, J., Ben Abdallah, N. M. B., Hensley, F. W., Weber, K. J., Hellweg, R. and Gass, P. (2010) 'Deletion of running-induced hippocampal neurogenesis by irradiation prevents development of an anxious phenotype in mice', *PLoS ONE*, 5(9).

Fuss, J., Ben Abdallah, N. M. B., Vogt, M. A., Touma, C., Pacifici, P. G., Palme, R., Witzemann, V., Hellweg, R. and Gass, P. (2010) 'Voluntary exercise induces anxiety-like behavior in adult C57BL/6J mice correlating with hippocampal neurogenesis', *Hippocampus*, 20(3).

Gage, F. H., Kempermann, G., Palmer, T. D., Peterson, D. A. and Ray, J. (1998) 'Multipotent Progenitor Cells in the Adult Dentate Gyrus', *Journal of Neurobiology*, 36(2).

Galea, L. A. M., Spritzer, M. D., Barker, J. M. and Pawluski, J. L. (2006) 'Gonadal hormone modulation of hippocampal neurogenesis in the adult', *Hippocampus*, 16(3).

Gangaraju, V. K. and Lin, H. (2009) 'MicroRNAs: Key regulators of stem cells', *Nature Reviews Molecular Cell Biology*, 10(2).

García-Capdevila, S., Portell-Cortés, I., Torras-Garcia, M., Coll-Andreu, M. and Costa-Miserachs, D. (2009) 'Effects of long-term voluntary exercise on learning and memory processes: dependency of the task and level of exercise', *Behavioural Brain Research*, 202(2).

Garcia-Valles, R., Gomez-Cabrera, M., Rodriguez-Mañas, L., Garcia-Garcia, F. J., Diaz, A., Noguera, I., Olaso-Gonzalez, G. and Viña, J. (2013) 'Life-long spontaneous exercise does not prolong lifespan but improves health span in mice', *Longevity & Healthspan*, 2(1).

Garrett, L., Lie, D. C., Angelis, M. H. de, Wurst, W. and Hölter, S. M. (2012) 'Voluntary wheel running in mice increases the rate of neurogenesis without affecting anxiety-related behaviour in single tests', *BMC Neuroscience*, 13(1).

Ge, X.-T., Lei, P., Wang, H.-C., Zhang, A.-L., Han, Z.-L., Chen, X., Li, S.-H., Jiang, R.-C., Kang, C.-S. and Zhang, J.-N. (2015) 'miR-21 improves the neurological outcome after traumatic brain injury in rats', *Scientific Reports*, 4(1).

Ghosh, T., Aprea, J., Nardelli, J., Engel, H., Selinger, C., Mombereau, C., Lemonnier, T., Moutkine, I., Schwendimann, L., Dori, M., Irinopoulou, T., Henrion-Caude, A., Benecke, A. G., Arnold, S. J., Gressens, P., Calegari, F. and Groszer, M. (2014) 'MicroRNAs Establish Robustness and Adaptability of a Critical Gene Network to Regulate Progenitor Fate Decisions during Cortical Neurogenesis', *Cell Reports*. The Authors, 7(6).

Gilley, J., Adalbert, R., Yu, G. and Coleman, M. P. (2013) 'Rescue of peripheral and CNS axon defects in mice lacking NMNAT2.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 33(33).

Gilley, J. and Coleman, M. P. (2010) 'Endogenous Nmnat2 Is an Essential Survival Factor for Maintenance of Healthy Axons', *PLoS Biology*, 8(1).

Gobatto, C. A., de Mello, M. A., Sibuya, C. Y., de Azevedo, J. R., dos Santos, L. A. and Kokubun, E. (2001) 'Maximal lactate steady state in rats submitted to swimming exercise', *Comp Biochem Physiol A Mol Integr Physiol*, 130(1).

Gomez-Pinilla, F. and Hillman, C. H. (2013) 'The Influence of Exercise on Cognitive Abilities', *Comprehensive Physiology*, 3(1).

Good, C. and Johnsrude, I. (2002) 'A voxel-based morphometric study of

ageing in 465 normal adult human brains', *Biomedical Imaging*, 36(1998).

Gould, E. (2014) 'Neurogenesis in the Neocortex of Adult Primates', *Science*, 548(1999).

Greenwood, B. N., Foley, T. E., Day, H. E. W., Campisi, J., Hammack, S. H., Campeau, S., Maier, S. F. and Fleshner, M. (2003) 'Freewheel running prevents learned helplessness/behavioral depression: role of dorsal raphe serotonergic neurons.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 23(7).

Griffin, É. W., Bechara, R. G., Birch, A. M. and Kelly, Á. M. (2009) 'Exercise enhances hippocampal-dependent learning in the rat: Evidence for a BDNF-related mechanism', *Hippocampus*, 19(10).

Griffin, É. W., Mullally, S., Foley, C., Warmington, S. A., O'Mara, S. M. and Kelly, Á. M. (2011) 'Aerobic exercise improves hippocampal function and increases BDNF in the serum of young adult males', *Physiology and Behavior*, 104(5).

Gu, S. and Kay, M. a (2010) 'How do miRNAs mediate translational repression?', *Silence*, 1(11).

De Guire, V., Robitaille, R., Tetreault, N., Guerin, R., Menard, C., Bambace, N. and Sapieha, P. (2013) 'Circulating miRNAs as sensitive and specific biomarkers for the diagnosis and monitoring of human diseases: promises and challenges.', *Clinical biochemistry*, 46(10–11).

Han, J., Lee, Y., Yeom, K., Kim, Y., Jin, H. and Kim, V. N. (2004) 'The Drosha – DGCR8 complex in primary microRNA processing', *Genes & Development*, 18(24).

Harada, C. N., Love, M. C. N. and Triebel, K. (2013) 'Normal Cognitive Aging', *Clin Geriatr Med.*, 29(4).

Hatse, S., Brouwers, B., Dalmasso, B., Laenen, A., Kenis, C., Schöffski, P. and Wildiers, H. (2014) 'Circulating MicroRNAs as easy-to-measure aging biomarkers in older breast cancer patients: Correlation with chronological age but not with fitness/frailty status', *PLoS ONE*, 9(10).

Hayes, S. M., Alosco, M. L. and Forman, D. E. (2014) 'The effects of aerobic exercise on cognitive and neural decline in aging and cardiovascular disease', *Current Geriatrics Reports*, 3(4).

Hébert, S. S., Horre, K., Nicolai, L., Papadopoulou, A. S., Mandemakers, W., Silahtaroglu, A. N., Kauppinen, S., Delacourte, A. and De Strooper, B. (2008) 'Loss of microRNA cluster miR-29a/b-1 in sporadic Alzheimer's disease correlates with increased BACE1/ -secretase expression', *Proceedings of the National Academy of Sciences*, 105(17).

Hébert, S. S. and De Strooper, B. (2009) 'Alterations of the microRNA network cause neurodegenerative disease', *Trends in Neurosciences*, 32(4).

Hedden, T. and Gabrieli, J. D. E. (2004) 'Insights into the ageing mind: a view from cognitive neuroscience', *Nature Reviews Neuroscience*, 5(2).

Herting, M. M. and Nagel, B. J. (2012) 'Aerobic fitness relates to learning on a virtual Morris Water Task and hippocampal volume in adolescents', *Behavioural Brain Research*. Elsevier B.V., 233(2).

Heydari, A. R., Unnikrishnan, A., Lucente, L. V. and Richardson, A. (2007) 'Caloric restriction and genomic stability', *Nucleic Acids Research*, 35(22).

Hikosaka, K., Ikutani, M., Shito, M., Kazuma, K., Gulshan, M., Nagai, Y., Takatsu, K., Konno, K., Tobe, K., Kanno, H. and Nakagawa, T. (2014) 'Deficiency of nicotinamide mononucleotide adenylyltransferase 3 (Nmnat3) causes hemolytic anemia by altering the glycolytic flow in mature erythrocytes', *Journal of Biological Chemistry*, 289(21).

Hillman, C. H., Erickson, K. I. and Kramer, A. F. (2008) 'Be smart, exercise your heart: exercise effects on brain and cognition', *Nature reviews. Neuroscience*, 9(1).

Howerton, C. L., Garner, J. P. and Mench, J. A. (2008) 'Effects of a running wheel-igloo enrichment on aggression, hierarchy linearity, and stereotypy in group-housed male CD-1 (ICR) mice', *Applied Animal Behaviour Science*, 115(1–2).

Huang, T., Larsen, K. T., Ried-Larsen, M., Møller, N. C. and Andersen, L. B. (2014) 'The effects of physical activity and exercise on brain-derived neurotrophic factor in healthy humans: A review', *Scandinavian Journal of Medicine and Science in Sports*, 24(1).

Hunter, M. P., Ismail, N., Zhang, X., Aguda, B. D., Lee, E. J., Yu, L., Xiao, T., Schafer, J., Lee, M. L. T., Schmittgen, T. D., Nana-Sinkam, S. P., Jarjoura, D. and Marsh, C. B. (2008) 'Detection of microRNA expression in human peripheral blood microvesicles', *PLoS ONE*, 3(11).

Imai, S. I. (2010) 'A possibility of nutriceuticals as an anti-aging intervention: Activation of sirtuins by promoting mammalian NAD biosynthesis', *Pharmacological Research*, 62(1).

Issa, J. P. J., Ottaviano, Y. L., Celano, P., Hamilton, S. R., Davidson, N. E. and Baylin, S. B. (1994) 'Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon', *Nature Genetics*, 7(4).

Jayakody, K., Gunadasa, S. and Hosker, C. (2014) 'Exercise for anxiety disorders: systematic review', *British Journal of Sports Medicine*, 48(3).

Jessberger, S., Clark, R. E., Broadbent, N. J., Gregory D Clemenson, J., Consiglio, A., Lie, D. C., Squire, L. R. and Gage, F. H. (2009) 'Dentate gyrus-specific knockdown of adult neurogenesis impairs spatial and object recognition memory in adult rats', *Learning & Memory*, 16(2).

Jin, J., Kim, S., Liu, X., Zhang, H., Zhang, C., Kim, Y., Sun, T., Avenue, Y. and Neuroscience, C. (2016) 'miR-17-92 cluster regulates adult hippocampal neurogenesis, anxiety and depression', *Cell Reports*, 16(6).

Jung, H. J. and Suh, Y. (2014) 'Circulating miRNAs in ageing and ageing-related diseases', *Journal of Genetics and Genomics*, 41(9).

Junn, E. and Mouradian, M. M. (2012) 'MicroRNAs in neurodegenerative diseases and their therapeutic potential', *Pharmacology and Therapeutics*, 133(2).

Kahl, P., Gullotti, L., Heukamp, L. C., Wolf, S., Friedrichs, N., Vorreuther, R., Solleder, G., Bastian, P. J., Ellinger, J., Metzger, E., Schüle, R. and Buettner, R. (2006) 'Androgen receptor coactivators lysine-specific histone demethylase 1 and four and a half LIM domain protein 2 predict risk of

prostate cancer recurrence', *Cancer Research*, 66(23).

Kareta, M. S., Botello, Z. M., Ennis, J. J., Chou, C. and Chédin, F. (2006) 'Reconstitution and mechanism of the stimulation of de novo methylation by human DNMT3L', *Journal of Biological Chemistry*, 281(36).

Ke, Z., Yip, S. P., Li, L., Zheng, X. X. and Tong, K. Y. (2011) 'The effects of voluntary, involuntary, and forced exercises on brain-derived neurotrophic factor and motor function recovery: A rat brain ischemia model', *PLoS ONE*, 6(2).

Kee, N., Sivalingam, S., Boonstra, R. and Wojtowicz, J. M. (2002) 'The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis', *Journal of Neuroscience Methods*, 115(1).

Kempermann, G., Kuhn, H. G. and Gage, F. H. (1997) 'More hippocampal neurons in adult mice living in an enriched environment', *Nature*, 386(6624).

Kennedy, K. M. and Raz, N. (2009) 'Aging white matter and cognition: Differential effects of regional variations in diffusion properties on memory, executive functions, and speed', *Neuropsychologia*, 47(3).

Kenyon, C. J. (2010) 'The genetics of ageing', *Nature*, 464(7288).

Kenyon, C. J., Chang, J., Gensch, E., Rudner, A. and Tabtlang, R. (1993) 'A c.elegans mutant that lives twice as long as wild type', *Nature Letters*, 366(2).

Kim, J., Inoue, K., Ishii, J., Vanti, W. B., Voronov, S. V, Murchison, E., Hannon, G. and Abeliovich, A. (2009) 'A microRNA feedback circuit in midbrain dopamine neurons', *Science*, 317(5842).

Kim, M. Y., Zhang, T. and Kraus, W. L. (2005) 'Poly (ADP-ribosyl)ation by PARP-1: "PAR-laying" NAD+ into a nuclear signal', *Genes and Development*, 19(17).

Klose, R. J. and Bird, A. P. (2006) 'Genomic DNA methylation: The mark and its mediators', *Trends in Biochemical Sciences*, 31(2).

Knapen, J., Vancampfort, D., Moriën, Y. and Marchal, Y. (2015) 'Exercise therapy improves both mental and physical health in patients with major depression', *Disability and Rehabilitation*, 37(16).

Knight, S. W. and Bass, B. L. (2001) 'A Role for the RNase III Enzyme DCR-1 in RNA Interference and Germ Line Development in Caenorhabditis elegans', *Science*, 293(8).

Kohli, R. M. and Zhang, Y. (2013) 'TET enzymes, TDG and the dynamics of DNA demethylation', *Nature*, 502(7472).

Koltai, E., Szabo, Z., Atalay, M., Boldogh, I., Naito, H., Goto, S., Nyakas, C. and Radak, Z. (2009) 'Exercise alters SIRT1, SIRT6, NAD and NAMPT levels in skeletal muscle of aged rats', *Mechanisms of Ageing and Development*, 131(1).

Komada, M., Takao, K. and Miyakawa, T. (2008) 'Elevated Plus Maze for Mice', *Journal of Visualized Experiments*, (22).

Kriaucionis, S. and Bird, A. (2003) 'DNA methylation and Rett syndrome',

Human Molecular Genetics, 12(suppl 2).

Kristin L. Szuhanya, Matteo Bugattia, M. W. O. (2015) 'A meta-analytic review of the effects of exercise on brainderived neurotrophic factor', *Journal of Psychiatric Research*, 60.

Kronenberg, G., Bick-Sander, A., Bunk, E., Wolf, C., Ehninger, D. and Kempermann, G. (2006) 'Physical exercise prevents age-related decline in precursor cell activity in the mouse dentate gyrus', *Neurobiology of Aging*, 27(10).

Kuhn, H. G. (2015) 'Control of cell survival in adult mammalian neurogenesis', *Cold Spring Harbor perspectives in biology*, 7(12).

Kuhn, H. G., Dickinson-Anson, H. and Gage, F. H. (1996) 'Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 16(6).

Kumar, P., Dezso, Z., MacKenzie, C., Oestreicher, J., Agoulnik, S., Byrne, M., Bernier, F., Yanagimachi, M., Aoshima, K. and Oda, Y. (2013) 'Circulating miRNA Biomarkers for Alzheimer's Disease', *PLoS ONE*, 8(7).

Kuphal, K. E., Fibuch, E. E. and Taylor, B. K. (2007) 'Extended Swimming Exercise Reduces Inflammatory and Peripheral Neuropathic Pain in Rodents', *Journal of Pain*, 8(12).

Kuzmichev, A., Nishioka, K., Edrjument-Bromage, H., Tempst, P. and Reinberg, D. (2002) 'Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein', *Genes and Development*, 16(22).

Kuznetsova, K. A., Maniega, S. M., Ritchie, S. J., Cox, S. R., Storkey, A. J., Starr, J. M., Wardlaw, J. M., Deary, I. J. and Bastin, M. E. (2016) 'Brain white matter structure and information processing speed in healthy older age', *Brain Structure and Function*, 221(6).

Lagace, D. C., Fischer, S. J. and Eisch, A. J. (2007) 'Gender and Endogenous Levels of Estradiol Do Not Influence Adult Hippocampal Neurogenesis in Mice', *Hippocampus*, 17(9).

Lan, F., Bayliss, P. E., Rinn, J. L., Whetstine, J. R., Wang, J. K., Chen, S., Iwase, S., Alpatov, R., Issaeva, I., Canaani, E., Roberts, T. M., Chang, H. Y. and Shi, Y. (2007) 'A histone H3 lysine 27 demethylase regulates animal posterior development', *Nature*, 449(7163).

Larson, E. B., Wang, L., Bowen, J. D., Mccormick, W. C., Teri, L. and Crane, P. (2006) 'Exercise is associated with reduced risk for incident dementia among persons 65 years of age and older', *Annals of internal medicine*, 144(2).

Lautenschlager, N. T., Cox, K. L., Flicker, L., Foster, J. K., van Bockxmeer, F. M., Xiao, J., Greenop, K. R. and Almeida, O. P. (2008) 'Effect of physical activity on cognitive function in older adults at risk for Alzheimer disease: a randomized trial.', *JAMA: the journal of the American Medical Association*, 300(9).

Leasure, J. L. and Jones, M. (2008) 'Forced and voluntary exercise differentially affect brain and behavior', *Neuroscience*, 156(3).

Lee, K. K. and Workman, J. L. (2007) 'Histone acetyltransferase complexes: One size doesn't fit all', *Nature Reviews Molecular Cell Biology*, 8(4).

Lee, R. C., Feinbaum, R. L. and Ambros, V. (1993) 'the C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14.', *Cell*, 75(5).

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Rådmark, O., Kim, S. and Kim, V. N. (2003) 'The nuclear RNase III Drosha initiates microRNA processing', *Nature*, 425(6956).

Lee, Y., Kim, M., Han, J., Yeom, K.-H., Lee, S., Baek, S. H. and Kim, V. N. (2004) 'MicroRNA genes are transcribed by RNA polymerase II', *The EMBO Journal*, 23(20).

Leger, M., Quiedeville, A., Bouet, V., Haelewyn, B., Boulouard, M., Schumann-bard, P. and Freret, T. (2013) 'Object recognition test in mice', *Nature Protocols*, 8(12).

Lencastre, A. De, Pincus, Z., Zhou, K., Kato, M., Sylvia, S. and Slack, F. J. (2011) 'MicroRNAs both promote and antagonize longevity in C. elegans', *Current Biology*, 20(24).

Li, B., Carey, M. and Workman, J. L. (2007) 'The Role of Chromatin during Transcription', *Cell*, 128(2).

Li, E. (2002) 'Chromatin modification and epigenetic reprogramming in mammalian development', *Nature Reviews Genetics*, 3(9).

Li, Y., Daniel, M. and Tollefsbol, T. O. (2011) 'Epigenetic regulation of caloric restriction in aging', *BMC Medicine*, 9(98).

Lie, D.-C., Colamarino, S. A., Song, H.-J., Désiré, L., Mira, H., Consiglio, A., Lein, E. S., Jessberger, S., Lansford, H., Dearie, A. R. and Gage, F. H. (2005) 'Wnt signalling regulates adult hippocampal neurogenesis', *Nature*, 437(7063).

Lim, D. A. and Alvarez-buylla, A. (2016) 'The Adult Ventricular – Subventricular Zone', *Cold Spring Harbor perspectives in biology*, 8(5).

Lund, E., Güttinger, S., Calado, A., Dahlberg, J. E. and Kutay, U. (2004) 'Nuclear Export of MicroRNA', *Science*, 303(January).

Ma, C. -y., Yao, M. -j., Zhai, Q. -w., Jiao, J. -w., Yuan, X. -b. and Poo, M. -m. (2014) 'SIRT1 suppresses self-renewal of adult hippocampal neural stem cells', *Development*, 141(24).

Ma, D. K., Marchetto, M. C., Guo, J. U., Ming, G., Gage, F. H. and Song, H. (2010) 'Epigenetic choreographers of neurogenesis in the adult mammalian brain', *Nature Neuroscience*, 13(11).

Maegawa, S., Hinkal, G., Kim, H. S., Shen, L., Zhang, L., Zhang, J., Zhang, N., Liang, S., Donehower, L. A. and Issa, J. J. (2010) 'Widespread and tissue specific age-related DNA methylation changes in mice', *Genome Research*, (713).

Maegawa, S., Lu, Y., Tahara, T., Lee, J. T., Madzo, J., Liang, S., Jelinek, J., Colman, R. J. and Issa, J. P. J. (2017) 'Caloric restriction delays age-related methylation drift', *Nature Communications*. Springer US, 8(1).

de Magalhães, J. P., Curado, J. and Church, G. M. (2009) 'Meta-analysis of age-related gene expression profiles identifies common signatures of aging', *Bioinformatics*, 25(7).

Manson, J. E., Hu, F. B., Rich-Edwards, J. W., Colditz, G. A., Stampfer, M. J., Willett, W. C., Speizer, F. E. and Hennekens, C. H. (1999) 'A prospective study of walking as compared with vigorous exercise in the prevention of coronary heart disease in women', *The New England journal of medicine*, 341(9).

Marlatt, M. W., Potter, M. C., Lucassen, P. J. and van Praag, H. (2012) 'Running throughout middle-age improves memory function, hippocampal neurogenesis, and BDNF levels in female C57BL/6J mice', *Developmental Neurobiology*, 72(6).

Martin, B., Mattson, M. P. and Maudsley, S. (2006) 'Caloric restriction and intermittent fasting: Two potential diets for successful brain aging', *Ageing Research Reviews*, 5(3).

Masoro, E. J. (2005) 'Overview of caloric restriction and ageing', *Mechanisms of Ageing and Development*, 126(9).

Massudi, H., Grant, R., Braidy, N., Guest, J., Farnsworth, B. and Guillemin, G. J. (2012) 'Age-associated changes in oxidative stress and NAD+ metabolism in human tissue', *PLoS ONE*, 7(7).

Mastroeni, D., Grover, A., Delvaux, E., Whiteside, C., Coleman, P. D. and Rogers, J. (2010) 'Epigenetic changes in Alzheimer's disease: Decrements in DNA methylation', *Neurobiology of Aging*, 31(12).

Mathews, K. J., Allen, K. M., Boerrigter, D., Ball, H., Shannon Weickert, C. and Double, K. L. (2017) 'Evidence for reduced neurogenesis in the aging human hippocampus despite stable stem cell markers', *Aging Cell*, 16(5).

Mattison, J. A., Colman, R. J., Beasley, T. M., Allison, D. B., Kemnitz, J. W., Roth, G. S., Ingram, D. K., Weindruch, R., De Cabo, R. and Anderson, R. M. (2017) 'Caloric restriction improves health and survival of rhesus monkeys', *Nature Communications*. Nature Publishing Group, 8(May 2016).

Mercken, E. M., Mitchell, S. J., *et al.* (2014) 'SRT2104 extends survival of male mice on a standard diet and preserves bone and muscle mass', *Aging Cell*, 13(5).

Mercken, E. M., Hu, J., Krzysik-Walker, S., Wei, M., Li, Y., Mcburney, M. W., de Cabo, R. and Longo, V. D. (2014) 'SIRT1 but not its increased expression is essential for lifespan extension in caloric-restricted mice', *Aging Cell*, 13(1).

Merom, D., Grunseit, A., Eramudugolla, R., Jefferis, B., Mcneill, J. and Anstey, K. J. (2016) 'Cognitive benefits of social dancing and walking in old age: The dancing mind randomized controlled trial', *Frontiers in Aging Neuroscience*, 8(26).

Mi S., Zhang J., Zhang W., H. R. S. (2013) 'Circulating miRNAs as biomarkers for inflammatory diseases', *MicroRNA*, 2(1).

Mitchell, P. S. *et al.* (2008) 'Circulating microRNAs as stable blood-based markers for cancer detection', *Proceedings of the National Academy of Sciences*, 105(30).

Mitchell, S. J., Martin-Montalvo, A., Mercken, E. M., Palacios, H. H., Ward, T. M., Abulwerdi, G., Minor, R. K., Vlasuk, G. P., Ellis, J. L., Sinclair, D. a., Dawson, J., Allison, D. B., Zhang, Y., Becker, K. G., Bernier, M. and De Cabo, R. (2014) 'The SIRT1 activator SRT1720 extends lifespan and improves health of mice fed a standard diet', *Cell Reports*. Elsevier, 6(5).

Mo, M. H., Chen, L., Fu, Y., Wang, W. and Fu, S. W. (2012) 'Cell-free Circulating miRNA Biomarkers in Cancer', *Journal of Cancer*, 3(1).

Mul, J. D. (2018) 'Voluntary exercise and depression-like behavior in rodents: Are we running in the right direction?', *Journal of Molecular Endocrinology*, 60(3).

Mustroph, M. L., Chen, S., Desai, S. C., Cay, E. B., DeYoung, E. K. and Rhodes, J. S. (2012) 'Aerobic exercise is the critical variable in an enriched environment that increases hippocampal neurogenesis and water maze learning in male C57BL/6J mice', *Neuroscience*. IBRO, 219.

Nguyen, J. C. D., Killcross, A. S. and Jenkins, T. A. (2013) 'Effect of low-intensity treadmill exercise on behavioural measures and hippocampal parvalbumin immunoreactivity in the rat', *Behavioural Brain Research*. Elsevier B.V., 256.

Nigro, A., Menon, R., Bergamaschi, A., Clovis, Y. M., Baldi, A., Ehrmann, M., Comi, G., De Pietri Tonelli, D., Farina, C., Martino, G. and Muzio, L. (2012) 'MiR-30e and miR-181d control Radial Glia cell proliferation via HtrA1 modulation', *Cell Death and Disease*. Nature Publishing Group, 3(8).

Nilsson, L.-G. (2003) 'Memory function in normal aging', *Acta Neurologica Scandinavica*, 107.

O'Callaghan, R. M., Ohle, R. and Kelly, Á. M. (2007) 'The effects of forced exercise on hippocampal plasticity in the rat: A comparison of LTP, spatial- and non-spatial learning', *Behavioural Brain Research*, 176(2).

Olivieri, F., Capri, M., Bonafè, M., Morsiani, C., Jung, H. J., Spazzafumo, L., Viña, J. and Suh, Y. (2017) 'Circulating miRNAs and miRNA shuttles as biomarkers: Perspective trajectories of healthy and unhealthy aging', *Mechanisms of Ageing and Development*, 165(B).

Olivieri, F., Rippo, M. R., Procopio, A. D. and Fazioli, F. (2013) 'Circulating inflamma-miRs in aging and age-related diseases', *Frontiers in Genetics*, 4(JUN).

Olivieri, F., Spazzafumo, L., Santini, G., Lazzarini, R., Albertini, M. C., Rippo, M. R., Galeazzi, R., Abbatecola, A. M., Marcheselli, F., Monti, D., Ostan, R., Cevenini, E., Antonicelli, R., Franceschi, C. and Procopio, A. D. (2012) 'Age-related differences in the expression of circulating microRNAs: miR-21 as a new circulating marker of inflammaging', *Mechanisms of Ageing and Development*. Elsevier Ireland Ltd, 133(11–12).

Olson, A. K., Eadie, B. D., Ernst, C. and Christie, B. R. (2006) 'Environmental enrichment and voluntary exercise massively increase neurogenesis in the adult hippocampus via dissociable pathways', *Hippocampus*, 16(3).

Ortiz-Matamoros, A., Salcedo-Tello, P., Avila-Muñoz, E., Zepeda, A. and Arias, C. (2013) 'Role of wnt signaling in the control of adult hippocampal functioning in health and disease: therapeutic implications.', *Current*

neuropharmacology, 11(5).

Paillard, T., Rolland, Y. and de Souto Barreto, P. (2015) 'Protective Effects of Physical Exercise in Alzheimer's Disease and Parkinson's Disease: A Narrative Review.', *Journal of clinical neurology (Seoul, Korea)*, 11(3).

Pal, S. and Tyler, J. K. (2016) 'Epigenetics and aging', *Science Advances*, 2(7).

Pan-Vazquez, A., Rye, N., Ameri, M., McSparron, B., Smallwood, G., Bickerdyke, J., Rathbone, A., Dajas-Bailador, F. and Toledo-Rodriguez, M. (2015) 'Impact of voluntary exercise and housing conditions on hippocampal glucocorticoid receptor, miR-124 and anxiety.', *Molecular brain*, 8(40).

Pannese, E. (2011) 'Morphological changes in nerve cells during normal aging', *Brain Structure and Function*, 216(2).

Parikh, I., Guo, J., Chuang, K. H., Zhong, Y., Rempe, R. G., Hoffman, J. D., Armstrong, R., Bauer, B., Hartz, A. M. S. and Lin, A. L. (2016) 'Caloric restriction preserves memory and reduces anxiety of aging mice with early enhancement of neurovascular functions', *Aging*, 8(11).

Patten, A. R., Sickmann, H., Hryciw, B. N., Kucharsky, T., Parton, R., Kernick, A. and Christie, B. R. (2013) 'Long-term exercise is needed to enhance synaptic plasticity in the hippocampus', *Learning & memory (Cold Spring Harbor, N.Y.)*, 20(11).

Pekcec, A., Baumgärtner, W., Bankstahl, J. P., Stein, V. M. and Potschka, H. (2008) 'Effect of aging on neurogenesis in the canine brain', *Aging Cell*, 7(3).

Pereira, A. C., Huddleston, D. E., Brickman, A. M., Sosunov, A. A., Hen, R., McKhann, G. M., Sloan, R., Gage, F. H., Small, S. A. and Brown, T. R. (2007) 'An in vivo correlate of exercise-induced neurogenesis in the adult dentate gyrus.', *Proceedings of the National Academy of Sciences of the United States of America*, 104(13).

Peters, R. (2006) 'Ageing and the brain', *Postgraduate medical Journal*, 82(964).

Pfaffl, M. W. (2001) 'A new mathematical model for relative quantification in real-time RT-PCR', *Nucleic Acids Research*, 29(9).

Pietrelli, A., Lopez-Costa, J., Goñi, R., Brusco, A. and Basso, N. (2012) 'Aerobic exercise prevents age-dependent cognitive decline and reduces anxiety-related behaviors in middle-aged and old rats', *Neuroscience*, 202.

Piper, M. D. W. and Partridge, L. (2017) 'Drosophila as a model for ageing', *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 1864(9).

Pollack, M., Phaneuf, S. and Dirks, A. (2002) 'The role of apoptosis in the normal aging brain, skeletal muscle, and heart', *Ann. N.Y. Acad. Sci.*, 959.

Portela, A. and Esteller, M. (2010) 'Epigenetic modifications and human disease', *Nature Biotechnology*. Nature Publishing Group, 28(10).

van Praag, H. (2005) 'Exercise Enhances Learning and Hippocampal Neurogenesis in Aged Mice', *Journal of Neuroscience*, 25(38).

van Praag, H. (2009) 'Exercise and the brain: something to chew on', *Trends in Neurosciences*, 32(April).

Van Praag, H. (2008) 'Neurogenesis and exercise: Past and future directions', *NeuroMolecular Medicine*, 10(2).

van Praag, H., Christie, B. R., Sejnowski, T. J. and Gage, F. H. (1999) 'Running enhances neurogenesis, learning, and long-term potentiation in mice.', *Proceedings of the National Academy of Sciences of the United States of America*, 96(23).

van Praag, H., Kempermann, G. and Gage, F. H. (1999) 'Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus', *Nat Neurosci*, 2(3).

Probst, A. V., Dunleavy, E. and Almouzni, G. (2009) 'Epigenetic inheritance during the cell cycle', *Nature Reviews Molecular Cell Biology*, 10(3).

Quina, A. S., Buschbeck, M. and Di Croce, L. (2006) 'Chromatin structure and epigenetics', *Biochemical Pharmacology*, 72(11).

Rafalski, V. A. and Brunet, A. (2011) 'Energy metabolism in adult neural stem cell fate', *Progress in Neurobiology*, 93(2).

Rao, M. S., Hattiangady, B., Abdel-Rahman, A., Stanley, D. P. and Shetty, A. K. (2005) 'Newly born cells in the ageing dentate gyrus display normal migration, survival and neuronal fate choice but endure retarded early maturation', *European Journal of Neuroscience*, 21(2).

Raz, N., Lindenberger, U., Rodrigue, K. M., Kennedy, K. M., Head, D., Williamson, A., Dahle, C., Gerstorf, D. and Acker, J. D. (2005) 'Regional brain changes in aging healthy adults: General trends, individual differences and modifiers', *Cerebral Cortex*, 15(11).

Reiner, M., Niermann, C., Jekauc, D. and Woll, A. (2013) 'Long-term health benefits of physical activity--a systematic review of longitudinal studies.', *BMC public health*, 13(1).

Revollo, J. R., Grimm, A. a. and Imai, S. I. (2004) 'The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates Sir2 activity in mammalian cells', *Journal of Biological Chemistry*, 279(49).

Rhee, I., Bachman, K. E., Park, B. H., Jair, K. W., Yen, R. W. C., Schuebel, K. E., Cui, H., Feinberg, A. P., Lengauer, C., Kinzler, K. W., Baylin, S. B. and Vogelstein, B. (2002) 'DNMT1 and DNMT3b cooperate to silence genes in human cancer cells', *Nature*, 416(6880).

Roedel, A., Storch, C., Holsboer, F. and Ohl, F. (2006) 'Effects of light or dark phase testing on behavioural and cognitive performance in DBA mice.', *Laboratory animals*, 40.

Roig, M., Nordbrandt, S., Geertsen, S. S. and Nielsen, J. B. (2013) 'The effects of cardiovascular exercise on human memory: a review with meta-analysis', *Neurosci Biobehav Rev*, 37(8).

Ropero, S. and Esteller, M. (2007) 'The role of histone deacetylases (HDACs) in human cancer', *Molecular Oncology*, 1(1).

Rossetto, D., Avvakumov, N. and Côté, J. (2012) 'Histone phosphorylation: A chromatin modification involved in diverse nuclear events', *Epigenetics*,

7(10).

Rossi, F. (2015) *NMNAT1 AND ITS ROLE ON AGEING AND AGE - RELATED DISEASES*.

Rossi, F., Geiszler, P. C., Meng, W., Barron, M. R., Prior, M., Herd-Smith, A., Loreto, A., Lopez, M. Y., Faas, H., Pardon, M. C. and Conforti, L. (2018) 'NAD-biosynthetic enzyme NMNAT1 reduces early behavioral impairment in the htau mouse model of tauopathy', *Behavioural Brain Research*. Elsevier, 339(November 2017).

Rueden, C. T., Schindelin, J., Hiner, M. C., DeZonia, B. E., Walter, A. E., Arena, E. T. and Eliceiri, K. W. (2017) 'ImageJ2: ImageJ for the next generation of scientific image data', *BMC Bioinformatics*, 18(1).

Saeidimehr, S., Ebrahimi, A., Saki, N., Goodarzi, P. and Rahim, F. (2016) 'MicroRNA-based linkage between aging and cancer: From epigenetics view point', *Cell Journal*, 18(2).

Salam, J. N., Fox, J. H., DeTroy, E. M., Guignon, M. H., Wohl, D. F. and Falls, W. A. (2009) 'Voluntary exercise in C57 mice is anxiolytic across several measures of anxiety', *Behavioural Brain Research*, 197(1).

Salami, A., Eriksson, J., Nilsson, L. G. and Nyberg, L. (2012) 'Age-related white matter microstructural differences partly mediate age-related decline in processing speed but not cognition', *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 1822(3).

Salthouse, T. A. (2009) 'When does age-related cognitive decline begin?', *Neurobiology of Aging*, 30(4).

Salthouse, T. A. (2010) 'Selective review of cognitive aging', *Journal of the International Neuropsychological Society*. University of Nottingham, 16(5).

Santos-Soto, I. J., Chorna, N., Carballeira, N. M., Vélez-Bartolomei, J. G., Méndez-Merced, A. T., Chornyy, A. P. and Peña De Ortiz, S. (2013) 'Voluntary running in young adult mice reduces anxiety-like behavior and increases the accumulation of bioactive lipids in the cerebral cortex', *PLoS ONE*, 8(12).

Saraulli, D., Costanzi, M., Mastrorilli, V. and Farioli-Vecchioli, S. (2017) 'The Long Run: Neuroprotective Effects of Physical Exercise on Adult Neurogenesis from Youth to Old Age', *Current Neuropharmacology*, 15(4).

Satoh, J. ichi (2012) 'Molecular network of microRNA targets in Alzheimer's disease brains', *Experimental Neurology*, 235(2).

Scahill, R. I., Frost, C., Jenkins, R., Whitwell, J. L., Rossor, M. N. and Fox, N. C. (2003) 'A Longitudinal Study of Brain Volume Changes in Normal Aging Using Serial Registered Magnetic Resonance Imaging', *Archives of neurology*, 60(7).

Schuff, N., Woerner, N., Boreta, L., Kornfield, T., Shaw, L. M., Trojanowski, J. Q., Thompson, P. M., Jack, C. R. and Weiner, M. W. (2009) 'MRI of hippocampal volume loss in early Alzheimers disease in relation to ApoE genotype and biomarkers', *Brain*, 132(4).

Schwarzenbach, H., Hoon, D. S. B. and Pantel, K. (2011) 'Cell-free nucleic acids as biomarkers in cancer patients', *Nature Reviews Cancer*. Nature Publishing Group, 11(6).

Scott, J. P. R., McNaughton, L. R. and Polman, R. C. J. (2006) 'Effects of sleep deprivation and exercise on cognitive, motor performance and mood', *Physiology and Behavior*, 87(2).

Seib, D. R. M., Corsini, N. S., Ellwanger, K., Plaas, C., Mateos, A., Pitzer, C., Niehrs, C., Celikel, T. and Martin-Villalba, A. (2013) 'Loss of dickkopf-1 restores neurogenesis in old age and counteracts cognitive decline', *Cell Stem Cell*, 12(2).

Seib, D. R. M. and Martin-Villalba, A. (2015) 'Neurogenesis in the Normal Ageing Hippocampus: A Mini-Review', *Gerontology*, 61(4).

Seibenhener, M. L. and Wooten, M. C. (2015) 'Use of the Open Field Maze to Measure Locomotor and Anxiety-like Behavior in Mice', *Journal of Visualized Experiments*, (February).

Sen, P., Shah, P. P., Nativio, R. and Berger, S. L. (2016) 'Epigenetic mechanisms regulating longevity and aging', *Cell*, 166(4).

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J. R., Cole, P. A., Casero, R. A. and Shi, Y. (2004) 'Histone demethylation mediated by the nuclear amine oxidase homolog LSD1', *Cell*, 119(7).

Shilatifard, A. (2012) 'The COMPASS Family of Histone H3K4 Methylases: Mechanisms of Regulation in Development and Disease Pathogenesis', *Annual Review of Biochemistry*, 81.

Shoji, H., Takao, K., Hattori, S. and Miyakawa, T. (2016) 'Age-related changes in behavior in C57BL/6J mice from young adulthood to middle age', *Molecular Brain*. Molecular Brain, 9(1).

Sibley, B. A. and Etnier, J. L. (2003) 'The relationship between physical activity and cognition in children: a meta-analysis', *Pediatric Exercise Science*, 15.

Silva, G. J. J., Bye, A., el Azzouzi, H. and Wisløff, U. (2017) 'MicroRNAs as Important Regulators of Exercise Adaptation', *Progress in Cardiovascular Diseases*, 60(1).

Silva, R. H., Kameda, S. R., Carvalho, R. C., Takatsu-Coleman, A. L., Niigaki, S. T., Abílio, V. C., Tufik, S. and Frussa-Filho, R. (2004) 'Anxiogenic effect of sleep deprivation in the elevated plus-maze test in mice', *Psychopharmacology*, 176(2).

Simpson, J. and Kelly, J. P. (2011) 'The impact of environmental enrichment in laboratory rats-Behavioural and neurochemical aspects', *Behavioural Brain Research*, 222(1).

Singal, R. and Ginder, G. D. (1999) 'DNA Methylation', *Blood*, 93(12).

Smith, P. J., Blumenthal, J. A., Hoffman, B. M., Strauman, T. A., Welsh-bohmer, K., Jeffrey, N. and Sherwood, A. (2011) 'Aerobic exercise and neurocognitive performance: a meta- analytic review of randomized controlled trials', *Psychosomatic Medicine*, 72(3).

Sohel, M. H. (2016) 'Extracellular / Circulating MicroRNAs : Release Mechanisms , Functions and Challenges', *Als*, 10(2).

Sorrells, S. F. *et al.* (2018) 'Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults', *Nature*. Nature

Publishing Group, 555(7696).

Spaan, P. E. J. (2015) 'Episodic and semantic memory functioning in very old age: Explanations from executive functioning and processing speed theories', *Cogent Psychology*. Cogent, 2(1).

Spalding, K. L., Bergmann, O., Alkass, K., Bernard, S., Salehpour, M., Huttner, H. B., Boström, E., Westerlund, I., Vial, C., Buchholz, B. A., Possnert, G., Mash, D. C., Druid, H. and Frisén, J. (2013) 'Dynamics of hippocampal neurogenesis in adult humans.', *Cell*, 153(6).

Stein, L. R. and Imai, S. I. (2014) 'Specific ablation of Nampt in adult neural stem cells recapitulates their functional defects during aging', *EMBO Journal*, 33(12).

Stott, D. (2006) 'Cognitive decline in ageing', *Asian Journal of Gerontology & Geriatrics*, 1(1).

Strickland, I. T., Richards, L., Holmes, F. E., Wynick, D., Uney, J. B. and Wong, L. F. (2011) 'Axotomy-induced mir-21 promotes axon growth in adult dorsal root ganglion neurons', *PLoS ONE*, 6(8).

Stubbs, B., Vancampfort, D., Rosenbaum, S., Firth, J., Cosco, T., Veronese, N., Salum, G. A. and Schuch, F. B. (2017) 'An examination of the anxiolytic effects of exercise for people with anxiety and stress-related disorders: A meta-analysis', *Psychiatry Research*. Elsevier, 249(January).

Svensson, M., Rosvall, P., Boza-Serrano, A., Andersson, E., Lexell, J. and Deierborg, T. (2016) 'Forced treadmill exercise can induce stress and increase neuronal damage in a mouse model of global cerebral ischemia', *Neurobiology of Stress*, 5(9).

Taki, Y., Goto, R., Evans, A., Zijdenbos, A., Neelin, P., Lerch, J., Sato, K., Ono, S., Kinomura, S., Nakagawa, M., Sugiura, M., Watanabe, J., Kawashima, R. and Fukuda, H. (2004) 'Voxel-based morphometry of human brain with age and cerebrovascular risk factors', *Neurobiology of Aging*, 25(4).

Tamburini, B. A. and Tyler, J. K. (2005) 'Localized Histone Acetylation and Deacetylation Triggered by the Homologous Recombination Pathway of Double-Strand DNA Repair', *Molecular and cellular biology*, 25(12).

Taurino, C., Miller, W. H., McBride, M. W., McClure, J. D., Khanin, R., Moreno, M. U., Dymott, J. A., Delles, C. and Dominiczak, A. F. (2010) 'Gene expression profiling in whole blood of patients with coronary artery disease', *Clinical Science*, 119(8).

Tissenbaum, H. A. (2015) 'Using C. elegans for aging research', *Invertebrate Reproduction and Development*. Taylor & Francis, 59.

Toescu, E. (2014) 'Ageing of the Brain', 123.

Tsukada, Y. I., Fang, J., Erdjument-Bromage, H., Warren, M. E., Borchers, C. H., Tempst, P. and Zhang, Y. (2006) 'Histone demethylation by a family of JmjC domain-containing proteins', *Nature*, 439(7078).

Turchinovich, A., Samatov, T. R., Tonevitsky, A. G. and Burwinkel, B. (2013) 'Circulating miRNAs: Cell-cell communication function?', *Frontiers in Genetics*, 4(JUN).

Urbán, N. and Guillemot, F. (2014) 'Neurogenesis in the embryonic and adult brain: same regulators, different roles', *Frontiers in Cellular Neuroscience*, 8(November).

Urdinguio, R. G., Sanchez-mut, J. V and Esteller, M. (2009) 'Epigenetic mechanisms in neurological diseases : genes , syndromes , and therapies', 8(November).

Váchová, L., Čáp, M. and Palková, Z. (2012) 'Yeast colonies: A model for studies of aging, environmental adaptation, and longevity', *Oxidative Medicine and Cellular Longevity*, 2012.

Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J. and Lötvall, J. O. (2007) 'Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells', *Nature cell biology*, 9(6).

Vandesompele, J., De Preter, K., Pattyn, ilip, Poppe, B., Van Roy, N., De Paepe, A. and Speleman, rank (2002) 'Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes', *Genome Biology*, 3(711).

Varela-Nallar, L. and Inestrosa, N. C. (2013) 'Wnt signaling in the regulation of adult hippocampal neurogenesis', *Frontiers in Cellular Neuroscience*, 7(June).

Varriale, A. and Bernardi, G. (2010) 'Distribution of DNA methylation, CpGs, and CpG islands in human isochores', *Genomics*, 95(1).

Waddington, C. H. (1942) 'The epigenotype', *Endeavour*.

Wahid, F., Shehzad, A., Khan, T. and Kim, Y. Y. (2010) 'MicroRNAs: Synthesis, mechanism, function, and recent clinical trials', *Biochimica et Biophysica Acta - Molecular Cell Research*, 1803(11).

Wang, J., Chen, T. and Shan, G. (2017) 'miR-148b Regulates Proliferation and Differentiation of Neural Stem Cells via Wnt/β-Catenin Signaling in Rat Ischemic Stroke Model', *Frontiers in Cellular Neuroscience*, 11(October).

Wang, Y.-Z., Plane, jennifer M., Jiang, P., Zhou, C. J. and Deng, W. (2011) 'Concise Review: Quiescent and Active states of endogenous Adult Neural stem cells: Identification and Characterization', *Stem Cell*, 29(6).

Waterhouse, E. G., An, J. J., Orefice, L. L., Baydyuk, M., Liao, G.-Y., Zheng, K., Lu, B. and Xu, B. (2012) 'BDNF Promotes Differentiation and Maturation of Adult-born Neurons through GABAergic Transmission', *Journal of Neuroscience*, 32(41).

Williams, J., Smith, F., Kumar, S., Vijayan, M. and Reddy, P. H. (2017) 'Are microRNAs true sensors of ageing and cellular senescence?', *Ageing Research Reviews*, 35.

Wilson, R. S., Beckett, L. A., Barnes, L. L., Schneider, J. A., Bach, J., Evans, D. A. and Bennett, D. A. (2002) 'Individual differences in rates of change in cognitive abilities of older persons', *Psychology and Aging*, 17(2).

Wu, X. and Zhang, Y. (2017) 'TET-mediated active DNA demethylation: Mechanism, function and beyond', *Nature Reviews Genetics*. Nature Publishing Group, 18(9).

Xia, H., Zhang, W., Zhang, B., Zhao, Y. and Zhao, Y. (2017) 'miR-21

modulates the effect of EZH2 on the biological behavior of human lung cancer stem cells in vitro', 8(49).

Yamamoto, M., Hikosaka, K., Mahmood, A., Tobe, K., Shojaku, H., Inohara, H. and Nakagawa, T. (2016) 'Nmnat3 is dispensable in mitochondrial NAD level maintenance in vivo', *PLoS ONE*, 11(1).

Yuede, C. M., Zimmerman, S. D., Dong, H., Kling, M. J., Bero, A. W., Holtzman, D. M., Timson, B. F. and Csernansky, J. G. (2009) 'Effects of voluntary and forced exercise on plaque deposition, hippocampal volume, and behavior in the Tg2576 mouse model of Alzheimer's disease', *Neurobiology of Disease*, 35(3).

Zec, R. F., Markwell, S. J., Burkett, N. R. and Larsen, D. L. (2005) 'A longitudinal study of confrontation naming in the “normal” elderly', *Journal of the International Neuropsychological Society*, 11(6).

Zhang, H. L., Yang, L. F., Zhu, Y., Yao, X. D., Zhang, S. L., Dai, B., Zhu, Y. P., Shen, Y. J., Shi, G. H. and Ye, D. W. (2011) 'Serum miRNA-21: Elevated levels in patients with metastatic hormone-refractory prostate cancer and potential predictive factor for the efficacy of docetaxel-based chemotherapy', *Prostate*, 71(3).

Zhang, J., Ji, F., Liu, Y., Lei, X., Li, H., Ji, G., Yuan, Z. and Jiao, J. (2014) 'Ezh2 Regulates Adult Hippocampal Neurogenesis and Memory', *Journal of Neuroscience*, 34(15).

Zhang, T., Berrocal, J. G., Frizzell, K. M., Gamble, M. J., DuMond, M. E., Krishnakumar, R., Yang, T., Sauve, A. A. and Lee Kraus, W. (2009) 'Enzymes in the NAD+ salvage pathway regulate SIRT1 activity at target gene promoters', *Journal of Biological Chemistry*, 284(30).

Zhang, X., Kurnasov, O. V., Karthikeyan, S., Grishin, N. V., Osterman, A. L. and Zhang, H. (2003) 'Structural characterization of a human cytosolic NMN/NaMN adenylyltransferase and implication in human NAD biosynthesis', *Journal of Biological Chemistry*, 278(15).

Zhang, Y., Ueno, Y., Liu, X. S., Buller, B., Wang, X., Chopp, M. and Zhang, Z. G. (2013) 'The MicroRNA-17-92 Cluster Enhances Axonal Outgrowth in Embryonic Cortical Neurons', *Journal of Neuroscience*, 33(16).

Appendix 1: PIPS Reflective Statement

Note to examiners:

This statement is included as an appendix to the thesis in order that the thesis accurately captures the PhD training experienced by the candidate as a BBSRC Doctoral Training Partnership student.

The Professional Internship for PhD Students is a compulsory 3-month placement which must be undertaken by DTP students. It is usually centred on a specific project and must not be related to the PhD project. This reflective statement is designed to capture the skills development which has taken place during the student's placement and the impact on their career plans it has had.

PIPS Reflective Statement

For my placement I worked at the Royal Institution of Great Britain, in the role of 'media assistant' for the Royal Institution Christmas Lectures 2016. This role involved aiding the media team during the busy run up to and follow up to the Christmas Lectures which aired over the holidays in 2016. I wrote a number of press releases to be sent to local, national and online news outlets to advertise tours based on the previous Lecture series, competitions for the current series and information on the upcoming program of events at the Ri. I also helped with website construction, formatting and writing information for various pages across the site and keeping the website up to date with the latest information on the lectures and other sci-comm events occurring at the Ri. There was also a bursary competition for underprivileged children to attend one of the Lectures, which I organised and arranged. This included sending the information out to numerous schools in the designated areas, receiving and sorting through the entries, and organising transport, accommodation and food for the winners. Finally, I helped out with various aspects of the behind the scenes preparation for the Lectures, including building sets, sourcing materials, organising guests and conducting behind the scenes tours of the building on Lecture night.

During my time at the Royal Institution, I developed a number of skills related to science communication. Firstly, my writing skills. I developed the ability to write press releases so that information about science and science-related events could be presented to a wider audience in a concise and accessible way. These press releases also had to grab the attention of journalists quickly in order for them to be published. I also further developed my skills in imparting information via the Royal Institution website in a format consistent with the Ri tone and made accessible for its target audience. I also developed communication and organisation skills by organising the student bursary to attend the Lectures. This included communicating with the schools and students themselves and ensuring their trip to and from the lectures was organised to a professional standard. Finally, I learned a significant amount about science communication in general, including setting the tone for target audiences and the amount of organisation required to set up key events.

From my placement at the Royal Institution, I would be interested in further connections with science communication, as I feel it is important to engage a wider audience in all areas of science and encourage future generations to pursue a career in science.