Ageing in the Mammalian Brain

SOHAIR MOHAMMED KHOJAH, M.Sc. B.Sc.

UNIVERSITY

Of

GLASGOW

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College of Medical, Veterinary and Life Sciences

School of Life Sciences

Laboratory of Human Anatomy

University of Glasgow

Abstract

With a globally ageing population diseases associated with this natural process are becoming major issues worldwide. Research into the process of ageing and its concomitant issues is rapidly expanding; the need for new tools and models to investigate this rapidly expanding arena of research is paramount. The discovery of a spontaneous mutation in the AS rat strain which introduces a premature stop mutation into the gene encoding protein kinase C γ (PKCγ) lead to the development of a model for one such age related disorder, Parkinson's disease. Consequently, this model has been selected to investigate age related changes in specific areas of the brain (the cerebellum, basal ganglia, cerebral cortex and brainstem). These regions were selected because they have previously been shown to demonstrate changes with age (cerebellum, cerebral cortex and basal Ganglia), they show differences between the AS and AS/AGU strains (basal ganglia) or they show differences in PKCγ knockout models (cerebellum). The Brainstem was selected as it shows little change due to age and shows no differences in PKCγ knockouts or AS/AGU rats. This study used established qPCR methods to measure a validated biomarker of ageing, CDKN2A (the transcript for $p16^{INK4a}$) in the brains of these rats to determine whether this model is in fact a genuine model for accelerated ageing. This thesis demonstrates that CDKN2A expression, in combination with senescence-associated β-galactosidase staining, provides clear evidence of accelerated ageing in the brains of AS/AGU rats when compared with the parent AS strain.

These investigations were furthered by an investigation of members of the Sirtuin family of proteins. The changes in expression of these Sirtuins indicates that there may be increased levels of cellular stress, disruption of metabolism and DNA damage in the AS/AGU rats, this would be congruent with the accelerated ageing phenotype present in this strain. Furthermore, the levels of these Sirtuins were in line with the predictions from the MTR trinity in regards to the accelerated ageing phenotype. Whilst some of the changes in senescence and metabolic disruption may be attributable to the PKCγ mutation in the AS/AGU rats, it would appear that there is some element of accelerated ageing that is independent of this mutation.

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Author's Declaration

I hereby declare that the work presented in this thesis is entirely my own original work and carried out under the supervision of Professors Anthony P. Payne and Paul G. Shiels. Where other sources of information have been used, they have been acknowledged.

Sohair Mohammed Khojah
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Dedication

I dedicate this thesis to my most loving parents and my family, especially my husband, who has been very patient and who has given up so much to support and encourage me throughout.

Definitions/Abbreviations

Abbreviation	Complete Phrase
5-hmC	5-hydroxymethylcytosine
5-HT	5-hydroxytryptamine/Serotonin
5-mc	5-methylcytosine
A	Absorbance
AS	Albino Swiss
AS/AGU	Albino Swiss / Anatomy Glasgow University
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
β-gal	β-galactosidase
BER	Base excision repair
BoA	Biomarkers of Ageing
C. elegans	*Caenorhabditis elegans*
CR	Calorie restriction
CPS1	Carbomyl phosphate synthase I
CO_2	Carbon dioxide
CVD	Cardiovascular disease
CKD	Chronic kidney disease
COPD	Chronic obstructive pulmonary disease
cDNA	Complementary DNA
CI	Confidence interval
Ct	Cycle threshold
CDK	Cyclin dependent kinase
CDKN2A	Cyclin dependent kinase inhibitor 2A
CDKN2B	Cyclin dependent kinase inhibitor 2B
^oC	Degrees Celsius
dH_2O	De-ionised water
dNTPs	Deoxynucleotide triphosphates
DNA	Deoxyribonucleic acid
DNMTs	Deoxyribonucleic acid methyltransferases
T2D	Diabetes Mellitus type II

DEPC	Diethylpyrocarbonate
DA	Dopamine
GABA	γ-amino-butyric acid
Gal	Galactose
GP	Globus Pallidus
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HRP	Horse radish peroxidase
HD	Huntington's disease
HGPS	Hutchinson-Gilford progeria syndrome
IGF	Insulin-like growth factor
KO	Knockout
l	litre
Mg	Magnesium
$MgCl_2$	Magnesium chloride
mTOR	Mammalian target of rapamycin
MetS	Metabolic syndrome
mtDNA	Mitochondrial DNA
MTR Theory	Mitochondrion, Telomere nucleo-protein complexes and the control of Ribosome biosynthesis theory
µg	micrograms
µl	microlitres
µm	micrometres
M	Molar
nm	nanometre
ng	nanograms
NK	Natural killer cell
NSC	Neural stem cell
NAM	Nicotinamide
NAD^+	Nicotinamide adenine dinucleotide (oxidising agent)
NADH	Nicotinamide adenine dinucleotide (reducing agent)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidising agent)
NADPH	Nicotinamide adenine dinucleotide phosphate (reducing

	agent)
NO	Nitric oxide
OD	Optical density
PD	Parkinson's disease
PNS	Peripheral nervous system
PPARγ	Peroxisome proliferator-activated receptor gamma
PBS	Phosphate buffered saline
PI3K	Phosphoinositide 3-kinase
Cp	Phosphorylated Cytosine
PCR	Polymerase chain reaction
K	Potassium
PKCγ	Protein Kinase C gamma
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
ROS	Reactive Oxygen species
Rb	Retinoblastoma
RBL1	Retinoblastoma like 1 (also known as p107)
Ra	Retinoic acid
RA	Rheumatoid arthritis
RNase	Ribonuclease
RNA	Ribonucleic acid
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
Rt	Room temperature
RT	Reverse Transcription
SAM	S-adenosyl methionine
SAMP	Senescence Accelerated Mouse Prone
SAMR	Senescence Accelerated Mouse Resistant
SA β-gal	Senescence associated β-galactosidase
Sirt	Sirtuin
Sirt1	Sirtuin 1
Sirt2	Sirtuin 2
Sirt3	Sirtuin 3

Sirt4	Sirtuin 4
Sirt5	Sirtuin 5
Sirt6	Sirtuin 6
Sirt7	Sirtuin 7
NaCl	Sodium chloride
SN	Substantia Nigra
SNC	Substantia Nigra Pars Compacta
SNR	Substantia Nigra Pars Reticulata
STN	Subthalmic Nucleus
SVZ	Subventricular zone
TET	Ten-eleven translocation proteins
TBS	Tris buffered saline
TNFα	Tumour necrosis factor alpha
T2D	Type II Diabetes mellitus
Th	Tyrosine hydroxylase
UV	Ultraviolet
UBF	Upstream binding factor
VTA	Ventral tegmental area
WS	Werner syndrome
WT	Wilm's tumours
WHO	World Health Organisation

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1. Introduction

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1.1 Ageing

The process of ageing in humans is broadly defined as the accumulation of changes in a person over time (Bowen and Atwood, 2004). These changes come in physical, biological, chemical and psychological forms, ultimately transforming the appearance and personality of the individual. In fact, a more recent definition by Fulop *et. al.*, has described it as a complex phenomenon encompassing a series of deficits which accumulate at different rates, in different ways, in each individual; furthermore within the individual this occurs at different rates in different organs and tissues (Fulop, *et. al.*, 2010). At the molecular level, a combination of factors which includes genes and environmental processes define the lifespan of an organism (Jazwinski, *et. al.*, 1998; Takahashi, *et. al.*, 2000). This has been reinforced by the generation of genetically engineered animal models which demonstrate an accelerated ageing phenotype (Kuro-o, *et. al.*, 1997). The influence of environmental factors is highlighted in the case of the biologically immortal hydra (e.g. *Hydra vulgaris*) (Martinez, 1998) which does not undergo senescence, although this has been recently disputed (Estep, 2010), but is still subject to trauma and infection. Similarly, the planarian flatworm possesses extensive telomere regenerative ability and a highly proliferative population of adult stem cells (Tan, *et. al.*, 2012), but again they are susceptible to trauma and infection indicating that they are not truly immortal. Additionally, lifespans of these worms vary greatly between species, demonstrating that age is a product of both genetic and environmental determinants. The term ageing is used to describe the process of progression through lifespan and the consequent loss of dynamic regulation of interactions at cellular, tissue and organ levels in the organism (Monaghan, *et. al.*, 2008).

The maximum lifespan or age at which an organism is considered elderly varies dramatically according to inherited differences amid species (Austad, 2009), for example an elderly mouse would be around 3 years old,

whereas a human would be 70-80 years old. However, high levels of variation in intra-species lifespan suggest that genetic factors alone are not sufficient to explain organismal ageing, thus environmental factors must play a major role.

The proportion of individuals aged 60 or over is currently increasing in almost every country in the world, resulting in an ageing population as a whole. Current estimates believe that the percentage of the world's population over 60 will increase to 22% by 2050, this is double the 11% level seen in 2000. This is a result of longer life expectancy, as we understand the ageing process more, our ability to provide new treatments and policies to enhance the lifespan further continues to increase. Despite the fact that old age is no longer considered a cause of mortality, due to the proximal reasons normally being identified and recorded, it is the most potent driver of mortality in the world today. The increase in our older generations brings with it an increase in problems, diseases and disorders associated with age; these are now beginning to dominate, becoming ever more prevalent and presenting novel challenges for healthcare and society in general. This increase has concomitantly resulted in increased research into these disorders, and into ageing in general. Consequently, as a result of our ageing society and the improvements in general health and healthcare, the World Health Organisation (WHO) included four age-related causes of death in the top ten leading causes of death globally in 2002: Ischaemic heart disease (12.6%), Cerebrovascular disease (9.7%), Chronic obstructive pulmonary disease (COPD, 4.8%) and airway/lung associated cancers (2.2%) (WHO, 2004).

The significance of ageing, and its influence on society, has been studied for over a century. August Weissman, who developed the germ-plasm theory of inheritance (Weissman, 1893) which suggested distinct germ and somatic cell lineages, suggested that senescence is beneficial for the population as it removed older and less productive members (Weissman, 1889). However, Weissman never addressed the issue of senescence as its existence was an assumption made within his theory. The evolution of senescence is still debated today, with three main theories of its

emergence, although strong ties between the theories are evident. The first theory, championed by Haldane (Haldane, 1941) and Medawar (Medawar, 1952), suggests that the force of evolutionary selection declines proportionately with age, in accordance with Fisher's reproductive value (Fisher, 1930) – a measure of contribution to future generations by any given individual. This includes the proposition that an accumulation of deleterious mutations would occur in older individuals, resulting in reduced survival and reproductive capability. The second theory is an extension of the first by Williams (Williams, 1957), this theory, typically termed antagonistic pleiotropy, suggests senescence is a consequence of positive selection of genetic factors which have a deleterious effect in later life, i.e. due to higher numbers of living individuals in younger age classes, mutations which present favourable fitness benefits during early years are selected for even if they are detrimental in old age. The third theory, termed 'the disposable soma theory' focuses on the balance between limited resources, self-maintenance and additional activities (in particular reproduction) (Kirkwood, 1977; Kirkwood, 2002; Kirkwood and Holliday, 1979). This theory postulates that unrepaired damage to molecules, cells and tissues as a result of metabolism and stress, as well as other factors, result in a decline in function. This is damage which accumulates with age (Sgro and Partridge, 1999; Westendorp and Kirkwood, 1998); this theory also emphasises the influence of biochemical mechanisms, which prevent or repair this damage, have on the rate of accumulation. Although these three theories have been proposed it seems evident that these are consequently an evolution of the first theory as more is understood about the processes involved.

The natural process of ageing in mammals follows a very similar overall trend, with increases in the biological functionality during the early stages of life, peaking in early adulthood, and thus prime reproductive age, before a slow decline. The rate of decline is determined by many factors, from diet and exercise to genetics and environmental factors. Ultimately, these factors mean that chronological age is not necessarily the optimal method to determine an individual's actual "health age" this has lead to research into "biological age", or bio-age, which is determined by biochemical, epigenetic and genetic factors. The definition of a biomarker

of ageing (BoA) is a marker which alone or in composite will be superior in predicting functional outcome at a later age than chronological age alone, in the absence of disease (Baker and Sprott, 1988). These BoA provide greater insight into the actual age of an individual. Again, animal models which demonstrate accelerated ageing phenotypes demonstrate the importance of bio-age versus chronological age (Kuro-o, *et. al.*, 1997). Furthermore, it has been demonstrated that psychosocial effects influence ageing in man, with higher prevalence of ageing related diseases being associated with lower socioeconomic status. For example links between the lower end of the socio-economic scale and increased levels of inflammatory markers, as well as increased biomarkers for cardiovascular disease (CVD) have been demonstrated (McGuinness, *et. al.*, 2012). This further reinforces the link between these socio-economic factors and accelerated bio-ageing. Matters are further complicated by the discovery that different organs/tissues can age at different rates, with respect to bio-age. For example, age-related disorders of the heart occur at much younger ages than those of the brain. It is also evident that the choices people make heavily influence how and when they, or specific parts of them, age. Over exposure to high levels of ultraviolet (UV) radiation for example induces premature ageing in the skin (also known as photo-ageing) (Gilchrest, 1990), studies in *C. elegans* have demonstrated a direct link between UV exposure and shortened lifespan (Klass, 1977). Smoking has been linked to increases in age-related disease susceptibility (Valdes, *et. al.*, 2005), however the true nature of the relationship between smoking and ageing is masked by environmental exposure to passive tobacco smoke and other environmental toxins including car exhaust fumes; this makes finding a true negative exposure control almost impossible. Poor diet choices can lead to metabolic and intestinal problems earlier in life than would normally be expected. The effect of over eating on lifespan has been demonstrated with shortening of life expectancy in several models including protozoans (Rudzinska, 1952), rotifers (Fanestil and Barrows, 1965), nematodes (Klass, 1977) and rats (Ross, 1961; Ross and Bras, 1975). In humans obesity has also being linked with risk for age-related diseases (Valdes, *et. al.*, 2005). Taking all of these factors into consideration it is now obvious that many of the day to day decisions we make may seem

trivial at the time, but ultimately they can have a monumental influence on our healthspan (years of healthy living).

Different tissues age in different ways, making age related comparisons between tissues difficult. By segregating body tissues into four main categories these differences can be minimised and catalogued. Firstly there is connective tissue; this tissue generally becomes stiffer increasing the rigidity surrounding the body's organs, blood supply and airways. Ultimately this reduces the flow of nutrients and oxygen to tissues and organs resulting in reduced capacity throughout. Secondly, muscle tissue, including all forms of muscle: smooth or involuntary muscle which is an integral part of the stomach, intestine and other organs; striated or voluntary muscle which is responsible for conscious movement of the limbs and skeleton; cardiac muscle, another type of involuntary muscle which is found exclusively in the heart. Muscle tissue tends to atrophy, or reduce in size with a concurrent diminishment of capacity. Thirdly, epithelial tissue which lines the internal passages of the body as well as covering the exterior and protecting other body tissue from direct exposure to the environment. Similarly to connective tissue, epithelial tissue becomes more rigid or less elastic and suffers from a reduced regenerative capacity. Finally Neuronal, or nerve, tissue which encompasses the brain, spinal cord and peripheral nervous system (PNS), this tissue rarely regenerates and is generally replaced with scar tissue when damaged. Similar to muscle neuronal tissue is at high risk from atrophy. There are also overall differences in mass of organs as these atrophy with age, the largest age related changes in reserve function occur in the heart, lungs and kidneys.

1.2 The biochemistry of ageing

Ultimately all of the changes apparent with ageing are explainable at the biochemical level, the occurrence of premature ageing syndromes and creation of animal models which appear to prematurely age is evidence of the overall genetic control of ageing (Kuro-o, *et. al.*, 1997). Cells, similarly to whole organisms, undergo symptoms of ageing with degradation of DNA over time, changes in morphology and ultimately senescence and apoptosis;

whereupon they stop growing and replicating, then finally "self-destruct". In general the process of cellular ageing can be analysed as two distinct phenotypes, the first being proliferative senescence where ordinarily diploid cells lose their ability to divide, *in* vitro this limit is around 50 cell divisions, this phenomenon is also known as the "Hayflick Limit" after the author, Dr Leonard Hayflick, who first described it (Hayflick and Moorhead, 1961). Although incapable of reproduction these senescent cells maintain metabolic activities and generally adopt the senescence associated secretory phenotype: flattening of cell, altered gene expression and secretion profile; as well as demonstrating positive staining for Senescence associated β galactosidase (SA β-gal) (Campisi, 2013). Removal of senescent cells has been demonstrated to protect against ageing disorders (Baker, *et. al.*, 2011), thus implicating cellular senescence in the generation of age related phenotypes. The second is ageing and death of an individual post-mitotic cell (Kurz, *et. al.*, 2000). These post-mitotic cells are found in specific areas of the body which are unable to replenish by replication, or mitosis, these areas replenish themselves from the germline, or stem cell population. However, there is one exception to this, the brain and nervous system, which is unable to replenish dead cells by either mitosis or from a stem cell population. Despite this apparent disadvantage the effects of cell depletion are not apparent until old age when neurodegenerative disorders and memory loss become more common.

Looking at age related changes in tissues and organs it becomes evident that the type of change is related to the biochemistry or senescent status of the cell. Those cells which undergo regular mitotic division suffer from a reduction in elasticity and reduced regenerative capacity; whilst cells which do not undergo mitosis, post-mitotic tissues, atrophy with age due to in inability to replace lost or damaged cells. The ultimate cause of atrophy is unknown, however it is most likely a combination of reduction in use (or decreased workload), reduced nutrient/oxygen supply or a reduction in stimulation (by nerves or hormones).

1.2.1 Cellular senescence

The process of cellular senescence gives us insights into the "ageing" of the individual cell and the pathways which govern it. The process of ageing and cellular senescence have become closely entwined (Jeyapalan and Sedivy, 2008), however it must be noted that senescence can occur without the necessary onset of age related changes. Believed to be a defensive mechanism against onset of neoplastic transformation, the term senescence is used to refer to age related phenotypic changes, incorporating many biological, developmental and maturational processes, which do not necessarily precipitate apoptosis. These changes include irregular morphology, a flattened appearance, and increased nuclei size (Suh, *et. al.*, 2002; Wagner and Jansen-Durr, 2000). Due to the range of these alterations the phenotype of cellular senescence is complex (Takahashi, *et. al.*, 2000). Furthermore, expression of another biomarker in the form of SA β–gal is also characteristic of senescent cells (Dimri, *et. al.*, 1995). Senescence is accompanied by an inflammatory secretome (Sikora, *et. al.*, 2010). The onset of cellular senescence can be triggered by a number of factors. Firstly, the Hayflick limit, after a set number of replications a cell effectively retires and becomes senescent, sometimes referred to as replicative senescence (Hayflick and Moorhead, 1961). This is extremely important, as the longer a cell is active and the more times it undergoes mitosis the more likely it is to incur increased DNA damage which may ultimately lead to disastrous consequences, for example tumourigenesis. This process is effectively over-watched by the telomere system. Telomeres are found at the ends of each chromosome and comprise a nucleo-protein complex around a region of repetitive nucleotide sequences (Blackburn and Gall, 1978). These protect the ends of the chromosomes from degradation, at the expense of becoming shortened themselves via the end replication problem (Counter, *et. al.*, 1992; Olovnikov, 1973). This shortening however, serves as a biological clock indicating the replicative history of the cell; once the telomeres have shortened to a specific length the cell undergoes senescence and/or apoptosis to protect the entire organism (von Zglinicki, 1998). This process was originally believed to be irreversible however some studies have

demonstrated that the key senescence regulator $p16^{INK4a}$ has the ability to induce reversible cellular senescence dependent on its expression and interaction with the Retinoblastoma (Rb) protein (Beausejour, *et. al.*, 2003). Telomere dysfunction, or accelerated shortening of telomeres, has also been demonstrated to be at the centre of a number of clinically diverse disorders which all ultimately demonstrate a catastrophic tissue failure (i.e. loss of function); for example, bone marrow failure or idiopathic pulmonary fibrosis (Armanios and Blackburn, 2012). The average length of telomeres in a given cell population can now be measured by quantitative polymerase chain reaction (qPCR), giving a rough guide to the bio-age and life expectancy of that cell population (Cawthon, 2002). However, it has also been demonstrated that the shortest telomere length in any given cell is critical for determining the lifespan of that cell (Hemann, *et. al.*, 2001). This shortening is overcome in stem cells, and other immortalised cell lineages, where telomere shortening is counter-acted by an enzyme called telomerase, which adds repeats back on to the ends of chromosomes to preserve the integrity of the genome in these cells (Blackburn, *et. al.*, 1989). In a similar fashion to normal cells, inhibiting telomerase activity in immortalised cells, for example stem cells or cancer cells, results in apoptosis (Zhang, *et. al.*, 1999).

The second factor influencing the onset of cellular senescence is oxidative stress (Serrano and Blasco, 2001). Increased levels of oxidative stress induce senescence associated mechanisms and can result in early onset of senescence as a protective mechanism within the cell. This oxidative stress most often derives from reactive oxygen species (ROS) (Lu and Finkel, 2008). This can of course be either temporary or permanent dependent on the level, duration and severity of the stress on the cell. In fact if the stress is sufficiently severe the cell can rapidly undergo apoptosis to prevent further damage to surrounding tissues (Johnson, *et. al.*, 1996). Tissue hypoxia has been demonstrated to inhibit senescence; increasing tissue hypoxia with increasing age provides a potential override for the tumour suppressor function of cellular senescence (Welford and Giaccia, 2011).

A third possibility for inducing senescence comes from toxicology, as specific toxins or environmental factors can induce senescence. For example, lipopolysaccharide has been demonstrated to cause cellular senescence in lung alveolar epithelial cells at sub-lethal doses (Kim, *et. al.*, 2011a). Bacterial heat-stable enterotoxin has also been demonstrated to induce cell cycle arrest (Basu, *et. al.*, 2013).

Finally, senescence can be induced by genetic, or more likely epigenetic, changes which result in aberrant expression of genes. This process is most often found in response to DNA damage and telomere erosion. Some genes are critical for senescence and their induction under specific circumstances can be used as a biomarker, p53 (Tyner, *et. al.*, 2002) and $p16^{INK4a}$ (Jacobs and de Lange, 2004) are two such genes. Recent studies have also suggested that p21, part of the p53 cellular senescence pathway, may also be able to induce senescence independently of p53 (Zuo, *et. al.*, 2012). Oncogenes have also been demonstrated to induce senescence, for example the oncogene *ras* can induce senescence via p53 and $p16^{INK4a}$ (Serrano, *et. al.*, 1997); another oncogene *raf* has also been shown to induce senescence in human fibroblasts (Zhu, *et. al.*, 1998). In some cases senescence can be induced not by aberrant expression, but by anomalous activation of specific proteins.

Originally presented as two independent hypotheses (Campisi and d'Adda di Fagagna, 2007), the process of cellular senescence is now widely accepted to be critical for two seemingly unrelated functions. These are tumour control and ageing, although more and more research is tying these phenomena together revealing overlapping pathways and mechanisms. The close links between these two functions can be epitomised by mouse models with hyperactive p53; these mice do not suffer from tumours but do exhibit severe and multiple symptoms of accelerated ageing (Tyner, *et. al.*, 2002).

1.2.2 Post mitotic cells – ageing and death

The exact definition of what constitutes a 'post mitotic cell' remains debated, with some believing it to cover both senescent and quiescent cells while others suggest that it only covers cells which do not exhibit mitosis and cell division after foetal development. However, the most common definition, and the one referred to from this point forward, is one which states that post mitotic cells are mature cells which are no longer capable of reproducing, or in fact re-entering the normal cell cycle (Campisi and d'Adda di Fagagna, 2007). This excludes quiescent cells which are capable of re-entering the cell cycle even after extended periods of inactivity in the cell cycle. Post mitotic cells are found throughout the body and include neurons, cardiac myocytes, skeletal muscle cells and terminally differentiated cells e.g. gut epithelial cells and peripheral blood cells (Terman, *et. al.*, 2009). Generally the major post mitotic tissues are considered to be neural, cardiac and skeletal muscle, as these tissues are rarely replaced and can, in some instances, be as old as the organism itself (Bhardwaj, *et. al.*, 2006). Due to the lack of normal proliferation and cell cycle, these tissues can only undergo limited repair and regeneration (Campisi and d'Adda di Fagagna, 2007). Furthermore this limited regenerative capacity has been linked to recruitment of mitotic stem cells (or their progeny) in the brain (Emsley, *et. al.*, 2005) and heart (Srivastava and Ivey, 2006). Skeletal muscle is capable of complete regeneration due to somatic stem cells resident within the skeletal muscle itself (Shi and Garry, 2006), these stem cells are also known as satellite cells (Cossu and Biressi, 2005; Mauro, 1961).

Within post mitotic cells, highly modified expression of anti-apoptotic and cell cycle regulators is apparent. In particular, high expression of B-cell lymphoma 2 (BCL2; a proto-oncogene) (Hockenbery, *et. al.*, 1991), an anti-apoptotic protein membrane protein found within mitochondria, has been noted (Hockenbery, *et. al.*, 1990). The cyclins, cyclin dependent kinases (CDKs) and CDK inhibitors are also differentially regulated in post mitotic cells providing the mechanism for maintaining the cells in a quiescent state (Yoshikawa, 2000). In fact it has been

demonstrated, both in neuronally differentiated cell lines and sympathetic neurons, that CDK inhibitors are necessary for cell survival (Park, *et. al.*, 1996). Retinoblastoma (Rb) and the proteins which control its phosphorylation appears to be key to maintaining the quiescent state (Lois, *et. al.*, 1995) and preventing apoptosis (Park, *et. al.*, 1998). The Rb protein itself is differentially expressed between areas of neurogenesis and post mitotic neurons (Slack, *et. al.*, 1993); as are the Rb family member p107 (also known as Retinoblastoma like 1, or RBL1) (Gill, *et. al.*, 1998) and p130 (a Rb related protein), which is expressed in quiescent cells and decreased in cells re-entering normal cell cycle (LeCouter, *et. al.*, 1996). This is to be expected as phosphorylation of Rb is essential for progression into the synthesis (S) and mitotic (Mi) phases of the cell cycle.

Due to their very nature, these post mitotic tissues are present throughout the lifespan of the organism (Terman, *et. al.*, 2009). In the case of skeletal muscle, its regenerative capacity maintains the population and replaces cells as necessary. However, in neural tissue regeneration is very limited and is restricted to only a few key neurogenic areas. There are two constitutive areas of neurogenesis: the hippocampal dentate gyrus (Altman and Das, 1965) and the olfactory bulb (Altman, 1969). However, there have also been other areas identified which possess potential regenerative cells: neural stem cells (NSCs). For example a primary cell line derived from the striatum of mice could be induced to proliferate and differentiate into cells which were morphologically and antigenically similar to neurons and astrocytes (Reynolds and Weiss, 1992). Another study demonstrated a similar phenomenon in rat cells in culture (Palmer, *et. al.*, 1995), however neither of these studies demonstrated this *in vivo*.

The regeneration of neuronal tissue is believed to result from small key areas of multipotent stem cells, some of which can migrate large distances across the brain (Lois and Alvarez-Buylla, 1993). These cells are capable of differentiating into different neuronal lineages including neurons, astrocytes and oligodendrocytes depending on the supporting signals present (Johe, *et. al.*, 1996). Some cell models have been developed to mimic these cells in order to study neurogenesis; two of the

most significant models to date are embryonal carcinoma (EC) cell lines, for example murine P19 cells treated with retinoic acid (Ra) (McBurney, *et. al.*, 1988) and Ra treated human NTera2 cells (Andrews, 1984). Ra treatment of P19 cells results in a differentiated population consisting of several neural post mitotic cells including: microglia-like phagocytes (Aizawa, *et. al.*, 1991), astrocytes-like cells (Jones-Villeneuve, *et. al.*, 1982) and oligodendrocytes (Staines, *et. al.*, 1996). The interplay between this regenerative capacity and the ageing process in the brain is still not fully understood.

1.2.3 DNA damage repair mechanisms

Due to the lack of replication, and thus dilution of post-mitotic tissues maintenance and repair become essential mechanisms for the healthy cell and disruption of these mechanisms can be catastrophic for post-mitotic cells and tissues. One such set of repair and maintenance mechanisms is DNA damage repair.

The integrity of the genome, and the DNA which encodes it, is absolutely critical for the survival of any organism. This is reflected in the level of resources attributed to its accurate replication, protection and repair mechanisms. In particular a very high level of resources and complexity is attributed to the repair and maintenance of DNA. The extensive nature of these mechanisms also reflects the potential insults the genome can endure for example from UV light (Ravanat, *et. al.*, 2001), radiation (Ward, 1988), hydrolysis (Lindahl, 1993), replication errors (McCulloch and Kunkel, 2008), incorporation of altered nucleotides (Shimizu, *et. al.*, 2003), ROS (Cadet, *et. al.*, 1997), RNS (Burney, *et. al.*, 1999), viruses (Chen, *et. al.*, 2014), toxins (Bedard and Massey, 2006), pharmaceuticals and mutagens (Irigaray and Belpomme, 2009; Wogan, *et. al.*, 2004). DNA is also inexorably linked to ageing, it has been postulated that the DNA repair mechanisms also play into the ageing mechanisms through their activation; it is also obvious that advancing age adversely affects the DNA repair mechanisms slowing them down and making them less efficient. These mechanisms are also closely linked to the cell

senescence and apoptosis pathways, for example cells can undergo reversible senescence to initiate repair or apoptosis in the event of catastrophic damage.

1.2.3.1 Mismatch repair

The mismatch repair system detects and repairs mismatched bases introduced during DNA replication and missed by the proof-reading component of the polymerases. This also incorporates loops that are generated by slippage in the replication of repetitive sequences. This system is one of the simpler processes and is accomplished in three steps: recognition, excision and repair (Fukui, 2010; Li, 2008). This system also demonstrates homology with the bacterial system found in E. coli (Modrich, 2006), with two protein complexes called MutS and MutL which are responsible for recognition of site (Su and Modrich, 1986) and initiation of repair respectively (Galio*, et. al.*, 1999).

1.2.3.2 Base excision repair

Base excision repair (BER) occurs when a single nucleotide, or a small section, becomes damaged and needs to be replaced, this system comes into effect when there is little overall disruption to the DNA helical structure (Zharkov, 2008). A key example of this repair mechanism is the excision and replacement of Uracil bases in DNA, but incorporates a broad range of base alterations and damage including some methylations. There have been twelve DNA glycosidases, the enzyme responsible for binding to the damaged base and excising it, identified to date each with a unique single or small set of targets representing a different type of base damage (Jacobs and Schar, 2011). This system effectively swings the damaged base outward from the helical structure allowing easy access and excision, creating an abasic/apyrimidic or AP site (Hitomi, *et. al.*, 2007). These AP sites are then treated by apurinc/apyrmidinic endonuclease 1 to create a single strand break flanked by 3' -OH and 5'-deoxyribose phosphate ready to accept a replacement base (Abbotts and Madhusudan, 2010). However, there are several potential 3' and 5' termini that cannot be treated by this

mechanism giving rise to a diverse set of enzymes and processes to create the 3'-OH and 5'-deoxyribose phosphate necessary for repair (Caldecott, 2008). Once the break is repaired the site is filled and the ends ligated, these repairs are segregated into short and long patch repairs, the former is initiated in response to single base replacements each type of repair has its own mechanism with long patch requiring more input from the DNA replication machinery (Fortini and Dogliotti, 2007).

1.2.3.3 Nucleotide excision repair

The Nucleotide excision repair (NER) pathway is a more versatile system capable of responding to larger scale DNA malformities, which induce helical disruptions. One of the most prominent of these repairs is pyrimidine dimers. Despite the principals being similar NER is far more complex than BER requiring around thirty proteins in a mutli-step approach involving recognition, local helical disruption, excision, repair and ligation (Shuck, *et. al.*, 2008). These repair pathways are subdivided into two categories: global genome NER and transcription-coupled NER, the former conducts repairs throughout the genome whereas the latter focusses solely on the coding strand of actively transcribed genes. The difference between these pathways is found in the recognition step and the proteins that accomplish this task (Dexheimer and Mathews, 2013).

1.2.3.4 Double stranded breaks

Double stranded breaks are potentially lethal and can induce chromosomal deletions and aberrations, making the repair of these breaks essential for cell survival and genome stability (Khanna and Jackson, 2001; van Gent, *et. al.*, 2001). There are two mechanisms for repairing this kind of break the first, homologous recombination, is more accurate and less error prone as it repairs the break using the intact sister chromatid as a template (Li and Heyer, 2008). The second type, non-homologous end joining, is quite error prone and involves direct ligation of the ends (Lieber, 2010).

As would be expected a great numbers of problems and diseases are a result of DNA damage and in particular of malfunction of these repair mechanisms, for example many cancers involve an alteration in the repair mechanisms, particularly defects in mismatch repair systems (Peltomaki, 2001). Furthermore, Xeroderma pigmentosum, Cockayne syndrome and Trichothiodystrophy are all linked to genetic abnormalities in nucleotide repair which all demonstrate overlapping symptoms with cancer and premature ageing (Cleaver, *et. al.*, 2009; Vermeulen, *et. al.*, 1997).

1.2.4 Biomarkers of ageing

Biomarkers represent useful tools for the *in vivo* and *in vitro* measurement of various phenomena, in this regard ageing is no different. Biomarkers in ageing are different to standard biomarkers which detect or monitor diseases. These biomarkers are by their very definition natural and normal, therefore molecules which naturally change with age are the only potential candidates. In order to assess the validity of any biomarker with regard to ageing a well accepted definition was published by Baker and Sprott in 1988: "a biological parameter of an organism that either alone or in some multivariate composite will, in the absence of disease, better predict functional capacity at some later age than will chronological age" (Baker and Sprott, 1988). This definition came with a more specific set of criteria for the determination and measurement of such a biomarker. These criteria were, the first four are based on the original set published by Reff and Schneider (Reff and Schneider, 1982), 1) non-lethal; 2) highly reproducible; 3) displays significant alterations during relatively short time periods; 4) critical to effective maintenance of health and prevention of disease; 5) reflects a measurable parameter that can be predicted at a later age; 6) reflects some basic biological process of ageing and metabolism; 7) should have high reproducibility in cross-species comparisons (Baker and Sprott, 1988; Reff and Schneider, 1982). These criteria are very useful but do have their limitations, for example with regard to criteria 1, it may not always be possible to measure a biomarker non-lethally particularly in sensitive tissues like the brain. Criteria 3, it is

possible that some biomarkers may experience bursts of activity/level changes due to environmental insults, but otherwise change at a different rate; this also leads to criteria 5 which implies that the level of the biomarker will change linearly over time and track with chronological age, by the very definition of a BoA neither of these would be true. Finally criteria 6 states that a BoA should be involved in ageing and metabolism, this may not necessarily be directly true, the molecule may influence metabolism indirectly e.g. through control of the cell cycle.

The search for biomarkers has been further hampered by the inter-species differences in ageing. It is obvious from lifespans that all organisms do not age equally, in fact some like the hydra are effectively immortal (Martinez, 1998), these differences in ageing suggest a mechanism or mechanisms which areimplemented and regulated differentially between species. Furthermore, the genetic variation between species suggests marked differences in the genes and proteins involved in the ageing processes may also be present. Despite this some of the ageing databases describe common elements present in ageing, for example GenAGE [http://genomics.senescence.info/genes/microarray.php, accessed on 12th January 2015]. This suggests that a common core of ageing processes may exist, with a variety of inputs and/or outputs which are species specific.

Due to the highly selective nature of the criteria for a BoA very few BoA exist. In fact only two meet the definition of a BoA and come close to completely fulfilling these criteria, CDKN2A product $p16^{INK4a}$ and telomere length, however in direct comparisons telomere length has been shown to be weaker than CDKN2A (Shiels, 2012). Additionally CDKN2A has been shown to be superior to chronological age (Koppelstaetter, *et. al.*, 2008; McGlynn, *et. al.*, 2009). In terms of age related changes many candidates have been identified, although few meet these stringent criteria, for example the AGEMAP programme in mice sought to examine the age related changes throughout the mouse lifespan. This study demonstrated that not only did many genes change in association with advancing age, but that changes were different between tissue types (Zahn, *et. al.*, 2007). Several databases also exist which list genes that have been associated

with ageing in a variety of species, including rat and human; for example GenAge (Human) [http://genomics.senescence.info/genes/allgenes.php, accessed on 3rd January, 2015], AnAge (animal species) [http://genomics.senescence.info/species/, accessed on 3rdJanuary, 2015)

1.3 Theories of ageing

There are several different theories of ageing and how the processes involved are related and controlled. In general, these theories can be divided into two classes, programmed and stochastic. Theories in the programmed classification use the biological clock basis, where all events and genetic changes are, in general, a result of a pre-determined sequence. The stochastic theories cite environmental effects on organisms which induce cumulative damage resulting in ageing; this can come in the form of DNA damage, tissue or cell damage as a result of free radicals.

From all of these theories of ageing it is evident that no single process or pathway is responsible for all of the effects and changes induced by advancing age, biological or chronological. Furthermore, these theories are not exclusively independent of one another; in fact several of these theories could be construed as advancements or amalgamations of previous theories. Currently, theories of ageing are classified into two categories: the programmed theories and the damage or error theories, the following theories are extrapolated from the categories suggested by Kunlin Jin (Jin, 2010).

1.3.1 The programmed theories

The programmed theories follow the basic premise that ageing is predetermined in some form or other. This pre-determination can be minimally influenced by other factors but in general follows a predetermined route with specific events on a defined schedule. There are three main theories within this sub-category, these are outlined below:

1.3.1.1 Programmed longevity theory

This theory stipulates that ageing, and all if its consequences, is a direct result of sequential activation/silencing of specific genes, in a programmed manner. This ultimately leads to senescence when the correct set of genes is activated due to age related defects becoming evident. Many believe that this theory includes provisions that explain the effects of ROS and other damage mechanisms (Longo, *et. al.*, 2005). It has also been proposed that this theory could be expanded to incorporate elements of altruism (Longo, *et. al.*, 2005), this suggests that an organism can pass advantages onto its kin or group members by remaining within the group. This in turn be selected for and would improve the survival of such genetic traits that promote longevity (Longo, *et. al.*, 2005).

This theory also incorporates many of the ideas found in the antagonistic pleiotropy theory of the origin of ageing; which postulates that some genes are inherited because they are beneficial to younger organisms, despite having deleterious effects in old age. In other words genes are selected for by their benefits in reaching and utilising the fertile period, which outweigh any later costs when an organism is no longer able to reproduce. This idea was first suggested by George Williams (Williams, 1957) and was based on an original idea presented by Peter Medawar (Medawar, 1952). In more recent times this view on the origin of ageing has been challenged due to a lack of clear cut examples (Kirkwood, 2005). Indeed one of the strongest classical examples of this was p53, however its position as a clear cut example has recently been challenged (Blagosklonny, 2010b). However, a new example in the form of mammalian target of rapamycin (mTOR) has emerged (Blagosklonny, 2010b). It has also been demonstrated that calorie restriction (CR) can be antagonistically pleiotropic, although in the opposite direction (harmful in early life, but beneficial in ageing) (Blagosklonny, 2010a). This indicates that many of the genes regulated by CR may be involved in the principal behind antagonistic pleiotropy. This theory does not however address the promotion of mutations which have no obvious fertility/early growth benefits but do present improvements in longevity (Longo, *et. al.*, 2005).

Examples of this are the deletion of a guanine nucleotide-binding protein known as *RAS2* in yeast or the insulin like growth factor 1 known as *daf-2* mutants in *Caenorhabditis elegans* (*C. elegans*) (Kenyon, 2011; Longo and Finch, 2003).

1.3.1.2 Endocrine based theory

The endocrine theory, or hormone theory of ageing, suggests that the most important factors involved in ageing are the changes induced by the endocrine system. This theory is supported by the influence of insulin and insulin-like growth factor (IGF) on longevity (Tatar, *et. al.*, 2003; van Heemst, 2010). This has been elegantly demonstrated in *C. elegans* by investigating longevity mutations and tracing the causal genetic mutation back to the IGF signalling cascade (IIS) (Honjoh, *et. al.*, 2009; Kenyon, *et. al.*, 1993). This effect, mediated through FOXO, has also been demonstrated in *Drosophila* models (Tatar, *et. al.*, 2001). Growth hormone (GH) has also been implicated via its receptor, growth hormone receptor (GHR); this integral component of the endocrine system has been demonstrated to be involved in longevity in a knock out mouse model (Arum, *et. al.*, 2009). The highly entwined nature of the IIS and GH pathways in higher mammals has made it extremely difficult to examine the roles of these molecules independently; however deletion of insulin receptor, specifically in the fat tissue of mice, increases life expectancy (Bluher, *et. al.*, 2003). GHR knockout mouse models have also demonstrated the same effects mediated through reduced GH/IGF-1 signalling (Chandrashekar, *et. al.*, 1999; Flurkey, *et. al.*, 2001). It is obvious from these reports that the hormonal system, particularly relating to metabolism, has a large impact on lifespan. This theory has also attributed control of circadian rhythms to the hormone system; however it is still disputable whether hormones drive or are driven by circadian rhythms.

1.3.1.3 Immunological theory

Some have postulated that the immune system is programmed reduce over time, thus increasing susceptibility to infection resulting in ageing and death. Given a high level of ROS involvement in the activity of the immune system it is possible to tie some of these effects into the damage and error theories, particularly the free radicals theory (see below, 1.3.2.4 Free Radicals Theory). However, it is well documented that immune function peaks at adolescence and then declines with age; resulting in reduced ability to fight disease, causing cellular stress (Cornelius, 1972).

The immunological theory of ageing was first proposed by Roy Walford, who suggested that ageing is related to faulty immune function (Walford, 1964). This is tentatively supported by clinical observations that some immune function traits are associated with early mortality, regardless of the ultimate cause of death (Wayne, *et. al.*, 1990; Wikby, *et. al.*, 1998). Further evidence for this theory can be gleaned from *C. elegans* mutants with a *daf-2* mutation which results in an extended lifespan; these mutants are also highly resistant to bacterial pathogens (Garsin, *et. al.*, 2003). However, it should be noted that the involvement of *daf-2* implicates the IGF pathways, which ties this theory with the endocrine theory (see above, 1.3.1.2 Endocrine based theory). Another major driver of this theory is T cell replicative senescence (Effros, 2004). Basically, the diversity of antigen recognition in the T cell population is driven by proliferation, with each T cell forming a unique combination of DNA encoding a T cell receptor. This combination is unique to each T cell and its progeny. Replicative senescence of T cells results in reduction and or loss of these unique receptors, thus reducing the available repertoire. Reduction in immunoregulatory molecules (cytokines and chemokines) from ageing natural killer (NK) cells further debilitates the immune system and its function. This is combined with a direct decline in the cytotoxicity of the same NK cells which results in impaired NK cell function (Hazeldine and Lord, 2013). This further ties this theory with the

'Hayflick limit' (Hayflick and Moorhead, 1961) or programmed longevity (see above, 1.3.1.1 Programmed longevity theory).

Additionally, the involvement of the immune system in diseases associated with ageing for example Alzheimer's disease (AD) provides further evidence that the immune system may be involved in ageing (Rozemuller, *et. al.*, 2005). The immunological theory, similarly to other theories, is ultimately a question of which comes first. It is obvious that reduced numbers of immunological cells are involved in establishing the phenotype of ageing. However, the question remains whether this reduction is a cause or a consequence of normal biological ageing?

1.3.2 The damage or error theories

The damage or error theories of ageing are based on the belief that ageing is a result of damage, both acute and chronic, or errors in repair mechanisms which accumulate over time. This ultimately results in an accumulation of biological problems as age increases, until these unresolved issues overwhelm the cell/organism and result in its death. In this context the senescence and apoptotic pathways are believed to protect against this by eliminating or halting cells that are too badly damaged, or full of errors, to be of further use to the organism. In actual fact, these cells may become a liability with errors that lead towards tumourigenesis.

1.3.2.1 The wear and tear theory

Exactly as it reads, this theory is based on the principal that all physical components of the body accumulate damage or 'wear', with specific traumatic events inducing more severe damage in a short space of time, simply put as 'tear'. This theory was first postulated by Dr August Weissman in 1882 and stipulates that overuse and abuse lead to damage which ultimately overcomes repair mechanisms and causes us to age. This theory covers many of the modern insults to our bodies, including over consumption of sugar, alcohol, fats and caffeine; as well as our use of

nicotine, over exposure to UV light and other physical, emotional and environmental stress which have become an everyday part of our lifestyles (Salvi, *et. al.*, 2006).

Whilst appealing to the general public because it fits with the visual observations of ageing, this theory is no longer a prominent one. In fact this theory has been superseded by more modern and explanative theories, which describe the same phenomenon in more depth and with an appropriate mechanism. Both the free radicals theory (see below, 1.3.2.4 Free radicals theory) and the disposable soma theory (see below, 1.3.2.5 Disposable soma theory) can be viewed as examples of this.

1.3.2.2 Rate of living theory

The rate of living theory of ageing suggests that the basal metabolism rate, particularly the use of oxygen in metabolism, is directly related to its lifespan. This is based on studies in *C. elegans* which demonstrated that increased metabolism resulted in shortened lifespan (Brys, *et. al.*, 2007). A modified version of the original theory (Pearl, 1928) has been proposed which incorporates the antagonism seen between growth and stress in normal ageing, demonstrated by TOR and FOXO signalling pathways (Rollo, 2010). More recently, this has been developed to address a range of age related non-communicable diseases which appear to have common underlying cellular and molecular components, despite disparate overt pathologies.

Despite some considering this theory as an independent entity, many consider this theory as synonymous with the free radicals theory (see below, 1.3.2.4 Free radicals theory), including Brys *et al*, the authors that originally demonstrated the links between metabolism and lifespan (Brys, *et. al.*, 2007). In fact, others have postulated that this idea forms the basis of an oxidative-stress theory of ageing which incorporates the concepts of increased ageing resulting from oxidative damage, metabolism and stress (Hulbert, *et. al.*, 2007). This in turn links to the lysosomal-mitochondria axis theory of ageing which postulates that the

damage from free radicals, and thus from mitochondria, progressively accumulates particularly in post-mitotic tissues resulting in ageing (Brunk and Terman, 2002; Terman, *et. al.*, 2006; Terman, *et. al.*, 2009). There is some evidence, from studies on *Saccharomyces*, that a substantial, almost catastrophic, drop in Nicotinamide adenine dinucleotide phosphate (NADPH) levels early in life initiates the ageing processes; furthermore it is suggested that activities which raise NADPH levels delay these processes, for example CR (Brandes, *et. al.*, 2013).

Notably, modulation of the Nicotinamide adenine dinucleotide (NADH) precursor Nicotinamide (NAM) *in vivo*, leads to an increase in healthspan and lifespan in mice (Schmeisser, *et. al.*, 2013). There have been a few reports which indicate that ROS production increases with age in various mammalian tissues; for example the brain, liver and heart of rats (Sohal, *et. al.*, 1990; Sohal and Brunk, 1992) and the brain, kidney and heart of mice (Sohal, *et. al.*, 1994). Furthermore it has been demonstrated that redox couple ratios (e.g. $NADH/NAD^+$ and $NADHP/NADP^+$) become more oxidising with age (Noy, *et. al.*, 1985). Interestingly, it has been demonstrated recently that phosphate homeostasis plays a role in healthspan (Stenvinkel and Larsson, 2013). This role appears to be substantial and is supported by numerous systems within the organism to maintain this homeostasis, e.g. FGF-23, FGF-7 and MEPE; as well as protective mechanisms to prevent phosphate toxicity, including parathyroid hormone (White, *et. al.*, 2006). Phosphate is a critical post-translational modification for many proteins which can effectively activate many enzymatic functions and an imbalance in levels can lead to a severe disruption of cellular function. Hyperphosphatemia is most commonly associated with chronic kidney disease, where it is considered a cardiovascular risk factor due to vascular calcification (Hruska, *et. al.*, 2008).

1.3.2.3 Cross-linking theory

First suggested by Johan Bjorksten in 1942, the cross-linking theory suggests that glycation interactions between proteins and lipids in the

presence of excess glucose, particularly proteins like collagen, disrupts the normal function of these proteins resulting in accelerated ageing (Bjorksten, 1968). The accelerated ageing seen in diabetics appears to support this particular theory. Further evidence has been presented that chemical crosslinking agents can be used to alter proteins from young human brains to make them resemble those found in age advanced samples (Bjorksten and Tenhu, 1990).

The evidence for this theory is rather limited and it may be considered more of a niche theory. This theory still fits comfortably into the premise that ageing is a result of a general accumulation of damage throughout the lifespan and that crosslinking is one form of that damage.

1.3.2.4 Free radicals theory

The free radicals theory is built on the observations of Gerschman et al. who demonstrated the toxic effects of ROS (Gerschman, *et. al.*, 1954). The theory is based on the principal that free ROS, produced as a result of normal metabolism, cause damage to macromolecules, organelles, cell membranes and DNA/RNA; it postulates that the process of ageing is a direct result of the cumulative damage that ROS inflict over time (Balaban, *et. al.*, 2005; Harman, 1956). There is a robust and substantial literature supporting this effect (Beckman and Ames, 1998). Furthermore, ROS signalling has emerged as a key moderator of both cellular senescence and whole organism ageing (Afanas'ev, 2010). However, there is some evidence that low-levels of ROS may have a positive influence on lifespan, derived from observations on C. elegans involving the consumption of a known chemical ROS generator, juglone (5-Hydroxy-1,4-naphthalenedione) (Heidler, *et. al.*, 2009). The addition of 2-Deoxyglucose, which effectively blocks glycolysis and glucose metabolism (Sols and Crane, 1954), increases mitochondrial respiration and ultimately results in an increase in ROS and extends lifespan. The addition of an antioxidant along with the 2-Deoxyglucose removes this life extending phenomenon (Schulz, *et. al.*, 2007). It should be noted that an alternative hypothesis, involving acetate toxicity and pH resulting in ageing as

opposed to oxidation, at least in yeast (Burtner, *et. al.*, 2009). Overall, the influence of ROS on ageing is no longer disputed, however whether the damage caused by high levels of them is a result or a process of ageing is still debated (Salmon, *et. al.*, 2010).

1.3.2.5 Disposable soma theory

The disposable soma theory takes Weissman's stipulation that mechanical and/or chemical damage could be repaired to a high degree of fidelity (Weissman, 1889) and balances it with energy requirements. Basically, the repair of such damage requires energy and this demand for energy must be balanced with other organismal requirements; ultimately this leads to compromises between repair mechanisms and lifespan, growth or reproductive ability (Kirkwood, 2005). This is supported by observations indicating decreased fertility/growth is found in many long-lived mutant organisms (Longo and Finch, 2003). This has led to the idea that damage repair is balanced with other needs, therefore the need for extensive repairs may lead to a lack of fidelity due to insufficient energy availability. Seemingly at odds with this theory is the discovery that CR increases lifespan in a broad range of organisms (Mitteldorf, 2001). However, it is also notable that CR restricts growth and reduces energy investment in reproductive capability; this may ultimately lead to an increase in the level of energy available for repair and maintenance mechanisms (Mitteldorf, 2001; Shanley and Kirkwood, 2000). CR also facilitates a more efficient state of energy production and hence lowers the rate of ROS production during metabolism, thus mitigating ROS damage over time.

1.3.2.6 Error catastrophe theory

This theory was proposed and developed by Leslie Orgel (Orgel, 1963; Orgel, 1970; Orgel, 1973) and covers the DNA damage that occurs in every organism due to replication and environmental insult (Saul and Ames, 1986). Despite extremely accurate and effective repair and protection mechanisms some damage occurs that is not repaired or

replaced. These mutations accumulate at an increasing rate with age resulting in loss of function and cell death. This forms the basis of this particular theory where these errors accumulate over time resulting in the characteristics and pathologies we associate with ageing. Furthermore, the accumulation of these errors would further restrict the repair of future errors resulting in an exponential increase in error occurrence and accumulation. This theory has been analysed and supported by mathematical models which suggest that the accumulation of errors below a specific threshold would be dealt with by the systems in place; however upon reaching and broaching the threshold errors would begin to accumulate ultimately resulting in an error catastrophe (Goel and Ycas, 1976).

1.3.3 Post mitotic cells and ageing

Post mitotic cells, by their very definition, manifest ageing differently than mitotic cells. They already exhibit some of the conditions considered to be senescent. In fact, many consider the process of ageing to be mainly focussed on the post-mitotic tissues, particularly the cardiac and neural tissues. In these cells, which don't actively divide and thus are incapable of replacing or diluting damaged tissues, there is believed to be an accumulation of detritus (damaged biological structures) (Terman, *et. al.*, 2006). Furthermore, post mitotic tissues like neurons and cardiac myocytes have higher energy requirements (Grote, 1989); this in turn leads to greater levels of oxygen metabolism and ROS production. The idea that metabolism is at the centre of ageing is supported by data which suggests that mitochondrial function declines with age (Byrne, *et. al.*, 1991). This is consistent with the mitochondrial environment being more prone to mutation due to the high local levels of ROS, less efficient DNA repair and having a high turnover (Byrne, *et. al.*, 1991). When ROS accumulation was investigated, it was demonstrated that this increase was not associated with disruption of oxygen metabolism in the mitochondria using a mutant mouse model (Trifunovic, *et. al.*, 2004). In fact these mutant mice demonstrated an increased ageing phenomenon without any associated increase in ROS production or oxidative stress, potentially indicating that

disruption of metabolism is enough to induce an increased ageing phenotype (Trifunovic, *et. al.*, 2005). This further supports the idea that it is accumulation of damaged structures which results in ageing; this could be a result of ROS or translation/transcription issues which would include DNA damage.

Another important component of this system is phosphate equilibrium. Phosphate is a critical component of the cellular balance, required for functions ranging from intracellular/extracellular signalling, lipid biosynthesis and the formation and maintenance of DNA both directly and indirectly (Bergwitz, *et. al.*, 2013). In mammals levels of phosphate in the blood and retention/excretion of phosphate is tightly regulated by Fibroblast growth factor 23 (FGF23), parathyroid hormone and 1,25-dihydroxy vitamin D ($1,25(OH)_2D$) (Bergwitz and Juppner, 2011; Bringhurst and Leder, 2006). Disruption of these mechanisms has been demonstrated to result in severe human disease (Bringhurst and Leder, 2006). Knockout *Fgf23* mice have drastic growth retardation and abnormal bone formation in addition to a greatly reduced lifespan (Shimada, *et. al.*, 2004). The toxicity of high phosphate levels (hyperphosphatemia) has been demonstrated by knockout mouse models for klotho, these mice have a shortened lifespan and show signs of premature ageing (Ohnishi and Razzaque, 2010). Furthermore, when this knockout was combined with a sodium-phosphate cotransporter knockout the premature ageing effects were ameliorated; feeding these mice a phosphate rich diet again reduced lifespan and induced a similar premature ageing phenotype (Ohnishi and Razzaque, 2010). Hyperphosphatemia is most commonly seen in patients with chronic kidney disease (CKD), where serum phosphate levels can be predictive of outcome (Razzaque, 2009). Similarly, hypophosphatemia has severe consequences, most commonly seen as a result of cancer or malnutrition this too can have lifespan reducing consequences (Bergwitz and Juppner, 2009).

Phosphate is so critical to cellular functions that it is stored, as poly P chains, in both bacterial and mammalian cells (Docampo, *et. al.*, 2005). In mammals the concentration of poly P can reach 1.5μM under normal

physiological conditions (Kumble and Kornberg, 1995). These intracellular stores of phosphate appear to stimulate the activity of mTOR, which is a critical component of the regulation system for cell growth and proliferation (Schmelzle and Hall, 2000). The importance of phosphate balance in the control of redox has been established through the NADH/NADPH mechanism which is essential not only as a signalling mechanism, but also as a form of resistance to oxidative stress. This is evidenced by the lifespan promoting effects of glucose-6-phosphate dehydrogenase (G6PD) a critical enzyme in NADPH biosynthesis (Legan, *et. al.*, 2008). The combination of these factors indicates that phosphate levels and regulation are critical for both energy production and usage, which is evident in the consequences of its disruption. Furthermore, this suggests that phosphate regulation may also contribute significantly to not only the generation of ROS but also to ageing related damage accumulation more directly.

Despite apparent evidence that ROS itself is not responsible for ageing, human studies have demonstrated a significant increase in ROS production from 40 years old upwards; thus demonstrating an association between increased ROS and age. It has been suggested that this increase may result from an up-regulated inflammatory response related to age. Increases in ROS are associated with inflammation and the destruction of healthy tissue (Winrow, *et. al.*, 1993); this is also part of a positive feedback loop where inflammation promotes ROS production which further increases inflammation associated gene transcription (Conner and Grisham, 1996). Although, there is some debate as to which comes first as ROS damage has been shown to initiate the inflammatory response which leads to ageing (Chung, *et. al.*, 2001). Alternatively, ageing may be associated with an age-related reduction in antioxidant defensive mechanisms, which gives some credence to the programmed theories of ageing.

1.3.4 Combined theories

Although no official combined theories have been presented, it is rapidly becoming the consensus that most if not all of these theories have

merit and that the true process of ageing is likely to be an amalgamation of these ideas. Some researchers have attempted to unify these theories under other auspices, for example a reduction in bioenergetics as we age, driven by the mitochondria, has been suggested as the central tenet for biological ageing (Trubitsyn, 2012). However, this particular theory states that the reduction in bioenergetics is programmed but does not address the possibility that this programmed reduction is a result of ageing or progressive damage.

The most successful combined theory to date is the MTR theory (mitochondrion, telomere nucleo-protein complexes and the control of ribosome biosynthesis) (Shiels and Davies, 2004), which effectively integrates energy production/usage with damage detection and responses. This covers three critical areas of ageing the ability to detect and respond to oxidative damage, the production of energy (and thus ROS) and the measurement of energy usage. The Sirtuin family of proteins are critical regulators of all three components of the MTR making them ideal markers. The demonstrable roles for the various Sirtuins in CR and enhancement of lifespan and healthspan add further credence to this theory. Furthermore, the AGEMAP study demonstrated that a geneset based around the NADH dehydrogenase system, which is related to mitochondrial and Sirtuin function, is differentially expressed in accordance with age this supports the mitochondrial arm of the MTR theory. Providing support for the ribosomal arm of the MTR theory is the discovery that gene sets related to the function of the ribosome also demonstrate age-related changes in the mouse model (Zahn, *et. al.*, 2007). Many of the genes found in the ageing databases also have functions related to the MTR trinity either directly or indirectly.

The association of specific genes and pathways with longevity or lifespan across multiple species strongly indicates that there is a genetic (or programmed) component. Likewise, the evidence for the influence of diet, toxins and oxidative stress in terms of longevity is similarly irrefutable. This leads to the optimal conclusion that ageing is programmed but heavily influenced by our environment and the damage/stress we undergo; as well as by internal mechanisms including

the action of the endocrine and immunological systems. This combined approach also helps to explain the discrepancies in both types of theory. In fact, the main dispute between these theories is whether programmed deterioration leads to increased susceptibility to damage (or wear and tear) or whether this damage leads to the activation/silencing of genes resulting in the consequences of ageing; i.e. which comes first.

1.4 Diseases of ageing

There are many diseases associated with ageing, some obvious and well known and others which are not generally considered as being so closely linked with ageing. However, when analysing the effects of cellular senescence and the molecules involved in it, it becomes obvious that diseases like cancer are closely linked to the ageing process. Some of the most well known and high incidence diseases related to advanced ageing now have well documented connections with the processes and molecules involved in the ageing process. The connections of these diseases with ageing is strengthened by the influence each has on the prevalence of others, most are considered co-morbidities with at least one or two other diseases of ageing. In fact, most of these ageing associated diseases share a low grade pro-inflammatory status (Olivieri, *et. al.*, 2013). Furthermore, age associated diseases may also be related to early life environmental influences, in particular extreme CR (Tarry-Adkins and Ozanne, 2014). The main diseases of ageing are outlined in Table 1.1 below.

Table 1.1 Age related diseases. The major age related diseases are described and their links to ageing presented here.

Disease	Description and aetiology
Arthritis	There are over 100 variations of this inflammatory joint disorder, although these tend to fall into three categories: auto-immune, infection and degenerative. It is the latter of these that is related directly to advanced ageing, however auto-immune forms have late onset and are also considered related to age. Common symptoms for all forms of arthritis include pain, swelling and stiffness of the affected joints. There are several forms of arthritis, most of which increase with age indicating that this is a prominent disease of ageing and consequently is a major issue for the provision of healthcare in an ageing society. Osteoarthritis: Obesity and age are the most common risk factors (Arden and Nevitt, 2006); may be a disease of "wear and tear". There are differences in occurrence between sexes and races (Arden and Nevitt, 2006); Localised premature ageing induced by oxidative stress and resulting in cellular senescence and/or apoptosis is possibly at the core of the aetiology (Loeser, 2009). Rheumatoid arthritis (RA): an auto-immune disorder which is reported to be far more common in women than men and demonstrates earlier onset in females. An early study suggested that its occurrence is equal in men and women, but men were far more likely to enter remission while women are more likely to develop chronic ongoing disease (Otten and Boerma, 1959). Other studies in the same time period reported ratios of nearly 2 to 3 times as many females to men (Rotes-Querol and Roig-Escofet, 1968) (Jacoby, *et. al.*, 1973) The occurrence and symptoms areincreased due to immunosenescence (Goronzy, *et. al.*, 2013; Weyand, *et. al.*, 2013). In particular, senescence in T-cells has been linked to chronic tissue inflammation similar to that seen in RA

	(Weyand, *et. al.*, 2013). Additionally, increased levels of $CD56^{+}$ monocytes have been demonstrated to increase with age; as well as being significantly elevated in RA, this was particularly evident in individuals under 40 years old (Krasselt, *et. al.*, 2013). Furthermore, patients with RA have been demonstrated to have defects in DNA repair (Hohensinner, *et. al.*, 2012), including telomerase and the kinase Ataxia telangiectasia mutated (ATM) which is involved in repair of double stranded DNA breaks; these are also associated with premature ageing (Hohensinner, *et. al.*, 2012). Gout induced arthritis: formation of urate crystals which cause inflammation in the joints, particularly in the western nations. This form of arthritis is related to age (Hootman and Helmick, 2006), lifestyle and metabolic syndrome (also age-related) which individually or in combination promote hyperuricemia. The involvement of glutamate transporters in the aetiology of this disorder (Vitart, *et. al.*, 2008) heavily implicates the metabolic system as a causative element; in turn this directly relates this disorder to age. The metabolic system has been shown to deteriorate with advancing age, particularly when put under great stress due to lifestyle choices, in this case high lifetime fructose intake may cause aberrant expression of the glutamate receptors, particularly for a specific genotypic variant (VanItallie, 2010).
Cardiovascular Disease (CVD)	Increased risk of CVD associated with increased age. Not only is CVD directly related to ageing (Finegold, *et. al.*, 2012) its incidence is also influenced by age related risk factors including metabolic syndrome (MetS) (Mazza and Morley, 2007), type II diabetes mellitus (T2D) (Snell-Bergeon, *et. al.*, 2013) and hypertension (Castelli, 1996). Furthermore key BoA and senescence markers are directly related to increased risk of CVD, for example the CDKN2A/2B locus (Congrains, *et. al.*, 2011; Landman, *et. al.*, 2012). It is also interesting to note that CVD is a common manifestation of the premature ageing phenotype seen in progeroid syndromes, for

	example Werner syndrome (WS) (Ishida, *et. al.*, 2013).
Cancer	Cancer is now widely accepted as a disease of ageing and the processes of tumourigenesis are very closely related to cellular senescence, the process behind ageing. This has been clearly demonstrated by a mouse model that is remarkably free of tumours but demonstrates extreme signs of advanced ageing (Tyner, *et. al.*, 2002). Furthermore, the involvement of critical cellular senescence markers in tumourigenesis provides further proof of the links between senescence and tumourigenesis. For example, p53 (Zuckerman, *et. al.*, 2009) is heavily involved in tumourigenesis and senescence, as is $p16^{INK4a}$ (Fung, *et. al.*, 2013), Rb (Sperka, *et. al.*, 2012) and p21 (Warfel and El-Deiry, 2012).
Cataracts	Symptoms include difficulty with accurate vision (recognition of faces or text), problems distinguishing colours and contrasts and an inability to cope with glare (Allen and Vasavada, 2006). Although cataracts can arise from genetic abnormalities (Chang, *et. al.*, 2013; Malt, *et. al.*, 2013) or heat/electrical damage (Reddy, 1999), age is considered the most common cause of this disease (Courtney, 1992). Advancing age results in a reduction in repair and protective mechanisms which defend against environmental insults to which the eye is exposed, for example UV radiation (Sliney, 2001). Cataracts can also be induced by other forms of radiation including microwave and ionising (Lipman, *et. al.*, 1988), again incidence of these is enhanced by slower repair and protection mechanisms characteristic of advancing age.
Diabetes Mellitus type II (T2D)	T2D is associated with ageing and poor diet, characterised by insulin resistance. Symptoms often develop gradually, in fact some cases do not demonstrate any symptoms in the early stages. Classic symptoms include frequent urination (polyuria), excessive thirst (polydipsia), weight loss and hunger (polyphagia), although these are often far subtler and develop more slowly than type 1 diabetes. T2D is the most prominent form accounting

	for approximately 90% of cases and the world has experienced a massive surge in the diagnosis of T2D in the past three decades (Smyth and Heron, 2006). Increases the risks for several other age-related diseases including CVD. MetS is closely related to T2D with both being considered a risk factor for each other (Mazza and Morley, 2007). T2D is also directly linked with the key ageing/senescence biomarkers encoded by the *CDKN2A* and *cyclin dependent kinase inhibitor 2B* (*CDKN2B*) loci (Landman, *et. al.*, 2012). Mouse models have also revealed that animals subjected to hyperglycaemia exhibit early senescence in endothelial cells, this was accompanied by a reduction in expression of all seven Sirtuins; diabetic mice exhibited similar changes with increased senescence in renal glomerulus and retinal blood vessels with a reduction in Sirt1 levels (Mortuza, *et. al.*, 2013).
Hypertension	Hypertension is a risk factor for many diseases and disorders, particularly for other diseases of ageing including CVD (Castelli, 1996) and MetS (Mazza and Morley, 2007). This increase in blood pressure is linked to the reduced elasticity in blood vessels that comes with advancing age (Rammos, *et. al.*, 2014; Sherratt, 2014). Unlike other diseases of ageing, hypertension appears to induce cellular senescence via the expression of $p16^{INK4a}$ rather than be caused or worsened by it (Westhoff, *et. al.*, 2008). This indicates that hypertension may accelerate ageing and would explain its role in the promotion of so many other age related disorders. Furthermore, genetic variations (Single nucleotide polymorphisms) at the *CDKN2A*/*CDKN2B* locus are linked to the incidence of cerebrovascular disease (stroke) (Wahlstrand, *et. al.*, 2009).
Metabolic syndrome (MetS)	Metabolic syndrome (MetS) consists of five chronic risk factors: central (visceral) obesity, hypertension, aberrant glucose metabolism (insulin resistance), proinflammatory state, abnormal lipid balance and prothrombotic state (Alberti, *et. al.*, 2006). Originally it also included sleep apnoea as well (Morley and

	Sinclair, 2009). The incidence of MetS rises rapidly with age with more than 40% of individuals over 60 years old exhibiting the symptoms (Mazza and Morley, 2007). Many factors influence the occurrence of MetS, however the most prominent risk factor appears to be abdominal obesity (Setayeshgar, *et. al.*, 2012). All of the other risk factors increase with age (Garner and Rubio-Ruiz, 2012). Age decreases the influence of central obesity as a risk factor for developing MetS (Okazawa, *et. al.*, 2013). MetS is a critical risk factor for several age related diseases including CVD (Aoqui, *et. al.*, 2014) and diabetes (Mazza and Morley, 2007). In fact some people believe that MetS is the precursor to CVD and non-alcoholic fatty liver disease (Monteiro, *et. al.*, 2014).
Osteoporosis	A progressive bone disorder characterised by reductions in bone mass/density, which ultimately leads to an increased risk of fracture. In general these fractures occur in locations that contain higher levels of cancellous or trabecular bone (also known as spongy bone), these are subject to higher turnover when bone remodelling is imbalanced as it is in osteoporosis. These sites depend upon the trabecular bone for strength, they develop microfractures which severly weaken the bones (Abed and Moreau, 2009; Chen and Kubo, 2014). These areas include the vertebrae (Old and Calvert, 2004), ribs (Prior, *et. al.*, 2014), hips (Mpalaris, *et. al.*, 2015) and wrists (Cummings, *et. al.*, 1985). It is generally diagnosed by measuring bone mineral density. The incidence and severity of this disorder increases with age, one proposed form (primary type 2) is suggested to occur in the over 75 population and is referred to as senile osteoporosis (Riggs, *et. al.*, 1982). The other proposed form is commonly known as postmenopausal osteoporosis or primary type 1; this has been linked to the reduction of hormones that occurs with menopause in women (Riggs, *et. al.*, 1982). However, the two forms hypothesis (Riggs, *et. al.*, 1982) has now been challenged with the general consensus being that osteoporosis is a continual process with several pathogenetic mechanisms that cause deterioration of the bones (Raisz, 2005). In women

	osteoporosis can be linked to oestrogen deficiency and in men it can be caused by a reduction in testosterone. Secondary osteoporosis can occur at any age and equally afflicts men and women; however this form is linked to pre-existing medical issues or prolonged use of glucocorticoids. A genetic element is also present with an inheritable increased risk (Raisz, 2005). Particularly, polymorphisms in oestrogen receptor alpha have been linked to risk of developing osteoporosis (Lee, *et. al.*, 2003). Like many other age related diseases, osteoporosis has well established links with metabolic dysfunction (Hu and Liu, 2014). The problems of bone fragility in age related osteoporosis are compounded by other age related problems including movement disorders (e.g. Parkinson's disease [PD]), eyesight problems, sarcopenia (loss of skeletal muscle) and dementia which may result in a greater propensity to fall.
Progeroid syndromes	Progeroid syndromes are a rare class of genetic disorders which present with the symptoms of ageing, i.e. they are diseases of accelerated ageing caused by a genetic defect. These defects are thus far believed to arise from mutations of single genes (Navarro, *et. al.*, 2006), usually involved in DNA repair mechanisms (Mohaghegh and Hickson, 2001) or nuclear stability/chromatin structures (McClintock, *et. al.*, 2007). This class of disease includes the familial forms of AD and PD, WS, Bloom syndrome (BS), Cockayne syndrome (CS), and Hutchinson-Gilford progeria syndrome (HGPS). All of these disorders have the commonality of resulting in a reduced lifespan (Navarro, *et. al.*, 2006); in some cases this is extreme for example HGPS ordinarily results from novel mutations as individuals rarely reach reproductive age. Furthermore, some of the ageing mechanisms seen in other age-related diseases mimic those seen in progerias, for example the laminopathy seen in HGPS is reminiscent of CKD (Shanahan, 2013).

1.5 The Mammalian Brain

1.5.1 The Brain and its Functions

The human brain has a similar structure to that of other mammals, although not the largest brain found in mammals in absolute terms it is the largest relative to the size of the body. In fact in terms of relative size the human brain is almost three times the size of our closest relative, the chimpanzee. The larger brain capacity appears to be mostly focussed in the cerebral cortex and particularly the frontal lobes, which are responsible for working memory (short term memory), problem solving and conscious thought. However, this does not account for the relative difference in size compared with the great apes, as research has demonstrated that they have a similarly large frontal cortex (Semendeferi, *et. al.*, 2002).

Evolution has provided the brain with highly effective and substantive protection in the form of the thick skull bones, suspension in cerebrospinal fluid and a blood-brain barrier; but despite this the brain is still susceptible to disease and damage. The anatomy of the human brain is extremely complex and research into the intricacies of its function and structure is still continuing. However, a great deal is known and the anatomy of the cerebrum of the brain has been segregated into six main areas, or lobes (Ribas, 2010; Strominger, *et. al.*, 2012) (four of which are shown in Figure 1.1) with each lobe having specific functions associated with it. The frontal lobe (shown in red - Figure 1.1) is primarily associated with cognitive abilities and conscious thought, which includes short term memory, planning, motivation, attention and ultimately action selection (Coutlee and Huettel, 2011). The temporal lobe (shown in yellow – Figure 1.1) is believed to play an important role in the organisation of sensory input, language comprehension, memories (particularly visual) and emotion. The parietal lobe (shown in green – Figure 1.1) is mostly associated with information processing, spatial orientation, speech, recognition and perception of stimuli. The occipital lobe (shown in blue – Figure 1.1) is associated with the sense of sight (Goodale and Milner, 1992), this is also demonstrated by disruptions in visual acuity associated with

damage to the occipital lobe (Zihl, *et. al.*, 1983). The limbic lobe is found on the interior of the brain and has borders with the frontal, parietal and temporal lobes, it is responsible for emotion and longer term memory functions. The insular lobe is also found on the interior of the brain between the frontal and temporal lobes and is believed to house the centres for pain and some other sensory systems.

Although each of these lobes has individual processes attributed to it the brain functions as one, making decisions, actions, thoughts and memories by interaction between lobes, for example an internal thought train is believed to result from cooperation between the frontal and parietal lobes (Smallwood, *et. al.*, 2012) and memory in social cognition involves interactions between the hippocampus, temporal and parietal lobes (Spreng and Mar, 2010). This interactivity could also be attributed to the arbitrary method of defining the four major lobes by the bones of the skull which they underlie. Despite this major connectivity and crosstalk the metabolic state, and ageing processes, proceed at different rates and in different manners between regions of the brain. This is most apparent when looking at functional areas of the brain.

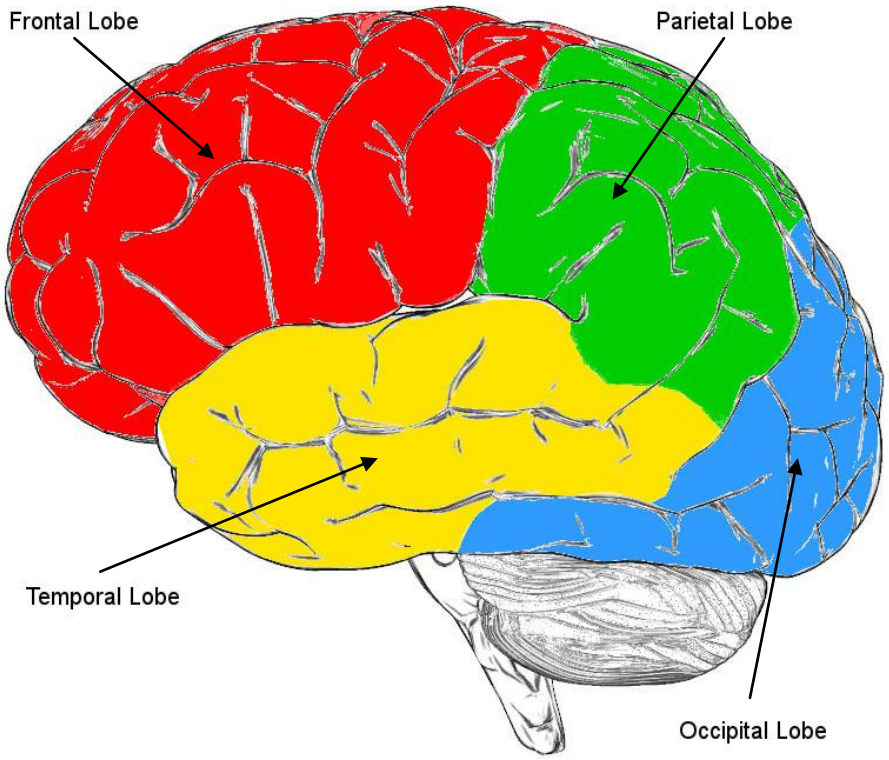


Figure 1.1 The General Anatomy of the Human Brain. Diagram of the human brain, with four of the six lobes of the brain highlighted: the frontal lobe (red), parietal lobe (green), temporal lobe (yellow) and occipital lobe (blue).

The brain and spinal cord are separated into three distinct categorical areas (Figure 1.2); the first is the forebrain which encompasses the cerebrum and internal structures including thalamus and hypothalamus. The forebrain is responsible for most of the functions of the brain, particularly conscious thought, memory, data collation and interpretation etc. The midbrain connects the forebrain and hindbrain; together with the hindbrain it forms the brainstem. This region of the brain is involved in auditory and visual responses and motor functions. The hindbrain extends from the spinal cord and includes the pons, cerebellum and medulla oblongata resulting in control of balance, equilibrium, movement coordination and autonomic functions (i.e. breathing, circulation and digestion).

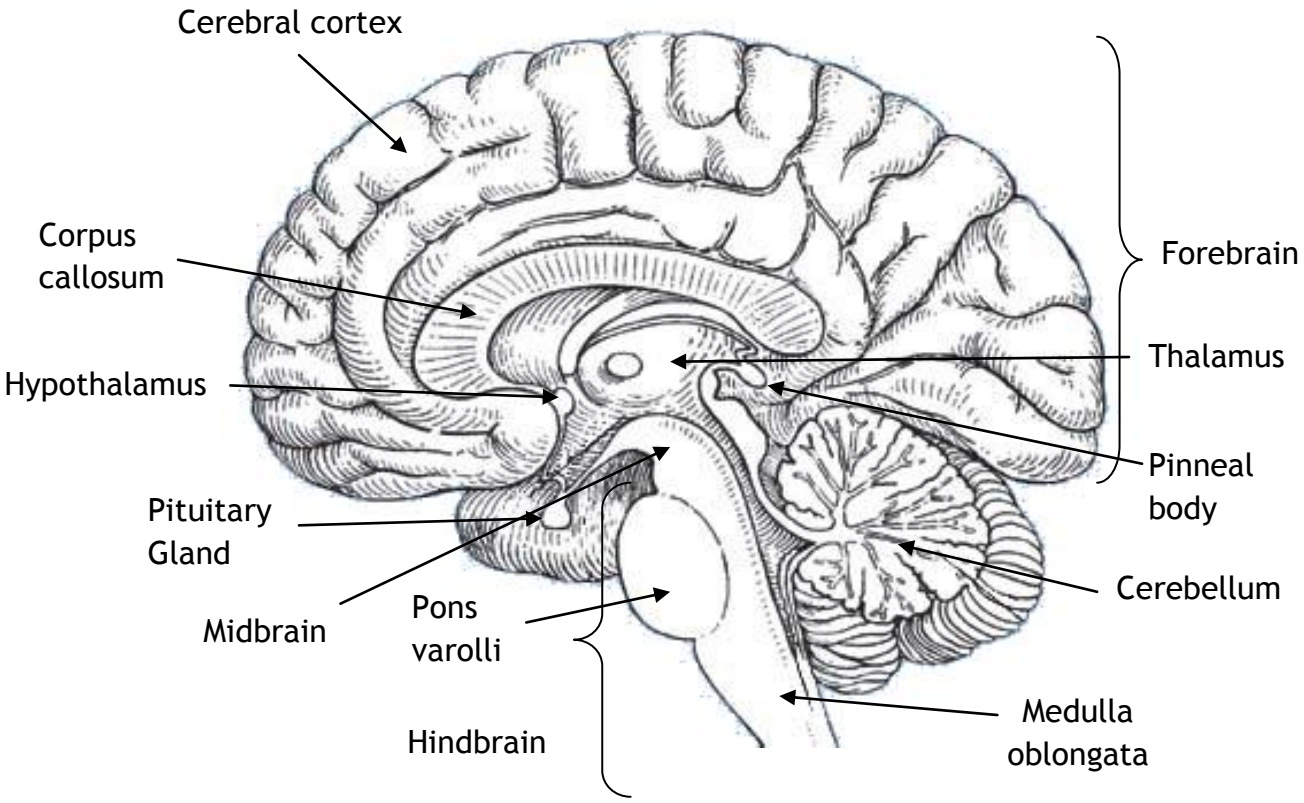


Figure 1.2 Major Structural Features of the Human Brain. A cross-sectional diagrammatic representation of the human brain divided on the sagital plane. The forebrain, midbrain and hindbrain areas are shown as well as several key components including the cerebellum and cerebral cortex.

1.5.1.1 The Cerebellum

The cerebellum (or little brain) so called for its similarity in appearance to the cerebral cortex, however the structure and function of this brain region is vastly different. It is located posterior in the cranium, beneath the cerebral cortex (Figure 1.2) and is a major component of the hindbrain. Its structure is similar to the bellows of an accordion, being a thin layer comprising a highly organised and structured arrangement of neurons, particularly Purkinje cells and granule cells, which is tightly

folded on itself. The cerebellum also contains stellate, basket and golgi neurons as well as mossy, climbing and parallel fibres (Ghez and Fahn, 1985).

The primary function of the cerebellum is believed to be the coordination of motor function; particularly it contributes to coordination, timing and accuracy by integrating signals from various parts of the CNS and PNS including the spinal cord and sensory systems (Fine, *et. al.*, 2002). In fact damage in this area of the brain results in problems with fine motor control, motor learning and balance (Fine, *et. al.*, 2002). However some evidence suggests it may be involved in personality, mood or psychosis. This is supported by links with the associative and paralimbic cortices (Schmahmann and Pandya, 1997) and also the prefrontal cortex (Ramnani, 2006); it appears that the non-motor functions of this area are similar to the motor functions in that it monitors signals for errors in thought (Schmahmann and Sherman, 1998). The "dysmetria of thought" postulated by Schmahmann (Schmahmann, 1998) and/or structural/functional disruption are believed to be involved in schizophrenia (Andreasen, *et. al.*, 1999; Reyes and Gordon, 1981; Tran, *et. al.*, 1998), depression (Pillay, *et. al.*, 1997; Videbech, *et. al.*, 2001) and bipolar disorder (Brambilla, *et. al.*, 2001; Kruger, *et. al.*, 2003). One study investigating damage within the cerebellum revealed associations with a variety of behavioural and personality problems (Wolf, *et. al.*, 2009).

1.5.1.2 The cerebral cortex

The cerebral cortex is the major component of the human brain (~40% of total mass) and constitutes the largest component of the forebrain; it forms the outermost layer of the cerebrum (Figure 1.2). It forms two cortices along the sagittal plane divided by the medial longitudinal fissure, dividing the two cerebral hemispheres. It is believed to be responsible for conscious thought, memory, perception, language skills, processing of sensory inputs and realisation of motor functions. It is divided into the neocortex, which is the major component of the cerebral

cortex in humans (~90%), and the allocortex (Strominger, *et. al.*, 2012). The neocortex in mammals comprises six individual cell layers labelled in roman numerals (I to VI) with the outermost layer being I (Lui, *et. al.*, 2011). This area is believed to be responsible for sensory perception, motor initiation, spatial awareness/reasoning, language skills and consciousness (Strominger, *et. al.*, 2012). The size, shape and general structure of the neocortex varies dramatically even within mammalian species with rodents demonstrating a small unfolded region and humans showing a much larger and highly folded neocortex (Herculano-Houzel, 2009). The neocortex mainly contains two types of neurons, pyramidal neurons (excitatory) and interneurons (inhibitory), in a 4:1 ratio respectively. In contrast to the neocortex the allocortex has 3-4 layers rather than 6. The hippocampus and olfactory system are the major components of this region (Strominger, *et. al.*, 2012).

The cerebral cortex region of the brain contains mostly grey matter; this is due to the high density of neuronal cell bodies, glial cells, capillaries and unmyelinated axons. This is in contrast to the white matter which is primarily composed of myelinated axons and few cell bodies. The primary excitatory neurotransmitter in the cerebral cortex is glutamate (Strominger, *et. al.*, 2012).

1.5.1.3 The basal ganglia

Found within the corpus callosum, the basal ganglia are nuclear complexes in the cerebrum and midbrain which consists of five discreet areas, all with unique structures and functions: the substantia nigra (SN), the ventral tegmental area (VTA), the striatum, the globus pallidus (GP) and the subthalmic nucleus (STN) (Strominger, *et. al.*, 2012). It has connections throughout the cerebral cortex which appear to be organised into discreet loops indicating a level of two-way communication that was unanticipated until recently (Middleton and Strick, 2000). These areas are collectively involved in somatomotor and motivation behaviours as well as adaptive behaviours, e.g. attention, learning, planning and memory (Reiner, *et. al.*, 1998). The basal ganglia can be further categorised into 3

distinct functional categories: a) the input nuclei which receive information from other areas of the brain and include the caudate nucleus, the putamen and the nucleus accumbens which are collectively known as the striatum; b) the intrinsic nuclei which form the intercommunication/processing link between the input nuclei and the output nuclei, consisting of the GP (lateral segment), the STN, the substantia nigra pars Compacta (SNC) and the VTA; c) the output nuclei which transmit inhibitory signals to other areas of the brain, these consist of the GP (medial segment), the substantia nigra pars reticulata (SNR) and the ventral pallidum (Strominger, *et. al.*, 2012). The major inputs are from the cerebral cortex and the thalamus using glutamate as a neurotransmitter (Strominger, *et. al.*, 2012).

The SN can be sub-divided into two regions the SNC and SNR. These areas use different neurotransmitters, dopamine (DA) for SNC and γ-amino-butyric acid (GABA) for SNR, and have different connectivity (Strominger, *et. al.*, 2012). The SN has a population of actively dividing progenitor cells which give rise to glial cells (Lie, *et. al.*, 2002). Interestingly, given the correct stimulation these cells appear to have the potential to differentiate into neurons (Lie, *et. al.*, 2002). The VTA is located medial to the SNC, uses the same neurotransmitter and appears to have a similar role (Strominger, *et. al.*, 2012).

The striatum, also referred to as the striate nucleus or the neostriatum, is the largest component of the basal ganglia consisting of the caudate nucleus and the putamen, collectively known as the dorsal striatum and also the nucleus accumbens (or ventral striatum) (Strominger, *et. al.*, 2012). It is located on the interior of the forebrain laterally to the thalamus. It has two major neuron populations: projection neurons and interneurons, projection neurons communicate out with the striatum, whereas the axons of interneurons remain within the striatum. The majority of neurons found in the striatum are medium spiny neurons (>90%) which use the neurotransmitter GABA (Kita and Kitai, 1988; Parent and Hazrati, 1995), but also carry DA receptors (Strominger, *et. al.*, 2012). It is the major receiving area (Parent, 1990) of the basal ganglia

with incoming connections from the three other areas of the basal ganglia. It receives dopaminergic connections from the SNC, and smaller connections from the GP and the STN. It also sends projections back out into the SNR and STN. These connections are mainly glutamatergic and come from many areas throughout the cerebral cortex (McGeorge and Faull, 1989).

The GP is also known as the pallidum or paleostriatum. This area consists of the Dorsal pallidum, containing medial (internal segment) and lateral (external segment) components, as well as the ventral pallidum (Strominger, *et. al.*, 2012). It maintains close ties with the SN and striatum (Smith and Villalba, 2008). It is part of the mechanism for regulating voluntary movements, evidenced by movement disorders which result from damage within this area, for example dystonia (Shakkottai, 2014); however deliberate creation of microlesions using implanted electrodes has been used to treat involuntary muscle movements, including dystonia and PD (Gross, *et. al.*, 1999; Singh, *et. al.*, 2012; Tisch, *et. al.*, 2007). Being largely inhibitory the main function of the GP is to provide regulation and fine control over the excitatory movement signals generated in the cerebellum. Other inputs come from the striatum and the collated data are transmitted to the thalamus (Strominger, *et. al.*, 2012).

The STN forms the major component of the subthalamus, located ventrally to the thalamus. It is primarily populated by long spiny dendrites which use glutamate as their neurotransmitter (Rafols and Fox, 1976); in humans there is a small percentage of interneurons which use GABA as a neurotransmitter. It receives excitatory signals from the somatosensory areas and the prefrontal and motor cortices of the cerebral cortex; it also receives inhibitory signals from the GP (Canteras, *et. al.*, 1990) and neuromodulatory inputs from the SNC via DA signalling (Cragg, *et. al.*, 2004). The STN has been implicated in obsessive compulsive disorder (OCD) and severe impulsive behaviour, it has also been suggested that stimulation of this region may be utilised as an alternative treatment for these problems (Mallet, *et. al.*, 2008).

1.5.1.4 Brainstem

The brainstem encompasses the midbrain to the medulla oblongata, including the pons (Figure 1.2). This area, with 10 of the 12 pairs of major cranial nerves, provides the main sensory and motor functions for the head and neck. Additionally, because of the inclusion of the medulla oblongata it is also responsible for many autonomic functions particularly respiratory and cardiac systems. It also regulates the sleep cycle and is a critical control point in the maintenance of consciousness.

The brainstem plays a major role in relaying information in both direction between the body and brain. It also plays a major role integrating and transmitting information for the autonomic functions, maintaining proper control of consciousness, awareness, pain sensitivity, respiratory and cardiovascular control. These integral roles underlie the potential disruption to bodily function due to damage within this region.

1.5.1.5 The differences between rat and human brains

There are several model species that can be used to study the mammalian brain, each with its own advantages/disadvantages (Clancy, *et. al.*, 2007). Studies have demonstrated that the early development of the brain is remarkably conserved in mammals (Finlay, *et. al.*, 2001). Furthermore, by using overlapping techniques, i.e. neuroinformatics, Carnegie stages and neuroanatomical comparisons an accurate mapping of development between several mammalian species has been established (Clancy, *et. al.*, 2007). The rat has emerged as a preferred species in many research laboratories for a few good reasons: 1) large litter sizes, 2) generally disease resistant, 3) easy to care for, 4) short gestation period (~22.5 days), and 5) well established neuroanatomy, neurophysiology and behavioural patterns (Clancy, *et. al.*, 2007). These reasons alone are enough to convince many researchers to utilise this as a model organism, further work which has mapped the development of rat brains onto the timescale for human brains (Bayer, *et. al.*, 1993) has added further to the appeal of this model organism.

There are some obvious and distinct differences in the appearance of rat brains when compared with human brains. Firstly, overall size the human brain is much larger, however all mammals have the same basic brain structures; therefore the only real differences between the brains of mammals are found in the size and proportion of each region. The proportional size of each region varies according to the mammal in question with larger olfactory areas found in rats than those seen in humans. Similarly, humans have a much larger cerebral cortex relatively speaking. Another major difference is found in the connection between the brain and the spinal cord due to the angular difference between standing upright and running on all fours. Overall the structure and function of the brain is very similar throughout mammals. Even in the motor cortex, which was thought to be much simpler in rats than the human equivalent, it has been demonstrated that the rat brain region is almost as complex both structurally and functionally (Smith and Alloway, 2013). This makes the rat an ideal model to study brain function.

1.5.2 Energy usage in the brain

The Brain is the most resource intensive part of the human body, utilising up to 20% of the body's resting energy production to maintain proper function (Rolfe and Brown, 1997), with action potentials (47%) and postsynaptic effects of glutamate (34%) accounting for the majority of energy usage (Attwell and Laughlin, 2001). Furthermore, the brain effectively singles out glucose as its source of energy sequestering around 65% of circulating glucose (Reinmuth, *et. al.*, 1965), while the rest of the body uses a combination of glucose, fats and proteins. The energy demand from the brain can drop up to 40% during deep sleep (Boyle, *et. al.*, 1994). However, energy requirement can dramatically increase during times of stress (Hitze, *et. al.*, 2010), even mild stress can increase the brain's requirements by 12% (Madsen, *et. al.*, 1995). The critical nature of the brain and its high energy demands are highlighted by its ability to switch energy sources during fasting and exercise, including the use of unusual energy sources, for example ketone bodies and lactate (Quistorff, *et. al.*,

2008; van Hall, *et. al.*, 2009), to ensure sufficient energy is made available to maintain brain function. This relative increase in energy demand for higher brain functions is believed to have been balanced by evolution in the form of a smaller gastro-intestinal tract, another high level metabolic tissue, which therefore requires a high quality diet to maintain (Foley and Lee, 1991). It is believed that higher brain function and the enhanced abilities it imparts were sufficient to provide the organism with access to a higher quality diet allowing it to maintain brain function at the expense of dietary processing capability. Ketone bodies have been shown to replace glucose as the brain's energy source during times of starvation (Davis and Tate, 2001; Owen, *et. al.*, 1967).

Of the 20% of total body energy and oxygen used by the brain 95% is consumed within the grey matter, indicating the extremely high energy demands of active neurons (Miller, *et. al.*, 1980). The mechanism by which the brain sequesters additional energy resources has been dubbed "brain pull" (Peters and Langemann, 2009). Two mechanisms have been suggested by which this energy pull occurs; the first involves the sympatho-nervous system (SNS) and the hypothalamus-pituitary-adrenal axis suppressing *de* novo insulin secretion. This action termed cerebral insulin suppression (CIS) results in insulin dependent uptake of glucose in the body periphery via GLUT4 glucose transporter being effectively suspended, thus increasing the available glucose in the blood which can cross the blood-brain barrier via the insulin independent GLUT1 glucose transporter. CIS has been shown to become active in a broad range of situations from circumstantial e.g. CR (Peters, *et. al.*, 2011), sleep deprivation (Spiegel, *et. al.*, 1999) and hypoxia (Chen, *et. al.*, 2007) to damage and disease states e.g. burns (Allison, *et. al.*, 1968), haemorrhage (Cerchio, *et. al.*, 1971; Kruyt, *et. al.*, 2011), stroke (Harada, *et. al.*, 2009; McPherson, *et. al.*, 2009) and heart attacks (Taylor, *et. al.*, 1969). The second mechanism is known as cerebral lactate demand (CLD), where lactate is used to satisfy increased energy demands during times of stress (Kubera, *et. al.*, 2012). SNS activation has been shown to increase lactate production in muscles (Meyer, *et. al.*, 2005), some studies have demonstrated a 17% reduction in glucose uptake in the brain as a result of increased lactate levels (Smith, *et. al.*, 2003). In

fact the availability of energy for use in the brain has been linked to brain size and thus intelligence (Aiello, 1995). In order to ensure swift and adequate supply of energy to the brain a suitably strong supply chain is required. Thus supply to the brain accounts for a large proportion of blood flow and oxygen usage as well. This places additional pressure on the mitochondria to produce larger levels of energy, as a result disruption or dysfunction in these organelles results in the manifestation of diseases and disorders, for example HD (Yano, *et. al.*, 2014), migraine attacks (Colombo, *et. al.*, 2014) and psychiatric disorders (Streck, *et. al.*, 2014). Mitochondrial dysfunction in combination with ROS damage (oxidative stress) has been postulated to play a major role in Amyotrophic lateral sclerosis (ALS), shown in mouse models (Pollari, *et. al.*, 2014).

1.6 Ageing in the brain

The brain, as with all organs in the body demonstrates signs of ageing, specifically reduced functional capacity associated with old age. This can be seen at the physiological and psychological level with obvious outward symptoms. The situation at the biological and chemical level is far more complicated, and unique. The brain still possesses some ability to repair and replace damaged cells. There are two areas of constitutive neurogenesis, the olfactory bulb and the hippocampal dentate gyrus (Emsley, *et. al.*, 2005). Additionally, there are several areas that have been demonstrated to be capable of neurogenesis, although in most cases this has not been demonstrated under normal conditions *in vivo* or in humans. These areas include the subventricular zone (SVZ) (Gritti, *et. al.*, 1996; Lois and Alvarez-Buylla, 1993), striatum (Palmer, *et. al.*, 1995; Reynolds and Weiss, 1992), cortex (Reynolds and Hardy, 1997), retina (Turner and Cepko, 1987), septum (Palmer, *et. al.*, 1995) and tectum (Galileo, *et. al.*, 1990).

Changes in brain biology and chemistry have been associated with the ageing process; although research on the ageing process in healthy brains is limited, there has been a plethora of research into neurodegenerative disorders and their effect on brain morphology. In many cases these neurodegenerative diseases are seen as a form of accelerated ageing, with

one particular area or cell type demonstrating more senescence or atrophy than others. Some magnetic resonance imaging based studies have demonstrated an overall reduction in long range connections, particularly the fronto-parietal and fronto-occipital links and higher connectivity in short range links, for example within the frontal, parietal and occipital lobes (Sala-Llonch, *et. al.*, 2014). Furthermore, there appears to be a general increase in the activity seen in the prefrontal cortex (Turner and Spreng, 2011), which may result from a progressive inefficiency seen in neural circuits with age placing an increased load on the frontal lobe (Park and Reuter-Lorenz, 2009). Socio-economic status has also been shown to be related to both BoA (Shiels, *et. al.*, 2011) and cerebellar volume (Cavanagh, *et. al.*, 2013). Additionally, other age related disease markers, particularly for CVD, have also been shown to relate to. Age related changes in connectivity between regions are seen throughout the brain with some areas increasing and others decreasing (Ferreira and Busatto, 2013). Whilst some neurochemical changes occur within the brain during ageing (Strong, 1998), the most significant changes appear to be related to neuronal and axonal loss. In fact, many of the neurochemical changes seen may be a direct result of these losses.

General studies have demonstrated that there are age related reductions in regional cerebral volumes (Raz, *et. al.*, 2005). However, these reductions do not appear to be uniform and can vary greatly from area to area, with some areas shrinking by up to 1% per year while others remain constant for most of the person's lifespan only to shrink rapidly in the latter stages of the life-span (Raz and Rodrigue, 2006). For example, studies have demonstrated that the frontal lobes of the cerebral cortex reach maturity around 20 years of age in humans (Giedd, *et. al.*, 1999) and in healthy, older individuals appear to shrink on average 0.5% per year (Coffey, *et. al.*, 1992; Fjell, *et. al.*, 2009). This age related volume loss appears to be highly variable and has been demonstrated in the cerebral cortex (Bigler and Raz, 1996; Platt and Haug, 1989) and cerebellum (Ellis, 1920; Sullivan, *et. al.*, 2000). Despite no apparent overall shrinkage in the brainstem (Bigler and Raz, 1996), the midbrain region also appears to show age-related volume loss (Doraiswamy, *et. al.*, 1992; Luft, *et. al.*, 1999; Weis, *et. al.*, 1993).

However, it has more recently demonstrated that the age related shrinkage in the cerebellum is also highly variable according to the region examined (Luft, *et. al.*, 1999). Jernigan *et. al.* demonstrated that there are marked differences in the rate of loss, or shrinkage, in subcortical structures as well; in particular the greatest losses were seen in the hippocampus followed by the caudate nucleus and nucleus accumbens, whereas no losses were observed in the amygdale, thalamus and lenticular nucleus (Jernigan, *et. al.*, 2001). The lack of change in the thalamus has been disputed with some researchers demonstrating an age-related reduction in size (Raz, *et. al.*, 2001; Raz, *et. al.*, 1997). Others have suggested that there is no reduction in the hippocampus with age (Sullivan, *et. al.*, 1995); or there is no differential loss in the amygdale and hippocampus relative to other grey matter (Good, *et. al.*, 2001). However, it has been speculated that these differences in opinion and results may be a result of different age ranges being sampled, with those studies utilising the oldest age ranges demonstrating differences (Walhovd, *et. al.*, 2005). There are some questions about whether these changes are normal ageing or indicative of early neurodegenerative diseases as many of the same losses or age associated issues are seen exaggerated in the likes of AD. However, demonstrations of similar changes in individuals with very low probability of having or developing AD have strongly suggested that neurodegenerative diseases are an accelerated ageing phenomenon and that these changes are normal (Fjell, *et. al.*, 2013; Fjell, *et. al.*, 2012). It has also been suggested that areas with high neuroplasticity are more prone to ageing phenotypes, for example dendritic spines involved in long term memory (Benavides-Piccione, *et. al.*, 2012; Sanders, *et. al.*, 2012). It has been speculated that increased requirements for plasticity in specific regions increases susceptibility to pathologies associated with ageing (Walhovd, *et. al.*, 2014). Unlike other areas of the body neurogenesis in adults is highly restricted (Rakic, 2004), thus it is unlikely that changes in neurogenesis are responsible for age-related phenomenon, particularly volume loss (Walhovd, *et. al.*, 2014). This age related shrinkage in the grey matter is most frequently related to neuronal loss (Anderton, 2002; Kolb and Whishaw, 1998)

The reduction, or lack thereof, in white matter is similarly debated with some researchers reporting an overall loss in volume with age (Guttmann, *et. al.*, 1998; Jernigan, *et. al.*, 2001); whilst others report no significant changes (Blatter, *et. al.*, 1995; Good, *et. al.*, 2001). In white matter this decrease in volume is believed to be a result of loss of axons and progressive demyelination (Sasson, *et. al.*, 2011; Schmierer, *et. al.*, 2007; Schmierer, *et. al.*, 2008; Song, *et. al.*, 2002). However, it has been reported that some of the volume reduction may result from cell loss, particularly in the SN (Hirai, 1968; Vaillancourt, *et. al.*, 2010). Interestingly, this cell loss has been reported to be greatest in the dorsal SN with normal ageing but is reversed with the ventral SN showing greater cell loss in PD (Fearnley and Lees, 1991). It has been noted, however, that deterioration of brain tissue in the temporal lobe is associated with ageing (Sasson, *et. al.*, 2013). This lobe has several major white matter projections, involved in many brain functions including working memory (Sepulcre, *et. al.*, 2009), attention (Nestor, *et. al.*, 2007), learning (Spiers, *et. al.*, 2001), language (Catani, *et. al.*, 2003), visual memory (Shinoura, *et. al.*, 2007), memory formation and retrieval (Levine, *et. al.*, 1998). Given that all of these functions can be impaired as a result of ageing it could be suggested that degradation of this white matter is a major component of the ageing pathology.

Although researchers originally believed that ageing resulted in reductions in both blood flow and metabolism, there is currently a great debate over whether or not brain metabolism is significantly altered during ageing. There have been several small scale studies directed at this phenomenon, reviewed by Cunnane *et. al.* (Cunnane, *et. al.*, 2010); which have proven to be inconclusive with around half showing some degradation in metabolism, particularly glucose usage, whilst the other half demonstrated no differences. However two such studies which showed decreases in brain metabolism with age reverted to no change when adjusted for ageing related brain atrophy (Cunnane, *et. al.*, 2010); thus indicating that any such change may simply be a result of general brain shrinkage and loss of cells as opposed to any significant reduction in metabolism in the remaining cells.

Interestingly, despite their permanent quiescent state neurons still express a number of cell cycle regulators which are essential for normal cell cycle progression, albeit at levels which vary from neural progenitors or tumourigenic cells (both of which are mitotic cells) (Yoshikawa, 2000). For example CDK2 is reduced while CDK4 is increased during neural differentiation (Kranenburg, *et. al.*, 1995); furthermore levels of Rb protein are also increased (Gill, *et. al.*, 1998). The differential expression of these cell cycle regulators appears to balance the post mitotic state of terminally differentiated neurons, with their disruption leading to problems; for example overexpression of cyclin D1 induces apoptosis (Freeman, *et. al.*, 1994; Kranenburg, *et. al.*, 1996).

It is obvious from the differences presented here, the differences in function of various areas, the differential reduction in size with age, neurogenesis limited to specific areas and different levels of energy usage in each area, that each region of the brain ages at a unique rate. This rate will be a factor which combines energy usage and function. Due to these obvious differences it seems prudent to examine each region of the brain independently to determine the specific effects of ageing within each area. This is particularly evident when discussing previous studies which appear to present conflicting findings, particularly when the brain is examined in its entirety. All of the regions used in this study demonstrate changes with ageing, albeit with different rates and manifestations.

1.6.1 Neurodegenerative disorders

The cellular processes linked to ageing can have a particularly detrimental effect in non-replacing tissues, e.g. brain, skeletal and cardiac muscle, therefore it is unsurprising that ageing is the main risk factor in neurodegenerative disease (Gonzales Mc Neal, *et. al.*, 2001; Lin and Beal, 2006). Furthermore, it has been demonstrated that the mitochondria play a key role in both ageing and neurodegeneration (Beal, 2005); with oxidative stress playing a major role in both (Mariani, *et. al.*, 2005). These neurodegenerative diseases include well known disorders such as AD, Cerebrovascular disease, PD, ALS and Huntington's disease (HD). All of

these have been correlated with a progressive loss of specific neurotrophic factors as a result of cell death in specific subsets of neurons (Gorman, *et. al.*, 1996); these neurotrophic factors are normally involved in cell proliferation, differentiation, migration, survival, neurite outgrowth and synapse formation (Skaper, 2012). The best known of these include brain derived growth factor, neurotrophin-3 and nerve growth factor, which have been studied both *in vivo* and *in vitro* (Skaper, 2012). The age related loss and/or deterioration of specific neurons has also been linked to the aetiology of neurodegenerative disorders, for example PD and the dopaminergic neurons of the SNC (Agid and Blin, 1987); more recently it has become apparent that dopamine signalling throughout the Basal ganglia may play a role in the development and manifestation of this disorder (Benazzouz, *et. al.*, 2014; Smith and Villalba, 2008).

1.7 Models of ageing in the brain

1.7.1 SAMP and SAMR mice

Originally, there were nine strains of senescence accelerated mouse prone (SAMP) models, and three of senescence accelerated mouse resistant (SAMR) (Takeda, *et. al.*, 1981). This has since been expanded to fourteen SAMP and four SAMR strains, all based on a AKR/J strain background (Takeda, 1999). The SAMP models present with various diseases and disorders that are associated with ageing including osteoporosis, degenerative joint disease, amyloidosis and deficits in learning and memory (Takeda, 1999; Takeda, *et. al.*, 1991). The SAMP and SAMR strains have similar growth rates and are sexually mature at the same age points but the SAMP mice demonstrate accelerated ageing phenotypes to varying degrees (Hosokawa, *et. al.*, 1984) and the animals have a shorter lifespan (Takeda, *et. al.*, 1991; Takeda, *et. al.*, 1981). These models have been extensively studied and characterised, and are now used as a model to study specific diseases associated with ageing. These mouse models have been analysed at the genetic level (Higuchi, 1997; Tanisawa, *et. al.*, 2013; Xia, *et. al.*, 1999). Furthermore they have been used in studies to demonstrate the effects of age related changes in the brain (Shimada and Hasegawa-Ishii,

2012), for example the influence of ROS on proteins and lipids (Butterfield, *et. al.*, 1998). The individual differences between the strains have also been used to elucidate novel information about the aetiology of neuronal age related disorders including using SAMP8 mice for the study of dementia (Okuma and Nomura, 1998) and glucose hypometabolism (Ohta, *et. al.*, 1996). Furthermore a comparison between two models which both demonstrate age related deterioration in memory and learning, SAMP8 and SAMP10, as they present with low and high levels of amyloidosis (respectively) demonstrates significant morphological differences (Kawamata, *et. al.*, 1997), they also demonstrate behavioural differences and abnormalities in circadian rhythms (Miyamoto, 1997). SAMP6 mice have been used for studying age related increases in S100β which has been linked to AD (Griffin, *et. al.*, 1998).

Although these mouse strains have proved to be useful tools for the investigation of age-related diseases, they are not without their problems. Many researchers question the validity of the model and its application to humans. Furthermore the SAMP mice are also somewhat immune compromised, requiring a low calorie diet and/or pathogen free environment to extend lifespan and allow research with these strains. Investigations into the relationship between this immune impairment and age-related neurological deterioration suggest that the two phenomena are independent, at least in the case of amyloidosis (Hosono, *et. al.*, 1997). These reports are however at odds with the general consensus that immunosenescence is related to ageing and age-related deterioration (Panda, *et. al.*, 2009); although the extent of the immune-compromisation seen in these mice has never been compared to the profile seen in natural immunosenescence.

1.7.2 AS and AS/AGU rats

The AS/AGU (Albino Swiss/Anatomy Glasgow University) rat strain arose from a spontaneous recessive mutation (Campbell, *et. al.*, 1996) which introduced a stop codon into the gene encoding Protein kinase C gamma (PKCγ) (Craig, *et. al.*, 2001), a member of the PKC family (Saito and

Shirai, 2002). PKCγ was originally believed to be solely neuronal in its distribution and activity, however it has now been demonstrated to be expressed in the heart (Rouet-Benzineb, *et. al.*, 1996) as well as in pulmonary fibroblasts (Ludwicka-Bradley, *et. al.*, 2000) and pulmonary epithelial cells (Lin, *et. al.*, 2000). In this model the stop mutant appears to have a largely neuronal impact, with no obvious cardiac or pulmonary effects. The stop mutant leads to a disruption in the release of neurotransmitters (Dopamine [DA] and Serotonin [5-hydroxytryptamine, 5-HT]) within the Basal Ganglia of these rats. Despite presenting with normal cerebral morphology the AS/AGU rat demonstrates reduced whole tissue levels of DA (20-30%) at 6 months old (Campbell, *et. al.*, 1996) and extracellular DA levels are reduced by 80-90% in the dorsal caudate putamen by 3 months old (Campbell, *et. al.*, 1998), both compared with similar age AS rats. Similarly 5-HT levels are significantly lower in the dorsal caudate putamen at 3 months old (Al-Fayez, *et. al.*, 2005). They also suffer from a loss of tyrosine hydroxylase in the SNC at 12 months old (Clarke and Payne, 1994). This disruption in neurotransmitter levels results in a progressive gait disturbance which increases with age. Although, rodent models with disrupted motor function is not novel, the SAMP1 mouse presents with motor dysfunction (Aoyama, *et. al.*, 2013), the AS/AGU rat is unique in that the motor impairment seen is a result of DA disruption. Furthermore, it has been demonstrated that glucose usage in the brains of AS/AGU rats, particularly in the basal ganglia, is significantly different from the AS strain (Lam, *et. al.*, 1998). However, the other areas of the brain investigated in this study demonstrated no significant alterations in glucose utilisation. Despite these major differences there are no gross morphological manifestations as a result of the stop mutant. The PD phenotype of this mutant rat, in the same manner as PD itself, can be reduced by the administration of L-Dopa (Campbell, *et. al.*, 1998). It is also worth noting that these AS/AGU rats have a much lower final body weight than comparably aged AS rats and a greatly reduced lifespan. Manifestation of symptoms is evident from a very early age including a staggering gait, a tendency to fall, difficulty initiating movement and whole body tremor (Clarke and Payne, 1994). The disruption of motor function, loss of DA in the SN of the Basal Ganglia, the characteristics of PD and a greatly

shortened lifespan leads to the possibility that this novel rat strain may represent a model of accelerated ageing when compared with its progenitor background, the AS rat. The early onset of symptoms and disruption of DA signalling suggests that the PD like phenotype is also early onset, this further implies that any differences later in life within this model is unlikely to be attributable to these early events and is more likely to be associated with ageing in general. The differences between the basal ganglia of these two rat strains make it a prime target for this study to investigate the differences in ageing.

This is not the first model to incorporate the knockout of PKCγ, another mouse model has also been studied and it presented with very mild differences, although several were noted 1) they exhibited alterations to the long term potentiation (LTP) in the hippocampus; 2) demonstrate mild disruption in spatial/contextual learning; 3) reduced effect of ethanol on type A GABA receptors; 4) reduced activation of opioid receptors (Saito and Shirai, 2002). Furthermore, this mouse model also demonstrated mild motor impairment, however this was attributed to innervations of the Purkinje cell climbing fibres, in this model Purkinje neurons posses multiple climbing fibres rather than one to one connections due to these not being eliminated early in the post-natal period (Chen, *et. al.*, 1995a), and not DA disruption. Additionally, AS/AGU rats do not demonstrate the LTP seen in the PKCγ KO mice (Shahraki and Stone, 2002). PKCγ also appears to play a critical role in the cerebellum as it is highly expressed in Purkinje cells, during development it plays a role in the elimination of unnecessary climbing fibres (Kano, *et. al.*, 1995). The role of PKCγ in the cerebellum indicates that this region may also present differences in the AS/AGU rat; therefore this region was investigated in this study.

1.7.3 Sex and ageing in animal models

Traditionally male rats have been used for most studies conducted in rats, although this has recently changed with the NIH (National Institute of Health) in the USA announcing that all projects which they fund must use both male and female rats. This predominant use of males is due to several

reasons mostly focussed around the differences between the sexes. This is almost solely attributable to the sex hormones and their variance during the menstrual cycle, which adds another level of variability to experiments. This variability is evident throughout the body including the immune system (Northern, *et. al.*, 1994; Oertelt-Prigione, 2011), introducing the possibility of far reaching changes exerted in a relatively short time span; in general, rats have a 4-5 day menstrual cycle which changes with age (Meites, *et. al.*, 1980). Other differences have also been cited, specifically it has been demonstrated that there are significant differences in memory and learning between the sexes (Belviranli, *et. al.*, 2012), further differences have been shown when diet is altered (Rajab, *et. al.*, 2014).

On a more study specific note, estrogen has been clearly demonstrated to influence methylation patterns (Vrtacnik, *et. al.*, 2014{Thakur, 1981 #6368)} and thus has the potential to affect global methylation levels. The expression and function of the sirtuins has also been shown to be effected by estrogen levels (Buler, *et. al.*, 2014). Furthermore, the Ras-p16-Rb pathway has been shown to be inhibited via an oestrogen receptor mediated mechanism (Zhu, *et. al.*, 2011). For these reasons alone the use of female rats is not suitable, in addition to this there have been no studies conducted in the AS/AGU rat model using female rats it is therefore plausible, although unlikely, that there are significant differences in the effects of the PKCγ mutation that are not evident in males. In this case dopamine levels, release and metabolism would have to be remeasured, as would glucose levels in various areas of the brain in order to construct a solid foundation for the measurements in female rats within the current study. It is therefore reasonable to conclude that only male rats would be suitable for use in this particular study.

In addition to this, males and females age differentially (Maklakov, 2013, Bioessays) possibly due to sex hormone (oestrogen and testosterone) regulation of various components of the ageing machinery. For example, Sirt5 is up-regulated in an oestrogen related receptor α dependent manner with over expression of peroxisome proliferator-activated receptor g coactivator 1-α (Buler, *et. al.*, 2014). The regulation of mitochondrial biogenesis by oestrogen, via the oestrogen related receptor α (Ranhotra,

2014) further serves to confirm that ageing pathways are heavily influenced by the sex hormones, particularly oestrogen.

These major differences precludes the possibility of using a combination of the sexes for direct comparison and would require separate experiments for each sex to determine the differences in each sex, requiring a dramatic increase in animal numbers used in these experiments and a further level of complexity for incomparable results.

1.8 Aims

Previous studies have demonstrated that ageing does indeed occur in the mammalian brain; however these studies investigated individual areas or time points in various models. Therefore, to investigate ageing in the mammalian brain this study investigated two temporally distinct age points, 2 months and 12 months, to allow intra-strain and inter-strain differences to be demonstrated. Furthermore, studies involving expression of biomarkers in four regions of the brain were carries out to address the contemporary theories that ageing manifest variably dependent on location. These four locations were selected because they have demonstrable histopathological differences between AS and AS/AGU rats or with age.

Specifically, it aimed to:

1. Determine if an enhanced ageing phenotype was present in the AS/AGU strain when compared to the parent strain, by measuring differential expression of a validated BoA (CDKN2A) at two age points (2 months and 12 months) and in four areas of the brain (cerebral cortex, brainstem, cerebellum and basal ganglia).
2. Determine if the accelerated ageing phenotype was related to metabolic disruption and/or increased DNA damage accumulation by measuring expression of MTR related metabolic stress/DNA damage markers (Sirtuins) at two age points (2 months and 12 months) and in four areas of the brain (cerebral cortex, brainstem, cerebellum and basal ganglia).
3. To decouple AS/AGU pathology from specific age related processes in the rat brain.

SOHAIR MOHAMMED KHOJAH, M.Sc. B.Sc.

Ageing in the Mammalian Brain

2. General Materials and Methods

2. General materials and methods

The general materials and methods used throughout this thesis are presented here. Any additional steps or reagents specific to a series of experiments will be outlined and detailed in the materials and methods section of the appropriate chapter.

2.1 Chemicals

All chemical reagents used throughout this thesis, unless otherwise stated, were purchased from Sigma-Aldrich (Dorset, UK), Fisher Scientific (Leicester, UK), Promega (Madison, WI, USA) and Life Technologies Ltd (Paisley, UK).

2.1.1 DEPC (Diethylpyrocarbonate) treated water

1l of DEPC treated water was prepared by mixing 0.1% v/v DEPC (Sigma-Aldrich, Dorset, UK) with de-ionised water (10ml DEPC and 990ml water), incubated for 2 hours at 37^{o}C then autoclaved at 121^{o}C for 15 minutes. Once cooled this was used or stored in a sealed container at room temperature (Rt, ~25^{o}C).

2.1.2 0.1M Sodium citrate in 10% v/v ethanol

1l of this solution was prepared by making a 10% v/v mixture of ethanol in DEPC treated water (100ml absolute ethanol and 900ml of DEPC treated water). To this 29.41g of Monosodium citrate was added to 950ml of the 10% v/v ethanol mixture and mixed well.

2.1.3 10x Tris buffered saline (TBS)

1l solution of 10x TBS was prepared by adding 60g of Tris (tris(hydroxymethyl)aminomethane) and 87.6g of Sodium chloride (NaCl) to 800ml of DEPC treated water, stir to dissolve. Using a fully calibrated pH meter concentrated Hydrochloric acid (HCl) was used to buffer the pH to

7.5, a less concentrated (1M) solution of HCl acid may be used when pH approaches the target pH. Use DEPC treated water to top up buffered solution to 1l.

2.1.4 1x TBS (1l)

Dilute 10x TBS stock solution in DEPC treated water: 100ml stock solution in 900ml of DEPC treated water.

2.1.5 400mM Potassium ferricyanide ($K_3[Fe(CN)_6]$)

5ml of a 400mM Potassium ferricyanide solution was prepared by the addition of 0.658g of Potassium ferricyanide to 5ml of de-ionised water (dH_2O) and mixed well.

2.1.6 400mM Potassium ferrocyanide ($K_4[Fe(CN)_6]$)

5ml of a 400mM Potassium ferrocyanide solution was prepared by the addition of 0.845g of Potassium ferricyanide to 5ml of dH_2O and mixed well.

2.1.7 200mM Magnesium chloride ($MgCl_2$)

4ml of a 200mM Magnesium chloride solution was prepared by the addition of 0.163g of Magnesium chloride to 4ml of dH_2O and mixed well.

2.1.8 1.5M Sodium chloride (NaCl)

40ml of a 1.5M Sodium chloride solution was prepared by the addition of 3.506g of Sodium chloride to 40ml of dH_2O and mixed well.

2.1.9 800mM Citric acid ($C_6H_8O_7$)

A 800mM solution of Citric acid was prepared by adding 4.61g of Citric acid to 30ml of dH_2O and mixing well.

2.1.10 800mM Sodium phosphate dibasic(Na_2PO_3)

A 800mM solution of Sodium phosphate was prepared by adding 3.408g of Sodium phosphate (dibasic) to 30ml of dH_2O and mixing well.

2.1.11 800mM Sodium citrate (Trisodium citrate; $Na_3C_6H_5O_7$)

A 800mM solution of Sodium citrate was prepared by adding 7.058g of Trisodium citrate (dihydrate) to 30ml of dH_2O and mixing well.

2.1.12 Nuclear fast red solution

Nuclear fast red counter stain solutions was prepared by adding 25g of Aluminium potassium sulphate dodecahydrate (Sigma Aldrich, Dorset, UK) to 500ml of dH_2O and mixing to dissolve. To this solution 0.5g of Nuclear Fast Red was added and the solution was heated to dissolve. Final solution was filtered using filter paper [3mm] (Whatman) before use.

2.2 Animal tissues

All animal tissues were obtained using humane methods in accordance with University of Glasgow and national regulations, all experiment methodologies were approved by the Ethical Review Process Application Panel, University of Glasgow. All animal husbandry and sacrifices were carried out by a professional home office licence holder within the guidelines and regulations set out by the University of Glasgow.

2.2.1 Preparation of brain tissue samples

Brains were cut in half mid-sagittally, one-half in preparation for Immunohistochemical analysis and the other half for gene expression and DNA methylation. Brains were rapidly removed from AS and AS/AGU rats aged 2 and 12 months (6 per group) after death by Carbon dioxide (CO_2) inhalation. All tissue samples were rapidly frozen in isopentane using liquid

nitrogen. The tissue was mounted onto a carefully labelled cryomold (25mmx20mmx5mm), using a solvent resistant marker, and frozen by insertion of the cryomold into the cooled isopentane. The cryomolds were then stored at -70^{o}C.

2.2.2 Section preparation and mounting

To obtain slides of brain sections, tissue was taken immediately after removal from the animal and mounted on a cryomold in preparation for sectioning. To mount the tissue a small amount of 22-oxacalcitriol (OCT) compound (BDH Laboratory Supplies, Merck Ltd, Leicester, UK) was placed onto the base of a cryomold. The tissue was oriented with the side of interest towards the bottom of a cryomold and gently placed on top of the OCT compound. The cryomold was then quick frozen by immersion in isopentane cooled with liquid Nitrogen until the OCT compound was fully frozen. 10µm sections were then cut on a cryostat (BRIGHT 5030). Sections were then mounted onto slides by placing a fresh super frost plus slide (Thermo Scientific, Wilmington, USA) under each section. Slides were fixed in 95% alcohol before storing at 4^{o}C. Prior to use, slides were removed from 4^{o}C and warmed to Rt before immersion in 95% ethanol for 10 minutes, air dried for 30 minutes followed by three 5 minute washes in Phosphate buffered saline (PBS; Gibco, Life Technologies, Paisley, UK).

2.3 Methodology

2.3.1 RNA Isolation from Tissue Samples

All equipment and reagents used in the isolation of Ribonucleic acid (RNA) from tissue samples were RNase-free, nuclease free reagents and consumables were used and all equipment was cleaned using RNase Away reagent (Fisher Scientific, Leicester, UK). Filtered pipette tips were used throughout to reduce the risk of contamination.

Tissue samples were homogenised in 1ml of TRIzol reagent (Ambion, Life Technologies, Paisley, UK) for every 50-100mg of tissue used in a tissue

homogeniser, frozen samples were homogenised immediately upon removal from -80°C storage. This step ensured that the RNase neutralising reagent (TRIzol) was thoroughly distributed and could eliminate RNase activity completely. Samples were then transferred into centrifuge tubes and incubated for 5 minutes at Rt. 200μl of Chloroform (Sigma-Aldrich, Dorset, UK) was added for every 1ml of TRIzol and vortexed for 15 seconds. This mixture was incubated at Rt for 2 to 3 minutes and then centrifuged at 12,500g for 15 minutes at 4°C. The colourless upper phase (aqueous phase) was carefully removed to a fresh microcentrifuge tube. An equal volume of a 1:1:1 ration mixture of phenol:chloroform:isoamyl alcohol (Ambion, Life Technologies, Paisley, UK) at pH 6.6 was then added to the samples and vortexed for 15 seconds. Samples were then centrifuged at 12,500g for 10 minutes at 4°C. Once again the upper aqueous phase was carefully transferred to a fresh microcentrifuge tube. To precipitate the purified RNA, an equal volume of ice cold isopropanol (Sigma-Aldrich, Dorset, UK) was added and mixed well this was incubated for 1 hour at -20°C. GlycoBlue, catalogue number AM9516, (Ambion, Life Technologies, Paisley, UK) can be added at this point to improve visualisation of the RNA pellet in smaller sample volumes. Samples were again centrifuged at 12,500g for 30 minutes at 4°C and the supernatant was carefully removed and discarded, removing as much as possible without disturbing the RNA pellet. 1ml of ice cold 95% v/v ethanol in DEPC treated water was then added to the pellet, vortexed briefly and then centrifuged at 12,500g for 20 minutes at 4°C. Once again the supernatant was carefully removed and discarded, taking care not to disturb the pellet. This ethanol wash was repeated once more and the pellets were air dried for 5 to 7 minutes. Pellets were resuspended in 30μl of RNase free or DEPC treated water and placed on ice. After at least 15 minutes a 1.6μl aliquot was removed for quantitation using a NanoDrop 1000 (Nanodrop, Thermo Scientific, Wilmington, USA) measuring absorbance at 260nm and 280nm. A high quality sample should have an absorbance ratio, A260/A280, close to 2 (ideally between 1.9 and 2). Samples below 1.9 were subjected to further purification using Lithium chloride precipitation or phenol-chloroform extraction, as a low reading suggests high levels of proteins which may inhibit reverse transcription and/or Polymerase Chain Reaction (PCR) procedures. Samples were stored

at -80°C until use. Samples were DNase treated using Turbo DNase I, according to manufacturer's instructions (Promega, Southampton, UK).

2.3.2 Generation of complementary DNA (cDNA) using reverse transcription (RT)

Reverse transcription (RT) was completed, using the Transcriptor Reverse Transcriptase kit (Roche Products Ltd, Welwyn Garden City, UK), by mixing 500ng of purified RNA with 2μl of random primers (Invitrogen, Life Technologies, Paisley, UK) with dH_2O to a final volume of 13μl for each sample tested. These were then incubated at 65°C for 10 minutes and cooled on ice. To each sample 4μl of 5x Transcriptor RT reaction buffer (Roche Products Ltd, Welwyn Garden City, UK), 0.5μl of RNase®OUT (20U) (Roche Products Ltd, Welwyn Garden City, UK), 2μl 10mM deoxynucleotide triphosphates (dNTPs) (Roche Products Ltd, Welwyn Garden City, UK) was added for a final reaction volume of 20μl. RT reaction was then completed using a PCR machine with the following thermal profile:

10 minutes at 25°C
30 minutes at 55°C
5 minutes at 85°C
2 minutes at 4°C

cDNA samples were then stored at -20°C until use, a negative control which contained all components except RNA was completed for every set of cDNA reactions.

2.3.3 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Quantitative Real-time PCR was completed using TaqMan® pre-designed assays and probes (Applied Biosystems Inc., Paisley, UK) consisting of specific primer-probe set with a conjugated quencher (MGB) and reporter tags (FAM). This kit is based on fluorescence detection and measurement of amplified PCR product. The specific TaqMan® assays (Applied Biosystems Inc., Paisley, UK) used are referenced in each chapter and in Appendix 1. The control/reference gene used for all qRT-PCR

reactions was Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Taqman® assay: Rn01775763_g1, Applied Biosystems Inc., Paisley, UK). qRT-PCR was performed in 96 well plates with a final reaction volume of 10μl using an ABI Fast 7500 Real Time PCR machine (Applied Biosystems Inc., Paisley, UK). Each sample was run in triplicate and a standard deviation between cycle threshold (Ct) values of less than 0.25 was considered as acceptable for further analyses. Each qRT-PCR reaction consisted of 1μl of cDNA, 0.5μl of specific primer/probe set, 5μl of Master Mix (Applied Bioscience Inc., Paisley, UK) and 3.5μl of nuclease free water. The thermal cycle profile applied for each gene amplification was:

50°C for 2 minutes
95°C for 10 minutes
then 40 cycles of: 95°C for 15 seconds
60°C for 1 minute

Relative gene expression was analysed using comparative threshold (Ct) method ($2^{-\Delta\Delta Ct}$ method) (Livak and Schmittgen, 2001) using GAPDH as the endogenous reference gene. The amplification of these genes has been previously validated in the host lab, and the efficiencies of amplification for genes of interest and the endogenous control were approximately equal. Controls included in each batch of samples were a no template (the no RNA control from cDNA generation) and an amplification control (no cDNA added).

SOHAIR MOHAMMED KHOJAH, M.Sc. B.Sc.

Ageing in the Mammalian Brain

3. Differential age related expression pattern of $p16^{INK4a}$ (CDKN2A) in rat brains.

3. Differential age related expression of $p16^{INK4a}$ (CDKN2A) in the rat brain.

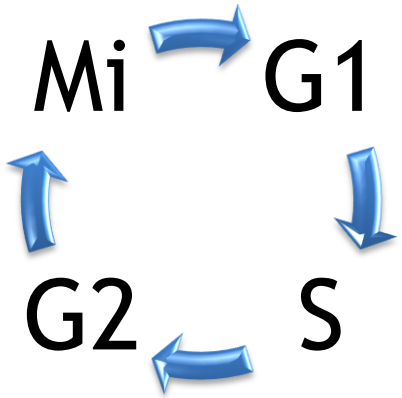
3.1 Introduction

3.1.1 Cell Cycle

The cell cycle is vital to all living organisms and follows the same sequence regardless of phylum or species. This cycle is highly conserved between species and employs many similar proteins to regulate it. The complete cell cycle consists of four phases: gap 1 (G1), synthesis (S), gap 2 (G2) and Mitotic (Mi) (Figure 3.1) (King, *et. al.*, 1994). These phases are essential for the methodical reproduction of the cell and its division into two identical daughter cells. The first phase, G1 or 1st growth phase (gap 1), involves the duplication of all cellular contents with the exception of the chromosomes (Deoxyribonucleic acid, DNA) (Donjerkovic and Scott, 2000). The second phase, S or synthesis phase, is when the chromosomes are replicated; this is one of the most conserved phases with higher organisms demonstrating a high level of homology with the budding yeast, *Saccharomyces cerevisiae* (Huberman, 1996). The next phase is G2, or 2nd growth phase (gap 2), where the cell checks for errors in the replication of chromosomal DNA and makes any necessary repairs before proceeding to the final phase (Cuddihy and O'Connell, 2003). The last phase is the Mi, or mitotic phase, where karyokinesis, or nuclear division occurs followed by cytokinesis where cell organelles, cytoplasm and membrane divide to form two new daughter cells each with its own nucleus (Koshland, 1994). Due to the nature of the cell cycle, controlling not only growth but cell division as well, it is central to the life and death of a cell. Furthermore, its control over growth and reproduction make it a central tenet of cellular senescence; therefore, all molecules involved in the control and regulation of the cell cycle are prime candidate markers for cellular senescence.

Figure 3.1 The cell cycle

Diagram of the general cell cycle found in all eukaryotic cells.



3.1.2 Cell Cycle Regulation

The framework of proteins required for the cell cycle is present in all cells, even though not all cells undergo complete cycles. Each type of cell has a tightly regulated cell cycle which is either a) cycling at a rate specific to that cell or cell type or b) blocked within a specific phase of the cell cycle. For example, post-mitotic cells are generally halted in the first growth phase, G1, and are deemed to be in G0 (gap 0, resting). These cells are senescent; therefore they normally do not undergo the processes required for duplication, growth or mitotic division, and they essentially remain frozen in time. However, there is a mechanism to switch G0 cells back into G1 phase if necessary (Heckmann, *et. al.*, 2012). The switch to G0 phase also occurs in cells that have undergone DNA damage and would therefore produce non-viable or dangerous offspring. The checkpoints present at each stage of the cell cycle are present to identify these cells and halt their progress, thus preventing mitosis. This mechanism prevents the replication of damaged cells, and thus cancer, making the proteins involved in these checkpoints key players in tumourigenesis. These regulatory molecules are subdivided into two key classes, cyclins and CDKs; between them they balance the progression of a cell through the cell cycle (Arellano and Moreno, 1997; John, *et. al.*, 2001; MacLachlan, *et. al.*, 1995; Suryadinata, *et. al.*, 2010). CDK inhibitors also play critical roles, for example $p16^{INK4a}$ which is found in the *CDKN2A* locus and is upregulated during cellular senescence (Martin, *et. al.*, 2013). Also known as Multiple

tumour suppressor 1, $p16^{INK4a}$ is a tumour suppressor with a vital role in regulating the cell cycle. It acts through the Rb pathway and inhibits the action of the CDKs; in particular it targets CDK4 and CDK6 to induce cellular senescence (Rayess, *et. al.*, 2012). Damage or modification of the *CDKN2A/p16*^{INK4a} gene increases the risk of developing cancer (Liggett and Sidransky, 1998; Rocco and Sidransky, 2001); this has been demonstrated in several types of cancer including pancreatic (Caldas, *et. al.*, 1994; Morrison, 2012), mesothelioma (Sekido, 2013), oesophageal (Igaki, *et. al.*, 1994), squamous cell carcinoma (Hillbertz, *et. al.*, 2012), colorectal (Bihl, *et. al.*, 2012) and head and neck cancers (Loyo, *et. al.*, 2013).

3.1.3 CDKN2A

The CDKN2A locus has three potential products, which vary in the first exon and produce the $p16^{INK4a}$, p12 and $p14^{ARF}$ protein products. It is located on the short arm of chromosome 9, region 2, band 1 (9p21). This locus, and in particular the $p16^{INK4a}$ product, is a well known susceptibility gene for melanoma estimated to account for up to 50% of familial melanoma cases (Marzuka-Alcala, *et. al.*, 2013). This link has been confirmed by studies demonstrating close links between variants in the *CDKN2A* locus and this familial melanoma risk (Maccioni, *et. al.*, 2013). The $p14^{ARF}$ product has also been linked to cancer, for example non-small-cell lung carcinoma (NSCLC) where specific knockout mice have demonstrated increases in tumour size, malignancy and morbidity (Busch, *et. al.*, 2013). In another study these mice also demonstrated higher levels of morbidity associated with haemangiomas and haemangiosarcomas in the liver (Busch, *et. al.*, 2012). The third transcript isoform from the *CDKN2A* locus, p12, overexpression of which leads to senescence (cell cycle arrest at the G1 phase) in human pancreatic cells, also has links with cancer in the pancreas (Poi, *et. al.*, 2013). Although heavily implicated in many cancers, the disease-mediating effects of the *CDKN2A* locus are far broader, being involved in type 2 diabetes (Landman, *et. al.*, 2012), and COPD (Acquaah-Mensah, *et. al.*, 2012) while $p16^{INK4a}$ has also been clearly linked as a risk factor for Primary Open-Angle Glaucoma (Ng, *et. al.*, 2013b). Interestingly, this locus has been shown to be heavily regulated by a variety of epigenetic

mechanisms including long non-coding RNAs (ANRIL) (Congrains, *et. al.*, 2013), promoter methylation (Popov and Gil, 2010) and histone modifications (Huang, *et. al.*, 2010). Epigenetic regulation of this locus has been implicated in the control of proliferation found in Schwann cells after nerve injury or neurofibroma challenge (Gomez-Sanchez, *et. al.*, 2013) as well as development of CVD (Congrains, *et. al.*, 2011; Pilbrow, *et. al.*, 2012) and tumourigenesis (Ushijima, *et. al.*, 2006).

3.1.4 $p16^{INK4a}$ expression, cellular senescence and ageing

Expression has been clearly demonstrated to increase with age, reducing the ability of stem cells to reproduce (Perez-Campo, *et. al.*, 2013; Yu and Kang, 2013). This has led to $p16^{INK4a}$ being regarded as a tissue BoA (Koppelstaetter, *et. al.*, 2008). This reduction in the ability of stem cells to reproduce is generally considered a defence against cancer; however it also increases the risks associated with cellular senescence. Forkhead box A1 has been shown to activate the transcription of $p16^{INK4a}$ by multiple pathways through a direct transcriptional activator role, a potential distal loop-back site (~150bp away) and by opposing polycomb function (Li, *et. al.*, 2013b). The senescence-promoting effects of $p16^{INK4a}$ can be reversed by the recruitment of polycomb repressive complexes (PRCs) to the *CDKN2A* locus by homeobox proteins, in particular HLX1 and HOXA9, which have been linked to leukaemia (Martin, *et. al.*, 2013). Jun dimerisation protein 2 has been demonstrated to promote the dissociation of PRCs from the *CDKN2A* locus, and thus promote cellular senescence through its histone modification properties (Huang, *et. al.*, 2010). Silencing of Yes-associated protein, a transcription coactivator, leads to reduced cell proliferation and premature senescence which is dependent on the Rb/$p16^{INK4a}$/p53 axis, further demonstrating the key role of $p16^{INK4a}$ in cellular senescence.

It has also been demonstrated that suppression of $p16^{INK4a}$ expression is a key target for some viruses, particularly the Epstein-Barr Virus which produces the EBNA3C protein to specifically reduce $p16^{INK4a}$ levels and overcome the cellular senescence it induces (Skalska, *et. al.*, 2013). The

same virus also produces another protein, EBNA3A, which specifically targets $p14^{ARF}$ (Maruo, *et. al.*, 2011). Knockout mice experiments have also demonstrated that over-expression of $p16^{INK4a}$ in renal allografts may be responsible for some negative outcomes, including tubular atrophy and interstitial fibrosis (Braun, *et. al.*, 2012). Significantly, given the ties of $p16^{INK4a}$ with cellular senescence, it is associated with many diseases associated with ageing including cancer, CVD, T2D, RA (Murakami, *et. al.*, 2012; Nonomura, *et. al.*, 2006), cataracts (Baker, *et. al.*, 2013; Cheong, *et. al.*, 2006) and osteoporosis (Kua, *et. al.*, 2012).

3.1.5 $p16^{INK4a}$ expression in the brain

Although there have been few studies of the expression of $p16^{INK4a}$ in the brain, it has been demonstrated that it is up-regulated in the ageing brains of mice (Abdouh, *et. al.*, 2012). Furthermore, it has been demonstrated that γ-irradiation of rat cerebromicrovascular cells (CMVECs) promotes cellular senescence by up-regulating $p16^{INK4a}$ (Ungvari, *et. al.*, 2013). The *CDKN2A* locus was also found to demonstrate abnormal gene expression in the brains of SAMP8 mice (Cheng, *et. al.*, 2013). Additional evidence for its role in the nervous system is available from the PNS, where $p16^{INK4a}$ expression is linked to regeneration in Schwann cells (Gomez-Sanchez, *et. al.*, 2013). Regarding tumours, diffuse intrinsic pontine gliomas (DIPGs) can present with a loss of $p16^{INK4a}$/$p14^{ARF}$ in a small number of cases; however, it would appear that rather than a direct involvement, regulation of CDK4 and CDK6 is necessary for this particular type of cancer to progress in the brain (Barton, *et. al.*, 2013). This strongly implies that the *CDKN2A* locus, and thus $p16^{INK4a}$, plays a major role in the regulation of senescence in the brain as loss of function can result in tumourigenesis.

Despite the paucity of direct evidence addressing the role of $p16^{INK4a}$ in the function and physiology of the brain, there is a considerable amount of indirect evidence given the roles of two key interactions with p53 and Rb. Rb has been demonstrated to force S-phase in neurons, with concomitant neuronal degeneration as a result of cell death (Oshikawa, *et. al.*, 2013). The role of p53 in neurodegenerative diseases and ageing in the

brain has been investigated in terms of its pro-oxidant activity (Chatoo, *et. al.*, 2011). p53 has also been shown to promote inflammation after brain trauma or disease stimuli; this neuroinflammation is linked to stroke and neurodegenerative diseases and is mediated through microRNAs, particularly mir-155 and its capacity to down regulate the anti-inflammatory c-Maf (Su, *et. al.*, 2013). After brain trauma, p53 promotes apoptosis resulting in neuronal loss and subsequently can cause further impairments (Rachmany, *et. al.*, 2013). In fact a similar role, promoting apoptosis, is believed to be critical in the progression of two of the most prevalent neurodegenerative diseases: PD and AD (Checler and da Costa, 2013; de la Monte, *et. al.*, 1998). These links between key $p16^{INK4a}$ interactive proteins, neurodegenerative disease, brain trauma and cancer in the brain suggest that $p16^{INK4a}$ may play a major role in normal brain physiology.

The close ties between CDKN2A/$p16^{INK4a}$ expression, cellular senescence and bio-ageing make it a key player in any study involving ageing or senescence. The work in this chapter sought to:

- confirm that CDKN2A was differentially expressed between two age points (2 months and 12 months) in four different regions of the brain (cerebral cortex, brainstem, cerebellum and basal ganglia) and in two related rat strains (AS and AS/AGU).
- Determine if CDKN2A was differentially expressed between two rat strains (AS and AS/AGU) at two time points (2 months and 12 months) and in four different brain regions (cerebral cortex, brainstem, cerebellum and basal ganglia).

3.2 Materials and methods

3.2.1 Animals used

The animals used for these experiments were raised and sacrificed in accordance with all regulations, both national and local, and all animal

husbandry/operations were performed by a qualified Home Office licence holder. Both strains of rat were fed a standard diet, with drinking water provided as required. Animals were reared and housed in the Joint Research Facility, University of Glasgow under standardised conditions – light/dark cycle 12/12 hours, temperature 22^{o}C +/- 2^{o}C, humidity 50% +/- 5% in plastic-metal cages.

A total of twenty four rats, six for each of the four experimental groups were used. The experimental groups used were AS rats at 2 months old, AS rats at 12 months old, AS/AGU rats at 2 months old and AS/AGU rats at 12 months old.

Sacrificed animals had their brains excised and rapidly frozen using liquid Nitrogen; these were then stored at -80^{o}C until required.

3.2.2 qRT-PCR for P16^{INK4a} mRNA

RNA was isolated from tissue samples as described in the general method (Chapter 2: General Materials and Methods, section 2.3.1). cDNA was generated as described previously (Chapter 2: General Materials and Methods, section 2.3.2).

qRT-PCR was then conducted in accordance with the general method set out in Chapter 2: General Materials and Methods, section 2.3.3. The following amendments to the protocol were used:

Taqman assay primers used - Rn00580664_m1 (Applied Biosystems Inc., Paisley, UK).

The sequence of these primers is provided in Appendix 1a.

The sequence of the housekeeping/reference gene used (GAPDH) is provided in Appendix 1c.

3.2.3 Immunohistochemistry Staining for $p16^{INK4a}$ protein

Following fixation of the frozen tissue sections with 95% v/v ethanol for 10 minutes and air drying, slides were allowed to warm up to Rt and then rehydrated with 1xPBS (Gibco, Life Technologies, Paisley, UK) for 5 minutes. Sections were treated with 3% v/v Hydrogen peroxide (Sigma-Aldrich, Dorset, UK) for 10 minutes to remove any endogenous peroxidase activity. Sections were then washed for 5 minutes in water and surrounded with a hydrophobic barrier pen, for example a DAKO Delimiting pen (S200230-2; DAKO, Ely, UK). Non-specific antibody binding was prevented by incubation in a blocking solution, 20% v/v normal goat serum (PCN5000; Invitrogen, Life Technologies, Paisley, UK) in TBS for 1 hour at 37°C. After the blocking step, sections were incubated with a primary antibody raised against CDKN2A/$p16^{INK4a}$ (M-156, cat no: sc-1207; Santa Cruz Biotechnology Inc., Heidelberg, Germany) at a dilution of 1:100 in DAKO antibody diluent (S0809; DAKO, Ely, UK) at 4°C overnight. Following incubation with primary antibody, sections were washed twice for 5 minutes in TBS. The secondary antibody, Horse radish peroxidase (HRP)-conjugated goat anti-rabbit (P0448; DAKO, Ely, UK), was diluted 1:200 in 20% v/v goat serum in TBS. Sections were incubated with secondary antibody for 30 minutes at 37°C and then washed twice for 5 minutes in TBS. Slides were then developed for chromogenic reactivity using 3,3'-Diaminobenzidine (DAB) as a substrate for HRP (SK- 4100; Vector Laboratories, Peterborough, UK). DAB solution was prepared by adding and mixing 5 ml of distilled water, 2 drops buffer stock solution (Vector Laboratories, Peterborough, UK), 4 drops of DAB and 2 drops of hydrogen peroxide as supplied with the kit. Sections were incubated with DAB substrate at Rt until a brown colour developed (~4 minutes). The chromogenic reaction was stopped by dipping slides in water. The slides were then washed for 5 minutes in water, followed by counterstaining with Harris Haematoxylin (Sigma-Aldrich, Dorset, UK) for 40 seconds, washed in running water and subsequently incubated with blueing agent (Scott's tap water substitute: 2g Sodium bicarbonate, 20g Magnesium sulphate in 1000ml distilled water; VWR International, Lutterworth, UK). Sections were again washed thoroughly using running water, before they were dehydrated in serial 1 minute Rt incubations of 70% ethanol, 90%

ethanol and absolute (100%) ethanol; followed by 2x1 minute incubations with Xylene (Fisher Scientific, Leicester, UK). After the final Xylene step, the sections were mounted with DPX histology mounting medium (Sigma-Aldrich, Dorset, UK). The negative control used only antibody diluent at the primary antibody step, and all subsequent steps including secondary antibody treatment were conducted as above.

3.2.4 Statistics

All datasets were tested for normality using the Kilmagorov-Smirnoff test, all datasets presented herein passed this normality test and were deemed of normal distribution for all further analyses.

The unpaired t test (2-tailed) was used to demonstrate differences between the means of experimental groups and between brain regions; p values are presented to 3 decimal places. A 95% CI ($p<0.05$) was used throughout to determine significance, however significance at the 99% CI ($p<0.01$) and 99.9% CI levels ($p<0.001$) are also denoted where appropriate.

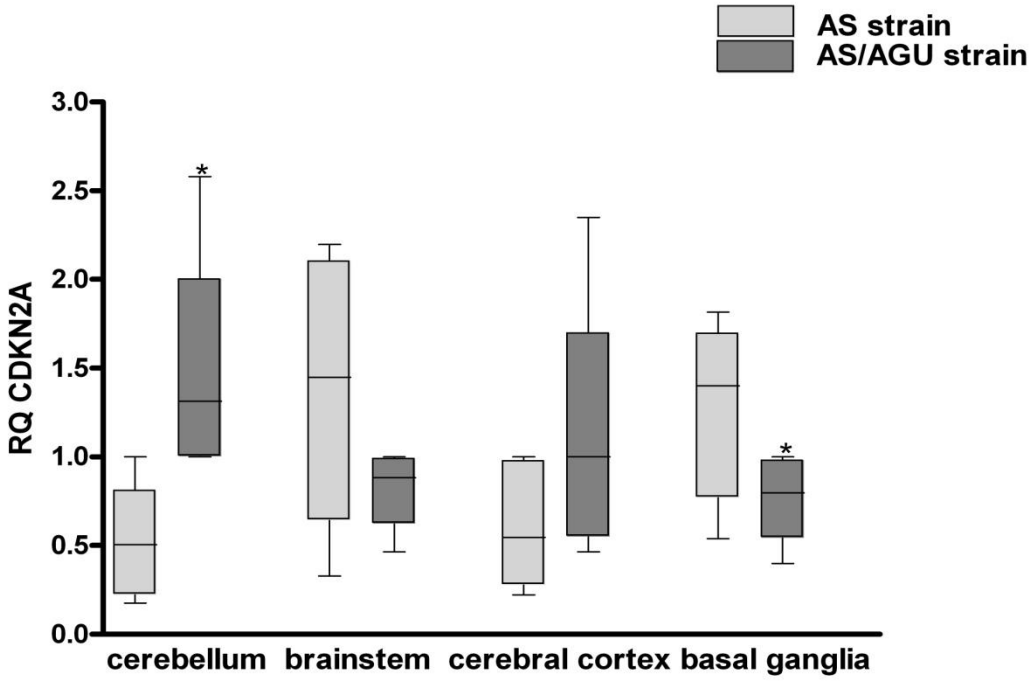
3.3 Results

3.3.1 CDKN2A expression in rat brains at 2 months

Expression levels for CDKN2A were determined using qPCR for 4 different brain regions (cerebellum, brainstem, cerebral cortex and basal ganglia) in each of the rat strains at 2 months old. The results for each experimental group (AS rats at 2 months old and AS/AGU rats at 2 months old) are presented in Figure 3.2.

Figure 3.2 *Expression of CDKN2A mRNA in brain regions of AS and AS/AGU rats at 2 months.*

Box and whisker plot demonstrating the distribution of results and mean CDKN2A expression levels. Results from both AS and AS/AGU rat brains in four regions (cerebellum, brainstem, cerebral cortex and basal ganglia) at 2 months of age are presented. The mean expression data were compared using the unpaired t test. Results significant at the 95% CI level ($p<0.05$) are marked with *.



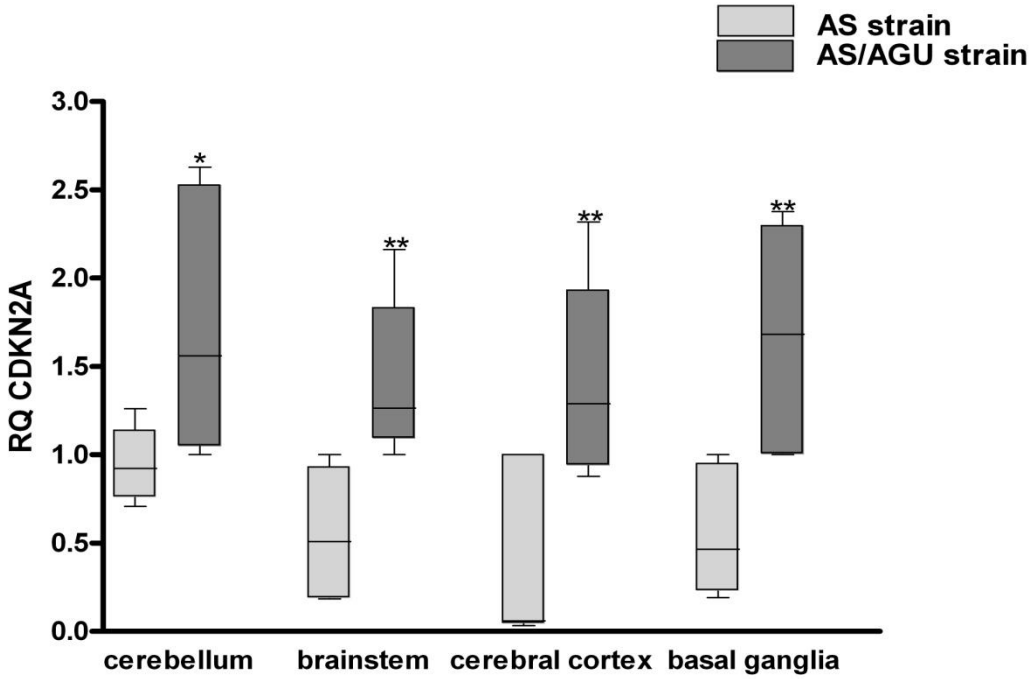
The results demonstrated differential expression in AS versus AS/AGU rats in all 4 regions at 2 months old, however only the differences seen in the cerebellum ($p=0.01$) and basal ganglia ($p=0.032$) were significant; those in the brainstem ($p=0.17$) and cerebral cortex ($p=0.171$) were not.

Though expression of CDKN2A was significantly higher in the cerebellum of AS/AGU rats when compared with AS rats; this pattern is reversed in the basal ganglia with AS/AGU rats demonstrating significantly lower levels of CDKN2A expression.

3.3.2 CDKN2A/p16^{INK4a} expression in rat brains at 12 months

Expression of CDKN2A was measured in both AS and AS/AGU rats aged 12 months old. The results are presented in a box and whisker plot below (Figure 3.3).

Figure 3.3 *Expression of CDKN2A in brain regions of AS and AS/AGU rats aged 12 months old.*
Box and whisker plot demonstrating the distribution and mean CDKN2A expression levels. Results from both AS and AS/AGU rat brains in four regions (cerebellum, brainstem, cerebral cortex and basal ganglia) are presented. Results significant at the 95% CI level ($p<0.05$) are marked with *. Results significant at the 99% CI level ($p<0.01$) are marked with **.

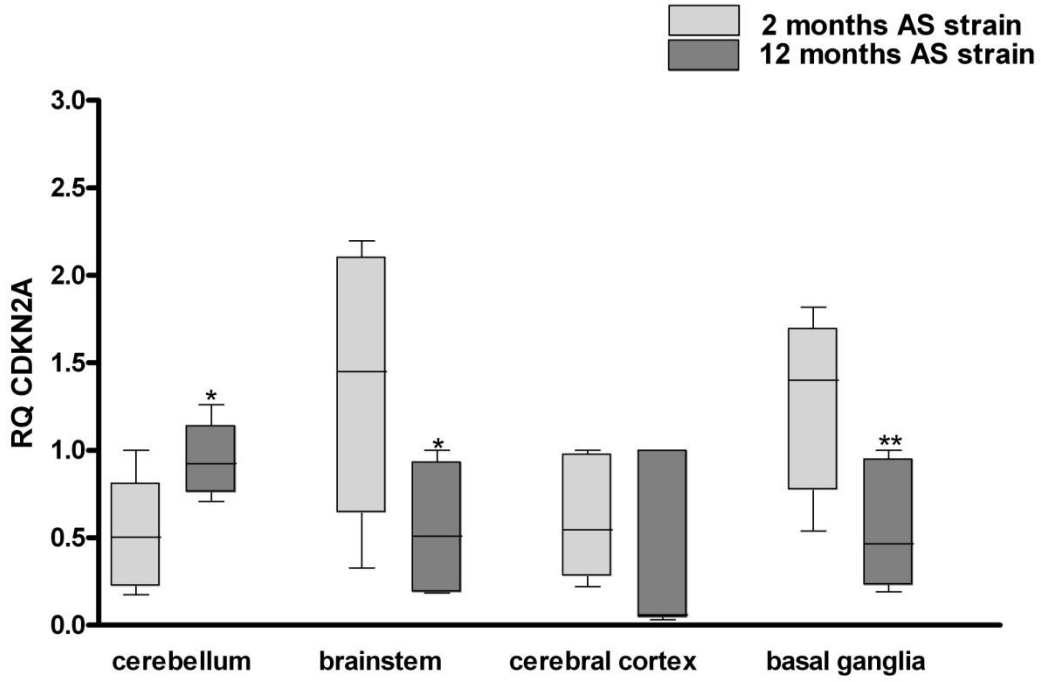


Significant differences were observed between AS and AS/AGU rats in all four brain regions: cerebellum ($p=0.032$), brainstem ($p=0.006$), cerebral cortex ($p=0.006$) and basal ganglia ($p=0.002$). Levels of p16^{INK4a} were significantly higher in AS/AGU rats in all regions of the brain tested.

3.3.3 CDKN2A expression in AS rat brains at 2 months and 12 months

A comparison of CDKN2A expression in regions of the AS rat brain at 2 months and 12 months was conducted. The results are presented as a box and whisker plot in figure 3.4.

Figure 3.4 *Comparison of expression of CDKN2A in brain regions of AS rats at 2 and 12 months.*
Box and whisker plot demonstrating the distribution and mean levels. Expression levels from AS rat brains in four regions (cerebellum, brainstem, cerebral cortex and basal ganglia) at both 2 months and 12 months old are presented. Results significant at the 95% CI level ($p<0.05$) are marked with *. Results significant at the 99% CI level ($p<0.01$) are marked with **.



In general the AS rat brain shows declines in CDKN2A levels, with the notable exception of the cerebellum and no significant change in cerebral cortex. In the instance of the brainstem ($p=0.03$) and basal ganglia ($p=0.009$) these reductions were significant, whereas in the cerebral cortex there was no significant change in either direction ($p=0.359$). The increase seen in the cerebellum was also significant ($p=0.014$).

Comparison of different brain regions in the AS rat at 2 months old using an unpaired t test revealed significant differences in CDKN2A expression levels. Levels in the cerebellum were significantly lower to those found in the brainstem ($p=0.022$) and in the basal ganglia ($p=0.006$), while levels in the brainstem were significantly higher to those found in the cerebral cortex ($p=0.038$) and levels in the cerebral cortex were lower than those found in the basal ganglia ($p=0.014$).

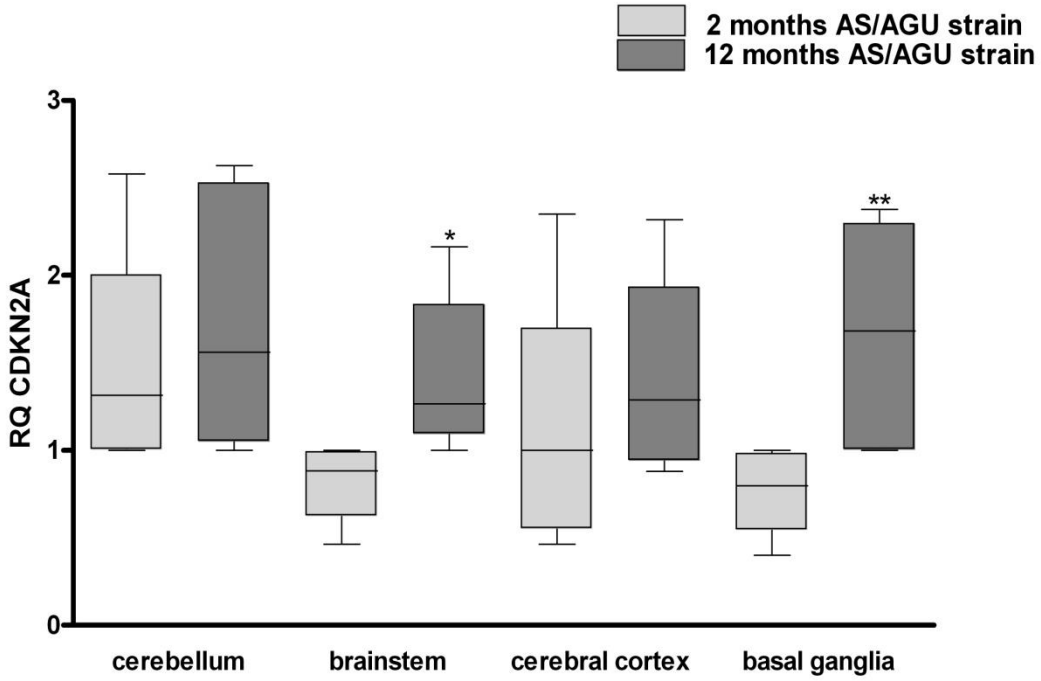
Expression levels of CDKN2A in brain regions of 12 month old AS rats also demonstrated significant differences, although these were restricted to the cerebellum. The cerebellum differed from all other measured regions of the brain: brainstem ($p=0.041$), cerebral cortex ($p=0.024$) and basal ganglia ($p=0.032$).

3.3.4 CDKN2A expression in AS/AGU rat brains at 2 months and 12 months

A Comparison of the CDKN2A expression levels in AS/AGU rat brains at 2 months old and 12 months old was conducted (Figure 3.5). Furthermore, levels in different brain regions were compared at each age to determine any variation between areas in mean expression levels.

Figure 3.5 *Comparison of expression of CDKN2A in brain regions of AS/AGU rats at 2 and 12 months.*

Box and whisker plot demonstrating the distribution and mean levels. Results from AS/AGU rat brains in four regions (cerebellum, brainstem, cerebral cortex and basal ganglia) at both 2 months and 12 months old are presented. Results significant at the 95% CI level ($p<0.05$) are marked with *. Results significant at the 99% CI level ($p<0.01$) are marked with **.



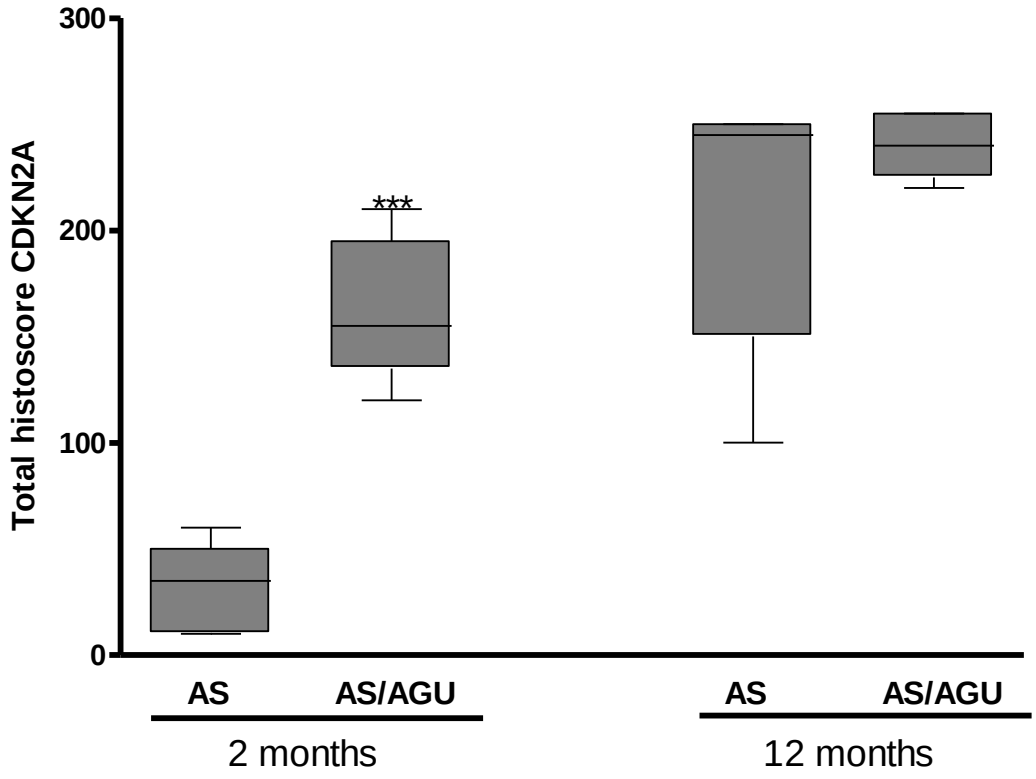
In general, CDKN2A levels are higher in the 12 month old rats when compared with 2 month old rats in all four brain regions. However, only the differences seen in the brainstem ($p=0.046$) and the basal ganglia ($p=0.006$) were significant.

The only differences in CDKN2A levels in 2 month old AS/AGU rats were observed between the cerebellum and the basal ganglia ($p=0.05$). There were no significant differences in levels between any of the four brain regions examined in 12 month old AS/AGU rats.

3.3.5 Immunohistochemical analysis of CDKN2A expression in rat brains

Following initial examination of CDKN2A transcript levels, an evaluation of the expression of the cognate protein, $p16^{INK4a}$ was undertaken. Immunohistochemical analysis of para-sagittal rat brain sections was then carried out to determine differences in overall expression of $p16^{INK4a}$ protein levels (Figure 3.6) using a clinically validated histoscoring technique.

Figure 3.6 *Total histoscores from immunohistochemical staining of rat brain sections.*
Histoscores were generated by double blind scoring of immunohistochemical staining for $p16^{INK4a}$ in brain sections from both strains of rat, at 2 time points (2 months and 12 months old). Highly significant differences ($p<0.001$) are marked with ***.



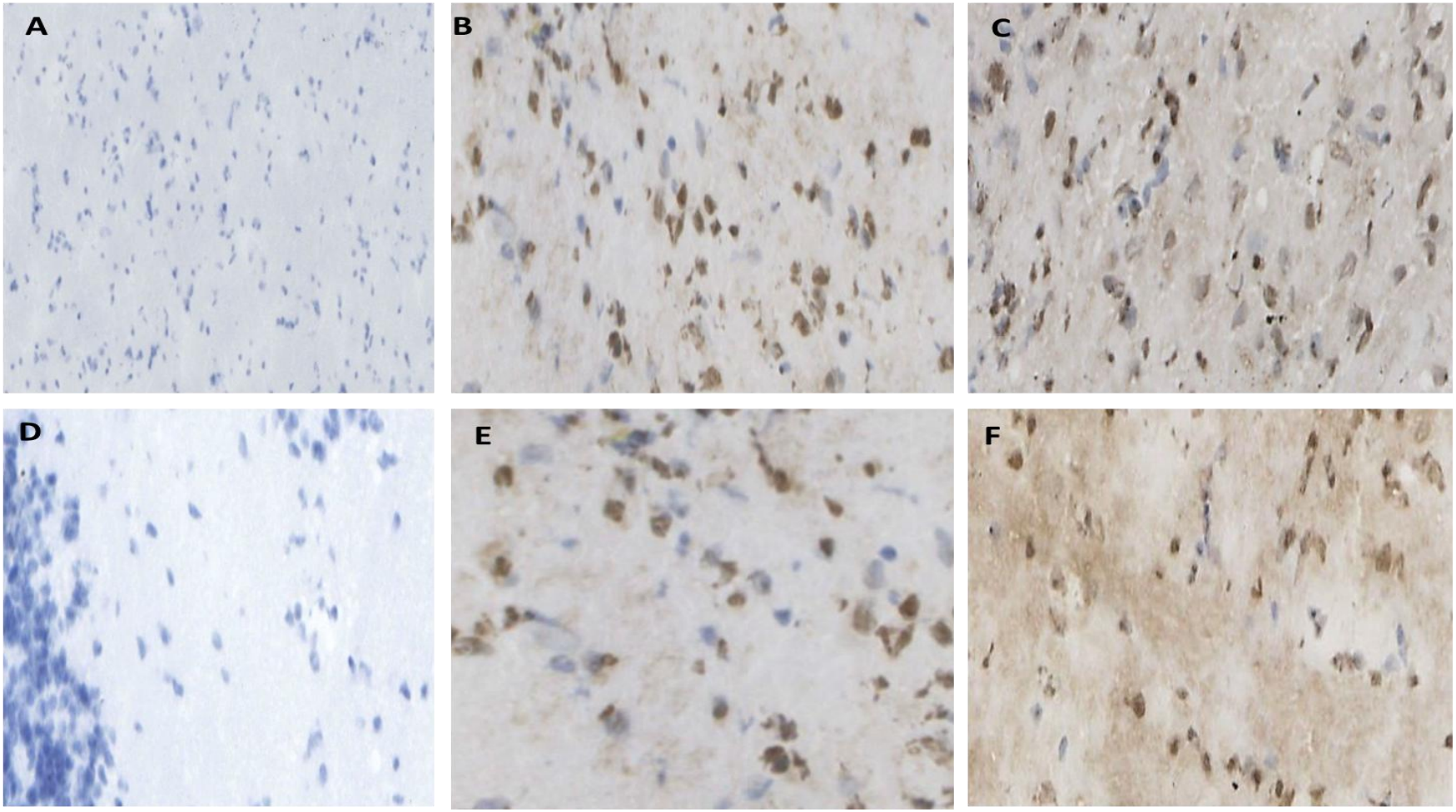
Analysis of the total histoscores revealed highly significant differences between the brains AS and AS/AGU rats, at 2 months old

($p<0.001$). Although the AS rats demonstrated a larger spread of $p16^{INK4a}$ protein expression, these differences were no longer significantly different to those seen in AS/AGU rats at 12 months of age. Overall expression levels were significantly different between 2 months and 12 months in both AS ($p<0.001$) and AS/AGU ($p<0.001$) rat strains.

An example of typical staining is presented in Figure 3.7, the negative controls demonstrate little or no perceptible background staining, confirming the specificity of the secondary antibody and showing no cross reaction with epitopes in rat brain sections. The results show expression of $p16^{INK4a}$ in both AS and AS/AGU rat brains at both time points: 2 months and 12 months. In the case of both strains of rat, AS and AS/AGU levels of specific reaction appears to increase at 12 months, which is consistent with the histoscore results.

Figure 3.7 *Immunohistochemical staining for p16^{INK4a} in para-sagittal brain sections from AS and AS/AGU rats at 2 months and 12 months old.*

Immunohistochemical staining using a CDKN2A/p16^{INK4a} specific antibody (Rabbit anti-mouse monoclonal Antibody) at 100x magnification; A: negative control (AS at 12 months old). B: 2 month old AS. C: 12 month old AS. D: negative control (AS/AGU at 2 months old). E: 2 month old AS/AGU. F: 12 month old AS/AGU. Blue staining represents the haematoxylin staining nuclear staining brown/blue staining represents specific HRP staining for p16^{INK4a}.



3.4 Discussion

The results presented in this chapter indicate distinct differences in the expression of CDKN2A/p16^{INK4a} in both rat strains in association with each age point. Both strains demonstrate an increase in expression of this BoA with increasing age, at both the transcriptional and translational levels, in keeping with its status as a validated BoA, fitting the Baker and Sprott criteria (Baker and Sprott, 1988; Koppelstaetter, *et. al.*, 2008). These two strains do, however, differ in the level and magnitude of these differences and the changes that occur within the 10 month period surveyed.

These findings are consistent with the current literature which suggests that p16^{INK4a} expression in the brain increases with age in mouse models (Abdouh, *et. al.*, 2012). However, they appear at odds with another similar study conducted in mice which compared expression of genes in two related strains of mice, the accelerated ageing model SAMP8 and its background strain SAMR1. In this comparative model, expression of CDKN2A was recorded as being reduced in the accelerated ageing model, SAMP8, compared with the control SAMR1 mice (Cheng, *et. al.*, 2013). Although, this disparity may be explained by a lack of specific information on each of the products of the *CDKN2A* locus, little is known of the expression of p14^{ARF} or p12 in the brain, it may be that overall expression of the entire locus may be lowered while an individual product may increase. Moreover, these data are incongruent with a range of other rodent studies showing increased CDKN2A expression/p16^{ink4a} expression with age (Krishnamurthy, *et. al.*, 2004). Notably, data in the present AS and AS/AGU studies, looked specifically at CDKN2A/p16^{INK4a}, not the entire CDKN2A locus transcript or protein set.

Furthermore, it should be noted that in the Cheng *et.al.* study, these differences were seen in the hippocampal regions, whereas they showed no significant differences in the other region they measured, the cerebral cortex. This is consistent with the earlier 2 month time point in the rat study; however a significant difference was noted between the strains at the 12 month time point. It may also be the case that the AS/AGU accelerated ageing phenotype is mediated through the actions of p16^{INK4a} and its related

pathways, while the SAMP8 phenotype may be a result of another pathway which does not require elevation of $p16^{INK4a}$. This would include alternative pathways and downstream activation, or over-expression of signalling components, of the $p16^{INK4a}$ pathway.

It must also be noted that the AS/AGU rat is considered a model for disruption of the dopaminergic system (Payne, *et. al.*, 2000) and dopamine disruption is linked to major depression (Kapur and Mann, 1992). Similarly, increased expression of $p16^{INK4a}$ is linked to depression (Teyssier, *et. al.*, 2012). Taken together this indicates that the level of $p16^{INK4a}$ may be "naturally" higher in AS/AGU rats compared with AS rats. In fact, expression appears to be higher in the AS/AGU rats in most cases. This is supported by overall histoscores of complete brain sections and the results from 12 month old AS/AGU rats which demonstrate significantly higher levels of $p16^{INK4a}$ in all brain regions compared with AS rats of a similar age. However, there are some areas where levels are significantly lower, for example the basal ganglia at 2 months of age demonstrate significantly lower levels of $p16^{INK4a}$ expression than equivalent AS rats. Furthermore, the age-related increase seen in expression levels suggests that, while dopamine disruption may lead to a low to moderate increase in overall levels, it is not wholly responsible for the levels or expression pattern of $p16^{INK4a}$ seen in AS/AGU rats. To investigate the effects of dopamine disruption on expression of $p16^{INK4a}$, further experimentation would be required utilising a similar experimental design as used here, but with concurrent measurement of dopamine levels and/or function.

Similarly, the Parkinson's disease like phenotype of the AS/AGU rat (Payne, *et. al.*, 2000), with highly disrupted dopamine levels leads to the possibility that abnormalities in comparative expression levels in AS and AS/AGU rats, i.e. the distribution of $p16^{INK4a}$ expression, may be a part of the locomotor disease phenotype. This hypothesis is supported by the role of p53 in Parkinson's disease (Checler and da Costa, 2013; Levine, *et. al.*, 2012; Venderova and Park, 2012). However, in order to test this hypothesis, further work would be required; for example, dopamine levels would be measured in conjunction with $p16^{INK4a}$ expression; p53 could also be

measured and compared as well as monitoring the physical manifestations of the disease. All of these measurements and comparisons should also be measured in the AS rat to provide a suitable control.

Interestingly, these two strains also exhibit different distributions of $p16^{INK4a}$, particularly in the AS strain where expression varies depending on brain region, especially in the 2 month old rats. This variation in expression is more muted in the 12 month rats, whilst the expression pattern found in the AS/AGU rats at 2 months is similar to that of 12 month old AS rats, in keeping with an accelerated ageing phenotype and a commensurate increase in the rate of molecular damage. At 12 months old the AS/AGU rats have almost ubiquitous expression of $p16^{INK4a}$. This would indicate that the AS/AGU rats have reached "old-age" where expression becomes ubiquitous and the cells are truly senescent and possibly beginning to degrade and apoptose. The only other study of this type measured the *CDKN2A* locus as a whole in the brains of normal and accelerated ageing phenotype mice (Cheng, *et. al.*, 2013), as part of a much larger gene expression study, and did not focus on the expression of specific products. Cheng and co-workers demonstrated no difference in gene expression from the *CDKN2A* locus in the only directly comparable region (the Cerebral cortex); however, as mentioned previously, this encompasses three distinct products which may have variable expression in this brain region and makes direct comparisons difficult.

Levels of $p16^{INK4a}$ have been clearly established as an age-related marker that increases expression with age (Koppelstaetter, *et. al.*, 2008; Krishnamurthy, *et. al.*, 2004; McGlynn, *et. al.*, 2009). Particularly, this biomarker is proposed to result in increased cellular senescence, a mechanism that has been suggested as a defence against cancer. As expected, expression of $p16^{INK4a}$ increases with age in the brains of both strains. The most profound increases occur in the non-mutant AS rat, this may indicate a more natural, or slower, ageing process, as opposed to the accelerated phenotype proposed to exist in the AS/AGU rat. The higher expression levels found at 2 months old in the AS/AGU strain, compared with 2 month old AS rats, further supports a more rapid onset of ageing or cellular

senescence in the brains of these rats. The data presented here supports the premise that AS/AGU rats demonstrate accelerated ageing compared to other strains; for example, biomarkers show that 12 month old AS rats appear to be of a similar age to 2 month old AS/AGU rats. However, variations in expression patterns suggest that this is either not an exact comparison or that further abnormalities in the ageing process of the AS/AGU rats are present.

Overall, both AS and AS/AGU rats follow a similar pattern of increasing $p16^{INK4a}$ levels consistent with its status as a BoA. The levels in AS/AGU rats are notably higher at the 12 month time point. This is consistent with their shortened lifespan and their neurodegeneration. However, these notable differences in expression may be involved in the Parkinson's-like phenotype displayed by these rats due to disruption of the dopaminergic system. The increase in CDKN2A levels seen in the basal ganglia of AS/AGU rats when comparing 2 month old and 12 month old rats may be dismissed as a change induced by DA disruption. However, when this is taken in context with the rest of the brain, particularly the brainstem which shows no overt differences in any PKCγ model it is more likely that these are a result of accelerated ageing and not the PKCγ mutation. It is also worthy to note that differences are seen between the levels in each brain section, this is more overt in the AS rat strain which is a more accurate model of normal ageing. Measurement of DA alongside CDKN2A levels may be able to separate this more conclusively. Similarly, these differences may be attributable to differences in the ageing process within these rats, and further experimentation would be required to elucidate the true reasons for these differences in $p16^{INK4a}$ expression.

3.5 Overall Conclusions

The results presented here are consistent with levels of CDKN2A increasing in association with advancing age, this is confirmed in both models. Furthermore, higher levels are seen in the AS/AGU rats indicating that these rats may represent an accelerated ageing phenotype. These results also confirm that differential expression is seen in different areas of

the brain, this is evident at two temporally distinct age points and in two species. This is unsurprising given the different functions performed by the different areas of the brain. This is consistent with other findings that different tissues age at different rates, however this does uniquely demonstrate intra-tissue differences in ageing. Furthermore, it has been shown that these differences vary between two closely related strains.

SOHAIR MOHAMMED KHOJAH, M.Sc. B.Sc.

Ageing in the Mammalian Brain

4. Expression of Sirtuins 5, 6 and 7 in the brains of AS and AS/AGU rat strains at two time points.

4. Expression of Sirtuins 5, 6 and 7 in the brains of AS and AS/AGU rat strains at two time points.

4.1 Introduction

4.1.1 The Sirtuin protein family

The Sirtuins are a family of highly conserved proteins, found in a diverse range of organisms ranging from bacteria to humans. First identified in the budding yeast *Saccharomyces cerevisiae*, the Silent information regulator 2 (Sir2) gene (Rine, *et. al.*, 1979) has served as the basis for detection of orthologs in all other species. SIR2 was the first discovery of 4 other similar protein products, which constitute the Sir family, in *Saccharomyces cerevisiae* (Ivy, *et. al.*, 1985; Ivy, *et. al.*, 1986; Shore, *et. al.*, 1984). Although originally identified as modulators of mating type in a role unique to yeast, identification of orthologs in other species rapidly identified new functions. For example, Sir2 was soon identified as suppressing recombination in ribosomal RNA tandem repeats (Gottlieb and Esposito, 1989). Further research has revealed that the Sir family of proteins are involved in lifespan determination and health not only in yeast, but also nematodes and flies (Dali-Youcef, *et. al.*, 2007). In fact this function appears to be mediated via CR, at least in lower organisms (Hamilton, *et. al.*, 2005; Hansen, *et. al.*, 2007; Kaeberlein, *et. al.*, 1999; Rogina and Helfand, 2004; Sinclair and Guarente, 1997). This has been challenged recently by Burnett *et. al.*, who suggest that, after controlling for genetic background, there is no apparent effect of CR in Sir2 transgenic studies with nematodes and flies (Burnett, *et. al.*, 2011). Despite this, studies in primates suggest similar roles for the Sirtuins and CR in higher organisms (Ramsey, *et. al.*, 2000). Human research also appears to suggest a role for the Sirtuins in improved lifespan, with allelic variants being linked to elongated lifespan (Bellizzi, *et. al.*, 2005; Kuningas, *et. al.*, 2007; Rose, *et. al.*, 2003). Sirtuins are also key players in the MTR theory of ageing (Shiels and Davies, 2004), by relating all three components of the theory together via their roles in modulating metabolism and ribosome biogenesis, as well as influencing DNA damage repair mechanisms.

In humans, seven members of the Sirtuin (Sirt) family have been identified, numbered 1 to 7 (Frye, 2000; Michishita, *et. al.*, 2005). All are orthologs of the original Sir2 gene (Rine, *et. al.*, 1979), each with unique interactions, targets and localisations (Table 4.1). Cellular energy production is at the centre of most Sirtuin function, mediated through Nicotinamide Adenine Dinucleotide (NAD^+)-dependent deacetylation reactions, as each contains a NAD^+ binding site and a catalytic domain (Haigis and Sinclair, 2010). The reliance of the deacetylase function on the presence of NAD^+ clearly links these proteins and their functions with cellular metabolism. Their function can be inhibited by a number of small molecules, particularly the NAD^+ intermediate product NAM, which may also be able to reverse the reaction (McGuinness, *et. al.*, 2011). These proteins are heavily implicated in ageing of mammals as well as lower organisms and appear to provide a key link between the regulation of metabolism and ageing (Haigis and Guarente, 2006; Westphal, *et. al.*, 2007). Their key roles in metabolism and ageing are reinforced by their deep involvement in diseases of ageing (Donmez and Guarente, 2010; Haigis and Sinclair, 2010; Srivastava and Haigis, 2011). Such as cancer (Schumacker, 2010), T2D (Avogaro, *et. al.*, 2009), atherosclerosis (Ota, *et. al.*, 2010) and major neurodegenerative diseases such as AD (Albani, *et. al.*, 2010) and PD (Esteves, *et. al.*, 2010).

Table 4.1 Sirtuins in humans, distribution and known interactions/functions (Donmez and Outeiro, 2013; Haigis and Guarente, 2006; Haigis and Sinclair, 2010; Li and Kazgan, 2011; McGuinness, *et. al.*, 2011; Sebastian, *et. al.*, 2012a; Westphal, *et. al.*, 2007)

Sirtuin	Localisation	Known interactions	Enzyme Activity	Biological Function
Sirt1	Nucleus (but has been found in cytosol)	FOXO, PGC-1α, SREBP-1c, PPARγ, UCP2, LXR, NF-κB, p53, MyoD, Ku70, LXR, p300, Tat, PCAF, ERα, AR, SMAD7, p73, Sox9, HES1, Hey2, NcoR/SMRT, E2F1	NAD^+ deacetylase	Cell survival/metabolism, lipogenesis, cholesterol metabolism, insulin secretion, inflammation, neurodegeneration
Sirt2	Cytoplasm (but known to interact with some nuclear proteins)	α-Tubulin, H4, NF-κB, FOXO	NAD^+ deacetylase	Cell cycle control, tumourigenesis, inhibits adipocyte differentiation, promotes gluconeogenesis, tubulin deacetylation
Sirt3	Mitochondria (but can localise to nucleus if Sirt5 is overexpressed)	AceCs2, HMGCS2, SDHA, ornithine carbamoylase, GDH complex 1	NAD^+ deacetylase	Thermogenesis/metabolism, fatty acid catabolism, promotes ketone body synthesis, upregulates electron transfer chain, upregulates urea cycle, ATP production

Sirt4	Mitochondria	GDH, IDE, ANT	ADP-ribosyl transferase	Insulin secretion/ metabolism, fatty acid oxidation (opposes Sirt3)
Sirt5	Cytoplasmic/ Mitochondria	CPS1	NAD^+ deacetylase, demalonylase, desuccinylase	Ammonia detoxification, Urea cycle
Sirt6	Nucleus	DNA polymerase β, Sirt1, FOXO3a, NRF-1, NF-κB, Hif1α, helicase	ADP-ribosyl transferase, NAD^+ deacetylase	DNA repair/metabolism, telomeres and telomeric function, lipid metabolism, lipid storage, glucose uptake and metabolism
Sirt7	Nucleus	RNA Polymerase type 1, E1A, SMAD6, UBF	NAD^+ deacetylase	rDNA, RNA polymerase I transcription

4.1.2 Sirtuins in the brain

The brain is the most energy hungry component of the body, in this regard the presence of Sirtuins is vital to its survival as they modulate not only metabolism but also mitigate the damage that can result from increased metabolic pressure i.e. oxidative stress. Sirt1 has been investigated in the mammalian brain and has been linked to many functions, particularly in the hypothalamus (Guarente, 2013). It has also been demonstrated to fluctuate in accordance with dietary change. In connection with this, it has been shown to localise specifically to pro-opiomelanocortin (POMC) neurons which are vital for the maintenance of glucose levels and normal body weight (Ramadori, *et. al.*, 2008). Its effects in POMC are mediated via the interaction between leptin and phosphoinositide 3-kinase (PI3K) (Ramadori, *et. al.*, 2010). Sirt1 has also been shown to be active in steroidogenic factor 1 neurons, where it heavily influences obesity due to diet (Ramadori, *et. al.*, 2011). Sirt1 has previously been shown to influence the circadian rhythm or clock via BMAL1 (Nakahata, *et. al.*, 2008) and Per2 (Asher, *et. al.*, 2008), which have been demonstrated to be linked to health and ageing (Pittendrigh and Minis, 1972). It has recently been demonstrated that this influence over the circadian clock occurs via the amplification of BMAL1 (Chang and Guarente, 2013). Furthermore, Sirt1 over-expression has been shown to be beneficial in models of neurodegenerative diseases, for example AD (Donmez, 2012; Donmez, *et. al.*, 2010; Min, *et. al.*, 2010), HD (Jeong, *et. al.*, 2012; Jiang, *et. al.*, 2012) and PD (Donmez, *et. al.*, 2012). Interestingly, poor short and long term memory functions are found in *Sirt1* knockout models (Michan, *et. al.*, 2010). The effect of Sirt1 in PD has been disputed, since no protection was observed in Sirt1 transgenic mice in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD model (Kakefuda, *et. al.*, 2009). Despite this, most studies demonstrate protective effects in PD and this is currently the prevailing hypothesis. This may indicate that MPTP-induced PD is not a truly representative of the mechanisms behind spontaneous PD aetiology and pathology.

Sirt2 has been demonstrated to both promote (Ji, *et. al.*, 2011) and retard (Li, *et. al.*, 2007) oligodendrocyte differentiation, as well as promoting Schwann cell myelin formation (Beirowski, *et. al.*, 2011). Sirt2 has also been shown to be detrimental in neurodegenerative disease (Donmez and Outeiro, 2013), particularly in HD using knock down models (Luthi-Carter, *et. al.*, 2010) and PD (Outeiro, *et. al.*, 2007).

Very little information is available on the localisation, influence and function of other Sirtuin family members in the brain. However, Sirt3 has been demonstrated to be important in neuron viability in cell models (Kim, *et. al.*, 2011b) and has been suggested to have a neuroprotective effect (Kincaid and Bossy-Wetzel, 2013). Whereas Sirt4 is highly expressed in post natal astrocytes and during embryogenesis in radial glia, a general decrease in expression is seen during development (Komlos, *et. al.*, 2013). There is no direct evidence for the role of Sirt5 in neural development or neural ageing; however its role in mitochondria and metabolism suggests that expression of this Sirtuin will be linked to neural ageing. Furthermore, the involvement of the other mitochondrial Sirtuins (Sirt3 and Sirt4) in neural ageing and development as well as the fact that Sirt5 can modulate the localisation of Sirt3 provides further support for a role for Sirt5. Sirt6 appears to act in a different manner in the brain compared with the rest of the body; full body knock outs die due to hypoglycaemia whereas neuron specific deletion does not have the same effect (Schwer, *et. al.*, 2010). Sirt6 knockouts also appear to demonstrate symptoms of extreme ageing (Mostoslavsky, *et. al.*, 2006).

Sirt7 plays a key role in ribosome biogenesis and protein synthesis via multiple pathways (Tsai, *et. al.*, 2013b), this indicates that this Sirtuin would be critical for the measurement of ribosomal biogenesis and thus the 'R' of the MTR trinity. Sirt6 binds to histones at the telomeric structures and influences telomere function (Michishita, *et. al.*, 2009). Additionally, null mouse models for Sirt6 experience severe metabolic disruption leading to death after only 1 month post-natal (Mostoslavsky, *et. al.*, 2006). Sirt6 also regulates ribosome metabolism via its influence on the activity of myc (Sebastian, *et. al.*, 2012b). This indicates that Sirt6 exerts an influence

over all components of the MTR trinity which may explain the severity of outcome when it is knocked out. While Sirt5's role in the mitochondria, regulation of the urea cycle, may not immediately appear to be directly related to metabolism it is however critical for the elimination of ROS promoting ammonia and this reducing oxidative stress. More importantly, however, are its novel demalonylation and desuccinylation enzyme functions (Du, *et. al.*, 2011). Both of the substrates for these enzyme activities are intermediates for the tricarboxylic acid (TCA) cycle in mitochondria (Peng, *et. al.*, 2011; Zhang, *et. al.*, 2010) which is at the core of energy production. This indicates that Sirt5 probably exerts an influence over the TCA cycle by injecting intermediates into the cycle thus increasing capacity and activity. More recently, Sirt5 has been shown to demonstrate lysine-glutarylation activity as well, however this appears to be directed at carbamoyl phosphate synthase 1 (CPS1) (Tan, *et. al.*, 2014). Therefore these enzymatic activities are at the core of the 'M' component of the MTR trinity.

The key roles for Sirtuins in ageing and stress response make them prime candidates for involvement in any disease of ageing. Furthermore the recent elucidation of the roles of Sirt1 and Sirt2 in the aetiology of several neurological disorders suggests that the action and expression of this family of proteins is going to be critical to our understanding, and ultimately treatment, of these disorders. The biology of ageing suggests roles for metabolism (Sirt5), DNA damage (Sirt6) and reduction of tandem repeats in ribosomal DNA (rDNA) (Sirt7). Therefore Sirtuins 5, 6 and 7 were selected for their roles in these areas to determine the potential differences in the ageing process between the two rat strains, AS and AS/AGU rats; as well as providing a vehicle to investigate the implications of these differences in the context of neurological disorders, for example PD.

4.2 Materials and Methods

4.2.1 Animals used

The animals used for these experiments were raised and sacrificed in accordance with all regulations, both national and local, and all animal husbandry/operations were performed by a qualified Home Office licence holder. Both strains of rat were fed a standard diet, with drinking water provided as required. Animals were reared and housed in the Joint Research Facility, University of Glasgow under standardised conditions – light/dark cycle 12/12 hours, temperature 22°C +/- 2°C, humidity 50% +/- 5% in plastic-metal cages.

A total of twenty four rats, six for each of the four experimental groups were used. The experimental groups used were AS rats at 2 months old, AS rats at 12 months old, AS/AGU rats at 2 months old and AS/AGU rats at 12 months old.

Sacrificed animals had their brains excised and rapidly frozen in Isopentane using liquid Nitrogen; these were then placed in a cryomold, clearly labelled and stored in Isopentane at -80°C until required.

4.2.2 qRT-PCR for Sirtuin mRNA

Material for qRT-PCR was obtained from frozen samples as described in Chapter 2: General Materials and Methods, section 2.3.1. cDNA was generated as described previously (Chapter 2: General Materials and Methods, section 2.3.2). Then qRT-PCR was conducted in accordance with the general method set out in Chapter 2: General Materials and Methods, section 2.3.3. The following amendments to the protocol were used:

Taqman assay primers used:
Sirt5: Rn01450559_ml (Applied Biosystems, Paisley, UK)
Sirt6: Rn01408249_ml (Applied Biosystems, Paisley, UK)
Sirt7: Rn01471420_ml (Applied Biosystems, Paisley, UK)

Control GAPDH: Rn01775763_g1 (Applied Biosystems, Paisley, UK)

Sequence of primers is given in Appendix 1b. Sequence for the housekeeping/reference gene used is provided in Appendix 1c.

4.2.3 Statistics

All datasets were tested for normality using the Kilmagorov-Smirnoff test, all datasets presented herein passed this normality test and were deemed of normal distribution for all further analyses.

The unpaired t test (2-tailed) was used to demonstrate differences between the means of experimental groups and between brain regions; p values are presented to 3 decimal places. A 95% CI ($p<0.05$) was used throughout to determine significance, however significance at the 99% CI ($p<0.01$) and 99.9% CI levels ($p<0.001$) are also denoted where appropriate.

4.3 Results

4.3.1 Sirtuin 5 expression in rat brains

The levels of Sirtuin 5 were measured in the brains of both AS and AS/AGU rats, and compared at 2 months old and 12 months old.

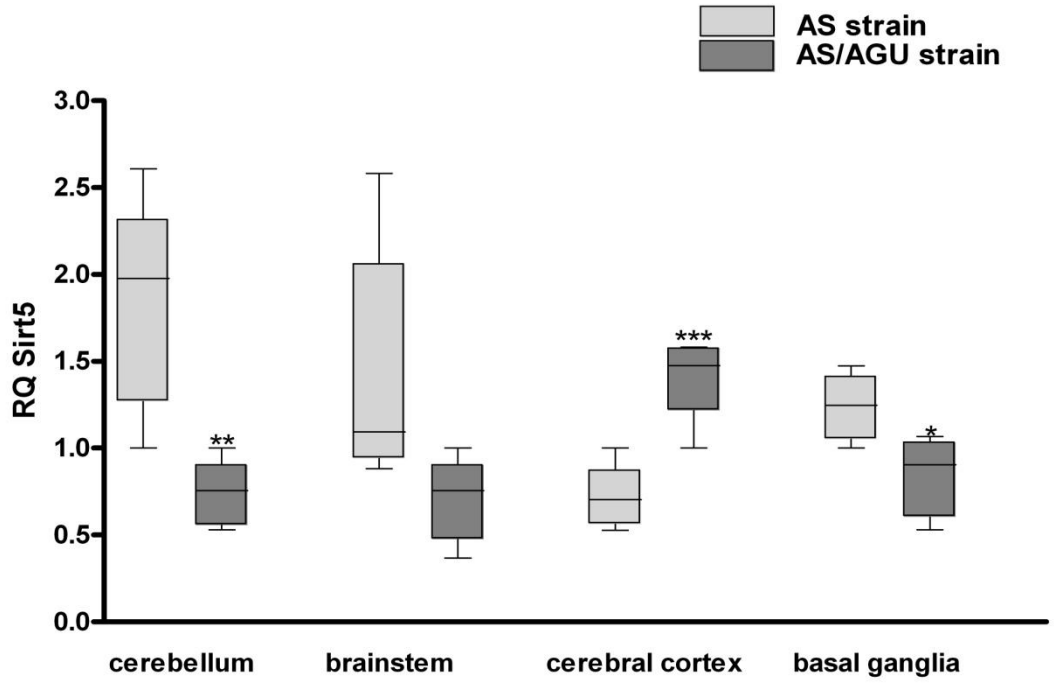
4.3.1.1 Comparison of expression levels of Sirtuin 5 at 2 months old in both AS and AS/AGU rats

Levels of Sirt5 expression in the brains of AS and AS/AGU rats were determined and compared in 2 month old rats (Figure 4.1). Levels in the cerebellum ($p=0.005$) and basal ganglia ($p=0.016$) were significantly higher in the AS rats than in the AS/AGU; although levels found in the brainstem ($p=0.061$) were also higher, these were not significant at the 95% CI level. Interestingly, the levels of Sirt5 found in the cerebral cortex were also significantly different ($p<0.001$), however higher levels were seen in the AS/AGU rats. Furthermore, cerebral cortex demonstrated higher levels in

general when compared to other areas of the brain in AS/AGU rats, whilst the cerebral cortex demonstrated the lowest levels of expression seen in AS rats.

Figure 4.1 ***Comparison of expression levels of Sirt5 in 2 month old rats of AS and AS/AGU backgrounds.***

Box and whisker plot demonstrating the distribution of results and mean levels. Results from both AS and AS/AGU rat brains in four regions (cerebellum, brainstem, cerebral cortex and basal ganglia) at 2 months of age are presented. Results significant at the 95% CI level ($p<0.05$) are marked with *, significance at 99% CI level ($p<0.01$) is denoted with ** and highly significant results ($p<0.001$, 99.9% CI level) are denoted with ***.



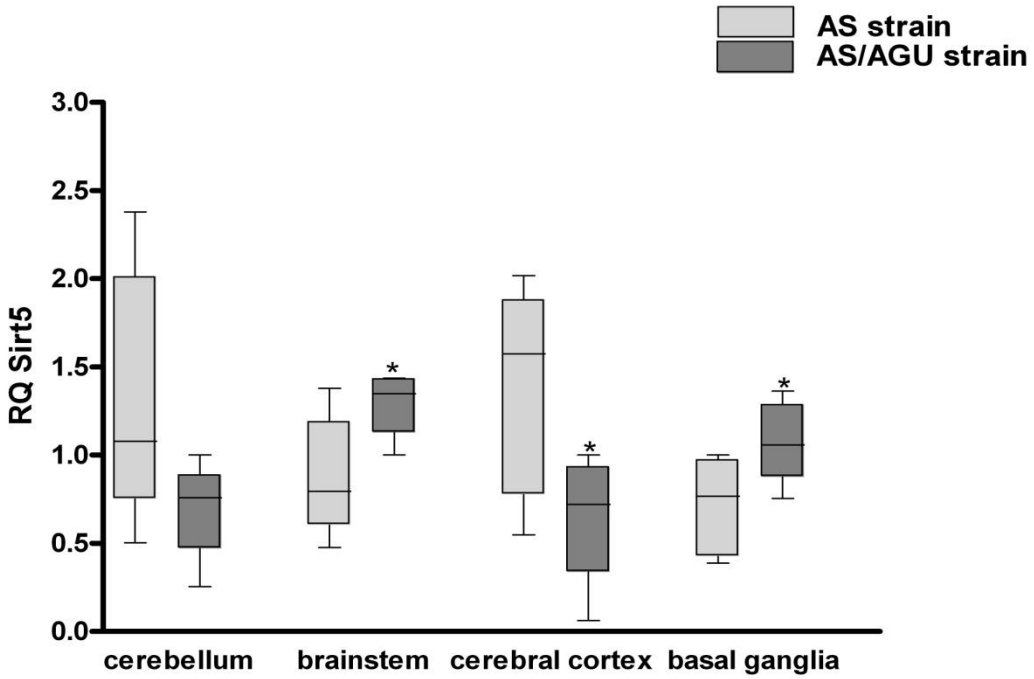
4.3.1.2 Comparison of expression levels of Sirtuin 5 at 12 months old in both AS and AS/AGU rats

Sirt5 Expression levels were determined in four regions of brains from both AS and AS/AGU rats at 12 months old (Figure 4.2). AS/AGU rats demonstrated significantly higher expression of Sirt5 in both the brainstem ($p=0.011$) and the basal ganglia ($p=0.027$). Levels found in the cerebral cortex ($p=0.048$) were significantly lower in the AS/AGU rats;

levels in the cerebellum also appeared to be lower in these rats but the difference was not significant (p=0.106).

Figure 4.2 *Comparison of expression levels of Sirt5 in 12 month old rats of AS and AS/AGU backgrounds.*

Box and whisker plot demonstrating the distribution of results and mean levels. Results from both AS and AS/AGU rat brains in four regions (cerebellum, brainstem, cerebral cortex and basal ganglia) at 12 months of age are presented. Results significant at the 95% CI level ($p<0.05$) are marked with *.



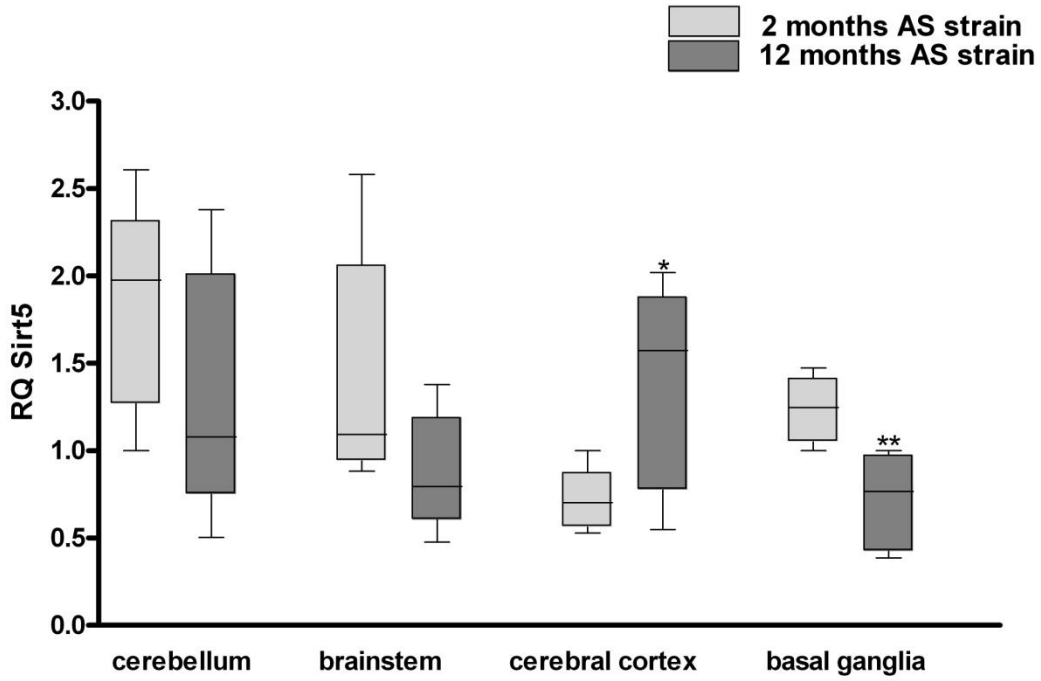
4.3.1.3 Comparison of expression levels of Sirtuin 5 at 2 months old and 12 months old in AS rat brain regions

A comparison of the expression levels found in AS rat brain regions at 2 months and 12 months was carried out; the results are presented in Figure 4.3. Levels in the cerebral cortex (p=0.044) were significantly higher in the older AS rats when compared to the 2 month old rat brains, whereas levels in the older rats were found to be significantly lower in the Basal ganglia (p=0.005). Levels in the brainstem appeared to be lower in the older rats , however this was not significant (p=0.108), and levels in

the cerebellum did not demonstrate any real differences in expression levels between 2 month old and 12 month old rats (p=0.258).

Figure 4.3 *Comparison of expression levels of Sirt5 in 2 month old and 12 month old rats from the AS background.*

Box and whisker plot demonstrating the distribution of results and mean levels. Levels from AS rat brains in four regions (cerebellum, brainstem, cerebral cortex and basal ganglia) at both 2 months old and 12 months old are presented. Results significant at the 95% CI level (p<0.05) are marked with * and significance at the 99% CI level (p<0.01) is denoted with **.



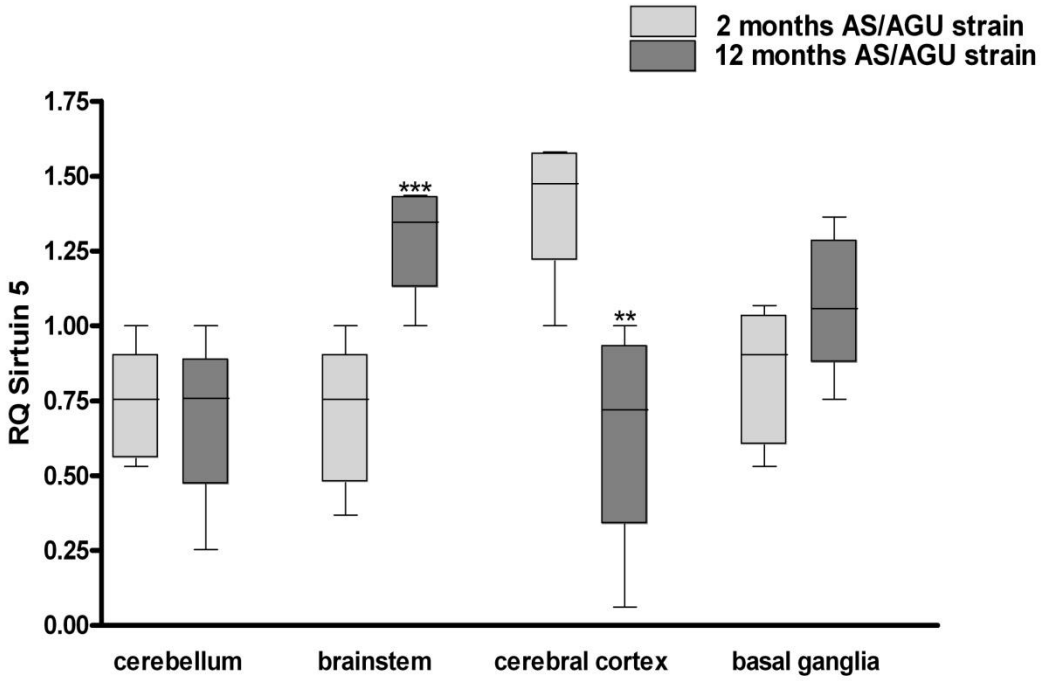
4.3.1.4 Comparison of expression levels of Sirtuin 5 at 2 months old and 12 months old in AS/AGU rat brain regions

The expression levels of Sirt5 found in AS/AGU rat brain regions at 2 months and 12 months were compared (Figure 4.4). Levels in the cerebral cortex (p=0.004) were significantly lower in the older AS/AGU rat brains when compared to the 2 month old rat brains. Levels in the older rats were found to be significantly higher in the brainstem (p<0.001). Levels in the basal ganglia appeared to be higher in older AS/AGU rats, however this was not significant (p=0.1), and levels in the cerebellum did

not demonstrate any difference in expression levels between 2 month old and 12 month old rats (p=0.795).

Figure 4.4 *Comparison of expression levels of Sirt5 in 2 month old and 12 month old AS/AGU rats.*

Box and whisker plot demonstrating the distribution of results and mean levels. Results from AS/AGU rat brains in four regions (cerebellum, brainstem, cerebral cortex and basal ganglia) at 2 months old and 12 months old are presented. Results significant at the 95% CI level ($p<0.05$) are marked with *, significance at the 99% CI level ($p<0.01$) is denoted with ** and highly significant results ($p<0.001$, 99.9% CI level) are denoted with ***.



4.3.2 Sirtuin 6 expression in rat brains

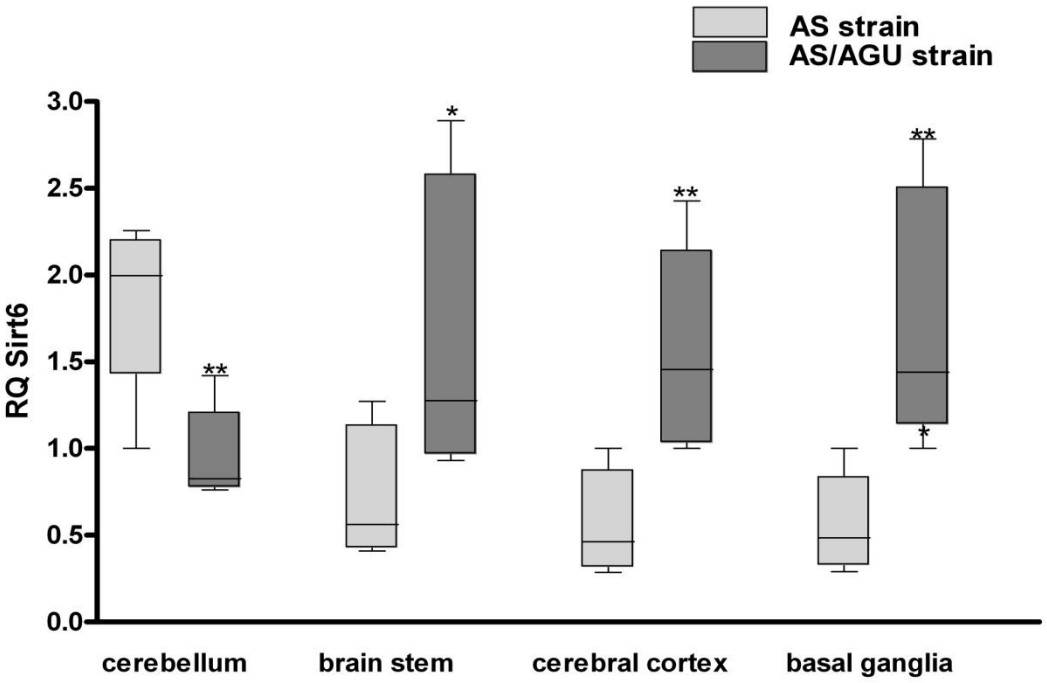
The levels of Sirtuin 6 were measured in the brains of both AS and AS/AGU rats, and compared at 2 months old and 12 months old.

4.3.2.1 Comparison of expression levels of Sirtuin 6 at 2 months old in both AS and AS/AGU rats

The levels of Sirt6 expression measured in regions of brain from AS and AS/AGU rats were graphed and compared (Figure 4.5). Levels were found to be significantly elevated in the brainstem (p=0.029), cerebral cortex (p=0.003) and basal ganglia (p=0.003) of the 2 month old AS/AGU rats when compared with AS rats of an equivalent age. This pattern was reversed with significantly higher expression in the AS rats found in the cerebellum brain region (p=0.003). Furthermore, the highest levels seen in the AS rats were found in this region, whilst the lowest levels from AS/AGU rats were seen here. Expression levels seen in the other three regions were similar, dependant on the strain of rat.

Figure 4.5 *Comparison of expression levels of Sirt6 in 2 month old rats of AS and AS/AGU backgrounds.*

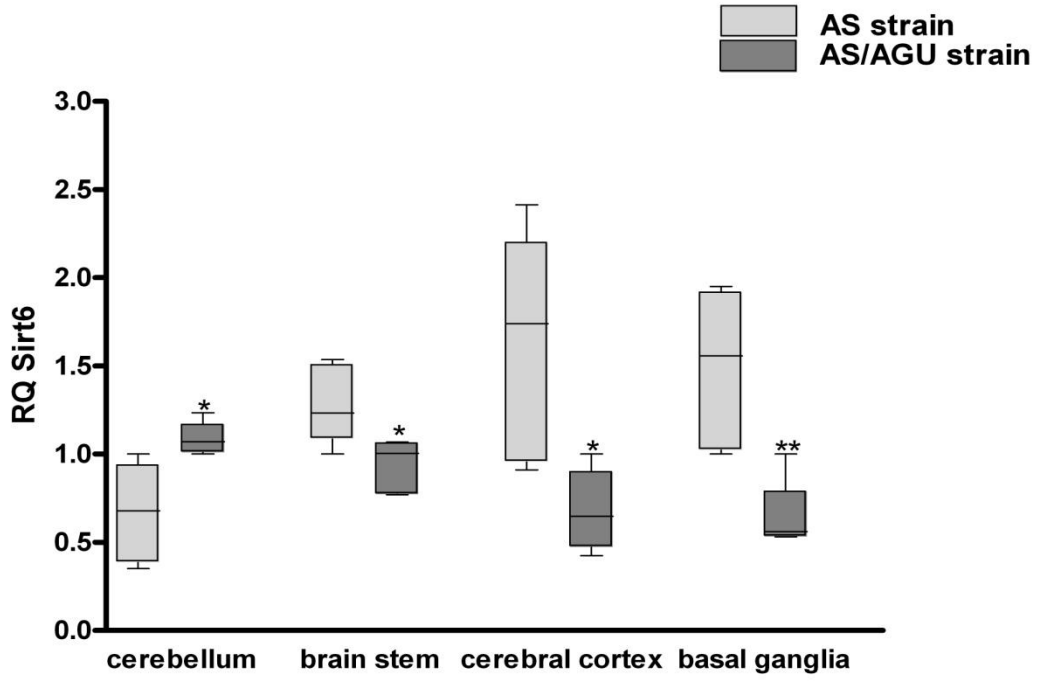
Box and whisker plot demonstrating the distribution of results and mean levels. Results from both AS and AS/AGU rat brains in four regions (cerebellum, brainstem, cerebral cortex and basal ganglia) at 2 months of age are presented. Results significant at the 95% CI level ($p<0.05$) are marked with * and significance at the 99% CI level ($p<0.01$) is denoted with **.



4.3.2.2 Comparison of expression levels of Sirtuin 6 at 12 months old in both AS and AS/AGU rats

Sirt6 levels of expression measured in the brains of 12 month old AS and AS/AGU rats were compared and graphed (Figure 4.6). The results demonstrate significantly lower expression levels found in AS/AGU rats in the brainstem (p=0.013), cerebral cortex (p=0.016) and basal ganglia (p=0.001), whilst higher expression is seen in the cerebellum (p=0.011). The cerebellum contains the lowest levels of Sirt6 expression seen in the brain regions tested of the AS rat, while levels in other areas are broadly similar. Levels in the AS/AGU rat at 12 months old appear to be similar in all brain regions.

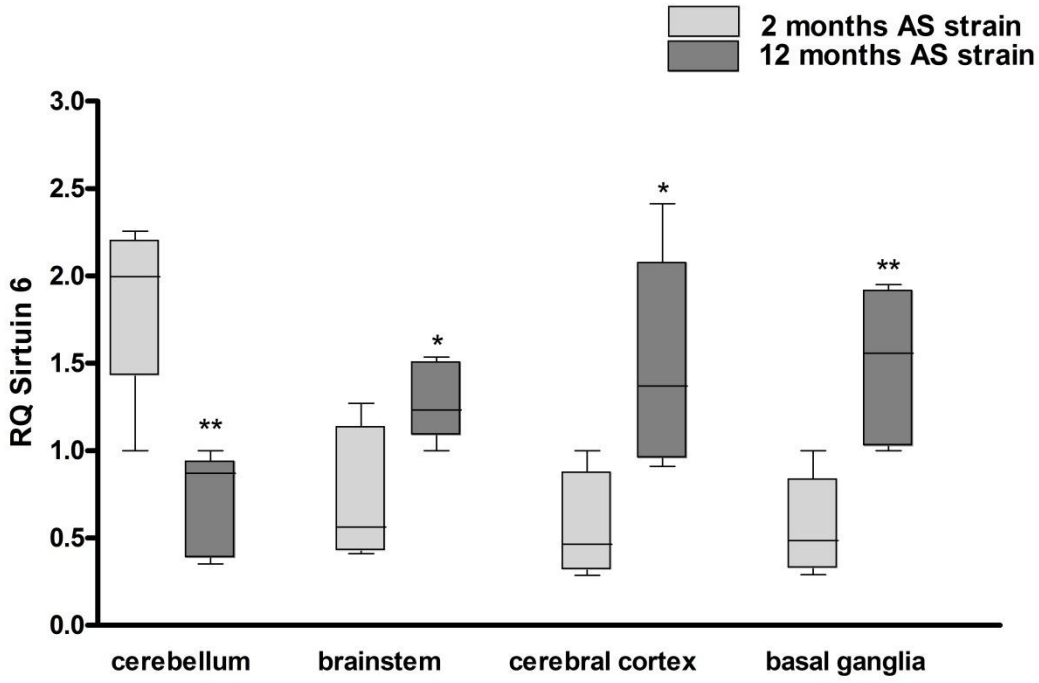
Figure 4.6 *Comparison of expression levels of Sirt6 in 12 month old rats of AS and AS/AGU backgrounds.*
Box and whisker plot demonstrating the distribution of results and mean levels. Results from both AS and AS/AGU rat brains in four regions (cerebellum, brainstem, cerebral cortex and basal ganglia) at 12 months of age are presented. Results significant at the 95% CI level ($p<0.05$) are marked with *.



4.3.2.3 Comparison of expression levels of Sirtuin 6 at 2 months old and 12 months old in AS rat brain regions

A comparison of the expression levels of Sirt6 in AS rats at two time points, 2 months and 12 months, revealed that expression in the Cerebellum (p=0.002), Brainstem (p=0.011), Cerebral Cortex (p=0.015) and Basal Ganglia (p=0.001) was significantly different (Figure 4.7).

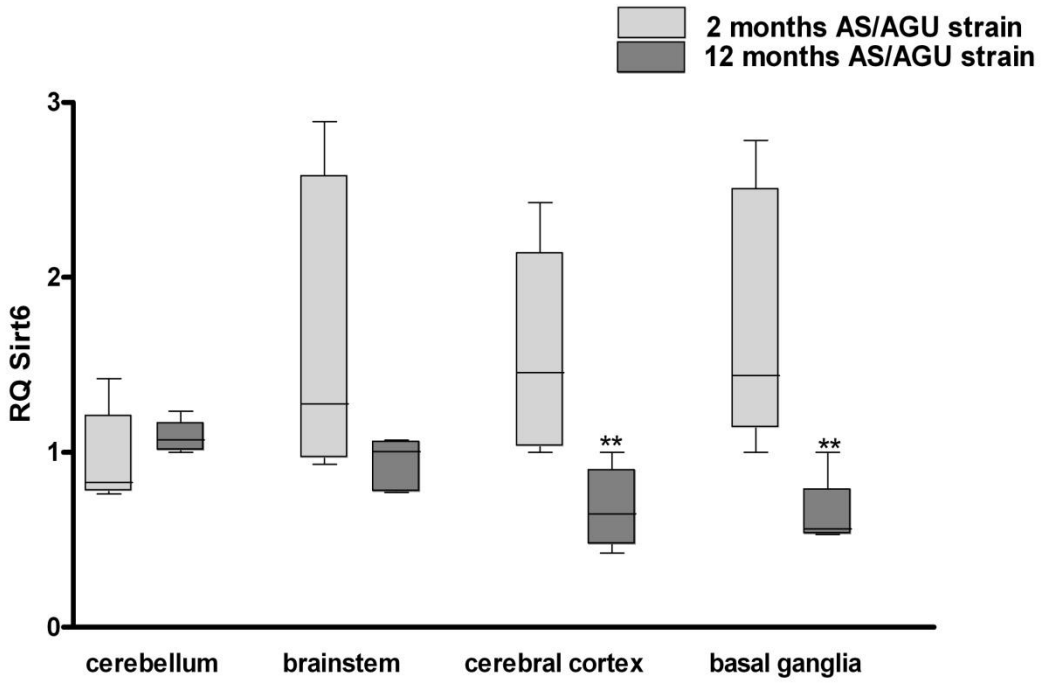
Figure 4.7 *Comparison of expression levels of Sirt6 in 2 month old and 12 month old AS rats.*
Box and whisker plot demonstrating the distribution of results and mean levels. Expression levels from AS rat brains in four regions (cerebellum, brainstem, cerebral cortex and basal ganglia) at 2 months and 12 months of age are presented. Results significant at the 95% CI level ($p<0.05$) are marked with * and significance at the 99% CI level ($p<0.01$) is denoted with **.



4.3.2.4 Comparison of expression levels of Sirtuin 6 at 2 months old and 12 months old in AS/AGU rat brain regions

Comparing the observed expression levels in AS/AGU rats at 2 months and 12 months (Figure 4.8) revealed that levels were generally lower in older rats, with the exception of the cerebellum although this was not significant (p=0.248). The lower expression seen in the cerebral cortex (p=0.009) and basal ganglia (p=0.004) was significant, however the reduction in the brainstem (p=0.071) was not.

Figure 4.8 *Comparison of expression levels of Sirt6 in 2 month old and 12 month old AS/AGU rats.*
Box and whisker plot demonstrating the distribution of results and mean levels. Results from AS/AGU rat brains in four regions (cerebellum, brainstem, cerebral cortex and basal ganglia) at 2 months old and 12 months old are presented. Results significant at the 95% CI level ($p<0.05$) are marked with * and significance at the 99% CI level ($p<0.01$) is denoted with **.



4.3.3 Sirtuin 7 expression in rat brains

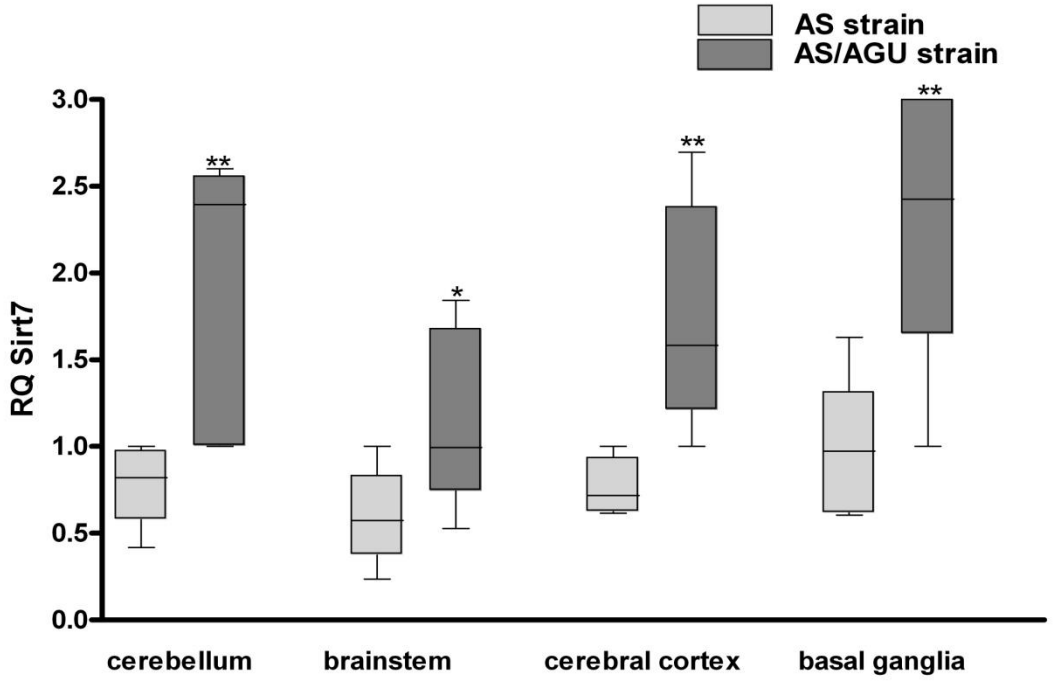
The levels of Sirt7 were measured in the brains of both AS and AS/AGU rats, and compared at 2 months old and 12 months old.

4.3.3.1 Comparison of expression levels of Sirtuin 7 at 2 months old in both AS and AS/AGU rats

Measured expression levels of Sirt7 in both AS and AS/AGU rats at 2 months of age were compared (Figure 4.9). Levels were found to be significantly higher in all brain regions of AS/AGU rats: cerebellum ($p=0.004$), brainstem ($p=0.03$), cerebral cortex ($p=0.003$) and basal ganglia ($p=0.008$).

Figure 4.9 *Comparison of expression levels of Sirt7 in 2 month old rats of AS and AS/AGU backgrounds.*

Box and whisker plot demonstrating the distribution of results and mean levels. Results from both AS and AS/AGU rat brains in four regions (cerebellum, brainstem, cerebral cortex and basal ganglia) at 2 months of age are presented. Results significant at the 95% CI level ($p<0.05$) are marked with * and significance at the 99% CI level ($p<0.01$) is denoted with **.

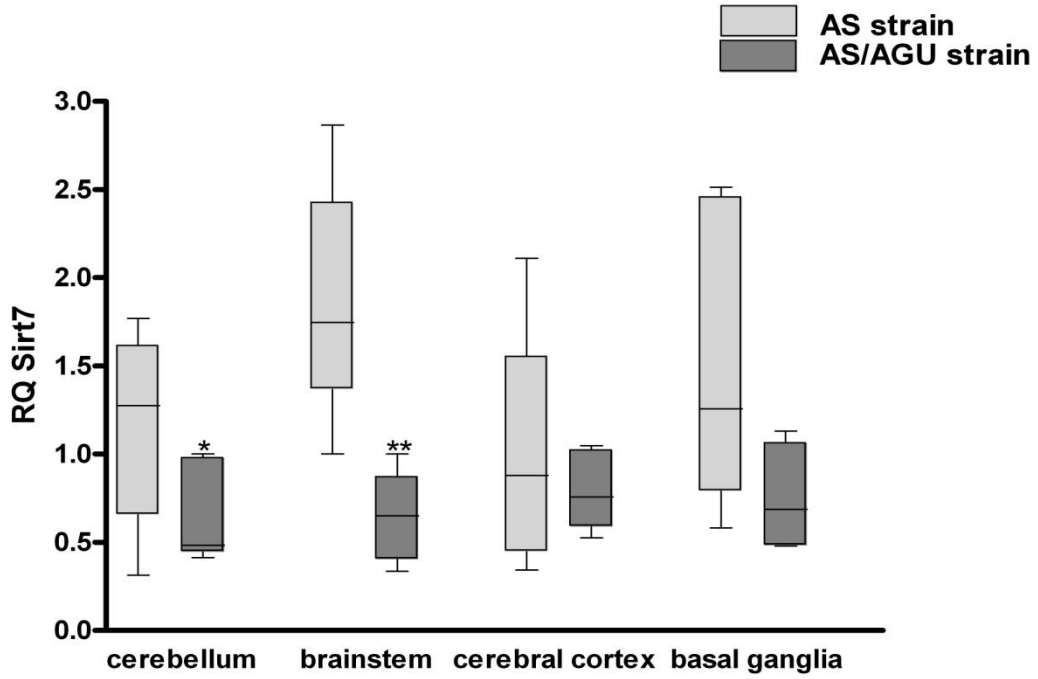


4.3.3.2 Comparison of expression levels of Sirtuin 7 at 12 months old in both AS and AS/AGU rats

The measured expression levels of Sirt7 at 12 months were compared between the two rat strains AS and AS/AGU (Figure 4.10). In general, levels in the AS/AGU rats were lower than those observed in the AS rats. However, this reduction was only significant in two regions: cerebellum ($p=0.039$) and the brainstem ($p=0.002$), it was not significant in the cerebral cortex ($p=0.537$) or the basal ganglia ($p=0.057$).

Figure 4.10 *Comparison of expression levels of Sirt7 in 12 month old rats of AS and AS/AGU backgrounds.*

Box and whisker plot demonstrating the distribution of results and mean levels. Results from both AS and AS/AGU rat brains in four regions (cerebellum, brainstem, cerebral cortex and basal ganglia) at 12 months of age are presented. Results significant at the 95% CI level ($p<0.05$) are marked with * and significance at the 99% CI level ($p<0.01$) is denoted with **.

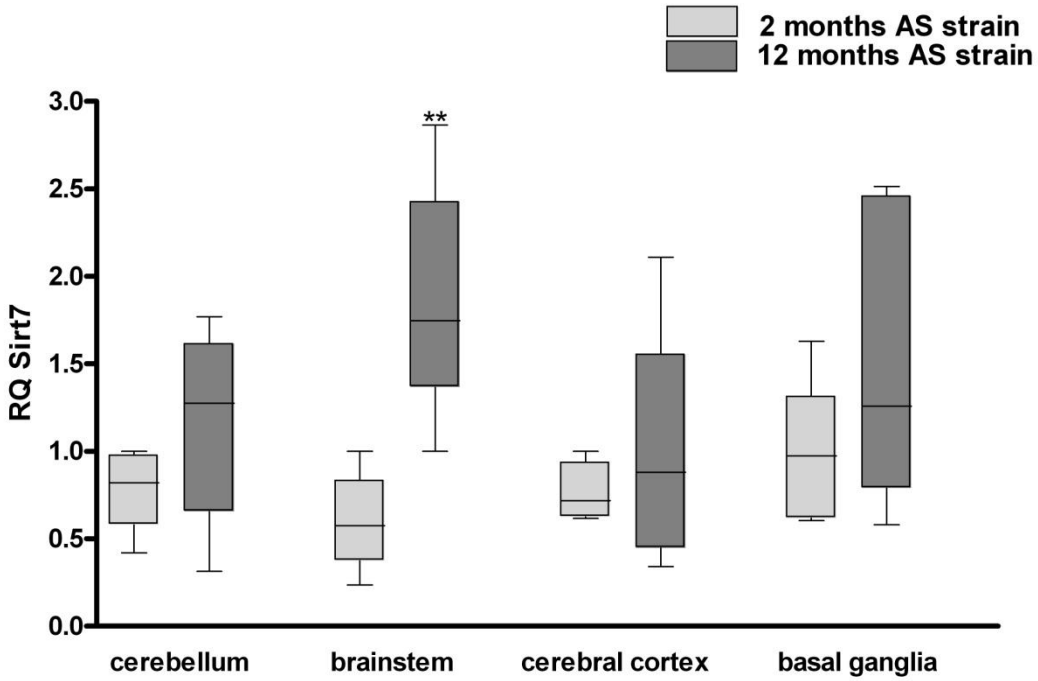


4.3.3.3 Comparison of expression levels of Sirtuin 7 at 2 months old and 12 months old in AS rat brain regions

A comparison of the expression levels of Sirt7 in both 2 month old and 12 month old AS rats was completed and graphed (Figure 4.11). Although levels were generally higher in the older rats, this was only found to be significant in the brainstem ($p=0.002$), which also contained the highest levels overall in older rats (12 month old) but one of the lowest levels in younger rats (2 months old). The other areas, cerebellum ($p=0.106$), cerebral cortex ($p=0.468$) and basal ganglia ($p=0.21$) were found not to be significantly different, although the cerebellum displayed a trend of increasing expression with age.

Figure 4.11 *Comparison of expression levels of Sirt7 in 2 month old and 12 month old AS rats.*

Box and whisker plot demonstrating the distribution of results and mean levels. Expression levels from AS rat brains in four regions (cerebellum, brainstem, cerebral cortex and basal ganglia) at 2 months and 12 months of age are presented. Results significant at the 95% CI level ($p<0.05$) are marked with * and significance at the 99% CI level ($p<0.01$) is denoted with **.

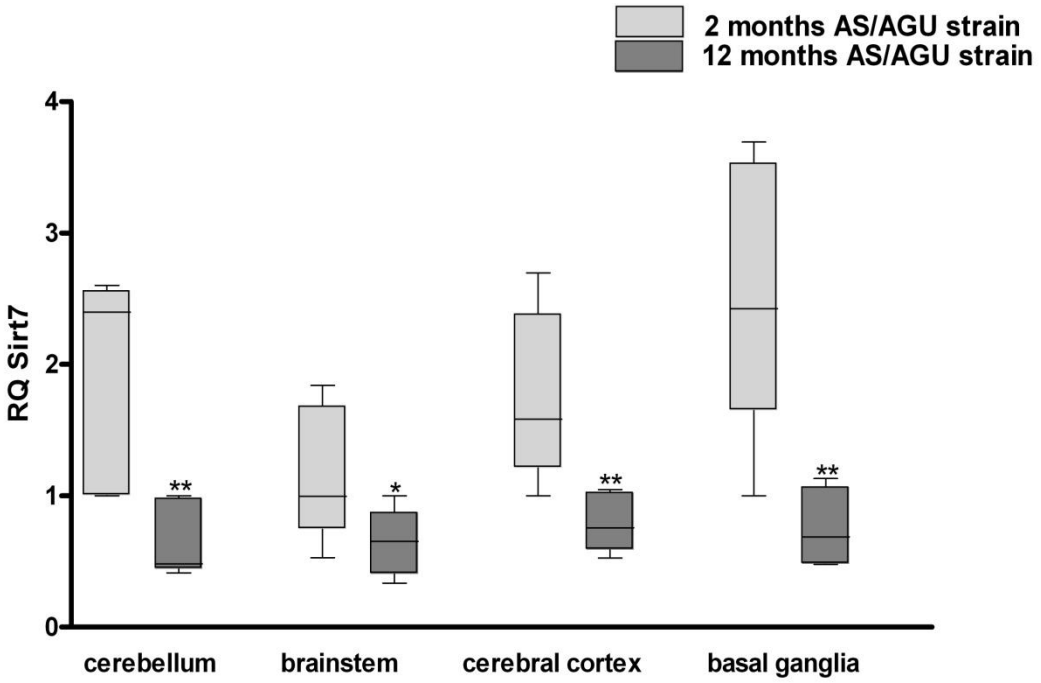


4.3.3.4 Comparison of expression levels of Sirtuin 7 at 2 months old and 12 months old in AS/AGU rat brain regions

Expression of Sirt7 in 2 month old and 12 month old AS/AGU rats was compared (Figure 4.12). Levels were found to be significantly reduced in older rats in all areas of the brain: cerebellum ($p=0.002$), brainstem ($p=0.041$), cerebral cortex ($p=0.004$) and basal ganglia ($p=0.001$). Levels in the 12 month old rats were consistently low in all regions and relatively ubiquitous throughout. Expression in younger rats appears to be more widely varied in addition to being higher, with the highest levels in the cerebellum and basal ganglia.

Figure 4.12 *Comparison of expression levels of Sirt7 in 2 month old and 12 month old AS/AGU rats.*

Box and whisker plot demonstrating the distribution of results and mean levels. Results from AS/AGU rat brains in four regions (cerebellum, brainstem, cerebral cortex and basal ganglia) at 2 months old and 12 months old are presented. Results significant at the 95% CI level ($p<0.05$) are marked with * and significance at the 99% CI level ($p<0.01$) is denoted with **.



4.4 Discussion

The results presented in this chapter demonstrate that the expression of all 3 Sirtuins measured here not only varies with age, but also shows highly distinctive differences between rat strains. These results are summarised in Table 4.2.

Table 4.2 Summary of Sirtuin Results

Table 4.2 summarises the results presented in this chapter as simple increase (green) or decrease (red), when comparing the first factor with the second in each case, with the significance marked with ns showing no significance, * representing p<0.05, ** denoting p<0.01 and *** showing significance p<0.001.

	AS 2 months vs 12 months	AS/AGU 2 months and 12 months	AS vs AS/AGU 2 months	AS vs AS/AGU 12 months
Sirt 5 Cerebellum	ns	ns	**	ns
Sirt5 Brainstem	ns	***	ns	*
Sirt5 Cerebral cortex	*	**	***	*
Sirt5 Basal ganglia	**	ns	*	*
Sirt 6 Cerebellum	**	ns	**	*
Sirt6 Brainstem	*	ns	*	*
Sirt6 Cerebral cortex	*	**	**	*
Sirt6 Basal ganglia	**	**	**	**
Sirt 7 Cerebellum	ns	**	**	*
Sirt7 Brainstem	**	*	*	**
Sirt7 Cerebral cortex	ns	**	**	ns
Sirt7 Basal ganglia	ns	**	**	ns

4.4.1 Sirt5 expression in the mammalian brain

The expression of Sirt5 mRNA in the various brain regions demonstrates significant and distinct differences not only due to age, but also due to strain. At both age points the expression pattern of Sirt5 mRNA appears to be opposite in the two rat strains. For example, at 2 months of age expression is high in the cerebellum, brainstem and basal ganglia of the AS rats, whereas low expression is seen in the AS/AGU rat in these areas. Furthermore where expression is lowest in the AS rats, in the cerebral cortex; it is highest in the AS/AGU rats. This contrasting nature of expression between the strains is repeated in the 12 month old rats. However, the pattern was altered such that low expression was now seen in the brainstem and basal ganglia in the AS rats and high expression is seen in the AS/AGU strain. The cerebellum appears to be the only region that does not fluctuate, with high expression of Sirt5 in AS rats and low in AS/AGU rats at both ages. The cerebral cortex, at 12 months, demonstrates high levels of expression in the AS rats with low expression levels in the AS/AGU strain. Interestingly, the cerebellum shows no significant changes in expression levels between 2 month and 12 month old AS or AS/AGU rats; consistent with the maintenance of the pattern alluded to above. This also suggests that, although there is significantly different expression between strains in the cerebellum, Sirt5 levels do not fluctuate greatly in this brain region in response to age. This stable state for Sirt5 possibly reflects a constant demand for energy, furthermore the differences between the strains is most likely a factor of the PKCγ mutation in the AS/AGU rats.

The brainstem demonstrates a significant increase in expression in the AS/AGU rats between the 2 month and 12 month time points, but no significant changes in the AS strain across the same time frame. The cerebral cortex shows changes in opposite directions with an increase in expression between 2 months and 12 months in the AS strain and a decrease in the AS/AGU rats. The switch in pattern for the basal ganglia appears to be as a result of a significant decrease in levels between 2 months and 12 months in AS rats, with no significant change in AS/AGU rats. These notable differences are not unheard of when comparing

animals, one study investigating the effect of breed using a fatty breed and a lean breed of pig on the levels of Sirtuins in the brains demonstrated that expression of all Sirtuins varied significantly, some increased and some decreased. However, this study also demonstrated that the levels of all Sirtuins decreased with age in both breeds (Ren, *et. al.*, 2013). This appears to be consistent with the AS strain, although a direct comparison is not possible without measuring total expression throughout the brain at both time points and determining whether it increases or decreases with age. However, this appears to be opposite to the situation found in the AS/AGU strain, although similarly to the AS strain total expression levels would be required to demonstrate this conclusively.

Taking the AS strain alone, levels of Sirt5 appear to lower as the rats age, with the notable exception of a large increase in the cerebral cortex. By contrast the AS/AGU rats show a different pattern, with levels dropping in the cerebral cortex and rising in both the brainstem and basal ganglia. Interestingly, research into Neural stem cells (NSCs) and a v-myc transformed NSC line derived from mouse cerebral cortex demonstrated significantly lower levels of Sirt5 in the transformed cell line, linking lower expression with cancer (Wang, *et. al.*, 2012a). This, taken with the lower levels in AS/AGU rats and higher levels in AS rats, may suggest a malfunction in Sirt5 regulation in AS/AGU rats. This malfunction however is likely to be a direct or indirect effect of the PKCγ mutation, suggesting that PKCγ is capable of influencing or altering the activities of Sirt5. However this would require further investigation, for example detection of succinylation/malonylation post-translational modifications of proteins. These experiments would have to be done in conjunction with Sirt5 levels to determine whether the PKCγ mediated effects, or lack thereof, are truly responsible for the modulation of Sirt5's behaviour. Sirt5 levels in the spinal cord have been linked to ALS (Korner, *et. al.*, 2012). However, it must be noted that neither increased susceptibility to cancer nor the occurrence of ALS has been noted or demonstrated in the AS/AGU rat model. However, investigation of Sirt5 polymorphisms in humans has suggested that decreased expression may promote PD and HD through mitochondrial dysfunction (Glorioso, *et. al.*, 2011). This is consistent with

very low levels of expression seen in the cerebral cortex of the AS/AGU rats at 12 months. The Parkinsonian phenotype associated with these rats (Payne, *et. al.*, 2000) may also be a result of the lowered Sirt5 expression levels seen here. This argument is strengthened by the increase in levels seen in the relatively healthy AS strain across the same time frame. These reports and the results presented here appear to suggest that Sirt5 levels in the brain are highly localised and specific with deviation up or down resulting in increased risk of disease, which is dependent on localisation. This could be investigated using specific Sirt5 agonists and antagonists, for example suramin (Schuetz, *et. al.*, 2007), to increase or decrease levels in specific areas of the brain.

In order to investigate the effects of age properly with respect to Sirt5 a third time point would be required, either in between for example 6 months or outside the longer time point, for example 15 months. This would allow a more focussed comparison to determine the pattern of changes due to ageing in both strains. It would also allow a more accurate comparison between the levels of Sirt5 and the onset/severity of symptoms in the AS/AGU rat. The current dataset is difficult to interpret with respect to age alone as the patterns of expression are opposite at both time points and not consistent with a linear deployment due to age, rather these data points would appear to indicate that expression of Sirt5 may demonstrate a bell curve of expression with levels rising during the first few months after birth then dropping with increased age. This hypothesis is supported by the high early levels seen in pigs (at 1 month), which drops as they age (Ren, *et. al.*, 2013), however this is at odds with the AS control rats which start low at 2 months and rise to 12 months and is more consistent with the pattern seen in the AS/AGU rats. Further evidence is provided by the lower levels of Sirt5 seen in transformed NSCs (Wang, *et. al.*, 2012a) which, in general, have unhinged themselves from the ageing process, or found a way to counter it, suggesting that lower levels are seen in very young cells. It is important to note that although Sirt5 is related to age and in particular to longevity, it is a highly responsive mitochondrial protein which drives the stress/starvation response. Therefore some of the variation demonstrated here may be due to dietary issues including feeding times, variety of diet

and calorie intake. It is also interesting to note at this juncture that AS/AGU rats have a slower growth rate and a lower final body weight that the AS control rats; however, no metabolic or feeding studies have, as yet, been conducted with these animals. Additionally, the effects of other mitigating factors cannot be discounted and it may be that the levels of Sirt5 are highly influenced by the disruption of the dopaminergic system via the PKCγ mutation and therefore these strains may not truly be comparable; the third time point suggested earlier would also aid in determining whether this is a root cause of the variation or not. Alternatively localising the expression of Sirt5 and determining whether it is primarily mitochondrial or cytosolic (Park, *et. al.*, 2013) may also allow determination of the cause of the variation.

4.4.2 The expression pattern of Sirtuin 6 found in the rat brain

Similar to Sirt5 expression, the pattern of distribution of Sirt6 mRNA levels appears to be opposite when comparing AS to AS/AGU rats, at both age points. Even though the levels switch between the age points, the opposing nature of expression is continued. At 2 months, AS rats demonstrate highest expression in the cerebellum, whereas AS/AGU rats demonstrate low expression in the same region. In the brainstem, cerebral cortex and basal ganglia, levels are low in AS rats and high in AS/AGU rats, all of these differences are significant at the 95% CI level.

At 12 months the situation is almost completely reversed, with AS rats having low levels in the cerebellum, where levels are highest in the AS/AGU rat. High levels in the brainstem, cerebral cortex and basal ganglia in the AS rat are countered with low levels in the AS/AGU rats. Once again, all of these differences were significant. However, all of these changes were significant in the AS rats indicating that transcription of Sirt6 fundamentally changes between the age points. Significant reductions were seen in the cerebral cortex and basal ganglia in AS/AGU rats, this indicates that the shift in expression patterns was driven by an increase in the cerebellum of AS/AGU rats, a reduction in the brainstem of AS rats and a

combination of reductions in the cerebral cortex and basal ganglia of the AS/AGU strain, with concurrent increases in the same regions in AS rats.

In the case of Sirt6, it would appear that the AS/AGU rats reduce levels of mRNA as the animals age; this is consistent with previous reports that levels of Sirt6 reduce with age (Ren, *et. al.*, 2013), However levels rise in three of the four areas in AS rats, possibly indicating that these animals are still maturing and have not reached the decline phase, this is consistent with increased expression of NADH hydrolysis gene sets with age in mice (Zahn, *et. al.*, 2007). Expression of Sirt6 is predominantly localised to neurons and not glial cells and decreases in response to ischaemia (Lee, *et. al.*, 2013). Increased levels are seen in transformed NSCs (Wang, *et. al.*, 2012a). Like Sirt5 levels, this would appear to suggest that neuronal cells in AS/AGU rats are highly disrupted, however it should be noted again that increased tumourigenesis has never been demonstrated in this model. Furthermore reduced levels in the brains of a diabetes mouse model were found to correlate with onset of T2D, and it was further proposed that this may also result in diabetes-induced neurodegeneration (Favero, *et. al.*, 2013). This report is consistent with the age-related status of T2D. Specific neuronal deletion of Sirt6 has been shown to promote obesity, as well as attenuate somatic growth, due to modulation of chromatin structures in neurons (Schwer, *et. al.*, 2010). This may suggest a mode of neural degeneration in the AS/AGU model, where levels are low at an early age resulting in slow neural development. The expression of Sirt6 mRNA may be even more complicated by the discovery of splice variants, one of which was present in the brain of pigs (Jin, *et. al.*, 2009); splice variants may represent different functions for this gene *in vivo*.

Although there is little direct evidence for the influence of Sirt6 in neuroprotection, its interactions with Sirt1 heavily implicate it in the aetiology of several neurodegenerative disorders including AD, HD and PD. Sirt6 can mediate the function of peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1α (PGC1α) by deacetylating the general control non-repressed protein 5 which regulates PGC1α (Beauharnois, *et. al.*, 2013); these proteins have been implicated in

the development and progression of PD. This role for Sirt6 in neurodegenerative disorders is reinforced by studies demonstrating lower levels of Sirt6 are present in mouse models of HD which can be counteracted by treatment with Rosiglitazone, a PPARγ agonist (Jin, *et. al.*, 2013). Once again, the lower levels of Sirt6 present in AS/AGU rats is consistent with these reports and suggests that lower levels of this Sirtuin are related to neurodegenerative disorders, including the Parkinsonian phenotype of the AS/AGU rats. Furthermore, this indicates that Sirt6 may exhibit a neuroprotective function in the brain. These findings are consistent with the role of Sirt6 in resistance to DNA damage and stress, both key factors in age-related diseases (Beauharnois, *et. al.*, 2013).

The data presented in this thesis are also consistent with abnormal neurometabolism in the AS/AGU brain with age, as Sirt6 mediates glycolytic metabolism. Lower levels of Sirt6 may indicate aerobic glycolysis, while higher levels indicate aerobic metabolism via the TCA cycle. In mitochondria damage is likely occurring with age in both the AS/AGU and the AS animals, albeit at different rates. This damage could be measured by qPCR (Hunter, *et. al.*, 2010; Santos, *et. al.*, 2006). It has been shown that Sirt6 rapidly responds to changes in diet, primarily due to stabilisation of protein and not through increased transcription (Kanfi, *et. al.*, 2008), however if this is continued long term it is reasonable to assume that a consistent change in diet would lead to altered transcription. Furthermore these data imply that SIRT6 is not upregulated in AS/AGU rats, perhaps because the pathology observed in these animals has a major mitochondrial component. Of note a dopaminergic specific phenotype could result from dopamine being trapped at synapses, unable to be exported in vesicles, with mitochondria concentrated well away from the nucleus and thus nuclear DNA. Therefore the ROS being generated by the mitochondria, being unable to be repackaged due to lack of vesicle transport, would primarily attack the mitochondrial DNA and ultimately destroy the mitochondria. This potential mechanism would explain both Sirt5 and Sirt6 levels in the AS/AGU rats, it would also suggest that the levels of these Sirtuins is driven by PKCγ, determination of damage to nuclear and mitochondrial DNA would answer this question.

4.4.3 Pattern of expression for Sirtuin 7 in the mammalian brain

Although the opposing pattern of expression seen in Sirt5 and Sirt6 is not apparent for Sirt7, the trend of levels moving in opposite directions with age is still present. Given that levels of Sirtuins generally decline with age, the rising levels seen in AS rats may represent a low level early in life which rises to a peak and then falls off with age. This may be due to a general increase in ribosome biogenesis throughout early life indicating healthy growth, followed by a decline in old age related to senescence and accumulated oxidative stress damage. This would be testable with older time points to determine if this is true.

Levels of Sirt7 consistently decrease significantly with age in all areas of the brain in the AS/AGU strain, whereas general increases are seen in the AS rats during the same time span; however, this is only significant in the brainstem. This may indicate a general decline in ribosome biogenesis as these rats age, this would be consistent with an accelerated ageing phenomenon. Furthermore, this is also consistent with the MTR theory for ageing, where ribosome biogenesis would decline in older animals as they become more damaged by oxidative stress and increase the levels of cellular senescence. Levels in the AS/AGU strain are significantly higher in all areas at the 2 month age point; whereas they are lower at the 12 month age point – only significant in the cerebellum and brainstem. Again this fits with the theory that these rats demonstrate an accelerated ageing phenotype from an early age. This early increase in Sirt7 indicates a higher rate of ribosomal biogenesis and thus a higher rate of living.

Decreasing levels in the AS/AGU rats in association with age is consistent with current understanding of the role of Sirt7 in ageing (Ghiraldini, *et. al.*, 2013), furthermore it may be a factor in the shortened life expectancy of these rats, as this has been demonstrated in mice (Vakhrusheva, *et. al.*, 2008b). While the data presented here for AS rats is not immediately consistent with these reports, and suggests an opposing rise in Sirt7 levels between 2 months and 12 months, it is consistent with

the levels demonstrated for Sirt5 and Sirt6 which suggests that these Sirtuins are found at low levels very early on in life, increase and then begin their decline into old age. However, more age points would be required to confirm this hypothesis. Additionally testing using agonists and antagonists may be informative here, by using agonists to drive levels very early or very late in life it may be possible to extend the lifespan of these rats. Similarly the use of antagonists in the central point of the lifespan may have detrimental effects.

The role of Sirt7 in ageing has been established in mouse models, where inactivation of the Sirt7 gene resulted in reduced lifespan (Vakhrusheva, *et. al.*, 2008b) and Sirt7 levels were associated with ageing in normal BALB/c mice (Ghiraldini, *et. al.*, 2013). Although there is little information on the expression or role of Sirt7 in the brain, the information that has been presented suggests that it will decrease with age (Ren, *et. al.*, 2013). It will also be lower in tumour cells compared with normal (Wang, *et. al.*, 2012a) and expression of Sirt7 has been inversely correlated with the tumourigenic potential of mouse derived cell lines (Vakhrusheva, *et. al.*, 2008a). These reports are consistent with reducing levels of Sirt7 in the brain with age and the status of cancer as a disease of ageing. This apparent role for Sirt7 in tumourigenesis in the brain is however contradictory to the current understanding of the role of Sirt7 in tumourigenesis where lower levels have been linked to inhibition of tumour growth (Tsai, *et. al.*, 2013a) and raised levels are associated with some cancers for example breast cancer (Ashraf, *et. al.*, 2006) and hepatocellular carcinoma (Kim, *et. al.*, 2012). Moreover, the deacetylation action of Sirt7 acting on Lysine 18 of Histone 3 (H3K18) has been shown to be critical for stabilising tumour cells (Barber, *et. al.*, 2012). An interesting point with these reports is that they all concern mitotic cells and not post-mitotic cells, therefore the situation in the brain may be unique. In fact the only data that has been reported for the brain suggests that lower levels are associated with tumourigenesis. The data presented here is consistent with the levels of other Sirtuins (5 and 6, also presented here) which suggest that levels in the AS/AGU rat, particularly at the advanced – 12 month – age point, may promote the development of diseases associated

with ageing. Once again, however, there have been no investigations which have demonstrated this in the AS/AGU rat model.

The role of modulating ribosomal RNA (rRNA) structures has been demonstrated in neurodegenerative disorders (Hwang, *et. al.*, 2013). This involves the Upstream Binding Factor (UBF), which has been demonstrated to interact with Sirt7 (Grob, *et. al.*, 2009). Furthermore, the interactions between Sirt7 and RNA Polymerase 1 (RNA pol 1) (Chen, *et. al.*, 2013) clearly implicate it in the regulation of rRNA structures and production (Tsai, *et. al.*, 2012). Regulation by the cell cycle machinery, the CDK1-cyclin B pathway during normal mitotic cell division function (Grob, *et. al.*, 2009), combined with findings that loss of Sirt7 results in replicative senescence (Kiran, *et. al.*, 2013), suggests that levels in the brain should be low or non-existent. However the findings here that Sirt7 is indeed expressed suggest that Sirt7 may either be constitutively active in post-mitotic or senescent cells or may be further regulated by other mechanisms in areas with high levels of post-mitotic cells, e.g. the brain.

4.4.4 Sirtuin expression in the mammalian brain

An interesting disparity between the data presented here and the current literature exists; low levels of Sirt6 in the brains of AS/AGU rats may disrupt metabolism and has been shown to promote obesity in other models. However, the AS/AGU rats present with much lower body weight than the AS control group which may result from the higher levels of Sirt6 seen at the earlier age point (2 months). The low levels of Sirt6 at the later age point and disrupted metabolism may in turn be a driver for Sirt5 level increases, as dietary input is processed differently due to altered Sirt6 expression. This highlights the interplay between these proteins and suggests a larger network of interactions which lie behind the differences between these rat strains. Furthermore this is also indicative of the complexity of neurological disorder phenotypes; in particular in this case it suggests that at least these three Sirtuins, and more than likely others, are heavily involved in the parkinsonian phenotype found in the AS/AGU rats.

Experiments involving the measurement of Sirtuins with and without the administration of L-Dopa may be more informative in this regard.

It must also be noted that dopamine involvement in appetite and CR has been previously demonstrated (Fernandes, *et. al.*, 2013; Hansen, *et. al.*, 2013; Manuel-Apolinar, *et. al.*, 2013). Given the involvement of the Sirtuins in CR it is possible that the disruption in dopamine levels observed in AS/AGU rats (Campbell, *et. al.*, 1996) may be responsible for higher than normal levels of Sirtuins, particularly in the brain. Furthermore, this disruption in levels becomes more evident with age, particularly after 6 months (Campbell, *et. al.*, 1997). This would ultimately lead to disruption of diet, resulting in differences in calorie intake. These changes might result in varied Sirtuin expression particularly at the 12 month age point, thus providing an explanation for the broad differences between the two related strains at the later time point and the opposing nature of expression seen. This phenomenon may also explain the high Sirtuin levels seen at early time points in the AS/AGU strain as calorie modulation in the mother's diet has been demonstrated to result in increased variation of dopamine receptor expression, with CR leading to increased numbers of receptors to promote weight gain post-natal (Manuel-Apolinar, *et. al.*, 2013). Given the disruption in dopamine signalling seen in AS/AGU rats and the observed slow weight gain it is possible that the increases in Sirt5 are a biological attempt to rebalance the metabolism of the AS/AGU rats.

Interestingly, direct roles in the modulation of nutrient usage, storage or uptake have been demonstrated for both Sirt5 and Sirt6 indicating the potential for variable levels of these Sirtuins to drive a change in diet or be induced by a change. Moreover, mice deficient in Sirt7 have been shown to develop chronic hepatosteatosis, similar to fatty liver disease in humans (Shin, *et. al.*, 2013). This introduces the possibility that much of the variation demonstrated here may be as a result of varied nutrient uptake and usage. In addition to this the role of Sirt1 in the regulation of diet via Ghrelin, which regulates weight gain (Karra, *et. al.*, 2013), is well established (Velasquez, *et. al.*, 2011; Wang, *et. al.*, 2012b), as is its role in the regulation of diet via the hypothalamus (Satoh, *et. al.*,

2010) and the interaction between Sirt1 and Sirt6 provides further weight to the argument that diet plays a key role in the levels of Sirtuins seen in the brains of the AS/AGU rats. However, it is not possible from this data set to demonstrate whether this is the case, or whether these changes are a result of an already modified appetite, via dopamine modulation, or whether the changes are the result of normal ageing. The most likely answer is a mixture of both but without additional measurements and an additional time point it is impossible to state at this juncture.

Interestingly, diet has been clearly linked to epigenetic status, in particular methylation (Lillycrop, *et. al.*, 2008). The results presented here with the Sirtuins suggest that there may also be epigenetic disruption in association with the accelerated ageing phenotype. In fact, there are studies which demonstrate that the maternal diet influences the epigenetic status of the offspring (Cooney, *et. al.*, 2002). Furthermore, the disruption of DA may indicate that there is altered calorie intake in the maternal rats, potentially indicating a disruption in methylation patterns in newborn AS/AGU rats. In this regard, epigenetic status and/or changes may be the key to the accelerated ageing phenotype seen in the AS/AGU rats. Measurement of methylation levels would be critical to understanding whether this is true or not.

Measuring dopamine levels or the level of dopamine receptor expression alongside levels of Sirtuins would provide more information on the influence of diet on the levels of Sirtuins in the brains of the AS/AGU rats and may provide a greater insight into the highly variable response seen here. Combining this with a third time point and monitoring of the physical abnormalities found in this strain (Campbell, *et. al.*, 1996) at each time point would provide invaluable information into the interaction of the dopaminergic system and the Sirtuins in both the development of the Parkinsonian phenotype and ageing or cellular senescence.

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5. Senescence associated β-galactosidase (SA β-gal) expression in the brains of AS and AS/AGU rat brains

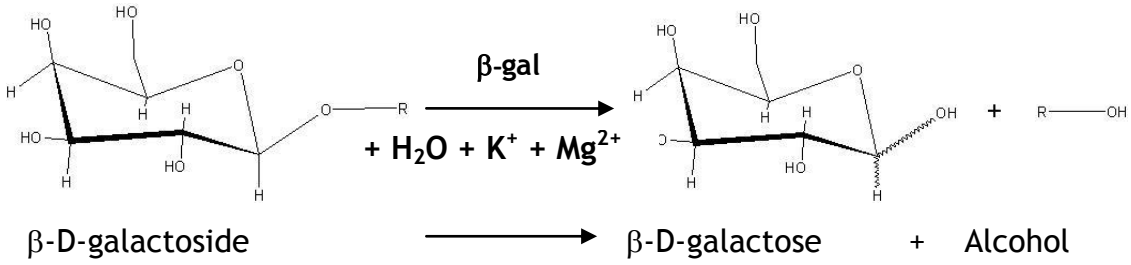
5. Senescence associated β-galactosidase (SA β-gal) expression in the brains of AS and AS/AGU rat brains

5.1 Introduction

5.1.1 β-galactosidase

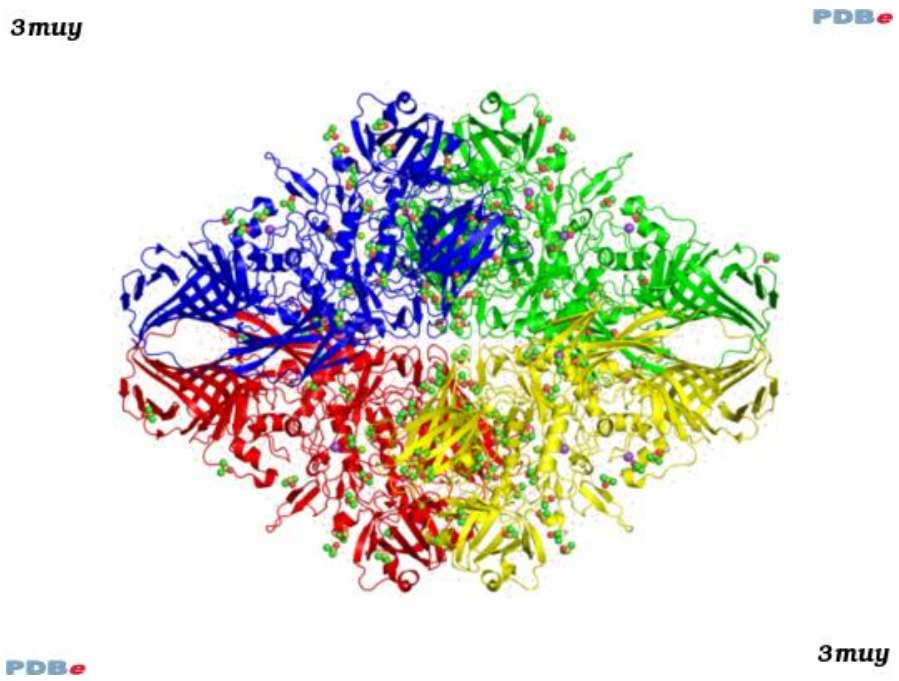
Also known as beta-gal or β-gal, the β-galactosidases are a class of enzymes involved in the hydrolysis of the β-glycosidic bond between a galactose (Gal) residue and another sugar residue (Figure 5.1). Targets of β-galactosidases include lactose, lactosylceramides, glycoproteins and gangliosides, although galactopyranosides have also been demonstrated to be useful substrates in the detection of β-gal activity (Buller, *et. al.*, 2003; Groth, *et. al.*, 1998; Martin, *et. al.*, 1996; Zhang, *et. al.*, 1991) both *in vitro* and *in vivo*. In *E. coli* the β-gal gene, *lacZ*, is part of the inducible *lac* system and becomes active when glucose levels are low but lactose is present. Deficiencies or disruptions in β-gal function have been attributed to lysosomal storage diseases including galactosialidosis (Koike, *et. al.*, 2008) and Morquio's syndrome (B variant) (Groebe, *et. al.*, 1980; Ishii, *et. al.*, 1995).

Figure 5.1 Cleavage of the glycosidic bond by β-gal. Schematic drawing indicating the reaction substrate, direction of reaction, additional components and reaction products. Magnesium (Mg) and Potassium (K) ions are required for maximum efficiency of the reaction although they are not essential.



Structural studies in *E. coli* have determined that the enzyme forms a tetramer with 3 other identical copies of itself (Appel, *et. al.*, 1965; Kalnins, *et. al.*, 1983), a structure that is essential to normal function. Each individual enzyme consists of 5 domains with the third domain containing the enzymatic active site (Figure 5.2). The enzymatic hydrolysis reaction has an optimal pH of 7.2, and the presence of Potassium (K) and Magnesium (Mg) cations have also been demonstrated to enhance the reaction efficiency.

Figure 5.2 The tetrameric structure of the *E. coli* LacZ protein. Image obtained from the Protein Data Bank in Europe (PDBe) (available at: http://www.ebi.ac.uk/pdbe-srv/view/entry/3muy/summary); PDB accession: 3MUY. Structure published by Dugdale *et. al.* (Dugdale, *et. al.*, 2010). Each monomeric unit is presented in a different colour.



β-gal is commonly used as a reporter gene in molecular biology to monitor transfected gene expression levels (Serebriiskii and Golemis, 2000). Its usefulness in molecular biology extended further with the discovery of α-complementation where the gene is split into two sequences each producing a peptide, denoted LacZα and LacZΩ; when expressed together in the same transfected cell these will produce a functional β-gal, but separately they are

non functional. This has been used as the basis for blue/white colony screening for plasmid transfection into *E.coli* (Fung, *et. al.*, 2006). Bacteria are grown on agar plates containing X-gal which produces a blue colour when cleaved by functional β-gal, giving a clear indication of successfully transfected colonies.

5.1.2 Senescence associated β-galactosidase (SA β-gal)

In 1995 Dimri *et. al.* identified what they believed to be a new isoform of β-gal which was detectable at pH 6.0, unlike other isoforms which were readily detectable at pH 4.2 and not at pH 6.0 (Dimri, *et. al.*, 1995). This novel activity is associated with senescent cells and it appeared to increase with age in human skin samples from people of different ages. This novel biomarker was termed senescence associated β-galactosidase (SA β-gal) (Dimri, *et. al.*, 1995; Gorbunova, *et. al.*, 2002), and new assays for its detection were implemented. It has now been shown that this activity is attributable to lysosomal β-gal accumulation (Lee, *et. al.*, 2006) and is accompanied by an increase in lysosomal mass (Kurz, *et. al.*, 2000), which is activated in response to several different cellular stresses (Severino, *et. al.*, 2000). Despite some claims that this renders the assay non-specific (Krishna, *et. al.*, 1999), it is still widely used and is deemed by most to be accurate (Satyanarayana and Rudolph, 2004). Reports continue to demonstrate the association between SA β-gal and ageing (Melk, *et. al.*, 2003). However, it has been demonstrated in astrocytes that SA β-gal accumulation during senescence is dependent on the presence of functional pRb and that there may be a $p21^{WAF1}$ senescence pathway that is independent of SA β-gal expression (Fanton, *et. al.*, 2001). The most commonly used assay for *in situ* detection of SA β-gal is based on its ability to cleave the X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) galactopyranoside substrate at pH 6 (Dimri, *et. al.*, 1995), although other methods have been proposed using flow cytometry (Kurz, *et. al.*, 2000), chemiluminescence (Bassaneze, *et. al.*, 2008) and fluorogenic substrates (Gary and Kindell, 2005; Yang and Hu, 2004).

5.1.3 SA β-gal expression in the brain

Most of the information available on SA β-gal expression in the brain results from its use as a senescence marker, where it is used to determine whether a factor induces cellular senescence or not. For example, beta amyloid drives expression of p16INK4a and SA β-gal, indicating a role for senescent astrocytes in the aetiology of AD (Bhat, *et. al.*, 2012). While this information is not immediately useful it does suggest that this marker is not ubiquitously expressed. It can be predicted that β-gal expression would increase overall in brain as it ages; this is due to the association of increasing levels of gangliosides during differentiation and their decrease in ageing, this decrease being the result of increased β-gal activity (Aureli, *et. al.*, 2011). In fact, it has been demonstrated that levels of SA β-gal can increase rapidly with age in the hippocampus of normal rats (Geng, *et. al.*, 2010). It has also been shown that levels of senescent neurons, and thus abundance of SA β-gal increases with age (Jurk, *et. al.*, 2012). Furthermore, due to this increasing expression with age SA β-gal, is rapidly emerging as a potentially valuable marker in the investigation of neurodegenerative diseases; however no direct associations between its expression and occurrence of neurodegenerative diseases have thus far been discovered.

The work in this chapter sought to investigate overall levels of SA β-gal to confirm increased senescence with age. Given the highly accepted nature of this marker it provides a solid grounding for the interpretation of other data presented in this thesis. For example, it serves to confirm and validate the findings for both CDKN2A and the Sirtuins by demonstrating an increase in cellular senescence in conjunction with increased biological ageing and its associated metabolic stress/DNA damage.

5.2 Materials and methods

5.2.1 Animals used

The animals used for these experiments were raised and sacrificed in accordance with all regulations, both national and local, and all animal husbandry/operations were performed by a qualified Home Office licence holder. Both strains of rat were fed a standard diet, with drinking water provided as required. Animals were reared and housed in the Joint Research Facility, University of Glasgow under standardised conditions – light/dark cycle 12/12 hours, temperature 22°C +/- 2°C, humidity 50% +/- 5% in plastic-metal cages.

A total of twenty four rats, six for each of the four experimental groups, were used. The experimental groups used were AS rats at 2 months old, AS rats at 12 months old, AS/AGU rats at 2 months old and AS/AGU rats at 12 months old.

Sacrificed animals had their brains excised and rapidly frozen in Isopentane using liquid Nitrogen; these were then placed in a cryomold, clearly labelled and stored in Isopentane at -80°C until required.

5.2.2 Immunohistochemical staining for SA β-gal

Slides were prepared as previously described (Chapter 2: Materials and Methods, section 2.3.3). The required slides were removed from 4°C and allowed to warm to RT before processing. They were then rehydrated in PBS (Gibco, Life Technologies, Paisley, UK) for 10 minutes.

5.2.2.1 Preparation of specific pH solutions

pH-specific base solutions were prepared by mixing 450ml of citric acid (400mM; Chapter 2: Materials and Methods 2.1.9) and 450ml of sodium phosphate (400mM; Chapter 2: Materials and Methods 2.1.10). The pH 4 base

solution was then made by taking half of this mix (450ml) and adjusting the pH to 4.0 by the addition of concentrated hydrochloric acid (to lower the pH) or concentrated sodium hydroxide (to increase the pH). The total volume was then made up to 500ml using a mixture of equal volumes citric acid (400mM) and sodium phosphate (400mM). The pH 6 base solution was made in the same manner using the other 450ml of citric acid/sodium phosphate and adjusting the pH to 6.0 using concentrated hydrochloric acid or sodium hydroxide.

To make 10ml of the final pH specific solutions 1 ml of each of these solutions was mixed with 7.45ml of PBS (Gibco, Life Technologies, Paisley, UK), 200μl of x-gal (50mg/ml stock solution; Sigma Aldrich, Dorset, UK), 125μl potassium ferricyanide (400mM; Chapter 2: Materials and Methods 2.1.5), 125μl potassium ferrocyanide (400mM; Chapter 2: Materials and Methods 2.1.6), 100μl magnesium chloride (200mM; Chapter 2: Materials and Methods 2.1.7), 1ml sodium chloride (1.5M; Chapter2: Materials and Methods 2.1.8)

5.2.2.2 SA β-gal staining

Slides were carefully dried and the tissue section was circled with a hydrophobic barrier pen (DAKO Delimiting pen: S200230-2; DAKO, Ely, UK). 200μl of the appropriate final pH specific solution was added to each slide (pH6 for test slides and pH4 for positive controls). Slides were then incubated for 48 hours.

Slides were then washed in running water to remove the SA β-gal staining solutions, and counterstained by immersion in Nuclear Fast Red solution (Chapter 2: Materials and Methods 2.1.11) for 30 seconds. Slides were again washed in running water.

Slides were dehydrated by immersing in 70% ethanol for 1 minute, then 90% ethanol for 1 minute followed by 2 immersions in 100% ethanol

each for 1 minute and finally 2 immersions in xylene each for 1 minute. Slides were then mounted with cover slips using DPX histology mounting medium (Sigma-Aldrich, Dorset, UK).

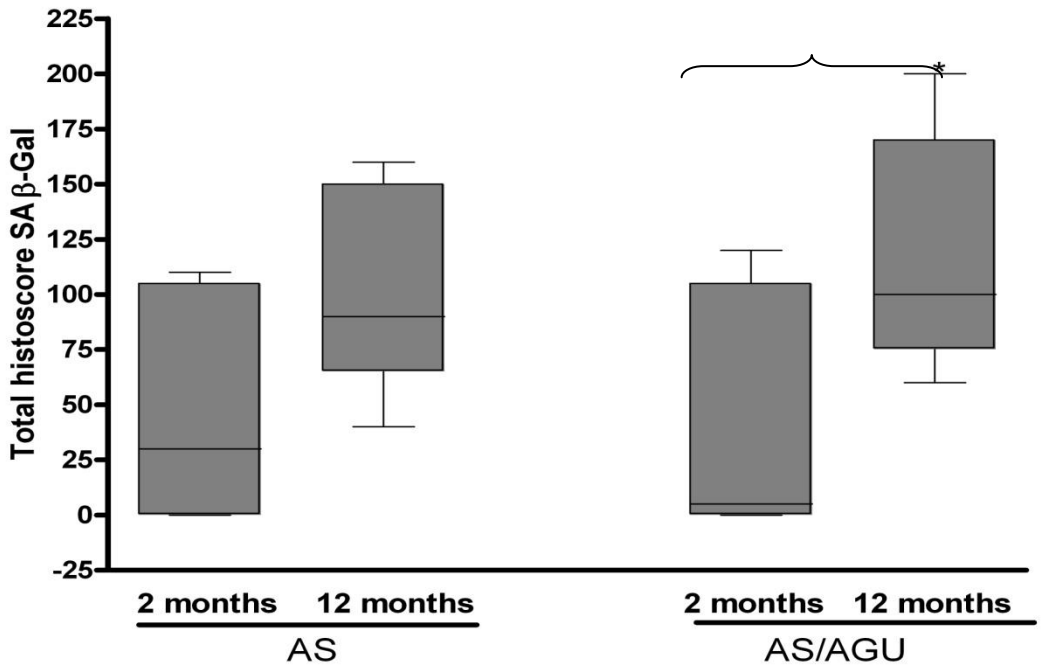
The number of positive cells was then evaluated using a microscope and a histoscore for each slide. This was calculated by counting the number of positive cells compared to the number of negative cells in several visual fields. The percentage of positive cells was calculated and averaged for each slide before being collated and presented as a mean for each experimental group.

5.3 Results

5.3.1 SA β-Gal expression in the brains of AS and AS/AGU rats aged 2 and 12 months

Expression levels of SA β-Gal were measured in para-sagittal sections of rat brains from both strains (AS and AS/AGU) at two time points, 2 months and 12 months (Figure 5.3). A significant difference in the protein expression levels of SA β-Gal in the brain was noted between 2 month and 12 month old AS/AGU rats ($p=0.025$); a trend was observed in AS rats but this was not quite significant at the 95% CI level ($0=0.066$). Additionally, no significant differences were noted between the strains at the different time points: 2 months ($p=0.791$) and 12 months ($p=0.627$).

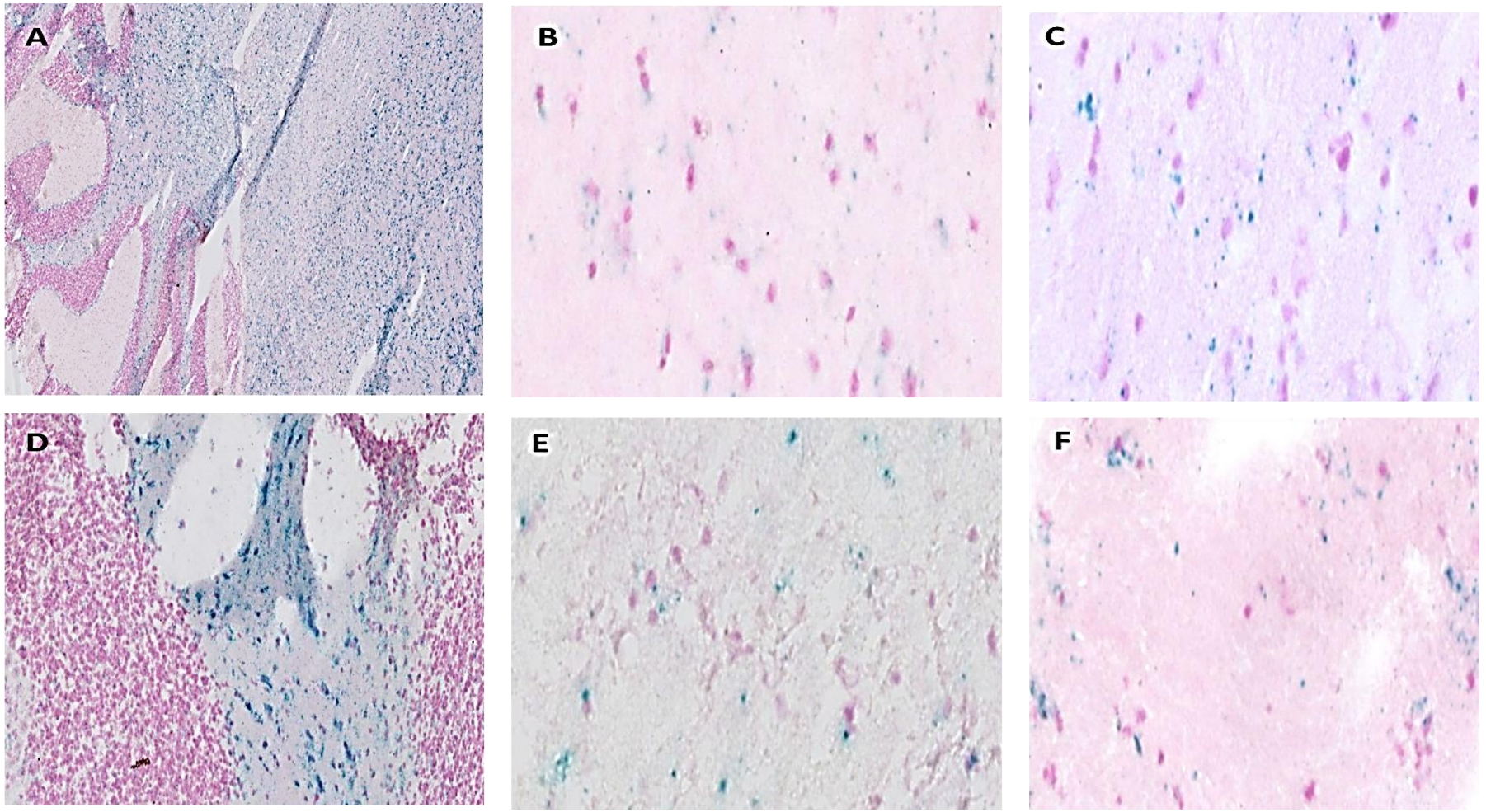
Figure 5.3 Senescence-associated β-galactosidase (SA β-gal) levels seen in sagittal sections of brain from AS and AS/AGU rat strains collected at 2 and 12 months of age. A significant difference in protein expression levels was observed between SA β-Gal level in AS/AGU brain sections between 2 and 12 months old rats (p=0.025). Significance at the 95% CI level is denoted by *.



5.3.2 Immunohistochemical analysis of SA β-Gal expression in rat brains

Immunohistochemical analysis of SA β-Gal protein expression was conducted in the para-sagittal cut brain sections of both rat strains at two time points (2 months and 12 months). Example staining is shown in Figure 5.4, demonstrating differences seen between the overall expression levels seen between the two points in both strains. This staining also shows the control groups at pH4 (Figure 5.4 panels A (AS rats) and D (AS/AGU rats) demonstrating normal β-Gal activity compared with the specific activity of SA β-Gal seen at pH6 in AS rats (Figure 5.4, panels B and C) and AS/AGU rats (Figure 5.4, panels E and F).

Figure 5.4 Immunohistochemical staining for Senescence Associated β galactosidase (SA β-Gal) activity at 100x magnification. A:12 months AS pH4 (control). B: 2months-AS pH6. C: 12months-AS pH6. D: 2months AS/AGU pH4 (control).E: 2months-AS/AGU pH6. F: 12months-AS/AGU pH6. Quantification of SA β-Gal staining was performed using automated slide-path method aided by a microscope equipped to quantify the amount of indigo-blue staining within the cells (cytoplasm) in whole rat brain frozen brain section cut para-sagittally. Red staining indicates presence of SA β-Gal.



5.4 Discussion

5.4.1 Expression pattern of SA β-gal found in the brains of AS and AS/AGU rats

The expression of SA β-gal in both rat strains appears to rise with age, but this is only significant in the AS/AGU rat. This trend is consistent with the current literature and understanding of ageing in the mammalian brain (Geng, *et. al.*, 2010; Jurk, *et. al.*, 2012). Interestingly, the differences between rat strains at each time point are non-significant indicating no real overall difference between the strains at each time point. However, the significant rise in AS/AGU rats suggests that the increase in expression levels between the two time points found in these rats is greater than the increase seen in AS rats. This would appear to indicate that levels rose faster in the AS/AGU rats, potentially indicating an increased rate of senescence and thus ageing in the brains of these AS/AGU rats.

This marker is purely a marker of senescence (Dimri, *et. al.*, 1995), but has become well established as a successful biomarker, particularly in post-mitotic tissues. It is not required for senescence, but is a result of the process making it an excellent marker for the presence of senescent cells (Lee, *et. al.*, 2006). In this regard it would appear that AS/AGU rats have increased cellular senescence levels over the 10 month period examined here. A lack of power in the experiment may be at fault for no significant differences being seen in the AS rats. Further time points, monitoring of DA levels and observation of phenotypic variance would result in a more informative dataset, it may also allow investigation of the relationships between SA β-gal expression and dopamine, ageing or the Parkinsonian phenotype of the AS/AGU rats.

The results presented here in conjunction with previous results, particularly CDKN2A expression serve to confirm the accelerated ageing phenotype found in the AS/AGU rats. This data also confirms that cellular

senescence is concurrent with the ageing process, measured by CDKN2A, indicating that accelerated ageing is a direct result of increased cellular senescence and not apoptosis of neurons. In this regard, these results suggest that measurement of overall grey or white matter volume is unlikely to be a useful measurement for the level of ageing in the brain. However, as volume loss is apparent in the brain with advancing age it does suggest that the combination of senescence and apoptosis may combine to give an additive effect in regards to neurodegeneration associated with ageing.

It has been shown that senescent cells produce high levels of ROS and contain higher levels of DNA damage due to this oxidative stress (Chen, *et. al.*, 1995b; Song, *et. al.*, 2005). In conjunction with the Sirtuins these results confirm that the changes in expression of the Sirtuins are most likely associated with cellular senescence and thus increasing age. Furthermore, this suggests that increased oxidative stress and DNA damage is the driving force behind the accelerated ageing. This also indicates that the increase in CDKN2A, Sirtuins and SA β-gal are all driven by accelerated biological ageing and not directly by the PKCγ mutation in the mutant AS/AGU rats.

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6. Global DNA Methylation levels in AS and AS/AGU rats at two time points (2 months and 12 months).

6. Global DNA Methylation levels in AS and AS/AGU rats at two time points (2 months and 12 months).

6.1 Introduction

DA may disrupt diet, however more importantly the aberrant expression of the Sirtuins may influence diet and clearly demonstrates a disruption in metabolism and nutrient storage/uptake in the AS/AGU rats. This disruption in the metabolic state suggests that there may be a critical impact on epigenetic markers. For these reasons global DNA methylation levels were investigated here to determine whether significant differences could be seen due to either the accelerated ageing phenotype or the metabolic disruption evident in AS/AGU rats.

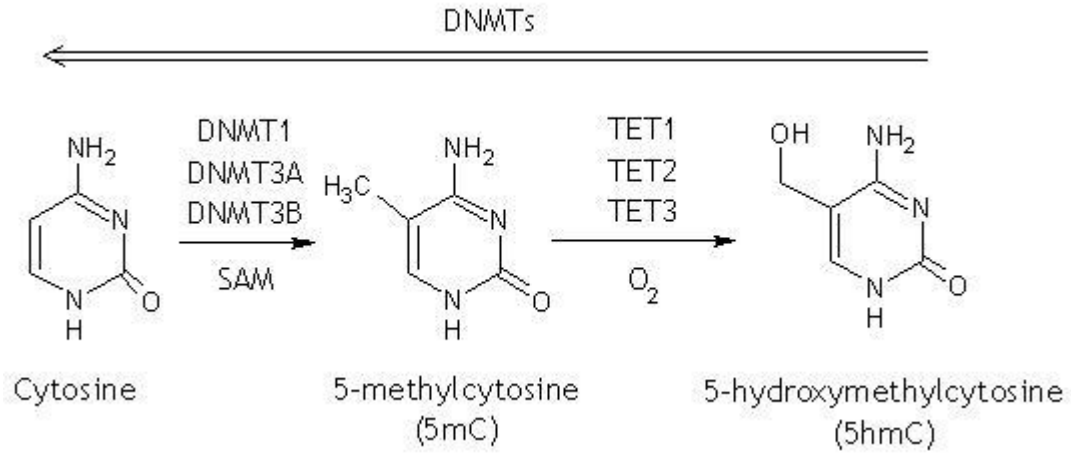
6.1.1 DNA methylation

Methylation of DNA is rapidly emerging as a critical epigenetic modification, with far reaching implications. Originally believed to be a stable, inheritable and long-term form of gene silencing it now appears that DNA methylation levels can adjust quickly in response to several factors (Wu and Zhang, 2010). In fact methylation combined with histone modifications tightly control storage and expression of the information encoded by eukaryotic DNA (Portela and Esteller, 2010). This relatively simple chemical alteration to the DNA structure is implicated in epigenetic regulation of many genes. It has been clearly demonstrated that maternal diet can have far reaching effects for progeny in terms of epigenetic markers (Dominguez-Salas, *et. al.*, 2014; Marco, *et. al.*, 2014). For example, people conceived just before or during the Dutch Hunger Winter (1944-1945) have now been demonstrated to have significantly lower DNA methylation on specific genes than same-sex siblings who were not exposed to this event (Heijmans, *et. al.*, 2008). Furthermore, the influence of diet and other socioeconomic factors have also been shown to influence global DNA methylation levels (McGuinness, *et. al.*, 2012). These variations in levels have been shown to coincide with increased risk for age related diseases, for example CVD (McGuinness, *et. al.*, 2012). It has also been

shown that global DNA methylation can be used to demonstrate ageing and/or senescence (Issa, 2014; Jin, *et. al.*, 2014; Sidler, *et. al.*, 2014).

This epigenetic modification of DNA, catalysed by the DNA methyltransferases (DNMTs) in the presence of S-adenosyl methionine (SAM) (Goll and Bestor, 2005), occurs specifically at the Carbon 5 position on phosphorylated Cytosine (Cp) residues found in Dinucleotide pairs with Guanosine (G), i.e. CpG. This occurs most frequently in sites which contain high levels of CpG repeats, also known as CpG islands (Figure 6.1) (Dahl, *et. al.*, 2011). The DNMTs fall into two categories, *de novo* (DNMT3A and DNMT3B) which establish the pattern of DNA methylation during embryogenesis (Okano, *et. al.*, 1999) and maintenance (DNMT1) which replicates the methylation status of DNA during mitosis by favouring hemi-methylated DNA (Hermann, *et. al.*, 2004). Further modification of 5-methylcytosine (5mC), by Ten-eleven translocation (TET) proteins in the presence of Oxygen (Iyer, *et. al.*, 2009; Tahiliani, *et. al.*, 2009), results in 5-hydroxymethylcytosine (5hmC) which may facilitate demethylation of the Cytosine residue (Figure 6.1). Approximately 1% of all bases in mammalian genomes appear to be 5mC, which is around 4% of the cytosine content of DNA resulting in 75% of CpGs being methylated in humans (Dahl and Guldberg, 2003). Levels of 5hmC have been shown to be highest in neurons (Globisch, *et. al.*, 2010). Several active mechanisms have been presented which may be responsible for demethylation of 5mC and 5hmC (Ooi and Bestor, 2008; Wu and Zhang, 2010), including enzyme activity, base excision repair (BER) and deamination. However, the most likely candidates appear to be the DNMTs themselves (Kangaspeska, *et. al.*, 2008; Metivier, *et. al.*, 2008).

Figure 6.1 *Cytosine methylation and demethylation*. Chemical schemata of the methylation, modification and demethylation of Cytosine residues by DNMTs and TET proteins (Dahl, *et. al.*, 2011; Kangaspeska, *et. al.*, 2008; Metivier, *et. al.*, 2008).



6.1.2 Physiological and pathological influence of DNA methylation

The pattern of DNA methylation is established during embryonic development, in a tissue specific manner which is believed to influence cellular differentiation in tissues (Meissner, 2010). Therefore, disruption of this pattern can have serious implications for the organism. In fact disruption of DNMT function has been shown to be lethal during embryogenesis or shortly after term (Okano, *et. al.*, 1999). Furthermore, many disease pathologies have now been linked to aberrant methylation patterns or disruption of the methylation machinery. For example, the neurodevelopment disorder, Rett syndrome, which causes mental retardation in females has been linked to a sex linked (X chromosome) mutation in the gene encoding methyl-CpG-binding protein 2 (MeCP2) (Amir, *et. al.*, 1999). Genetic imprinting disorders are linked to both hypo and hyper-methylated sequences found in imprinting centres (ICs) within the genome. These ICs contain several genes which become methylated during embryonic development; aberrant methylation here has been demonstrated to be responsible for a variety of disorders. These include two diseases with opposing clinical features from the same IC, firstly Beckwith-Wiedemann syndrome (BWS) (Lim and Maher, 2010), which results

in overgrowth either pre or post-natally with a variety of clinical pathologies (Cooper, *et. al.*, 2005; Elliott, *et. al.*, 1994); Secondly, Silver-Russell syndrome (SRS) (Abu-Amero, *et. al.*, 2008) which demonstrates growth retardation. It has been shown that these opposing phenotypes can be the result of opposing epimutations with hypomethylation at the *H19* differentially methylated region (DMR) found in 60% of SRS cases and hypermethylation at the same site in 5% of BWS cases (Lim and Maher, 2010). Transient neonatal diabetes mellitus, occurring within the first 6 weeks post-natal and disappearing by 18 months, is also caused by hypomethylation at another DMR which contains the *PLAGL1* gene (Lim and Maher, 2010). Furthermore, mutations in DNMT3B are responsible for immunodeficiency, centromere instability and facial anomalies, also known as ICF syndrome (Xu, *et. al.*, 1999), it is characterised by low levels of serum antibodies which usually results in death by infection at a young age.

Methylation is heavily linked to tumourigenesis, with a general loss of methylation globally resulting in hypomethylation but with highly localised increases (hypermethylation) of specific loci (Jones and Baylin, 2007). For example, hypermethylation of gene trap locus 2 (GTL2) promoter or the associated DMR has been shown in neuroblastoma, phaechromocytoma, Wilm's tumours (WT) (Astuti, *et. al.*, 2005) and pituitary adenomas (Gejman, *et. al.*, 2008; Zhao, *et. al.*, 2005). Additionally, hypermethylation of the PLAGL1 promoter has been linked to several forms of cancer including breast (Bilanges, *et. al.*, 1999), ovarian (Kamikihara, *et. al.*, 2005), pituitary adenomas (Pagotto, *et. al.*, 2000) and basal cell carcinomas (Basyuk, *et. al.*, 2005). However some tumours have been linked to hypomethylation, for example loss of imprinting, i.e. expression of both alleles at once due to hypomethylation, on the *IGF2* gene has been linked a broad range of sporadic embryonic and adult onset cancers (Jelinic and Shaw, 2007), as well as demonstrating specific ties to ovarian (Kamikihara, *et. al.*, 2005), colorectal (Kaneda and Feinberg, 2005) cancers and WT (Yuan, *et. al.*, 2005). Disruption of the methylation machinery has also been discovered in myeloid malignancies, where TET2 was found to be mutated in many cases (Abdel-Wahab, *et. al.*, 2009). Although whether these altered methylation patterns are a result or a

driver of tumourigenesis is still the subject of some debate (Baylin and Bestor, 2002). It has also been proposed that age-related methylation changes are highly similar to those found in cancer, leading to the possibility that changes in methylation are the drivers behind the increased cancer risk associated with ageing (Ahuja and Issa, 2000). Mapping of a large number of CpG sites (1505) in human samples revealed that there was a progressive hypermethylation in promoter based CpG islands and hypomethylation in non-CpG island promoters (Fernandez, *et. al.*, 2011).

6.1.3 Environmental influences on DNA methylation

The influence of environmental factors on methylation patterns has been confirmed by studies comparing methylation patterns in monozygotic twins. Young twins show a large degree of homozygosity in terms of DNA methylation pattern, whereas older twins demonstrated a much higher level of heterogeneity (Fraga, *et. al.*, 2005). Another Icelandic study measuring global DNA methylation levels in the same population on average 11 years apart demonstrated less than 10% difference in 70% of 111 individuals, the remaining 30% demonstrated more than 10% changes in DNA methylation levels (Bjornsson, *et. al.*, 2008). This study confirms not only that DNA methylation changes over time but that it changes at different rates, this is most likely attributable to environmental influences. This variation appears to be restricted to very specific loci, a comparison of blood and buccal cells across a period of several years demonstrated significant variation in 3 of 8 examined sites (Talens, *et. al.*, 2010). Examination of two imprinted sites (IGF2/H19 and IGF2R) over 20 years demonstrated no significant alterations in methylation pattern (Sandovici, *et. al.*, 2003).

In terms of dietary effects folate (Jacob, *et. al.*, 1998); (Rampersaud, *et. al.*, 2000); (Pufulete, *et. al.*, 2005a) and alcohol ((Choi, *et. al.*, 2009a); (Zhu, *et. al.*, 2010)) have both been demonstrated to modulate DNA methylation levels via the one carbon metabolism system (Choi, *et. al.*, 2009b). Physical exercise has also been linked to methylation of specific sites (Nakajima, *et. al.*, 2010); (Alibegovic, *et. al.*, 2010), although some express doubts over these connections due to lack of data.

Tentative data also exists showing that specific sites maybe related to obesity (Gemma, *et. al.*, 2010), however one large scale study found no relationship between body mass index and global methylation of repeat elements (Zhu, *et. al.*, 2010). Oestrogen intervention in post-menopausal women in a small pilot study (n=8) led to increased global methylation (Friso, *et. al.*, 2007).

DNA methylation levels have been shown to be lower in smokers than non-smokers in leukocytes (Monick, *et. al.*, 2012) and the mucosa of the colon (Pufulete, *et. al.*, 2005b). Other environmental factors have also be linked to fluctuations in methylation levels for example ionising radiation and some carcinogens (Mathers, *et. al.*, 2010); (Pavanello, *et. al.*, 2009).

6.1.4 Role of DNA methylation in ageing

DNA methylation has been demonstrated to change in accordance with increased age in general (Boyd-Kirkup, *et. al.*, 2013) and at specific CpG sites (Issa, 2000). This has been demonstrated in several sites including endometrium (Kim, *et. al.*, 2005b), colon (Yatabe, *et. al.*, 2001) and the small intestine (Kim, *et. al.*, 2005a). In general, it has been accepted that ageing induces global hypomethylation, with specific hypermethylation (van Otterdijk, *et. al.*, 2013). Additionally, it has been demonstrated that global methylation levels are negatively correlated with measurements of frailty in the over 65s (Bellizzi, *et. al.*, 2011). The direct measurement of age by DNA methylation is confounded by the discovery that global methylation levels vary according to mitochondrial DNA (mtDNA) haplotype, in particular haplotype J is different from non J haplotypes (Bellizzi, *et. al.*, 2012). In fact, to demonstrate the utility of DNA methylation in determining age, a model measuring 353 CpG sites has been proposed that interprets methylation state in multiple tissue types as a measure of ageing (Horvath, 2013). However, it has been demonstrated that some sites do not demonstrate increases in methylation due to ageing in non-mitotic tissues (Chu, *et. al.*, 2007). Despite this studies in the mouse hippocampal region have revealed an age-related increase in 5hmC levels which is independent of oxidative stress and is not associated with increased expression of the TET enzymes (Chen, *et. al.*, 2012). Interestingly, it has been shown that

levels of 5hmC decrease in mitochondrial DNA found in the frontal cortex in association with age, but not in the cerebellum (Dzitoyeva, *et. al.*, 2012), indicating that different regions of the brain age differently with respect to DNA methylation. This suggests that, at least in terms of DNA methylation, non-mitotic tissues do not exhibit normal ageing characteristics; this is consistent with these cells being senescent. Some believe that epigenetic drift, caused by errors in the maintenance of epigenetic marks as a result of ageing or senescence, leads to various pathologies including cancer (Issa, 2014). This is certainly supported by a growing body of evidence underlining the role of 5mC (Esteller, 2005), 5hmC (Tan and Shi, 2012), DNMTs (Li, *et. al.*, 2013a) and TET proteins (Bian, *et. al.*, 2014) in the development and progression of tumours.

The relationship between DNA methylation and diseases of ageing underscores the direct link between this epigenetic modification and the progression of time. From cancer (Esteller, 2005) to neurodegenerative disorders (Jakovcevski and Akbarian, 2012; Lu, *et. al.*, 2013), methylation is rapidly becoming the critical genomic link that was missing. Other diseases which are now understood to be related to age have also been tied to aberrant DNA methylation, for example heart disease (Duygu, *et. al.*, 2013), metabolic syndrome (Bruce and Cagampang, 2011) and autoimmune disorders (Grolleau-Julius, *et. al.*, 2009).

6.1.5 Role of DNA methylation in the brain

It is generally accepted that DNA methylation is critical for development in the embryo (Reik, *et. al.*, 2001; Wossidlo, *et. al.*, 2011); this is particularly true in the brain (Lister, *et. al.*, 2013). A methylation map has now been published charting the changes in methylation patterns during different stages of brain development in mice and humans (Lister, *et. al.*, 2013). The information gleaned from this study illustrates the role of methylation in directing and controlling brain development. This map also allows for new research into neurodegenerative diseases by comparing changes in methylation patterns. Studies have already began to show this in four major neurodegenerative disorders: AD, PD, HD and ALS (Lu, *et. al.*,

2013), as well as others (Urdinguio, *et. al.*, 2009). Other studies measuring methylation of CpG sites have confirmed this by demonstrating specific methylation fingerprints in neurological disorders (Fernandez, *et. al.*, 2011). In fact consistent changes in methylation patterns, involving hypermethylation of specific genomic regions resulting in silencing of three genes (*TBXA2R*, *SORBS3* and *SPTBN4*), have been found in both mice and humans in relation to AD (Sanchez-Mut, *et. al.*, 2013). In general it has been found that there is a general hypomethylation associated with AD, but with specific sites hypermethylated (Sung, *et. al.*, 2011). However, one recent study has demonstrated overall global hypermethylation, when incorporating both 5mC and 5hmC, and that these levels were specifically raised in neurons (Coppieters, *et. al.*, 2014). α-synuclein, the main component of lewy bodies which are responsible for the PD pathology, has also been shown to be related to DNA methylation albeit in studies of alcoholism (Bonsch, *et. al.*, 2005). This link has now been established directly in Parkinson's disease (Jowaed, *et. al.*, 2010). Furthermore, hypomethylation was detected in the substantia nigra of Parkinson's disease patients, post mortem (Matsumoto, *et. al.*, 2010); reduction of nuclear DNMT1 levels was also observed in post-mortem brain samples (Desplats, *et. al.*, 2011). Another potential influence of methylation on the aetiology of Parkinson's disease may be related to hypomethylation of the tumour necrosis factor alpha (TNFα) promoter region in SN cells (Pieper, *et. al.*, 2008). In Huntington's disease studies have revealed that 5mC may increase the expansion of triplet repeat sequences (CTG/CAG) which result in Huntington's disease (Behn-Krappa and Doerfler, 1994). This is supported by other studies which demonstrated global hypomethylation could make these triplet repeats unstable (Gorbunova, *et. al.*, 2004). Substantial changes in methylation levels have also been recently linked with expression of the huntingtin protein (Ng, *et. al.*, 2013a), which is heavily implicated in the pathology of Huntington's disease. Amyotrophic lateral sclerosis may also be linked to changes in DNA methylation, altered DNA methylation on specific genes has been found in the brains of individuals with sporadic ALS (Morahan, *et. al.*, 2009). Additionally, methylation in the *ATXN2* gene promoter region may be linked to the pathogenesis of ALS (Lahut, *et. al.*, 2012).

5hmC is found in relatively high levels in Purkinje neurons (0.6%) and granule cells (0.2%) (Kriaucionis and Heintz, 2009). The high levels found in the brain suggest a role for 5hmC in neuronal function via epigenetic regulation (Kriaucionis and Heintz, 2009). An alternative theory has also been proposed, where 5hmC marks sites during developmental stages which will later be demethylated (Lister, *et. al.*, 2013). This was discovered in foetal brains; however there is no reason why this could not be possible in adult brains at a lower or less plastic level. Interestingly, it appears that DNA methylation is also involved in memory formation (Miller and Sweatt, 2007) and synaptic plasticity in conjunction with histone acetylation (Miller, *et. al.*, 2008). Further studies have demonstrated that it may also affect neuronal function resulting in altered behavioural patterns (Day, *et. al.*, 2013; Day and Sweatt, 2010). The DNMTs have also been linked to learning, memory and synaptic plasticity through their ability to maintain methylation integrity and modulate neural gene expression (Feng, *et. al.*, 2010). It has also been demonstrated that CpG methylation is significantly more prevalent in CpG island shores in neurons (Kozlenkov, *et. al.*, 2013).

Previous studies have demonstrated differential methylation patterns according to brain region, which is believed to be associated to specialised function in each region (Ladd-Acosta, *et. al.*, 2007), although this may be attributable to initial or ongoing brain development. Previous studies have linked global methylation content, as a simple physical measure of overall epigenetic status, to healthspan and ageing in humans.

In this thesis, it was decided to see if this was observable in the AS/AGU rat and AS rat and if there was any difference in the physical quantity of methylated DNA that might reflect poorer healthspan and more rapid ageing in the AS/AGU rat brain tied to the loss of PKCγ function.

6.2 Materials and Methods

6.2.1 Animals used

The animals used for these experiments were raised and sacrificed in accordance with all regulations, both national and local, and all animal husbandry/operations were performed by a qualified Home Office licence holder. Both strains of rat were fed a standard diet, with drinking water provided as required. Animals were reared and housed in the Joint Research Facility, University of Glasgow under standardised conditions – light/dark cycle 12/12 hours, temperature 22°C +/- 2°C, humidity 50% +/- 5% in plastic-metal cages.

A total of twenty four rats, six for each of the four experimental groups were used. The experimental groups used were AS rats at 2 months old, AS rats at 12 months old, AS/AGU rats at 2 months old and AS/AGU rats at 12 months old.

Sacrificed animals had their brains excised and rapidly frozen in Isopentane using liquid Nitrogen, these were then placed in a cryomold, clearly labelled and stored in Isopentane at -80°C until required.

6.2.2 Measuring global DNA methylation levels

DNA global methylation levels were measured using the Methylamp™ Global DNA Methylation Quantification Ultra Kit (Epigentek, Farmingdale, NY, USA). ELISA plates were coated with samples by adding 2 μl of a 100 μg/ml genomic DNA sample (i.e. 200 ng) and 28 μl of DNA binding solution (Solution GU2 provided in the Methylamp™ Global DNA Methylation Quantification Ultra Kit; Epigentek, Farmingdale, NY, USA) to each well, ensuring the entire bottom surface of the well was covered by liquid. Samples, negative control (solution NC provided in the Methylamp™ Global DNA Methylation Quantification Ultra Kit; Epigentek, Farmingdale, NY, USA) and positive control (solution GU3 provided in the Methylamp™ Global DNA Methylation Quantification Ultra Kit; Epigentek, Farmingdale, NY, USA)

were all run in duplicate, whereas the standard curve points were ran in triplicate. The standard curve was obtained by serial dilution of the positive control provided (solution GU3 provided in the Methylamp™ Global DNA Methylation Quantification Ultra Kit; Epigentek, Farmingdale, NY, USA) into six distinct concentration points: 0.4, 1, 2, 5, 10 and 20 ng/well. Plates were then dried by 40 minute incubation at 37°C, followed by a 60 minute incubation at 60°C both in a hybridisation oven (low humidity environment).

150 μl of blocking solution (Solution GU4 provided in the Methylamp™ Global DNA Methylation Quantification Ultra Kit; Epigentek, Farmingdale, NY, USA) was added to each well and plates were incubated for 30 minutes at 37°C. Plates were then washed in a 10x dilution of wash solution (solution GU1 provided in the Methylamp™ Global DNA Methylation Quantification Ultra Kit; Epigentek, Farmingdale, NY, USA) three times before adding 50 μl of the primary antibody (solution GU5 provided in the Methylamp™ Global DNA Methylation Quantification Ultra Kit; Epigentek, Farmingdale, NY, USA) at a 1:1000 dilution in diluted solution GU1 (final concentration was 1 μg/ml) to each well. Plates were incubated for 60 minutes at RT. Plates were washed in 150 μl of the diluted wash solution (GU1) four times before adding 50μl per well of the secondary antibody (solution GU7 provided in the Methylamp™ Global DNA Methylation Quantification Ultra Kit; Epigentek, Farmingdale, NY, USA) diluted 1:5000 with diluted wash solution (GU1) to a final concentration of 80 ng/ml, plates were incubated at RT for 30 minutes. Plates were then washed five times in 150 μl of diluted wash solution (GU1), before the addition of 100 μl of developing solution (solution GU8 provided in the Methylamp™ Global DNA Methylation Quantification Ultra Kit; Epigentek, Farmingdale, NY, USA). Plates were kept in a darkened environment and monitored for the development of a blue colour at a medium density in the positive controls, at this point the reaction was stopped by addition of 50 μl of the stop solution (solution GU9 provided in the Methylamp™ Global DNA Methylation Quantification Ultra Kit; Epigentek, Farmingdale, NY, USA). At this point the colour changed from blue to yellow to indicate the reaction had

stopped, samples were then quantified by measuring absorbance at 450nm using a microplate reader within 15 minutes of stopping the reaction.

The standard curve was generated by plotting final concentration of the positive control (GU3) against the Optical Density at 450nm (OD_{450nm}) obtained. This gives a slope measuring OD_{450nm}/ng. This slope value was then used in the following equation to determine the amount of methylated DNA in each sample (x):

$$\text{Methylated DNA in sample (ng)} = \frac{xOD_{450nm} - \text{negative control}}{\text{Slope}}$$

The percentage of methylated DNA in each sample was calculated by correcting for the GC content of normal genomic DNA, which is 42% in rats (value provided by manufacturer, Epigentek for use with this kit and protocol), using the following formula:

$$\%\ \text{methylated DNA in sample} = \frac{\text{Methylated DNA in sample (ng)}/0.42}{\text{Sample DNA Amount Added (200ng)}} * 100\%$$

6.2.3 Statistics

All datasets were tested for normality using the Kilmagorov-Smirnoff test, all datasets presented herein passed this normality test and were deemed of normal distribution for all further analyses.

The unpaired t test (2-tailed) was used to demonstrate differences between the means of experimental groups and between brain regions; p values are presented to 3 decimal places. A 95% CI ($p<0.05$) was used throughout to determine significance, however significance at the 99% CI ($p<0.01$) and 99.9% CI levels ($p<0.001$) are also denoted where appropriate. All analyses and graphs were completed and prepared using Graphpad®Prizm Software (GraphPad Software Inc., La Jolla, CA, USA).

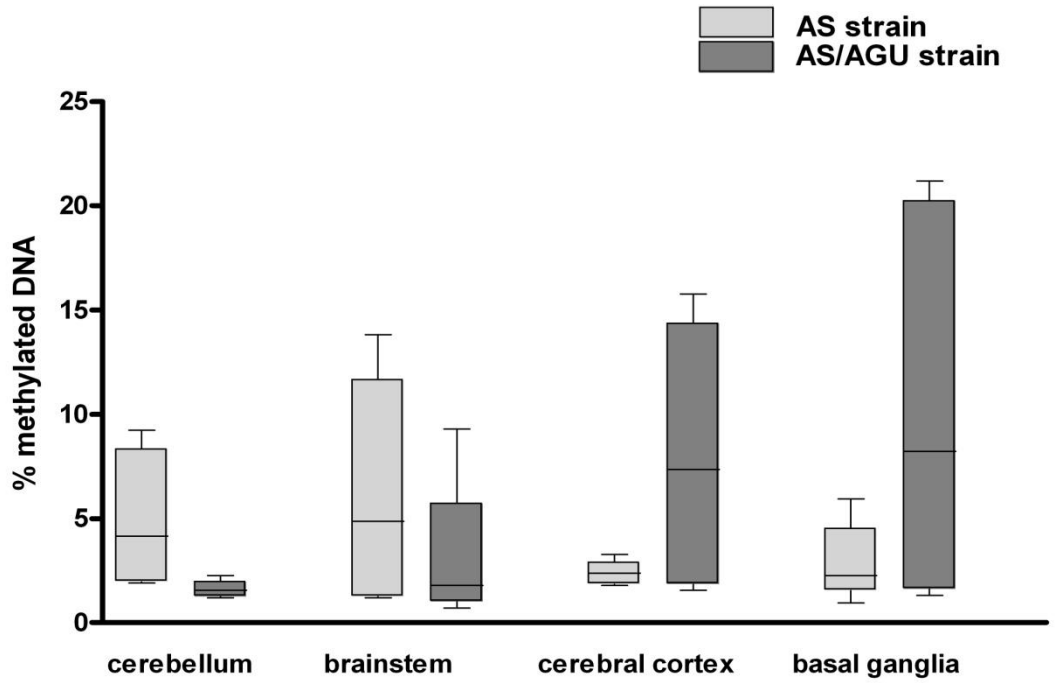
6.3 Results

6.3.1 Global DNA methylation levels at 2 months of age in AS and AS/AGU rats

Global DNA Methylation levels in the forebrain were not significantly different in the AS/AGU rats compared with AS rats (cerebral cortex, p=0.0624, basal ganglia, p=0.110, cerebellum (p=0.057) and brainstem (p=0.595) (Figure 6.2).

Figure 6.2 *Global DNA methylation levels found in both AS and AS/AGU rats at 2 months in four different brain regions.*

Box and whisker plot demonstrating the distribution and mean levels. Global DNA methylation levels from AS and AS/AGU rat brains in four regions (cerebellum, brainstem, cerebral cortex and basal ganglia) at 2 months old are presented.

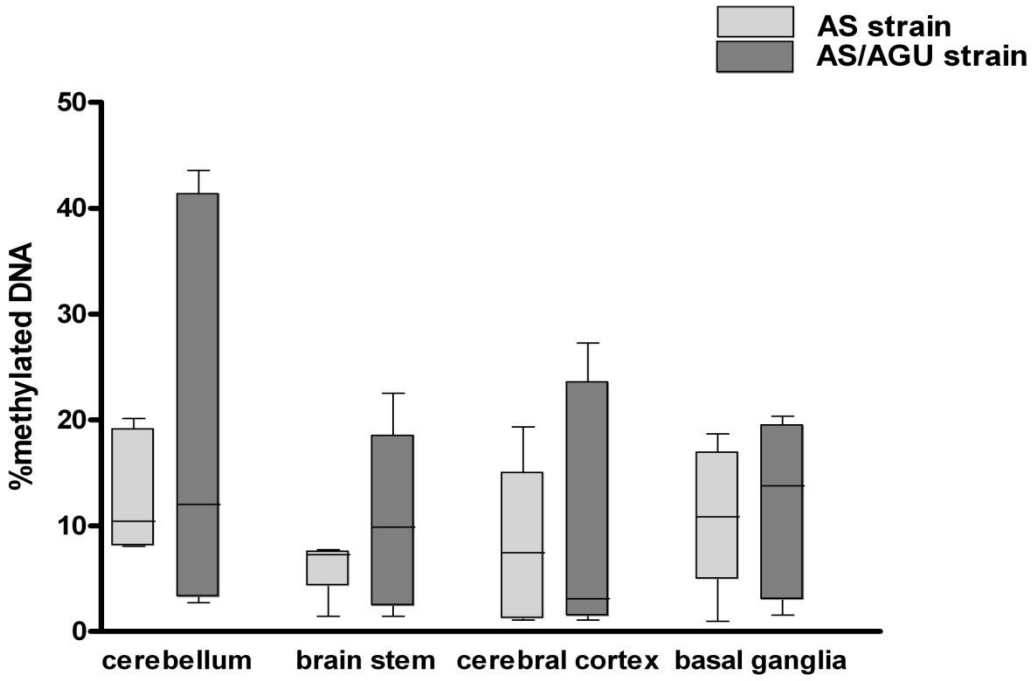


6.3.2 Levels of global DNA methylation at 12 months of age in AS and AS/AGU rats

At 12 months of age levels of global DNA methylation are almost equivalent in all areas of the brain, showing no differences (cerebellum, p=0.505; brainstem, p=0.301; cerebral cortex, p=0.647; and basal ganglia, p=0.775), with no comparison within areas being significantly different between the AS and AS/AGU rats and no obvious directional trends noticeable (Figure 6.3).

Figure 6.3 *Global DNA methylation levels found in both AS and AS/AGU rats at 12 months in four different brain regions.*

Box and whisker plot demonstrating the distribution and mean levels. Global DNA methylation levels from AS and AS/AGU rat brains in four regions (cerebellum, brainstem, cerebral cortex and basal ganglia) at 12 months old are presented.

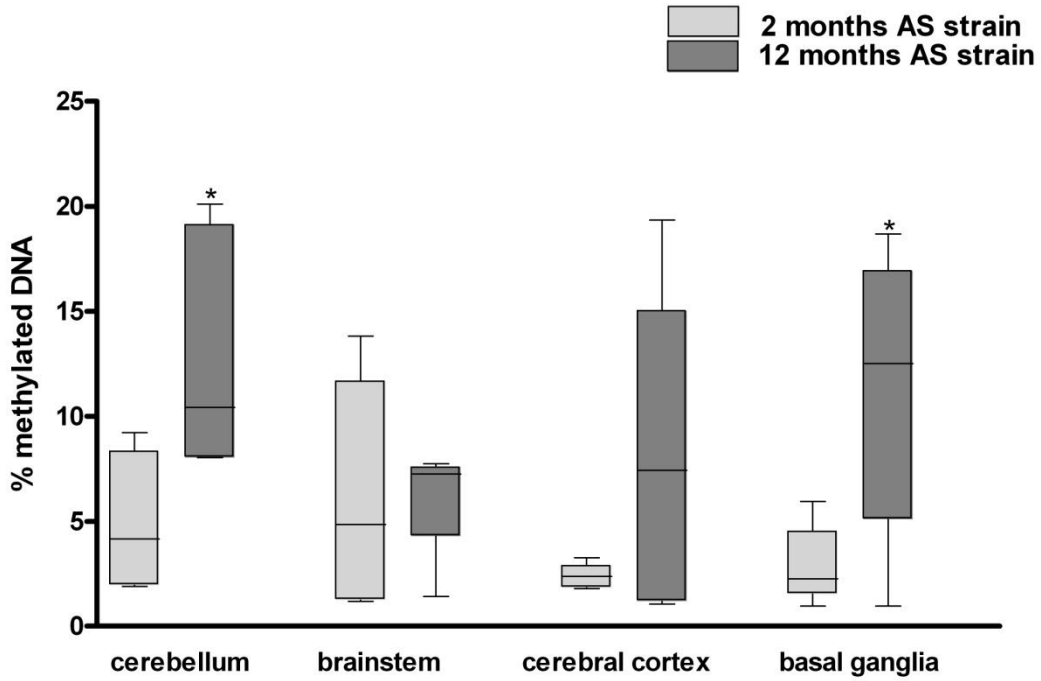


6.3.3 Comparison of global DNA methylation levels in AS rats between 2 months old and 12 months old

In the AS rat strain global DNA methylation levels increase throughout between 2 months and 12 months of age (Figure 6.4). These rises are significant in the cerebellum ($p=0.016$) and basal ganglia ($p=0.028$), but not in the brainstem ($p=0.916$) or the cerebral cortex ($p=0.107$).

Figure 6.4 *Global DNA methylation levels found in AS rats at both 2 months and 12 months old in four different brain regions.*

Box and whisker plot demonstrating the distribution and mean levels. Global DNA methylation levels from AS rat brains in four regions (cerebellum, brainstem, cerebral cortex and basal ganglia) at both 2 months and 12 months old are presented. Results significant at the 95% CI level ($p<0.05$) are marked with *.

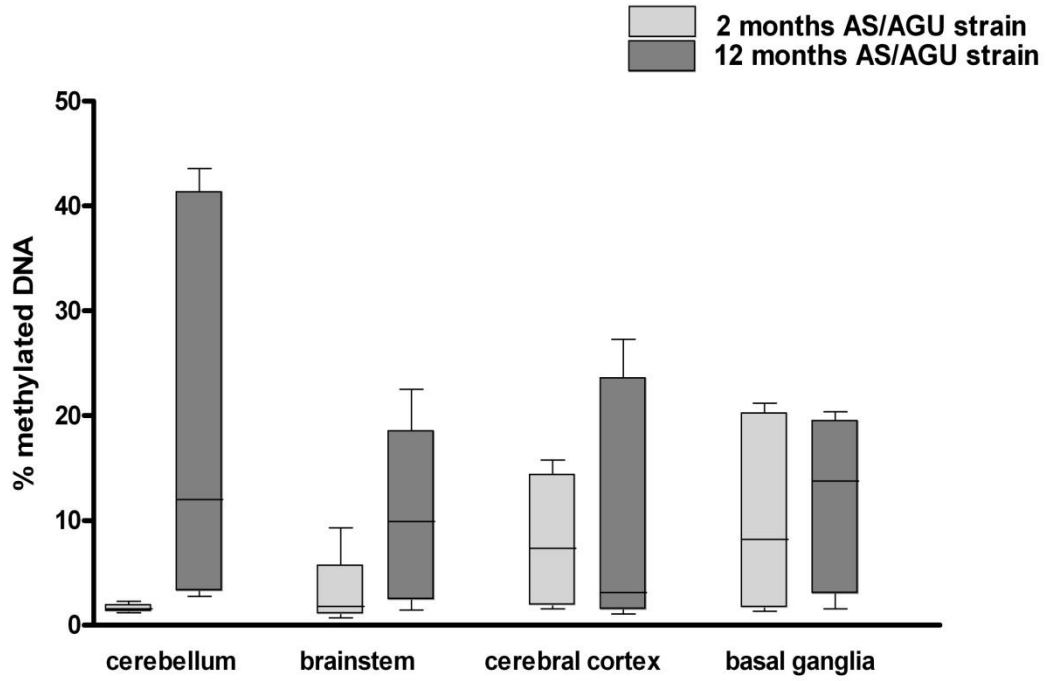


6.3.4 Comparison of global DNA methylation levels in AS/AGU rats between 2 months old and 12 months old

There appears to be no difference in global DNA methylation levels in the forebrain (Figure 6.5), cerebral cortex (p=0.0628) and basal ganglia (p=0.676). However levels appear to increase in the other areas of the brain, although these are not significant at the 95% CI level, cerebellum (p=0.064) and brainstem (p=0.091).

Figure 6.5 *Global DNA methylation levels found in AS/AGU rats at both 2 months and 12 months old in four different brain regions.*

Box and whisker plot demonstrating the distribution and mean levels. Global DNA methylation levels from AS/AGU rat brains in four regions (cerebellum, brainstem, cerebral cortex and basal ganglia) at 2 months and 12 months old are presented.



6.4 Discussion

6.4.1 Global DNA methylation levels associated with increased age in AS and AS/AGU rats

Although no significant differences between the strains were apparent, the results did demonstrate a general trend. At 2 months methylation was higher in the cerebellum and brainstem (the hindbrain) of AS rats compared to AS/AGU. This was reversed in the midbrain and forebrain (basal ganglia and cerebral cortex) where levels were higher in the AS/AGU rats. It is possible that the lower levels found in the hindbrain of AS/AGU rats is symptomatic of accelerated ageing. Furthermore, higher levels found in the basal ganglia may be characteristic of the AS/AGU rat strain resulting in disruption of the substantia nigra and thus dopamine production, as methylation has been shown to be critical in this area (Pieper, *et. al.*, 2008; Smidt, *et. al.*, 2004).

At 12 months very little difference is seen in the levels of DNA methylation in any region. Given that significant differences are apparent when comparing time periods in AS rats it is most likely that this equalisation effect is as a result of changes in the AS rats. This suggests that levels in the AS/AGU rats may have reached a plateau which the AS rats catch up with 10 months later. In order to examine this more closely further time points would be required, possibly 6 and 18 months, to determine any further differences and fully examine the trends involved. The data presented here is sufficient to say that levels appear to rise with age and that they may plateau once a certain age has been reached. Whether methylation levels begin to decline at advanced age points would have to be evaluated in a future study.

The data presented here involving two rat strains, which were raised in the same environment allowing a fair comparison of the two strains. However, it should be noted that dopamine affects mood and food uptake, this introduces potential confounding factors. Although, dietary intake initially seems irrelevant it can have far reaching consequences by

providing altered nutrient flow, even in the brain, thus influencing the immediate neural environment tested here. Additionally, mood has a dramatic effect on brain chemistry once again providing for potential disruption to the local environment. Even considering both of these factors both strains demonstrated hypermethylation over time and although the influence of these environmental exposures cannot be ruled out in terms of differences in magnitude or individual areas they were not sufficient to completely disrupt the overall pattern of hypermethylation in the AS or AS/AGU rats .

6.4.2 Differences in global DNA methylation levels in AS rats between 2 months and 12 months old

There were significant increases in global DNA methylation levels detected in the cerebellum and basal ganglia of AS rats between 2 months old and 12 months old. Very little is known about specific methylation patterns in the cerebellum or basal ganglia, in fact this is the first study to show differences related to age in these regions. The levels in the cerebral cortex also tended towards higher levels, although this failed to reach the cut-off significance value for 95% CI. These results show a general hypermethylation across all of the sites measured in the AS strain, although no real differences were seen in the brainstem over time. This is not consistent with the general belief that global hypomethylation is associated with age (van Otterdijk, *et. al.*, 2013), nor is it consistent with the hypothesis that methylation is relatively stable in brain tissues (Gravina, *et. al.*, 2013). However, it is consistent with the notion that specific sites may be hypermethylated, a phenomenon that would vary depending on the tissue or cell type being examined (Reinius, *et. al.*, 2012) and any environmental exposures. The differences in cell types/tissues may also explain the differences in methylation found in each of the brain regions. The differing functions of these regions may also explain some of the differences seen here. For example, the role of methylation in memory and learning (Miller and Sweatt, 2007) may result in changes in methylation pattern in the cerebral cortex.

6.4.3 Differences in global DNA methylation levels in AS/AGU rats between 2 months and 12 months old

The AS/AGU rats demonstrated increases in DNA methylation in the cerebellum, however this was not significant. The AS/AGU rats also demonstrated a trend towards higher DNA methylation levels in the brainstem with age, indicating a general increase in the hindbrain, whereas levels in the forebrain and midbrain did not vary significantly, which is consistent with reports of stable epigenetic marks in the brain (Gravina, *et. al.*, 2013). The trends of increased methylation in the Cerebellum and brainstem is not consistent with the general hypomethylation seen as a result of increased age (van Otterdijk, *et. al.*, 2013). However it is consistent with reports that non-mitotic cells do not demonstrate some of the age related changes in methylation (Chu, *et. al.*, 2007). It should be noted however that the differences shown here did not reach significant levels.

The AS/AGU rats did not demonstrate the anticipated hypomethylation in the basal ganglia. It was expected that levels here would decrease for two reasons, firstly ageing should induce a global hypomethylation (van Otterdijk, *et. al.*, 2013) which in this accelerated ageing model should have been exacerbated; and secondly PD demonstrates a global DNA hypomethylation (Jowaed, *et. al.*, 2010). Although it has been suggested that methylation patterns in post-mitotic tissues are relatively stable over time (Gravina, *et. al.*, 2013). It is possible that aberrant DNA methylation patterns in the basal ganglia, or more specifically the SN, may be related to silencing the expression of paired-like homeodomain transcription factor 3, via methylation of brain-derived neurotrophic factor (Martinowich, *et. al.*, 2003), which is responsible for the development of Mesodiencephalic dopaminergic (mdDA) neurons specifically in the SN (Jacobs, *et. al.*, 2011; Smidt, *et. al.*, 2004). Furthermore, a critical enzyme in the generation of DA from tyrosine, tyrosine hydroxylase (Th), is regulated by tissue specific methylation of CpG sites (Okuse, *et. al.*, 1993). This raises the possibility that the Parkinsonian phenotype seen in the AS/AGU rats is a result of aberrant

methylation during development or maturation. The lack of variation of levels in the brainstem would appear to suggest that the ongoing suppression of Th is a distinct possibility. This is supported by the lack of a reduction in levels from 2 months to 12 months as this would be more likely to result from high levels disrupting development of the SN. Furthermore, the fact that no reduction in levels is evident, unlike PD, suggests that the methylation levels may be an integral and continuous component of the phenotype. However, the SN represents only one part of the basal ganglia, therefore it is entirely possible that hypermethylation in the other components masks hypomethylation in this region. Further studies looking directly at the SN alone would be required to confirm whether methylation patterns are similar to those found in PD.

Further studies measuring levels of 5hmC would provide more valuable information about the state of global methylation levels in the brain. For example, if these demonstrated a global decrease in 5hmC levels then it may be speculated that 5hmC is being reduced to 5mC resulting in the increased levels associated with age seen here. Alternatively, the same decrease in levels may be the result of deamination resulting in DNA mismatches, which are then repaired from Uracil to Cytosine in the genetic code by mismatch repair enzymes whereupon the Cytosine residues would be remethylated by DNMTs.

The lack of differences seen in the cerebral cortex is again unexpected, as this is the area the ageing phenotype would be most likely to be apparent. More recent studies have demonstrated a similar finding by analysing 100 CpG sites in the brain and found that DNA methylation is stable over time (Gravina, *et. al.*, 2013).

6.4.4 The relationship of DNA methylation and ageing in brain regions of AS and AS/AGU rats

As very little information is available on the region specific levels of DNA methylation this study represents a step forward. Not only does it present data related to four key areas of the brain, it also demonstrates

the changes seen in these regions over a 10 month period. Furthermore, this study has measured these levels in two related strains which reinforces the findings and also grants an insight into the differences between these strains. Ultimately, these data demonstrate that a general hypermethylation is evident with increasing age in the four brain regions studied as seen in the AS rats. The lack of increases seen in the cerebral cortex and basal ganglia in AS/AGU rats may be evident of an increased ageing phenotype where levels stabilise or plateau with age. Further studies involving additional time points would be required to determine whether this is true or not. The alternative possibility is that these lack of changes in levels are a cause/result of the parkinsonian phenotype evident in these rats, this may include impairment of dopamine production or modulation by dopamine, or lack thereof. It is not possible that the disruption of one gene, and thus the epigenetic marks on that gene, is responsible for the differences seen in methylation patterns between the AS and AS/AGU rats. The only possibility in regards to this is that the gene itself has a direct effect on the methylation pattern of the animal, to date there are no studies demonstrating any direct methylation abilities for PKCγ, therefore it must be concluded that these differences are a result of the accelerated ageing phenotype and not a direct result of the mutation present in AS/AGU rats.

Histones are also responsible for epigenetic changes to gene transcription, these can be mediated through the actions of the Sirtuins. Histones are key regulators of gene transcription and modification of these can make them more or less stable. The Sirtuins all possess histone deacetylase (HDAC) activity this allows them to regulate gene transcription, particularly in response to changes in metabolism. Recently, succinylation and malonylation sites have been identified on histones (Xie, *et. al.*, 2012). This suggests that Sirt5 may play an even more critical role in the regulation of histones as it possesses both desuccinylation and demalonylation enzyme activities (Du, *et. al.*, 2011).

6.5 Overall Conclusions

Ultimately, the lack of significant differences between brain regions, time points and rat strains leaves the data here difficult to interpret. The previous discussion is based mostly on trends and possible explanations, but in conclusion the number of animals used represents insufficient statistical power to interpret the lack of difference between groups, due to an inability to reject the null hypothesis. Further measurement is required in a larger group to increase the statistical power and allow these findings to be investigated and interpreted fully. In conclusion, these results are intriguing and provide an excellent basis for further investigation, but do not provide a solid basis for further conclusions.

SOHAIR MOHAMMED KHOJAH, M.Sc. B.Sc.

Ageing in the Mammalian Brain

7. Conclusions

7. Conclusions

7.1 An enhanced ageing phenotype is apparent in AS/AGU rats when compared with AS rats

CDKN2A, or more precisely $p16^{INK4A}$, is now a well established biomarker of ageing (Collado, *et. al.*, 2007; Krishnamurthy, *et. al.*, 2004), proving to be more accurate than more traditional biomarkers including telomeres (Gingell-Littlejohn, *et. al.*, 2013; McGlynn, *et. al.*, 2009; Shiels, 2010). In fact this biomarker has been shown to associate with senescence and age even when telomeres demonstrate no shortening (Melk, *et. al.*, 2003). This biomarker has been demonstrated and validated in multiple human tissues including skin (Ressler, *et. al.*, 2006), kidney (Chkhotua, *et. al.*, 2003; Melk, *et. al.*, 2004), thymus (Kanavaros, *et. al.*, 2001), fibroblasts (Alcorta, *et. al.*, 1996) and T cells (Liu, *et. al.*, 2009) as well as in several mouse and rat tissues (Krishnamurthy, *et. al.*, 2004). The expression of $p16^{INK4A}$ within the tissues of the body is also highly selective, being confined to cells which are not involved in replenishment or replacement of dead cells (Nielsen, *et. al.*, 1999), this further reinforces the tight relationship between its expression and cellular senescence. Evidence also suggests that the senescence-associated secretory phenotype is not linked to $p16^{INK4A}$ expression (Coppe, *et. al.*, 2011); indicating that $p16^{INK4A}$ expression is likely to be an early event in cellular senescence improving its use as a biomarker through the potential for early detection of senescence. However there are some studies which suggest that the increased levels of CDKN2A associated with age may not be as apparent as some researchers believe, in fact one such study claims that CDKN2A only increased in cardiac tissue and actually decreased in the cortex in association with advancing age in mice (Swindell, 2009). Replicative cellular senescence can be divided into two categories (Jeyapalan and Sedivy, 2008), the first involving telomere shortening or dysfunction or other stress leading to DNA damage and is mediated via p53 (d'Adda di Fagagna, *et. al.*, 2004; Herbig, *et. al.*, 2004); the second involves $p16^{INK4A}$, which can be induced by a variety of stresses, this may represent a more natural phenomenon resulting directly from ageing rather than damage.

Probably the most widely used senescence marker is SA β-gal (Dimri, *et. al.*, 1995; Itahana, *et. al.*, 2007), however there have been some reports of non-senescent cells being stained using this method (Cristofalo, 2005; Severino, *et. al.*, 2000; Yang and Hu, 2005) potentially leading to false positives. Despite this it is still one of the most relied upon and accurate markers of cellular senescence available to date.

The raised levels of both CDKN2A (presented in chapter 3) and SA β-gal (presented in chapter 5) in whole brain sections indicate that cellular senescence increases overall in rat brains in association with increased age. The concurrent use of these markers to demonstrate senescence has been used in the past, for example in pancreatic islet cells (Halvorsen, *et. al.*, 2000). Furthermore, this increase appears to occur at an earlier time point in AS/AGU rats, i.e. is accelerated compared to the background AS strain. These data taken together suggest that an accelerated ageing phenotype is present in the brains of AS/AGU rats. Dual staining for both $p16^{INK4A}$ and SA β-gal may further enhance the data presented here by confirming the presence of both in the same cell population; however the increase seen for both in accordance with increasing age is highly convincing alone given what is known about both of these markers.

Sirtuin 5 has been suggested to be involved in the cellular stress response (McGuinness, *et. al.*, 2011), the high levels of this transcript seen at the earlier age point (2 months) in AS/AGU rats (presented in chapter 4) may indicate greater stress or genomic instability which has the potential to manifest as accelerated ageing. Furthermore this may be a progenitor to the increase in $p16^{INK4A}$ seen in the older AS/AGU rats. The higher levels seen at the older age point (12 months) in the AS rats (presented in chapter 4) may indicate that they have reached a similarly critical age point which would ultimately lead to a similar ageing phenotype as seen in the younger AS/AGU rats. The influence of both Sirtuins 6 and 7 on genomic stability (McGuinness, *et. al.*, 2011) suggests that they may have the potential to be early warning signs for oncoming cellular senescence and thus ageing. Higher

levels seen at the earlier age point (2 months) in AS/AGU rats (presented in chapter 4) would appear to suggest that this possibility has some merit.

Differential expression of CDKN2A (presented in chapter 3) throughout the brain (in the four regions evaluated here) suggests that different areas of the brain age at different rates. The similarities in regional expression between 2 month old AS/AGU rats and 12 month old AS rats suggests that the brains of these young AS/AGU rats are of a similar bio-age to the older AS rats. This provides further evidence for an accelerated ageing phenotype in AS/AGU brains. This data also suggests that this accelerated ageing occurs throughout the brain and is not isolated to specific regions; for example areas that differ between strains (e.g. the basal ganglia), areas that are susceptible to PKCγ KOs (e.g. the cerebellum), or areas that are known to show differences with age (e.g. the cerebral cortex, basal ganglia and the cerebellum) all demonstrate the same accelerated ageing phenotype. This indicates that the enhanced ageing phenomenon seen here is not simply the result of strain differences, PKCγ mutation or normal ageing. Given the ubiquitous nature of this accelerated ageing phenomenon in AS/AGU rat brains it is reasonable to assume that this may occur in other post-mitotic tissues as well, for example cardiac tissue or skeletal muscle, however this would require further rigorous examination to determine if this is the case. Investigating this would determine whether this model combination (AS and AS/AGU rats) is a general accelerated ageing model, i.e. throughout the entire organism, or if this phenomenon is present only in the brain.

PKCγ disruption/down regulation has been shown to be involved in the pathology of neurodegenerative disorders (Battaini and Pascale, 2005). Although one experiment in rabbits suggests that disruption of subcellular localisation is enough to induce memory impairment (Van der Zee*, et. al.*, 2004). This is similar to the disruption seen in the AS/AGU rats, however in neurodegenerative disorders this is accompanied by neuronal cell death. This does not appear to be the case in these rats; it appears that there is an increase in senescent cells as shown by SA β-gal and to a lesser extent by the increases in CDKN2A levels. This would suggest that the accelerated ageing seen in these rats is not directly related to the PKCγ mutation. Furthermore,

the appearance of symptoms and physiology at a very early age (Clarke and Payne, 1994), combined with the low levels of extracellular DA (Campbell, *et. al.*, 1998), mean that it is unlikely that any progressive change in markers is due to loss of DA or 5-HT. In addition , no obvious direct links exist between DA levels and transcription of the markers measured herein indicating that disruption of DA is unlikely to be linked to the accelerated ageing phenotype.

The similarities between AS rats at 12 months and AS/AGU rats at 2 months also suggests that ageing in these rats is accelerated and not disrupted, in other words the method of ageing is the same its just faster in AS/AGU rats. This suggests that this is simply a case of accelerated ageing and not a disruption to the ageing mechanisms induced by the PKCγ mutation.

7.2 Metabolic disruption and DNA damage accumulation as measured by Sirtuin levels in the brain may be part of the accelerated ageing phenotype seen in AS/AGU rats

The critical role for Sirt5 in the urea cycle modulating CPS-1, by deacetylation (Nakagawa, *et. al.*, 2009), demalonylation and desuccinylation (Du, *et. al.*, 2011), which catalyses the reaction converting ammonia to urea has the potential to reduce cellular levels of ammonium which promotes oxidative stress. Therefore Sirt5 has a critical role in the reduction of oxidative stress and thus reduces the potential for DNA damage accumulation, as well as having a more direct role in the modulation of metabolism.

It has been demonstrated that levels of cellular NAD^+ can fluctuate dramatically dependent on tissue type during CR (Chen, *et. al.*, 2008). This suggests that the activity and expression of Sirtuins is tissue specific. Sirt1 overexpression can mimic many of the phenotypes associated with CR from lower body weight to greater metabolic activity (Bordone, *et. al.*, 2007). The close ties between Sirt1 and Sirt6 implicates this DNA repair related Sirtuin with metabolic disruption and stress. In fact Sirt6 has been directly

related to metabolic homeostasis, particularly glucose uptake and glycolysis (Zhong, *et. al.*, 2010). This raises the possibility that increased levels of Sirt6 in young (2 month old) AS/AGU rats is associated with reduced glucose uptake which may be involved in the lower body mass and lower glucose usage seen in these rats (Lam, *et. al.*, 1998). Although this does not appear to fit well with reports that a mouse model with neural deletion of Sirt6 results in early growth retardation; it does fit with ultimate outcome of these neuronally deleted mice in that they ultimately become obese (Schwer, *et. al.*, 2010), whereas the AS/AGU rats remain underweight throughout. However, overexpression of Sirt6 has been shown to extend lifespan in mice (Kanfi, *et. al.*, 2012), this is clearly not the case in the AS/AGU rats, although the reduction seen in older rats (12 months) with a rise seen in the controls may indicate that this Sirtuin still has a role to play in the determination of lifespan. Overexpression of Sirt6 has also been associated with increased fatty acid oxidation (in liver cells), additionally liver specific deletion leads to fatty liver disease (Kim, *et. al.*, 2010). Overexpression also reduced the accumulation of visceral fat when on a high-fat diet (Kanfi, *et. al.*, 2010). Although this is not immediately applicable to the brain it demonstrates the potential for metabolic disruption from this particular Sirtuin. The major role for Sirt6 involves modulation of DNA repair mechanisms which presents a direct role for this Sirtuin in the prevention of DNA damage accumulation. The early increased levels of this Sirtuin in AS/AGU rats may represent an increase in activity in the DNA damage repair mechanisms, suggesting instability is present. Lower later levels suggest an age-related reduction in DNA damage repair either resulting from or due to accelerated ageing.

Sirt7 has been demonstrated to possess a role in protection against stress in cardiomyocytes (Vakhrusheva, *et. al.*, 2008b) and in activation of RNA polymerase 1 (Ford, *et. al.*, 2006). This indicates a role for Sirt7 in the maintenance of DNA integrity and prevention of DNA damage.

The Histone deacetylase function of all Sirtuins has been suggested to be an important factor in the DNA repair mechanisms, indicating that expression of all Sirtuins may be linked to DNA damage accumulation

(Kruszewski and Szumiel, 2005). Due to the dependence of all Sirtuins on the presence of NAD^+ they can all be considered metabolic sensors which respond to the metabolic status of the cell (Leibiger and Berggren, 2006). Taken together these factors make the Sirtuins ideal sensors for the damage or disruption that may lead to accelerated ageing.

The decreased later levels of Sirtuins seen in this study are consistent with reports that all Sirtuins decrease with age (Ren, *et. al.*, 2013). The data presented here would suggest that levels of the three Sirtuins examined start low, build through life, and then reach a tipping point where expression wanes in response to old age. This reduction may be indicative of DNA damage accumulation and may be a precursor to apoptosis in heavily affected cells. Data on apoptotic markers in these cells would provide confirmation as to whether this hypothesis is plausible or not.

Overall, the expression of Sirtuins appears to follow a pattern which suggests that expression in AS/AGU rats is increased early in life. Given the roles of these three Sirtuins in the prevention of oxidative stress, repair of DNA damage and modulation of metabolism it is highly suggestive that at least one of these factors is involved in the accelerated ageing phenotype of the AS/AGU rats. Further data involving the generation of ROS or the metabolic state would be required to verify this conclusion.

The data presented here for the Sirtuins, in combination with CDKN2A and SA β-gal, strongly indicates that the AS/AGU rats are more advanced in age, biologically speaking, at both time points. This further indicates that the three components of the MTR trinity are also involved. Early Sirt5 elevation potentially indicates an increase in mitochondrial activity, particularly within the urea cycle. This may in turn indicate increased energy generation from the citric acid cycle and thus a large increase in ROS activity. This activity may result in more oxidative stress from the mitochondria, in conjunction with early elevation in Sirt7, this suggests that ribosomal activity is also increased. Again this suggests an increase in energy usage and thus greater levels of ROS. Finally the elevation in Sirt6 suggests increased DNA damage and a reduction of telomere lengths. Taken together

this confirms the accelerated ageing phenotype and the involvement of the MTR trinity. It is unlikely that this is a direct result the PKCγ mutation, however it is not possible to rule out a potential influence by a more circuitous route – alteration of dietary intake via DA disruption. CR or disruption to diet is well known to alter the expression of Sirtuins in general. Further experimentation involving metabolic cages would be required to completely rule out this possibility.

7.3 The accelerated ageing phenotype seen in AS/AGU rats is concurrent with DNA methylation changes at the global level.

The global DNA methylation data generated within this project is inconclusive, with very few differences reaching significance. The general trends are interesting but drawing solid conclusions from these given the insignificance of the data is impossible. Further and more refined studies would be required to fully explore this research question and determine the full methylation status in regards to age.

The lack of change seen here is consistent with some reports which suggest that methylation in the brain can be relatively stable over time (Gravina, *et. al.*, 2013). Furthermore, it is entirely possible that a general hypomethylation associated with advancing age (van Otterdijk, *et. al.*, 2013) may be masked by specific hypermethylation of selected sites (Reinius, *et. al.*, 2012). Specific genes can demonstrate highly variable methylation levels throughout the lifespan, for example methylation of the Sirt6 promoter is relatively low during the early (<19) and late phases (>85) of human life and peaks in between (Sahin, *et. al.*, 2014). More focused and specific investigation of methylation will be required to determine whether the trends demonstrated here are indicative of the true nature of accelerated ageing in the AS/AGU rat strain.

7.4 Final conclusions

Overall, this study demonstrated that an accelerated ageing phenotype is present in the AS/AGU rats compared with their parent strain

the AS rat. This ageing phenotype manifests itself with increased cellular senescence and a molecular response by the Sirtuins to at least one major driver of ageing: DNA damage accumulation or metabolic disruption. Attempts to measure the epigenetic effects of this accelerated ageing phenotype were inconclusive. The presence of this phenotype is consistent with the known PD phenotype of these rats; it also confirms the usefulness of this model as an animal model for the development and progression of PD. This work provides scope for future work further categorising and developing our knowledge of both ageing and PD.

7.5 Future directions

The data presented here leads to great potential for further research in new and novel directions. These potential studies fall into two categories, firstly improvements or enhancements to the data provided herein and secondly novel projects leading from these results. A brief summary of the potential in both categories is provided here.

7.5.1 Improvements and enhancements to the current study

First and foremost a further age point would greatly enhance all of the data presented here, it would provide linearity to the results and an improved view of the data with regards to changes associated with ageing. The optimal configuration would be to use 3-4 monthly sampling points however this may prove to be prohibitive due to the number of animals that would be required to achieve.

Another improvement to this study would be to use dual staining to demonstrate the concurrence of increased levels of $p16^{INK4a}$ and SA β-gal. This method of multiple staining could also enhance the Sirtuin results by showing where high levels of each occur and whether these are linked or independent.

Incorporation of metabolic studies with Sirtuin expression studies would determine whether changes in diet, intake or glucose usage result in

altered Sirtuin expression. Furthermore, this would give valuable insight into the effects of metabolic factors on not only the expression of Sirtuins but also on the effects of Sirtuin expression on lifespan in this particular model.

Further co-localisation studies involving the co-expression of the three Sirtuins investigated here would provide and insight into whether the changes in expression seen are in the same locations or whether these alterations occur in adjacent/distant cells. Furthermore, co-localisation with $p16^{INK4a}$ and/or SA β-gal would determine whether changes in expression levels are associated with cellular senescence or not.

Further enhancements include conducting investigations in more brain regions, or more localised brain regions for example looking at the SNC independently rather than as a component of the basal ganglia. However, this requires extreme precision and equipment that may only be within the grasp of a few labs in the world.

Measurement of specific sites for methylation (Koch*, et. al.*, 2012) would further enhance the data on senescence, it might also provide early insight into the initiation of senescence. Measurement of 5hmC may also provide insights into the true nature of the methylation changes seen here.

Site specific methylation studies and measurement of 5hmC would improve the data received from this study and provide valuable insight into the level and nature of DNA methylation in this ageing model.

A repeat of this experimental setup, with additional measurements of DA and glucose usage with female rats would provide a much more complete dataset. This may also allow further examination of the differences between the sexes in regard to ageing. Furthermore, it would provide valuable information on the AS/AGU model in general allowing for further experimentation with this rat model.

7.5.2 Potential research projects

With the demonstration of altered $p16^{INK4a}$ expression from the CDKN2A locus it becomes apparent that the products of this locus may be involved in the phenotypes present in this rat strain. Therefore it would be prudent to incorporate the other products ($p14^{ARF}$ and p12) of this gene locus in further studies. Furthermore, it may also be advisable to incorporate relating genes and loci into future studies including CDKN2B, Rb and p53 to generate a more complete picture of the status of ageing in these rats. This data would also provide invaluable insight into the pathways involved in the PD phenotype if it was coupled with a more in depth investigation of localization, for example by examining key locales of the Basal ganglia to determine expression differences associated with the Dopamine disruption.

The obvious differences seen in the expression of the three Sirtuins examined here warrants further investigation. An expanded study involving all seven mammalian Sirtuins may reveal more interactions between these molecules and would enhance our understanding of the action and effect of these molecules with regard to ageing and PD. The high level of information available for Sirt1 in these contexts would also provide a necessary guideline for a more complete and accurate evaluation of the role of the other Sirtuins. This study may also involve a greater number of smaller areas of the brain in order to determine micro-differences in expression patterns, particularly focused around the SN.

More complete and thorough investigation of the methylation status throughout would require a completely new study aimed at elucidating the presence and effects of epigenetic changes with regards to the accelerated ageing phenotype demonstrated in the AS/AGU rat. This study should incorporate multiple time points and more accurate methodology for example bisulphate sequencing, which would allow the analysis of individual promoters as well as a more accurate measurement of global DNA methylation levels (Chatterjee, *et. al.*, 2012). Another possibility is to

use Methylated DNA immunoprecipitation (MeDIP) methods to enrich methylated sequences (Weber, *et. al.*, 2005).

The presence of an accelerated ageing phenotype within the AS/AGU rat opens numerous possibilities for future studies, particularly related to the PD phenotype it also presents with. In fact, these are more than likely closely related however further studies that conclusively demonstrate this would be required. More accurate methylation data specifically within the Basal ganglia would provide a greater insight into localized changes. Determination of the metabolic status of these animals in relation to age-related changes would also enhance our knowledge of the ageing process as it manifests in this rat strain. Once these studies have been completed it would be possible to examine the effects of L-Dopa in the context of altering expression of Sirtuins, $p16^{INK4a}$ and methylation status to determine whether this effective treatment for PD has any effect on the ageing phenotype or whether it merely overcomes and masks the effects of the loss of DA.

SOHAIR MOHAMMED KHOJAH, M.Sc. B.Sc.

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8. Appendices

8. Appendices

8.1 Appendix 1

8.1.1 Appendix 1a – CDKN2A

Gene Symbol	Assay ID	Gene Name and Alternative names	Gene Aliases	NCBI Chromosomal Location	Organism	Forward primer DNA sequence 5'-3'	Reverse primer DNA sequence 3'-5'
CDKN2A	Rn00580664_m1	cyclin-dependent kinase inhibitor 2A (CDKN2A); p16; ARF; MTS1; INK4A; p19ARF;	Arf, INK4A, MTS1, p16, p16Cdkn2a, p19ARF	5q32	Rat	ATGGAGTCCTCTGCAGATAGA	ATCGGGGGTACGACCGAAAGTGTT

8.1.2 Appendix 1b - Sirtuins

Gene Symbol	Assay ID	Gene Name and Alternative names	Gene Aliases	NCBI Chromosomal Location	Organism	Forward primer DNA sequence 5'-3'	Reverse primer DNA sequence 3'-5'
Sirt5	Rn01450559_m1	Sirtuin 5	-	17p12	Rat	GTCTTTCCCAAGATGCAAAGGT	GACGGTTTCAGGATCACAACAG
Sirt6	Rn01408249_m1	Sirtuin 6	-	7q11	Rat	GCAGTGGTGTGTGGAGTGTGTA	ACACCCAGTGTTCAGCACAGTT
Sirt7	Rn01471420_m1	Sirtuin 7	-	10q32.3	Rat	GCCGCATACCCAATACAGAAA	CAAGGACACCTCCATCCTATCC

8.1.3 Appendix 1c – Housekeeping gene (reference gene)

Gene Symbol	Assay ID	Gene Name and Alternative names	Gene Aliases	NCBI Chromosomal Location	Organism	Forward primer DNA sequence 5'-3'	Reverse primer DNA sequence 3'-5'
Gapdh	Rn01775763_g1	Glyceraldehyde-3-Phosphate dehydrogenase	Gapd	4q42	Rat	TGATGACATCAAGAAGGTGGTGAG	TTCTTGGAGGCCATGTAGGCCAT

SOHAIR MOHAMMED KHOJAH, M.Sc. B.Sc.

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9. References

9. References

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