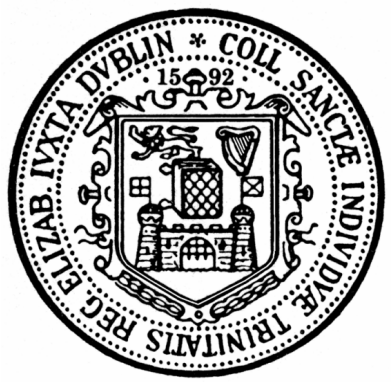
COMPARING AND CONTRASTING THE EFFECTS OF AEROBIC EXERCISE AND THE EXERCISE MIMETIC, RESVERATROL, ON NEUROCOGNITIVE FUNCTION

ROSALIND CLARE HUSSEY



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A thesis submitted for the degree of
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DECLARATION

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TABLE OF CONTENTS

ABBREVIATIONS

5-HPETE	Arachidonic acid 5-hydroperoxide
5-LO	Arachidonate 5-lipoxygenase
AA	Arachidonic acid
AC	Adenylate cyclise
AD	Alzheimer's disease
ADP	Adenosine-5'-diphosphate
AMP	Adenosine-5'-monophosphate
AMPK	5' AMP-activated protein kinase
ANOVA	Analysis of Variance
AP-1	Activator protein-1
ATP	Adenosine-5'-triphosphate
BAT	Brown adipose tissue
BBB	Blood-brain barrier
BCA	Bicinchoninic Acid
BDNF	Brain-derived neurotrophic factor
bFGF	Basis fibroblast growth factor
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CA1	Region I cornu ammonis
CA2	Region II cornu ammonis
CA3	Region III cornu ammonis
CA4	Region IV cornu ammonis

CAM	Cell adhesion molecule
ICAM	Intercellular cell adhesion molecule
VCAM	Vascular cell adhesion molecule
CaMKII	Ca^{2+}/calmodulin-dependent protein kinases II
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
C/EBPα	CCAAT/enhancer binding α
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
CoA-SH	Coenzyme A
COX	Cyclooxygenase
COX1	a cyclooxygenase isoenzyme
COX2	a cyclooxygenase isoenzyme
CR	Calorie restriction
CREB	Cyclic AMP response element-binding protein
CS	Citrate synthase
DALY	Disability-adjusted life year
DG	Dentate gyrus
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
DNMS	Delayed-non-matching-to-sample
DTNB	Ellman’s reagent
ECL	Enhanced chemiluminescence
ELISA	Enxyme-linked immunosorbent assay
FaO	a differentiated rat liver cancer line
FDG-PET	Fludeoxyglucose-positron emission tomography
fMRI	Functional magnetic resonance

FOXO	Forkhead box O
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
GPx	Glutathione peroxidase
HDL	High-density lipoprotein
HeLa	immortal human cell line derived from cancer cells in patient, Henrietta Lacks
HIPP	Hippocampus
H.M.	a famous patient of Scoville and Milner
HO	Haem oxygenase
HPA	Hypothalamic-pituitary-adrenal
i.c.v.	Intracerebroventricularly
IGF	Insulin-like growth factor
IL	Interleukin
i.p.	Intraperitoneally
IQ	Intelligence quotient
IRF-1	Interferon regulatory factor 1
ITI	Intertrial interval
IKK	IκB kinase
i.v.	Intravenously
LDL	Low-density lipoprotein
LKB1	Serine-threonine kinase B1
LPS	Lipopolysaccahride
LTA_4	Leukotriene A_4
LTB_4	Leukotriene B_4
LTP	Long-term potentiation
MA	Middle-aged

MAO	Monoamine oxygenase
MAPK	Mitogen-activated protein kinase
MCI	Mild cognitive impairment
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
mtTFA	Mitochondrial transcription factor A
NA	Noradrenaline
NAD^+	Nicotinamide adenine dinucleotide
NBM	Nucleus basalis magnocellularis
NCD	Noncommunicable disease
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NO	Nitric oxide
NOR	Novel object recognition
NOS	Nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
NRF	Nuclear respiratory factor
NSAIDs	Non-steroidal anti-inflammatory drugs
NST	Non-shivering thermogenesis
O_2	Oxygen
O_2^-	Superoxide
$ONOO^-$	Peroxynitrite
p75NTR	p75 neurotrophin receptor
PC	Perirhinal cortex
PDE4	Phosphodiesterase 4
PDH	Pyruvate dehydrogenase E1α

PGC-1α	Peroxisome proliferator-activated receptor γ coactivator-1α
PG	Prostaglandin
PKA	Protein kinase A
PKC	Protein kinase C
PLA_2	Phospholipase A_2
p.o.	Peroral
PPAR	Peroxisome proliferator-activated receptor
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatise
PTZ	Pentylenetetrazole
RM	Repeated measures
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-PCR	Real-time polymerase chain reaction
RunCTL	Running control group
RunRES	Running resveratrol-treated group
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SedCTL	Sedentary control group
SedRES	Sedentary resveratrol-treated group
sir2	Silent information regulator 2
SIRT1	Silent information regulator two protein 1
SKM	Skeletal muscle
SOD	Superoxidase dismutase
CuSOD	Copper superoxidase dismutase
MnSOD	Manganese superoxidase dismutase
ZnSOD	Zinc superoxidase dismutase
STAT-1α	a signal transducer and activator of transcription protien

Thr	Threonine residue
TNF-α	Tumour necrosis factor-α
Trk	Tropomyosin receptor kinase
TTR	Transthyretin
TXA_2	Thromboxane A_2
UCP	Uncoupling protein
UCP-1	Uncoupling protein 1
UCP-3	Uncoupling protein 3
YLD	Years lived with disability
YLL	Years of life lost
VEGF	Vascular endothelial growth factor
VEGFR-2	VEGF receptor-2
WAT	White adipose tissue
WSCT	Wisconsin Card Sorting Task
YG	Young

Chapter One

LITERATURE REVIEW

"Mens sana in corpora sano"

1.1 INTRODUCTION

Although taken out of context from their use in the opening line of Juvenal's poem, Satire X, from the late 1st century, these words have often been repeated to promote the belief that only a healthy body can produce or sustain a healthy mind – a healthy mind in a healthy body. It is uncertain who introduced the use of this phrase with its modern day interpretation; however, it extends long before scientific proof was advanced enough to support such a theory. Luckily, we are presently in an epoch that has made, and continues to make, leaps and bounds in research investigating the connection between physical and mental health. There is compelling evidence that a healthy mind is often linked to a healthy body. In humans it is difficult to interpret whether a healthy body encourages mental wellbeing, or whether a healthy mind promotes lifestyle choices that encourage fitness. However, animal studies indicate that forced or voluntary exercise directly enhances cognitive function.

The concept of this thesis was to explore how elements of physical and mental health are connected; particularly focusing on the effects that aerobic exercise and resveratrol ingestion have on learning and memory. Research to date indicates that factors that enhance the metabolic system also relieve certain elements of cognitive decline. The extent to which this occurs is still to be determined, with a

vast number of scientists investigating different aspects of cognitive decline and methods of altering metabolism. The aim of this thesis is to determine the extent of cognitive enhancement associated with regular resveratrol ingestion and aerobic exercise, and to explore and compare the pathway adjustments that are involved in these actions. A direct comparison study of these two factors will be used in order to highlight more clearly any differences between their actions on the metabolic system and the varying outcome on cognition.

The following literature review will discuss the effects of ageing both physiologically and neurologically, the growing problem of noncommunicable diseases, and the therapeutic potential of aerobic exercise and resveratrol as tools against noncommunicable diseases, particularly in relation to cognitive decline.

1.2 THE AGEING BODY AND BRAIN

The leading risk factor for physical and neurological degeneration is ageing. All humans experience some degree of physical and cognitive decline as we grow older; for many these will become debilitating. Developments in scientific understanding and medical care have improved both general health and average life expectancy, particularly in developed countries. Average worldwide life expectancy at birth has increased from 48 years in 1955, to 66 years in 2009, with predictions that this figure will reach 73 years by 2025 (Wise, 1998). These values are greater in developed countries, with life expectancy in Ireland at 80.5 years in 2011 (World Bank, 2012). Life expectancy is commonly used as a measure of overall population health, with predicted length of life being indicative of mortality rates at a given time, and this dramatic improvement in mortality is seen as one of the most notable achievements of the past century (World Health Organisation, 1998). However, with these increases in the ageing population, mortality rates no longer present an accurate picture of the population's health status; indicators of morbidity, such as the prevalence of chronic diseases and disabilities, present a more appropriate impression of global health. One attempt to give a more accurate measure of the global burden of disease is the disability-adjusted life year (DALY) that was created as a measure of overall disease burden, expressed as number of years lost due to ill-health, disability or early death (Murray and Lopez, 1997).

DALY	**=**	**YLL**	**+**	**YLD**
Disability-adjusted life year		Years of life lost		Years lived with disability

The swing from high to low mortality was largely facilitated by the development of immunisations against infectious and parasitic diseases, such as smallpox, polio and measles; with these previously the leading causes of disease and

death. Nowadays, the largest global health burden is the prevalence of chronic noncommunicable diseases (NCD) (World Health Organisation, 2011). Although often associated with ageing, these can affect both young and old, with 25% of deaths in 2008 attributable to these disorders occurring in people under the age of 60. However, age is certainly a key risk factor for these debilitating and life-threatening conditions, such as cardiovascular disease, cancer and neurodegeneration; thus, these are increasing in prevalence along with lengthened life expectancies. It is becoming increasingly important for future medicine to improve mental and physical health in old age.

1.2.1 Degeneration of Ageing

Ageing is the most prominent aetiological factor of physiological and neurological decline. It is an accumulation of damaging alterations at molecular and cellular levels that result in increased risk of morbidity and mortality. Ageing is defined by (a) the increased probability of mortality with increasing age and (b) the characteristic changes in phenotype that occur in all individuals due to the limiting of certain processes over time (Johnson et al., 1999). These phenotypic changes associated with ageing occur to a certain degree in all individuals of a population; they are distinct from changes related to diseases of ageing which affect only a subset of a population.

Symptoms of Ageing	Associated NCD
Atherosclerosis, arteriosclerosis, hypertension	Cardiovascular diseases
Loss of brain tissue, memory decline	Neurodegenerative diseases
Demyelination	Neurodegenerative diseases
Reduced gland size, hormone dysregulation	Diabetes
Increased hyperplasia and macromolecular aggregates	Cancer
Weakening of connective tissue	Rheumatoid arthritis
Reduced bone mineral density	Osteoporosis
Lung capacity and elasticity lessens	Respiratory diseases
Higher autoimmunity	
Decreased metabolism	
Muscular atrophy	
Anosmia	
Presbycusis	
Ageusia	
Alopecia, canities	
Wrinkling of skin	
Reduced thermoregulation	

Table. 1-1. The phenotypic changes associated with ageing and the key noncommunicable disease that they increase the risk of attaining.

Although distinct from disease-related changes (Hayflick, 2007), these modifications that occur with ageing leave the body more susceptible to noncommunicable diseases and increase the risk of mortality. There are direct

correlations between certain symptoms of ageing and specific NCDs, as outlined in table. 1-1.Other symptoms of ageing, such as alopecia and wrinkling of skin, are generally thought to be unrelated to an increased risk of mortality, but some argue that the underlying mechanisms controlling these processes may also affect the mortality of other organs (Schnohr et al., 1995). Ageing is a phenomenon that is not fully understood; here several molecular models of ageing that have been developed in attempts to explain this abating aspect of life will be discussed.

Age-related autoimmunity

Inflammation is involved in the development of most diseases because it puts the entire body under metabolic stress, inducing symptoms and causing morbidity. Targeting altered metabolic pathways in inflammation may enhance our understanding of disease pathogenesis and point the way to new therapies. The endogenous inflammatory response is initiated as a result of trauma or infections which activate cellular mediators, such as macrophages. These cells release pro-inflammatory cytokines, such as interleukins (IL) and tumour necrosis factor-α (TNF-α), which are responsible for the progression of the response to a systemic level. This inflammatory response is designed to destroy microbial pathogens, initiate tissue repair processes, and promote a return to physiological homeostasis (Gabay and Kushner, 1999). In response to the presence of pro-inflammatory cytokines, a number of transcription factors are regulated to promote the synthesis of the inducible isoform of nitric oxide synthase (iNOS) in many cells and produces large levels of nitric oxide (NO) as a defence mechanism. Induction of iNOS usually occurs in an oxidative environment, allowing the NO produced to react with superoxide (O_2^-) to produce the anion, peroxynitrite ($ONOO^-$), causing cell toxicity (Fig. 1-1). The activated transcription factors also promote the synthesis of more cytokines, such as IL-1β, IL-8 and TNF-α.

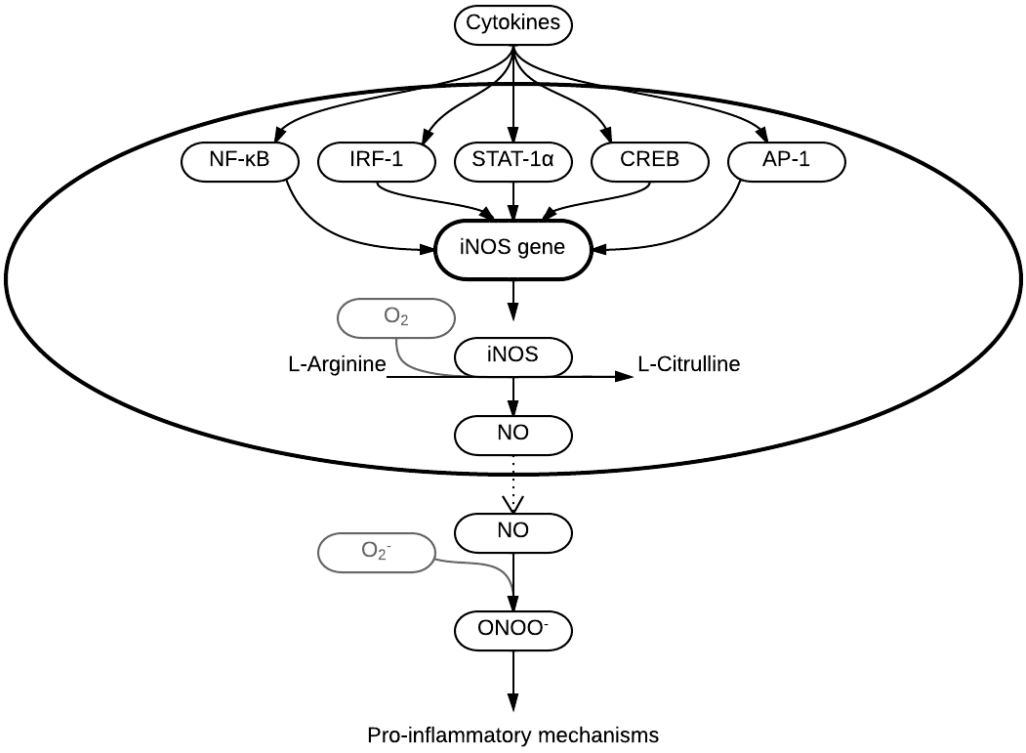


Fig. 1-1. The inflammatory mechanism by which the presence of cytokines encourages the production of nitric oxide as a defence mechanism in many cells.

In a parallel process, cell membrane phospholipids are hydrolysed by phospholipase A_2 (PLA_2) to produce arachidonic acid (AA) and lysophospholipids (Svensson and Yaksh, 2002). AA is then converted by cyclooxygenase (COX) to prostaglandin H_2 (PGH_2), which is a precursor to important biological mediators called prostanoids. Prostanoids, such as prostaglandins and thromboxane, act as pro-inflammatory molecules (Fig. 1-2). Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin and ibuprofen, exert their anti-inflammatory effects by blocking this pathway through the inhibition of COX. The compounds produced through these pathways lead to a number of pro-inflammatory mechanisms that protect the body from invading pathogens and encourage the body to return to homeostatic normality. As a result of production during an inflammatory response, peroxynitrite enhances apoptosis and necrosis in order to fight off the invading

pathogen, whilst prostanoids enhance angiogenesis and induce many of the symptoms associated with inflammation, such as pain, fever, and hypertension.

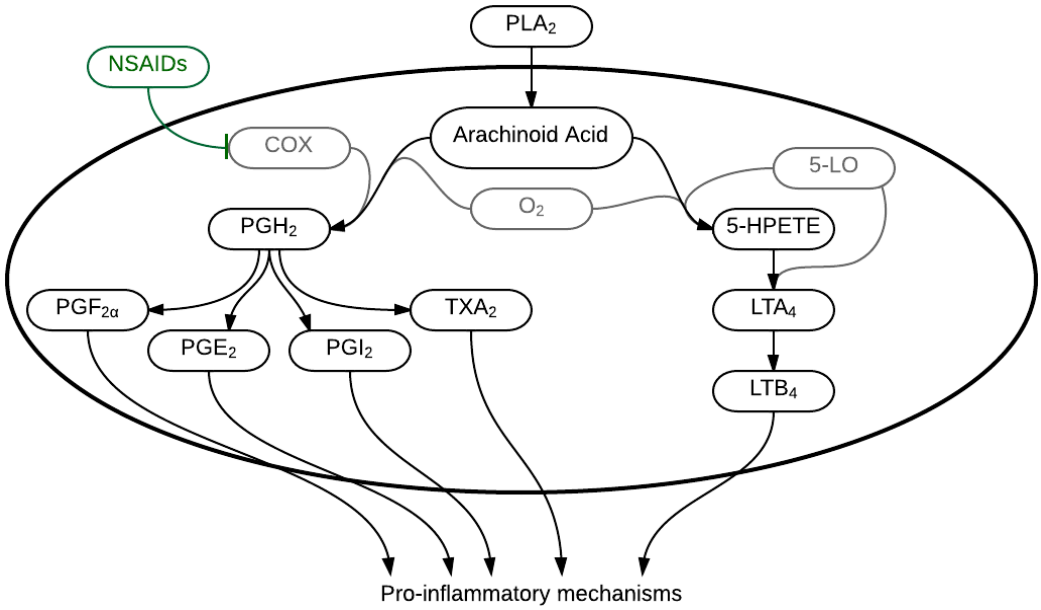


Fig. 1-2. The inflammatory pathway targeted by non-steroidal anti-inflammatory drugs.

As we age, levels of pro-inflammatory mediators typically increase, even in the absence of acute infection or other stressors. Studies investigating levels of antibodies present in healthy elderly people, have found that levels of non-organ specific (Manoussakis et al., 1987; Bruunsgaard et al., 2002) and organ specific (Candore et al., 1997) antibodies are much higher than those found in younger populations, with some antibody levels increased up to 25-fold. The autoimmune response to ageing is a chronic, low level, subclinical process mediated by the same molecules, but differing in degree (Tracy, 2003). Although this response is important for preventing and neutralising dangerous infectious agents, in aged individuals it

becomes a meaningful stress leading to altered immunoregulation and unbalanced responses. The mechanisms and meaning of autoimmunity during ageing is not clear, but it seems that this is a mere reflection of the advanced organ damage taking place with ageing, resulting in a chronic immune response. One example is the adipose tissue dysfunction associated with ageing. This results in impairment of adipogenesis and an accumulation of senescent pre-adipocytes, which attract immune cells that secrete pro-inflammatory cytokines, such as interleukins and TNF-α. This starts a vicious cycle, as TNF-α and IL-6 repress the adipogenic transcription factors, peroxisome proliferator activated receptor gamma (PPARγ) and CCAAT/enhancer binding α (C/EBPα), whose key roles are involved in adipogenesis (Kirkland et al., 2002). Ageing is associated with increased circulating levels of TNF-α, IL-6, cytokine antagonists, and acute phase proteins (Bruunsgaard et al., 2001). Increased levels of circulating pro-inflammatory mediators may be responsible for many aspects of degeneration associated with ageing, with feedback loops explaining the gradual increase over time.

The autoimmune process in the brain is unique due to the blood-brain barrier (BBB). This layer of tightly packed endothelial cells prevents the permeation of pro-inflammatory agents and only allows select nutrients and small molecules into the central nervous system (CNS). However, the integrity of the BBB becomes compromised with chronic systemic inflammation induced by stimuli such as ageing, cigarette smoking, and poor diet (Mattson et al., 2002). This allows irritants to enter the brain, leading to increased production of pro-inflammatory cytokines, such as the interleukins, IL-6 and IL-18 which impair neurogenesis (Vallières et al., 2002; Qiu et al., 2006), and IL-1β, IL-6 and TNF-α, which damage and destroy existing neurons (Acarin et al., 2000; Pringle et al., 2001; Griffin et al., 2002). These biomarkers of inflammation, in particular IL-6, have been linked to cognitive impairment in healthy elderly people (Weaver et al., 2002), and patients with neurodegenerative diseases (Licastro et al., 2000). A number of complex inter-related mechanisms are thought to contribute to age-related autoimmunity, which will be further discussed now.

Oxidative stress

Endogenous reactive oxygen species (ROS) and reactive nitrogen species (RNS) are predicted to play a key role in molecular, cellular and structural damage over time (Harman, 1981). Under normal physiological conditions, ROS are formed as a by-product of oxygen metabolism and have vital roles in signal transduction cascades by acting as molecular on/off switches through the oxidisation or reduction of protein cysteine thiol groups (Brandes et al., 2009). ROS act in concert with intracellular Ca^{2+} in signalling pathways that regulate the balance of cell proliferation and cell death (Sauer et al., 2001). Low levels of ROS serve as important signalling molecules in processes such as gene transcription, apoptosis, and metabolism (Stadtman and Berlett, 1998; Finkel and Holbrook, 2000; D'Autréaux and Toledano, 2007). Low concentrations of RNS also play significant roles as redox active molecules (Moncada et al., 1991). High levels of ROS and RNS are toxic and can cause oxidative damage to deoxyribonucleic acid (DNA), lipids, and proteins; putting organisms in a state of oxidative stress (Sies, 1991). Severe oxidative damage eventually leads to apoptosis and cell death. One example of when this is beneficial to a cell is production of the previously mentioned RNS, peroxynitrite ($ONOO^-$), through the inflammatory pathway in order to increase cell toxicity and fight off invading pathogens (Fig. 1-1). To maintain redox homeostasis under conditions where ROS concentrations begin to increase, cells utilise a range of enzymatic and non-enzymatic defence and repair strategies (Brandes et al., 2009). For rapid ROS detoxification and scavenging, most cells use a combination of antioxidant enzymes with very high catalytic activity, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), or of high cellular abundance, such as peroxiredoxins (Rhee et al., 2005).

Accumulating ROS have been shown to directly regulate a number of transcription factors (Kamata and Hirata, 1999); these include the activator protein-1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Meyer et al., 1993). This ability to rapidly adapt gene expression to different environmental conditions is crucial for the growth and survival of every organism. Redox regulation of these proteins is mediated by the modification of one or more

cysteine residues, leading to either the activation or inactivation of the respective transcription factor (Meyer et al., 1993). With ROS both activating the AP-1 and NF-κB transcription factors directly and indirectly (Fig. 1-3), ROS accumulation can activate the inflammatory pathway (Fig. 1-1).

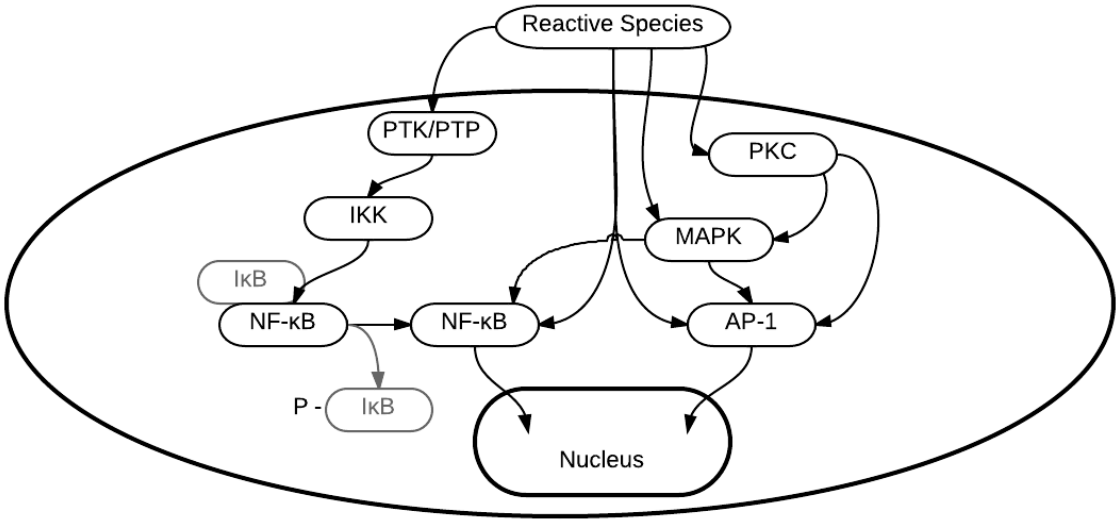


Fig. 1-3. Reactive species directly and indirectly regulate a number of transcription factors, including NF-κB and AP-1 which activate genes which upregulate pro-inflammatory mechanisms, amongst other pathways.

Animals with higher metabolic rates often have shorter lifespans; however, animals that produce fewer ROS and RNS from metabolism, such as birds and primates, tend to live longer than would be predicted by their metabolic rates (Ku et al., 1993). This has led to the free-radical theory of ageing (Harman, 1957). As we age, levels of reactive species increase in many tissues (Drew and Leeuwenburgh, 2002), with levels of antioxidant enzymes, such as SOD, catalase and GPx, showing different expression levels between studies and tissues (Rao et al., 1990; Hussain et

al., 1995; Ínal et al., 2001). Additionally, transgenic *Drosophila* overexpressing SOD and catalase live 34% longer than controls (Orr and Sohal, 1994), whilst conversely, mice knocked out for genes encoding GPx or SOD do not display a phenotype of rapid ageing (Reaume et al., 1996; Ho et al., 1997). Although increased antioxidant enzyme activity can promote longevity, loss of these factors does not appear to be detrimental to lifespan. Age-related increases in reactive species lead to over-oxidation and irreversible changes in protein structure and function. It appears that progressive oxidative damage is a conserved, central mechanism of age-related functional decline (Muller et al., 2007), with many species showing an age-dependent upregulation of oxidative stress-response genes (Yanker et al., 2008). It is the damaging effect of increasing oxidant concentrations that many believe is the underlying culprit of eukaryotic ageing (Finkel and Holbrook, 2000).

The brain is particularly susceptible to oxidative damage due to its high oxygen consumption, roughly 20% of the oxygen used by the entire body, and due to high concentrations of phospholipids which are especially prone to oxidative damage (Wu et al., 2004). With ageing, there is a significant and progressive increase in the level of oxidatively damaged DNA and lipids in the brain (Head, 2009), with dietary antioxidants shown to prevent oxidative damage of the brain in aged rats and reduce cognitive decline (Liu et al., 2002; Wu et al., 2004).

Hormonal imbalance

The endocrine system experiences an age-related decline in function, with an imbalance of hormonal production. One hormone that increases in production with ageing is the catabolic hormone, cortisol, due to over-stimulation of the hypothalamic-pituitary-adrenal (HPA) axis (Luz et al., 2003). Conversely, the production of many anabolic hormones decreases with ageing. The anabolic hormones, dehydroepiandrosterone (DHEA), melatonin and growth hormone,

enhance the proliferation and activity of cellular mediators of immunity, with DHEA also reducing pro-inflammatory cytokine production (Inserra et al., 1998). With ageing, reduction in levels of these hormones, and a parallel increase in cortisol levels, leads to increased chronic inflammation (Buford and Willoughby, 2008). Of the anabolic hormones, androgens such as oestrogen and testosterone in particular, greatly decline. These hormones have been shown to modulate the production of pro-inflammatory cytokines, such as IL-6 (Pottratz et al., 1994), at least partially through inhibition of NF-κB activity (Keller et al., 1996), and lower levels of these hormones is associated with increased production of pro-inflammatory cytokines (Maggio et al., 2006). This interaction occurs in both directions, with the immune system shown to modulate the endocrine system. Exogenous cytokines have been used to highlight the marked change that these can have on the HPA axis in rats (Besedovsky et al., 1977; Besedovsky and del Rey, 1987) and humans (Jablons et al., 1989; Mastorakos et al., 1993; Späth-Schwalbe et al., 1994).

In the brain, anabolic hormone receptors are distributed throughout and assist in regulating the transcription of a vast array of genes involved in cognition and behaviour. Sufficient activation of these receptors is essential for normal brain functioning; when hormonal imbalances or deficiencies disrupt this activation, cognitive deficits occur as a result (Sonntag et al., 2005). Studies in rats suggest that the hormone, oestrogen, also has the ability to function directly as a neurotransmitter in the CNS (Balthazart and Ball, 2006); with increased levels of androgens linked to improved cognitive function (Bagger et al., 2005; Newman et al., 2005), and decreases linked to lower functional capacity (Gray et al., 2005; Daniel, 2006). The connection between these hormones and cognitive function appears to be partly due to the role androgens play in maintaining synaptic density; with hippocampal synaptic maintenance shown to be androgen-dependent (MacLusky et al., 2006). Lower levels of DHEA, which is particularly active in the CNS, have also been tied to impaired cognitive performance; a deficit which can be improved with DHEA supplementation (Huppert and Niekerk, 2006).

Mitochondrial dysfunction

Gene expression studies suggest that a reduction in the expression of mitochondrial genes with ageing is strongly conserved from *C. elegans* to humans (Zahn et al., 2007; Yanker et al., 2008). A decline in mitochondrial function seems to be an important modulating factor on the ageing process in all species examined, and it can have positive or negative effects on lifespan, depending on the context (Sedensky and Morgan, 2006). Reduction in mitochondrial function has been shown to shorten lifespan in a number of species (Trifunovic et al., 2004; Kujoth et al., 2005; Rea et al., 2007), while augmentation of mitochondrial function has been shown to extend lifespan (Lin et al., 2002; Schriner et al., 2005). Mitochondria are highly dynamic organelles that fuse and divide in response to environmental stimuli, developmental status, and energy requirements (Seo et al., 2010). A recent wave of studies demonstrates the pleiotropic role of these mechanisms in many cellular processes, such as mitochondrial metabolism, redox signalling, maintenance of mitochondrial DNA (mtDNA), and autophagy (Chan, 2006; Twig et al., 2008; Seo et al., 2010; Zorzano et al., 2010). Among other functions, they provide energy for anabolic reactions by securing adenosine-5'-triphosphate (ATP) produced from catabolic reactions (Fig. 1-4). Hydrolysis of ATP to adenosine-5'-diphosphate (ADP) or adenosine-5'-monophosphate (AMP) provides energy for most biological processes making mitochondria essential for normal cell function and maintenance of redox homeostasis and programmed cell death (Marzetti et al., 2010).

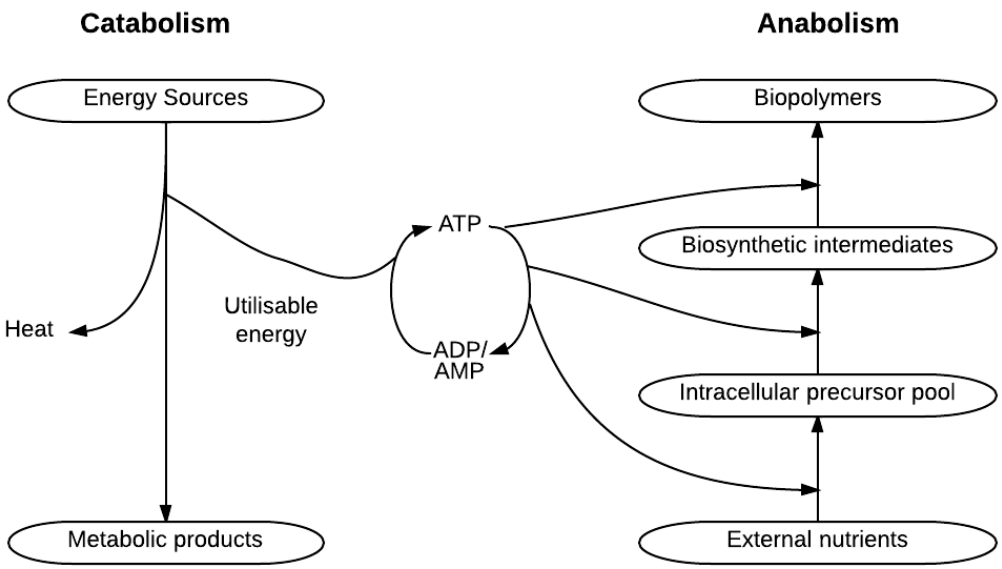


Fig. 1-4. Mitochondria provide energy, in the form of ATP, for biosynthetic anabolic reactions by securing ATP produced in energy-yielding catabolic reactions.

In quiescent cells, the fission machinery is active and mitochondria are present as distinct small spheres or short rods. This machinery contributes to the elimination of irreversibly damaged mitochondria through autophagy. In response to stresses, such as inflammation, a mitochondrial shift towards fusion favours the generation of interconnected mitochondria, which contribute to a rapid provision of energy to the cell by maximising ATP synthesis. An ongoing fusion-fission cycle allows mitochondrial functional and genetic complementation, generating the appropriate distribution of new organelles during cell division (Fig. 1-5). Dysfunctional regulation of these mechanisms is thought to be one of the intrinsic causes of mitochondrial dysfunction, which contributes to oxidative stress and cell death during the ageing process (Seo et al., 2010). An imbalance between mitochondrial fusion, fission, biogenesis and autophagy events may cause substantial changes in mitochondrial number, biomass, shape and function.

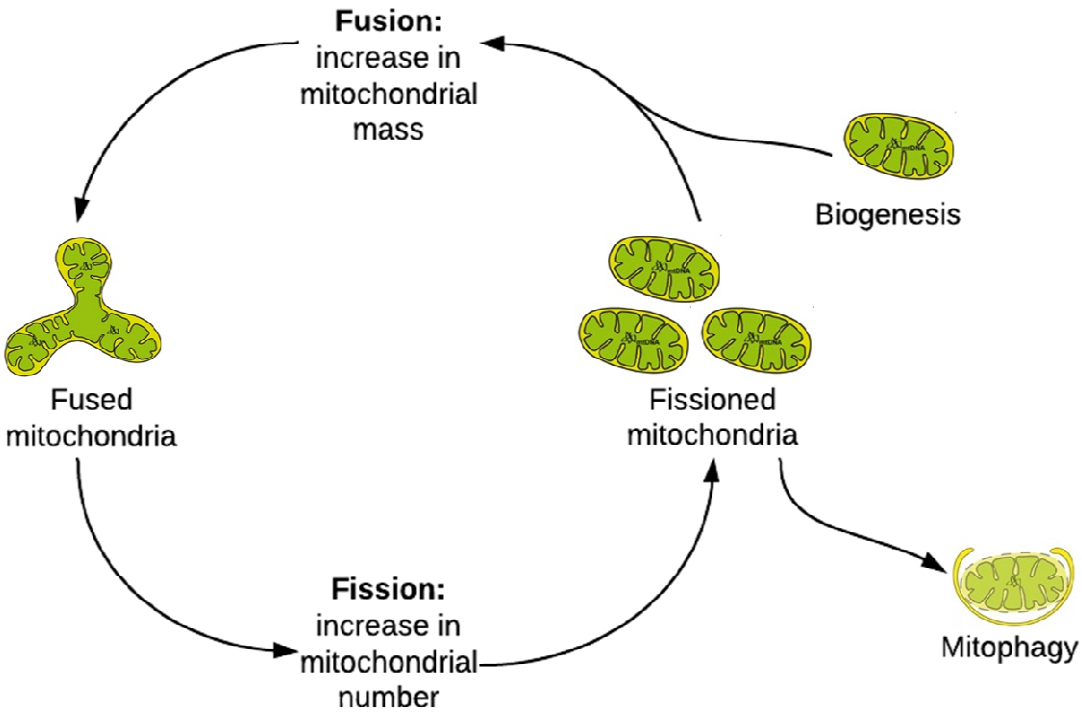


Fig. 1-5. The mitochondrial fusion-fission cycle. This mechanism allows mitochondrial functional and genetic complementation, generating the appropriate distribution of new organelles during cell division

As key regulators of redox homeostasis, mitochondria are the main source of reactive species and, therefore, play a prominent role in oxidative stress, whilst oxidative damage to mitochondrial DNA (mtDNA) results in dysregulation of cell and organ function leading to overall system decline, recognised as ageing (Bua et al., 2006). Tissues affected by age-related mtDNA damage, such as muscle, adrenal organs, ovaries and testes, exhibit loss of function with age (Wei and Lee, 2002). There is a clear decline in mitochondrial function in humans and other animals with ageing, particularly in postmitotic tissues (Boffoli et al., 1994), with a variety of mtDNA alterations, such as point mutations, deletions, and oxidative modifications, increasing with age (Lee et al., 1997). Although, it remains uncertain as to whether mitochondrial dysfunction is the primary cause of ageing, with mutations in genes that confer longevity slowing the accumulation of mtDNA deletions compared to wild-type (Melov et al., 1995).

Evidently these key theories of ageing can all be attributable to each other – autoimmunity, oxidative stress, hormonal imbalance, and mitochondrial dysfunction. These are four thoroughly entwined mechanisms that raise similar considerations as the chicken or the egg causality dilemma, and the importance of investigating upstream factors.

1.2.2 Memory Decline Associated with Ageing

Cognitive decline does not affect all individuals equally; some degree of cognitive depreciation as we age is considered normal, but often this decay becomes debilitating over time. As stated in the previous section, structural and neurophysiological changes occur in the brain with ageing, along with associated variable decline in cognition. Far from exhibiting uniform depreciation, it appears that all cognitive faculties do not show the same age-related decline (Fig. 1-6). There is much evidence to suggest that certain functions are relatively resistant to the effects of ageing. Knowledge or facts about the world, semantic memory, is relatively spared in ageing (Light, 1991; Wingfield and Stine-Morrow, 2000; Beier and Ackerman, 2001), with some evidence to suggest that older adults can, in fact, outperform younger adults on tests of semantic memory (Wegesin, 2000) and priming (Laver and Burke, 1993). Skill acquisition also remains intact into old age (Brown et al., 2009), and it seems that there are no age-related effects on skill performance, provided the skill is kept in practice (Krampe and Ericsson, 1996). Contrary to this, episodic and working memories typically show a pronounced age-related impairment (Salthouse and Babcock, 1991; Mäntylä and Nilsson, 1997; Park et al., 2002). Decline in episodic memory is a hallmark of dementia onset, but certain types of episodic memory tasks have been shown to be markedly impaired with normal ageing also. Memory for associations is typically more affected than memory for item information (Castel and Craik, 2003), and source and temporal order judgements also show increased vulnerability to age-related deficits (Cabeza et al., 2000). Working memory also declines with age (Dobbs and Rule, 1989; Park et al.,

2002) with this often being attributed to the inability of older adults to actively inhibit competing information or that which is no longer relevant, rendering them more susceptible to interference (Hasher and Zacks, 1988).

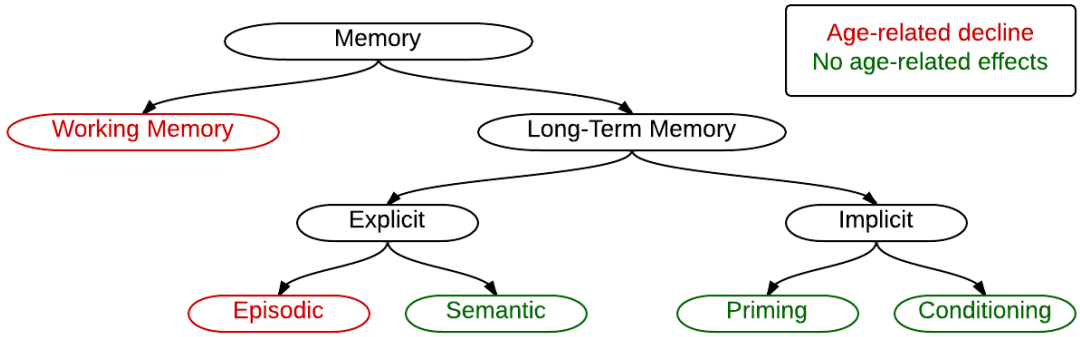


Fig. 1-6. A diagram showing the sub-types of memory, with those affected by ageing highlighted in red.

Whilst adaptations in the brain can be observed and compared to young subjects, it is not always clear whether these alterations have a degenerative effect on cognition or if they have occurred to compensate for the adverse effects of other modifications. Different studies help to shed light on which alterations are debilitating and which are compensatory. It has been observed that with ageing the interaction between various brain regions associated with higher-order cognitive functions becomes less co-ordinated (Andrews-Hanna et al., 2007) and that neural activity becomes less localised in some regions (Cabeza et al., 2002; Park and Reuter-Lorenz, 2009). Interestingly, aged individuals with delocalised activity exhibit better cognitive performance than those with more localised activity, suggesting that this delocalisation is a compensatory response to less co-ordinated interactions (Cabeza et al., 2002). Often ageing is associated with neurodegenerative diseases, such as Alzheimer's disease, other forms of dementia and Parkinson's

disease. Functional magnetic resonance imaging (fMRI) studies suggest that measurements of activity in the hippocampus and associated cortical regions can distinguish normal ageing from pathological ageing (Bishop et al., 2010). Normal ageing is associated with reduced metabolic activity in the subiculum and dentate gyrus, whereas reduced activity in the entorhinal cortex is thought to be an early indicator of Alzheimer's disease (Small et al., 2002). Neuronal loss is minimal in most cortical regions with normal ageing (Morrison and Hof, 1997), and it is thought that the breakdown in these brain systems may be at least partially due to disruption of myelinated fibres on these connective neurons (Andrews-Hanna et al., 2007). In Alzheimer's disease, there is evident neuronal loss, particularly in the entorhinal cortex and region I cornu ammonis (CA1) of the hippocampus, together with volume loss in the medial temporal lobe (Braak and Braak, 1991; West et al., 1994; Gomez-Isla et al., 1996; Price et al., 2001; Rodrigue and Raz, 2004). Focussing on alterations in the brain regions important for learning and memory, and further distinguishing healthy ageing from abnormal degeneration, will help us further understand age-related cognitive decline.

The medial temporal lobes and memory

Initial understanding of the function of the medial temporal lobes came through studies involving patients with epileptic seizures that underwent medial temporal lobotomy to remove damaged tissue and prevent further seizures. One such patient, known as H.M., attracted the attention of Scoville and Milner (1957) when it was observed that removal of the medial temporal lobes had little effect on perceptual abilities, intelligence quotient (IQ), or personality, but left H.M. with an inability to form new memories (Corkin, 2002); H.M. was experiencing anterograde amnesia. Although exhibiting intact working memory and long-term memory, H.M. was no longer able to consolidate explicit information from working memory to long-term memory and, hence, was unable to form new memories after his surgery. Based on this work and similar studies (Victor et al. 1961; Drachman and Ommaya,

1964), it became apparent that the structures within the temporal lobe were important for the formation and consolidation of explicit memories.

Subsequent research has confirmed that damage specifically to the hippocampal formation causes deficits in spatial and learning and memory in rats (Morris et al., 1982), monkeys (Zola-Morgan and Squire, 1990; Zola et al., 2000), and humans (Eichenbaum, 2001). Hippocampal lesions impair spatial learning and highlight an essential involvement of the hippocampal formation in allocentric spatial tasks. O'Keefe and Nadel (1978) proposed that the hippocampus mediates a neuronal representation of the physical environment; they termed this "cognitive mapping", and it has been found that different types of cells interact extensively to produce this cognitive map in rats. "Place cells" were found to fire bursts of action potentials when an animal occupies a particular position in space (O'Keefe and Dostrovsky, 1971), "head direction cells" increase firing rates when an animal's head points in a certain direction (Taube et al., 1990), and "grid cells" fire within spatial firing fields arranged in a triangular grid thought to encode a cognitive representation of Euclidean space (Hafting et al., 2005). Dusek and Eichenbaum (1997) extended the properties of cognitive mapping to non-spatial dimensions of memory organisation in animals, indicating that the role of the hippocampal region in explicit memory expression may be as general in rats as it is in humans. It is believed that these cells are present in humans too, but it is not yet possible to determine this. More recently, the hippocampus has received increasing attention for its potential role in energy regulation (Davidson et al., 2007). Hippocampal damage interferes with energy and body weight regulation, with this thought to be due to disruption of higher-order learning and memory processes contributing to the control of appetite and consumptive behaviour.

Broadly speaking the hippocampal formation comprises of the hippocampus proper, dentate gyrus (DG), subiculum, with some also including the presubiculum, parasubiculum and entorhinal cortex. The hippocampus has four main histological divisions: region I cornu ammonis (CA1), region II cornu ammonis (CA2), region III cornu ammonis (CA3) and region IV cornu ammonis (CA4) (Fig. 1-7). Hippocampal

pyramidal cells have extensive apical and basal dendrites and axons that diverge, sending projections both anterior and posterior in the alveus. CA1 pyramidal cells mainly project to the septal nuclei, subiculum, as well as other structures. The subiculum receives afferents mainly from the CA1 of the hippocampus and the entorhinal cortex. Subicular cells project to the entorhinal cortex, deep layers of perirhinal cortex, and also to subcortical structures such as mammillary bodies, hypothalamus, amygdala and nucleus accumbens. The mammillary body projections to the pons may provide an important link between the hippocampus and the cerebellum, permitting hippocampal influences on motor behaviour. The major afferents to the hippocampal formation (the dentate gyrus and Ammon's horn) come from the entorhinal cortex via the perforant pathway, which in turn receives input from the entire neocortex. Finally, there are commissural afferents from the pyramidal cells of contralateral hippocampus and from contralateral entorhinal cortex. Spatial memory was found to have many sub-regions in the hippocampus, in particular the dentate gyrus.

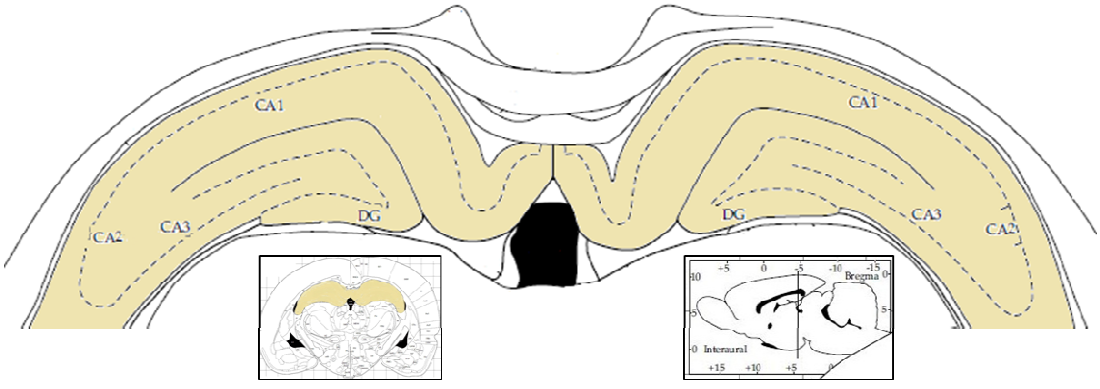


Fig. 1-7. A diagram showing the key regions of the hippocampal formation in the rat brain, at the designated coronal slice. Adapted from Figure 38 in Paxinos and Watson (1998).

Another standard feature linked to classical anterograde amnesia is a loss of recognition memory, a role designated to the perirhinal cortex rather than the hippocampus. Studies involving rats and monkeys all agree that the severity of impairment in standard tests of recognition memory in these species is greater following perirhinal lesions than hippocampal lesions. The perirhinal cortex is involved in discriminating the familiarity and recentness of items (Davachi, 2004). A lesion to the perirhinal cortex in both monkeys and rats leads to the impairment of visual recognition memory, disrupting stimulus-stimulus associations and object-recognition abilities (Murray and Mishkin, 1986; Zola-Morgan et al., 1989; Suzuki et al., 1993; Meunier et al., 1993; Buckley and Gaffan, 1998). The role of the perirhinal cortex in the formation and retrieval of stimulus-stimulus associations suggest that it is part of a larger semantic system that is crucial for endowing objects with meaning (Murray, 2007).

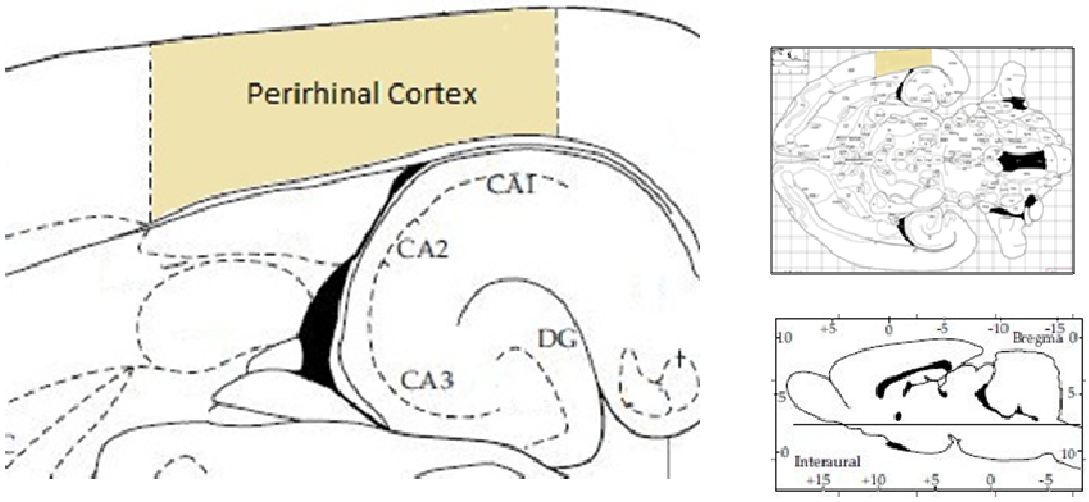


Fig. 1-8. A diagram showing the location of the perirhinal cortex in relation to the hippocampal formation in the rat brain, at the designated horizontal slice. Adapted from Figure 98 in Paxinos and Watson (1998).

The perirhinal cortex is comprised of two regions, Brodmann areas 35 and 36, with both divided into three subdivisions; making this a six-layered structure (Fig. 1-8). Layer IV, in area 35, lacks any cells. The perirhinal cortex projects to distal CA1 pyramidal cells, overlapping projections from the entorhinal cortex (Suzuki and Amaral, 1994; van Hoesen and Pandya, 1975). The same CA1 cells send return projections back to the perirhinal cortex. Inputs from the subiculum terminate in both superficial and deep layers. The majority of inputs come from high-level sensory areas that vary between animals: in the monkey these come from high-level visual areas, whereas, in rat these are primarily from olfactory and, to a lesser extent, auditory areas.

Neurogenesis and neurotrophins

One of the key features that differentiates the hippocampus from other brain regions is an ability to constantly produce new neurons. It is thought that this ability to renew neurons allows this region to carry out its role in learning and memory. Although the vast majority of neurons in the mammalian brain are formed prenatally, parts of the adult brain retain the ability to grow new neurons from neural stem cells in a process known as neurogenesis. Such replication is a normal process for many cell types, but for many years it was believed that neurons could not replicate (Gage, 2002). This is understandable due to the fact that neurons in most brain regions cannot replicate, however, it has been discovered that neurogenesis can occur in adult cells in the hippocampus, particularly in the dentate gyrus and the subventricular zone. Hippocampal neurogenesis has been shown to occur in birds (Barnea and Nottebohm, 1994), rodents (Kempermann et al., 1998), monkeys (Kornack and Rakic, 1999) and humans (Eriksson et al., 1998). Neurogenesis has been measured by immunohistological detection of the incorporation of bromodeoxyuridine (BrdU) into the DNA of proliferating cells (Kempermann et al., 1998) or use of a retroviral vector expressing green fluorescent protein (GFP) to label dividing cells (van Praag et al., 2002). The latter method also highlighting the possibility that these new neurons probably arise from progenitors in the subgranular

zone, and that the newly generated neurons are functionally similar to mature dentate granule cells. One of the implications of a role for adult neurogenesis in learning and memory is that neurogenesis can be regulated by numerous factors associated with an animal's behavioural and cognitive states (Deng et al., 2010). An animal's experiences, including hippocampal-dependent learning (Gould et al., 1999), environmental enrichment (Nilsson et al., 1999) and aerobic exercise (van Praag et al., 1999), can affect the rate of neurogenesis and enhance cognition. Inhibition of neurogenesis in the rat brain, using low dose irradiation, has been shown to interfere with hippocampal-dependent memory function (Winocur et al., 2006). With ageing, the rate of neurogenesis decreases (von Bohlen und Halbach, 2010), in parallel to loss of several aspects of cognitive function. Assessment with BrdU labelling shows that reduced proliferation in the hippocampus is not attributable to a general aged-related metabolic impairment, because the density of BrdU-positive cells is not altered in other brain regions with known mitotic activity (Kuhn et al., 1996). Thus, the age-related decline in neurogenesis can be related to a decreasing proliferation of granule cell precursors. Although all is not so clear cut, with age-related decline in neurogenesis not always correlated to impaired learning and memory (Bizon et al., 2004).

Neurotrophins play an important role in neurogenesis, as these are a set of gene products that are required for cell growth, proliferation, and cellular differentiation (Rohrer, 1990). Of these, brain-derived neurotrophic factor (BDNF) and its major receptor, tropomyosin receptor kinase B (TrkB), have the most abundant and widespread expression in the developing and adult mammalian brain (Murer et al., 2001); with the highest expression levels found in the hippocampus. BDNF is most widely expressed in the CA2, CA3, dentate gyrus and cortex (Ernfors et al., 1990; Schmidt-Kastner et al., 1996) and has been shown to promote the differentiation and survival of neurons during development and in the adult brain, as well as in cultured cells (Memberg and Hall, 1995; Takahashi et al., 1998). BDNF levels initially increase with age, suggesting that on top of development, these neurotrophins regulate the structure and function of the mature nervous system (Yan et al., 1992; Friedman et al., 1993; Thoenen, 1995). BDNF is involved in spine formation, density and morphology of neurons (Ethell and Pasquale, 2005), and has

also been shown to have a critical role in long-term potentiation (LTP), a form of synaptic plasticity which is still widely considered a cellular model of long-term memory formation (Bliss and Collingridge, 1993; Martin et al., 2000). It is not possible to induce LTP in BDNF-null mice (Korte et al., 1995) and *in vitro* studies have established that BDNF is required for the late phase of LTP which requires synthesis of new messenger ribonucleic acid (mRNA) and protein (Korte et al., 1998). BDNF is stored in platelets and circulates in plasma, with peripheral effects in angiogenesis observed (Kermani and Hempstead, 2007). Environmental enrichment (Gobbo and O'Mara, 2004) and aerobic exercise (Gobbo and O'Mara, 2005) have been shown to increase BDNF levels in the hippocampus, whilst also showing memory improvements; similar to studies investigating neurogenesis.

Other neurotrophins, such as vascular endothelial growth factor (VEGF) and nerve growth factor (NGF), have also been demonstrated as critical mediators of hippocampal neurogenesis and improved cognition in rats (Fiore et al., 2002; Cao et al., 2004). Hippocampal gene transfer of VEGF resulted in almost twice the rate of neurogenesis compared to normal rats, with resulting increases in cognition. Furthermore, when VEGF expression was inhibited by RNA interference, neurogenesis levels decreased (Cao et al., 2004). VEGF promotes angiogenesis by initiating a tyrosine signalling cascade in endothelial cells (Prior et al., 2004). Binding to VEGF receptor-2 (VEGFR-2) starts a tyrosine kinase signalling cascade that stimulates the production of factors, such as endothelial nitric oxide synthase (eNOS) which activates NO and stimulates vessel permeability, basis fibroblast growth factor (bFGF) which promotes cell proliferation/survival, and cell adhesion molecules (ICAM and VCAM) which promote migration and differentiation into mature blood vessels. Studies show that VEGF acts as a neurotrophic factor (Jin et al., 2000; Matsuzaki et al., 2001), and enhances neurogenesis (Jin et al., 2002). On the other hand, NGF is involved in maintenance of hippocampal LTP (Kelly and Lynch, 1998), with blockade of endogenous NGF reducing LTP and impairing spatial memory (Conner et al., 2009). NGF expression has a positive correlation to performance in learning and memory tasks (Pham et al., 2002; O'Callaghan et al., 2009), whilst infusion of NGF enhances memory in rats (Walz et al., 2000). NGF has been shown to play an important role in hippocampal-dependent memory

(Fischer et al., 1991; Klein et al., 2000; De Rosa et al., 2005) and consolidation (Woolf et al., 2001) It is thought that NGF carried out this beneficial effect on cognition through its major receptor, tropomyosin receptor kinase A (TrkA) (Woolf et al., 2001).

Neurotrophins act to upregulate a number of parallel pathways through attachment to their major tropomyosin receptor kinase (Trk) receptors and the p75 neurotrophin receptor (p75NTR). This initiates a number of cascades in a complex pathway that promotes cell growth and survival (Kaplan and Miller, 2000). There is strong evidence to suggest that BDNF, VEGF and NGF exert a critical role in consolidation by upregulating the mitogen-activated protein kinase (MAPK) pathway (Matsuzaki et al., 2001; Bozon et al., 2003). As seen in figure 1-3, this leads to upregulation of the transcription factors NF-κB and AP-1, as well as another important transcription factor, cyclic AMP response element-binding protein (CREB). NF-κB, AP-1, and CREB are all cellular transcription factors that bind certain DNA sequences when activated and cause an increase or decrease in the transcription of downstream genes; these transcription factors are also implicated in learning and memory. NF-κB is understood to be responsible for cytokine production and cell survival, and has been implicated in synaptic plasticity (Albensi and Mattson, 2000; Meffert et al., 2003) and memory consolidation (Merlo et al., 2005). CREB has a well-validated role in neuronal plasticity and long-term memory formation (Silva et al., 1998; Bozon et al., 2003). In addition to these functions, these transcription factors also regulate transcription of BDNF (Kassel and Herrlich, 2007; Kairisalo et al., 2009).

Morphological changes associated with ageing include less dendritic branching, reduction in spine and fibre densities projecting into the hippocampus (von Bohlen und Halbach, 2010). Although it is unlikely that one single factor is responsible for all these age-related changes, neurotrophins stand out as a critical factor in this process. Plasma levels of BDNF initially increase with ageing, but then show a marked decrease following middle-age (Lommatzsch et al., 2005; Erickson et al., 2010), with VEGF and NGF levels and expression in the brain decreasing with

old age (Lärkfors et al., 1987; Shetty et al., 2005). Interestingly, in the hippocampus, decreases in the expression of trkB are more significant than changes in BDNF levels with ageing (Webster et al., 2006).

1.2.3 Memory Decline Associated with Noncommunicable Diseases

"Noncommunicable diseases" (NCD) is an umbrella term for diseases that are non-infectious and non-transmittable between people (World Health Organisation, 2011). These are often, but not solely, of long duration and slow progression. The four most abundant NCDs leading to mortality and morbidity are cardiovascular diseases, cancer, respiratory diseases and diabetes. Often these disorders are associated with other afflictions - a simple reminder that all aspects of our body are thoroughly entwined and working together. For example, having diabetes is a risk factor for developing cardiovascular disease (Elkeles et al., 1998) and can increase risk of dementia (Ott et al., 1999). Often associated with old age, an important aspect to realise about these afflictions is that they have not only grown in prevalence due to an ageing population - the globalisation of unhealthy lifestyles has played an equally important role (World Health Organisation, 2000). Mitochondrial dysfunction, systemic inflammation, hormonal imbalance, and oxidative stress are at the root of most NCDs, as well as ageing. Severe oxidative damage, that will eventually lead to apoptosis and cell death, has been shown to accompany many pathological conditions, such as cancer, neurodegenerative diseases, and diabetes (Cook et al., 2004; Lowell et al., 2005). Risk factors attributed to the development of NCDs include unhealthy diets, physical inactivity, alcohol abuse, and exposure to tobacco smoke. Although factors such as genetics and ageing play an important part, the above risk factors are all modifiable, and so, it is possible that many mortalities and morbidities associated with NCDs may be preventable. NCDs, even those not directly affecting the brain, often show an associated decline in cognitive function. This shows the interconnectivity between the pathways and molecules associated with these and brain regions associated with higher-cognitive function. It may prove

more useful to explore the larger class of NCDs, in order to further understand mechanisms involved in neurodegeneration.

Neurodegenerative disorders

Dementia is a term for severe loss of global cognitive function without previous impairments, beyond those associated with normal ageing. Alzheimer's disease (AD) is the most common form of dementia, with most cases diagnosed in people over 65 (World Health Organisation and Alzheimer's Disease International, 2012). The earliest and main symptom of AD is progressive memory decline, with symptoms able to be divided into three stages. Stage 1: Pre-dementia stages of the disease involve mild cognitive impairment (MCI); this decline is no more severe than with natural ageing but increasing severity may indicate the onset of AD. Stage 2: The memory impairment becomes more severe leading to decline in independence, requiring need for structure, reminders and assistance with everyday activities. Stage 3: Late stage AD can be characterised by a loss of episodic memory, a reduction in language, and severe impairment of overall cognitive function. This leaves the patient unable to care for themselves as they require intensive care and support (Teng et al., 2007). There is a wealth of research indicating that the pathological hallmarks of Alzheimer's disease, such as synaptic loss, β-amyloid plaques and neurofibrillary tangles of hyperpolarised microtubule-associated protein tau, correlate with cognitive decline (Terry et al., 1991; Näslund et al., 2000; Cleary et al., 2004; Nelson et al., 2009); however, some of these pathological changes have also been detected to varying degrees in healthy aged people with no sign of cognitive decline (Guillozet et al., 2003). In Alzheimer's disease, the hippocampus is one of the first regions to suffer damage, with memory loss and disorientation amongst the early symptoms (De Leon et al., 1989). Low levels of neurotrophins and decreased neurogenesis are observed in a variety of brain disorders, including cognitive decline (Sugaya et al., 1998; Bizon et al., 2004) and depression (Schmidt and Duman, 2007), as well as dementia (Jacobs et al., 2000; Karege et al., 2002) and Alzheimer's disease (Mattson, 2008). Additionally, resistance to insulin and insulin-

like growth factor (IGF) have been implicated as a key part in the progression of Alzheimer's disease (Gasparini and Xu, 2003).

Cardiovascular diseases

Cardiovascular disease is the leading cause of morbidity and mortality worldwide. Although cases have been declining in developed countries since the 1960s, they have increased more rapidly in developing countries during this time (Reddy and Yusuf, 1998). Atherosclerosis and hypertension are the most common antecedents of cardiovascular diseases and begin in early life, making primary preventions against these important. Platelet aggregation forms thrombi and leads to subsequent blockages in blood vessels which can lead to transient ischemia, myocardial infarction or stroke (Fuster et al., 1992). Interestingly, cardiovascular disease has been associated to increased levels of circulating neurotrophins, such as BDNF and VEGF, thought to be produced in a rehabilitating effort to promote angiogenesis (Kermani and Hempstead, 2007). This increased neurotrophic level would be expected to correlate with improved memory, but cardiovascular disease is linked to higher incidence of dementia (Newman et al., 2005). This may be due to lower blood levels reaching the brain due to weaker heart function, and targeting of these neurotransmitters to area of particular need. Heart failure, previous vascular events, presence of plaques in the carotid arteries, and evidence of atherosclerosis are all associated with decreases in cognitive performance (Breteler et al., 1994; Vogels et al., 2006).

Diabetes

Diabetes, a condition in which the body does not properly process glucose, is the most common endocrine disease in the world. This inability to process glucose properly is due to a lack of insulin production (diabetes mellitus type 1) or

functioning (diabetes mellitus type 2) (Biessels et al., 2008). High-calorie diets cause numerous pathological conditions including increased glucose and insulin levels which lead to the development of diabetes and cardiovascular disease (Siebler and Galle, 2006). Due to the high metabolic demand for energy in the brain, even small perturbations in glucose metabolism can noticeably impact cognitive performance. Diabetes has been linked with lower levels of neurotrophins (Tomlinsin et al., 1997; Krabbe et al., 2007) and higher incidence of dementia (Ott et al., 1999). Markedly different patterns of glucose utilisation and brain activity have been observed between healthy and diabetic/pre-diabetic patients using fludeoxyglucose-positron emission tomography (FDG-PET) (Baker et al., 2010). Blood vessels to the brain become more constricted in diabetic patients, with impaired blood flow, and increased progression of brain atrophy (Biessels et al., 2002). All these changes in the brain have been associated with poor cognitive performance in diabetic patients compared to healthy controls, with a greater decline evident with poorly-managed diabetes compared to well-managed patients (Biessels et al., 2008).

1.2.4 A Healthy Mind in a Healthy Body

It is evident that physical degeneration is often linked to cognitive degeneration whether through normal ageing or disease-related impairment. With much research in neuropharmacology focussing solely on the brain and its functioning, here the concern is raised that it may be necessary to extend this vision beyond the brain. In order to truly promote a healthy life, in both youth and old age, it is necessary to ensure well-being in both mind and body. Let’s now look at two potential candidates for ensuring overall health.

1.3 AEROBIC EXERCISE BENEFITS BODY AND BRAIN

Due to the increasing prevalence of chronic noncommunicable diseases, it is necessary to develop primary preventative interventions to prolong the period of healthy life (Everitt et al., 2002). The use of metabolic adjusting factors could, therefore, have great potential in countering the degeneration of ageing and debilitation of noncommunicable diseases – both physiologically and neurologically. As outlined in the previous section, there are clear links between autoimmunity, oxidative stress, hormonal imbalance, mitochondrial dysfunction, and cognitive decline. Ageing is a process that affects the entire body, and so, it is important to focus on therapeutic interventions that can benefit the body as a whole. The rate of ageing is not inevitable; conserving cognitive vigilance into late life requires early and aggressive intervention to preserve the brain in its youthful physical and functional state (Westendorp, 2006). Clear associations exist between the rate and severity of cognitive decline and a variety of modifiable factors, as have been mentioned above; proactive lifestyle changes and nutritional interventions have been shown to decrease the rate of intellectual decay and potentially reverse age-related and NCD-related cognitive decline. Here the evidence supporting the potential therapeutic use of aerobic exercise for this purpose will be discussed. A lack of physical activity is related to a large quantity of NCDs. Regular exercise, whether intense or moderate, is essential for people's health and well-being (World Health Organisation, 2010). Even amongst frail and elderly, mobility and functioning can be improved through physical activity (Butler et al., 1998). Currently, physical inactivity is the fourth leading risk factor for global mortality with 6% of deaths attributed to a lack of physical activity (World Health Organisation, 2010).

1.3.1 Therapeutic Action of Aerobic Exercise on Ageing

Ageing is the largest aetiological factor of physiological and neurological decline through an accumulation of damaging alterations at molecular and cellular levels that result in increased risk of morbidity and mortality. Although ageing is a phenomenon that is not fully understood, it is clear that the ageing process can be

delayed by undergoing a suitable physical activity programme. Exercise has positive effects on a number of different aspects related to the age-related degeneration.

Targeting the models of ageing

With reduction in mitochondrial function strongly associated with ageing and longevity, aerobic exercise has strong effects on mitochondrial biogenesis. Exercise increases hepatic mitochondrial number (Nisoli et al., 2005; López-Lluch et al., 2006) as well as in skeletal muscle. Mitochondrial biogenesis in response to exercise is reliant, at least in part, on adaptive changes in the expression of the transcriptional coactivator, peroxisome proliferator-activated receptor γ coactivator-1alpha (PGC-1α) (Jäger et al., 2007). This increased expression of PGC-1α is associated with phosphorylation by 5' AMP-activated protein kinase (AMPK) (Jäger et al., 2007) and acetylation by the histone deacetylase enzyme, silent information regulator two protein 1 (SIRT1) (Nemoto et al., 2005; Rodgers et al., 2005; Amat et al., 2009). It is thought that SIRT1 may function, at least partially, by activating AMPK (Zang et al., 2006; Hou et al., 2008). AMPK in skeletal muscle declines with ageing (Reznick et al., 2007). Serine-threonine kinase B1 (LKB1) is the principal AMPK kinase that activates AMPK by catalysing the phosphorylation of a threonine residue (Thr^{172}) in response to a decrease in energy state, such as that produced by increased energy expenditure, such as during exercise (Ruderman et al., 2010). AMPK can be activated in skeletal muscle during acute bouts of exercise through the modulation of the AMP/ATP ratio, with SIRT1 activated through changes in nicotinamide adenine dinucleotide (NAD^+) levels within the cell (Fulco et al., 2008). Those metabolic alterations activate these molecules which then interact with each other to regulate the phosphorylation and deacetylation of PGC-1α and subsequent mitochondrial biogenesis (Handschin et al., 2003) (Fig. 1-9). The phosphorylated PGC-1α protein then enters the cell nucleus and helps transcribe nuclear respiratory factor (NRF) genes and peroxisome proliferator-activated receptor (PPAR) genes. Increased expression of these proteins leads to increased activation of mitochondrial transcription factor A (mtTFA) and uncoupling protein (UCP) which enhance mitochondrial biogenesis (Aubert et al., 1997; Hood, 2001). Voluntary exercise can

induce mitochondrial biogenesis in a SIRT1-independent manner (Chabi et al., 2009), which may be due to improved calcium (Ca^{2+}) handling associated with aerobic exercise (Ferreira et al., 2010). Improved Ca^{2+} handling leads to upregulation of the transcription factor, CREB, which transcribes PGC-1α (Handschin et al., 2003). CREB is also upregulated by increased AMP/ATP ratios (Zhang et al., 2004). Other studies show that activation of SIRT1 and mitochondrial proteins are not always associated with increased PGC-1α expression (Suwa et al., 2008).

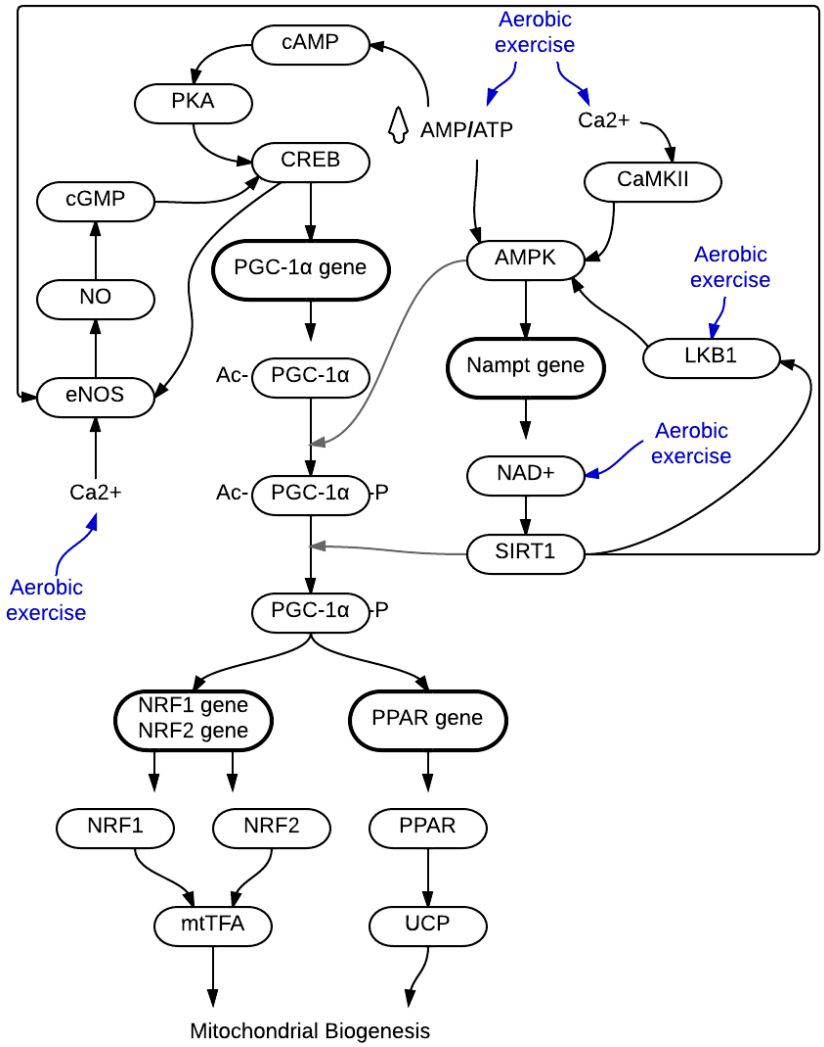


Fig. 1-9. A diagram showing the action of aerobic exercise on the AMPK/SIRT1 pathways, leading to increased mitochondrial biogenesis.

Regular aerobic exercise not only increases mitochondrial biogenesis, but also decreases various manifestations of oxidative stress on the brain (Boveris and Navarro, 2008). With improved Ca^{2+} handling and increased AMP/ATP ratios leading to upregulation of CREB, and increasing transcription of PGC-1α, this becomes an important mechanism in the antioxidant effect of aerobic exercise. The acetylated PGC-1α protein, before phosphorylation by AMPK and subsequent acetylation by SIRT1, acts to transcribe the antioxidant enzymes, SOD and catalase (St-Pierre et al., 2006) (Fig. 1-10). These enzymes act to rapidly scavenge and detoxify the reactive species, ROS and RNS (Rhee et al., 2005). These antioxidant enzymes can also be upregulated by AMPK and SIRT1, as these proteins activate forkhead box O (FOXO) by phosphorylation by AMPK and subsequent acetylation by SIRT1 (Calnan and Brunet, 2008), allowing FOXO to transcribe SOD and catalase (Chiribau et al., 2008). FOXO also increases transcription of the PGC-1α protein, thereby additionally feeding into that pathway (Rodgers et al., 2005).

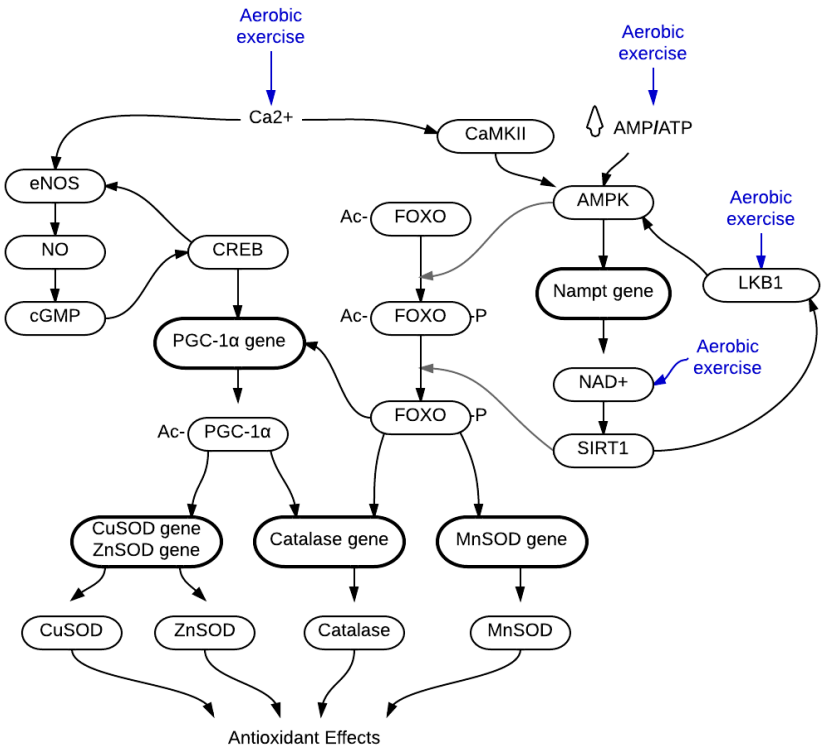


Fig. 1-10. A diagram showing the action of aerobic exercise on the AMPK/SIRT1 dependent and independent pathways, leading to antioxidant effects.

Aerobic exercise puts stress on the body and this leads to increased release of pro-inflammatory cytokines, although, the cytokine response to exercise differs from that elicited by invading pathogens (Pedersen and Hoffman-Goetz, 2000). Levels of TNF-α do not typically increase with exercise, usually IL-6 is the first cytokine present during exercise (Petersen and Pedersen, 2005). The level of circulating IL-6 increases up to 100-fold in response to exercise and declines soon after exercise desists. As marked increases in IL-6 have not lead to muscle damage in a large number of studies (Nieman et al., 1998; Ostrowski et al., 2000; Starkie et al., 2001), it appears that this pro-inflammatory cytokine may have a more important role in initiating a rehabilitative anti-inflammatory response. Circulating levels of IL-6 stimulate an anti-inflammatory response, increasing levels of anti-inflammatory cytokines, such as IL-1 receptor agonist and IL-10, and lowering levels of pro-inflammatory cytokines (Petersen and Pedersen, 2005). This could potentially occur through the same mechanisms as those leading to mitochondrial biogenesis and antioxidant effects. AMPK activity in muscle and adipose tissue caused by swimming is decreased in IL-6 knockout mice (Kelly et al., 2003; Ruderman et al., 2006), suggesting that IL-6 may be involved in AMPK activation in several tissues (Richter and Ruderman, 2010). AMPK activation in human skeletal muscle also correlates closely with IL-6 release during cycling (MacDonald et al., 2003). PGC-1α has been shown to promote anti-inflammatory activity, believed to be through its upregulation of antioxidant factors (Handschin and Spiegelman, 2008). In addition, PPAR helps lower levels of pro-inflammatory cytokines and COX through negative regulation of NF-κB and AP-1 (Neve et al., 2000).

With the body encountering stress due to external factors and reactive species, it has developed a number of endogenous mechanisms that act rapidly to counter the harmful effects. Putting the body under frequent, mild stress, such as with regular aerobic exercise, encourages the activation of these pathways. It is believed that aerobic exercise works to delay ageing as these pathways are more readily activated in response to mechanisms of degeneration (McArdle and Jackson, 2002).

1.3.2 Therapeutic Action of Aerobic Exercise on Noncommunicable Diseases

Physical inactivity is the main cause for approximately 21-25% of breast and colon cancers, 27% of diabetes and 30% of ischaemic heart disease (World Health Organisation, 2009, 2010). An individual's fitness level is a more important predictor of mortality than established risk factors, such as smoking, high blood pressure, high cholesterol, and diabetes (Kodama et al., 2009). A regular exercise regime has therapeutic potential of against a number of key NCDs.

Cardiovascular diseases

A strong, graded inverse relationship is observed between energy expenditure during aerobic exercise and the incidence of coronary heart disease (Manson et al., 1999). Regular exercise promotes weight loss, thereby, lowering blood pressure which reduces damaging low-density lipoprotein (LDL) and total cholesterol levels in the blood, whilst increasing beneficial high-density lipoprotein (HDL) levels in the blood (Stefanick et al., 1998). Aerobic exercise has been shown to have direct benefits on the heart and coronary vasculature, including myocardial oxygen demand, endothelial function, coagulation and clotting factors, and the development of coronary collateral vessels (Clausen and Trap-Jensen, 1976; Hambrecht et al., 2000). The precise mechanism by which exercise therapy improves mortality in patients with coronary heart disease has not been elucidated fully (Thompson et al., 2003). When oxygen becomes limiting, as with cardiovascular disease, mitochondrial oxidative metabolism is restricted. This process can be countered by the increased oxygen consumption promoted through aerobic exercise. It is possible that exercise helps counter cardiovascular diseases by improving mitochondrial function, blocking the immune response and lowering the release of reactive species. Indeed, the action of aerobic exercise on delaying the ageing process may also prove influential in the action against NCDs. Additionally, increased levels of circulating neurotrophins, such as BDNF and VEGF, are thought to promote angiogenesis (Kermani and Hempstead, 2007), thereby promoting blood flow.

Neurodegenerative disorders

Many studies have found that incidence of cognitive impairment and dementia are considerably lower in elderly populations that undergo a regular physical activity regime (Laurin et al., 2001; Verghese et al., 2003; Colcombe et al., 2004; Larson et al., 2006). Other studies have found that aerobic exercise can have rehabilitating effects on dementia (Heyn et al., 2004) and AD (Kramer and Erickson, 2007) patients. In fact, an exercise regime is already often prescribed alongside other treatments for patients with Alzheimer's disease, and other NCDs (Armstrong, 2006). Aerobic exercise has been shown to improve AD-related memory decline in animal models (Hoveida et al., 2011) and humans (Kramer and Erickson, 2007). Relating back to the pathways in figures 1-9 and 1-10, SIRT1 has been reported to provide neuronal protection in rodents with Alzheimer's disease (Anekonda and Reddy, 2006; Kim et al., 2007). AMPK is abnormally activated in neurons displaying AD pathological hallmarks (Vingtdeux et al., 2011), believed to be activated in response to β-amyloid exposure (Thornton et al., 2011). With PGC-1α expression also lower in brains of AD patients compared to healthy controls (Qin et al., 2009). Upregulation of these pathways through aerobic exercise may explain the rehabilitative effects against AD. There is also strong evidence for neuroprotective effects of neurotrophins (Siegel and Chauhan, 2000; Murer et al., 2001; Nagahara et al., 2009). Additionally, cerebral blood flow is increased with regular exercise, meaning this increased supply of oxygen may help counter the degeneration of these disorders (Dustman et al., 1984; Scarmeas et al., 2003; Ainslie et al., 2008).

Diabetes

Since current methods of treating diabetes are inadequate, prevention is preferable (Harris et al., 1998). It is predicted that diabetes mellitus type 2 is preventable (Knowler et al., 1995), as supported by observational and clinical studies assessing adjustments of diet, exercise, or both in people at high risk of this disease (Pan et al., 1997; Tuomilehto et al., 2001). In diabetic patients, regular exercise improves the body's ability to use insulin to control glucose levels in the blood. One study found the incidence of diabetes was reduced by 58% with a lifestyle

intervention involving dietary changes and an exercise regime; with only a 31% reduction with administration of the anti-diabetic drug, metformin (Lifshitz and Hall, 2002). PGC-1α and polymorphisms of associated proteins are often downregulated in type 2 diabetes (Ek et al., 2001; Hara et al., 2002; Mootha et al., 2003) as are neurotrophins (Tomlinson et al., 1997; Krabbe et al., 2007).

1.3.3 Therapeutic Potential of Aerobic Exercise on Memory Decline

Along with the physiological benefits associated with maintaining a regular exercise regime, there is prevailing research suggesting that aerobic exercise improves cognitive function. Preserving cognitive health and plasticity during life is an important public health goal, and it is progressively more evident that physical activity can facilitate this (Cotman & Berchtold, 2002). Human studies have shown the benefits of exercise on cognitive function, principally in ageing populations (Laurin et al., 2001; Verghese et al., 2003; Colcombe et al., 2004; Larson et al., 2006). Physical activity has been shown to have desirable effects on working memory (Clarkson-Smith and Hartley, 1989), long-term memory (Griffin et al., 2009) and spatial learning (Kobilo et al., 2011). Evidence suggests that neurogenesis in the hippocampus increases in response to aerobic exercise (Kempermann, 1998; van Praag et al., 1999; Cotman and Berchtold, 2002), and this is predicted to result in overall improvement in learning and memory. Activity-dependent increases in hippocampal neurogenesis have been proposed to underlie the “neurogenic reserve” hypothesis (Kempermann, 2008; Mirochnic et al., 2009), suggesting that increased neurogenesis may support adaptation of the hippocampal network and aid cognitive flexibility with ageing (Nithianantharajah and Hannan, 2009). It is thought that during running on wheels, rodents may “replay” previously learnt spatial maps in regions such as the hippocampus (Czurkó et al, 1999) because the relevant cognitive systems have evolved to associate running with rapid transition through space (Nithianantharajah and Hannan, 2009).

Aerobic exercise has been shown to increase neurotrophin expression and levels in the rodent hippocampus (Cotman, 2002; Ang et al., 2003; Griffin et al., 2009) and circulating levels in humans (Griffin et al., 2011). Increased neurotrophin activity is suggested to be important for cognitive enhancement associated with aerobic exercise (Kramer et al., 2006; Hennigan et al., 2007). The beneficial effects of exercise on cognition may be directly related to the duration of exercise, with a study in rats finding a direct correlation between distance run and BDNF expression (Oliff et al., 1998). As with action against ageing and NCDs, the AMPK/SIRT1 pathways have important roles in healthy cognitive functioning. SIRT1 (Michán et al., 2010) and AMPK (Spasić et al., 2009) are necessary for healthy cognition, whilst PGC-1α is downregulated in disorders involving cognitive decline (Qin et al., 2009). Activity of these pathways and neurotrophins are not completely dissociated. CREB has been shown to upregulate BDNF (Tao et al., 1998) and NGF (Riccio et al., 1999), whilst PGC-1α upregulates VEGF (Arany et al., 2008).

With aerobic exercise causing so many therapeutic effects against ageing, NCDs, and memory decline, it is clear to see the interest in discovering orally active compounds that can evoke similar actions on the body. As the action of aerobic exercise is so widespread, it is difficult to develop such activity in one compound. Fortunately, plants have developed potential compounds for us to use, or even improve. Resveratrol is one of these compounds.

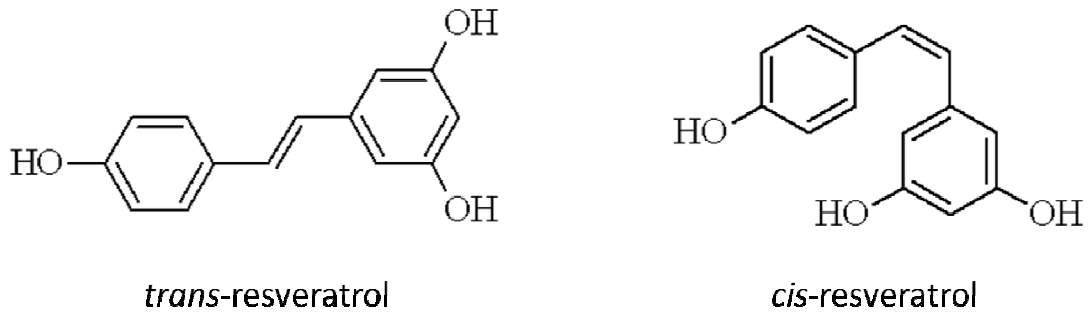
1.4 RESVERATROL ACTION ON BODY AND BRAIN

The benefits of aerobic exercise on general health make it desirable to identify orally active agents that would mimic or potentiate the effects of exercise in delaying the degenerative effects of ageing and treat NCDs. Plant-derived phenolic compounds have potential in this area as they interact with numerous targets and signalling pathways that may be useful in the management of metabolic conditions. Several proposed mechanisms for these effects are through direct antioxidant activity, attenuation of cellular stress, blockade of pro-inflammatory cytokines, and blockade of transcription factors related to metabolic diseases. These are similar mechanisms as already alluded to with aerobic exercise. Most polyphenols modulate oxidative stress and inflammatory responses and it may be these interactions that represent potential mechanisms for the prevention of metabolic disturbances. Moreover, polyphenols attenuate the metabolic effects of high-fat, high-cholesterol diets when administered continuously at high doses. Although the mechanisms of action are not fully understood and clinic trials with such compounds are only recently underway, many of these polyphenols are widely available in capsules for supplementation. These studies will focus on one particular polyphenol - resveratrol.

1.4.1 Resveratrol – understanding the compound

Resveratrol (3,5,4'-trihydroxystilbene) is a natural polyphenol produced by plants in response to environmental stress (Signorelli and Ghidoni, 2005). This polyphenol belongs to the stilbene class of aromatic phytochemicals, and exists in both *cis*- and *trans*- form (Fig. 1-11). The *trans*- form of this compound is found in significant concentrations in a number of nuts and berries but most abundantly on the skin of red grapes and, therefore, red wine (Siemann and Creasy, 1992). *trans*-Resveratrol rose to attention in the mid-90s as a possible explanation for the "French Paradox" when it was thought that the levels of resveratrol found in red wine may work to counteract the unhealthy Mediterranean diet in order to explain the impressive health of elderly people in France (Kopp, 1998). The antioxidant effects and inhibition of platelet aggregation that was observed with resveratrol ignited

interest in this compound (Frankel et al., 1993; Bertelli et al., 1994). Although this initial proposition has been dispelled, interest in resveratrol has since grown due to its possible role in the prevention of diverse pathological processes. Produced by plants in response to environmental stress, such as fungal infection or injury, it is thought that resveratrol synthesis is related to the activation of the plant's defence mechanism (Jeandet et al., 1995). Resveratrol also seems to evoke similar defence mechanisms in animals and humans.



trans-resveratrol *cis*-resveratrol

Fig. 1-11. The chemical structures of *trans*- and *cis*-resveratrol from the stilbene class of aromatic phytochemicals.

1.4.2 Therapeutic Action of Resveratrol on Ageing

Most studies investigating the action of resveratrol have focussed on its action as a CR mimetic. Calorie restriction has been well known since the 1930s (McCay and Crowell, 1934) for its ability to slow the rate of ageing in mammals (Weindruch and Sohal, 1997; Koubova and Guarente, 2003) and delaying the onset of numerous NCDs, such as cardiovascular disease (Lane et al., 1999), cancer (Hursting et al., 2003), diabetes (Kelley et al., 1993; Lane et al., 1999) and neurodegeneration (Mattson et al., 2001). This involves a reduction in calorie intake to a level that has been shown to increase lifespan and stress-resistance in multiple

species, without causing malnutrition. Research investigating resveratrol's action as a calorie restriction (CR) mimetic found that this compound has positive effects on a number of different aspects related to the age-related degeneration; mechanisms which correspond to those evoked through aerobic exercise.

Targeting the models of ageing

Resveratrol has been shown to extend the lifespan of *S. cerevisiae* (Howitz et al., 2003), *C. elegans* (Wood et al., 2004; Viswanathan et al., 2005) and *D. melanogaster* (Bauer et al., 2004; Wood et al., 2004), but only if the gene that encodes silent information regulator 2 (sir2; the closest homologue to SIRT1 in these animals) is present. Resveratrol consistently recapitulates the protective effects of SIRT1 overexpression in cell culture (Howitz et al., 2003; Araki et al., 2004). Resveratrol improves the health and extends the lifespan of mice on a high fat diet but, although showing health benefits in normal mice, it does not extend lifespan (Pearson et al., 2008). With reduction in mitochondrial function strongly associated with ageing and longevity, resveratrol acts on similar pathways as aerobic exercise to evoke strong effects on mitochondrial biogenesis. In one study, the livers of resveratrol-treated high-calorie mice had considerably more mitochondria than those of high-calorie controls and were not significantly different compared to those of the standard-diet group (Baur et al., 2006). Culturing cancer cells, such as FaO hepatoma or HeLa, in the presence of resveratrol also increased mitochondrial number, similar to the reported effect of culturing cells in serum from calorie restricted rats (Lopez-Lluch et al., 2006). Acetylated PGC-1α in resveratrol treated mice was 3-fold lower than the diet matched controls. Although, as no increase in SIRT1 protein levels was detected, the authors suggested that SIRT1 enzymatic activity was enhanced by resveratrol. An alternative proposition has been the influence of AMPK in these effects. Chronic activation of AMPK occurs on a calorie restrictive diet and has been proposed as a longevity strategy for mammals (McCarty, 2004), with additional copies of the AMPK gene proving sufficient to extend lifespan in *C. elegans* (Apfeld et al., 2004). Resveratrol has been shown to activate AMPK in cultured cells through

an indirect mechanism (Baur et al., 2006), and AMPK-null mice did not have increased insulin sensitivity, glucose tolerance, mitochondrial biogenesis, or physical endurance following resveratrol administration, although wild-type controls showed all these improvements (Um et al., 2010). A number of studies demonstrate that LKB1 may act as an upstream kinase responsible for resveratrol-induced AMPK activation (Hou et al., 2008; Shin et al., 2009), whilst inhibitors of cAMP-specific phosphodiesterase 4 (PDE4) have been shown to mimic the action of resveratrol through elevated Ca^{2+} levels (Park et al., 2012b). Resveratrol has been shown to upregulate compounds further down the AMPK/SIRT1 pathways, such as PGC-1α (Lagouge et al., 2006) and MnSOD (Robb et al., 2008). Although the principal method by which resveratrol activates these pathways is unclear, it clearly has a profound effect on mitochondrial biogenesis (Fig. 1-12).

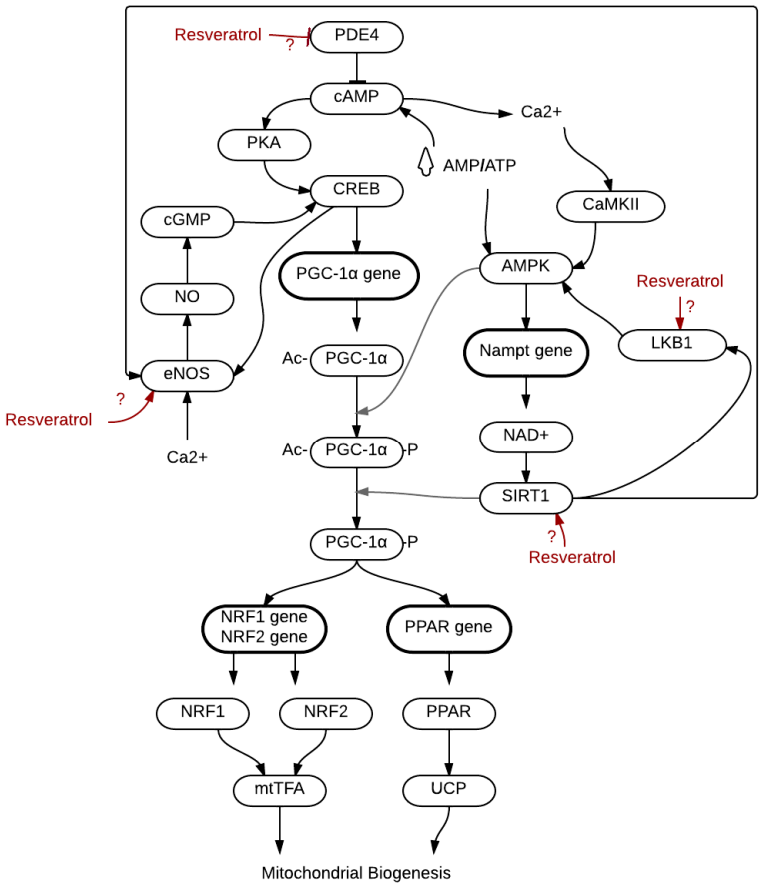


Fig. 1-12. A diagram showing the proposed actions of resveratrol on the AMPK/SIRT1 pathways, leading to increased mitochondrial biogenesis.

Resveratrol not only increases mitochondrial biogenesis, but also decreases various manifestations of oxidative stress *in vitro* (Chanvitayapongs et al., 1997) and *in vivo* (Gupta et al., 2002a). Additionally, resveratrol directly scavenges free radicals (Frankel et al., 1993). With resveratrol shown to upregulate previously mentioned proteins in the AMPK/SIRT1 pathways, and additional upregulation of FOXO (Chen et al., 2009) and MnSOD (Kitada et al., 2011), the same pathways as in aerobic exercise may be implicated in these antioxidant effects (Fig. 1-13).

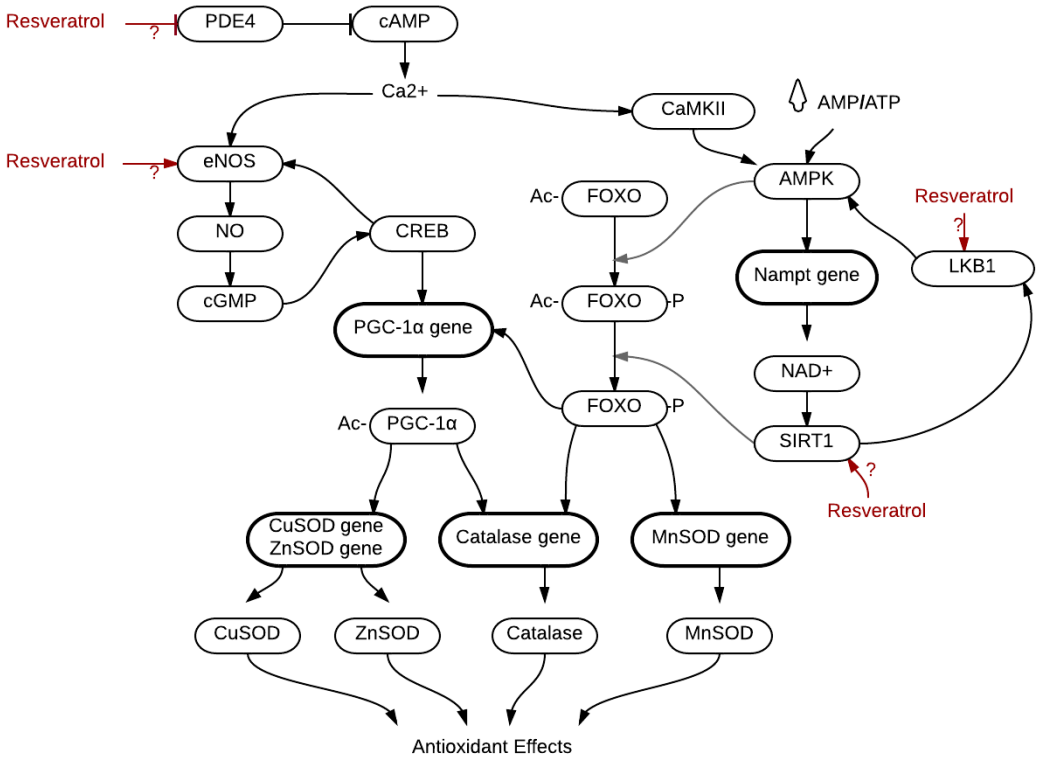


Fig. 1-13. A diagram showing the action of resveratrol on the AMPK/SIRT1 dependent and independent pathways, leading to antioxidant effects.

Resveratrol has also been reported to act as a phytooestrogen in some systems (Gehm et al., 1997). As oestrogen replacement therapy has been shown to reduce the risk of cardiovascular disease and osteoporosis in postmenopausal women (Lobo, 1995), it has been suggested that this property of resveratrol might mediate its cardioprotective effects. However, both the oestrogenic effects of resveratrol *in vivo* and the cardioprotective effects of oestrogen replacement (Hodis et al., 2003; Bluming, 2004) have since become subjects of debate, and a firm connection remains to be established (Baur and Sinclair, 2006). It is thought that much of resveratrol's action on the body occurs through upregulation of PGC-1α, whether through SIRT1, AMPK, or another mechanism.

1.4.3 Therapeutic Action of Resveratrol on Noncommunicable Diseases

Although resveratrol has clear protective effects, the exact metabolic targets remain elusive. *In vitro* experiments have universally shown favourable effects, leading to the identification of multiple direct targets for resveratrol. As referred to in the previous section, aerobic exercise acts to prevent and treat a number of NCDs. The therapeutic effects of resveratrol treatment on NCDs will now be compared to those of aerobic exercise in order to highlight the potential of this compound as an exercise mimetic.

Cardiovascular diseases

Resveratrol prevents platelet aggregation *in vitro* (Bertelli et al., 1995) and *in vivo* (Zini et al., 1999; Wang et al., 2002) which suggests therapeutic potential against transient ischemia, myocardial infarction or stroke by preventing thrombus formation and subsequent blockages in blood vessels. The predicted mechanism for

this preventative method involves its differential action on the cyclooxygenases, COX1 and COX2, because the balance of prostaglandins synthesised by these cyclooxygenase isoforms regulates vascular homeostasis (Jang et al., 1997; Baur and Sinclair, 2006). COX1 synthesises thromboxane A2 (TxA_2) which acts as a potent inducer of platelet aggregation and as a vasoconstrictor (Hamberg et al., 1975), whereas COX2 synthesises prostacyclin which acts as an anti-platelet aggregator and vasodilator (Moncada et al., 1976). Resveratrol has been shown to exhibit preferential inhibition of COX1 over COX2 activity (Jang et al., 1997) which, therefore, promotes blood flow and decreases clot formation. Oxidation of low-density lipoprotein (LDL) particles is strongly associated with the risk of coronary heart disease and myocardial infarction (Holvoet, 2004). Resveratrol prevents LDL oxidation in vitro by chelating copper, as well as by directly scavenging free radicals (Frankel et al., 1993). Treatment of healthy rats with resveratrol does not affect LDL oxidation (Lobo, 1995), but in stroke-prone rats this significantly reduces markers of oxidative stress, such as glycated albumin in serum and 8-hydroxyguanosine in urine (Mizutani et al., 2001). Additionally, resveratrol has proved to be effective at protecting isolated rat hearts against ischaemia/reperfusion injury, resulting in improved recovery of developed pressure and aortic flow, reduction of malondialdehyde concentrations and reduction of infarct size (Ray et al., 1999; Bradamante et al., 2000; Sato et al., 2000; Fulda and Debatin, 2004). These actions are thought to take place through activation of NOS, as inhibitors of NOS have been shown to block the protective effects of resveratrol in isolated rat hearts (Hattori et al., 2002) while hearts from iNOS-null mice are not protected (Imamura et al., 2002). Increases in the expression of both eNOS and iNOS (Das et al., 2005), as well as increases in serum NO (Hung et al., 2000), were observed in mice treated with resveratrol, demonstrating that this mechanism could be relevant *in vivo*.

Neurodegenerative disorders

With cholinergic neurons and pathways playing widespread roles in the regulation of learning, memory and cerebral blow flow to the nervous system

(Mesulam et al., 2002), resveratrol has been shown to act through the cholinergic pathway to alleviate memory decline associated with Alzheimer's disease (AD) when administered intraperitoneally (i.p.) (Gacar et al., 2011). Recently it was established that specific binding sites for resveratrol exist in the brains of rats, most densely found in the choroid plexus and subfornical organ of the brain (Han et al., 2006; Radkar et al., 2008). The primary protein produced by the choroid plexus is transthyretin (TTR) which is the main binding protein for β-amyloid, and it is proposed that activation of TTR production may be a mechanism for resveratrol's action against AD (Bastianetto et al., 2007). Numerous studies have raised the possibility that resveratrol may be useful in protecting against brain damage following cerebral ischaemia. Resveratrol administered intracerebroventricularly (i.c.v.) during and after transient global cerebral ischaemia lowers delayed neuronal cell death and glial cell activation in the hippocampus (Wang et al., 2002). Administered intravenously (i.v.), resveratrol decreased ischaemic volume and brain water content following middle cerebral artery occlusion in rats (Wang et al., 2004). Resveratrol administered i.p. also prevented seizures induced by $FeCl_3$ (Gupta et al., 2001), kainic acid (Gupta et al., 2002a) or pentylenetetrazole (Gupta et al., 2002b), and partially restored cognition in rats receiving streptozotocin i.c.v. (Sharma and Gupta, 2002). These results suggest that resveratrol is capable of penetrating the blood-brain-barrier and exerts strong neuroprotective effects, even at low doses. Rats given resveratrol i.p. for 21 days showed less motor impairment and significantly smaller infarct volume after middle cerebral artery occlusion (Sinha et al., 2002). Similar effects were seen in wild-type, but not in PPAR-null mice (Inoue et al., 2003).

Diabetes

High-calorie fed mice show alterations in plasma levels of markers that predict the onset of diabetes and a shorter lifespan, including increased levels of insulin, glucose and IGF-1 (Baur et al., 2006). When treated with resveratrol, mice showed significantly lower levels of these markers. An oral glucose tolerance test

indicated that the insulin sensitivity of the resveratrol-treated mice was considerably higher than controls. A number of other studies have found similar effects with resveratrol administration (Ramadori et al., 2009; Kitada et al., 2011). With PGC-1α and polymorphisms of associated proteins (Ek et al., 2001; Hara et al., 2002; Mootha et al., 2003) and neurotrophins (Tomlinson et al., 1997; Krabbe et al., 2007) often downregulated in type 2 diabetes, it may be through these pathways that resveratrol counteracts the symptoms of this disorder.

1.4.4 Therapeutic Potential of Resveratrol on Memory Decline

Along with the physiological benefits associated with resveratrol administration, there is evidence to suggest that resveratrol penetrates the blood-brain-barrier and promotes desirable improvements in cognition and memory (Baur, 2010). Resveratrol ingestion can improve performance in working memory tasks of healthy aged rats (Joseph et al., 2008) and aged mice with induced neuroinflammation (Abraham and Johnson, 2009). Long-term resveratrol treatment improved spatial memory in aged animals, assessed using a Y-maze task (Oomen et al., 2009). In non-human primates, resveratrol treatment was shown to enhance both working and spatial memory using an 18-month regime (Dal-Pan et al., 2011), while in humans, a single dose of oral resveratrol increased cerebral blood flow, but did not improve cognitive function (Kennedy et al., 2010). Resveratrol potentially enhances memory by suppressing the formation of inflammatory metabolic products within the brain and optimising overall brain metabolism, with dose-dependent enhancement of cerebral blood flow and oxygenation. Although questions have been raised over the bioavailability of oral resveratrol *in vivo* (Baur and Sinclair, 2006), evidence of other orally available compounds that improve cognitive functioning in the hippocampus maintain hope (Townsend et al., 2006; O'Hare et al., 2013).

Cognitive enhancement with resveratrol is often related to action on the AMPK/SIRT1 pathways. SIRT1-null mice exhibit impaired learning and memory, suggesting the SIRT1 is of importance in those processes (Gao et al., 2010; Michán et al., 2010). However, Michán and colleagues (2010) found that boosting natural levels of SIRT1 did not enhance cognition in the SIRT1-null mice, leading them to question the cognitive benefit of supplementation with SIRT1 activators. This also raises the question of how important the SIRT1 protein is in the cognitive enhancement observed with resveratrol treatment. Within the same pathways, resveratrol has been shown to stimulate AMPK (Dasgupta and Milbrandt, 2007) and PGC-1α (Mudò et al., 2012) activity in neurons. Activation of these pathways may indeed prove vital for the cognitive enhancement associated with resveratrol. Some studies have also detected increased levels of neurotrophins following resveratrol administration (Rahvar et al., 2011; Pang and Hannan, 2012). With neurotrophins thought to play an important role in neuronal repair and plasticity, resveratrol may enhance memory by encouraging early cell survival in brain regions associated with learning and memory (Frielingsdorf et al., 2007; van Praag, 2009). Contrary to this, one study found that resveratrol administered i.p. could lower levels of BDNF and inhibit hippocampal neurogenesis (Park et al., 2012a). These results clearly raise questions about resveratrol's action as an exercise mimetic which must be further explored. Understanding the principal action of both aerobic exercise and resveratrol action may allow the development of suitable, potentially better, pharmacological agents which can evoke similar improvements both physiologically and neurologically.

1.5 THESIS AIMS AND STRUCTURE

The concept of this thesis was to explore how elements of physical and mental health are connected; particularly focusing on the effects that aerobic exercise and resveratrol ingestion have on learning and memory. Research to date indicates that these factors that enhance the metabolic system also relieve certain elements of cognitive decline. The extent to which this occurs is still to be determined, with a vast number of scientists investigating different aspects of cognitive decline and methods of altering metabolism.

The first aim of this thesis was to determine the extent of cognitive enhancement associated with regular resveratrol ingestion and aerobic exercise in healthy memory, memory decline associated with ageing, and AD-related amnesia. A range of *in vivo* techniques were chosen to conduct a direct comparison study of these two factors in order to highlight more clearly the potential of resveratrol ingestion as an exercise mimetic. Specific focus was placed on the effects of ingesting a relatively small quantity of resveratrol, with the intention of replicating the action of taking resveratrol tablets as a supplement.

The second aim was to determine the potential mechanisms through which both aerobic exercise and resveratrol ingestion may act to evoke their beneficial action on learning and memory. Analysis of endogenous proteins involved in the neurotrophin pathway and the AMPK/SIRT1 pathways was conducted in hippocampal and perirhinal cortex. Further analysis of mitochondrial functioning in tissues that expend large levels of energy and require higher numbers of mitochondria was carried out.

It was hypothesised that resveratrol ingestion and aerobic exercise would improve healthy memory, and memory decline associated with ageing and AD, in a similar manner. It was expected that activation of the AMPK/SIRT1 pathways would be involved in any detected cognitive enhancement, leading to enhanced

mitochondrial functioning. These findings would promote the use of resveratrol and aerobic exercise in enhancing cognition as well as in delaying degeneration associated with ageing and disease.

Chapter Two

AEROBIC EXERCISE AND RESVERATROL IMPROVE LONG-TERM MEMORY THROUGH AN AMPK/SIRT1-INDEPENDENT PATHWAY

"You can't help getting older, but you don't have to get old."

- *George Burns*

2.1 ABSTRACT

The polyphenol, resveratrol, rose to attention in the mid-1990s as a possible explanation for the "French Paradox" and has since attracted further interest due to discoveries revealing many beneficial effects on human end-organ function. These include antioxidant properties, cardioprotection, anti-tumour effects, enhanced longevity and boosted whole body metabolism. These improvements are also elicited through aerobic exercise; leading to the hypothesis that resveratrol may be considered an exercise mimetic. Few studies have directly compared the action of resveratrol to that of physical activity, though the results of separate studies indicate that both may act through identical pathways to produce such similar alterations.

To determine how comparable these mechanisms are, it was examined in healthy young (YG; 3 months) and middle-aged (MA; 14 months) male Wistar rats whether a regular oral dose of resveratrol (20 mg/kg; 5 d/week) would have a similar effect on long-term recognition memory as 1 h treadmill running at 17 m/min (5 d/week), following a 4 week protocol. To assess this, a novel object recognition (NOR) task with a 24 h delay was used. The potential underlying mechanisms facilitating cognitive enhancement were investigated using hippocampal and perirhinal tissue samples.

After the 4 week treatment/exercise protocol, YG and MA rats that underwent resveratrol treatment and treadmill running performed significantly better than sedentary control rats in the NOR task. Protein analysis showed that improved cognitive ability was associated with elevated levels of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), whilst levels of key proteins in the AMPK/SIRT1 pathways remained steady in hippocampal tissue.

These results indicate that regular 20 mg/kg oral resveratrol treatment produces similar hippocampal-dependent cognitive enhancement in the NOR task as regular 1 h treadmill running, in both young and middle-aged rats. Associated increases in BDNF and NGF levels suggest that it may be the action of resveratrol and exercise on these neurotrophins that elucidate their beneficial effects on cognition, and perhaps other aspects of end-organ function. These findings highlight the potential use of resveratrol and aerobic training as a memory aid both in enhancing normal memory and in ameliorating age-related decline.

2.2 INTRODUCTION

Chronic noncommunicable diseases (NCDs) are currently the leading global health burden with high morbidity and mortality rates. Although non-modifiable factors such as ageing and genetics contribute to the prevalence of these afflictions, globalisation of unhealthy lifestyles has played a major role in this rise. Alcohol and tobacco abuse, physical inactivity, and unhealthy diets all form modifiable risk factors for the development of many NCDs, such as diabetes type 2, cardiovascular diseases, and some cancers (Mendis and Fuster, 2009). Thus, making healthier lifestyle choices in these areas will help prevent many cases of NCD. Lack of physical activity is related to a large quantity of NCDs, with this predicted to be the main cause for approximately 21-25% of breast and colon cancers, 27% of diabetes type 2, and 30% of ischaemic heart disease cases (World Health Organisation, 2009; 2010). Aerobic exercise is, therefore, a key primary preventative intervention for many of these diseases.

Regular exercise encourages favourable structural and metabolic alterations which improve both endurance and general health. Such adaptations are triggered by a network of molecular pathways that are not yet fully understood. Developments in research aimed at targeting components predicted to be involved in this circuitry have implicated the importance of a number of proteins, such as silent information regulator two protein 1 (SIRT1) (Ferrara et al., 2008), 5' AMP-activated protein kinase (AMPK) (Durante et al., 2002), peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1α) (Handschin and Spiegelman, 2008), manganese superoxide dismutase (MnSOD) (French et al., 2008) and neurotrophins (Neeper et al., 1995, 1996; Richardson et al., 2000). Regular aerobic exercise improves mitochondrial dysfunction, hormonal imbalances, inflammatory mechanisms, and oxidative stress associated with NCDs as well as with normal ageing. These improvements are thought to occur at least partially through increases in the previously mentioned proteins (Jäger et al., 2007); with these pathways all thoroughly entwined. The benefits of aerobic exercise make it desirable to identify orally active agents that mimic or potentiate these effects.

Plant-derived phenolic compounds, such as resveratrol, have the potential to treat NCDs and delay age-related degeneration, with numerous studies showing therapeutic effects against cardiovascular disease (Jäger and Nguyen-Duong, 1999; Ray et al., 1999; Zini et al., 1999), stroke (Gupta et al., 2002; Wang et al., 2002; Inoue et al., 2003), diabetes type 2 (Thirunavukkarasu et al., 2007; Szkudelska and Szkudelski, 2010), some cancers (Jang et al., 1997; Bove et al., 2002) and neurodegeneration (Marambaud et al., 2005; Sun et al., 2010). Resveratrol (3,5,4'-trihydroxystilbene) is a natural polyphenol produced by plants in response to environmental stress (Signorelli and Ghidoni, 2005). The *trans*- form of this compound is found in significant concentrations in a number of nuts and berries but most abundantly on the skin of red grapes and, therefore, red wine (Siemann and Creasy, 1992). *trans*-Resveratrol rose to attention in the mid-90s as a possible explanation for the "French Paradox" when it was thought that the levels of resveratrol found in red wine may work to counteract the unhealthy Mediterranean diet in order to explain the impressive health of elderly people in France (Kopp, 1998). The antioxidant effects and inhibition of platelet aggregation that was observed with resveratrol ignited interest in this compound (Frankel et al., 1993; Bertelli et al., 1994). Although this initial proposition has been dispelled, interest in resveratrol has since grown due to discoveries revealing many beneficial effects on human end-organ function. Resveratrol has been shown to activate some of the same pathways as aerobic exercise, with studies finding increased activity of SIRT1 (Howitz et al., 2003), AMPK (Um et al., 2010), PGC-1α (Lagouge et al., 2006), MnSOD (Robb et al., 2008) and neurotrophins (Thirunavukkarasu et al., 2007; Rahvar et al., 2011). In relation to the similarity of these widespread actions, resveratrol may be considered an exercise mimetic.

Alongside physical adaptations, there is evidence suggesting that aerobic exercise and resveratrol penetrate the blood-brain-barrier and promote desirable improvements in cognition and memory (Joseph et al., 2008; van Praag, 2009). Although questions have been raised over the bioavailability of oral resveratrol *in vivo* (Baur and Sinclair, 2006), evidence of other orally available compounds that improve cognitive functioning in the hippocampus maintain hope (Townsend et al., 2006; O'Hare et al., 2013). It remains unclear which pathway either aerobic exercise

or resveratrol act upon that leads to cognitive enhancement. Many studies have highlighted that neurotrophins, such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), are implicated in the cognitive enhancement evident with aerobic exercise. Research shows that levels of these neurotrophins are increased with exercise (Neeper et al., 1995; Cotman and Berchtold, 2002; O'Callaghan et al., 2007). Additionally, BDNF (Griffin et al., 2009) and NGF (Tuszynski et al., 2005) administration alone has been shown to enhance memory. Neurotrophins are thought to play an important role in neuronal repair and plasticity, and may enhance cognition by promoting hippocampal neurogenesis (Frielingsdorf et al., 2007; van Praag, 2009). Most studies involving resveratrol focus on the action of this polyphenol on SIRT1 and, more recently, AMPK, with administration of both proteins linked with cognitive enhancement (Michán et al., 2010; Kobilo et al., 2011). It is possible that both aerobic exercise and resveratrol act on these pathways to enhance hippocampal memory, though both aerobic exercise and resveratrol have wide-spread actions on a number of molecular pathways and their effects on cognitive enhancement may work through an alternative pathway.

To confirm the beneficial effects on learning and memory, and contrast the mechanisms of action, a direct comparison of behaviour following regular aerobic exercise and oral resveratrol treatment was conducted, with follow up analysis of biological markers. This should provide greater insight to the pathways through which aerobic exercise and resveratrol act to enhance memory, and the potential use of resveratrol as an orally active compound against NCDs and age-related degeneration. For this study, cohorts of young (YG) and middle-aged (MA) male Wistar rats underwent either a training protocol of treadmill running, 1 h increasing from 10-17 m/min, 5 d/week, or led a sedentary lifestyle. These groups were again sub-divided so that half the animals were administered resveratrol orally at a dose of 20 mg/kg on training days, a dose shown to cause no adverse effects with daily dosage (Juan et al., 2002). Middle-aged rats were assessed because loss of cognitive processing during ageing is a complex process that starts to become evident during middle-age in humans and rats even in the absence of specific neurodegenerative diseases (Kluger et al., 1997). Following a 4 week protocol of regular treadmill

running and resveratrol administration, animals were tested in a substitution novel object recognition (NOR) task. This is a well-validated behavioural measure of rodent memory useful for evaluating experimental manipulations on cognition and has been shown to highlight cognitive differences between trained and untrained rats (Griffin et al., 2009). Using a 24 h delay, effects of treatment on long-term recognition memory was examined.

These results show that, in both young and middle-aged rats, treatment with resveratrol and regular aerobic exercise for 4 weeks led to significantly better performance in the NOR task compared to untreated, sedentary controls. These findings were paralleled to increased levels of BDNF and NGF in brain regions associated with learning and memory. AMPK, SIRT1, PGC-1α, and MnSOD expression in the hippocampus and perirhinal cortex did not increase with treadmill running or resveratrol administration, compared to sedentary, untreated controls. This suggests that aerobic exercise and the polyphenol, resveratrol, may act through an AMPK/SIRT1-independent pathway to enhance cognition.

2.3 MATERIALS AND METHODS

2.3.1 Animals

Young (YG; 3 months at study start, *n* = 24) and middle-aged (MA; 14 months at study start, *n* = 24) male Wistar rats were obtained from the BioResources Unit, Trinity College Dublin. They were housed in pairs (standard hard-bottomed, polypropylene cages; 44 × 28 × 18 cm) in a temperature-controlled vivarium (20 to 22 °C), with a 12:12-hour light-dark cycle. Animals were provided with food and water *ad libitum*. Experiments were carried out in strict accordance with regulations laid out by LAST Ireland and were compliant with the European Union directives on animal experimentation (86/609/EEC).

2.3.2 Drug and Dosing Regime

All rats were handled for one week pre-drug treatment and fed 0.5 ml of maple syrup (Maple Joe, Bernard Michaud) to familiarise them with feeding by syringe. *trans*-Resveratrol (>99% purity) from Sigma-Aldrich, UK was administered orally mixed in a solution of maple syrup. Treated animals were given an oral dose of maple syrup and resveratrol suspension (20 mg/kg; p.o.) 5 days per week, with controls given maple syrup only. Rats were dosed 30 min before exercise protocol.

2.3.3 Exercise Programme

Rats (*n* = 48) were familiarised to motorised treadmills (Exer 3/6 treadmill, Columbus Instruments) by walking on the treadmill for 15 min (belt speed, 7 m/min) every other day for one week (3 d). Rats were divided into YG and MA, and then sub-divided into 4 groups: sedentary controls (SedCTL), running controls (RunCTL), sedentary resveratrol-treated (SedRES) and running resveratrol-treated (RunRES) (*n* = 6 in each). YG rats were assigned between groups according to matched performance on the plank walk to ensure no motor differences between

groups; MA rats were assigned between groups at random (see Psychomotor Behaviour). The exercise protocol consisted of running one hour per day for 5 consecutive days per week (belt speed, gradually increased over the training period from 10 m/min to a maximum of 17 m/min, which is equivalent to 1 km/h). The treadmill is equipped with wire loops at one end of the belt through which a mild electric shock can be delivered; these act to motivate the rats to run continuously and were activated at low levels (on average an intensity of three on a scale of 0–10; this represents a current of 1 mA with an inter-pulse interval of 2 s) throughout all exercise sessions. Rats were observed while exercising to ensure they ran continuously and also to monitor for signs of stress. Sedentary rats were placed on stationary treadmills with shock loops activated at low levels for the same duration. Training in the NOR task began on the final day of the exercise programme, during the 4th week, following 18 days treadmill running.

2.3.4 Psychomotor Behaviour

Before treadmill running and dosing began, all rats were assessed in their ability to undertake the plank walk task. Balance and co-ordination were measured by exposing the rats to one trial on each of three horizontal planks (wide = 30 mm, medium = 25 mm, narrow = 15 mm), all 1 m in length and placed 30 cm above the table top. These complex motor behaviours should be excellent in young healthy rats and steadily decline with ageing (Shukitt-Hale et al., 1998). However, none of the MA rats were able to balance on the narrow planks and so no data is recorded for them. The order of plank widths was randomised and counterbalanced across groups. Latency to fall (max score = 60 sec) and number of turns on the planks were recorded and averaged for each trial.

2.3.5 Open Field Exploration

An open-field test was conducted in a black circular open field (diameter, 90 cm; height, 45 cm) placed in a dimly lit-room. Rats were examined in this empty arena on their first day of habituation (following 16 days treadmill running and treatment) to measure for general exploration and spontaneous behaviour in response to a novel environment. Observation of such behaviours is of particular importance in drug trials as these compounds may have unexpected or previously undiscovered behavioural effects (Kršiak and Borgesova, 1972). Rats were allowed to move freely for 5 min with tracks recorded and analysed using a computer-based tracking system (Ethovision, Noldus Co. Ltd., The Netherlands). Habituation and reactions to spatial changes were evaluated by the exploration of objects in the same open field arena during the initial 5 min NOR training trial. This was to examine the sensitivity of the rats to an environmental change, by recording the interaction each rat made with the individual objects.

2.3.6 Novel Object Recognition Task

Rats were well-handled and habituated to the experimental apparatus (see Open Field Exploration), with 20 min of exploration in the absence of objects each day for 2 days before the task was performed. Objects were constructed from toy bricks and were fixed to the floor of the open field, 15 cm from the walls. Objects and arena were cleaned thoroughly between trials to ensure the absence of olfactory cues. Scoring for exploration was strictly based on active exploration, where rats had to be touching the object with at least their noses. An object substitution task was used to assess non-spatial recognition memory. For the training phase, three distinct objects (A, B and C) were positioned in the open field in a room with prominent extramaze cues which could be used for efficient allocentric orientation. Rats were allowed to explore the objects for 3 x 5 min trials with an intertrial interval (ITI) of 5 min. For the testing phase, 24 h later, object C was replaced with novel object D (Fig. 2-1). Rats were reintroduced to the open field for a single 5 min trial. Measurement of the time spent exploring each object was recorded and expressed as

a discrimination ratio (novel object interaction/total interaction with all objects) (Bevins and Besheer, 2006). Object recognition is reflected by spending more time interacting with the novel object D over familiar objects A and B, shown here with an object discrimination ratio above 0.333.

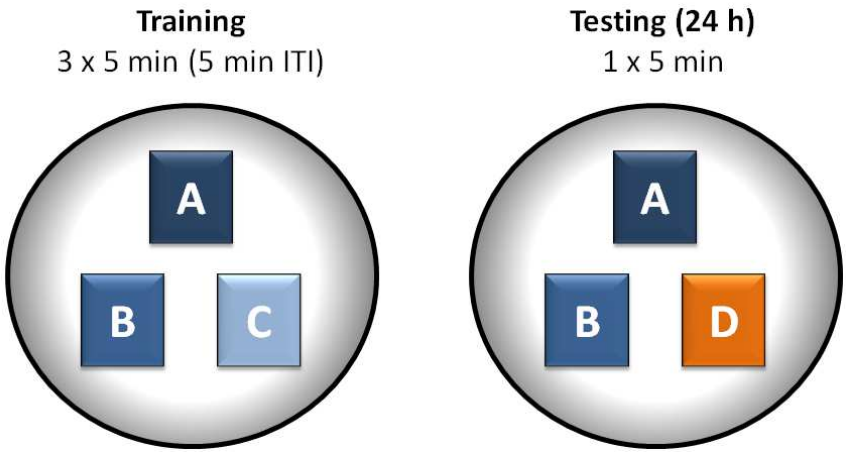


Fig. 2-1. Overview of novel object recognition task used to assess long-term recognition memory. Rats were exposed to an arena containing 3 distinct objects for three 5 min periods. Following a 24 h delay, rats were reintroduced to the same arena with 2 of the same objects in the same locations (A and B) and one object substituted with another distinct object (C with D).

2.3.7 Tissues and Serum Samples

Rats were sacrificed by decapitation 1 h following the testing phase of the NOR task. Their brains were removed and tissue was taken from the dentate gyrus (DG), remainder of hippocampus (HIPP) and perirhinal cortex (PC). These samples were homogenised in lysis buffer, with a small portion separated for mRNA analysis, and stored at -80°C until further analysis.

2.3.8 Analysis of Protein Levels by Enzyme-Linked Immunosorbent Assay (ELISA)

Samples homogenised in lysis buffer were thawed, assayed for protein content using a Micro BCA Protein Assay Kit (Thermo Scientific, Hampshire, UK), and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, Delaware, USA). Samples were all diluted to a final volume of 100 µl to equalise for protein content and then stored at -20°C until further analysis.

BNDF levels in dentate gyrus, hippocampus and perirhinal cortex were quantitatively assessed using *Chemikine*™ BDNF Sandwich ELISA kit (Millipore S.A.S., Molsheim, France). NGF levels in dentate gyrus, hippocampus, and perirhinal cortex were quantitatively assessed using *Chemikine*™ Nerve growth factor Sandwich ELISA kit (Millipore S.A.S., Molsheim, France). All ELISAs were conducted according to instructions provided by the manufacturer. All samples assayed by ELISA were done in triplicate.

2.3.9 Analysis of Protein Expression by Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA from brain tissue was extracted from snap-frozen samples using the NucleoSpin RNA II isolation kit (Machery-Nagel Inc., Germany) following manufacturer's instructions, and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, Delaware, USA). For RT-PCR, total RNA was retro-transcribed to cDNA. cDNA synthesis was performed on 1 - 2 µg RNA using a High Capacity cDNA RT Kit (Applied Biosystems, USA). Following this, total cDNA was submitted to RT-PCR for SIRT1, AMPKα1, AMPKα2, PGC-1α, MnSOD, BDNF and NGF. Rat β-actin was used as an endogenous control (cDNA samples were not normalised prior to RT-PCR) and expression was conducted using a gene expression assay containing forward and

reverse primers and a VIC-labelled MGB TaqMan probe (Applied Biosystems, USA). All RT-PCR measurements were conducted using an ABI Prism 7300 instrument (Applied Biosystems, USA). Forty cycles were run as follows: 10 min at 95 °C and for each cycle, 15 sec at 95 °C and 1 min at 60 °C. Fluorescence was read during the annealing and extension phase (60 °C) throughout the program and gene expression was calculated relative to the endogenous control. Analysis was performed using the $2^{-\Delta\Delta}$CT method. Data are presented as mean relative quotient (RQ) values that represent fold changes relative to the mean value for controls using StepOne™ Software v2.1 (Applied Biosystems, USA).

2.3.10 Statistical Analysis

All data was analysed using GraphPad Prism (GraphPad Software, Inc.) and Statistical Package for the Social Sciences (SPSS). One-way ANOVA, two-way ANOVA or unpaired two-tailed Student's t-test were conducted as appropriate. *Post hoc* comparisons were made using the Tukey's HSD test. A significance level of $p = 0.05$ was accepted for all comparisons: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Data are presented as mean ± SEM.

2.4 RESULTS

2.4.1 Psychomotor Behaviour Comparisons for Group Matching

To confirm a fair distribution of animals between treatment groups, all rats were initially assessed for psychomotor behaviour using a plank walk test. Using results from this task, young animals were evenly matched between 4 groups. One-way ANOVA revealed no difference between YG groups before treadmill running and dosage regime on latency to fall ($F_{3,20}$ = 0.151, p = 0.928) (Fig. 2-2A), and number of turns ($F_{3,20}$ = 1.20, p = 0.335) (Fig. 2-2B). All YG rats were healthy with good balance and co-ordination before experimentation began. These results were used to match YG rats evenly between the 4 treatment groups: sedentary controls (SedCTL), running controls (RunCTL), sedentary resveratrol-treated (SedRES) and running resveratrol-treated (RunRES) (n = 6 in each). Middle-aged animals were larger and found it too difficult to balance on the plank, so treatment groups were randomly assigned for these animals (data not represented).

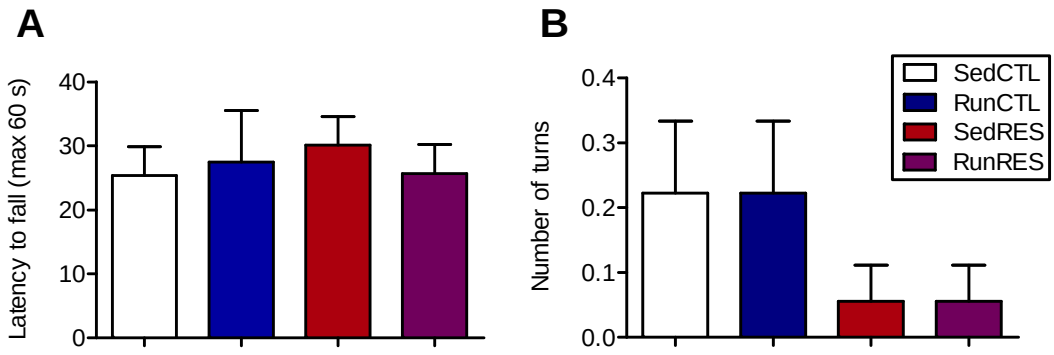


Fig. 2-2. There was no difference between young groups in psychomotor behaviour at beginning of experiment. Balance and co-ordination was assessed using a plank walk by measuring [A] latency to fall and [B] number of turns on three horizontal planks (wide = 30 mm, medium = 25 mm, narrow = 15 mm), all 1 m in length, 30 cm above the table top. For each rat performance on the 3 planks was averaged and group means are presented above. n = 6 per group. Groups were determined following performance in this task. Middle-aged animals were too large to remain on the planks and so the data for them is not presented. Results are presented as mean ± SEM.

2.4.2 Effect of Exercise Compared to Resveratrol on Open Field Behaviour

The impact of 3 weeks forced treadmill running and resveratrol ingestion on emotion and anxiety were compared using an open field assessment. Placed in an empty arena, qualitative and quantitative measurements of general locomotor activity and willingness to explore were taken. Assessed for the initial 5 min of the first habituation session, following 16 days of running and resveratrol treatment, one-way ANOVA analyses revealed no significant differences between YG groups for ambulation in terms of distance travelled ($F_{3,20} = 0.3492$, $p = 0.7902$) (Fig. 2-3A) and velocity ($F_{3,20} = 0.9438$, $p = 0.4381$) (Fig 2-3B). There were also no significant differences between MA groups for ambulation in terms of distance travelled ($F_{3,20} = 0.4748$, $p = 0.7033$) (Fig. 2-3C) and velocity ($F_{3,20} = 0.2346$, $p = 0.8712$) (Fig 2-3D). With no differences determined between age-matched groups, there were no sedative or stimulant effects of resveratrol treatment or aerobic exercise. Unpaired two-tailed t-test revealed that there was an age-related decline in distance travelled ($t_{46} = 2.143$, $^*p < 0.05$) and velocity ($t_{46} = 2.344$, $^*p < 0.05$) (data not represented).

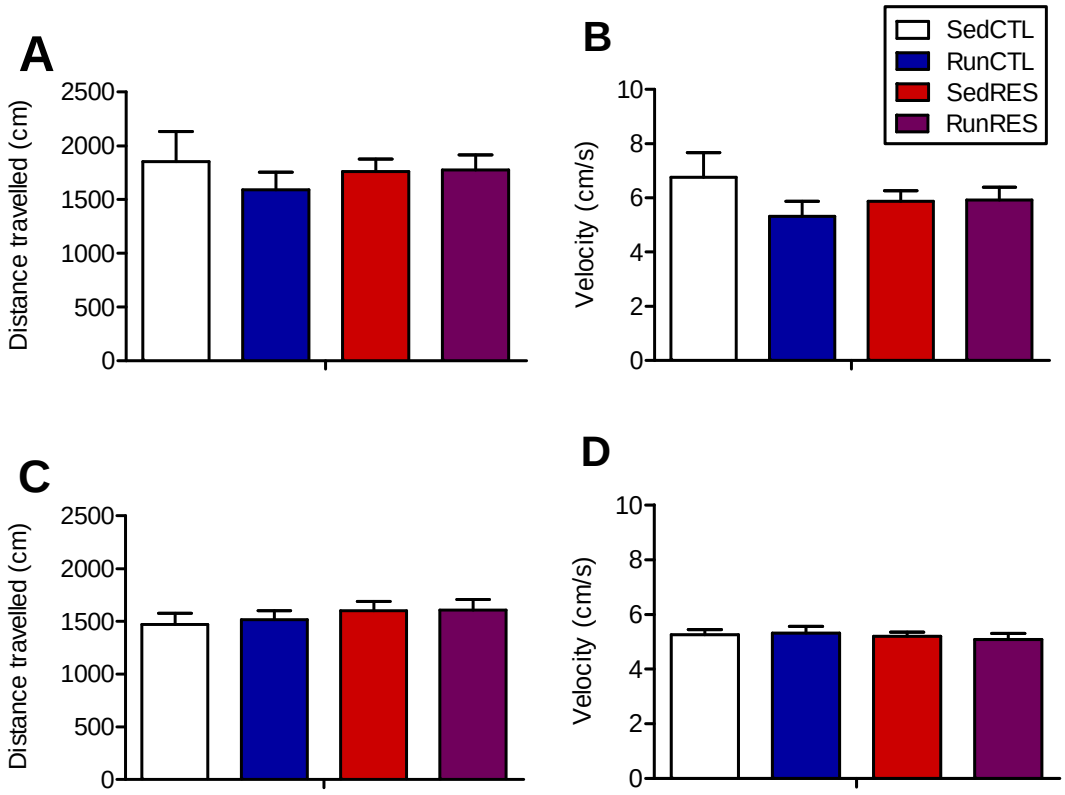


Fig. 2-3. No differences between groups were found in spontaneous locomotor behaviour in an open field during fourth week of regime. Locomotor activity was recorded for 5 min. SedCTL: pre-treatment with maple syrup alone (0.5 ml/d) and exposure to immobile treadmills (1h /d) for 16 d; RunCTL: pre-treatment with maple syrup alone (0.5 ml/d) and forced treadmill running (1h /d) for 16 d; SedRES: pre-treatment with resveratrol (20 mg/kg/d) in maple syrup and exposure to immobile treadmills (1h /d) for 16 d; RunRES: pre-treatment with resveratrol (20 mg/kg/d) in maple syrup and forced treadmill running (1h /d) for 16 d. Results are presented as mean ± SEM of the [A] distance travelled in centimetres (cm) and [B] velocity (cm/s) of young rats, and [C] distance travelled in centimetres (cm) and [D] velocity (cm/s) of middle-aged rats. *n* = 6 per group. No differences were found between groups.

Further analysis of general locomotor activity and willingness to explore was carried out and compared between groups when objects were added to the arena for the first NOR trial. All 4 groups in both YG and MA animals showed similar

sensitivity to changes in the local environment when the 3 objects were introduced to the open field environment for the initial training trial as assessed by the total exploration in YG ($F_{3,20}$ = 1.149, p = 0.3536) (Fig. 2-4A) and MA ($F_{3,20}$ = 1.114, p = 0.3667) (Fig. 2-5A). This further confirms that there were no sedative or stimulant effects of resveratrol treatment or aerobic exercise during the fourth week of the treatment regimes.

2.4.3 Treadmill Running and Resveratrol Ingestion have Similar Effects on NOR Performance

The impact of 4 weeks forced treadmill running with oral resveratrol treatment on recognition memory was assessed with an object substitution task (Fig. 2-1). Rats explored the objects on the training day for 3 x 5 min with a 5 min ITI. Over training trials 1 – 3, all YG groups showed habituation to their environment as expected, with total exploration time decreasing across trials (Fig. 2-4A). A two-way ANOVA revealed a significant effect of trial ($F_{2,40}$ = 23.407 , ***p < 0.001), but no significant trial x treatment interaction ($F_{6,40}$ = 0.95, p = 0.471), and no significant effect of treatment ($F_{3,20}$ =1.131, p = 0.361). This asymptotic habituation curve indicates normal encoding of the environmental features and spatial configuration, suggesting that any alterations to the environment should evoke increased exploratory behaviour.

A comparison of time spent exploring all objects during the NOR training trials and testing trial showed differences between groups. Differences between groups during the testing trial after a 24 h ITI were determined by one-way ANOVA analyses ($F_{3,20}$ = 6.304, *p < 0.01), with further *post hoc* analysis showing that resveratrol-treated animals explored more than sedentary controls (**p < 0.01) (Fig. 2-4A). Comparing exploration during the testing trial to the training trials, unpaired two-tailed t-test revealed that all YG treated groups showed similar exploration in the test trial compared to exploration in the initial training trial (T1), whereas

sedentary controls showed significantly less exploration (SedCTL: t_{10} = 2.134, *p < 0.05; RunCTL: t_{10} = 0.3392, p = 0.7415; SedRES: t_{10} = 0.4489, p = 0.6631; RunRES: t_{10} = 0.1032, p = 0.9198). Alternatively, sedentary controls showed similar exploration in the test trial compared to exploration in the final training trial (T3), whereas all treated groups showed significantly more exploration in testing compared to this trial (SedCTL: t_{10} = 0.09359, p = 0.9273; RunCTL: t_{10} = 3.282, **p < 0.01; SedRES: t_{10} = 4.013, ***p < 0.001; RunRES: t_{10} = 3.018, *p < 0.05) (data not represented).

Animals were assessed for exploration of object C (to be substituted in the testing trial) relative to objects A and B, during the 3 training trials. This is classed as the object discrimination ratio (ODR). ODR was averaged across the trials within treatment groups. All 4 groups spent approximately one third of the total exploration time exploring the object that was later replaced (SedCTL: t_{10} = 1.084, p = 0.304, ODR = 0.311; RunCTL: t_{10} = 0.01475, p = 0.9885, ODR = 0.333; SedRES: t_{10} = 0.4778, p = 0.6431, ODR = 0.354; RunRES: t_{10} = 0.02681, p = 0.9791, ODR = 0.333), signifying no preference for any one of the 3 objects (Fig. 2-4B). Young animals showed no particular interest for any specific object or object location during NOR training trials.

To measure long-term recognition memory using a 24 h ITI, during a 5 min testing trial the time spent exploring novel object D was compared to time spent exploring novel objects A and B. As rats show a natural tendency to explore new objects, greater exploration of object D indicates that animals remember objects A and B from the training trials. An unpaired two-tailed t-test showed that there was no indication of recognition memory in the YG sedentary controls following the 24 h delay as assessed by preferential exploration of novel object, D, suggesting that these rats were unable to learn this task (t_{10} = 0.8384, p = 0.4214, ODR = 0.298). All other groups showed increased exploration of novel object, D, relative to familiar objects, A and B (RunCTL: t_{10} = 3.473, **p < 0.01, ODR = 0.427; SedRES: t_{10} = 2.829, *p < 0.05, ODR = 0.432; RunRES: t_{10} = 2.456, *p < 0.05, ODR = 0.428), indicating an

enhancement in performance of this task in young animals following 4 weeks of resveratrol ingestion and treadmill running (Fig. 2-4C).

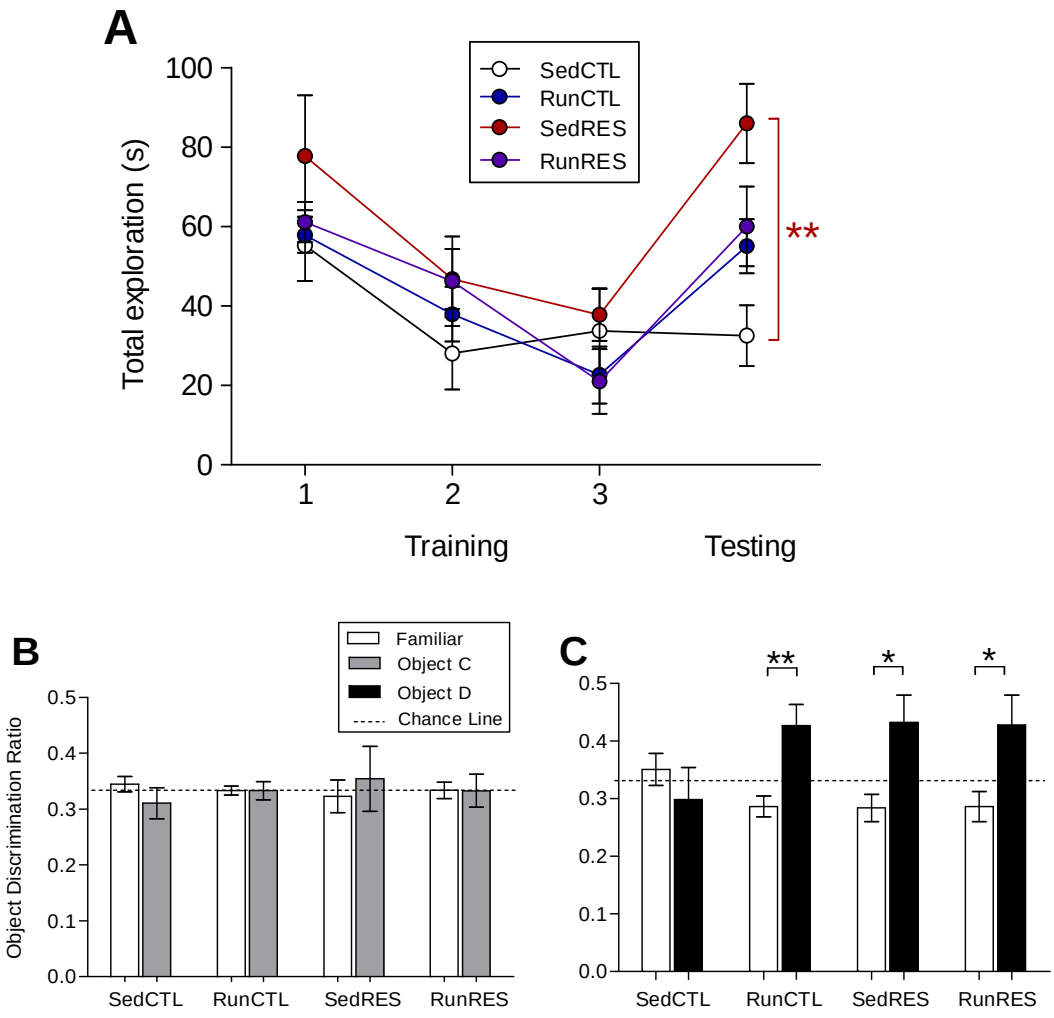


Fig. 2-4. A four week regime of resveratrol ingestion and treadmill running improved long-term recognition memory in young rats. [A] All groups showed habituation to their environment with total exploration time decreasing across training trials 1 – 3. Rats that underwent training or resveratrol ingestion explored objects more than sedentary controls following a 24 h delay. [B] Measurement of the time spent exploring each object was recorded and expressed as a discrimination ratio (novel object interaction/total interaction with all objects) for each training trial and averaged for each rat. No group showed any preference for object C over objects

A and B. [C] Following a 24 h delay animals were reintroduced to the arena with familiar objects A and B, and novel object D. Rats that underwent training or resveratrol ingestion explored novel object D more than familiar objects A and B. Sedentary control rats did not explore novel object D more than the familiar objects. $n = 6$ per group. Results are presented as mean ± SEM. Values significantly different from the sedentary control exploration and familiar object exploration are indicated with an asterisk (Tukey's HSD: *p < 0.05, **p < 0.01), with the running group in blue, resveratrol-treated in red, and those undergoing running and resveratrol-treatment in purple.

Middle-aged animals were assessed in the same task of long-term recognition memory. Over training trials 1 – 3, all MA groups showed habituation to their environment as expected, with total exploration time decreasing across trials (Fig. 2-5A). A two-way ANOVA revealed a significant effect of trial ($F_{2,40} = 83.364$, ***p < 0.001), but no significant trial x treatment interaction ($F_{6,40} = 0.412$, p = 0.867), and no significant effect of treatment ($F_{3,20} =1.038$, p = 0.397). This asymptotic habituation curve indicates normal encoding of the environmental features and spatial configuration, suggesting that any alterations to the environment should evoke increased exploratory behaviour.

Time spent exploring all objects during the NOR training trials and testing trial were compared to highlight any difference between groups. Differences were found during the testing trial after a 24 h ITI with one-way ANOVA analysis ($F_{3,20} = 3.947$, *p < 0.05). Further *post hoc* analysis showed that the running group explored more than sedentary controls (*p < 0.05) (Fig. 2-5A). Comparing exploration during the testing trial to the training trials, unpaired two-tailed t-test revealed that all MA treated groups showed similar exploration in the test trial compared to exploration in the initial training trial (T1), whereas sedentary controls showed significantly less exploration (SedCTL: $t_{10} = 2.528$, *p < 0.05; RunCTL: $t_{10} = 0.4484$, p = 0.6634; SedRES: $t_{10} = 1.378$, p = 0.1981; RunRES: $t_{10} = 0.612$, p =0.5542). Alternatively,

sedentary controls showed similar exploration in the test trial compared to exploration in the final training trial (T3), whereas all treated groups showed significantly more exploration in testing compared to this trial (SedCTL: t_{10} = 1.891, p = 0.0879; RunCTL: t_{10} = 3.774, **p < 0.01; SedRES: t_{10} = 3.302, **p < 0.01; RunRES: t_{10} = 3.086, *p < 0.05) (data not represented).

Animals were assessed for exploration of object C (to be substituted in the testing trial) relative to objects A and B, during the 3 training trials. ODR was averaged across the trials within treatment groups. All 4 groups spent approximately one third of the total exploration time exploring the object that was later replaced (SedCTL: t_{10} = 0.1283, p = 0.9004, ODR = 0.328; RunCTL: t_{10} = 0.2112, p = 0.8369, ODR = 0.327; SedRES: t_{10} = 1.298, p = 0.2234, ODR = 0.354; RunRES: t_{10} = 0.4419, p = 0.668, ODR = 0.356), signifying no preference for any one of the 3 objects (Fig. 2-5B). Middle-aged animals showed no particular interest for any specific object or object location during NOR training trials.

To measure long-term recognition memory using a 24 h ITI, during a 5 min testing trial the time spent exploring novel object D was compared to time spent exploring novel objects A and B. As rats show a natural tendency to explore new objects, greater exploration of object D indicates that animals remember objects A and B from the training trials. An unpaired two-tailed t-test indicated no recognition memory in the MA sedentary controls following the 24 h delay as assessed by preferential exploration of novel object, D, suggesting that these rats were unable to learn this task (t_{10} = 1.131, p = 0.2843, ODR = 0.316). All other groups showed increased exploration of novel object, D, relative to familiar objects, A and B (RunCTL: t_{10} = 2.463, *p < 0.05, ODR = 0.4401; SedRES: t_{10} = 3.078, *p < 0.05, ODR = 0.4785; RunRES: t_{10} = 3.201, **p < 0.01, ODR = 0.4155), indicating an enhancement in performance of this task in middle-aged animals following 4 weeks of resveratrol ingestion and treadmill running (Fig. 2-5C).

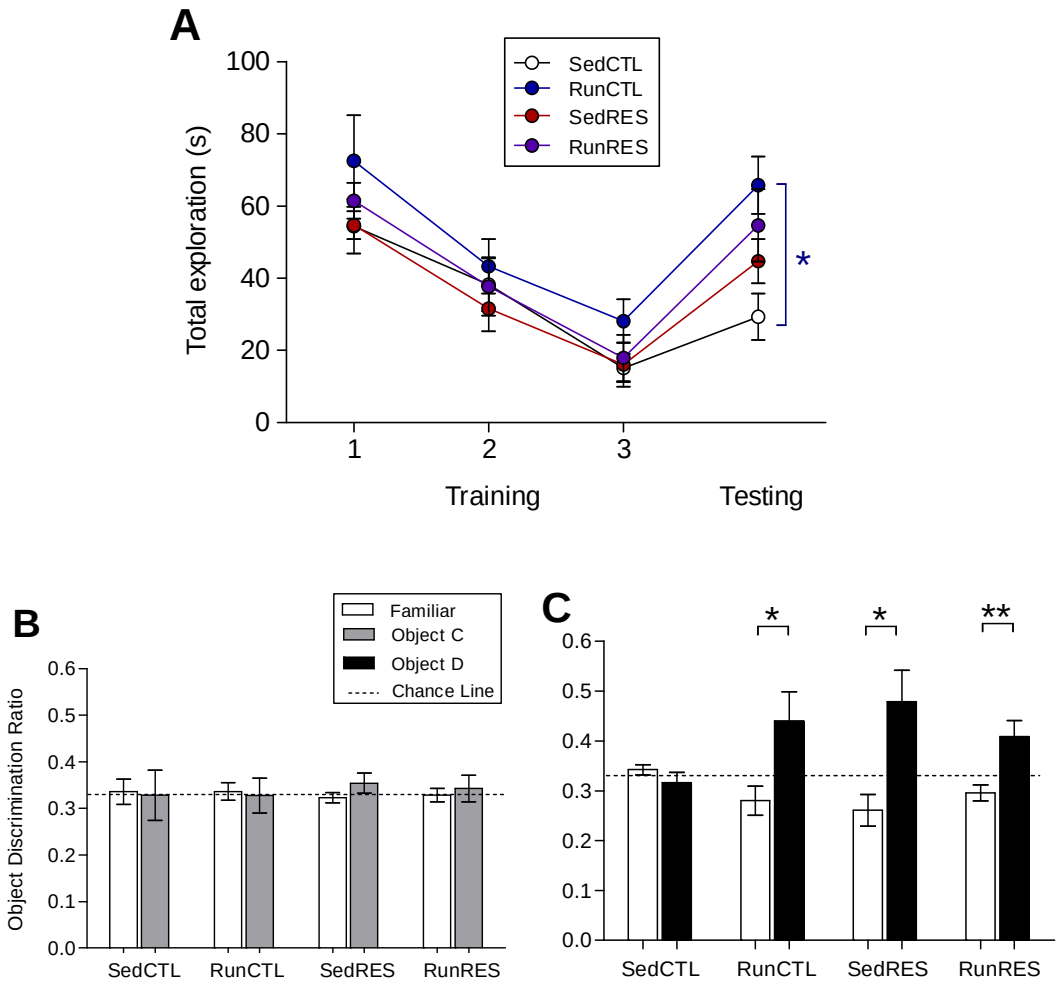


Fig. 2-5 A four week regime of resveratrol ingestion and treadmill running improved long-term recognition memory in middle-aged rats. [A] All groups showed habituation to their environment with total exploration time decreasing across training trials 1 – 3. Rats that underwent training or resveratrol ingestion explored objects more than sedentary controls following a 24 h delay. [B] Measurement of the time spent exploring each object was recorded and expressed as a discrimination ratio (novel object interaction/total interaction with all objects) for each training trial and averaged for each rat. No group showed any preference for object C over objects A and B. [C] Following a 24 h delay animals were reintroduced to the arena with familiar objects A and B, and novel object D. Rats that underwent training or resveratrol ingestion explored novel object D more than familiar objects A and B. Sedentary control rats did not explore novel object D more than the familiar objects. n = 6 per group. Results are presented as mean ± SEM. Values significantly different from the sedentary control exploration and familiar object exploration are indicated with an asterisk (Tukey's HSD: *$p < 0.05$, **$p < 0.01$), with the running group in blue, resveratrol-treated in red, and those undergoing running and resveratrol-treatment in purple.

2.4.4 Effect on Protein Expression in Hippocampus and Perirhinal Cortex

Hippocampal and perirhinal tissue was extracted from the brains of all rats and assessed for expression of proteins from the AMPK/SIRT1 pathways. This would indicate if proteins implicated in many beneficial actions of resveratrol and aerobic exercise physiologically were also involved in the cognitive enhancement observed with these treatment regimes. Expression of SIRT1, AMPKα1, AMPKα2, PGC-1α, MnSOD, BDNF, and NGF was assessed in these brain structures associated with learning and memory using real-time PCR. In young animals, one-way ANOVA analysis revealed significant differences between groups in BDNF mRNA levels in dentate gyrus ($F_{3,20}$ = 8.156, ***p < 0.001) with further *post hoc* analysis revealing significant increases with all treatment regimes relative to YG sedentary controls (RunCTL: **p < 0.01; SedRES: **p < 0.01; RunRES: **p < 0.01). One-way ANOVA analysis also revealed significant differences between groups in NGF mRNA levels in dentate gyrus ($F_{3,20}$ = 7.958, **p < 0.01) with further *post hoc* analysis revealing significant increases with all treatment regimes relative to YG sedentary controls (RunCTL: **p < 0.01; SedRES: **p < 0.01; RunRES: **p < 0.01). Additional one-way ANOVA analyses revealed no significant differences between YG groups for dentate gyrus expression of SIRT1 ($F_{3,20}$ = 0.1774, p = 0.915), subunit AMPKα1 ($F_{3,20}$ = 0.2238, p = 0.8787), subunit AMPKα2 ($F_{3,20}$ = 0.4257, p = 0.7367), PGC-1α ($F_{3,20}$ = 0.05454, p = 0.9827), and MnSOD ($F_{3,20}$ = 0.8136, p = 0.5014) (Fig. 2-6A).

Analysis of the rest of the hippocampus and perirhinal cortex showed similar enhancement of neurotrophins in young animals. One-way ANOVA analysis revealed significant differences between groups of BDNF mRNA levels in hippocampus ($F_{3,20}$ = 8.358, ***p < 0.001) with further *post hoc* analysis revealing significant increases with all treatment regimes relative to YG sedentary controls (RunCTL: **p < 0.01; SedRES: *p < 0.05; RunRES: **p < 0.01). One-way ANOVA analysis also revealed significant differences between groups of NGF mRNA levels in hippocampus ($F_{3,20}$ = 7.199, **p < 0.01) with further *post hoc* analysis revealing significant increases with all treatment regimes relative to YG sedentary controls (RunCTL: **p < 0.01; SedRES: *p < 0.05; RunRES: **p < 0.01).

Additional one-way ANOVA analyses revealed no significant differences between YG groups for hippocampal expression of SIRT1 ($F_{3,20} = 0.182$, $p = 0.9074$), subunit AMPKα1 ($F_{3,20} = 0.03317$, $p = 0.9916$), subunit AMPKα2 ($F_{3,20} = 0.5854$, $p = 0.6315$), PGC-1α ($F_{3,20} = 0.06511$, $p = 0.9777$), and MnSOD ($F_{3,20} = 0.9665$, $p = 0.4279$) (Fig. 2-6B). One-way ANOVA analysis revealed significant differences between groups of BDNF mRNA levels in perirhinal cortex ($F_{3,20} = 8.132$, ***$p < 0.001$) with further *post hoc* analysis revealing significant increases with all treatment regimes relative to YG sedentary controls (RunCTL: *$p < 0.05$; SedRES: **$p < 0.01$; RunRES: **$p < 0.01$). One-way ANOVA analysis also revealed significant differences between groups of NGF mRNA levels in perirhinal cortex ($F_{3,20} = 9.067$, ***$p < 0.001$) with further *post hoc* analysis revealing significant increases with all treatment regimes relative to YG sedentary controls (RunCTL: **$p < 0.01$; SedRES: **$p < 0.01$; RunRES: **$p < 0.01$). Additional one-way ANOVA analyses revealed no significant differences between YG groups for perirhinal expression of SIRT1 ($F_{3,20} = 0.2890$, $p = 0.8328$), subunit AMPKα1 ($F_{3,20} = 0.5255$, $p = 0.6698$), subunit AMPKα2 ($F_{3,20} = 0.1487$, $p = 0.9293$), PGC-1α ($F_{3,20} = 0.2083$, $p = 0.8894$), and MnSOD ($F_{3,20} = 0.09508$, $p = 0.9619$) (Fig. 2-6C).

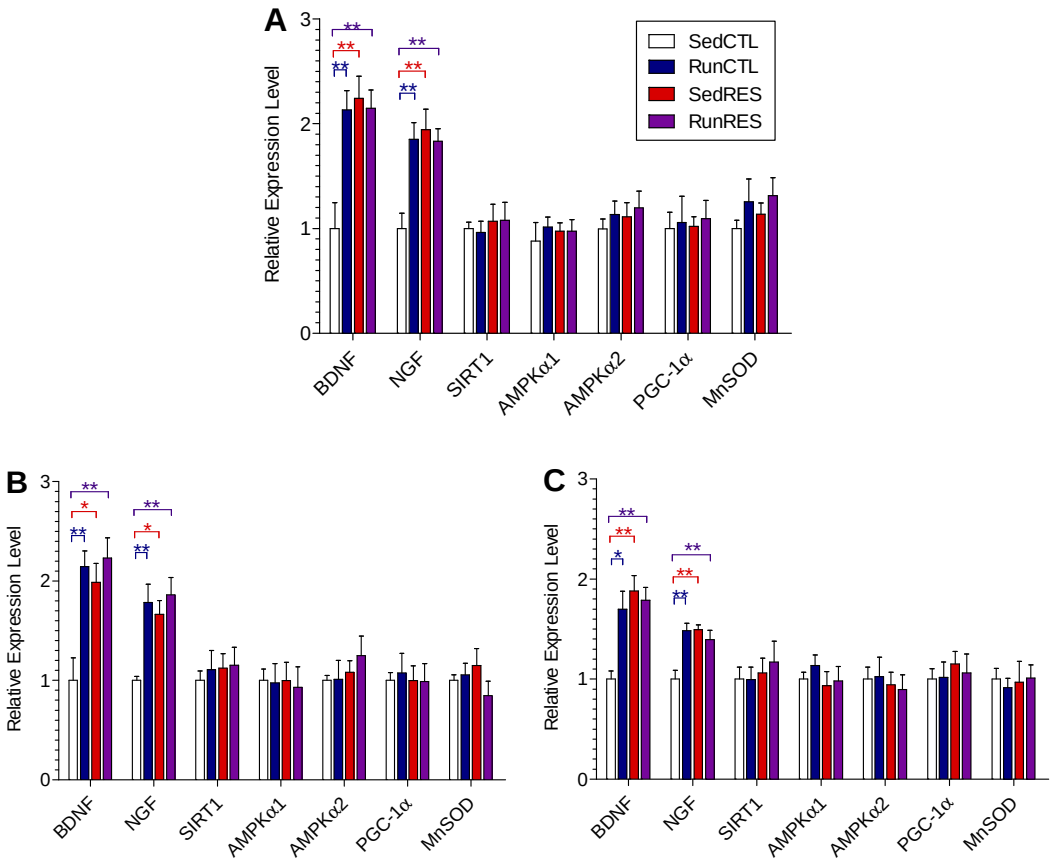


Fig. 2-6. In young rats, neurotrophin expression increased in perirhinal and hippocampal tissue following 4 week regimes of resveratrol ingestion and treadmill running. mRNA levels in hippocampus and perirhinal cortex were measured by RT-PCR analysis. Levels of BDNF and NGF mRNA were increased in [A] dentate gyrus, [B] rest of hippocampus and [C] perirhinal cortex of rats that underwent training or resveratrol ingestion, relative to sedentary controls. For expression of other analysed protein mRNAs no differences were found between groups. n = 6 per group. Results are presented as mean ± SEM. Values significantly different from the sedentary control expression level are indicated with an asterisk (Tukey's HSD: *p < 0.05, **p < 0.01), with the running group in blue, resveratrol-treated in red, and those undergoing running and resveratrol-treatment in purple.

Similar effects on expression levels were found in middle-aged animals as in young. In middle-aged animals, one-way ANOVA revealed significant differences between groups of BDNF mRNA levels in dentate gyrus ($F_{3,20}$ = 6.7, **p < 0.01)

with further *post hoc* analysis revealing significant increases with all treatment regimes relative to MA sedentary controls (RunCTL: *p < 0.05; SedRES: **p < 0.01; RunRES: **p < 0.01). One-way ANOVA analysis similarly revealed significant differences between groups of NGF mRNA levels in dentate gyrus ($F_{3,20}$ = 4.264, *p < 0.05) with further *post hoc* analysis revealing significant increases with all treatment regimes relative to MA sedentary controls (RunCTL: *p < 0.05; SedRES: *p < 0.05; RunRES: *p < 0.05). Additional one-way ANOVA analyses revealed no significant differences between MA groups for dentate gyrus expression of SIRT1 ($F_{3,20}$ = 0.01406, p = 0.9976), subunit AMPKα1 ($F_{3,20}$ = 0.5151, p = 0.6766), subunit AMPKα2 ($F_{3,20}$ = 0.6910, p = 0.6910), PGC-1α ($F_{3,20}$ = 0.4444, p = 0.7239), and MnSOD ($F_{3,20}$ = 0.2165, p = 0.8838) (Fig. 2-7A).

Analysis of the rest of the hippocampus and perirhinal cortex showed similar enhancement of neurotrophins in middle-aged animals. One-way ANOVA analysis revealed significant differences between groups of BDNF mRNA levels in hippocampus ($F_{3,20}$ = 7.878, **p < 0.01) with further *post hoc* analysis revealing significant increases with all treatment regimes relative to MA sedentary controls (RunCTL: **p < 0.01; SedRES: **p < 0.01; RunRES: *p < 0.05). One-way ANOVA analysis also revealed significant differences between groups of NGF mRNA levels in hippocampus ($F_{3,20}$ = 6.074, **p < 0.01) with further *post hoc* analysis revealing significant increases with all treatment regimes relative to MA sedentary controls (RunCTL: *p < 0.05; SedRES: *p < 0.05; RunRES: **p < 0.01). Additional one-way ANOVA analyses revealed no significant differences between MA groups for hippocampal expression of SIRT1 ($F_{3,20}$ = 0.09882, p = 0.9597), subunit AMPKα1 ($F_{3,20}$ = 0.3014, p = 0.8240), subunit AMPKα2 ($F_{3,20}$ = 0.1725, p = 0.9137), PGC-1α ($F_{3,20}$ = 0.155, p = 0.9252), and MnSOD ($F_{3,20}$ = 0.07967, p = 0.9703) (Fig. 2-7B). One-way ANOVA analysis revealed significant differences between groups of BDNF mRNA levels in perirhinal cortex ($F_{3,20}$ = 4.509, *p < 0.05) with further *post hoc* analysis revealing significant increases with all treatment regimes relative to MA sedentary controls (RunCTL: *p < 0.05; SedRES: *p < 0.05; RunRES: *p < 0.05). One-way ANOVA analysis also revealed significant differences between groups of NGF mRNA levels in perirhinal coretex ($F_{3,20}$ = 4.797, *p < 0.05) with further *post hoc* analysis revealing significant increases with

all treatment regimes relative to MA sedentary controls (RunCTL: *p < 0.05; SedRES: *p < 0.05; RunRES: *p < 0.05). Additional one-way ANOVA analyses revealed no significant differences between MA groups for perirhinal expression of SIRT1 ($F_{3,20}$ = 0.02649, p = 0.994), subunit AMPKα1 ($F_{3,20}$ = 0.364, p = 0.7797), subunit AMPKα2 ($F_{3,20}$ = 0.2367, p = 0.8697), PGC-1α ($F_{3,20}$ = 0.02265, p = 0.9952), and MnSOD ($F_{3,20}$ = 0.2391, p = 0.8680) (Fig. 2-7C). The improvements detected in long-term recognition memory with these 4 week treatment regimes of oral resveratrol and aerobic exercise were associated with increased expression of neurotrophins in brain regions involved in learning and memory. It may be increased levels of these proteins that are important for the cognitive enhancement associated with resveratrol treatment and aerobic exercise.

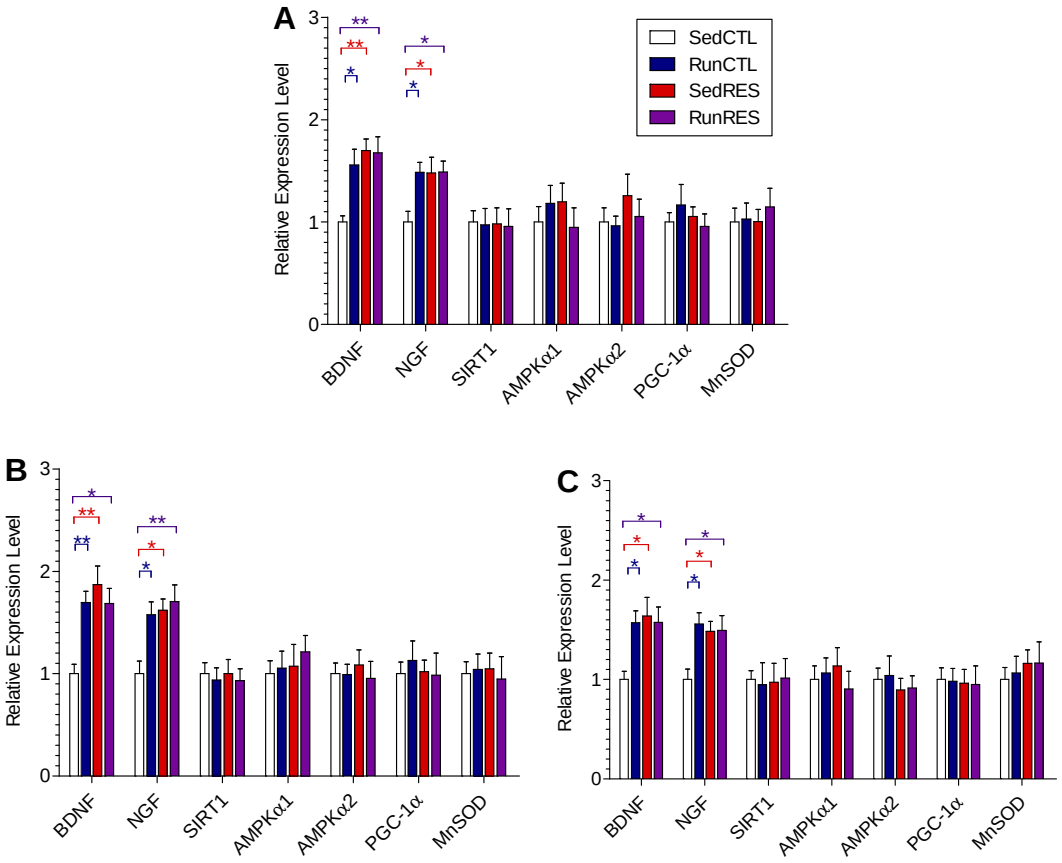


Fig. 2-7. In middle-aged rats, neurotrophin expression increased in perirhinal and hippocampal tissue following 4 week regimes of resveratrol ingestion and treadmill running. mRNA levels in hippocampus and perirhinal cortex were measured by RT-

PCR analysis. Levels of BDNF and NGF mRNA were increased in [A] dentate gyrus, [B] rest of hippocampus and [C] perirhinal cortex of rats that underwent training or resveratrol ingestion, relative to sedentary controls. For expression of other analysed protein mRNAs no differences were found between groups. $n = 6$ per group. Results are presented as mean ± SEM. Values significantly different from the sedentary control expression level are indicated with an asterisk (Tukey's HSD: *p < 0.05, **p < 0.01), with the running group in blue, resveratrol-treated in red, and those undergoing running and resveratrol-treatment in purple.

2.4.5 Effect on BDNF and NGF Levels in Hippocampus and Perirhinal Cortex

With increased expression of neurotrophins in hippocampal and perirhinal tissue in groups administered resveratrol and treadmill running, the potential importance of these proteins in long-term memory enhancement was further explored by measuring protein levels in these tissues. Levels of BDNF and NGF were measured in dentate gyrus, rest of the hippocampus, and the perirhinal cortex using ELISA. In young animals, one-way ANOVA revealed significant differences in the levels of BDNF ($F_{3,20} = 7.309$, **p < 0.01) and NGF ($F_{3,20} = 8.306$, ***p < 0.001) in the dentate gyrus. *Post hoc* analysis revealed significant increases with all treatment regimes relative to YG sedentary controls of BDNF (RunCTL: *p < 0.05; SedRES: **p < 0.01; RunRES: *p < 0.05) and NGF (RunCTL: **p < 0.01; SedRES: **p < 0.01; RunRES: *p < 0.05) (Fig. 2-8A). One-way ANOVA also revealed significant differences in the levels of BDNF ($F_{3,20} = 5.571$, **p < 0.01) and NGF ($F_{3,20} = 6.653$, **p < 0.01) in the remainder of the hippocampus. *Post hoc* analysis revealed significant increases with all treatment regimes relative to YG sedentary controls of BDNF (RunCTL: *p < 0.05; SedRES: *p < 0.05; RunRES: **p < 0.01) and NGF (RunCTL: **p < 0.01; SedRES: **p < 0.01; RunRES: *p < 0.05) (Fig. 2-8B). One-way ANOVA also revealed significant differences in the perirhinal levels of BDNF ($F_{3,20} = 7.226$, **p < 0.01) and NGF ($F_{3,20} = 6.515$, **p < 0.01). *Post hoc* analysis revealed significant increases with all treatment regimes relative to YG

sedentary controls of BDNF (RunCTL: **p < 0.01; SedRES: **p < 0.01; RunRES: *p < 0.05) and NGF (RunCTL: *p < 0.05; SedRES: *p < 0.05; RunRES: **p < 0.01) (Fig. 2-8C).

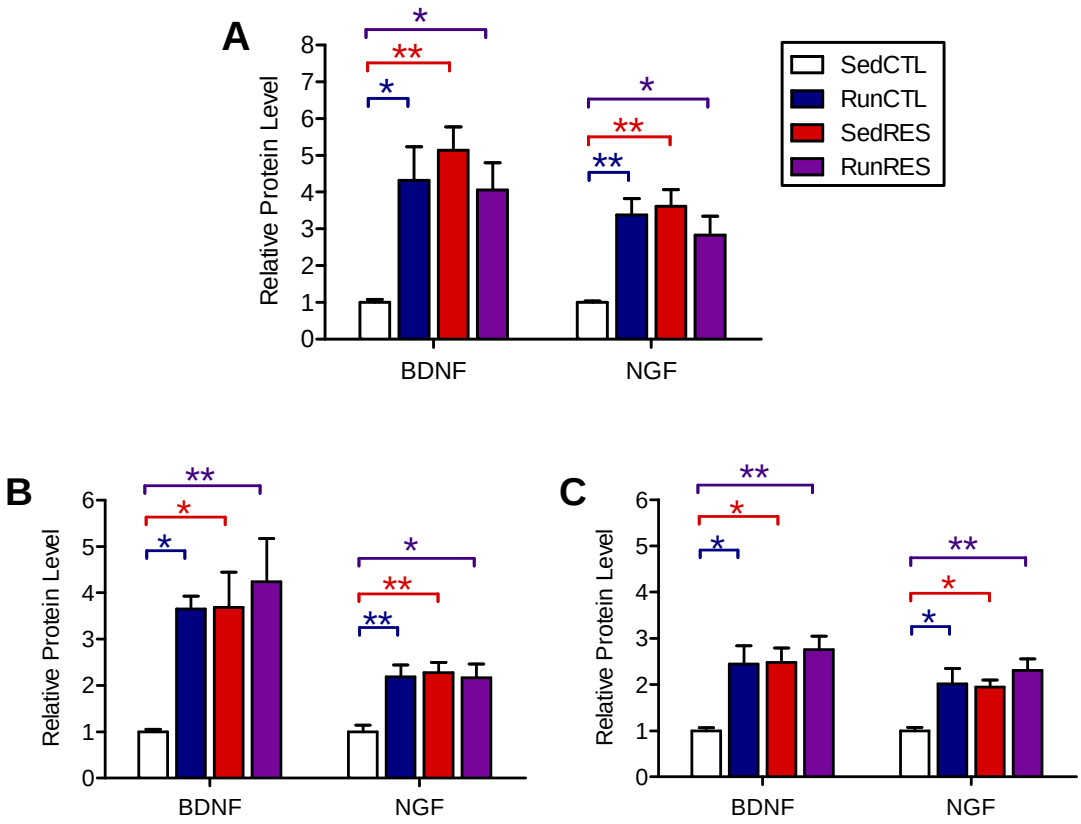


Fig. 2-8. In young rats, neurotrophin levels increased in perirhinal and hippocampal tissue following 4 week regimes of resveratrol ingestion and treadmill running. Protein levels in hippocampus and perirhinal cortex were measured by ELISA. Levels of BDNF and NGF were increased in [A] dentate gyrus, [B] rest of hippocampus and [C] perirhinal cortex of rats that underwent training or resveratrol ingestion, relative to sedentary controls. *n* = 6 per group. Results are presented as mean ± SEM. Values significantly different from the sedentary control protein level are indicated with an asterisk (Tukey's HSD: *p < 0.05, **p < 0.01), with the running group in blue, resveratrol-treated in red, and those undergoing running and resveratrol-treatment in purple.

Similar effects on neurotrophin levels were found in middle-aged animals as in young. In middle-aged animals, one-way ANOVA revealed significant differences in the levels of BDNF ($F_{3,20} = 6.537$, **p < 0.01) and NGF ($F_{3,20} = 7.375$, **p < 0.01) in the dentate gyrus. *Post hoc* analysis revealed significant increases with all treatment regimes relative to MA sedentary controls of BDNF (RunCTL: *p < 0.05; SedRES: **p < 0.01; RunRES: **p < 0.01) and NGF (RunCTL: **p < 0.01; SedRES: **p < 0.01; RunRES: *p < 0.05) (Fig. 2-9A). One-way ANOVA also revealed significant differences in the levels of BDNF ($F_{3,20} = 9.08$, ***p < 0.001) and NGF ($F_{3,20} = 7.647$, **p < 0.01) in the remainder of the hippocampus. *Post hoc* analysis revealed significant increases with all treatment regimes relative to MA sedentary controls of BDNF (RunCTL: **p < 0.01; SedRES: **p < 0.01; RunRES: **p < 0.01) and NGF (RunCTL: *p < 0.05; SedRES: **p < 0.01; RunRES: **p < 0.01) (Fig. 2-9B). One-way ANOVA also revealed significant differences in the perirhinal levels of BDNF ($F_{3,20} = 4.948$, **p < 0.01) and NGF ($F_{3,20} = 5.482$, **p < 0.01). *Post hoc* analysis revealed significant increases with all treatment regimes relative to MA sedentary controls of BDNF (RunCTL: **p < 0.01; SedRES: *p < 0.05; RunRES: *p < 0.05) and NGF (RunCTL: *p < 0.05; SedRES: *p < 0.05; RunRES: *p < 0.05) (Fig. 2-9C). The improvements detected in long-term recognition memory with these treatment regimes of oral resveratrol and aerobic exercise were associated with increased neurotrophin levels in brain regions involved in learning and memory. These findings further support the belief that these 4 week treatment regimes of resveratrol ingestion and treadmill running may improve cognition through increased levels of these proteins.

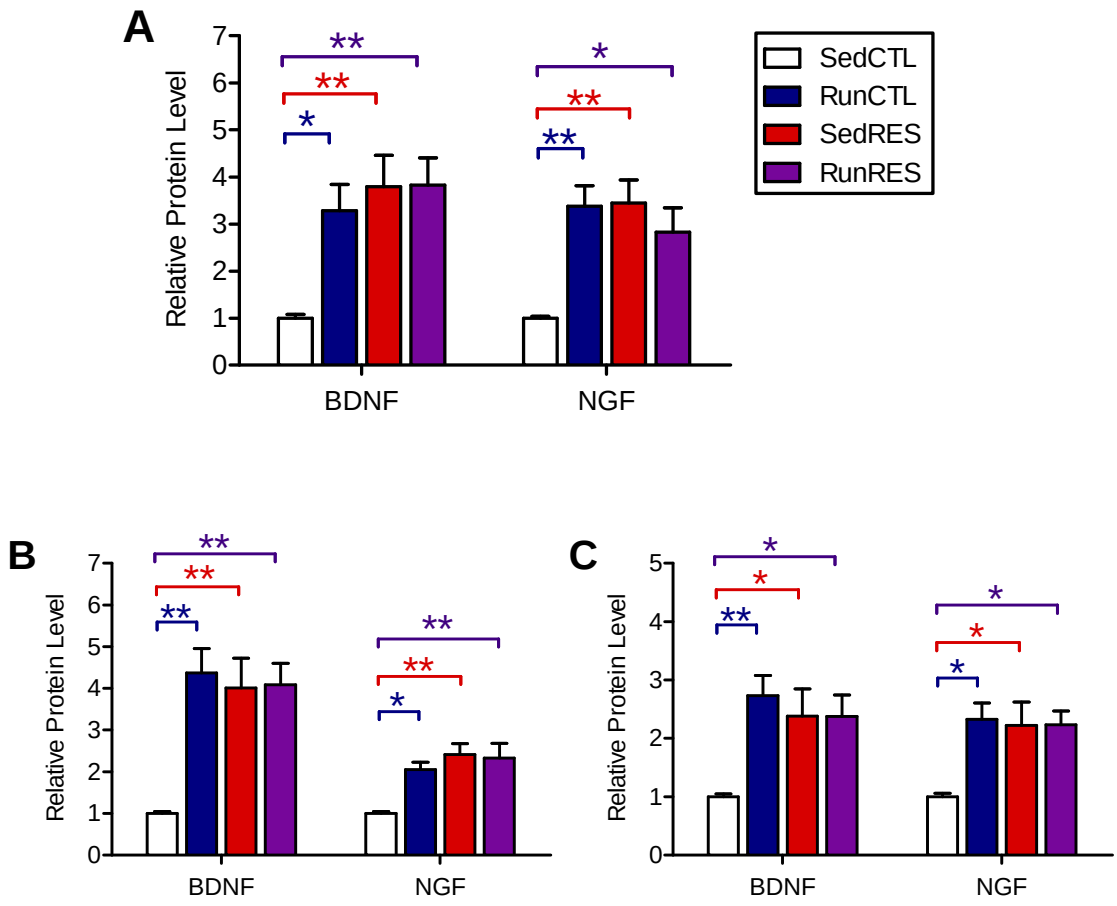


Fig. 2-9. In middle-aged rats, neurotrophin levels increased in perirhinal and hippocampal tissue following 4 week regimes of resveratrol ingestion and treadmill running. Protein levels in hippocampus and perirhinal cortex were measured by ELISA. Levels of BDNF and NGF were increased in [A] dentate gyrus, [B] rest of hippocampus and [C] perirhinal cortex of rats that underwent training or resveratrol ingestion, relative to sedentary controls. n = 6 per group. Results are presented as mean ± SEM. Values significantly different from the sedentary control protein level are indicated with an asterisk (Tukey's HSD: *p < 0.05, **p < 0.01), with the running group in blue, resveratrol-treated in red, and those undergoing running and resveratrol-treatment in purple.

2.5 DISCUSSION

There were two major aims of this study. The first aim was to evaluate the similarities of 4 weeks of resveratrol ingestion and aerobic exercise on healthy long-term memory and natural memory decline associated with ageing. These findings indicate that with 4 weeks (5d/week) of resveratrol treatment (20 mg/kg; p.o.) and treadmill running (1 h/day), either combined or individually, there are significant improvements in long-term recognition memory in a healthy model and a model of natural memory decline. The second aim was to determine the potential mechanisms through which both aerobic exercise and resveratrol ingestion may act to evoke their beneficial action on learning and memory. Memory enhancement following 4 weeks of resveratrol ingestion and aerobic exercise was associated with increased BDNF and NGF expression and levels in the hippocampus and perirhinal cortex. Proteins involved in the AMPK/SIRT1 pathways were not upregulated in these brain regions associated with learning and memory.

Many of the physiological benefits of aerobic exercise are well understood in the general population, but there are additional cognitive benefits that are not as well documented in the media. Aerobic exercise has a desirable effect on improving working memory (Clarkson-Smith and Hartley, 1989), long-term memory (Griffin et al., 2009) and spatial learning (Kobilo et al., 2011). This cognitive enhancement is thought to be linked to increased neurogenesis (van Praag, 1999) and neurotrophin levels (Neeper et al., 1995, 1996; O'Callaghan et al., 2007; Griffin et al., 2009). The benefits of aerobic exercise make it desirable to identify orally active agents that mimic or potentiate these effects; one such candidate is the polyphenol, resveratrol. Resveratrol can be termed an exercise mimetic due to its similar effects on mitochondrial biogenesis, endurance, metabolism and the cardiovascular system (Um et al., 2010). This polyphenol has also been shown to have protective properties against animal models of noncommunicable diseases, such as Alzheimer's disease (Marambaud et al., 2005; Kim et al., 2007), diabetes type 2 (Thirunavukkarasu et al., 2007; Szkudelska and Szkudelski, 2010), Huntington's disease (Kumar et al., 2006; Ho et al., 2010), cardiovascular disease (Jäger and Nguyen-Duong, 1999; Ray et al., 1999; Zini et al, 1999), and some cancers (Jang et al., 1997; Bove et al., 2002). To

confirm the beneficial effects on learning and memory, and compare the similarity of action, a direct comparison of behaviour following regular aerobic exercise and oral resveratrol treatment was conducted, with follow up analysis of biological markers.

Before treatment, all groups expressed normal health, with good balance and co-ordination measured in the young animals using a plank walk task. Middle-aged animals were larger and found it too difficult to balance on the plank. These complex motor behaviours have been shown to be excellent in young healthy rats and steadily decline with ageing (Shukitt-Hale et al., 1998); however, this could not be confirmed here due to the confounding aspect of the size of the older animals. Young animals were evenly matched between groups according to performance in this assessment of complex motor behaviours, with middle-aged animals randomly assigned to treatment groups. During the fourth week of resveratrol ingestion and treadmill running, general exploration and spontaneous behaviour in response to a novel environment was assessed in an open field. Measurements of general locomotor activity and willingness to explore novel areas allow assessment of animal emotion and anxiety (Stanford, 2007). All young and middle-aged groups spontaneously explored the arena normally, in terms of distance travelled and velocity. Groups did not differ in terms of initial exploration in the task (training trial 1), indicating similar visuomotor function and exploratory tendencies. With no differences determined between age-matched groups, there were no sedative or stimulant effects of resveratrol treatment or aerobic exercise. This supports findings from other studies investigating the sedative effects of resveratrol administration. No sedative effects were found with acute doses of 20, 40 and 80 mg/kg i.p. in rats with pentylenetetrazole (PTZ) induced seizures (Gupta et al., 2002b) or 20 mg/kg i.p. in healthy rats (Gupta et al., 2004). Interestingly, studies using higher doses of resveratrol have found contradictory findings following much longer treatment regimes, with one stating decreased spontaneous locomotor activity in rats with a 15 week treatment regime (200 and 400 mg/kg p.o. daily) (Lagouge et al., 2006) and another finding increased spontaneous locomotor activity in grey mouse lemurs with an 18 month regime (200 mg/kg p.o. daily) (Dal-Pan et al., 2011). In addition, all groups showed normal habituation to their spatial environment, with gradually decreasing exploration of objects across training trials in young and middle-aged

animals. This asymptotic habituation curve indicates normal encoding of the environmental features and spatial configuration, suggesting that any alterations to the environment should evoke increased exploratory behaviour.

A substitution version of the novel object recognition task was used (Fig. 2-1). It has previously been shown that this is too difficult for healthy young rats to perform following a 24 h delay with just 3 x 5 min training trials (Griffin et al., 2009). This task was chosen to highlight any enhancement associated with resveratrol ingestion and treadmill running. Resveratrol has been shown to evoke neuroprotective effects against traumatic brain injury with a single 100 mg/kg i.p. dose administered immediately after induction of brain injury (Sönmez et al., 2007), against Alzheimer's disease symptoms *in vitro* (Wu et al., 2008), and against seizures using a single dose of 40 mg.kg i.p. administered 5 min before kainic acid-induced seizure (Gupta et al., 2002a). 7 weeks of voluntary wheel running also evokes neuroprotection against brain injury in rats (Will et al., 2004), and 6 to 8 months of aerobic exercise improves cognitive scores in elderly people with mild cognitive impairment (MCI) and dementia (Ahlskog et al., 2011). In this study, no recognition memory was evident with the young or middle-aged untreated, sedentary controls after a 24 h delay as assessed by the object discrimination ratio of novel object, D. All other groups showed increased exploration of novel object, D, relative to familiar objects, A and B, in young and middle-aged animals. It was important to rule out preference for the object itself rather than the fact it was novel, as shown by disinterest from the sedentary controls 24 h later, as well as preference for object location, indicated by assessment of the object discrimination ratio of object C in training sessions relative to objects A and B. These findings indicate that after 4 weeks of resveratrol treatment and aerobic exercise, either combined or individually, there are significant improvements in long-term recognition memory in the healthy young rat and the middle-aged rat that has natural memory decline associated with ageing. Resveratrol administration is able to improve cognition in healthy young animals with already high cognitive function, as well as improving natural (Oomen et al., 2009) and enforced (Gupta et al., 2002a; Sönmez et al., 2007; Wu et al., 2008) memory decline, as shown in other studies. 7 consecutive days of treadmill running has previously been shown to improve object recognition memory in healthy rats

(O'Callaghan et al., 2007; Griffin et al., 2009). Although no cognitive studies with resveratrol have been carried out in healthy young animals, resveratrol has been shown to improve cognitive function in aged animals, with mice given 150 µg resveratrol per gram of food for 6 to 8 months performing better in a Y-maze task (Oomen et al., 2009). With questions raised over the bioavailability of oral resveratrol *in vivo* (Baur and Sinclair, 2006), it is of importance to determine whether supplementation of this compound is a realistic cognitive therapy. These results support the belief that resveratrol supplementation and aerobic exercise may prove to be suitable cognitive therapies.

Compared to sedentary control animals, the three treated groups, although exhibiting the natural habituation curve during training trials, 24 h later showed renewed interest in all objects with a total exploration time similar to those shown in training trial 1 in both young and middle-aged animals. In fact, it appears that both young and middle-aged untreated, sedentary control rats lost interest in all objects immediately following their first exposure to the objects, and this disinterest remained 24 h later even when one of the objects was substituted. The lack of exploration of control rats during the testing phase indicates that the rats had some recollection of being exposed to the objects before but their memory was not vivid enough to realise that one of the objects was novel or this would have been indicated by an object discrimination ratio above 0.333. All other groups showed renewed interest in objects, with most attention directed towards the novel object D, further indicating better long-term memory than young and middle-aged sedentary controls.

Hippocampal and perirhinal tissue was extracted from the brains of all rats and assessed for expression and levels of proteins previously implicated in the beneficial action of resveratrol and aerobic exercise on the body. Many studies investigating cognition refer to resveratrol as a SIRT1 activator (Kim et al., 2007; Pallas et al., 2009; Baur, 2010) since this compound was found to consistently recapitulate the protective effects of SIRT1 overexpression in cell culture (Howitz et al., 2003; Araki et al., 2004). However, with no evidence to indicate that resveratrol is a direct activator of SIRT1, it may prove misleading to refer to resveratrol as

simply a SIRT1 activator. More recently, AMPK has been suggested as an alternative target for resveratrol that may be important for upregulating the beneficial pathways associated with resveratrol action (Narkar et al., 2008), with an interdependence of these proteins highlighted in another study (Price et al., 2012). In this study, results of the polymerase chain reaction further indicated that proteins involved in the AMPK/SIRT1 pathways were not upregulated in brain regions associated with learning and memory following 4 weeks of these treatment regimes. No increases in levels of SIRT1, AMPKα1, AMPKα2, PGC-1α or MnSOD mRNA were found in the dentate gyrus, rest of hippocampus, or perirhinal cortex in either young or middle-aged animals following resveratrol ingestion or treadmill running. Other *in vivo* studies have found increased levels of proteins involved in the AMPK/SIRT1 pathways with more localised and higher dosing regimes. SIRT1 expression in the hippocampus has been shown to increase with resveratrol administered intracerebroventricularly (5 µg/µl) (Kim et al., 2007), with MnSOD levels increasing with oral administration of 200 mg/kg resveratrol in rats on a high-fat diet, but not rats on a standard diet (Robb et al., 2008). The findings here suggest that although resveratrol may work through the AMPK/SIRT1 pathways to evoke many beneficial physiological effects, these pathways do not appear to be involved in the memory enhancement associated with resveratrol administration or aerobic exercise. As AMPK overactivation has recently been associated with neurofibrillary tangles of hyperpolarised microtubule-associated protein tau, such as found with Alzheimer's disease (Vingtdeux et al., 2011), it is of benefit to discover that cognitive enhancement through aerobic exercise and resveratrol does not require activation of this protein. There were significant increases in expression of the neurotrophins, BDNF and NGF, in these brain regions; with levels of these proteins also increased relative to sedentary controls. Additionally, increased BDNF and NGF expression has been measured in the hippocampus following 2, 4 and 7 nights of voluntary wheel running in rats (Neeper et al., 1996), with acute bouts of exercise increasing vascular endothelial growth factor (VEGF) levels in untrained human skeletal muscle, but not trained skeletal muscle (Richardson et al., 2000). Increased circulating VEGF levels have been measured with 2.5 mg/kg p.o. resveratrol daily for 15 days in streptozotocin-induced diabetic rats (Thirunavukkarasu et al., 2007) and dose-dependent increases in hippocampal BDNF levels were found when comparing resveratrol doses ranging from 2.5 to 20 mg/kg p.o. daily for 3, 10 and 30

days (Rahvar et al., 2011). It appears that resveratrol and aerobic exercise may evoke their effects on cognition through neurotrophins, or other proteins implicated in these pathways. This supports the suggestion that these regimes may enhance memory by encouraging early cell survival in brain regions associated with learning and memory (Frielingsdorf et al., 2007; van Praag, 2009).

The data presented here suggest that a four week regime of resveratrol ingestion and aerobic exercise have similar positive impacts on functioning of the hippocampal formation and perirhinal cortex as measured by long-term recognition of object novelty in the novel object recognition task. This enhancement is effective in both the healthy young rat and the middle-aged rat that has natural memory decline associated with ageing. These methods of memory enhancement have never before been combined in a study to compare similarities. It appears that it is not action through the AMPK/SIRT1 pathways that elicit these effects on learning and memory, but possibly through increased levels of neurotrophins that promote neuronal growth and survival in brain regions associated with memory. Further assessment of resveratrol and aerobic exercise action on the brain must be conducted for greater understanding of this action. It is possible future studies shall use resveratrol and other exercise mimetics to gain further insight as to how exercise exerts its adaptations physiologically and cognitively. First, more research must be conducted to ascertain whether these treatment methods act through identical pathways, as suggested by a wide range of studies to date.

Chapter Three

ORALLY ADMINISTERED RESVERATROL ALLEVIATES SCOPOLAMINE-INDUCED AMNESIA

"A man's health can be judged by which he takes two at a time – pills or stairs."

- *Joan Welsh*

3.1 ABSTRACT

Resveratrol is a highly active polyphenol found in certain plants, such as grapes and peanuts, that provides protection from invading pathogens and environmental stressors. Since its discovery as an antioxidant in red wine, research has revealed many beneficial effects on human end-organ function. These include action against noncommunicable diseases, such as anti-diabetic properties, cardioprotection, anti-cancer effects, and neuroprotection. Administered intraperitoneally, resveratrol has been shown to act through the cholinergic pathway to alleviate memory decline associated with Alzheimer's disease (AD). With questions raised over the bioavailability of oral resveratrol in vivo, it is of importance to determine whether supplementation of this compound is a realistic cognitive therapy.

To determine if oral resveratrol administration could counter the memory decline of AD and whether aerobic exercise evoked the same effect, it was assessed if a daily oral dose of resveratrol (20 mg/kg), given to young male Wistar rats, would have comparable alleviating effects on scopolamine-induced amnesia as 1 h treadmill running (17 m/min) after 7 days of daily treatment. To examine this, a novel object recognition (NOR) task was used, with one of the objects being displaced following a 24 h delay to assess long-term spatial recognition memory. The potential underlying mechanisms facilitating cognitive enhancement were investigated using hippocampal and perirhinal tissue samples.

Assessed in this task with a 24 h delay; sedentary controls were able to recognise the displaced object, but this memory was disrupted in animals administered scopolamine 30 min before NOR training. Pre-treatment with treadmill running did not prevent the scopolamine-induced amnesia; however, pre-treatment with resveratrol did prevent this amnesia associated with scopolamine administration. These animals were able to recognise the displaced object following a 24 h delay just as well as their counterparts administered vehicle only. Protein analysis showed that improved cognitive ability with one week of these regimes was associated with elevated levels of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), whilst levels of key proteins in the AMPK/SIRT1 pathways remained steady in hippocampal tissue.

These results indicate that daily pre-treatment with 20 mg/kg oral resveratrol provides a strong defence against scopolamine-induced amnesia after just 7 days. These findings were not replicated with pre-training on a treadmill for 1 h per day, suggesting that resveratrol action on the cholinergic pathway is stronger than that of 7 days regular treadmill running. Although showing similar enhancement with healthy memory, resveratrol has more potential than regular aerobic exercise in relieving amnesia. Increased BDNF and NGF levels with resveratrol and exercise suggest that these may help elucidate beneficial effects on healthy cognition, but are not the main factor in improving AD-related memory decline. These findings highlight the potential use of resveratrol ingestion to counter memory decline associated with Alzheimer's disease.

3.2 INTRODUCTION

Resveratrol (3,5,4'-trihydroxystilbene), a compound naturally found in a number of nuts, berries, and the skin of red grapes, is produced in plants during times of environmental stress (Signorelli and Ghidoni, 2005). Resveratrol has been demonstrated to increase the activity of silent information regulator two protein 1 (SIRT1) (Howitz et al., 2003), 5' AMP-activated protein kinase (AMPK) (Um et al., 2010), peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1α) (Lagouge et al., 2006), and manganese superoxide dismutase (MnSOD) (Robb et al., 2008) in a number of tissues. It is believed that upregulation of the AMPK/SIRT1 pathways are important for resveratrol's positive action against cardiovascular disease (Jäger and Nguyen-Duong, 1999; Ray et al., 1999; Zini et al, 1999), stroke (Gupta et al., 2002; Wang et al., 2002; Inoue et al., 2003), diabetes type 2 (Thirunavukkarasu et al., 2007; Szkudelska and Szkudelski, 2010), and some cancers (Jang et al., 1997; Bove et al., 2002). With aerobic exercise demonstrated to upregulate the same pathways (Durante et al., 2002; Ferrara et al., 2008; French et al., 2008; Handschin and Spiegelman, 2008) and evoke similar end-organ function (Manson et al., 1999; Tuomilehto et al., 2001; Colcombe et al., 2004), resveratrol may be considered an exercise mimetic (Narkar et al., 2008).

Alongside physical adaptations, there is evidence suggesting that aerobic exercise and resveratrol penetrate the blood-brain-barrier to promote desirable improvements in cognition and memory (van Praag, 2009; Baur, 2010), and to improve symptoms of neurodegeneration (Marambaud et al., 2005; Sun et al., 2010). Many studies investigating cognition refer to resveratrol as a SIRT1 activator (Kim et al., 2007; Pallas et al., 2009; Baur, 2010) since this compound was found to consistently recapitulate the protective effects of SIRT1 overexpression in cell culture (Howitz et al., 2003; Araki et al., 2004). However, with no evidence to indicate that resveratrol is a direct activator of SIRT1, it may prove misleading to refer to resveratrol as simply a SIRT1 activator. More recently, AMPK has been suggested as an alternative target for resveratrol that may be important for upregulating the beneficial pathways associated with resveratrol action (Narkar et al., 2008), with an interdependence of these proteins highlighted in another study (Price

et al., 2012). In a previous study it was discovered that upregulation of the AMPK/SIRT1 pathways in the hippocampus and perirhinal cortex were not necessary for cognitive enhancement associated with resveratrol ingestion or treadmill running (see Chapter Two). Cognitive enhancement was associated with increased brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) expression and levels in these brain regions, indicating that these proteins may be influential in the cognitive enhancement observed. Increased neurotrophin expression has previously been reported in a number of studies after aerobic exercise (Neeper et al., 1995, 1996; Richardson et al., 2000) and resveratrol administration (Thirunavukkarasu et al., 2007; Rahvar et al., 2011).

Resveratrol has also been shown to act through the cholinergic pathway to alleviate memory decline associated with Alzheimer's disease (AD) when administered intraperitoneally (Gacar et al., 2011). Cholinergic neurons and pathways play widespread roles in the regulation of learning, memory and cerebral blood flow to the nervous system (Mesulam et al., 2002). In AD patients, acetylcholine-releasing neurons selectively degenerate, whilst drugs that block central acetylcholine (ACh) muscarinic receptors have long been known to disrupt higher cognitive functions (Coyle et al., 1983). The hippocampus is one of the first regions to suffer damage in AD, with memory loss and disorientation amongst the early symptoms (De Leon et al., 1989). Clear differences have been determined between cognitive decline associated with normal ageing and cognitive decline associated with AD (Small et al., 2002; Bishop et al., 2010). Previous studies have indicated potential use of physical activity in prevention (Verghese et al., 2003; Colcombe et al., 2004) and rehabilitation (Heyn et al., 2004; Kramer and Erickson, 2007) for AD patients, including many animal model studies (Adlard et al., 2005; Kronenberg et al., 2006; Cracchiolo et al., 2007). However, this is often an easier prospect in theory than in practice. Physical activity for those with restricted mobility is an arduous task requiring constant hands-on assistance from another person. This is where a pharmacological agent functioning as an exercise mimetic may have the greatest potential for improving people's lives.

Administration of scopolamine, a centrally active muscarinic receptor blocker, in rats is commonly used as a rodent model of AD-related cognitive decline.

Scopolamine-induced amnesia works as a pharmacological tool to model AD-related cognitive decline by addressing the cholinergic system dysfunction associated with the disorder (Smith, 1988). With resveratrol shown to have therapeutic potential against scopolamine-induced amnesia when administered intraperitoneally (Gacar et al., 2011), this study was designed to ascertain whether an oral dose that showed cognitive improvement in healthy rats without upregulating the AMPK/SIRT1 pathways could elicit the same positive effects against AD-related amnesia. This model was also used to determine the therapeutic potential of regular aerobic exercise against AD-related amnesia and compare it to the effects of regular resveratrol ingestion. To confirm the beneficial effects on AD-related cognitive decline, and compare the similarity of action, a direct comparison of behaviour following regular aerobic exercise and oral resveratrol treatment was conducted. This should provide greater insight regarding the pathways through which aerobic exercise and resveratrol enhance memory, and the potential of resveratrol as an orally active compound against AD.

For this study, cohorts of young male Wistar rats underwent either a training protocol of treadmill running, 1 h/day, increasing from 10-17 m/min, or led a sedentary lifestyle. These groups were again sub-divided so half of the animals were administered resveratrol orally at a dose of 20 mg/kg on training days, a dose shown to cause no adverse effects with daily dosage (Juan et al., 2002). Following 7 days of daily treadmill running and resveratrol administration, animals were tested in a novel object recognition (NOR) task. This is a well-validated behavioural measure of rodent memory useful for evaluating experimental manipulations of cognition and has been shown to highlight cognitive differences between trained and untrained rats (Griffin et al., 2009).

In an initial study to ascertain the effects of oral resveratrol and treadmill running on normal memory, young rats were introduced to a substitution NOR task, following the 7 day regimes. Using a 24 h delay, the effects of treatment on long-term recognition memory were assessed. Treatment with resveratrol and regular aerobic exercise, separately or combined, for 7 consecutive days led to significantly

better performance in the NOR task compared to untreated, sedentary controls. These findings were paralleled to increased BDNF and NGF levels in brain regions associated with learning and memory. AMPK, SIRT1, PGC-1α, and MnSOD expression in the hippocampus did not increase with treadmill running or resveratrol administration, compared to sedentary, untreated controls. These results support observations using longer treatment regimes of 4 weeks (see Chapter Two). In the study to determine the effects of oral resveratrol and treadmill running on AD-related amnesia, half of the rats in each group were administered scopolamine (0.8 mg/kg; i.p.) 30 min prior to introduction to a displacement NOR task, following the 7 day regimes. Using a 24 h delay, the effects of treatment regimes on long-term spatial recognition memory were assessed. Treatment with resveratrol for 7 consecutive days led to significantly better performance in the NOR task compared to scopolamine-administered controls. There was no improvement in NOR performance following daily treadmill running for 7 consecutive days. Aerobic exercise and resveratrol ingestion both show similar upregulation of neurotrophins in the hippocampus and perirhinal cortex with these regimes, indicating that increased levels of these proteins are not sufficient to improve AD-related cognitive decline. These findings suggest that aerobic exercise and the polyphenol, resveratrol, may act through the same pathway to enhance normal cognition but resveratrol ingestion has a stronger action on the cholinergic pathway and a stronger therapeutic potential against AD-related amnesia.

3.3 MATERIALS AND METHODS

3.3.1 Animals

Male Wistar rats (225 ± 55 g; *n* = 72) were obtained from the BioResources Unit, Trinity College Dublin. They were housed in pairs (standard hard-bottomed, polypropylene cages; 44 × 28 × 18 cm) in a temperature-controlled vivarium (20 to 22 °C), with a 12:12-hour light-dark cycle. Animals were provided with food and water *ad libitum*. Experiments were carried out in strict accordance with regulations laid out by LAST Ireland and were compliant with the European Union directives on animal experimentation (86/609/EEC).

3.3.2 Drug and Dosing Regime

All rats were handled for one week pre-drug treatment and fed 0.5 ml of maple syrup (Maple Joe, Bernard Michaud) to familiarise them with feeding by syringe. *trans*-Resveratrol (>99% purity) from Sigma-Aldrich, UK was administered orally mixed in a solution of maple syrup. Treated animals were given an oral dose of maple syrup and resveratrol suspension (20 mg/kg; p.o.) for 7 consecutive days, with controls given maple syrup only. Rats were dosed 30 min before exercise protocol.

Scopolamine hydrobromide trihydrate ($C_{17}H_{21}NO_4.HBr.3H_2O$; Sigma-Aldrich, UK) was dissolved in a 0.9% saline solution at a concentration of 0.8 mg/kg, similar to previous studies (Okaichi et al., 1989; Biggan et al., 1996; Ormerod and Beninger, 2002). Rats were administered the scopolamine solution 30 min before NOR training. Vehicle controls were injected with an equivalent volume of saline solution (1 ml/kg).

3.3.3 Exercise Programme

Rats (n = 72) were familiarised to motorised treadmills (Exer 3/6 treadmill, Columbus Instruments) by walking on the treadmill for 15 min (belt speed, 7 m/min) on 3 consecutive days. For the first experiment to assess the effects of a 7 day protocol on young healthy rats, 24 animals were randomly assigned to 4 groups: sedentary controls (SedCTL), running controls (RunCTL), sedentary resveratrol-treated (SedRES) and running resveratrol-treated (RunRES) (n = 6 in each). For the second experiment to assess the therapeutic potential of a 7 day protocol against scopolamine-induced amnesia, animals were randomly assigned to the same 4 groups (n = 12 in each) with half of the animals in each group randomly administered scopolamine before novel object exposure and the rest administered vehicle.

The exercise protocol consisted of running one hour per day for 7 consecutive days (belt speed, gradually increased over the training period from 10 m/min to a maximum of 17 m/min, which is equivalent to 1 km/h). The treadmill is equipped with wire loops at one end of the belt through which a mild electric shock can be delivered; these act to motivate the rats to run continuously and were activated at low levels (on average an intensity of three on a scale of 0–10; this represents a current of 1 mA with an inter-pulse interval of 2 s) throughout all exercise sessions. Rats were observed while exercising to ensure they ran continuously and also to monitor for signs of stress. Sedentary rats were placed on stationary treadmills with shock loops activated at low levels for the same duration. Training in the NOR task began on the final day of the exercise programme, following 7 days treadmill running.

3.3.4 Open Field Exploration

An open-field test was conducted in a black circular open field (diameter, 90 cm; height, 45 cm) placed in a dimly lit-room. Rats were examined in this empty

arena on their first day of habituation (following 5 days treadmill running and treatment) to measure for general exploration and spontaneous behaviour in response to a novel environment. Observation of such behaviours is of particular importance in drug trials as new drugs may have unexpected or previously undiscovered behavioural effects. Rats were allowed to move freely for 5 min with tracks recorded and analysed using a computer-based tracking system (Ethovision, Noldus Co. Ltd., The Netherlands). Habituation and reactions to spatial changes were evaluated by the exploration of objects in the same open field arena during the initial 5 min NOR training trial. This was to examine the sensitivity of the rats to an environmental change, by recording the interaction each rat made with the individual objects.

3.3.5 Novel Object Recognition Task

Rats were well-handled and habituated to the experimental apparatus (see Open field exploration); with 20 min of exploration in the absence of objects each day for 2 days before the task was performed. Objects were constructed from toy bricks and were fixed to the floor of the open field, 15 cm from the walls. Objects and arena were cleaned thoroughly between trials to ensure the absence of olfactory cues. Scoring for exploration was strictly based on active exploration, where rats had to be touching the object with at least their noses. For the first experiment to assess the effects of a 1 week protocol on young healthy rats, an object substitution task was used to assess non-spatial recognition memory (Fig. 3-1A). It has been shown previously that young healthy rats require more training phases to learn this task (Griffin et al., 2009), and so, this is an appropriate task for highlighting memory enhancement in these subjects. For the training phase, three distinct objects (A, B and C) were positioned in the open field in a room with prominent extramaze cues which could be used for efficient allocentric orientation. Rats were allowed to explore the objects for 3 x 5 min trials with an intertrial interval (ITI) of 5 min. For the testing phase, 24 h later, object C was replaced with novel object D.

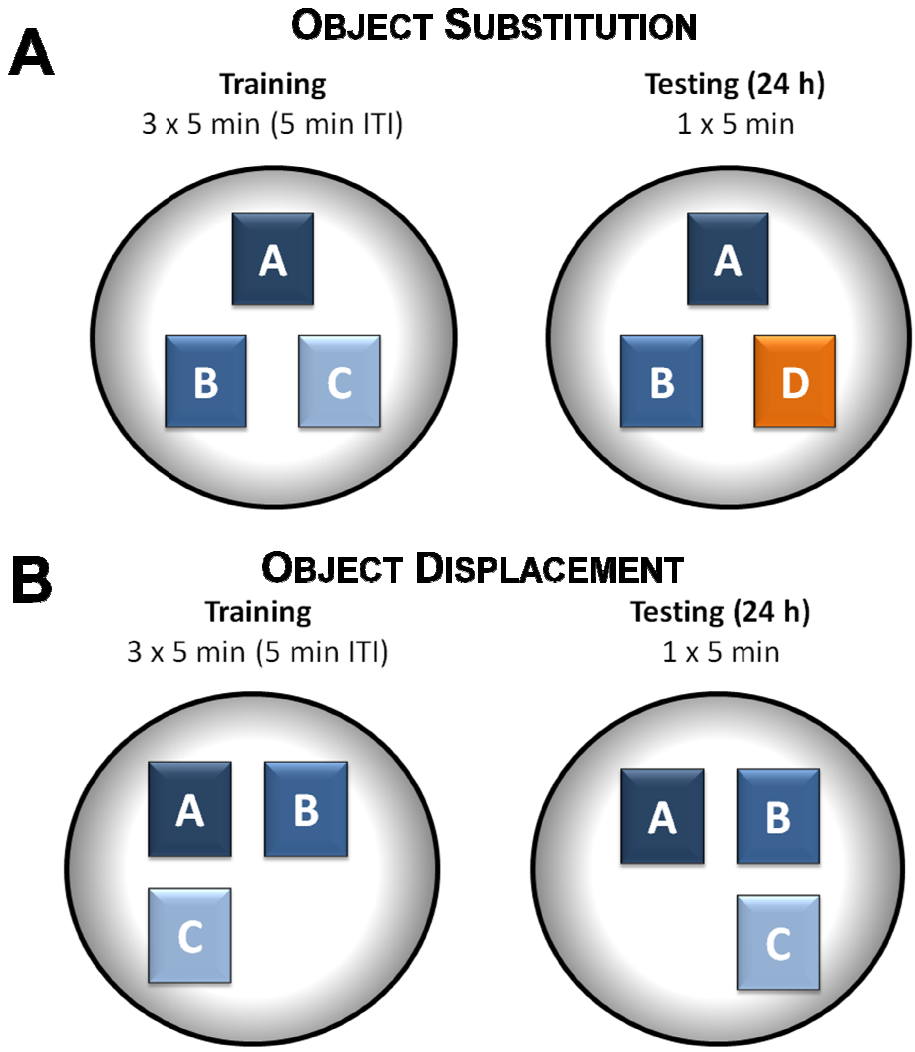


Fig. 3-1. Overview of novel object recognition tasks used to assess long-term recognition memory. For both versions of the task, rats were exposed to an arena containing 3 distinct objects for three 5 min periods. In [A] the object substitution task, rats were reintroduced to the same arena, following a 24 h delay, with 2 of the same objects in the same locations (A and B) and one object replaced with another distinct object (C with D). In [B] the object displacement task, rats were reintroduced to the same arena, following a 24 h delay, with 2 of the objects in the same locations (A and B) and one object displaced within the arena (C). This version of the task requires long-term spatial memory and is an easier task for the rats.

For the second experiment to assess the therapeutic potential of a 7 day protocol against scopolamine-induced amnesia, an object displacement task was used to assess spatial recognition memory (Fig. 3-1B). It has been shown previously that young healthy rats can complete this task (Griffin et al., 2009), and so this was a more appropriate version of the NOR task to highlight scopolamine-induced amnesia relative to healthy memory. For the training phase, three objects (A, B and C) were positioned in the open field in a room with prominent extramaze cues which could be used for efficient allocentric orientation. Rats were allowed to explore the objects for 3 x 5 min trials with an intertrial interval (ITI) of 5 min. For the testing phase, 24 h later, object C was displaced within the arena. In both versions of the NOR task, rats were reintroduced to the open field for a single 5 min trial. Measurement of the time spent exploring each object was recorded and expressed as a discrimination ratio (novel object interaction/total interaction with all objects) (Bevins and Besheer, 2006). Object recognition is reflected by spending more time interacting with the novel object D, or displaced object C, over familiar objects A and B, shown here with an object discrimination ratio above 0.333.

3.3.6 Tissues and Serum Samples

Rats were sacrificed by decapitation 1 h following the testing phase of the NOR task. Their brains were removed and tissue was taken from the dentate gyrus (DG), remainder of hippocampus (HIPP) and perirhinal cortex (PC). These samples were homogenised in lysis buffer, with a small portion separated for mRNA analysis, and stored at -80°C until further analysis.

3.3.7 Analysis of Protein Levels by Enzyme-Linked Immunosorbent Assay (ELISA)

Samples homogenised in lysis buffer were thawed, assayed for protein content using a Micro BCA Protein Assay Kit (Thermo Scientific, Hampshire, UK), and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, Delaware, USA). Samples were all diluted to a final volume of 100 µl to equalise for protein content and then stored at -20°C until further analysis.

BNDF levels in dentate gyrus, hippocampus, and perirhinal cortex were quantitatively assessed using *Chemikine*™ BDNF Sandwich ELISA kit (Millipore S.A.S., Molsheim, France). NGF levels in dentate gyrus, hippocampus, and perirhinal cortex were quantitatively assessed using *Chemikine*™ Nerve Growth Factor Sandwich ELISA kit (Millipore S.A.S., Molsheim, France). All ELISAs were conducted according to instructions provided by the manufacturer. All samples assayed by ELISA were done in triplicate.

3.3.8 Analysis of Protein Expression by Real-Time Polymerase Chain Reaction (RT-PCR)

Total RNA from brain tissue was extracted from snap-frozen samples using the NucleoSpin RNA II isolation kit (Machery-Nagel Inc., Germany) following manufacturer's instructions, and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, Delaware, USA). For RT-PCR, total RNA was retro-transcribed to cDNA. cDNA synthesis was performed on 1 - 2 µg RNA using a High Capacity cDNA RT Kit (Applied Biosystems, USA). Following this, total cDNA was submitted to RT-PCR for SIRT1, AMPKα1, AMPKα2, PGC-1α, MnSOD, BDNF and NGF. Rat β-actin was used as an endogenous control (cDNA samples were not normalised prior to RT-PCR) and expression was conducted using a gene expression assay containing forward and

reverse primers and a VIC-labelled MGB TaqMan probe (Applied Biosystems, USA). All RT-PCR measurements were conducted using an ABI Prism 7300 instrument (Applied Biosystems, USA). Forty cycles were run as follows: 10 min at 95 °C and for each cycle, 15 sec at 95 °C and 1 min at 60 °C. Fluorescence was read during the annealing and extension phase (60 °C) throughout the program and gene expression was calculated relative to the endogenous control. Analysis was performed using the $2^{-\Delta\Delta}$CT method. Data are presented as mean relative quotient (RQ) values that represent fold changes relative to the mean value for controls using StepOne™ Software v2.1 (Applied Biosystems, USA).

3.3.9 Statistical Analysis

All data was analysed using GraphPad Prism (GraphPad Software, Inc.) and Statistical Package for the Social Sciences (SPSS). One-way ANOVA, two-way mixed-factorial ANOVA or unpaired two-tailed Student's t-test were conducted as appropriate. *Post hoc* comparisons were made using the Tukey's HSD test. A significance level of $p = 0.05$ was accepted for all comparisons: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Data are presented as mean ± SEM.

3.4 RESULTS

Experiment One: Effects on Healthy Memory

3.4.1 Effect of Exercise Compared to Resveratrol on Open Field Behaviour

The impact of 5 days forced treadmill running and resveratrol ingestion on emotion and anxiety were compared using an open field assessment. Placed in an empty arena, qualitative and quantitative measurements of general locomotor activity and willingness to explore were taken. Assessed for the initial 5 min of the first habituation session, following 5 days of training and treatment, one-way ANOVA analyses revealed no significant differences between groups for ambulation in terms of distance travelled ($F_{3,20}$ = 0.3051, p = 0.8213) (Fig. 3-2A) and velocity ($F_{3,20}$ = 0.9474, p = 0.1199) (Fig. 3-2B). With no differences determined between groups, there were no sedative or stimulant effects of resveratrol treatment or aerobic exercise.

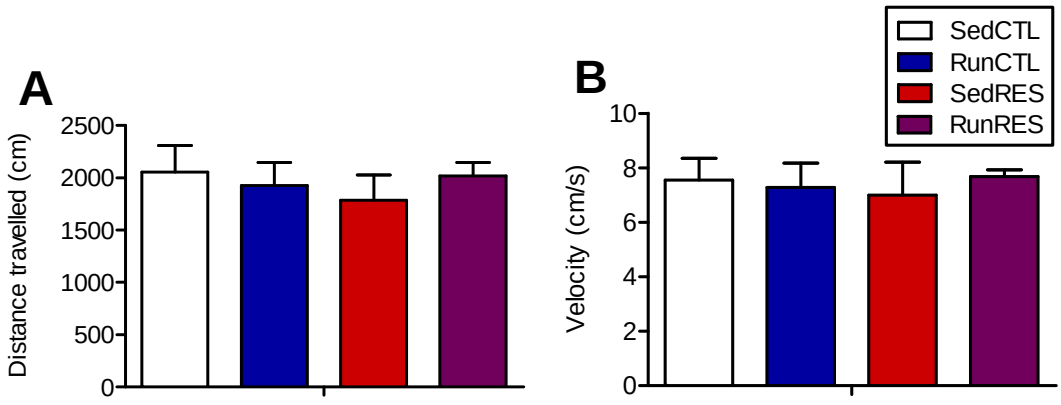


Fig. 3-2. No differences between groups were found in spontaneous locomotor behaviour in an open field after 5 days of regime. Locomotor activity was recorded for 5 min. SedCTL: pre-treatment with maple syrup alone (0.5 ml/d) and exposure to immobile treadmills (1h /d) for 5 d; RunCTL: pre-treatment with maple syrup alone (0.5 ml/d) and forced treadmill running (1h /d) for 5 d; SedRES: pre-treatment with

resveratrol (20 mg/kg/d) in maple syrup and exposure to immobile treadmills (1h /d) for 5 d; RunRES: pre-treatment with resveratrol (20 mg/kg/d) in maple syrup and forced treadmill running (1h /d) for 5 d. Results are presented as mean ± SEM of the [A] distance travelled in centimetres (cm) and [B] velocity (cm/s). *n* = 6 per group. No differences were found between groups.

Further analysis of general locomotor activity and willingness to explore was carried out and compared between groups when objects were added to the arena for the first NOR trial. All 4 groups showed similar sensitivity to changes in the local environment when the 3 objects were introduced to the open field environment for the initial training trial as assessed by the total exploration ($F_{3,20}$ = 0.9108, p = 0.4534) (Fig. 3-3A). This further confirms there were no sedative or stimulant effects of resveratrol treatment or aerobic exercise following 7 days of these treatment regimes.

3.4.2 Treadmill Running and Resveratrol Treatment have Similar Effects on NOR Performance

The impact of 7 days of forced treadmill running with oral resveratrol treatment on recognition memory was assessed with an object substitution task (Fig. 3-1A). Rats explored the objects on the training day for 3 x 5 min with a 5 min ITI. Over training trials 1 – 3, all groups showed habituation to their environment as expected, with total exploration time decreasing across trials (Fig. 3-3A). A two-way mixed-factorial ANOVA revealed a significant effect of trial ($F_{2,40}$ = 44.387 , ***p < 0.001), but no significant trial x treatment interaction ($F_{6,40}$ = 0.598, p = 0.730), and no significant effect of treatment ($F_{3,20}$ = 0.360, p = 0.783). This asymptotic habituation curve indicates normal encoding of the environmental features and

spatial configuration, suggesting that any alterations to the environment should evoke increased exploratory behaviour.

A comparison of time spent exploring all objects during the NOR training trials and testing trial showed no difference between groups, as determined by one-way ANOVA analysis ($F_{3,20} = 1.797$, $p = 0.1802$) (Fig 3-3A). Comparing exploration during the testing trial to the training trials, unpaired two-tailed t-test revealed that sedentary resveratrol-treated rats showed significantly less exploration in the test trial compared to exploration in the initial training trial (T1), whereas all other group showed exploration similar to T1 (SedCTL: $t_{10} = 0.9486$, $p = 0.3652$; RunCTL: $t_{10} = 0.64$, $p = 0.5366$; SedRES: $t_{10} = 2.238$, $^{*}p < 0.05$; RunRES: $t_{10} = 0.6327$, $p = 0.5411$). Alternatively, sedentary resveratrol-treated rats showed similar exploration in the test trial compared to exploration in the final training trial (T3), whereas all other groups showed significantly more exploration in testing compared to this trial (SedCTL: $t_{10} = 2.489$, $^{*}p < 0.05$; RunCTL: $t_{10} = 2.599$, $^{*}p < 0.05$; SedRES: $t_{10} = 1.275$, $p = 0.2312$; RunRES: $t_{10} = 6.355$, $^{***}p < 0.001$) (data not represented).

Animals were assessed for exploration of object C (to be substituted in the testing trial) relative to objects A and B, during the 3 training trials. This is classed as the object discrimination ratio (ODR). ODR was averaged across the trials within treatment groups. All 4 groups spent approximately one third of the total exploration time exploring the object that was later replaced (SedCTL: $t_{10} = 0.3503$, $p = 0.7334$, ODR = 0.327; RunCTL: $t_{10} = 0.2827$, $p = 0.7832$, ODR = 0.344; SedRES: $t_{10} = 0.5702$, $p = 0.5811$, ODR = 0.315; RunRES: $t_{10} = 0.9038$, $p = 0.3873$, ODR = 0.35), signifying no preference for any one of the 3 objects (Fig. 3-3B). Animals showed no particular interest for any specific object or object location during NOR training trials.

To measure long-term recognition memory using a 24 h ITI, during a 5 min testing trial the time spent exploring novel object D was compared to time spent exploring novel objects A and B. As rats show a natural tendency to explore new

objects, greater exploration of object D indicates that animals remember objects A and B from the training trials. An unpaired two-tailed t-test showed there was no indication of recognition memory in the sedentary controls following the 24 h delay as assessed by preferential exploration of novel object, D, suggesting that these rats were unable to learn this task (t_{10} = 0.0554, p = 0.9569, ODR = 0.332). All other groups showed increased exploration of novel object, D, relative to familiar objects, A and B (RunCTL: t_{10} = 4.281, *p < 0.01, ODR = 0.496; SedRES: t_{10} = 4.387, *p < 0.01, ODR = 0.443; RunRES: t_{10} = 6.212, ***p < 0.001, ODR = 0.476), indicating an enhancement in performance of this task following 7 days of resveratrol ingestion and treadmill running (Fig. 3-3C).

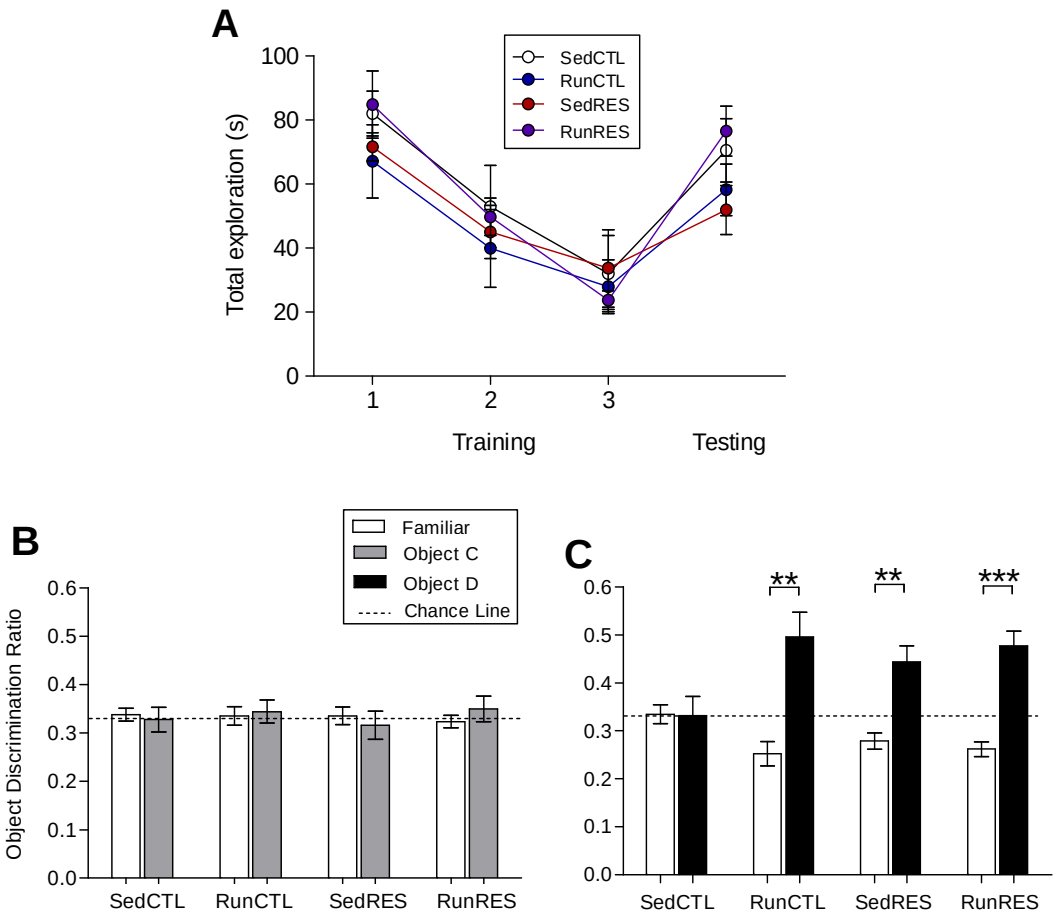


Fig. 3-3. Assessment of long-term recognition memory following a 7 day regime of resveratrol ingestion and treadmill running. [A] All groups showed habituation to their environment with total exploration time decreasing across training trials 1 – 3. [B] Measurement of the time spent exploring each object was recorded and

expressed as a discrimination ratio (novel object interaction/total interaction with all objects) for each training trial and averaged for each rat. No group showed any preference for object C over objects A and B. [C] Following a 24 h delay animals were reintroduced to the arena with familiar objects A and B, and novel object D. Rats that underwent training or resveratrol ingestion explored novel object D more than familiar objects A and B. Sedentary control rats did not explore novel object D more than the familiar objects. *n* = 6 per group. Results are presented as mean ± SEM.

3.4.3 Effect on Protein Expression in Hippocampus and Perirhinal Cortex

Hippocampal and perirhinal tissue was extracted from the brains of all rats and assessed for expression of proteins from the AMPK/SIRT1 pathways. This would indicate if proteins implicated in many beneficial actions of resveratrol and aerobic exercise physiologically were also involved in the cognitive enhancement observed with these treatment regimes. Expression of SIRT1, AMPKα1, AMPKα2, PGC-1α, MnSOD, BDNF, and NGF was assessed in brain structures associated with learning and memory using real-time PCR. One-way ANOVA analysis revealed significant differences between groups of BDNF mRNA levels in dentate gyrus ($F_{3,20}$ = 5.622, **p < 0.01) with further *post hoc* analysis revealing significant increases with all treatment regimes relative to sedentary controls (RunCTL: *p < 0.05;SedRES: **p < 0.01; RunRES: *p < 0.05) (Fig. 3-4A). One-way ANOVA analysis also revealed significant differences between groups of NGF mRNA levels in dentate gyrus ($F_{3,20}$ = 4.903, *p < 0.05) with further *post hoc* analysis revealing significant increases with all treatment regimes relative to sedentary controls (RunCTL: *p < 0.05;SedRES: *p < 0.05; RunRES: *p < 0.05) (Fig. 3-4A). Additional one-way ANOVA analyses revealed no significant differences between groups for dentate gyrus expression of SIRT1 ($F_{3,20}$ = 0.1549, p = 0.9253), subunit AMPKα1 ($F_{3,20}$ = 0.4936, p = 0.6907), subunit AMPKα2 ($F_{3,20}$ = 0.0881, p = 0.9657),

PGC-1α ($F_{3,20}$ = 0.06401, p = 0.9783), and MnSOD ($F_{3,20}$ = 0.1703, p = 0.9152) (Fig. 3-4A).

Analysis of the rest of the hippocampus and perirhinal cortex showed similar enhancement of neurotrophins. One-way ANOVA analysis revealed significant differences between groups of BDNF mRNA levels in hippocampus ($F_{3,20}$ = 6.456, **p < 0.01) with further *post hoc* analysis revealing significant increases with all treatment regimes relative to sedentary controls (RunCTL: *p < 0.05; SedRES: **p < 0.01; RunRES: **p < 0.01) (Fig. 3-4B). One-way ANOVA analysis also revealed significant differences between groups of NGF mRNA levels in hippocampus ($F_{3,20}$ = 6.151, **p < 0.01) with further *post hoc* analysis revealing significant increases with all treatment regimes relative to sedentary controls (RunCTL: *p < 0.05; SedRES: *p < 0.05; RunRES: **p < 0.01) (Fig. 3-4B). Additional one-way ANOVA analyses revealed no significant differences between groups for hippocampal expression of SIRT1 ($F_{3,20}$ = 0.3420, p = 0.7952), subunit AMPKα1 ($F_{3,20}$ = 0.8771, p = 0.4696), subunit AMPKα2 ($F_{3,20}$ = 0.827, p = 0.4945), PGC-1α ($F_{3,20}$ = 1.175, p = 0.3442), and MnSOD ($F_{3,20}$ = 1.371, p = 0.2805) (Fig. 3-4B). One-way ANOVA analysis revealed significant differences between groups of BDNF mRNA levels in perirhinal cortex ($F_{3,20}$ = 6.039, **p < 0.01) with further *post hoc* analysis revealing significant increases with all treatment regimes relative to sedentary controls (RunCTL: *p < 0.05; SedRES: **p < 0.01; RunRES: *p < 0.05) (Fig. 3-4C). One-way ANOVA analysis also revealed significant differences between groups of NGF mRNA levels in perirhinal cortex ($F_{3,20}$ = 4.952, **p < 0.01) with further *post hoc* analysis revealing significant increases with all treatment regimes relative to sedentary controls (RunCTL: *p < 0.05; SedRES: *p < 0.05; RunRES: *p < 0.05) (Fig. 3-4C). Additional one-way ANOVA analyses revealed no significant differences between groups for perirhinal expression of SIRT1 ($F_{3,20}$ = 0.2018, p = 0.8939), subunit AMPKα1 ($F_{3,20}$ = 0.3095, p = 0.8182), subunit AMPKα2 ($F_{3,20}$ = 0.07554, p = 0.9725), PGC-1α ($F_{3,20}$ = 0.07496, p = 0.9728), and MnSOD ($F_{3,20}$ = 0.2737, p = 0.8437) (Fig. 3-4C). The improvements detected in long-term recognition memory with these 7 day treatment regimes of oral resveratrol and aerobic exercise were associated with increased expression of neurotrophins in brain regions involved in learning and memory. These findings correspond to previous

results using 4 weeks of these treatment regimes in young and middle-aged animals (see Chapter Two). This supports the suggestion that increased levels of these neurotrophins may be important for the cognitive enhancement associated with resveratrol treatment and aerobic exercise.

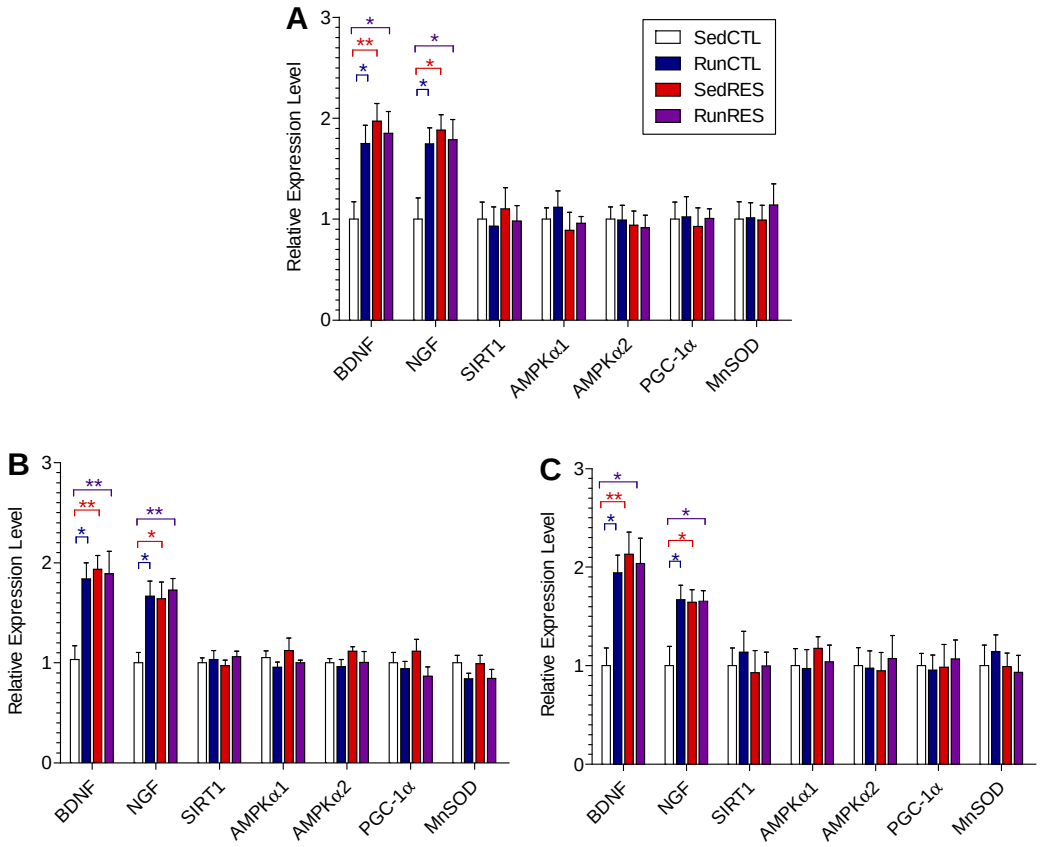


Fig. 3-4. Neurotrophin expression increased in perirhinal and hippocampal tissue following 7 day regimes of resveratrol ingestion and treadmill running. mRNA levels in hippocampus and perirhinal cortex were measured by RT-PCR analysis. Levels of BDNF and NGF mRNA were increased in [A] dentate gyrus, [B] rest of hippocampus and [C] perirhinal cortex of rats that underwent training or resveratrol ingestion, relative to sedentary controls. For expression of other analysed protein mRNAs no differences were found between groups. n = 6 per group. Results are presented as mean ± SEM. Values significantly different from the sedentary control expression level are indicated with an asterisk (Tukey's HSD: *$p < 0.05$, **$p < 0.01$), with the running group in blue, resveratrol-treated in red, and those undergoing running and resveratrol-treatment in purple.

3.4.4 Effect on BDNF and NGF Levels in Hippocampus and Perirhinal Cortex

With increased expression of neurotrophins in hippocampal and perirhinal tissue in groups administered resveratrol and treadmill running, to further determine the potential importance of these proteins in learning and memory enhancement, protein levels were measured in these tissues. Levels of BDNF and NGF were measured in brain structures associated with learning and memory using ELISA. One-way ANOVA revealed significant differences in the levels of BDNF ($F_{3,20}$ = 6.201, **p < 0.01) and NGF ($F_{3,20}$ = 4.777, *p < 0.05) in the dentate gyrus. *Post hoc* analysis revealed significant increases with all treatment regimes relative to YG sedentary controls of BDNF (RunCTL: *p < 0.05; SedRES: **p < 0.01; RunRES: *p < 0.05) and NGF (RunCTL: *p < 0.05; SedRES: *p < 0.05; RunRES: *p < 0.05) (Fig. 3-5A). One-way ANOVA also revealed significant differences in the levels of BDNF ($F_{3,20}$ = 5.391, **p < 0.01) and NGF ($F_{3,20}$ = 4.298, *p < 0.01) in the remainder of the hippocampus. *Post hoc* analysis revealed significant increases with all treatment regimes relative to sedentary controls of BDNF (RunCTL: *p < 0.05; SedRES: *p < 0.05; RunRES: *p < 0.05) and NGF (RunCTL: *p < 0.05; SedRES: *p < 0.05; RunRES: *p < 0.05) (Fig. 3-5B). One-way ANOVA also revealed significant differences in the perirhinal levels of BDNF ($F_{3,20}$ = 8.659, ***p < 0.001) and NGF ($F_{3,20}$ = 4.728, *p < 0.05). *Post hoc* analysis revealed significant increases with all treatment regimes relative to sedentary controls of BDNF (RunCTL: **p < 0.01; SedRES: *p < 0.05; RunRES: **p < 0.01) and NGF (RunCTL: *p < 0.05; SedRES: *p < 0.05; RunRES: *p < 0.05) (Fig. 3-5C). The improvements detected in long-term recognition memory with these treatment regimes of oral resveratrol and aerobic exercise were associated with increased neurotrophin levels in brain regions involved in learning and memory. These findings further support the belief that these 1 and 4 week treatment regimes of resveratrol ingestion and treadmill running may improve cognition through increased levels of these proteins.

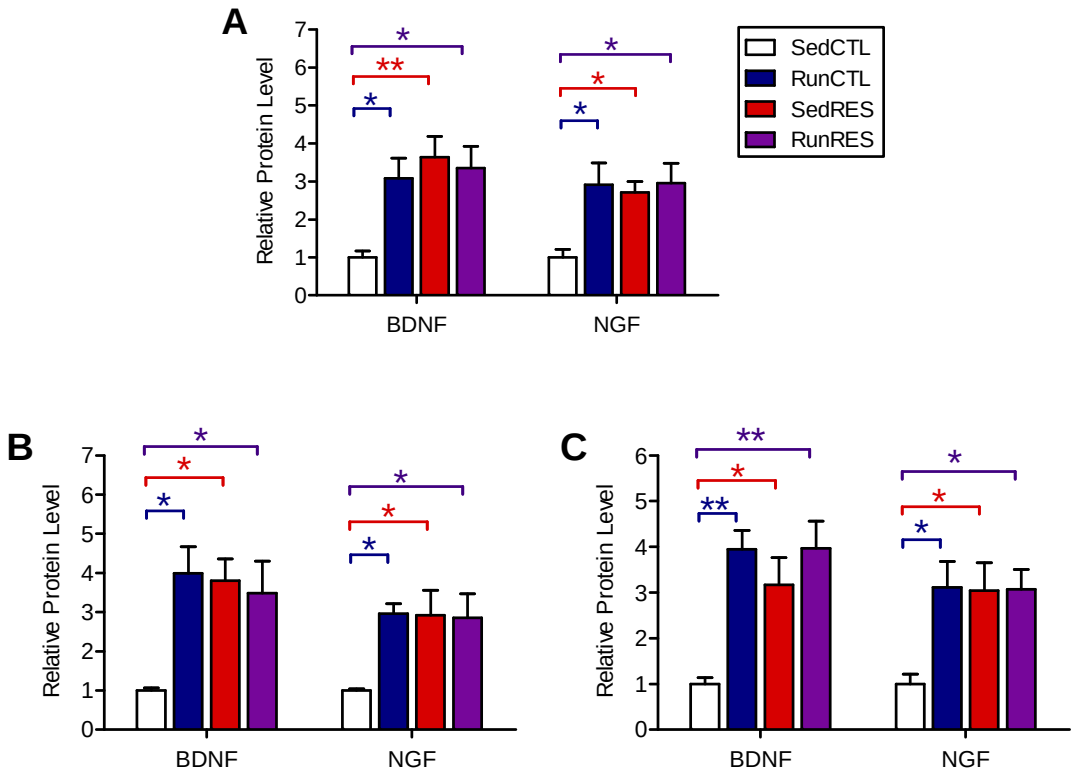


Fig. 3-5. Neurotrophin levels increased in perirhinal and hippocampal tissue following 7 day regimes of resveratrol ingestion and treadmill running. Protein levels in hippocampus and perirhinal cortex were measured by ELISA. Levels of BDNF and NGF were increased in [A] dentate gyrus, [B] rest of hippocampus and [C] perirhinal cortex of rats that underwent training or resveratrol ingestion, relative to sedentary controls. n = 6 per group. Results are presented as mean ± SEM. Values significantly different from the sedentary control protein level are indicated with an asterisk (Tukey's HSD: *p < 0.05, **p < 0.01), with the running group in blue, resveratrol-treated in red, and those undergoing running and resveratrol-treatment in purple.

3.4.5 Effect of Exercise Compared to Resveratrol on Open Field Behaviour

The impact of 5 days forced treadmill running and resveratrol ingestion on emotion and anxiety were compared using an open field assessment. Placed in an empty arena, qualitative and quantitative measurements of general locomotor activity and willingness to explore were taken. Assessed for the initial 5 min of the first habituation session, following 5 days of training and treatment, one-way ANOVA analyses revealed no significant differences between groups for ambulation in terms of distance travelled ($F_{3,20}$ = 0.3536, p = 0.7867) (Fig. 3-6A) and velocity ($F_{3,20}$ = 0.4346, p = 0.7293) (Fig. 3-6B). With no differences determined between groups, there were no sedative or stimulant effects of resveratrol treatment or aerobic exercise.

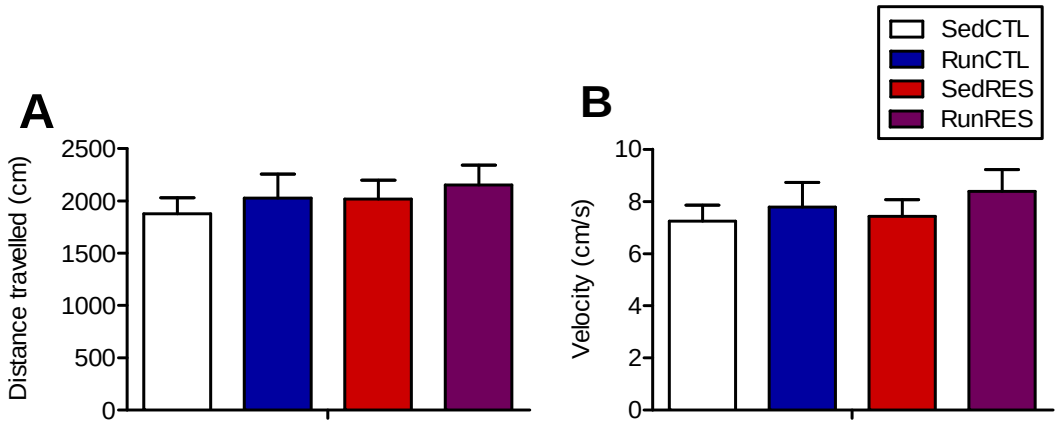


Fig. 3-6. No differences between groups were found in spontaneous locomotor behaviour in an open field after 5 days of regime. Locomotor activity was recorded for 5 min. SedCTL: pre-treatment with maple syrup alone (0.5 ml/d) and exposure to immobile treadmills (1h /d) for 5 d; RunCTL: pre-treatment with maple syrup alone (0.5 ml/d) and forced treadmill running (1h /d) for 5 d; SedRES: pre-treatment with resveratrol (20 mg/kg/d) in maple syrup and exposure to immobile treadmills (1h /d) for 5 d; RunRES: pre-treatment with resveratrol (20 mg/kg/d) in maple syrup and forced treadmill running (1h /d) for 5 d. Results are presented as mean ± SEM of the [A] distance travelled in centimetres (cm) and [B] velocity (cm/s). *n* = 12 per group. No differences were found between groups.

Further analysis of general locomotor activity and willingness to explore was carried out and compared between groups when objects were added to the arena for the first NOR trial. All groups showed similar sensitivity to changes in the local environment when the 3 objects were introduced to the open field environment for the initial training trial as assessed by the total exploration ($p = 0.887$) (Fig. 3-7A). This further confirms there were no sedative or stimulant effects of resveratrol treatment or aerobic exercise following 7 days of the treatment regimes.

3.4.6 Effect of Scopolamine on Learning and Memory

The rehabilitative effects of 7 days forced treadmill running were compared to 7 days of oral resveratrol treatment on scopolamine-induced amnesia, as assessed with an object displacement task (Fig. 3-1B). Rats were introduced to an arena with distinct objects A, B and C, and allowed to freely explore for 3 x 5 min with a 5 min ITI. Following a 24 h delay, animals were reintroduced to the arena with objects A and B in the same locations, and object C displaced within the arena. Over training trials 1 – 3, both groups administered scopolamine and those administered vehicle showed habituation to their environment as expected, with total exploration time decreasing across trials (Fig. 3-7A). In addition, no significant effect of treatment was seen on any of the habituation trials following unpaired two-tailed t-test for each trial (T1: $t_{46} = 0.1483$, $p = 0.887$; T2: $t_{46} = 0.937$, $p = 0.3537$; T3: $t_{46} = 0.7464$, $p = 0.4592$). Differences between groups during the testing trial after a 24 h ITI were determined by unpaired two-tailed t-test ($t_{46} = 3.982$, ***$p < 0.001$) (Fig. 3-7A).

To assess whether resveratrol ingestion or aerobic training had any effect on scopolamine-induced amnesia it first had to be determined whether or not healthy animals could perform well in this long-term spatial recognition task and if this dose of scopolamine administered 30 min prior to NOR exposure was sufficient to block long-term memory. Animals were assessed for exploration of object C (to be displaced in the testing trial) relative to objects A and B, during the 3 training trials.

ODR was averaged across the trials within groups. This exploration was averaged across the trials and treatment groups. Vehicle-administered and scopolamine-administered sedentary control groups spent approximately one third of the total exploration time exploring the object that was later displaced (SedCTL*Veh: t_{10} = 0.7318, p = 0.4811, ODR = 0.346; SedCTL*Scop: t_{10} = 0.9897, p = 0.3457, ODR = 0.350), signifying no preference for any one of the 3 objects (Fig. 3-7B).

To measure long-term recognition memory using a 24 h ITI, during a 5 min testing trial the time spent exploring displaced object C was compared to time spent exploring unmoved objects A and B. As rats show a natural tendency to explore new objects, greater exploration of object D indicates that animals remember objects A and B from the training trials. During a 5 min testing trial 24 h later, sedentary vehicle-treated controls showed preferential exploration of the displaced object, C, suggesting that these rats exhibited long-term spatial recognition memory in this task (t_{10} = 3.449, **p < 0.01, ODR = 0.477). In rats administered scopolamine 30 min prior to the NOR task, there was no indication of recognition memory following the 24 h delay, suggesting that scopolamine-induced amnesia meant these rats were unable to learn this task (t_{10} = 0.398, p = 0.699, ODR = 0.362) (Fig. 3-7C).

3.4.7 Improvements to Scopolamine-Induced Amnesia with Resveratrol Ingestion but not Treadmill Running

Assessed for exploration of object C (to be displaced in the testing trial) relative to objects A and B, during the 3 training trials, ODR was averaged across the trials within treatment groups. All 8 groups spent approximately one third of the total exploration time exploring the object that was later replaced (SedCTL*Veh: t_{10} = 0.7318, p = 0.4811, ODR = 0.346; SedCTL*Scop: t_{10} = 0.9897, p = 0.3457, ODR = 0.350; RunCTL*Veh: t_{10} = 1.375, p = 0.1992, ODR = 0.347; RunCTL*Scop: t_{10} = 0.2159, p = 0.8334, ODR = 0.328, SedRES*Veh: t_{10} = 0.2688, p = 0.7936, ODR = 0.341; SedRES*Scop: t_{10} = 0.6128, p = 0.5537, ODR = 0.347; RunRES*Veh: t_{10} =

0.6941, p = 0.6941, ODR = 0.348; RunRES*Scop: t_{10} = 0.1951, p = 0.8492, ODR = 0.344), signifying no preference for any one of the 3 objects (Fig. 3-7B).

During the 5 min testing trial 24 h later to measure long-term recognition memory, groups pre-treated with oral resveratrol or a combination of resveratrol and treadmill running showed preferential exploration of the displaced object, C, whether administered with vehicle or scopolamine 30 min prior to the NOR task (SedRES*Veh: t_{10} = 3.599, **p < 0.01, ODR = 0.479; SedRES*Scop: t_{10} = 3.081, *p < 0.05, ODR = 0.427; RunRES*Veh: t_{10} = 3.8, **p < 0.01, ODR = 0.464; RunRES*Scop: t_{10} = 2.899, *p < 0.05, ODR = 0.412) (Fig. 3-7C). There was no indication of recognition memory in groups that were administered scopolamine following no pre-treatment regime or a treadmill running regime alone, although rats from those treatment groups that were administered vehicle instead of scopolamine were able to perform the task (SedCTL*Veh: t_{10} = 3.449, **p < 0.01, ODR = 0.479; SedCTL*Scop: t_{10} = 0.398, p = 0.699, ODR = 0.362; RunCTL*Veh: t_{10} = 6.712, ***p < 0.001, ODR = 0.541; RunCTL*Scop: t_{10} = 1.143, p = 0.2798, ODR = 0.300), suggesting that these rats were unable to learn this task following scopolamine administration (Fig. 3-7C). An unpaired two-tailed t-test revealed that scopolamine-treated animals showed significantly more exploration in the testing trial than vehicle-treated animals (t_{46} = 3.982, ***p < 0.001), with one-way ANOVA revealing no difference between scopolamine-treated groups ($F_{3,20}$ = 1.571, p = 0.2277). These results indicate that pre-treatment with oral resveratrol for 7 days can block the deficits observed with scopolamine-induced amnesia, but 7 days treadmill running cannot evoke a similar action on the pathway.

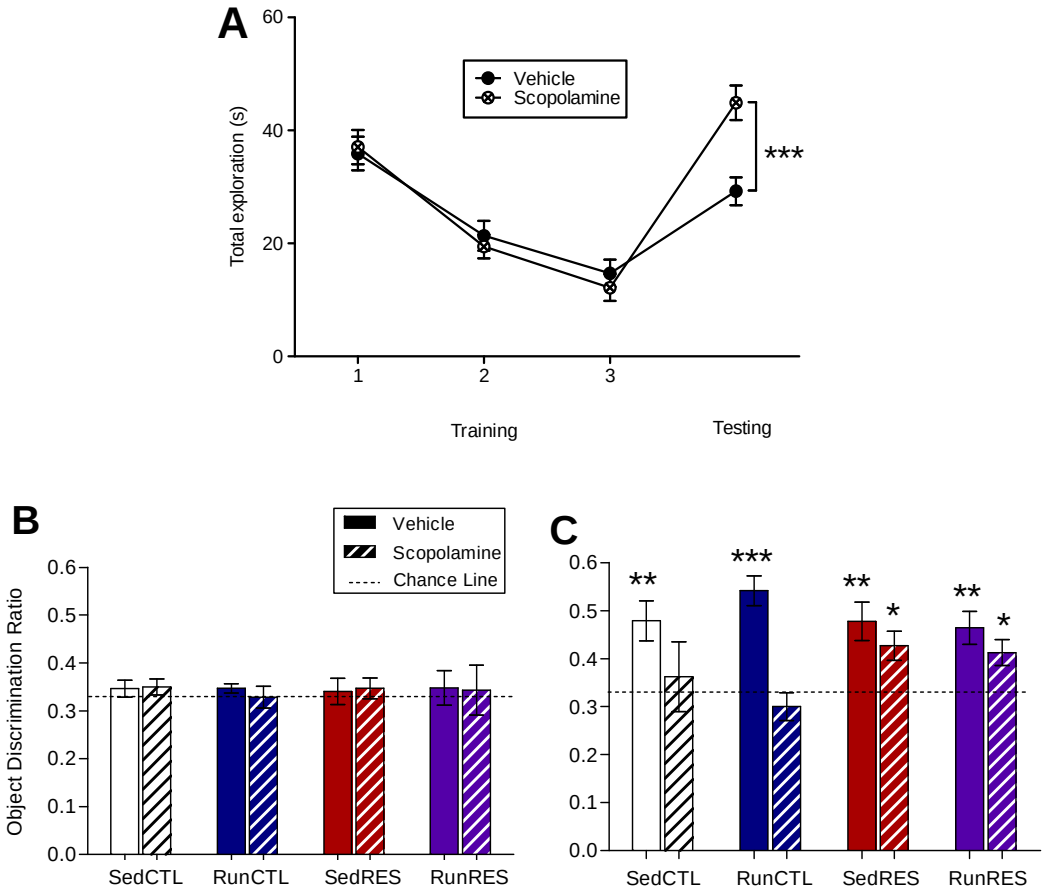


Fig. 3-7. A 7 day regime of resveratrol ingestion improved scopolamine-induced amnesia. Long-term spatial recognition memory was assessed using a displacement novel object recognition task. [A] Groups showed habituation to their environment with total exploration time decreasing across training trials 1 – 3. Scopolamine-administered explored more than vehicle administered in the testing trial. [B] Measurement of the time spent exploring each object was recorded and expressed as a discrimination ratio (novel object interaction/total interaction with all objects) for each training trial and averaged for each rat. No group showed any preference for object C above chance. [C] Following a 24 h delay animals were reintroduced to the arena with objects A and B in the same location, and object C displaced. Rats that had pre-treatment with resveratrol ingestion explored displaced object C more than objects A and B. Sedentary control and treadmill running rats and did not explore displaced object C more than the familiar objects. n = 6 per group. Results are presented as mean ± SEM. Values significantly different from the sedentary control exploration and familiar object exploration are indicated with an asterisk (Tukey's HSD: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.5 DISCUSSION

There were three major aims of this study. The first aim was to evaluate the similarities of 7 days resveratrol ingestion and 7 days aerobic exercise on healthy long-term memory. These findings indicate that with 7 days resveratrol treatment (20 mg/kg p.o.) and treadmill running (1 h/day), either combined or individually, there are significant improvements in long-term recognition memory in a healthy rat model. The second aim was to determine the potential mechanisms through which both aerobic exercise and resveratrol ingestion may act to evoke their beneficial action on learning and memory. Memory enhancement following 7 days of resveratrol ingestion and aerobic exercise was associated with increased neurotrophic expression and levels in the hippocampus and perirhinal cortex. Proteins in the AMPK/SIRT1 pathways were not upregulated in these brain regions associated with learning and memory. The third aim was to ascertain the therapeutic potential of oral resveratrol treatment and regular aerobic exercise in aiding memory decline associated with Alzheimer's disease. 7 days of resveratrol ingestion, either alone or combined with treadmill running, relieved scopolamine-induced amnesia. Treadmill running alone could not counteract the amnesic effects of scopolamine administration.

In a previous study, it was found that oral resveratrol administration and regular treadmill running in a 4 week programme showed similar memory enhancement in both healthy young and middle-aged male Wistar rats (see Chapter Two). An exercise regime is already often prescribed alongside other treatments for patients with cardiovascular disease, diabetes type 2, Alzheimer's disease, and other noncommunicable diseases (Armstrong, 2006). Additionally, physical activity has been shown to have desirable effects on working memory (Clarkson-Smith and Hartley, 1989), long-term memory (Griffin et al., 2009) and spatial learning (Kobilo et al., 2011). It is of interest to find edible compounds that evoke the same widespread, beneficial action physiologically that aerobic exercise evokes because physical inactivity is one of the leading causes of many noncommunicable diseases despite the vast knowledge about the benefits of taking regular exercise (World Health Organisation, 2009, 2010). Plant-derived phenolic compounds, such as

resveratrol, are potential candidates for this general therapeutic use. Resveratrol has been shown to have protective properties *in vitro* and *in vivo* against animal models of a variety of Alzheimer's disease symptoms when administered locally or intraperitoneally (Marambaud et al., 2005; Kim et al., 2007; Gacar et al., 2011). This study specifically focussed on the effects of ingesting a relatively small quantity of resveratrol, with the intention of replicating the action of taking resveratrol tablets as a supplement. This treatment regime of 20 mg/kg resveratrol orally for 7 consecutive days showed similar novel object recognition performance improvements as those observed with a forced treadmill running programme of 1 h daily for 7 consecutive days. Assessed in a model depicting the amnesia associated with Alzheimer's disease, resveratrol ingestion showed greater therapeutic potential than regular aerobic exercise over this time-scale.

Before treatment, all groups expressed normal health and, following 5 days of resveratrol ingestion and treadmill running, general exploration and spontaneous behaviour in response to a novel environment was assessed in an open field. Measurements of general locomotor activity and willingness to explore novel areas allow assessment of emotion and anxiety (Stanford, 2007). All groups in experiment one and two spontaneously explored the arena normally, in terms of distance travelled and velocity. Groups did not differ in terms of initial exploration in the task (training trial 1), indicating similar visuomotor function and exploratory tendencies. This supports findings from other studies investigating the sedative effects of resveratrol administration. No sedative effects were found with acute doses of 20, 40 and 80 mg/kg i.p. in rats with pentylenetetrazole (PTZ) induced seizures (Gupta et al., 2002b) or 20 mg/kg i.p. in healthy rats (Gupta et al., 2004). No sedative or stimulant effects of resveratrol administration were observed after 4 weeks using the same treatment regimes in this study (see Chapter Two). Interestingly, studies using higher doses of resveratrol have found contradictory findings following much longer treatment regimes, with one stating decreased spontaneous locomotor activity in rats with a 15 week treatment regime (200 and 400 mg/kg p.o. daily) (Lagouge et al., 2006) and another finding increased spontaneous locomotor activity in grey mouse lemurs with an 18 month regime (200 mg/kg p.o. daily) (Dal-Pan et al., 2011). In addition, all groups showed normal habituation to their spatial environment, with

gradually decreasing exploration of objects across training trials. This asymptotic habituation curve indicates normal encoding of the environmental features and spatial configuration, suggesting that any alterations to the environment should evoke increased exploratory behaviour.

In experiment one, a substitution version of the novel object recognition task was used (Fig. 3-1A). It has previously been shown that this is too difficult for healthy young rats to perform following a 24 h delay with just 3 x 5 min training trials (Griffin et al., 2009). This task was chosen to highlight any enhancement associated with resveratrol ingestion and treadmill running. Resveratrol has been shown to evoke neuroprotective effects against traumatic brain injury with a single 100 mg/kg i.p. dose administered immediately after induction of brain injury (Sönmez et al., 2007), against Alzheimer's disease symptoms *in vitro* (Wu et al., 2008), and against seizures using a single dose of 40 mg.kg i.p. administered 5 min before kainic acid-induced seizure (Gupta et al., 2002a). There is also evidence to suggest that resveratrol can improve cognitive function in aged animals, with mice given 150 µg resveratrol per gram food for 6 to 8 months performing better in a Y-maze task (Oomen et al., 2009). 7 consecutive days of treadmill running has been shown to improve object recognition memory in healthy rats (O'Callaghan et al., 2007; Griffin et al., 2009), with 7 weeks of voluntary wheel running showing neuroprotection against brain injury in rats (Will et al., 2004), and 6 to 8 months of aerobic exercise improving cognitive scores in elderly people with mild cognitive impairment (MCI) and dementia (Ahlskog et al., 2011). The treatment regimes of resveratrol ingestion and treadmill running used here have been shown to improve NOR performance over a longer epoch of 4 weeks (see Chapter Two). Here, no recognition memory was evident with the sedentary controls after a 24 h delay as assessed by the object discrimination ratio of novel object, D. All other groups showed increased exploration of novel object, D, relative to familiar objects, A and B. It was important to rule out preference for the object itself rather than the fact it was novel, as shown by disinterest from the sedentary controls 24 h later, as well as preference for object location, indicated by assessment of the object discrimination ratio of object C in training sessions relative to objects A and B. These findings indicate that after 7 days of resveratrol treatment and aerobic exercise, either

combined or individually, there are significant improvements in long-term recognition memory in healthy young rats. These results are similar to findings using a 4 week regime, showing that neither a long regime of treadmill running nor resveratrol ingestion are required to evoke potent effects on cognition. This corresponds to other studies investigating object recognition memory using a similar aerobic exercise regime of 7 days forced treadmill running (O'Callaghan et al., 2007; Griffin et al., 2009). Another study found that a single dose of 100 mg/kg resveratrol i.p., albeit much higher in concentration than ours, lessened neuronal loss in rat pups subjected to contusion injury (Sönmez et al., 2007), whilst another found that a dose of 250 and 500 mg resveratrol in capsule form increased cerebral blood flow in humans, but did not improve cognitive function (Kennedy et al., 2010). Acute bouts of exercise are deemed to improve cognition unless the fatigue levels from that session are too great (Tompoowski, 2003). The cerebral effects of resveratrol are potent, fast-acting, and comparable to the effects of aerobic exercise on healthy memory.

Hippocampal and perirhinal tissue was extracted from the brains of all rats in experiment one and assessed for expression and levels of proteins previously implicated in the beneficial action of resveratrol and aerobic exercise on the body. Many studies investigating cognition refer to resveratrol as a SIRT1 activator (Kim et al., 2007; Pallas et al., 2009; Baur, 2010) since this compound was found to consistently recapitulate the protective effects of SIRT1 overexpression in cell culture (Howitz et al., 2003; Araki et al., 2004). However, with no evidence to indicate that resveratrol is a direct activator of SIRT1, it may prove misleading to refer to resveratrol as simply a SIRT1 activator. More recently, AMPK has been suggested as an alternative target for resveratrol that may be important for upregulating the beneficial pathways associated with resveratrol action (Narkar et al., 2008), with an interdependence of these proteins highlighted in another study (Price et al., 2012). In a previous study it was discovered that upregulation of the AMPK/SIRT1 pathways in the hippocampus and perirhinal cortex were not necessary for cognitive enhancement associated with resveratrol ingestion of treadmill running (see Chapter Two). In this study, results of the polymerase chain reaction further indicated that proteins involved in the AMPK/SIRT1 pathways were

not upregulated in brain regions associated with learning and memory following 7 days of these treatment regimes. As with a 4 week regime, no increases in levels of SIRT1, AMPKα1, AMPKα2, PGC-1α or MnSOD mRNA were found in the dentate gyrus, rest of hippocampus, or perirhinal cortex following 7 days of resveratrol ingestion or treadmill running. This further supports the suggestion that the cognitive enhancement associated with these treatment regimes is not evoked through the AMPK/SIRT1 pathways. There were significant increases in expression of the neurotrophins, BDNF and NGF, in these brain regions; with levels of these proteins also increased relative to sedentary controls. The findings from this protein analysis are further supported by results using a 4 week regime (see Chapter Two). Additionally, increased BDNF and NGF expression has been measured in the hippocampus following 2, 4 and 7 nights of voluntary wheel running in rats (Neeper et al., 1996), with acute bouts of exercise increasing vascular endothelial growth factor (VEGF) levels in untrained human skeletal muscle, but not trained skeletal muscle (Richardson et al., 2000). Increased circulating VEGF levels with 2.5 mg/kg p.o. resveratrol daily for 15 days in streptozotocin-induced diabetic rats (Thirunavukkarasu et al., 2007) and dose-dependent increases in hippocampal BDNF levels comparing resveratrol doses ranging from 2.5 to 20 mg/kg p.o. daily for 3, 10 and 30 days (Rahvar et al., 2011). It appears that resveratrol and aerobic exercise may evoke their effects on cognition through neurotrophins, or other proteins implicated in these pathways. This supports the suggestion that these regimes may enhance memory by encouraging early cell survival in brain regions associated with learning and memory (Frielingsdorf et al., 2007; van Praag, 2009).

In experiment two, a displacement version of the novel object recognition task was used (Fig. 3-1B). This is a spatial memory task which targets long-term memory which is intact in young healthy rats (Griffin et al., 2009). This task was appropriate to draw attention to any deficits caused by scopolamine administration 30 min prior to novel object task exposure, and to highlight any enhancement associated with resveratrol ingestion and treadmill running. Scopolamine-induced amnesia is a pharmacological tool to model AD-related cognitive decline by addressing the cholinergic system dysfunction associated with the disorder (Smith, 1988). This study was designed to ascertain whether an oral dose that showed

cognitive improvement in healthy rats without upregulating the AMPK/SIRT1 pathways could elicit the same positive effects against AD-related amnesia. Sedentary controls treated with vehicle prior to novel object recognition task exposure exhibited recognition memory after a 24 h delay as assessed by the object discrimination ratio of displaced object, C, relative to familiar objects, A and B. Sedentary controls administered scopolamine showed no increase in exploration of displaced object, C. This shows that administration of scopolamine (0.8 mg/kg; i.p.) 30 min prior to introduction to a displacement novel object recognition task interfered with learning and left the rats unable to remember the situation 24 h later. This dosage has been shown in previous studies to have similar effects on cognition (Okaichi et al., 1989; Biggan et al., 1996; Ormerod and Beninger, 2002). This action occurs by blocking central acetylcholine (ACh) muscarinic receptors (Coyle et al., 1983), as cholinergic neurons and pathways play widespread roles in the regulation of learning, memory and cerebral blow flow to the nervous system (Mesulam et al., 2002).

Pre-treatment with oral resveratrol or a combination of resveratrol and treadmill running eradicated the scopolamine-induced amnesia, with animals administered scopolamine performing as well as animals given vehicle in these groups. Resveratrol has been shown to dose-dependently improve scopolamine-induced amnesia in rats when 12.5, 25 and 50 mg/kg i.p. resveratrol was administered 30 min before amnesia was induced with 0.6 mg/kg scopolamine i.p. 30 min before the probe trial in a Morris water maze task (Gacar et al., 2011). Conversely, pre-treatment with 10 and 20 mg/kg i.p. daily for 21 days did not improve scopolamine-induced (1 mg/kg; i.p.) amnesia in mice as measured in a Morris water maze task (Gupta et al., 2012). This 7 day regime of treadmill running did not counter the memory deficits induced by scopolamine administration; whilst those given vehicle showed preferential exploration of the displaced object, C, those administered scopolamine 30 min prior to the novel object recognition task showed no indication of recognition. In another study, treadmill running for 60 days (1 h/day) has been shown to improve AD-related amnesia caused by lesioning the nucleus basalis magnocellularis (NBM) in rats (Hoveida et al., 2011), with incidence of AD lower in more active people (Colcombe et al., 2004; Larson et al., 2006).

Although other studies have shown that aerobic exercise can improve AD-related memory decline, this 7 day regime was not enough to evoke such effects. It was important to rule out preference for the object itself rather than the fact it was displaced, as shown by disinterest from the sedentary controls 24 h later, as well as preference for object location, indicated by assessment of object discrimination ratio of object C in training sessions relative to objects A and B. These results indicate that pre-treatment with oral resveratrol for 7 days can block the deficits observed with scopolamine-induced amnesia, but 7 days treadmill running cannot evoke a similar action on this pathway. It is of use to know that these effects are potent through oral administration, as this will provide an agreeable supplementation route in humans. These results suggest that short-term resveratrol treatment has more potential as a therapeutic agent against AD than short-term aerobic exercise. Resveratrol ingestion appears to have a stronger action on the cholinergic pathway than is evoked by aerobic exercise.

Scopolamine-treated animals had much lengthier exploration times compared to vehicle-treated animals in the testing trial of the novel object recognition task. These animals had exploration times longer than the initial training trial the previous day. This supports previous studies that indicate that scopolamine induces hyperactivity (Shannon and Peters, 1990). With no differences measured between scopolamine-treated groups, it appears that neither resveratrol ingestion nor treadmill running had an impact on this hyperactivity, as supported by results from the open field task. However, this did not interfere with resveratrol's therapeutic action on scopolamine-induced amnesia. As these aerobic exercise and resveratrol regimes have been shown to evoke similar changes on the proteins assessed here in hippocampal and perirhinal tissue, it appears that the AMPK/SIRT1 pathways do not play a primary role in this action of resveratrol. Additionally, aerobic exercise and resveratrol ingestion both show similar upregulation of neurotrophins in the hippocampus and perirhinal cortex with these 7 day regimes, indicating that increased levels of these proteins are not sufficient to improve AD-related cognitive decline. These findings suggest that aerobic exercise and the polyphenol, resveratrol, may act through the same pathway to enhance normal cognition but resveratrol

ingestion has a stronger action on the cholinergic pathway and a stronger therapeutic potential against AD-related amnesia.

To summarise, resveratrol ingestion and regular aerobic exercise both evoke a strong enhancement of healthy memory using 7 day regimes. With associated activation of the neurotrophins, BDNF and NGF in the hippocampus and perirhinal cortex, it appears that these play a role in the memory enhancement measured. Short-term resveratrol administration has stronger action against scopolamine-induced amnesia than short-term aerobic exercise, therefore, promoting the therapeutic potential of resveratrol against the amnesia associated with Alzheimer's disease.

Chapter Four

ORAL RESVERATROL AND AEROBIC EXERCISE REGIMES CAN IMPROVE MEMORY WITHOUT INCREASING GENERAL MITOCHONDRIAL FUNCTION

"My mitochondria comprise a very large proportion of me. I cannot do the calculation, but I suppose there is almost as much of them in sheer dry bulk as there is the rest of me."

- *Lewis Thomas*

4.1 ABSTRACT

Mitochondria are extremely important organelles essential for normal cell function, maintenance of redox homeostasis and programmed cell death. Mitochondrial dysfunction is considered one of the main reasons for cognitive decline related to normal ageing as well as many neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and Huntington's disease. Mitochondrial function is heavily entwined with other degenerative processes, such as oxidative stress and hormonal imbalance. Resveratrol treatment and aerobic exercise have both been shown to improve mitochondrial function in skeletal muscle and other tissues. Previous studies have shown that low doses of oral resveratrol and daily treadmill running for just 7 days enhances normal

memory in healthy young adult Wistar rats. It is not yet clear how resveratrol or aerobic exercise evoke such effects on cognition, however, it could possibly be through action on mitochondrial function directly, or the downstream effect on oxidative stress or the endocrine system.

To further explore the changes that occur in the body with low doses of resveratrol ingestion and short aerobic exercise regimes that evoke strong cognitive enhancement, 12 day regimes of regular resveratrol ingestion (20 mg/kg) and 1 h treadmill running (17 m/min) were utilised in healthy male Wistar rats to assess if these induced changes in mitochondrial function. To assess this, oxygen consumption, uncoupling protein expression and mitochondrial abundance were measured in tissues that expend large quantities of energy and require higher numbers of mitochondria - brown adipose tissue (BAT) and skeletal muscle (SKM). Mechanisms that enhance mitochondrial function in BAT are of interest to determine as this is proposed to play a future role in the treatment of obesity in humans.

After the 12 day regimes of resveratrol ingestion and aerobic exercise, neither resveratrol nor treadmill running increased thermogenesis in brown adipocytes by oxidative phosphorylation, had any effect on uncoupling protein 1 (UCP-1) in BAT or uncoupling protein 3 (UCP-3) in SKM mitochondria, nor any effect on mitochondrial abundance in these tissues.

These results indicate that neither daily 20 mg/kg oral resveratrol treatment nor 1 h treadmill running for 12 consecutive days has a notable effect on mitochondrial function. Although from the literature it can be predicted that higher doses and longer training regimes may increase mitochondrial function in these tissues. These findings suggest that the memory enhancement associated with these short-term regimes is not associated with improvements to mitochondrial function.

4.2 INTRODUCTION

Mitochondria are highly dynamic organelles that fuse and divide in response to environmental stimuli, developmental status, and energy requirements (Seo et al., 2010). Among other functions, they provide energy for anabolic reactions by holding adenosine-5'-triphosphate (ATP) produced from catabolic reactions. Hydrolysis of ATP to adenosine-5'-diphosphate (ADP) or adenosine-5'-monophosphate (AMP) provides energy for most biological processes making mitochondria essential for normal cell function and maintenance of redox homeostasis and programmed cell death (Marzetti et al., 2010). A decline in mitochondrial function seems to be an important modulating influence on the ageing process in many species, and it can have positive or negative effects on lifespan (Sedensky and Morgan, 2006). A reduction in the expression of mitochondrial genes with ageing is strongly conserved from *C. elegans* to humans (Zahn and Kim, 2007), with a reduction in mitochondrial function shown to shorten lifespan (Trifunovic et al., 2004; Kujoth et al., 2005) and augmentation of mitochondrial function extending lifespan (Lin et al., 2002; Schriner et al., 2005) in a number of species. The mechanisms of mitochondrial function play a role in many cellular processes, such as mitochondrial metabolism, redox signalling, maintenance of mitochondrial DNA (mtDNA), and autophagy.

The highest densities of mitochondria are found in skeletal muscle (SKM), in particular SKM with a high proportion of slow twitch (Type I) fibres relative to fast twitch (Type II) fibres. The gastrocnemius is found in the lower leg and is packed with type I fibres. This muscle is rich in mitochondria, myoglobin and capillaries, making it red in appearance and more suitable for oxidative energy production (Wang et al., 2004). These SKM fibres produce ATP through oxidative phosphorylation, while type II fibres produce ATP through anaerobic mechanisms, such as glycolysis. Oxidative phosphorylation is thought to occur in SKM through upregulation of uncoupling protein 3 (UCP-3) which leads to a higher degree of uncoupling in mitochondria (Nedergaard and Cannon, 2003), though this is not certain. The proportion of type I to type II fibres in SKM can be adjusted through different types of training. Aerobic training will produce higher numbers of type I fibres to facilitate energy release for long periods (Howald et al., 1985). Strength

training produces higher numbers of type II fibres to facilitate fast energy release. As such, aerobic training increases the mitochondrial density in SKM to a greater extent than strength training. Exercise increases hepatic mitochondrial number (Chabi et al., 2005; Lopez-Lluch et al., 2006) as well as mitochondrial numbers in skeletal muscle. Regular exercise increases mitochondrial biogenesis and decreases various manifestations of oxidative stress (Holloszy, 1967; Oh-Ishi et al., 1997; Boveris and Navarro, 2008). Administration of high doses of resveratrol intraperitoneally to mice has also been shown to increase transcription of genes associated with oxidative phosphorylation and mitochondrial biogenesis in SKM (Lagouge et al., 2006).

Another tissue in mammals that is rich in mitochondria is brown adipose tissue. Brown adipose tissue (BAT) is a unique organ that has provided mammals with an evolutionary advantage. The primary function of BAT is to generate body heat in animals that do not shiver, allowing mammals to survive and be active during periods of nocturnal or hibernal cold and survive the cold stress of birth. It is particularly abundant in newborns and hibernating mammals (Cannon and Nedergaard, 2004). Brown adipocytes produce heat through non-shivering thermogenesis (NST), which is controlled by uncoupling oxidative phosphorylation in the inner mitochondrial membrane, allowing for proton leakage (Lončar et al., 1986; Porter and Brand, 1995). Noradrenaline (NA) released from the hypothalamus regulates this pathway by controlling the expression of uncoupling protein 1 (UCP-1) which catalyses oxidative phosphorylation. NA binds to β_3-adrenoreceptors on the surface of brown adipocytes, activating an attached G protein-coupled receptor (GPCR) which binds to adenylate cyclase (AC), activating it to produce cyclic adenosine monophosphate (cAMP) from ATP. cAMP then activates protein kinase A (PKA) which activates a number of transcription factors, such as cAMP response element-binding protein (CREB), leading to increased expression of UCP-1 (Richard et al., 2010). Upregulation of UCP-1 leads to a higher degree of uncoupling in mitochondria by uncoupling oxidative phosphorylation, leading to more energy is dissipated as heat. UCP-1 is the mitochondrial protein responsible for the unique function of BAT (Heaton et al., 1978; Aquila et al., 1985). For years it was thought that BAT was no longer present in adult humans because it was no longer needed as the individual grew and put on weight. This matter has received increased attention

since the discovery that adult humans also have BAT (Kramarova et al., 2008); with levels significantly lower in overweight or obese subjects. There is a negative relationship between BAT and both body mass index and percentage of body fat (van Marken Lichtenbelt et al., 2009). Whilst the more familiar white adipose tissue (WAT) can be viewed as fat which stores energy in the form of triglycerides, BAT can be viewed as fat which burns energy. BAT cells are able to do this as they have multilocular lipid droplets and are rich in mitochondria. UCP-1 production is a key marker that differentiates between the adipose tissues, as well as the presence of more noradrenergic fibres in brown adipocytes. Brown adipocytes are more similar to skeletal muscle than white adipocytes, and developmental studies have shown that proteins involved in muscle differentiation are also expressed in BAT (Sadurskis et al., 1995). However, studies have shown that through increased levels of the hormone, irisin, aerobic exercise can increase mitochondrial abundance in WAT, making it more like BAT (Boström et al., 2012). Potential activation, and increased levels, of BAT has been postulated to play a role in the treatment of obesity in humans (Seale and Lazar, 2009).

Resveratrol and aerobic exercise are strong candidates to play a role in the treatment of obesity in humans by controlling mitochondrial biogenesis and metabolism; most likely through activation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). PGC-1α is a transcriptional coactivator that is regarded to be one of the key metabolic controllers in cells. It is activated by signals that are related to energy and nutrient homeostasis (Liang and Ward, 2006). Upon activation, PGC-1α induces and coordinates gene expression that controls various pathways depending on the tissue and other coactivators. Mitochondrial biogenesis, fibre-type switching in SKM, and the thermogenic response in BAT are under the control of PGC-1α (Liang and Ward, 2006). The specific interaction of PGC-1α with ubiquitous transcription factors that bind to the promoter region of those target genes activates the expression of metabolic genes (Rodgers et al., 2005). The activity of PGC-1α is under specific and very tight control because is involved in the regulation of so many crucial pathways (Fernandez-Marcos and Auwerx, 2011). It is thought that much of resveratrol's

action in the body occurs through upregulation of PGC-1α, whether through activation of nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase sirtuin-1 (SIRT1), AMP kinase (AMPK), or another mechanism (Nemoto et al., 2005; Rodgers et al., 2005; Jäger et al., 2007; Amat et al., 2009). There is also evidence that aerobic exercise upregulates PGC-1α (Handschin and Spiegelman, 2008), which is also believed to occur through activation of the SIRT1/AMPK pathways. These metabolic alterations regulate the phosphorylation and deacetylation of PCG-1α and subsequent mitochondrial biogenesis (Handschin et al., 2003), predicted to be through increasing UCP expression (Boström et al., 2012). As such, resveratrol administration and aerobic exercise are strong candidates to play a future role in the treatment of obesity.

With the mitochondria proving central to many biochemical processes in cells, it is unsurprising that these organelles are important for cognitive processing. Mitochondrial dysfunction is considered one of the main reasons for normal memory decline with ageing (Harman, 1972) and lower cognitive performance associated with Alzheimer's disease (Mancuso et al., 2008), Parkinson's disease (Farrer, 2006), and Huntington's disease (Browne and Beal, 2004); with additional effects on other degenerative processes, such as oxidative stress and hormonal imbalance (Bua et al., 2006). In previous experiments, a regular oral dose of maple syrup and resveratrol suspension (20 mg/kg) for a 1 or 4 week regime have been shown to enhance normal long-term recognition memory in Wistar rats. Treadmill running (1 h/day; belt speed increasing from 10 - 17 m/min) over the same epochs has a similar action on this form of cognition. Upregulation of the SIRT1/AMPK pathways in the hippocampus and perirhinal was not associated with memory enhancement in these studies (see Chapter Two and Three), suggesting that these favourable effects were not through improved mitochondrial function in brain regions associated with learning and memory. The aim of this study was to address whether or not this concentration of oral resveratrol or programme of aerobic exercise would have distinct effects on mitochondrial function in tissues with high energy expenditure and, therefore, mitochondrial density. Using these treatment regimes over a 12 day epoch, neither resveratrol nor treadmill running increased thermogenesis in brown adipocytes by oxidative phosphorylation. In addition, there was no effect on UCP-1 in BAT or

UCP-3 in SKM mitochondria, nor any effect on mitochondrial abundance in these tissues. This data suggests that quantities of oral resveratrol treatment and aerobic exercise that have no impact on general mitochondrial function have positive effects on functioning of the hippocampal formation.

4.3 MATERIALS AND METHODS

4.3.1 Animals

Male Wistar rats (435 ± 50 g; *n* = 18) were obtained from the BioResources Unit, Trinity College Dublin. They were housed in pairs (standard hard-bottomed, polypropylene cages; 44 × 28 × 18 cm) in a temperature-controlled vivarium (20 to 22 °C), with a 12:12-hour light-dark cycle. Animals were provided with food and water *ad libitum*. Experiments were carried out in strict accordance with regulations laid out by LAST Ireland and were compliant with the European Union directives on animal experimentation (86/609/EEC).

4.3.2 Drug and Dosing Regime

All rats were handled for one week pre-drug treatment and fed 0.5 ml of maple syrup (Maple Joe, Bernard Michaud) to familiarise them with feeding by syringe. *trans*-Resveratrol (>99% purity) from Sigma-Aldrich, UK was administered orally mixed in a solution of maple syrup. Treated animals were given an oral dose of maple syrup and resveratrol suspension (20 mg/kg; p.o.) for 12 consecutive days, with controls given maple syrup only. Rats were dosed 30 min before exercise protocol.

4.3.3 Exercise Programme

Rats (*n* = 18) were familiarised to motorised treadmills (Exer 3/6 treadmill, Columbus Instruments) by walking on the treadmill for 15 min (belt speed, 7 m/min) on 3 consecutive days. Rats were randomly assigned to 3 groups: sedentary controls (SedCTL), running controls (RunCTL), and sedentary resveratrol-treated (SedRES) (*n* = 6 in each). The exercise protocol consisted of running one hour per day for 12 consecutive days (belt speed, gradually increased over the training period from 10

m/min to a maximum of 17 m/min, which is equivalent to 1 km/h). The treadmill is equipped with wire loops at one end of the belt through which a mild electric shock can be delivered; these act to motivate the rats to run continuously and were activated at low levels (on average an intensity of three on a scale of 0–10; this represents a current of 1 mA with an inter-pulse interval of 2 s) throughout all exercise sessions. Rats were observed while exercising to ensure they ran continuously and also to monitor for signs of stress. Sedentary rats were placed on stationary treadmills with shock loops activated at low levels for the same duration. Rats were sacrificed 24 h after the final exposure to treadmills, with programmes staggered so that tissue from only 3 animals (one per group) was processed in one day.

4.3.4 Tissues and Serum Samples

Rats were sacrificed by decapitation and the inter-scapular brown adipose tissue was removed first. Fur in the inter–scapular region was dampened with 70% ethanol and the skin removed with scissors. All visible white adipose and connective tissue was removed with fine scissors before removing whole BAT tissue. This was placed in BAT Ringer's solution (5 ml) and stored at room temperature. BAT samples were processed the same day for functional studies or stored at -80^{o}C until citrate synthase (CS) analysis was complete.

The lateral surface of the lower left leg was dampened with 70% ethanol and skin was removed with scissors. The gastrocnemius muscle was extracted with fine scissors, placed in an Eppendorf tube (1.5 ml) and stored at -80^{o}C for later CS analysis.

4.3.5 Isolation of Mature Brown Adipocytes

Following extraction, tissue was stored at room temperature in BAT Ringer's solution in separate falcon tubes (15 ml) with the lid removed (except in transit). Tissue was processed in the same order of removal: SedRES, RunCTL, and then SedCTL. BAT was cut in half, placed in a Petri dish, covered with BAT Ringer's solution, and any remaining WAT, muscle and connective tissue was removed. The whole tissue was carefully diced using fine scissors in BAT Ringer's solution supplemented with Type II collagenase from *Clostridium histolyticum* (1mg/ml). The sample was then placed on a rock and roller in an incubator at 37^{o}C for 60 min until pulpy, with the sample checked regularly during this period. After removal from the incubator, the sample was filtered through a 70 µm sieve and topped up with BAT Ringer's solution to stop the reaction. The BAT was then gently pushed through the sieve using the rubber end of a 3 ml syringe plunger, while washing with BAT Ringer's solution. The cells were spun at 625 rpm for 2 min in a centrifuge (Sorvall H400) at room temperature. The tubes were then extracted, opened, and allowed to stand for 60 s to prevent cells from becoming anoxic. Using a plastic Pasteur pipette, the floating dark brown layer underneath the uppermost white layer was aspirated off, leaving the pellet, collagenase solution and white adipose. The dark brown layer was then added to a clean falcon tube and washed with BAT Ringer's solution. This was again spun at 625 rpm for 2 min, and then BAT was aspirated off and added to a clean Eppendorf tube (1.5 ml). This was left open and was stored at room temperature until use.

4.3.6 Measurement of Oxygen Consumption in Brown Adipocytes

All measurements of respiration were made using an Oxygraph-2k respirometer (Oroboros Instruments, Austria), and oxygen flux was resolved with DATLAB 4.0 software. The machine was calibrated for at least 30 min with BAT Ringer's solution at 37^{o}C. The brown adipocytes were counted using a haemocytometer. Isolated brown adipocytes were added (50,000 cells/ml) to the

machine and allowed to stabilise for ~30 min so that a stable basal rate could be observed. Once the stable rate was determined and recorded, 2 µM NA in 2 mM sodium ascorbate solution was added to the chamber via syringe to stimulate oxygen consumption rate, and the change in oxygen consumption was noted. The rate was left to stabilise before recording. 3 µM antimycin A was then added to the chamber via syringe to inhibit the mitochondrial complex II and therefore mitochondrial oxygen consumption, and the steady rate was recorded. The measurement of oxygen consumption in brown adipocytes was completed in triplicate for each animal (*n* = 18).

4.3.7 Preparation of BAT and SKM Mitochondria for Citrate Synthase

BAT samples that had been isolated and used to measure oxygen consumption were then stored at -80^{o}C until further analysis. On the day of the citrate synthase assay procedure, brown adipocytes were removed from -80^{o}C and stored on ice. The brown adipocytes were defrosted and homogenised using 7 passes with a loose (0.26 inches) clearance pestle followed by 7 passes of a tight clearance pestle. The homogenate was spun at 2,300 rpm for 10 min at 4^{o}C. The supernatant was then removed and added to a fresh Eppendorf tube. The supernatant was then stored on ice until ready for use on the same day.

Whole gastrocnemius samples were removed from storage at -80^{o}C and kept on ice. On the day of use, a prepared stock of sucrose muscle homogenisation buffer (100 ml) was added to sucrose (8.54 g). A skeletal muscle sample between 30 and 50 mg was obtained and finely diced using scissors. The finely diced muscle sample was then added to muscle homogenisation buffer (1 ml) and was homogenised, while on ice, with 7 passes of the automatic homogeniser. The samples were then spun at 2,300 rpm for 10 min at 4^{o}C. The supernatant was then removed and added to a fresh Eppendorf tube (1.5 ml) and stored on ice until ready for use on the same day.

4.3.8 Bicinchoninic Acid (BCA) Assay to Determine Total Protein Concentration

Bovine serum albumin (BSA) stock solution (0.5 µg/ml) was defrosted and BSA standards were set up in triplicate on a 96-well plate (3 x 10 µl). BAT samples were diluted 1:50 with distilled water. SKM samples were diluted 1:15 with distilled water. Each sample was added in triplicate to a well on the plate (3 x 10 µl). BCA working solution (200 µl) and BCA (9800 µl) were added to a falcon tube and mixed. The mixture was added to each well containing a BSA standard, BAT or SKM sample (200 µl). The plate was covered and placed in an incubator at 37^{o}C for 40 min. A multi-plate reader was then used to read the plate at 550 nm. The protein calculations were noted, and adjusted to account for dilution factors.

4.3.9 Citrate Synthase (CS) Assay to Determine Mitochondrial Abundance

Following determination of the final protein concentration using a BCA assay, a CS assay was carried out to determine the mitochondrial abundance per mg of cellular protein (µmol/min/mg). The CS assay works on the principle that citrate synthase generates citrate and coenzyme A (CoA-SH) which is coupled to the reaction between CoA-SH and Ellman's reagent (DTNB) using substrates oxaloacetate and acetyl-CoA. The formation of DTNB-CoA can then be measured photometrically. Here, a spectrophotometer (Shimadzu, UVmini-1240) was used to measure the formation of DTNB-CoA which has an absorbance at 412 nm.

The spectrophotometer was connected to a chart recorder (1 cm/min, full scale deflection 20 cm (0-1 Abs)). The spectrophotometer was zeroed with TRIS-Sucrose: 0.1 M TRIS 0.15 M sucrose pH 8.1 with HCl (2785 µl) which was kept on ice. 4 mg/ml 5,5-dithio-bis-nitrobenzoic acid (DTNB; 50 µl; Sigma-Aldrich, UK), 2 mM acetyl-CoA (50 µl; Sigma-Aldrich, UK), 10% Triton X (15 µl) and sample (25 µl) were added to each plastic cuvette (3 ml). The non-specific activity was then

recorded, ensuring that the chart recorder was set to record. The blank rate was recorded for 3 min. 60 mM oxaloacetate (75 µl; Sigma-Aldrich, UK) was then added to the cuvette to initiate the reaction, giving a total volume of 3 ml, and the observed rate was recorded. The process was repeated in triplicate for each BAT and SKM sample. The activity was then calculated and graphed.

4.3.10 Analysis of UCP-1 in BAT and UCP-3 in SKM Mitochondria using SDS-PAGE and Western Blot Analysis

Samples of BAT and SKM were solubilised in sample buffer; 0.0625 M Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 1.66% β-mercaptoethanol (added directly prior to use) and 0.001% bromophenol blue. Prior to loading on gel, samples were vortexed and pulsed in a minifuge before boiling at 100 °C for 5 min. Gels were run using the Laemmli system for denaturing gels (Laemmli, 1970). Slight modifications were used: 0.375 M Tris–HCl, pH 8.7 and 0.1% SDS were used in solutions. Linear 12% polyacrylamide gels were prepared from a stock solution containing 30% (w/v) acrylamide; 0.4% (w/v) bis-acrylamide. Samples were prepared using Laemmli sample buffer containing either 5% (w/v) β-mercaptoethanol or 20 – 100 mM dithiothreitol, and subsequently incubated for 5 min at 100 °C or 20 min at 70 °C. The gel dimensions were 90 mm x 70 mm x 0.75 mm and gels were run on a Protean II (Bio-Rad) gel system.

Following polyacrylamide gel electrophoresis, proteins were transferred to nitrocellulose or polyvinylidene difluoride (PVDF) (Millipore, Ireland). Transfer was achieved using a semi-dry apparatus for 2 h at 110 mA or a Bio-Rad TransBlot Transfer Cell for 1 – 4 h at 20 V/cm. After transfer was complete, blots were blocked for 1 h in phosphate-buffered saline (PBS)-Tween (0.14 M NaCl, 2.7 mM KCl, 11.5 mM Na_2PO_4, 1.8 mM KH_2PO_4, pH 7.4 with 0.1% Tween-20) containing 5% non-fat dried milk (Marvel). This was followed by three 10 min washes with PBS-Tween. Blots were probed with primary antibody overnight at 4 °C in the same solution

containing anti-UCP-1 antibody (Sigma; 1:1000) and anti-PDH-E1 alpha (Mitosciences, 1:2000) in BAT samples, and anti-UCP-3 antibody (Eurogenetec; 1:1000) and anti-PDH-E1 alpha (Mitosciences, 1:2000) in SKM samples. These steps were followed by three 10 min washes in PBS-Tween. Blots were then probed with secondary antibody incubated in 1:10,000 dilution of a donkey anti-sheep immunoglobulin G (IgG) horseradish peroxide (HRP) conjugate (for full-length UCP-1 antibody) or a goat/donkey anti-rabbit IgG HRP conjugate (for the others) secondary antibody in PBS-Tween containing 5% non-fat dried milk for 1 h. After a further three 10 min washes in PBS-Tween, the blots were developed using an enhanced chemiluminescence (ECL) detection system (Amersham-Biosciences, UK) and visualised by exposure to X-ray film. Following Western blot development, the relative abundance of UCP-1 in BAT, UCP-3 in SKM, and PDH as a loading agent in both were determined using densitometry. The band intensities of the exposed film were analysed using ImageJ Software.

4.3.11 Statistical Analysis

All data was analysed using GraphPad Prism (GraphPad Software, Inc.) and Statistical Package for the Social Sciences (SPSS). One-way ANOVA or unpaired Student's t-test were conducted as appropriate. *Post hoc* comparisons were made using the Tukey's HSD test. A significance level of $p = 0.05$ was accepted for all comparisons: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Data are presented as mean ± SEM.

4.4 RESULTS

4.4.1 No Effect on Oxygen Consumption in Brown Adipocytes

The impact of 12 days daily treadmill running was compared to daily oral resveratrol treatment on rate of oxygen consumption in brown adipocytes. Use of an Oxygraph-2k respirometer allowed assessment of the effects of these treatment regimes on freshly extracted tissue. Assessed at basal oxygen consumption rates, one-way ANOVA analyses revealed no significant differences between sedentary controls, exercised or resveratrol treated animals ($F_{2,15} = 0.7597$, $p = 0.485$) in brown adipocytes (Fig. 4-1). Once basal rate was determined, NA was added to assess oxygen consumption of these cells after activation of the pathway controlling the expression of UCP-1. One-way ANOVA revealed no significant differences between groups following activation by NA ($F_{2,15} = 1.726$, $p = 0.2116$) (Fig. 4-1). All groups showed a significant elevation in oxygen consumption rate following the addition of NA, as expected, compared to basal rates (SedCTL: ***$p < 0.001$; RunCTL: ***$p < 0.001$; SedRES: ***$p < 0.001$) (data no represented). With UCP-1 known to regulate oxidative phosphorylation (Heaton et al., 1978; Aquila et al., 1985), it is clear that these treatment regimes have no impact on oxidative phosphorylation in brown adipocytes. Finally antimycin A was added to the cells in order to ascertain how much of the oxygen consumption measured in brown adipocytes was through mitochondrial respiration. One-way ANOVA revealed no significant differences between groups following complete inhibition of the mitochondrial complex III by antimycin A ($F_{2,15} = 0.1009$, $p = 0.9046$) (Fig. 4-1). All groups showed significant decreased oxygen consumption following addition of antimycin A (SedCTL: ***$p < 0.001$; RunCTL: ***$p < 0.001$; SedRES: ***$p < 0.001$) with these rates also significantly lower than basal level (SedCTL: ***$p < 0.001$; RunCTL: ***$p < 0.001$; SedRES: ***$p < 0.001$), as expected (data not represented). Most oxygen consumption taking place in these cells, for all groups, was through mitochondrial respiration. Therefore, if these levels of oral resveratrol or aerobic exercise were to have any effect on thermogenesis in brown adipocytes, they would have to directly interfere with mitochondrial activity within the cell.

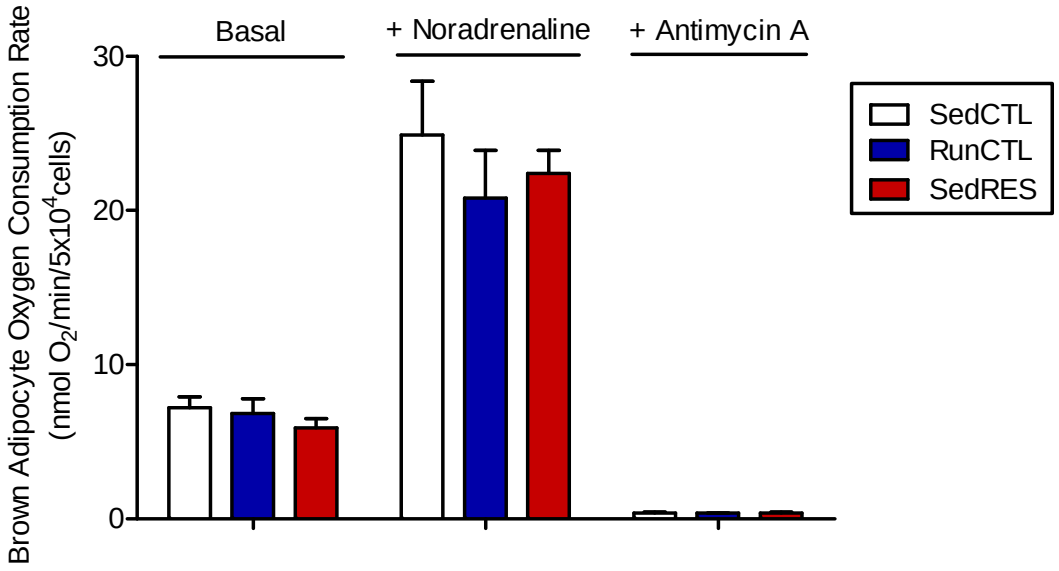


Fig. 4-1. No changes in oxygen consumption in brown adipocytes after 12 d resveratrol ingestion or treadmill walking. The mean level of oxygen consumption in isolated brown adipocytes was measured with an Oxygraph-2k respirometer. There was no difference between groups, with all groups reacting as expected to the addition of noradrenaline and antimycin A. Results are presented as mean ± SEM.

4.4.2 No Effect on Mitochondrial Abundance in BAT or SKM

To determine if this level of oral resveratrol or aerobic exercise were to have any effect on thermogenesis in brown adipocytes and skeletal muscle fibres, mitochondrial abundance was measured. Mitochondrial abundance in isolated brown adipocytes and skeletal muscle fibres was determined by citrate synthase (CS) assay, with activity of the citrate synthase enzyme serving as a marker for mitochondrial abundance. One-way ANOVA analyses revealed that there was no effect on mitochondrial abundance by the oral treatment with resveratrol or with treadmill running in BAT ($F_{2,15} = 0.1534$, $p = 0.8591$) (Fig. 4-2A) or SKM ($F_{2,15} = 0.3902$, $p = 0.6852$) (Fig. 4-2B). As it has just been illustrated that neither 12 days of exercise nor resveratrol ingestion affected thermogenesis in brown adipocytes, and that any thermogenesis that did occur was mitochondrial, the observation that mitochondrial number is not altered with these regimes is consistent with these results.

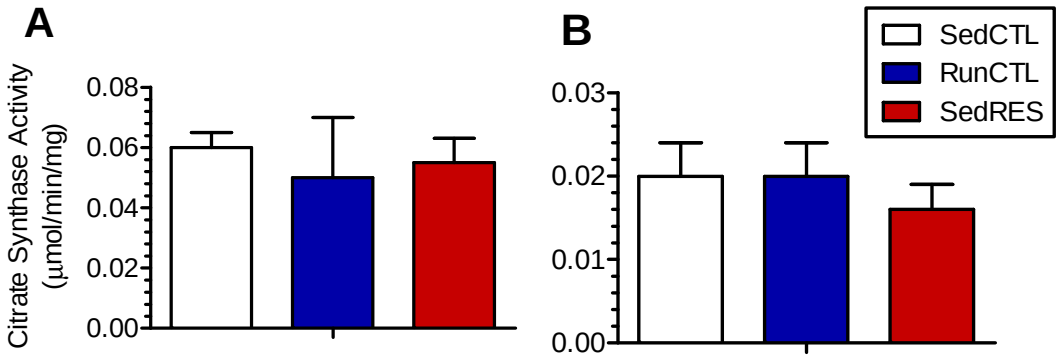


Fig. 4-2. No changes in mitochondrial abundance in [A] brown adipocytes and [B] gastrocnemius muscle fibres after 12 d resveratrol ingestion or treadmill walking. The mean mitochondrial abundance in isolated brown adipocytes and skeletal muscle fibres was determined by citrate synthase assay. No differences were found between groups. Results are presented as mean ± SEM.

4.4.3 No Effect on UCP-1 expression in BAT and UCP-3 in SKM Mitochondria

Resveratrol and aerobic exercise have previously been shown to evoke an enhancement of mitochondrial biogenesis through upregulation of PGC-1α, and subsequent expression of uncoupling proteins (Lagouge et al., 2006; Hood, 2009). To further determine any effects of these treatment regimes on oxidative phosphorylation by measuring expression of uncoupling protein-1 in BAT mitochondria and uncoupling protein-3 in SKM mitochondria. Figure 4-3A shows a representative immunoblot of UCP-1 (32kDa) and pyruvate dehydrogenase E1α (PDH; 44kDa) as a loading control in BAT mitochondria of control, exercised and resveratrol-treated rats. It is clear, that there was no difference in the expression level of UCP-1 between the three groups. To quantify the expression levels of UCP-1 in BAT mitochondria, densitometry measurements were performed and the UCP-1 signal normalised with the PDH signal for each group. One-way ANOVA analyses confirmed that UCP-1 expression levels were not changed in BAT mitochondria by the oral treatment with resveratrol or with treadmill running ($F_{2,15}$ = 0.02874, p = 0.9717) (Fig. 4-3B).

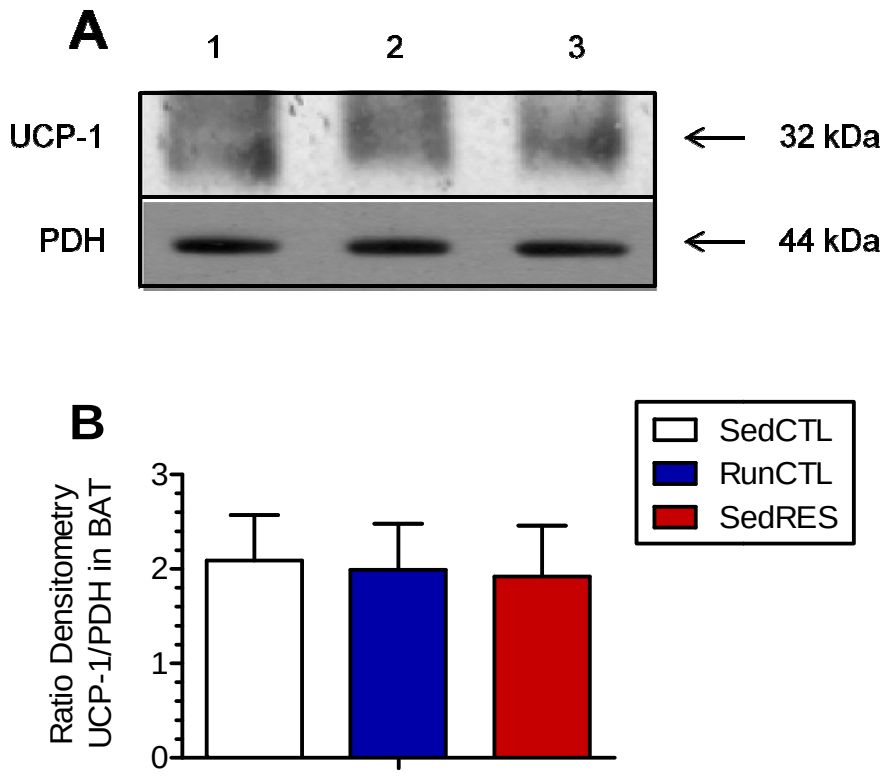


Fig. 4-3. No changes in uncoupling protein-1 expression in brown adipocytes after 12 d resveratrol ingestion or treadmill walking. [A] An immunoblot probed with 1:1000 dilution of anti-UCP-1 peptide antibody and an immunoblot probed with 1:2000 dilution of anti-PHD-E1α peptide antibody. Lane 1 represents sedentary controls, lane 2 exercised and lane 3 resveratrol-treated. Immunoblots were performed in triplicate. [B] Bands were quantified by densitometry using ImageJ software and presented as expression level ratios of UCP-1/PDH. The mean expression of UCP-1 in isolated brown adipocytes did not differ between groups. Results are presented as mean ± SEM.

Figure 4-4A shows a representative immunoblot of UCP-3 (32kDa) and PDH (44kDa) as a loading control in SKM mitochondria of control, exercised and resveratrol-treated rats. As with BAT mitochondria, there was no difference in the expression level of UCP-3 between the three groups. To quantify the expression levels of UCP-3 in SKM mitochondria, densitometry measurements were performed and the UCP-3 signal normalised with the PDH signal for each group. One-way

ANOVA analyses confirmed that UCP-3 expression levels were not changed in SKM mitochondria by the oral treatment with resveratrol or with treadmill running ($F_{2,15}$ = 0.2197, p = 0.8053) (Fig. 4-4B). These findings further support the belief that these treatment regimes of resveratrol ingestion and treadmill running did not promote uncoupling oxidative phosphorylation or mitochondrial biogenesis in these tissues.

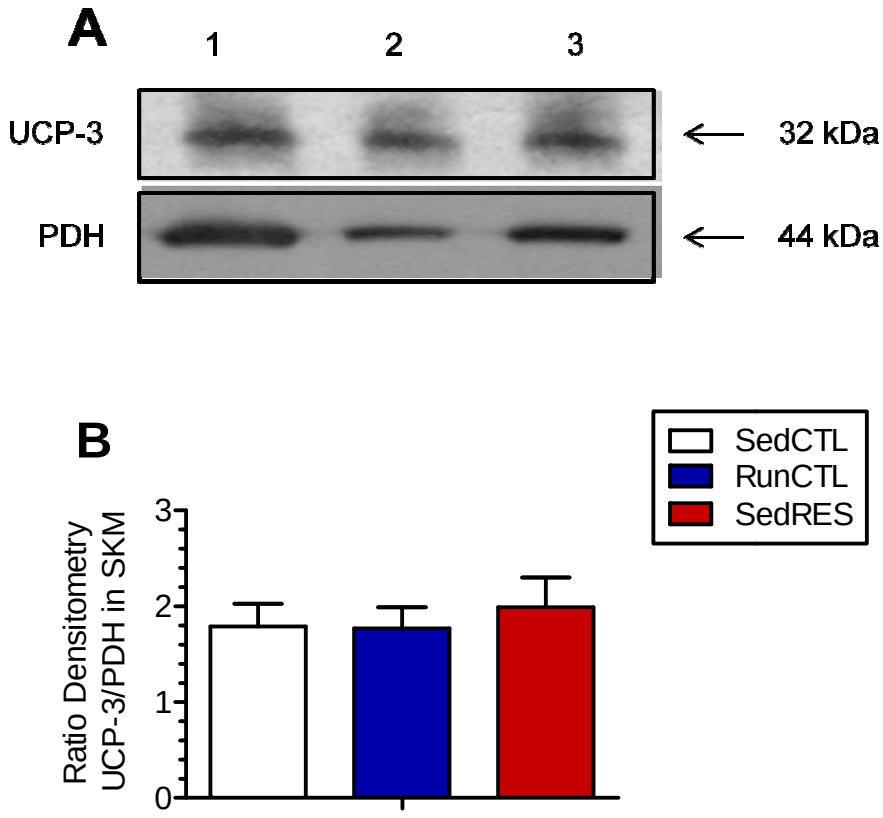


Fig. 4-4. No changes in uncoupling protein-3 expression in gastrocnemius muscle fibres after 12 d resveratrol ingestion or treadmill walking. [A] An immunoblot probed with 1:1000 dilution of anti-UCP-3 peptide antibody and an immunoblot probed with 1:2000 dilution of anti-PHD-E1α peptide antibody. Lane 1 represents sedentary controls, lane 2 exercised and lane 3 resveratrol-treated. Immunoblots were performed in triplicate. [B] Bands were quantified by densitometry using ImageJ software and presented as expression level ratios of UCP-3/PDH. The mean expression of UCP-3 in isolated skeletal muscle fibres did not differ between groups. Results are presented as mean ± SEM.

4.5 DISCUSSION

The aim of this study was to address whether or not a concentration of oral resveratrol or programme of aerobic exercise that have previously been shown to enhance normal cognitive functioning would have distinct effects on mitochondrial function in tissues with high energy expenditure and, therefore, mitochondrial density. These treatment regimes of 20 mg/kg resveratrol ingestion and treadmill running for 12 consecutive days did not increase mitochondrial abundance in brown adipose tissue or skeletal muscle, oxygen consumption, nor thermogenesis in brown adipocytes by uncoupling oxidative phosphorylation.

In previous experiments a regular oral dose of maple syrup and resveratrol suspension (20 mg/kg) for a 1 or 4 week regime has been shown to enhance normal long-term recognition memory in Wistar rats. Treadmill running (1 h/day; belt speed increasing from 10 - 17 m/min) over the same epochs has a similar action on this form of cognition (see Chapter Two and Three). Mitochondrial dysfunction is considered one of the main reasons for normal memory decline with ageing (Harman, 1972) and impaired cognitive performance associated with Alzheimer's disease (Mancuso et al., 2008), Parkinson's disease (Farrer, 2006), and Huntington's disease (Browne and Beal, 2004); with additional effects on other degenerative processes, such as oxidative stress and hormonal imbalance (Bua et al., 2006). The memory enhancement observed with these treatment regimes was not associated with upregulation of the AMPK/SIRT1 pathways in brain regions associated with learning and memory, suggesting that they do not elicit their favourable effects on cognition by countering mitochondrial dysfunction and improving the activity of these organelles. However, aerobic exercise has been shown to increase mitochondrial activity and levels in skeletal muscle (Holloszy, 1967; Howald et al., 1985) and brown adipose tissue (Boström et al., 2012). It may be that this training regime was not long enough to evoke such actions, with the mentioned studies using more strenuous protocols. Holloszy (1967) exercised rats on a treadmill for 12 weeks with the speed progressively increasing until in the final week the rats were running for 120 min at 31m/min with twelve 30 sec sprints at 42m/min interspersed through this session. Similar adaptations were detected in normally sedentary humans

exercised for 6 weeks (5 d/week) on bicycle ergometers for 30 min each session (Howald et al., 1985). Three weeks of free wheel running or swimming evoked such changes in adipose tissue (Boström et al., 2012). Resveratrol administration has also been shown to increase mitochondrial activity and levels in skeletal muscle using a dose of 200 and 400 mg/kg administered with food chow daily for 15 weeks (Lagouge et al., 2006). These doses are much higher than the dose used here that has been shown to enhance cognition (see Chapter Two and Three).

Results of oxygen consumption measured with an Oxygraph-2k respirometer indicated that these levels of oral resveratrol or aerobic exercise are not capable of enhancing thermogenesis in brown adipocytes. A common trend in this study was observed with basal rates and rates after the addition of NA generally proving higher than previously recorded in the literature (Bukowiecki et al., 1980; Marette and Bukowiecki, 1991; Cannon and Nedergaard, 2004), with a time-dependent effect on the magnitude of NA activation in brown adipocytes. The first few sets of brown adipocytes to be treated with NA showed a 5-7 fold increase, however, cells processed later in the day only showed a 2-4 fold increase. Despite this decrease in magnitude of activation by NA, basal rates remained statistically unchanged. This change in magnitude is likely because of receptor damage due to isolation of the cells. Therefore, one would conclude that there is a time-dependent effect with regard to NA sensitivity in isolated brown adipocytes. The control rates of oxygen consumption in isolated brown adipocytes were consistent with previous studies and served as a valid control to compare the effects of 12 days resveratrol ingestion (20 mg/kg/day) and 12 days treadmill running (1 h/day). Antimycin A significantly lowered oxygen consumption below basal and NA-stimulated rates, which confirmed that the vast majority of the respiration that occured within the brown adipocytes is mitochondrial respiration. Antimycin A acts by inhibiting the oxidation of ubiquinol in the electron transport chain. Through this action it prevents the formation of a proton gradient across the inner mitochondrial membrane, and subsequently inhibits oxidative phosphorylation from occurring (Dairaku et al., 2004). As a direct result, any oxygen consumption that occurs after addition of antimycin A is attributed to non- mitochondrial sources, such as enzyme activity through haem oxygenase (HO) (Evans et al., 2008) and monoamine oxygenase (MAO) (Audi et al., 2001).

Therefore, if this level of oral resveratrol or aerobic exercise were to have any effect on thermogenesis in brown adipocytes, they would have to directly interfere with mitochondrial activity within the cell. Mitochondrial abundance was measured to determine this.

Mitochondrial abundance per milligram of cellular protein can be measured by citrate synthase assay. The activity of the citrate synthase enzyme, serves as a marker for mitochondrial abundance. These results indicate that there is no change in mitochondrial abundance in BAT cells as a result of these resveratrol ingestion or aerobic exercise regimes. As it has just been illustrated that neither 12 days of exercise nor resveratrol ingestion affected thermogenesis in brown adipocytes, and that any thermogenesis that did occur was mitochondrial, the observation that mitochondrial number is not altered with these regimes is consistent with these results. The rates of citrate synthase activity in isolated brown adipocytes were lower than citrate synthase rates that have been reported in the literature (Nadal-Casellas et al., 2011), though there is a high degree of variation in the literature. These low values may be due to isolation and freezing of the BAT cells before thawing for mitochondrial isolation. This may have damaged the mitochondria and subsequently reduced citrate synthase activity. These sedentary control values for gastrocnemius cells are comparable to those in the literature (Pfeifer et al., 2001; Vila et al., 2001), however, mitochondrial abundance did not increase following the 12 day regime of treadmill running, as expected. In support of these findings, although vigorous aerobic exercise regimes have been shown to increase mitochondrial abundance in SKM (Holloszy, 1967; Howald et al., 1985), Leek and colleagues (2001) found that an acute bout of exercise resulted in numerous swollen mitochondria 1 h after exercise which can clearly confound training responses and artificially elevate citrate synthase values. A 12 day regime of aerobic training was too short to increase mitochondrial abundance, and the measurements were taken several hours following the final treadmill session, so an artificial citrate synthase increase was not detected either. When testing more vigorous regimes it will be important to leave sufficient time between the final training session and carrying out the citrate synthase assay. This will rule out artificial elevated levels due to temporarily swollen mitochondria. 12 days of treatment with low dose (20 mg/kg) resveratrol orally had no effect on

mitochondrial abundance in SKM either, although higher doses of oral resveratrol (200 and 400 mg/kg for 15 weeks) have been shown to increase mitochondrial numbers in this tissue (Lagouge et al., 2006).

Resveratrol action on brown adipocytes has not been directly assessed before, however, a number of studies have investigated the effects of exercise on brown adipocytes (Boström et al., 2012). Interestingly, a study using a similar exercise regime (7 d) with mice found increases in UCP-1 and PGC-1α expression, along with other key components for mitochondrial biogenesis and BAT activity (Slocum et al., 2012). If PGC-1α and these other components are being upregulated as a result of regular aerobic exercise, one would expect to see an increase in mitochondrial biogenesis and subsequently an increase in mitochondrial abundance and respiration (Uldry et al., 2006). An increase in mitochondrial or non-mitochondrial respiration was not observed here. In addition, there was no increased UCP-1 expression in BAT cells or UCP-3 expression in SKM which supports the findings that these regimes of resveratrol ingestion and treadmill running do not promote thermogenesis in brown adipocytes by uncoupling oxidative phosphorylation. In support of these findings, another study reported that aerobic exercise in rats reduced UCP expression in BAT, indicating that 9 weeks of this training regime had no overt effect on thermogenic activity (Segawa et al., 1998); with another group finding no effect of treadmill running, in young or aged rats, on UCP expression in BAT (Scarpace et al., 1994). Slocum and colleagues (2012) used diet-induced obese mice, whereas all the other studies have examined BAT samples in healthy rats. Maybe the action of aerobic exercise on UCP-1 expression varies between these species, or the high-fat diet may have been an adjusting factor in the action of aerobic exercise.

The data presented here suggests that oral resveratrol treatment and aerobic exercise that have similar positive impacts on functioning of the hippocampal formation do not evoke this action by improving mitochondrial function. The dose of resveratrol assessed here is considered a relatively low concentration, which nonetheless evokes a strong effect on normal cognition and AD-related amnesia (see

Chapter Three). Studies using higher doses, such as 0.3, 1 and 3 g/kg resveratrol administered intraperitoneally for 28 days (Hebbar et al., 2005), or 200 and 400 mg/kg orally for 15 weeks (Lagouge et al., 2006), have found an effect on mitochondrial function in skeletal muscle and other tissues. It is possible that if a higher dose of oral resveratrol was used here similar effects may have been found, and also an effect in brown adipose tissue. However, these results support the suggestion that action on mitochondrial function does not appear to be involved in memory enhancement associated with either of these factors (see Chapter Two and Three). These findings correspond with the fact that memory improvement with these regimes was not associated with upregulation of the AMPK/SIRT1 pathways in brain regions associated with learning and memory. It is possible that higher doses of resveratrol and an appropriate aerobic exercise programme that will increase oxygen consumption in brown adipose cells may prove a useful weight loss mechanism; however, such levels are not necessary to elicit beneficial effects on cognition.

Chapter Five

AEROBIC EXERCISE AND RESVERATROL AS PREVENTATIVE INTERVENTIONS IN WORKING MEMORY DECLINE

"Old age is like everything else. To make a success of it, you've got to start young."

- *Theodore Roosevelt*

5.1 ABSTRACT

Regular physical activity encourages favourable structural and metabolic alterations which can delay the ageing process, and the progression of age-related diseases. With an ageing population, and increases in the prevalence of noncommunicable diseases, it is becoming more desirable to identify orally active agents that mimic or potentiate the effects of aerobic exercise. One such candidate is the polyphenol, resveratrol, which may be termed an exercise mimetic due to its similar action on mitochondrial biogenesis, endurance, metabolism, long-term memory and the cardiovascular system.

To explore and compare the potential of long-term resveratrol ingestion and aerobic exercise in delaying the degeneration of cognitive function associated with ageing, middle-aged male Wistar rats were given a regular oral dose of resveratrol (20 mg/kg) to examine if this dose would have comparable

effects on learning, working memory and recovery of cognitive impairment as regular 1 h forced wheel running (17 m/min). For this, ongoing assessment with a delayed-non-matching-to-sample (DNMS) task was used, with delays randomised between 1 and 30 sec, to identify cognitive differences between treatment regimes. Animals were trained regularly in this task assessing spatial-temporal working memory, with progress compared between groups. Effects on recovery from cognitive impairment, induced by long intervals between DNMS sessions, were also measured.

All animals reached plateau level at the same stage of training, however, regular resveratrol ingestion and aerobic exercise enhanced performance in the DNMS task, compared to sedentary controls. Treated groups also showed improved recovery in performance after a 7 day interval between sessions, with clear differences in performance with longer delays, compared to sedentary controls. Additionally, no adverse effects were evident from 14 weeks of oral resveratrol treatment.

These results indicate that regular 20 mg/kg oral resveratrol treatment produces similar hippocampal-dependent cognitive enhancement in the DNMS task as regular 1 h bouts of forced wheel running, in middle-aged rats. Both regular resveratrol ingestion and aerobic exercise improved the learning of this task, working memory, and ability to recover memory after impairment. These findings highlight the potential use of resveratrol ingestion and aerobic exercise in relieving age-related cognitive decline.

5.2 INTRODUCTION

The global population is ageing, with average worldwide life expectancy predicted to reach 73 years by 2025 (Wise, 1998). Medical advances over the past century have improved mortality rates dramatically, and these continue to rise (World Health Organisation, 1998). This striking improvement is seen as one of the most notable achievements of the past century, but longer lives are not enough, we must strive for long, healthy lives. Ageing is the most prominent aetiological factor of physiological and neurological decline. It is an accumulation of damaging alterations at molecular and cellular levels that result in increased risk of morbidity and mortality (Johnson et al., 1999). With people living longer, this is currently creating a growing burden on the health sector; it is of interest to individuals themselves and society to discover and promote methods of improving long-term health and delaying the degeneration of ageing. The ageing process is not fully understood and is not something we can prevent, but there are lifestyle choices that can delay this degenerative process.

Regular physical activity encourages favourable structural and metabolic alterations which can delay the ageing process. Aerobic exercise promotes the activity of a number of proteins that have beneficial actions against degeneration, such as silent information regulator two protein 1 (SIRT1) (Ferrara et al., 2008), 5' AMP-activated protein kinase (AMPK) (Durante et al., 2002), peroxisome proliferator-activated receptor γ coactivator-1alpha (PGC-1α) (Handschin and Spiegelman, 2008), manganese superoxide dismutase (MnSOD) (French et al., 2008) and neurotrophins (Neeper et al., 1995, 1996; Richardson et al., 2000). Regular aerobic exercise improves mitochondrial dysfunction, hormonal imbalances, inflammatory mechanisms, and oxidative stress, all of which are associated with ageing (Johnson et al., 1999). The physiological alterations promoted through regular aerobic exercise are associated with improvements in long-term memory in young and middle-aged rats (see Chapter Two). The benefits of aerobic exercise also lower the incidence of noncommunicable diseases (NCD) and improve an individual's chances of living a long, healthy life (Vuori, 2001; Kemi and Wisløff, 2010; Lee et al., 2012). Unfortunately, although understanding of the physiological

value to undertaking regular physical activity is well known, the global prevalence of NCDs is high and continues to rise (Daar et al., 2007), indicating that many people are not following the guidelines recommended by the World Health Organisation (World Health Organisation, 2000). With this understanding it is desirable to identify orally active agents that mimic or potentiate the effects of aerobic exercise, in the hope of promoting long, healthy lives.

Plant-derived phenolic compounds, such as resveratrol, appear to have similar physiological actions as aerobic exercise. Resveratrol has been shown to activate some of the same pathways, with studies finding increased activity of SIRT1 (Howitz et al., 2003), AMPK (Um et al., 2010), PGC-1α (Lagouge et al., 2006), MnSOD (Robb et al., 2008) and neurotrophins (Thirunavukkarasu et al., 2007; Rahvar et al., 2011). Activation of these proteins has been associated with a number of physiological benefits (Moalem et al., 2000; Oberley, 2005; Cantó et al., 2009) and it is hoped that regular ingestion with such a protein may evoke similar therapeutic potential as regular physical activity. In previous studies, it has been shown that both resveratrol ingestion and aerobic exercise can improve long-term memory in young and middle-aged rats (see Chapter Two), whilst resveratrol ingestion has additional potential against amnesia associated with Alzheimer's disease (see Chapter Three) following short-term regimes. The memory improvement observed was not associated with upregulation of the AMPK/SIRT1 pathways, implicating that the upregulation of neurotrophins plays a more important role in cognitive enhancement associated with resveratrol ingestion and aerobic exercise.

To explore and compare the potential of long-term resveratrol ingestion and aerobic exercise in delaying the degeneration of cognitive function associated with ageing, a working memory task that requires a large quantity of training was utilised - the delayed non-matching-to-sample (DNMS) task. The DNMS task requires flexibly modulating behaviour over time, where the animal is rewarded for choosing the component not present in the initial exposure (Fig. 5-1). This task is sensitive to subtle changes in hippocampal function which may not be visible in behaviour until

later in life (Hok et al., 2012), with lesions of the hippocampus or prefrontal cortex shown to impair performance (Wiig and Bilkey, 1994; Murray and Mishkin, 1998; Clark et al., 2001). For this study, middle-aged Wistar rats underwent either a training protocol of regular forced wheel running, 1 h increasing from 8 – 17 m/min, 5 d/week, or led a sedentary lifestyle. These groups were again sub-divided so that half of the animals were administered resveratrol orally at a dose of 20 mg/kg on training days, a dose shown to cause no adverse effects with daily dosage (Juan et al., 2002). In parallel, all animals were regularly trained and assessed on performance in the DNMS task. Time to learn the task and performance in this working memory task was compared between groups. In week 13 of resveratrol ingestion, wheel running, and DNMS training, animals were given a 7 day interval between DNMS sessions to assess long-term memory for the task. Animals were then assessed for a further 7 sessions before DNMS training, and the treatment regimes were terminated, after 14 weeks. In week 22, eight weeks following termination of the treatment protocols, animals were again assessed in the DNMS task and in the number of sessions required to return to plateau performance.

No difference was found between groups in progression through the training stages of the DNMS task, and in the number of sessions required to reach plateau performance. However, regular resveratrol ingestion and aerobic exercise enhanced performance in the DNMS task, compared to untreated, sedentary controls. Treated groups showed improved recovery in performance after the 7 day interval, with clear differences in performance with longer delays, compared to sedentary controls. Following an 8 week break from DNMS training and treatment regimes, the effects of the treatment regimes were not so potent on long-term and working memory. No adverse effects were evident from 14 weeks of oral resveratrol treatment. These findings suggest that aerobic exercise and the polyphenol, resveratrol, enhance working memory as well as long-term memory. This highlights the potential benefit of resveratrol ingestion and aerobic exercise in delaying age-related cognitive decline.

5.3 MATERIALS AND METHODS

5.3.1 Animals

Male Wistar rats (14 months at study start; *n* = 48) were obtained from BioResources Unit, Trinity College Dublin. They were housed in pairs (standard hard-bottomed, polypropylene cages; 44 × 28 × 18 cm) in a temperature-controlled vivarium (20 to 22 °C), with a 12:12-hour light-dark cycle. Animals were provided with restricted food pellets and water *ad libitum*. Average weights were 595 ± 70 g at the start of experiment and animals were maintained at 85% of free-feeding weight throughout the study. Experiments were carried out in strict accordance with regulations laid out by LAST Ireland and were compliant with the European Union directives on animal experimentation (86/609/EEC).

5.3.2 Drug and Dosing Regime

All rats were handled for one week pre-drug treatment and fed 0.5 ml of maple syrup (Maple Joe, Bernard Michaud) to familiarise them with feeding by syringe. *trans*-Resveratrol (>99% purity) from Sigma-Aldrich, UK was administered orally mixed in a solution of maple syrup. Treated animals were given an oral dose of maple syrup and resveratrol suspension (20 mg/kg; p.o.) 5 days per week, with controls given maple syrup only. Rats were dosed 30 min before exercise protocol and DNMS training, prior to wheel running, for 14 weeks throughout the study.

5.3.3 Exercise Programme

Rats (*n* = 48) were familiarised to exercise wheels on a motorised wheel bed (Model 80805A, Lafayette Instrument) by walking for 15 min (bed speed, 4 m/min) every other day for a week (3 d). They were then assigned to 4 groups: sedentary controls (SedCTL), running controls (RunCTL), sedentary resveratrol-treated

(SedRES) and running resveratrol-treated (RunRES) (n = 12 in each). The exercise protocol consisted of running one hour per day for 5 days per week (bed speed, gradually increased over the training period from 8 m/min to a maximum of 17 m/min, which is equivalent to 1 km/h), for 14 weeks throughout the study. The motorised wheel bed was controlled by a keypad that allowed the speed and duration of exercise to be pre-determined. The bed held 6 exercise wheels. Rats were observed while exercising to ensure they ran continuously and also to monitor for signs of stress. Sedentary rats were placed in stationary wheels for the same duration. Training in the DNMS task began on the first day of the exercise programme.

5.3.4 Delayed Non-Matching-to-Sample Apparatus

Experiments were carried out in a standard modular test chamber (Med Associates; 30.5 cm L x 24.1 cm W x 21.0 cm H), with aluminium front and back walls, clear acrylic sides and top, and a grid floor (0.48 cm stainless steel rods spaced 1.6 cm apart). Each chamber was enclosed in a ventilated wooden sound-insulating box and was equipped with: three retractable levers, two on the front wall and one on the back; a food trough placed between the two front levers, and a house light positioned above the back lever. A ventilation fan providing masking noise (70 dB) would remain on throughout the entire session. 45 mg dustless sucrose pellets (TestDiet™, 5TUT formula) were delivered as a reward to the food trough. Behavioural programmes were controlled by an interface (Med Associates) connected to a computer, which allowed data storage and analysis via Med-PC.

5.3.5 Delayed Non-Matching-to-Sample Task Design

The DNMS task was chosen for assessment of the effects of long-term resveratrol ingestion and aerobic training as it allows us to repeatedly test each rat in

the same task. This allows each animal to serve as its own control for cognitive assessment throughout these regimes. The DNMS task requires the animal to press the retractable lever extended on a random basis on the left or right (sample response) to initiate a trial. This instigates a delay phase of random length between 1 and 30 sec. Following this delay the back lever extends and the animal must press this to release the 2 front levers. The animal now has a choice of levers to press (match/non-match choice phase). The correct response requires a press on the opposite lever than the one pressed in the sample phase (constituting a non-match response), which is rewarded by delivery of a sucrose pellet to the food trough. An incorrect response (pressing the same lever as in the sample phase) initiates a 5 sec time-out in which the house light was switched off and no sucrose pellet is delivered (Fig. 5-1). Each trial was separated by an intertrial interval (ITI) of 10 sec. A previous study has shown that overall performance on the task declines in a linear manner as the duration of the delay interval is increased from 1 to 30 sec (Callaghan et al., 2012).

5.3.6 Delayed Non-Matching-to-Sample Training Protocol

Animals were well-handled and habituated to the experimental apparatus. Rats were initially habituated to the chamber with the three levers already extended. They were trained to lever press for food reward on a continuous reinforcement schedule (i.e. pressing any lever resulted in delivery of a sucrose pellet to the food trough). Once animals expressed sufficient interest in pressing all 3 levers for reward, they were habituated to the extension and retraction of the levers with another continuous reinforcement based schedule: upon pressing, the lever would retract, a pellet would be delivered to the food trough, and the lever would extend again. Each session was limited to a total of 20 rewards. Once an animal completed this task in 5 times consecutive sessions, they progressed to the next training step.

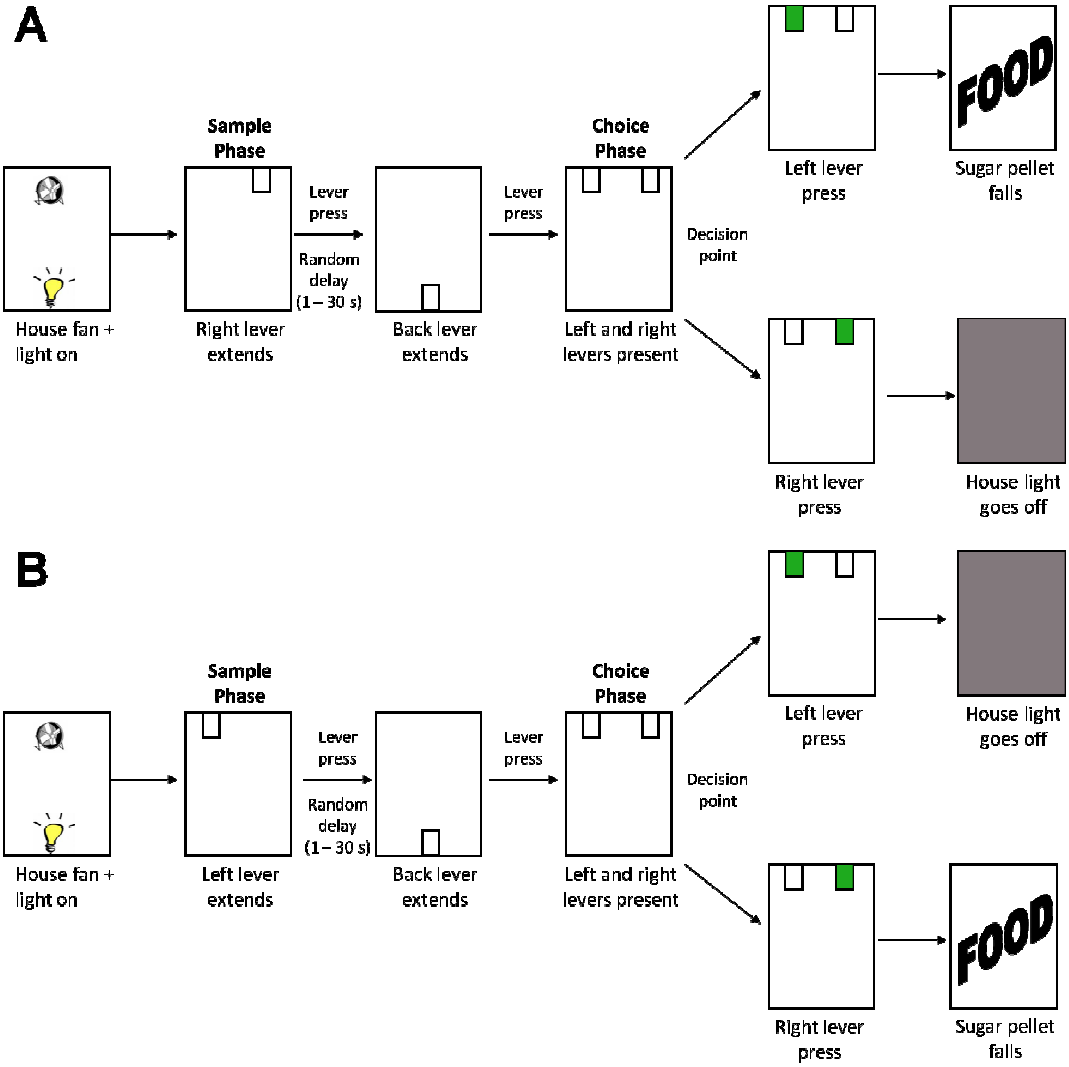


Fig. 5-1. Overview of the delayed-non-matching-to-sample task used to assess spatial-temporal working memory. Rats were placed in a test chamber with 3 retractable levers and a food trough which provided sucrose pellets as reward for correct trials. Each trial started with a sample phase in which one of the front levers extended at random, either [A] right or [B] left. Upon pressing, this lever would retract and after a random delay of between 1 and 30s the back lever would extend. Upon pressing this lever, both of the front levers would extend and the rat had the option to press one (i.e. the choice phase). The rat would be rewarded with a sucrose pellet upon pressing the non-matching lever, or the house light would be turned off for 5 sec and no pellet would present upon pressing the matching lever. Each daily session comprised of 90 trials in 90 min, with an ITI of 10 sec.

The next phase of training involved randomised presentation of the front lever (left or right), which triggered extension of the back lever upon pressing. Upon pressing the back lever, a reward pellet was released into the trough. These lever combinations were repeated 60 times (30 left/back and 30 right/back) with 10 sec intervals. Animals progressed from this training stage to the non-matching-to-sample task when they successfully completed this task in 5 consecutive sessions.

Training in the non-matching-to-sample task comprised of 90 trials in a 90 min session daily. Each session began with the house light switched on and the levers in the retracted position. Rats were initially trained on the task intricacies with no enforced delay between the sample and choice phase. Upon automatic initiation of trial, one front lever (left or right) was randomly selected and extended into the chamber (the "sample"). Once this lever was pressed, the lever retracted and the back lever extended. Upon pressing the back lever, the two front levers extend and the rat has the option to press one (the "choice"). The correct response required a press on the non-matching-to-sample lever, upon which both levers retracted, a sucrose pellet was delivered to the food trough, the house light remained on, and an ITI of 10 sec was initiated before the next trial began. An incorrect response was recorded upon pressing of the matching-to-sample lever, both levers retracted, no pellet was delivered, the house light switched off, and the ITI of 10 sec was initiated before the next trial began. Rats were required to meet a criterion of 80% correct responses for 3 consecutive sessions on this programme before the delay was introduced. As resveratrol ingestion and wheel running was ongoing throughout this DNMS training process, the number of sessions it took each rat to achieve this standard of correct trials was recorded and compared between groups.

In the final phase of training, the random delay was extended to a maximum of 30 sec, requiring the rat to wait for the extension of the rear lever before progressing to the choice phase. Training in the DNMS task continued until a plateau was reached for each rat. Once this plateau in performance has been reached, continual DNMS training does not alter an animal's output score of percentage correct responses for at least 4 weeks (Callaghan et al., 2012). The level of

performance plateau and the number of sessions it took each rat to achieve its plateau of correct trials was recorded and compared between groups.

To assess long-term memory with a longer delay, rats were given a 7 day delay between sessions, whilst still undergoing resveratrol ingestion and wheel running, and assessed in the DNMS task for a further 7 days. To further assess the long-lasting effects of resveratrol ingestion and wheel running, these regimes were then terminated (after 14 weeks) and a further 8 weeks later the rats were assessed in the DNMS for another 10 sessions.

5.3.7 Statistical Analysis

All data was analysed using GraphPad Prism (GraphPad Software, Inc.) and Statistical Package for the Social Sciences (SPSS). Repeated measures (RM) one-way or two-way ANOVAs were used for within animal analysis and regular one- or two-way ANOVAs were used for between group analysis where indicated. Linear regressions were run to determine the slope of lines. *Post hoc* comparisons were made using the Tukey's HSD test. A significance level of $p = 0.05$ was accepted for all comparisons: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Data are presented as mean ± SEM.

5.4 RESULTS

5.4.1 Effects of Exercise and Resveratrol on Length of Time to Learn DNMS Task

The impact of regular forced wheel running was compare with regular oral resveratrol treatment on working memory, in a long-term regime. Use of the delayed-non-matching-to-sample task allowed assessment of the effects on working memory regularly throughout the treatment regimes. Initially the animals were put through a DNMS training regime in order to teach them how to perform the task (see Delayed Non-Matching-to-Sample Training Protocol). As resveratrol ingestion and wheel running was ongoing throughout the DNMS training process, the number of sessions required for each rat to progress to the DNMS task itself was recorded and compared between groups. One-way ANOVA revealed no difference between groups on the number of sessions required to proceed through the training stages to the DNMS task itself ($F_{3,44} = 0.7043$, $p = 0.5545$) (Fig. 5-2).

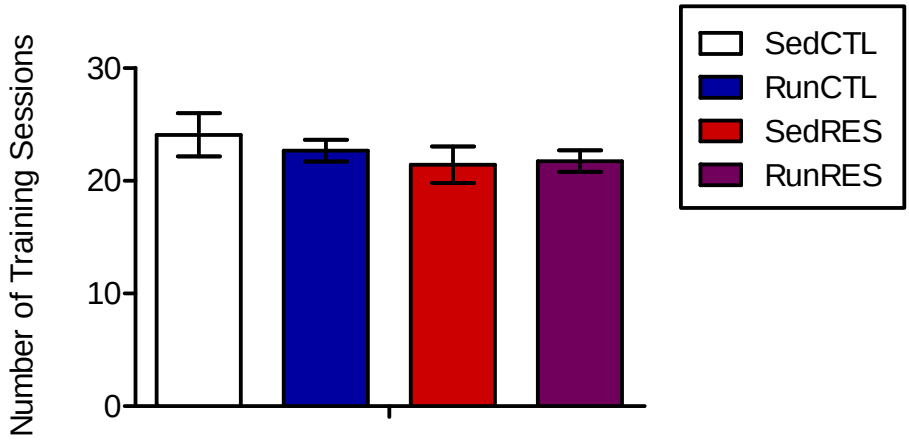


Fig. 5-2. There was no difference between groups in progression through the training stages of the DNMS task. Ability to learn new tasks was measured by recording the number of sessions required for each rat to progress through the DNMS training before beginning the task itself. The mean number of sessions for each group was compared, with no differences found between groups. *n* = 12 per group. Results are presented as mean ± SEM.

5.4.2 Wheel Running and Resveratrol Ingestion Enhance Working Memory

Once progressing to the DNMS task with a random delay between 1 and 30 sec, the number of sessions required for each rat to reach its plateau of correct responses in the task was recorded and compared between groups. Normalisation of plateau level of percentage correct responses showed that all groups reached plateau in an average of 37 sessions. No difference between groups on the number of sessions required to reach plateau level was found (Fig. 5-3A). Differences between groups in rate of learning were found, with two-way ANOVA analysis up to DNMS session 36 revealing a significant effect of treatment ($F_{3,1584} = 77.40$, ***$p < 0.001$), and a significant effect of session ($F_{35,1584} = 66.48$, ***$p < 0.001$), with no significant treatment x session interaction ($F_{105,1584} = 0.2492$, $p = 1$) (Fig. 5-3A). Further analysis reveals a different rate of learning between groups, with animals undergoing regular resveratrol ingestion and forced wheel running improving in the task more rapidly. A linear regression was run for each group to determine the slope of the relevant curves as an indication of the rate of learning (SedCTL: $\beta = 0.618 \pm 0.02118$, RunCTL: $\beta = 0.7248 \pm 0.03245$; SedRES: $\beta = 0.767 \pm 0.0288$; RunRES: $\beta = 0.726 \pm 0.03392$). Unpaired two-tailed t-test showed that there was significant difference in the rate of learning of all treated groups compared to sedentary controls (RunCTL: $t_{74} = 3.004$; **$p < 0.01$; SedRES: $t_{74} = 4.292$; ***$p < 0.001$; RunRES: $t_{74} = 2.669$; **$p < 0.01$) (data not represented). Regular resveratrol ingestion and wheel running, alone or combined, rapidly improved animals performance in the task compared to sedentary controls.

Chronic resveratrol ingestion and wheel running had a significant effect on overall performance in the DNMS task, with two-way ANOVA analysis of plateau performance over 7 sessions showing significant effect of treatment ($F_{3,308} = 28.24$, ***$p < 0.001$), and no significant effect of session ($F_{8,308} = 0.01711$, $p = 1$), or treatment x session interaction ($F_{24,308} = 0.02421$, $p = 1$). All groups had learned the task upon reaching plateau performance, with one-way ANOVA analysis revealing a significant difference between average plateau performance for groups and chance level ($F_{4,59} = 45.43$, ***$p < 0.001$). Further *post hoc* analysis showed that all groups performed above chance level, indicating that they had all learned the task when they

reached plateau performance (SedCTL: ***p < 0.001; RunCTL: ***p < 0.001; SedRES: ***p < 0.001; RunRES: ***p < 0.001) (data not represented) and all treated groups performed significantly better than sedentary controls (RunCTL: *p < 0.05; SedRES: **p < 0.01; RunRES: *p < 0.05) (Fig. 5-3A). All groups reached their plateau level at similar times, but the level of plateau was higher in rats that underwent resveratrol ingestion and wheel running, compared to sedentary controls. Regular resveratrol ingestion and wheel running, alone or combined, allowed animals to learn at a steeper rate and perform better in this assessment of working memory.

DNMS trials were further sorted by performance on these 7 sessions at plateau level according to length of delay on individual trials and were grouped according to 5 sec intervals (1-5, 6-10, 11-15, 16-20, 21-25, and 26-30). The enhanced DNMS performance evident with regular resveratrol ingestion and wheel running was clear across the different delay intervals. Two-way ANOVA revealed a significant effect of treatment ($F_{3,264} = 16.11$, ***p < 0.001), and a significant effect of delay ($F_{5,264} = 48.17$, ***p < 0.001), with no significant treatment x delay interaction ($F_{15,264} = 0.2444$, p = 0.9985) (Fig. 5-3B). Further *post hoc* analysis showed that all treatment groups performed significantly better than sedentary controls at delay block 11 – 15 sec (RunCTL: *p < 0.5; SedRES: **p < 0.01; RunRES: *p < 0.05), with resveratrol-treated animals also performing significantly better at delay block 16 – 20 sec (SedRES: **p < 0.01). With all groups performing significantly above chance, even at the longest delays, they showed an ability to learn the task. These findings indicate that with regular resveratrol treatment and aerobic exercise, either combined or individually, there are significant improvements in spatial-temporal working memory in the middle-aged rat that has natural memory decline associated with ageing.

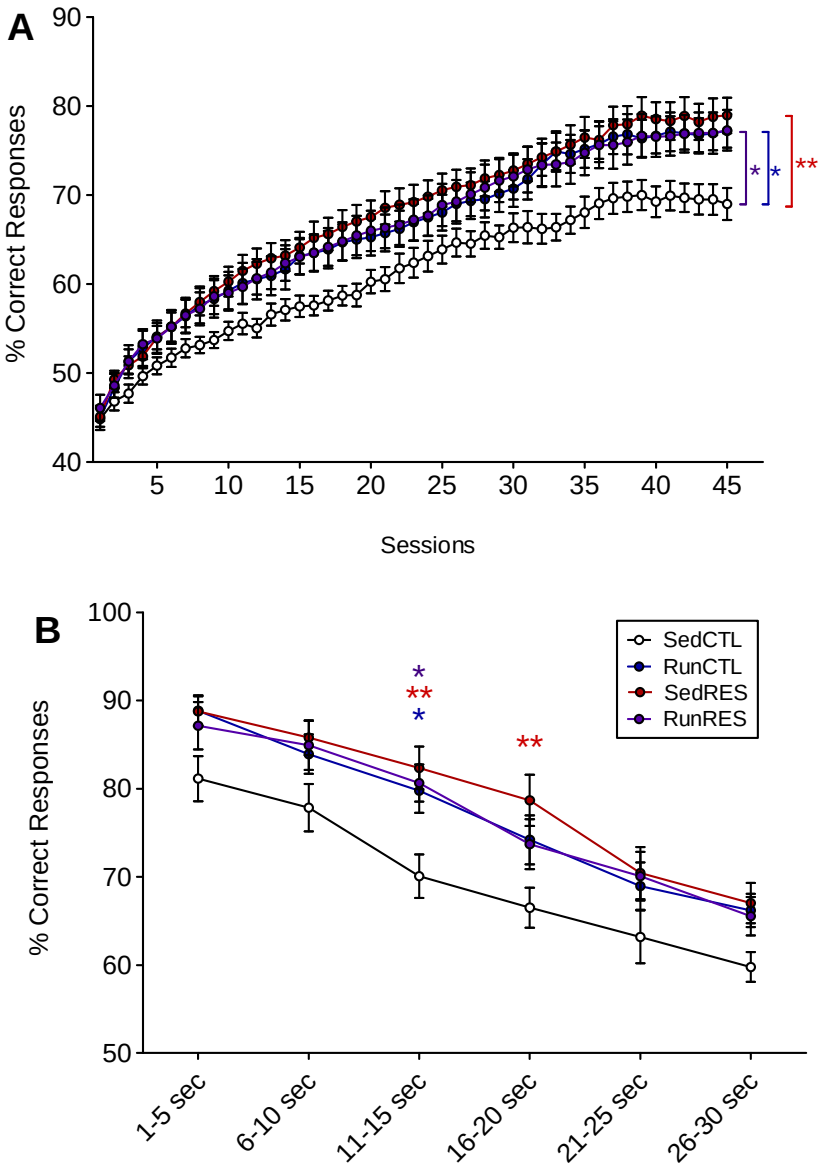


Fig. 5-3. Resveratrol ingestion and forced wheel running improve DNMS performance. [A] The number of sessions required for a rat to reach plateau of overall percentage correct responses was recorded. All groups reached plateau at similar times, but the level of plateau was higher in rats that underwent resveratrol ingestion and wheel running, compared to controls. [B] DNMS trials were sorted by performance according to length of delay on individual trials and were grouped according to 5 sec intervals (1-5, 6-10, 11-15, 16-20, 21-25, and 26-30). Performance at plateau level (n = 9 sessions) was averaged across trials, sessions, and animals. Each point thus represents the mean (± SEM) percent of correct trials performed within each delay across sessions. The enhanced performance with these treatment regimes was evident across various time delays. n = 12 per group. Results are presented as mean ± SEM. Values significantly different from the sedentary control performance are indicated with an asterisk (Tukey's HSD: *p < 0.05, **p < 0.01), with the running group in blue, resveratrol-treated in red, and those undergoing running and resveratrol-treatment in purple.

5.4.3 Wheel Running and Resveratrol Ingestion Improve Recovery in DNMS Performance

Performance in the DNMS task is sensitive to long delays between sessions. To maintain performance in this task whilst achieving plateau level of correct responses it is advisable to carry out sessions regularly, with at least 4 sessions per week, and no longer than a 2 day gap at any stage. With longer gaps between sessions, animals require a number of extra sessions before reaching plateau once again. To compare the impact of regular forced wheel running with regular oral resveratrol treatment on long-term memory, the effects of a 7 day interval between sessions on performance was determined following a 13 week regime of resveratrol ingestion and forced wheel running. There was a significant effect for all groups of a 7 day interval between sessions on plateau performance as shown by comparison of the final session before the 7 day interval (session 45) and the initial session following this delay (session 46). Unpaired two-tailed t-tests showed that there were significant decreases in percentage correct responses for all groups following the 7 day interval between sessions (SedCTL: $t_{22} = 4.883$; ***$p < 0.001$; RunCTL: $t_{22} = 4.392$; ***$p < 0.001$; SedRES: $t_{22} = 4.271$; ***$p < 0.001$; RunRES: $t_{22} = 4.03$; ***$p < 0.001$) (Fig. 5-4A).

DNMS trials were further sorted by performance on the initial session following this delay (session 46) according to length of delay on individual trials and were grouped according to 5 sec intervals (1-5, 6-10, 11-15, 16-20, 21-25, and 26-30). This data was compared between groups in order to determine ability to perform in the DNMS task following a long interval between training sessions, with a decrease in DNMS performance primarily associated with longer delay intervals. Two-way ANOVA revealed a significant effect of treatment ($F_{3,264} = 10.42$, ***$p < 0.001$), and a significant effect of delay ($F_{5,264} = 32.74$, ***$p < 0.001$), and no significant treatment x delay interaction ($F_{15,264} = 0.6121$, $p = 0.8643$) (data not represented). Two-way ANOVA comparing groups to chance revealed a significant effect of treatment ($F_{4,330} = 22.51$, ***$p < 0.001$), and a significant effect of delay ($F_{5,330} = 32.74$, ***$p < 0.001$), and treatment x delay interaction ($F_{20,330} = 2.620$, ***$p < 0.001$) (data not represented). Further *post hoc* analysis showed that all groups performed significantly above chance level at shorter delay blocks (SedCTL:

1-5 sec, ***p < 0.001, 6 – 10 sec, *p < 0.05; RunCTL: 1-5 sec, ***p < 0.001, 6 – 10 sec, ***p < 0.001, 11 – 15 sec, *p < 0.05; SedRES: 1-5 sec, ***p < 0.001, 6 – 10 sec, ***p < 0.001, 11 – 15 sec, ***p < 0.001; RunRES: 1-5 sec, ***p < 0.001, 6 – 10 sec, ***p < 0.001), but did not perform above chance at longer delays (data not represented). Although animals still remembered the non-matching-to-sample rule, evident through DNMS performance with short delays, the 7 day interval between sessions disrupted performance at longer delays.

Animals were assessed in the DNMS task for 7 sessions following the 7 day delay. A linear regression was run for each group to determine the slope of the relevant curves as an indication of the rate of performance improvement, with all groups showing improvements over these sessions (SedCTL: $r^2 = 0.1871$, $F_{1,82} = 18.87$, ***p < 0.001; RunCTL: $r^2 = 0.1094$, $F_{1,82} = 10.08$, **p < 0.01; SedRES: $r^2 = 0.1317$, $F_{1,82} = 12.43$, **p < 0.01; RunRES: $r^2 = 0.1483$, $F_{1,82} = 14.28$, **p < 0.01). One-way ANOVA analysis showed there were no significant differences between groups in the slope of performance improvement following the 7 day interval ($F_{3,27} = 0.1529$, $p = 0.9268$) (Fig. 5-4A). All groups showed significant improvements in DNMS performance over the 7 sessions.

To further determine the impact of regular forced wheel running with regular oral resveratrol treatment on ability to recover performance within 7 sessions, DNMS performance on the final session before the 7 day interval (session 45) was compared to performance on the seventh session following this delay (session 52). Unpaired two-tailed t-tests showed that groups that received regular resveratrol and wheel running had no differences in performance between the final session at plateau (session 45) and the seventh session following the 7 day interval (session 52) (RunCTL: $t_{22} = 1.530$; $p = 0.1403$; SedRES: $t_{22} = 0.6124$; $p = 0.5466$; RunRES: $t_{22} = 0.6981$; $p = 0.4924$), but sedentary controls remained significantly impaired (SedCTL: $t_{22} = 2.989$; **p < 0.01) (data not represented) (Fig. 5-4A). All groups showed recovery in DNMS performance over the 7 sessions, with regular resveratrol ingestion and wheel running enabling animals to reach plateau performance by this stage.

DNMS trials were further sorted by performance on the seventh session after the 7 day delay (session 52) according to length of delay on individual trials and were grouped according to 5 sec intervals (1-5, 6-10, 11-15, 16-20, 21-25, and 26-30). This data was compared between groups in order to determine the extent of recovery in DNMS performance. Two-way ANOVA revealed a significant effect of treatment ($F_{3,264}$ = 17.74, ***p < 0.001), and a significant effect of delay ($F_{5,264}$ = 18.84, ***p < 0.001), with no significant treatment x delay interaction ($F_{15,264}$ = 0.6922, p = 0.7916). Two-way ANOVA comparing groups to chance revealed a significant effect of treatment ($F_{4,330}$ = 85.69, ***p < 0.001), and a significant effect of delay ($F_{5,330}$ = 56.28, ***p < 0.001), and treatment x delay interaction ($F_{20,330}$ = 3.884, ***p < 0.001) (data not represented). Further *post hoc* analysis showed that all groups performed significantly above chance level at shorter delay blocks of 1 – 5 sec (***p < 0.001) and 6 – 10 sec (***p < 0.001) (data not represented). Sedentary controls did not perform above chance level at longer delays, with all treatment groups performing above chance level at all delays, except for the running group that did not perform above chance at the longest delay block of 26 – 30 sec. Although performance improved in sedentary controls over the 7 sessions, most of this was with the shortest delays. Aerobic exercise and resveratrol treatment enabled quicker recovery with long delays in this task.

DNMS trials were further sorted by performance on these 7 sessions following the 7 day interval, according to the length of delay on individual trials and were grouped according to 5 sec intervals (1-5, 6-10, 11-15, 16-20, 21-25, and 26-30). This data was compared between groups in order to determine ability to perform in sessions following a long interval between DNMS training sessions. There was no significant difference between groups with a short delay between 1 and 5 sec. Enhanced DNMS performance in groups undergoing regular resveratrol ingestion and wheel running was clear across longer delay intervals except, interestingly, the longest delay block, 25 – 30 sec. Two-way ANOVA revealed a significant effect of treatment ($F_{3,264}$ = 27.96, ***p < 0.001), and a significant effect of delay ($F_{5,264}$ = 56.28, ***p < 0.001), with no significant treatment x delay interaction ($F_{15,264}$ = 0.3905, p = 0.9808). Further *post hoc* analysis showed that all treatment groups performed significantly better than sedentary controls at delay block 11 – 15 sec

(RunCTL: *p < 0.5; SedRES: ***p < 0.001; RunRES: **p < 0.01) and 16 – 20 sec (RunCTL: *p < 0.5; SedRES: ***p < 0.001; RunRES: *p < 0.05), with resveratrol-treated and running combined also performing significantly better at delay block 6 – 10 sec (*p < 0.05), and resveratrol-treated alone also performing better at delay blocks 6 – 10 sec (**p < 0.01) and 21 -25 sec (***p < 0.001) (Fig. 5-4B). Two-way ANOVA comparing groups to chance revealed a significant effect of treatment ($F_{4,330}$ = 64.24, ***p < 0.001), and a significant effect of delay ($F_{5,330}$ = 18.84, ***p < 0.001), and treatment x delay interaction ($F_{20,330}$ = 1.826, *p < 0.05) (data not represented). All groups performed significantly above chance at shorter delay blocks of 1 – 5 sec (***p < 0.001) and 6 – 10 sec (***p < 0.001). Sedentary control animals did not perform significantly above chance with longer delays. Other groups performed significantly above chance over the middle time delay blocks of 11 – 15 sec (***p < 0.001) and 16 – 20 sec (***p < 0.001), but only resveratrol-treated animals were able to perform significantly above chance at the longer delay blocks of 21 – 25 sec (***p < 0.001) and 26 – 30 sec (*p < 0.05) (data not represented). Differences between groups were presumably not evident because all groups were performing at or near chance level percentage correct responses.

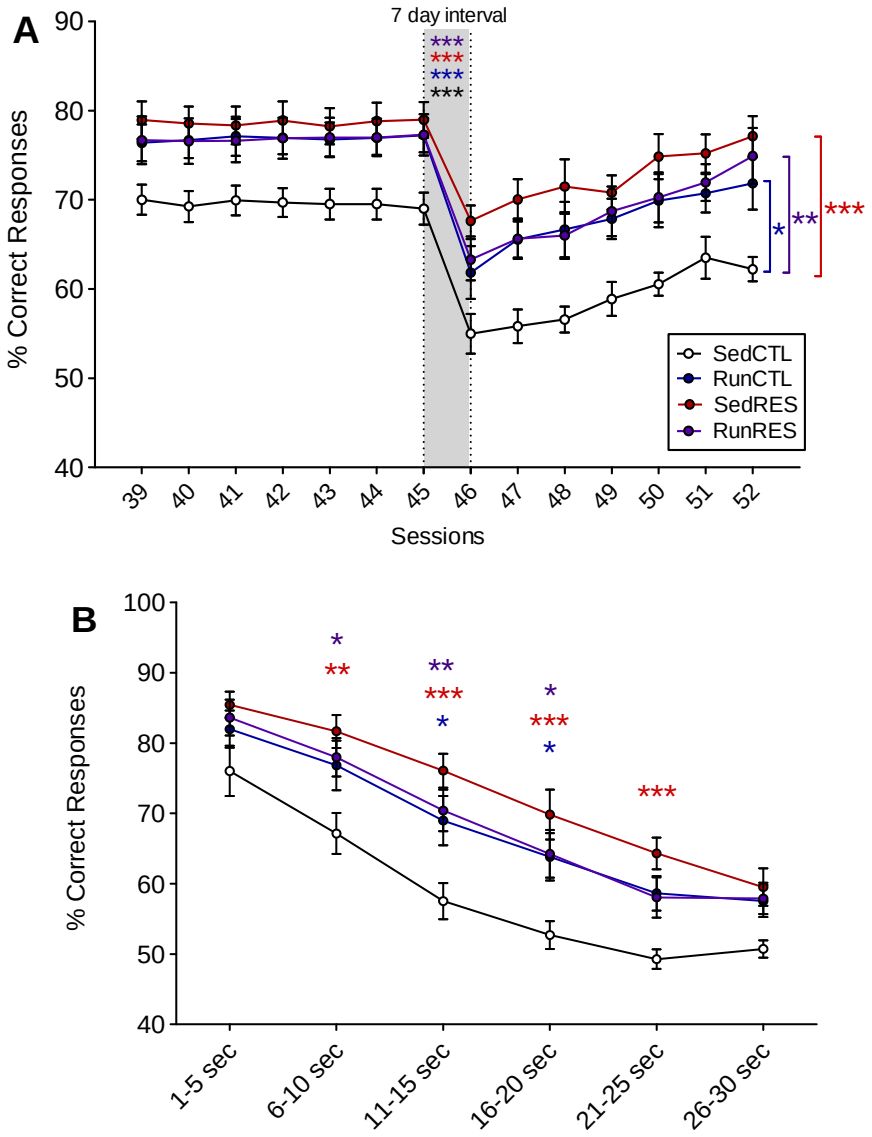


Fig. 5-4. Resveratrol ingestion and forced wheel running improve recovery in DNMS performance following delay-induced impairment. [A] The effect of a 7 day interval on overall percentage correct responses was measured. All groups showed similar decline in performance following this delay, and improved with more DNMS sessions. Treated groups reached plateau level by 7th session, sedentary controls did not. [B] DNMS trials were sorted by performance according to length of delay on individual trials and were grouped according to 5 sec intervals (1-5, 6-10, 11-15, 16-20, 21-25, and 26-30). Performance after the 7 day interval (n = 7 sessions) was averaged across trials, sessions, and animals. Each point thus represents the mean (± SEM) percent of correct trials performed within each delay across sessions. There was no significant difference with a short delay, but sedentary controls performed worse with longer delays. n = 12 per group. Results are presented as mean ± SEM. Values significantly different from the sedentary control performance are indicated with an asterisk (Tukey's HSD: *p < 0.05, **p < 0.01; ***p < 0.001) , with the running group in blue, resveratrol-treated in red, and those undergoing running and resveratrol-treatment in purple.

5.4.4 Effects of Resveratrol and Wheel Running Less Potent Following 8 Week Break

To determine the long-lasting effects of long-term resveratrol ingestion and aerobic exercise throughout middle-age, these regimes were terminated following the seventh training day after the 7 day interval (after a 14 week treatment regime). Animals remained in their home cage with food and water *ad libitum*, only taken out for handling. 8 weeks after the regimes were terminated, animals (20 month old) were again assessed in DNMS performance. To compare the long-lasting effects of these treatment regimes on long-term memory, the effect of an 8 week interval between sessions on performance was determined following a 14 week regime of resveratrol ingestion and forced wheel running. There was a significant effect for all groups of an 8 week interval between sessions on plateau performance as shown by comparison of the final session at plateau (session 45) and the initial session following this delay (session 53). Unpaired two-tailed t-tests showed that there were significant decreases in percentage correct responses for all groups following the 8 week interval between sessions (SedCTL: $t_{22} = 5.361$; ***$p < 0.001$; RunCTL: $t_{22} = 7.453$; ***$p < 0.001$; SedRES: $t_{22} = 6.649$; ***$p < 0.001$; RunRES: $t_{22} = 6.986$; ***$p < 0.001$) (Fig. 5-5A).

DNMS trials were further sorted by performance on the initial session following the 8 week delay (session 53) according to length of delay on individual trials and were grouped according to 5 sec intervals (1-5, 6-10, 11-15, 16-20, 21-25, and 26-30). This data was compared between groups in order to determine ability to perform in the DNMS task following a long interval between training sessions. Two-way ANOVA revealed a significant effect of treatment ($F_{3,264} = 3.248$, *$p < 0.05$), and a significant effect of delay ($F_{5,264} = 15.66$, ***$p < 0.001$), with no significant treatment x delay interaction ($F_{15,264} = 0.9952$, $p = 0.4605$) (data not represented). Further *post hoc* analysis showed that groups that underwent 14 weeks of resveratrol ingestion or forced wheel running did not perform any better than sedentary control rats following this 8 week break in DNMS training and treatment regimes.

Animals were assessed in the DNMS task for 10 sessions following the 8 week delay and break in treatment regimes to compare the impact of 14 weeks regular forced wheel running with regular oral resveratrol treatment, 8 weeks after regime termination, on length of recovery in performance following an 8 week interval between sessions. A linear regression was run for each group to determine the slope of performance improvement, with all groups showing improvements over these sessions (SedCTL: $r^2 = 0.211$, $F_{1,118} = 31.56$, ***$p < 0.001$; RunCTL: $r^2 = 0.4372$, $F_{1,118} = 91.66$, ***$p < 0.001$; SedRES: $r^2 = 0.2972$, $F_{1,118} = 49.89$, ***$p < 0.001$; RunRES: $r^2 = 0.3252$, $F_{1,118} = 56.87$, ***$p < 0.001$). One-way ANOVA analysis showed there were no differences between groups in the slope of performance improvement following the 8 week interval ($F_{3,39} = 2.413$, $p = 0.0826$) (Fig. 5-5A). All groups showed significant improvements in DNMS performance over the 10 sessions.

To further determine the lasting effects of regular forced wheel running with regular oral resveratrol treatment on ability to recover performance within 10 sessions, DNMS performance on the final session at plateau (session 45) was compared to performance on the tenth session following this delay (session 62). Unpaired two-tailed t-tests showed that there were no differences from the final session at plateau (session 45) in percentage correct responses by the tenth session following the 8 week interval (session 62) (SedCTL: $t_{22} = 0.4968$; $p = 0.6242$; RunCTL: $t_{22} = 0.3798$; $p = 0.7077$; SedRES: $t_{22} = 0.4793$; $p = 0.6365$; RunRES: $t_{22} = 0.2286$; $p = 0.8213$) (data not represented). All groups showed recovery in DNMS performance over the 10 sessions, with all groups reaching plateau performance by this stage.

DNMS trials were further sorted by performance on the tenth session after the 8 week interval (session 62) according to length of delay on individual trials and were grouped according to 5 sec intervals (1-5, 6-10, 11-15, 16-20, 21-25, and 26-30). This data was compared between groups in order to determine the extent of recovery in DNMS performance for groups. Two-way ANOVA revealed a significant effect of treatment ($F_{3,264} = 3.616$, *$p < 0.05$), and a significant effect of

delay ($F_{5,264}$ = 16.05, ***p < 0.001), with no significant treatment x delay interaction ($F_{15,264}$ = 1.51, p = 0.1011). Further *post hoc* analysis showing that there were no differences between groups at the shortest delay blocks, 1 – 5 sec and 6 – 10 sec, or the longest delay blocks, 21 – 25 sec and 26 -30 sec. Resveratrol-treated animals performed better than sedentary controls at delays in the 11 – 15 sec (*p < 0.05) and 16 – 20 sec (*p < 0.05) blocks, whilst the group treated with resveratrol and forced wheel running performed better in the 11 – 15 sec delay block (*p < 0.05) (data not represented). The effects of 14 weeks resveratrol ingestion and forced wheel running were not so potent following an 8 week break, when compared to sedentary controls.

DNMS trials were further sorted by performance on these 10 sessions following the 8 week interval, according to length of delay on individual trials and were grouped according to 5 sec intervals (1-5, 6-10, 11-15, 16-20, 21-25, and 26-30). This data was compared between groups in order to determine ability to perform in sessions following a long interval between DNMS training sessions and a break in treatment regimes. Two-way ANOVA revealed a significant effect of treatment ($F_{3,264}$ = 7.564, ***p < 0.001), and a significant effect of delay ($F_{5,264}$ = 48.33, ***p < 0.001), with no significant treatment x delay interaction ($F_{15,264}$ = 0.4302, p = 0.9694). Further *post hoc* analysis showed that resveratrol-treated animals alone performed better than sedentary controls at the delay block, 16 – 20 sec (**p < 0.01), otherwise there were no differences between groups (Fig. 5-5B). With resveratrol-treated groups showing significant performance compared to controls with mid-range delays, whilst the running group did not, this may signify that resveratrol has more long-lasting beneficial effects on cognition.

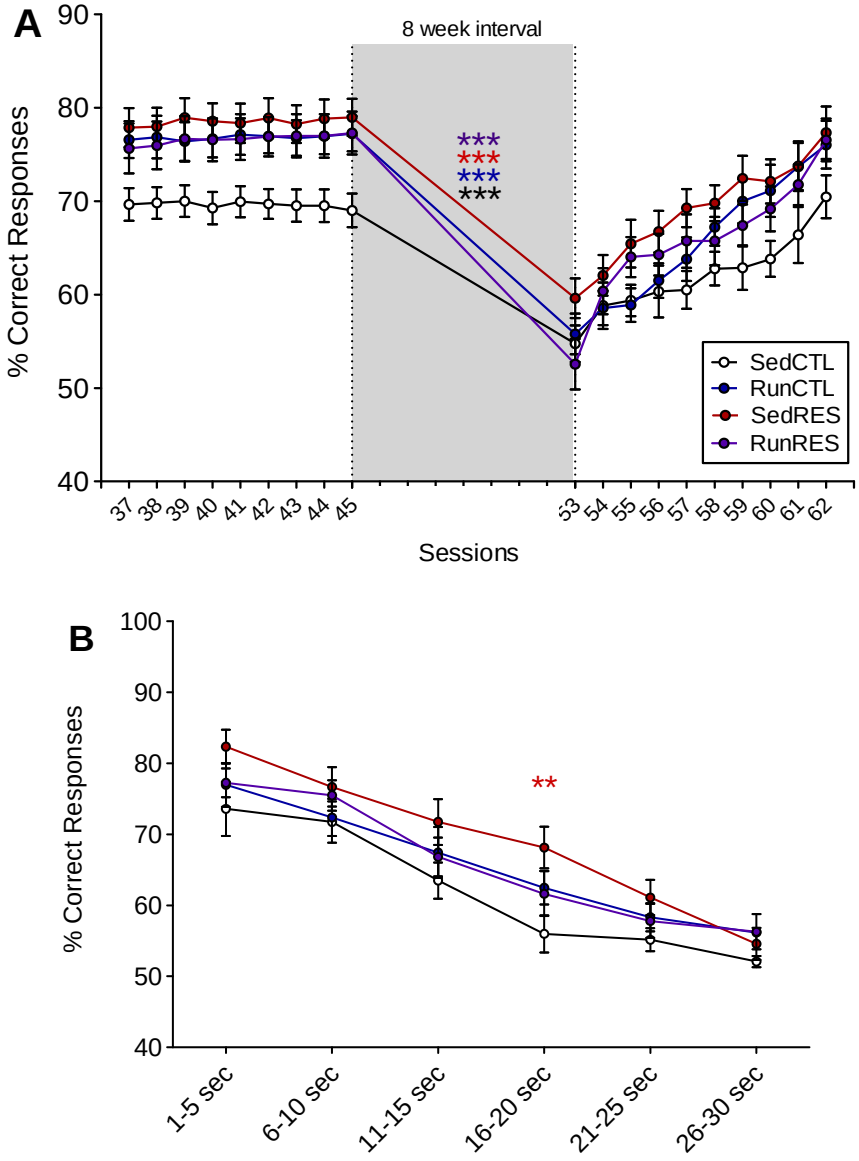


Fig. 5-5. Effects of resveratrol ingestion and forced wheel running less potent 8 weeks after treatment termination. [A] The effect of an 8 week interval on overall percentage correct responses was measured. All groups showed similar decline in performance following this delay, and improved with more DNMS sessions. All groups reached plateau levels by the tenth session. [B] DNMS trials were sorted by performance according to length of delay on individual trials and were grouped according to 5 sec intervals (1-5, 6-10, 11-15, 16-20, 21-25, and 26-30). Performance after the 8 week interval (n = 10 sessions) was averaged across trials, sessions, and animals. Each point thus represents the mean (± SEM) percent of correct trials performed within each delay across sessions. There was no significant difference with a short delay, but controls performed worse with longer delays. n = 12 per group. Results are presented as mean ± SEM. Values significantly different from the sedentary control performance are indicated with an asterisk (Tukey's HSD: *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$) , with the running group in blue, resveratrol-treated in red, and those undergoing running and resveratrol-treatment in purple.

5.5 DISCUSSION

There were three major aims of this study. The first aim was to evaluate similarities between the effects of a long-term regime of aerobic training and oral resveratrol administration on cognitive function in the middle-aged rat that has natural memory decline associated with ageing. These findings indicate that regular resveratrol treatment and aerobic exercise, either combined or individually, promote significant improvements in spatial-temporal working memory in this model of natural memory decline. The second aim was to determine the effects of these treatment regimes on recovery from cognitive impairment, induced by long intervals between DNMS sessions. 13 weeks of resveratrol treatment and aerobic exercise, either combined or individually, improved recovery of performance in this spatial-temporal working memory task. The third aim was to evaluate the long-lasting effects of a 14 week resveratrol and aerobic exercise regime on DNMS performance. The effects of long-term resveratrol ingestion and forced wheel running were less potent following an 8 week break.

Regular physical activity encourages favourable structural and metabolic alterations which can delay the ageing process (Evans and Cyr-Campbell, 1997; Kramer et al., 1999; Seals et al., 2008), and the progression of age-related diseases (Vuori, 2001; Kemi and Wisløff, 2010; Lee et al., 2012). With an ageing population, and increases in the prevalence of noncommunicable diseases (Daar et al., 2007), it is becoming more desirable to identify orally active agents that mimic or potentiate the effects of aerobic exercise. One such candidate is the polyphenol, resveratrol, which may be termed an exercise mimetic due to its similar action on mitochondrial biogenesis, endurance, metabolism and the cardiovascular system (Um et al., 2010). In previous studies, it has been shown that both resveratrol ingestion and aerobic exercise can improve long-term memory in young and middle-aged rats (see Chapter Two), whilst resveratrol ingestion has additional potential against amnesia associated with Alzheimer's disease (see Chapter Three) following short-term treatment regimes. In order to explore and compare the potential of long-term resveratrol ingestion and aerobic exercise in delaying the degeneration of cognitive function associated with ageing, a working memory task that requires a large quantity of

training was utilised - the delayed non-matching-to-sample (DNMS) task. This task is sensitive to subtle changes in hippocampal function which may not be visible in behaviour until later in life (Hok et al., 2012), with lesions of the hippocampus or prefrontal cortex impairing performance (Wiig and Bilkey, 1994; Murray and Mishkin, 1998; Clark et al., 2001). It was found that an ongoing treatment regime of 20 mg/kg resveratrol (5d/week) showed similar delayed non-matching-to-sample performance improvements in middle-aged rats as those observed with a forced wheel running programme of 1 h (5d/week).

The DNMS task requires flexibly modulating behaviour over time, where the animal is rewarded for choosing the component not present in the initial exposure (Fig. 5-1). As working memory declines with age (Dobbs and Rule, 1989; Park et al., 2002), performance in this task has been found to decline as rats age (Callaghan et al., 2012). Middle-aged rats were used because loss of cognitive processing during ageing is a complex process that first becomes evident during middle-age in humans and rats, even in the absence of specific neurodegenerative diseases (Kluger et al., 1997). This means that there is the possibility for these animals to improve beyond their current capabilities, making this a suitable model for therapeutic testing. First, rats must be habituated to the test chambers and learn to press levers for a sugar pellet reward before progressing through a series of training stages in order to learn the non-matching-to-sample rule. Training must be conducted on a regular basis in order to promote learning, or the rule is forgotten and the animal requires additional sessions in order to reach its previous level of performance. Once the animal showed sufficient understanding of the non-matching-to-sample rule, by meeting a criterion of 80% correct responses for 3 consecutive sessions, the delay was introduced. As resveratrol ingestion and wheel running was ongoing throughout this DNMS training process, the number of sessions required for each rat to progress to the DNMS task itself was recorded and compared between groups. Groups did not differ in terms of progression to the DNMS task itself, with most animals progressing to the task in week 5 of the treatment regime. Resveratrol ingestion and forced wheel running did not enable animals to modulate their behaviour in order to perform the task sooner than untreated, sedentary controls. These results are similar to a human study that found that aerobic exercise did not improve performance of stroke patients in the

Wisconsin Card Sorting Task (WSCT), a task designed to measure rule learning and resistance to perseveration (Quaney et al., 2009). Whereas, in another resveratrol administered orally (150 µg/g food) in mice improved speed of learning in a Y-maze task (Oomen et al., 2009).

Once progressing to the DNMS task with a random delay between 1 and 30 sec, training continued until all animals reached a plateau level of correct responses. Groups did not differ in number of sessions required to reach plateau level of correct performances, similar to progression through the training programmes, resveratrol ingestion and forced wheel running did not enable animals to proceed to their optimal performance at an earlier stage. However, during this time, animals that underwent regular resveratrol ingestion and forced wheel running improved in the task more rapidly, allowing them to reach a higher level of performance in this assessment of spatial-temporal working memory, compared to untreated, sedentary controls. Performance at plateau level over 7 sessions was compared between groups, with plateau level of correct responses significantly higher for all 3 treated groups, compared to sedentary controls. Once this plateau in performance has been reached, continual DNMS training does not alter an animal's output score of percentage correct responses for at least 4 weeks (Callaghan et al., 2012). Resveratrol and aerobic exercise groups performed better than sedentary controls at different delay intervals ranging from short delay of 1 – 5 sec to the longest delays of 25 – 30 sec. All groups performed significantly above chance, even at the longest delays, showing that all groups were able to learn the task. These findings indicate that with regular resveratrol treatment and aerobic exercise, either combined or individually, there are significant improvements in spatial-temporal working memory in the middle-aged rat that has natural memory decline associated with ageing. These findings corroborate human studies that have found that aerobic exercise improves performance in working memory tasks, with young adults undertaking high levels of physical activity performing better in a reading span task than people undertaking low levels of physical activity (Lambourne, 2006), and similar effects found in elderly people (Clarkson-Smith and Hartley, 1989). Another study, using a different version of the DNMS task, did not find improvements in performance following a 6 week forced running regime, with running rats

performing worse than sedentary (Braszko et al., 2001). Effects of resveratrol on DNMS performance have not previously been reported, but resveratrol treatment has been shown to improve performance in other working memory tasks. Dietary supplementation with resveratrol (0.4% of food chow) improved performance in a spatial working memory version of the Morris water maze in aged mice treated peripherally with lipopolysaccahride (LPS) (Abraham and Johnson, 2009) and resveratrol administered 90 mg/kg i.p. improved performance in a similar task using a model of hypoxia-induced brain injury (Karalis et al., 2011). With lesions of the hippocampus or prefrontal cortex impairing performance (Wiig and Bilkey, 1994; Murray and Mishkin, 1998; Clark et al., 2001), it is thought that wheel running and resveratrol ingestion induce changes in the hippocampus and prefrontal cortex that enable this improved performance.

Performance in the DNMS task is sensitive to long delays between sessions and to maintain performance in this task it is advisable to carry out training regularly. With long intervals between training sessions, animals require a number of extra sessions before reaching plateau once again. In week 14 of these treatment regimes of resveratrol ingestion and forced wheel running, animals were further assessed in DNMS performance following a 7 day interval between sessions. There was a clear dip in performance with all groups following this 7 day interval, and all groups showing recovery in DNMS performance over the following 7 sessions. In the session immediately following the 7 day interval, all groups could still perform above chance at the shorter delays, between 1 and 10 sec, showing that animals still remembered the non-matching-to-sample rule. Differences between groups were evident with longer delays. It seems that the encoding of trial-specific information is functionally distributed between two brain structures, depending on the length of delay. The subiculum encodes information mainly during shorter delays (1 – 15 sec), whilst the hippocampus is required during longer delays (16 – 30 sec) (Deadwyler and Hampson, 2004). The enhanced performance at longer delays observed in this study is potentially through increased levels of neurotrophins in the hippocampus, as measured with previous studies using these treatment regimes (see Chapter Two and Three). Administration of nerve growth factor (NGF) locally to the hippocampus in rats over 28 days, with a total dose of 0.25 µg, has been shown to improve spatial

working memory in a delayed matching-to-position task with a maximal delay of 15 min (Jakubowska-Doğru and Gümüşbaş, 2005). In this study, although all groups showed recovery in DNMS performance over the 7 sessions, sedentary controls did not perform above chance at longer delays by the seventh session. All other groups showed gradually improving performance at all delays with each session after the 7 day interval. All treated groups reached their plateau performance level by the seventh session, sedentary controls did not. These findings indicate that regular resveratrol treatment and aerobic exercise, either combined or individually, can improve recovery of performance in the middle-aged rat that has natural memory decline in a difficult task assessing spatial-temporal working memory. This suggests enhanced long-term memory with these regimes as seen with shorter training regimes of 1 and 4 weeks (see Chapter Two and Three). Improvements in long-term memory have been observed after an acute bout of aerobic exercise in young adults, assessed using the Brown-Peterson test, although showing no enhancement of working memory was observed in that study (Coles and Tomporowski, 2008).

Following the 7 sessions in week 14, training in the DNMS task, resveratrol ingestion and forced wheel running stopped. Animals remained in their home cage with food and water *ad libitum*, only taken out for handling. 8 weeks later, animals (now 20 months old) were again assessed in DNMS performance, in order to determine the long-lasting effects of a 14 week regime of oral resveratrol and forced wheel running. There was a clear dip in performance in all groups following this 8 week interval, with all groups showing recovery in DNMS performance over the 10 following sessions. In the session immediately after the 8 week interval and in average performance across the 10 sessions, there was little difference in DNMS performance between groups. All groups showed gradually improving performance at all delays with each session after the 8 week interval, and reached their plateau performance level by the tenth session. No differences were measured between treated groups and sedentary controls in the shorter delays, between 1 and 10 sec, and longer delays, between 21 and 30 sec. Groups treated with resveratrol (SedRES and RunRES) performed better at mid-range delays, between 11 and 20 sec, whilst the running group did not perform better than sedentary controls. The effects of long-term resveratrol ingestion and forced wheel running were less potent following an 8

week break, which may be due to neurotrophins returning to baseline after resveratrol ingestion and aerobic exercise regimes had ended. Three weeks of wheel running has been shown to elevate levels of hippocampal brain-derived neurotrophic factor (BDNF) in mice, with levels remaining elevated until returning to baseline 3-4 weeks after exercise ended (Berchtold et al., 2010). In that study, a corresponding decrease in performance in the radial-arm water maze was observed with declining levels of hippocampal BDNF until performance was at control level 4 weeks after exercise had stopped. It is difficult to ascertain whether better performance in the DNMS task at this stage is due to more successful trials during training, or due to prolonged cognitive enhancement due to these treatment regimes. With resveratrol-treated groups showing significant performance compared to controls with mid-range delays, whilst the running group did not, this may signify that resveratrol has more long-lasting beneficial effects on cognition. Further work would need to be conducted to be certain of this. Studies on humans have shown the benefits of exercise on brain health and function, particularly in ageing populations (Cotman and Berchtold, 2002). Such findings increase interest in identifying orally active agents that would mimic or potentiate the effects of exercise (Narkar et al., 2008). Resveratrol is becoming a convincing potential candidate.

The data presented here suggest that a regime of regular resveratrol ingestion and aerobic exercise have similar positive impacts on functioning of the hippocampal formation in relation to working memory as measured by use of the delayed non-matching-to-sample task. This enhancement is effective in the middle-aged rat that has natural memory decline associated with ageing. Both regular resveratrol ingestion and aerobic exercise improved the learning of this task, working memory, and ability to recover memory after impairment. With sedentary control rats performing well at shorter delays, differences were most striking at longer delays during the DNMS trials. There is potential that long-term resveratrol ingestion has longer-lasting effects than a long-term aerobic exercise regime. Throughout the 6 months in which this study was carried out, from middle-age to old-age, animals were monitored regularly for signs of ill-health. No ill-effects were evident from long-term treatment or aerobic exercise, all animals expressed normal decline in activity as expected with old age, which is important to determine if

regular supplementation of a compound is being promoted. In non-human primates, no adverse effects were found using an 18-month regime of resveratrol treatment (Dal-Pan et al., 2011). Along with studies showing enhancement of long-term memory with resveratrol ingestion and aerobic exercise, there is strong evidence that these treatments activate a multifaceted action on cognitive processes. Additionally, this indicates that regular supplementation with resveratrol during middle-age may have positive effects on cognition during old-age.

Chapter Six

CONCLUSIONS AND RECOMMENDATIONS

"Youth is wasted on the young"

- George Bernard Shaw

6.1 SYNOPSIS OF RESULTS

The concept of this thesis was to explore how elements of physical and mental health are connected; particularly focusing on the effects that aerobic exercise and resveratrol ingestion have on learning and memory. Many studies have found that resveratrol provides potent effects against mechanisms associated with ageing, such as oxidative stress (Frankel et al., 1993; Chanvitayapongs et al., 1997; Gupta et al., 2002a), mitochondrial biogenesis (Lagouge et al., 2006; López-Lluch et al., 2008; Um et al., 2010) and autoimmunity (Baur and Sinclair, 2006). Aerobic exercise also encourages healthy ageing, evoking similar effects on these degenerative mechanisms (Kramer et al., 1999; Nisoli et al., 2005). Alongside physical adaptations, there is evidence suggesting that aerobic exercise and resveratrol penetrate the blood-brain-barrier and promote desirable improvements in cognition and memory (Joseph et al., 2008; van Praag, 2009). With an ageing population, much of the medical sector is focussing on methods to prolong healthy life. In order to truly promote a healthy life, in both youth and old age, it is necessary to ensure well-being in both mind and body. Resveratrol and aerobic exercise both have great potential in prolonging healthy life by working against the mechanisms associated with ageing, as well as lowering the incidence of noncommunicable disease (Manson et al., 1999; Lifshitz and Hall, 2002; Baur and Sinclair, 2006). The

full potential of these factors in improving cognition is still being determined, with many studies investigating therapeutic effects on various aspects of cognitive decline.

The first aim of this thesis was to determine the extent of cognitive enhancement associated with regular resveratrol ingestion and aerobic exercise in healthy memory, memory decline associated with ageing, and AD-related amnesia. Previous studies investigating the effects of resveratrol on cognition have focussed on memory decline associated with ageing (Joseph et al., 2008; Oomen et al., 2009) and induced memory decline (Gupta et al., 2001, 2002a, 2002b; Abraham and Johnson, 2009). The effects on healthy memory have not yet been investigated. These studies specifically focussed on the effects of ingesting a relatively small quantity of resveratrol, with the intention of replicating the action of taking resveratrol tablets as a supplement. Many of the *in vivo* studies mentioned have used more concentrated doses and alternative methods of administration. With questions raised over the bioavailability of oral resveratrol *in vivo* (Baur and Sinclair, 2006), it is of importance to determine whether supplementation of this compound is a realistic cognitive therapy. A range of *in vivo* techniques were utilised to conduct a direct comparison study of these two factors in order to highlight more clearly the potential of resveratrol ingestion as an exercise mimetic. Resveratrol has long been thought of as a calorie restriction mimetic (Baur et al., 2006), but it is more recently that it has come to light that these physiological effects are similar to those evoked through aerobic exercise (Narkar et al., 2008). The treatment regime of oral resveratrol used here improved long-term recognition memory in healthy young rats in a similar way to the forced running regime. Memory was significantly improved using these regimes after 1 and 4 weeks. This was the first time that resveratrol treatment has been measured in young animals and it is of interest to discover that this polyphenolic compound can lead to potent cognitive enhancement in memory that is intact. In a 4 week regime, regular resveratrol ingestion and aerobic exercise both improved long-term recognition memory in middle-aged rats, showing that bio-availability of resveratrol was not a confounding factor using this ingestion regime. Resveratrol acted as an exercise mimetic in enhancing normal long-term memory and in ameliorating age-related decline. Using scopolamine to block muscarinic

receptors in the cholinergic pathways, the action of resveratrol ingestion and aerobic exercise on amnesia was assessed. Clear deficits in long-term recognition memory were found in scopolamine-administered animals, compared to vehicle-treated controls. This deficit did not occur in scopolamine-administered animals that underwent 7 days of resveratrol ingestion alone, or combined with aerobic exercise, beforehand. 7 days of treadmill running alone beforehand did not relief the deficit in long-term memory induced by scopolamine administration. Other studies have shown that aerobic exercise can improve AD-related memory decline in animals models (Hoveida et al., 2011) and that incidence of AD is lower in more active people (Colcombe et al., 2004; Larson et al., 2006), suggesting that resveratrol ingestion may have a more powerful action on the cholinergic pathway than is evoked by aerobic exercise. In a long-term treatment regime of resveratrol ingestion and forced wheel running, middle-aged animals were assessed regularly in performance in a spatial-temporal working memory task. Regular resveratrol ingestion and aerobic exercise, either combined or individually, improved task performance in this model of natural memory decline. Thirteen weeks of these regimes improved recovery in task performance after impairment was induced with a long delay. Resveratrol (Abraham and Johnson, 2009; Karalis et al., 2011) and aerobic exercise (Clarkson-Smith and Hartley, 1989; Lambourne, 2006) have been shown to improve performance in other working memory tasks. Improved performance following these regimes provides further evidence of the bio-availability of this oral dose of resveratrol, and permeability of the blood-brain-barrier. No adverse effects were evident from 14 weeks of oral resveratrol treatment, which is important to determine if regular supplementation of a compound is being promoted. In non-human primates, no adverse effects were found using an 18-month regime of resveratrol treatment (Dal-Pan et al., 2011). These regular regimes of resveratrol ingestion and aerobic exercise both work to enhance healthy memory and age-related memory decline, with resveratrol ingestion showing greater potential as a therapeutic agent in aiding AD-related cognitive decline.

The second aim of this thesis was to determine the potential mechanisms through which both aerobic exercise and resveratrol ingestion may act to evoke their beneficial action on learning and memory. Many studies investigating cognition refer

to resveratrol as a SIRT1 activator (Kim et al., 2007; Pallas et al., 2009; Baur, 2010) since this compound was found to consistently recapitulate the protective effects of SIRT1 overexpression in cell culture (Howitz et al., 2003; Araki et al., 2004). However, with no evidence to indicate that resveratrol is a direct activator of SIRT1, it is misleading to refer to resveratrol as simply a SIRT1 activator. More recently, AMPK has been suggested as an alternative target for resveratrol that may be important for upregulating the beneficial pathways associated with resveratrol action (Narkar et al., 2008), with an interdependence of these proteins highlighted in another study (Price et al., 2012). To determine and dissociate the importance of these proteins in the cognitive enhancement associated with resveratrol and aerobic exercise, the expression of endogenous proteins involved in the SIRT1/AMPK pathways in brain regions associated with learning and memory was measured. With improved long-term memory, no increases in expression of a number of proteins involved in these pathways in the hippocampus or perirhinal cortex were found, leading to questions about the action of these pathways in cognitive enhancement. Resveratrol been shown to upregulate AMPK (Dasgupta and Milbrandt, 2007) and SIRT1 (Della-Morte et al., 2009) in neurons with higher doses and alternative administration methods, but these findings suggest that upregulation of these proteins may not explain cognitive enhancement. This is supported by another study that found that boosting natural levels of SIRT1 did not enhance cognition in the SIRT1-null mice (Michán et al., 2010). Although aerobic exercise has also been shown to increase upregulation of SIRT1 (Fulco et al., 2008) and AMPK (Jäger et al., 2007), it is often increased levels of neurotrophins in the hippocampus that are thought to explain the cognitive enhancement associated with aerobic exercise (Cotman, 2002; Ang et al., 2003; Griffin et al., 2009). Some studies have also detected increased levels of neurotrophins following resveratrol administration (Rahvar et al., 2011; Pang and Hannan, 2012). To determine the importance of neurotrophins in the cognitive enhancement associated with resveratrol and aerobic exercise, the expression of neurotrophins in brain regions associated with learning and memory was measured. Increased expression of several neurotrophins were measured in the hippocampus and perirhinal cortex following forced running and resveratrol ingestion, leading to the suggestion that increased levels of neurotrophins in these brain regions may promote the improvement in cognition found with resveratrol administration as well as aerobic exercise. With most studies

investigating the action of resveratrol focussed on tissues outwith the brain, it was of interest to explore the effects of these treatment regimes on other tissues. Further analysis of mitochondrial functioning in tissues that expend large levels of energy and require higher numbers of mitochondria was carried out. Regular exercise has been shown to increase mitochondrial biogenesis and decrease various manifestations of oxidative stress in skeletal muscle (Holloszy, 1967; Howald et al., 1985; Irrcher et al., 2003) and brown adipose tissue (Boström et al., 2012), with administration of high doses of resveratrol promoting similar effects in skeletal muscle (Hebbar et al., 2005; Lagouge et al., 2006). Potential activation, and increased levels, of brown adipose tissue has been postulated to play a role in the treatment of obesity in humans (Seale and Lazar, 2009). If these regimes of aerobic exercise and resveratrol ingestion could improve mitochondrial function in brown adipose tissue and skeletal muscle it would extend the potential use of resveratrol supplementation beyond cognitive enhancement. Assessment of mitochondrial abundance, oxygen consumption, and levels of uncoupling proteins in these tissues indicated no effects of these regimes after 12 days. Levels of oral resveratrol and aerobic exercise that can induce cognitive enhancement, does not improve mitochondrial function in skeletal muscle or brown adipose tissue. This is supported by the fact that levels of proteins involved in the AMPK/SIRT1 pathways were not increased in brain regions associated with learning and memory when cognitive was enhanced. As AMPK overactivation has recently been associated with neurofibrillary tangles of hyperpolarised microtubule-associated protein tau, such as found with Alzheimer's disease (Vingtdeux et al., 2011), it is of benefit to discover that cognitive enhancement through aerobic exercise and resveratrol does not require activation of this protein.

6.2 FUTURE WORK

The treatment regimes used here evoked clear cognitive enhancement without upregulating the AMPK/SIRT1 pathways. It is evident from other studies

that different regimes, using higher doses and alternative methods of administration for resveratrol, can upregulate these pathways in a number of tissues, including neurons. With the possibility that upregulation of these pathways may not be recommended in the brain, it would be of interest to determine both the minimal dose of oral resveratrol that can enhance memory, and the maximal dose that can enhance memory without upregulating the AMPK/SIRT1 pathways. Additionally, it would be of interest to determine the action of resveratrol and aerobic exercise that enhances memory. Previous aerobic exercise studies indicate that upregulation of neurotrophins and neurogenesis are important for the cognitive enhancement associated with treadmill running. These regimes of treadmill running and resveratrol ingestion promoted neurotrophin expression and levels in brain regions associated with learning and memory. Further studies involving blocking neurotrophic action would provide further understanding of the impact of this upregulation on the cognitive enhancement observed here. Looking into alternative action of resveratrol may also prove necessary, such as determining changes in cerebral blood flow and white matter integrity, evoked through these regimes.

With this resveratrol regime showing more profound effects against scopolamine-induced amnesia than treadmill running, it is possible that the assessment of long-term and working memory used in healthy subjects was not difficult enough to distinguish the level of enhancement provided between treatments. It would be of interest to conduct a more difficult version of these tasks, possibly by introducing a 48 h interval between novel object recognition training and testing trials, to see if resveratrol ingestion also evokes a stronger enhancement of memory in healthy subjects than treadmill running. Progression from 4 week regimes in young rats to 4 week regimes in middle-aged rats to 1 week regimes in young rats was expected to highlight any differences between groups. A more difficult task to assess working memory also found no differences between treatments using long-term regimes. It was suggested that aerobic exercise and resveratrol ingestion evoke the same cognitive enhancement on healthy subjects, but further experiments may determine if this is the complete story or if there is more to tell.

In the search for a treatment of obesity in humans, it would be of interest to assess higher doses and longer treatment regimes in order to determine a level of aerobic exercise and resveratrol ingestion that can activate and increase levels of brown adipose tissue. As this activity is expected to occur through upregulation of the AMPK/SIRT1 pathways, it will also be of importance to determine cognitive effects of such regimes, and assess the effects on tangles of the protein tau.

6.3 CONCLUDING REMARKS

Several interesting discoveries have been made here that improve our understanding about how resveratrol affects cognition and the extent of this compound's function as an exercise mimetic. Other studies have shown cognitive improvements with resveratrol administration; here these effects have been replicated in long-term and working memory with a relatively small oral dose representing the action of taking resveratrol tablets as a supplement. Additionally, these results indicate that resveratrol and aerobic exercise improve cognition through an AMPK/SIRT1-independent pathway. Resveratrol was shown to evoke stronger cognitive enhancement in a model of amnesia, when compared to aerobic exercise. Overall, with no adverse effects evident with long-term treatment, suitable doses of oral resveratrol and levels of aerobic exercise throughout life may help people live longer, healthier lives.

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