

Does the activity of the proteasome decline during human ageing and in the brains of Parkinson's disease patients?

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List of Abbreviations

SNpc	Substantia nigra pars compacta
Cer	Cerebellum
CauN	Caudate nucleus
Hipp	Hippocampus
CC	Corpus Callosum
PFC	Pre-frontal cortex
Thal	Thalamus
ROS	Reactive oxygen species
NAD(P)H	Nicotinamide adenine dinucleotide phosphate-oxidase
ETC	Electron transport chain
Q	Ubiquione
•Q-	Semiquinone anion
GPx	Glutathione reductase
CAT	Catalase
NO•	Nitric oxide
NOS	Nitric oxide synthase
RNS	Reactive nitrogen species
TOR	Target of rapamycin
MA	Macroautophagy
CMA	Chaperone-mediated autophagy
HSP70	Heat shock cognate protein of 70KDa
LAMP-2A	Lysosome-associated membrane protein type 2A
Atg	Autophagy related protein
UbIc	Ubiquitin-like conjugation
FIP200	Focal adhesion kinase family-interacting protein of 200kDa
PI3k	Phosphoinositide 3-kinase
LC3	Light chain 3
PE	Phosphatidylethanolamine
PtdIns	Phosphatidylinositol

AVM	Putophagic vacuolar myopathies
GSD II	Glycogen storage disease type II
GAA	Acid alpha 1,4-glucosidase
A β	Amyloid β eta
CP	Core particle
RP	Regulatory particle
CF	Cystic fibrosis
CFTR	Cystic fibrosis conductance regulator
HSP70	Heat shock protein 70
AD	Alzheimer's disease
PD	Parkinson's disease
A β	Amyloid beta
APP	Amyloid precursor protein
HD	Huntington's disease
SNpc	Substantia nigra pars compacta
LRRK2	Leucine-rich-repeat-kinase2
α -synuclein	Alpha synuclein
LB	Lewy body
PB	Pale body
PINK1	PTEN-induced kinase 1
PVDF	Polyvinylidene fluoride

Abstract

Background: It is well documented that the global population is steadily rising. A major factor for the rising population is the increase in life expectancy. As medical advancements are made we are now living longer than at any point in human history. As a result of this, levels of neurodegeneration are at an all-time high and propose a significant health burden. Parkinson's disease (PD) is the world's second most prevalent neurodegenerative disease, which has its pathology linked to a range of complex interactions resulting in accumulation of protein aggregates, increased cellular oxidative stress, impaired mitochondrial function and dysfunction within the cellular maintenance systems, the ubiquitin proteasome system (UPS) and the lysosomal degradation pathway. This project aims to assess potential decline in UPS activity in PD and in conventional ageing, whilst also investigating dysregulation of important homeostatic cellular proteins as a result of oxidative stress.

Method: Post-mortem PD (n=11) and Control (n=10) brain tissue samples, alongside an ageing study group (n=40 23 years-93 years) underwent a fluorometric analysis of UPS activity utilising the fluorogenic substrate n-succinyl-leu-leu-val-tyr-7-amido-4-methylcoumarin targeting the 20S proteasome chymotrypsin-like activity in seven different brain regions instigated in the pathology of PD. Western blot analysis of the ageing study group targeting proteins impacted in oxidative stress; SOD1, alpha tubulin and beta tubulin.

Results: The UPS activity assay suggests that there is no significant change within any region assessed within PD/control samples and in the ageing brain ($p > 0.05$ in all cases). We noted a significant decrease in a range of integral cellular proteins in the ageing study, as SOD1, alpha and beta tubulin all showed visual decline. However, only alpha tubulin decline was statistically significant ($p = 0.0206$).

Interpretation: The lack of change within the rate of 20S proteasome activity in PD or in ageing within any assessed region suggests that there may be a more fundamental underlying cause for the decrease in UPS activity within PD that was not assessed within this study. The overall decrease in SOD1 and alpha and beta tubulin are indicative of an increased level of oxidative damage within ageing cells. Reduced levels of these proteins can lead to significant cellular stress, impacting the levels of oxidative stress within the intracellular environment and functionality of the autophagy-lysosomal pathway.

Chapter 1 Introduction

1.1 An introduction to the prevalence of neurodegeneration in an ageing population

It is well documented that the world's population is steadily rising and will continue to do so over the next 12 years. The United Nations has reported a projected increase of up to 1 billion inhabitants, resulting in the overall population reaching 9.6 billion by the year 2050.

As a direct result of this growing populace, life expectancy will increase, and individuals over the age of 65 will be the highest they have ever been globally (Wale, 2011).

1.1.1 The Impact of Ageing

Ageing is generally described biologically as a gradual loss of cellular function over time, leading to the inevitable mortality of the organism.

The ageing of cells and cellular apoptosis are fundamental physiological processes, important in maintaining a healthy and stable metabolic environment. There are however, several major risk factors attributed with cellular ageing that can lead to the development of a range of diseases, including cancer, cardiovascular and neurodegenerative diseases (Squier, 2001).

The long-term process of biological ageing eventually results in the complete or partial loss of cellular functionality, which in turn triggers programmed cellular death or apoptosis. It is often theorised that ageing and subsequently age associated mortality is an evolutionary trait in humans; as it is not present throughout the entirety of nature, and therefore must set to serve evolutionary benefit in some form other than biological deterioration over the time course (Kirkwood and Austad, 2000).

1.1.2 Common theories of Ageing

Whilst still largely unknown, there have been a range of theories stipulating the potential evolutionary role of ageing within the human population.

It has been proposed that Biological deterioration via cellular division with age may be a programmed evolutionary trait, inherent to prevent over population of the species, whilst simultaneously accelerating the turnover of subsequent generations (Prinzinger, 2005). This in turn

benefits the species in being more readily able to adapt to changes in the environment and could explain cellular ageing as a means of advancing natural selection at a consistent pace. However, it has been noted that senescence as a means of mortality is a rare occurrence within wild populations, who will often perish as a result of environmental hazards or medical conditions and are at the highest risk of this at a young age. There is in fact, scarce occurrence of significant ageing within wild animal populations, making this theory somewhat obsolete as a potential explanation (Kirkwood and Austad, 2000).

Another alternative theory of ageing is 'pleiotropy' often referred to as 'antagonistic pleiotropy', the idea that a single gene can control more than one trait. It has been suggested that Pleiotropic genes, which benefit the organism at an earlier stage of life are often favoured by natural selection, even if they may also result in more undesirable effects later in life (Williams, 2001). A good example of pleiotropy at work is the p53 tumour suppressor protein, wherein it can become functionally active in response to stress signals and DNA damage triggering apoptosis, transient cell cycle arrest or senescence, playing an important role as a potent anti-tumour suppressor, whilst also being able to act on renewing tissues preventing proliferation of progenitor or stem cells, which can alter the structure and function of tissues; a common component of ageing (Rodier et al., 2007).

Williams theory suggests that organisms favour early life stage genetic advantages over long term benefits as a means of increasing survival probability, even if the trade-off is a deleterious effect which can eventually lead to senescence or death later within the organisms life span. This balancing of the life-history trade-off, is also a central theme in another influential theory of ageing, the 'disposable soma' (Lithgow and Kirkwood, 1996).

Disposable soma is based on the idea that an ageing organism is in a perpetual state of balancing somatic maintenance (utilising energy to repair or avoid damage) and other important biological functions (such as reproduction). Since long term survival chance is low in wild populations, it is often seen to be favourable for an organism to expend energy resource on thermogenesis or reproduction aiding early stage survival, rather than cellular maintenance or repair which may eventually lead to the accumulation of reactive oxygen species (ROS) and other forms of cellular oxidative stressors (Lorenzini et al., 2011), which in turn have been shown to be responsible for a major component of biological ageing and a risk factor for a range of diseases, including cancer, cardiovascular disease and neurodegeneration (Dröge, 2005).

1.2 Oxidative Stress and The Impact of ROS During Biological Ageing

Having established the link between biological ageing and the development of these disease states, it is of vital importance that we are able to gain a thorough understanding of the underlying mechanisms involved with the generation of ROS and the subsequent oxidation and nitration of intracellular proteins leading to loss of function.

1.2.1 Oxidative Stress and the Role of Reactive Oxygen species

Cells which utilise oxidative metabolism as a means of energy production will subsequently release a plethora of reactive oxygen species into the cell as a by-product. ROS are highly chemically reactive oxygen containing species, with a high affinity for binding and potentially damaging a range of intercellular macromolecules and cellular components such as nucleic acids and lipids (Geng and Li, 2016).

Superoxide radicals are formed by nicotinamide adenine dinucleotide phosphate-oxidases (NAD(P)H), xanthine oxidase enzymes or non-enzymically via redox-reactive compounds (Droge, 2002), and can be converted into nonradical and subsequently less damaging forms via cellular defence mechanisms which prevent the build-up of ROS within the cell, such as the enzymes superoxide dismutase (SOD) and catalase which are able to convert superoxide into hydrogen peroxide or non-enzymically into hydrogen peroxide and singlet Oxygen ($^1\text{O}_2$) (Dröge, 2005), glutathione and glutathione peroxidase which reduce H_2O_2 to H_2O , and the vitamins E and C which are able to prevent lipid chain reactions which involve peroxy radicals (Squier, 2001). Excess levels of intracellular ROS can lead to a state of oxidative stress, leading to damage, loss of function or cell death. The cell however, is well adapted to regulating the levels of ROS present utilising highly adapted antioxidant defense mechanisms (Toren and Nikki, 2000).

1.2.2 Reactive Oxygen Species and Redox Signaling in Maintenance of Redox Homeostasis

Whilst damaging in abundance and uncontrolled within the cellular environment, ROS have been shown to act as an effective defense mechanism against environmental pathogens, produced and activated via neutrophils and macrophages leading to 'oxidative burst' of the invading pathogen (Lee et al., 2012) and as important signaling molecules at lower concentrations; upregulating the

production of interleukin-2; an immunologically important T cell protein for example (Dröge, 2005). As a highly metabolically active organelle, it is believed that the majority of all ROS production within the cell comes from the mitochondria, specifically at two points in the electron transport chain (ETC) complex I (NADH dehydrogenase) and complex III (ubiquinone-cytochrome c reductase) (Cahill-Smith and Li, 2014). The process of ROS generation stems from complex I and II dehydrogenases of the ETC wherein these dehydrogenases are transferred to ubiquinone also known as coenzyme Q (Q), after which the reduced form of coenzyme Q (QH₂) undergoes two sequential one electron reductions using reduced and oxidised forms of cytochrome b (Cyt b) and cytochrome c (Cyt C), this process is termed the Q cycle and the intermediate produced within this step the semiquinone anion species ($\bullet$ Q⁻) is able to cause superoxide generation via transfer of electrons to molecular oxygen (Muller, 2000). In order to combat this highly reactive molecule, the enzyme SOD is able to dismutase the $\bullet$ Q⁻ anion into hydrogen peroxide, which can then in turn be metabolised via the action of the enzymes glutathione reductase (GPx) or catalase (CAT) resulting in the formation of molecular O₂ and H₂O (Muller, 2000). It is important to note that this process of superoxide formation within the mitochondria is non-enzymatic and therefore the rate of metabolic activity within the cell results in relative levels of ROS production. This is of particular importance within highly metabolically active cells such as neurons which are highly susceptible to oxidative damage due to their high levels of mitochondria, non-replicative nature and lower antioxidant capacity (Lee et al., 2012).

Another source of oxidative stress within the cellular environment; and leading to oxidative damage of the mitochondria are derivatives of nitric oxide (NO $\bullet$) forming reactive nitrogen species (RNS) (Kowaltowski et al., 2009).

NO $\bullet$ formation occurs through enzymatic generation via a group of nitric oxide synthases (NOS); endothelial NOS, inducible NOS and neuronal NOS (nNOS) (Giulivi, 2003). This family of enzymes synthesize NO $\bullet$ utilising the substrate L-arginine and NADPH as an electron source (Moncada and Higgs, 1993). NO $\bullet$ has been demonstrated to be an important signaling molecule with a number of its targets being present within mitochondria, however, it is a highly reactive molecule and in abundance and uncontrolled can cause oxidation and modification to the proteins and lipids found within mitochondria which can lead to loss of function (Gadelha et al., 1997).

Whilst endogenous generation of ROS as a direct result of metabolic activity forms a major component of ROS generation, there are also a range of environmental stimulants which can also lead to the production of ROS such as; ultraviolet radiation and chemotherapeutic agents (Toren and Nikki, 2000), it is for this reason that a highly specialised and adapted range of antioxidant defence mechanisms are present within the cell to establish and attempt to maintain a stable redox environment.

1.2.3 The impact of impaired autophagy on mitochondria and ROS induced cellular dysfunction

Being both responsible for the generation and as a target for modification by ROS the mitochondria, and its ability to function play a major role in maintaining a healthy redox environment within the cell.

An important aspect of maintaining this healthy internal environment is linked to the biological turnover of mitochondria which is heavily reliant on the process of 'autophagy'.

Autophagy plays a key role in maintaining stable intracellular homeostasis through the removal of damaged or undesirably modified cellular components utilising a lysosome mediated degradation process (Vidal et al., 2014). The process of autophagy is therefore closely linked to the action and levels of ROS within the cell, and dysfunction within this system have been closely linked to an increase in ROS accumulation, cellular protein modification and protein aggregation, leading to further mitochondrial dysfunction and eventual cell death; aspects of the ageing phenotype (Yang et al., 2013).

1.2.4 The mechanisms and importance of cellular quality control, autophagy and the ageing phenotype

In order to maintain a healthy and stable internal metabolic environment, cells utilise a range of care taking systems which identify damaged proteins and organelles, leading to repair or removal (Morimoto, 2008). Dysfunction within the cell is first identified by surveillance mechanisms, after which molecular chaperones are then able to aid in the refolding and repair of the damaged protein (Bhattacharya et al., 2014). Should the damage be too severe, molecular chaperones are also able to assist in the transport of damaged proteins to the two-major cellular proteolytic systems ready for removal; the lysosomal system and the ubiquitin proteasome system (Cuervo, 2008).

1.2.5 The lysosomal system

Within the cellular environment, the lysosomes are tasked with the degradation of unwanted or damaged macromolecules, leading to their removal from the cell (Vidal et al., 2014). In order to undertake this task they are specially adapted with lumen containing a vast cellular concentration of hydrolases; lipases, glycases, proteases, alongside a membrane containing high levels of permeases, specifically adapted for breaking down the constituents of proteins into the cytosol (Pitt, 1975).

Lysosomes play a key role in maintenance of the cell by recycling both damaged and functional proteins. This process of constant renewal allows the cell to utilise amino acids and other constituent parts taken from the breakdown of proteins to be synthesised into new cellular components and as an energy source. This resulting renewal of cellular components and the removal of potentially toxic accumulation of misfolded and dysfunctional cellular components ensures stable intracellular homeostasis (Pitt, 1975).

Lysosome mediated autophagy is categorised into three types; microautophagy, macroautophagy and chaperone-mediated autophagy, based on the cellular component involved, the chaperone and delivery to the lysosome and the activation process (Mizushima, 2010).

1.2.6 Chaperone Mediated Autophagy

Chaperone-mediated autophagy (CMA) is distinct from other forms of autophagy (macroautophagy, microautophagy) in that it targets single cytosolic proteins for degradation, rather than whole regions of cytosol via a process involving the mediation to the lysosomal surface and translation across the lysosomal membrane (Kon and Cuervo, 2010). The importance of CMA is in its selectivity, targeting highly specific soluble protein for degradation (Cai et al., 2015). In order to carry out this highly selective functionality the autophagic cargo must be completely unfolded prior to reaching the lysosomal lumen, as unlike the aforementioned methods of autophagy, CMA utilises substrates which are translocated across the membrane, rather than being sequestered (Kaushik and Cuervo, 2008). Two distinct protein complexes are required in order to undertake this translocation across the lysosomal membrane; cytosolic chaperones which are able to recognise the specific cargo (the cargo recognition complex) and mediating lysosomal proteins situated over the membrane (the translocation complex) (Salvador et al., 2000).

The action of the cargo recognition complex is still subject to discovery and as of yet not all aspects of the process are fully understood (Kon and Cuervo, 2010). What is known however is that all substrates involved in the CMA contain a motif biochemically related to the pentapeptide KFERQ within their amino acid sequence which allows for their lysosomal targeting (Kaushik and Cuervo, 2012). This important interaction has been demonstrated in studies involving the removal of KFERQ after which lysosomal degradation is completely halted (Dice et al., 1986). The pentapeptide motifs are mediated by the heat shock cognate protein of 70KDa (hsc70) (Kaushik and Cuervo, 2012). HSC70 is a cytosolic chaperone with multiple functions, including protein disassembly and refolding and the most influential component of the CMA recognition complex (Sala et al., 2014). Upon recognition of the pentapeptide motif, a substrate-chaperone is formed and the next stage of CMA

takes place at the lysosomal membrane (Kon and Cuervo, 2010).

The translocation complex is the second stage of the CMA and involves the binding of the substrate to the lysosomal membrane, ready for membrane translocation, utilising the lysosome-associated membrane protein type 2A (LAMP-2A) a splice variant of the *lamp2* gene that also mediates the rate of CMA (Bandyopadhyay et al., 2008). Upon binding to LAMP-2A, subsequent transfer into the lysosome lumen is then carried out, with the substrate complex interaction driving the formation of LAMP-2A into the multimeric complexes necessary for translocation of the substrate across the lysosomal membrane (Bandyopadhyay et al., 2008). Upon transfer of the membrane, the unfolded protein is then subject to degradation via luminal proteases (Kiffin et al., 2004).

1.2.7 Microautophagy

Microautophagy is a lesser known version of autophagy when compared to its counterparts (macroautophagy, chaperone-mediated autophagy) which are well documented (Mijaljica et al., 2011). It differentiates itself from other forms in that it is able to utilise the lysosomal membrane itself through a process of deformation and rearrangement to engulf protein for degradation (Shpilka and Elazar, 2011). The process itself can be split into five distinct stages; macroautophagic invagination and production of autophagic tubes, vesicle formation, vesicle expansion, vesicle scission and degradation of the vesicle within the lumen (Li et al., 2012).

The primary stage of microautophagy involves the invagination of the lysosomal membrane and formation of the autophagic tubes (Sahu et al., 2011). This stage requires the bulging of the lysosomal membrane into the lysosome itself via the lateral segregation of lipids and exclusion of transmembrane proteins (McPherson, 2015). The process in creating this invagination is driven by lipids and lipid modifying proteins, and regulated by a dynamin GTPase Vps1p (Uttenweiler et al., 2005). The extending of the invagination of the lysosomal membrane then forms a tubular shape, the 'autophagic tube', and there is a dramatic decrease in intramembrane density towards the top of the tube itself (Shpilka and Elazar, 2011). The process of tube formation is an energy mediated step requiring ATP, which implicates mitochondrial impairment as a potential source of dysfunction within this process (Todde et al., 2009).

Autophagic tube generation is mediated via autophagy related gene pathways APG and Aut (Uttenweiler et al., 2005). In order to initiate membrane tethering and vesicle fusion, two ubiquitin-like conjugation systems (Ublc) are required. The first system utilises Atg8 which is coupled with the membrane lipid phosphatidyl-ethanolamine (PE), a process requiring the action of E2-like enzyme Atg3 and the E1-like enzyme Atg7 following subsequent lipidation via Atg4 (Sou et al., 2006). The

second Ubic system required for membrane fusion utilises E1-like enzyme Atg7 and E2-like enzyme Atg10, which conjugate with Atg5 and Atg12. The formation of the Atg5-Atg12 dimer then oligomerizes with Atg16 forming Atg8-PE (Nakatogawa et al., 2007).

The second stage of microautophagy; vesicle formation, utilises the unique levels of high density lipids and low density protein at the top end of the autophagic tube, which when sorted result in a natural separation and subsequent vesicle formation (Müller et al., 2000). The next stage involves the expansion of the newly formed vesicle. This is an enzyme mediated process which produces a pre-vesicular structure contained within the lysosomal lumen (Sattler and Mayer, 2000). At this point within the cycle the macroautophagic vesicle is not completely separated from the autophagic tube and contains high levels of lipids and no protein (Sattler and Mayer, 2000). Enzymes are then able to enter and aid in the formation of the final form of the vesicle by binding the inside of the structure, after which two Ubic systems finalise the vesicle structure and mediate scission (Mayer, 2008).

Upon completion of scission from the autophagic tube the vesicle is free to move into the lysosomal lumen, where it is subject to a degradation process through the action of a range of hydrolases and then recycled by the permease Atg22p into beneficial cellular nutrients and as a cellular energy source (Yang et al., 2006).

1.2.8 Macroautophagy

Of the three forms of lysosome mediated autophagy discussed above (microautophagy, macroautophagy and chaperone-mediated autophagy), macroautophagy is the most prominent inducible pathway in mammalian cells utilised in the cycling of cytoplasmic components (Klionsky and Emr, 2000).

The process of macroautophagy involves portions of the cytoplasm becoming sequestered within a lifting membrane formed of vesicles known as autophagic vacuoles, sealing into an autophagosome, which is then subject to degradation via the appropriate lysosomes, acquired by fusion with secondary lysosomes (Yorimitsu and Klionsky, 2005).

Macroautophagy and the processes involved in the lysosomal mediated breakdown of cellular components can be broken down into four major steps; induction, formation of the autophagosome, autophagosome docking and fusion with the lysosome or vacuole and autophagic body breakdown (Klionsky and Emr, 2000).

In the first instance, the process of induction is regulated both nutritionally and developmentally. This is of particular importance as nonspecific autophagy has been shown to be induced under conditions of starvation and inhibited in nutrient rich conditions (He and Klionsky, 2009). At the basal level and under normal homeostatic conditions the rate of macroautophagy is low, therefore the process requires an intricate level of control in order for the cell to maintain cellular function in times of cellular stress, such as high levels of oxidative stress (Gottlieb and Carreira, 2010). The initial stage of induction occurs when TOR (target of rapamycin) is inhibited as a result of starvation, this causes the dephosphorylation of autophagy related protein 13 (Atg13). The dephosphorylated Atg13 then associates with Atg1 homologues ULK1 and ULK2 kinases which leads to the recruitment of FIP200 (focal adhesion kinase family-interacting protein of 200kDa). The formation of the complex ULK-ATG13-FIP200 then leads to the elongation of the isolation membrane and the finalised autophagosomal structure (Lee et al., 2012, Webster et al., 2014).

The second stage of macroautophagy, the generation of the autophagosome, initially utilises the beclin-1-class III PI3k (phosphoinositide 3-kinase) complex and the production of LC3 (light chain 3)-II which is inserted into the membrane of the autophagosome (Chen and Klionsky, 2011). The ubiquitin like protein complex Atg8/LC3 undergoes posttranslational modification as a result of LC3-I becoming LC3-II (Gallagher et al., 2016). Subsequently, the proteasome Atg4 cleaves the C-terminal region of newly generated LC3 becoming LC3-I, important in this case as it has an acquired exposed C-terminal glycine residue (He and Klionsky, 2009). LC3-I is then modified by phosphatidylethanolamine (PE) becoming LC3-II. This modification utilises a ubiquitination like reaction controlled by the enzymes Atg3 (E2 like conjugating enzyme), Atg7 (E1 like activating enzyme) and the ATG16L complex (E3 like ligase enzyme) (Lee et al., 2012). Atg7 promotes the conjugation of Atg3 to LC3-I and Atg12 to Atg10, following which the E2 like enzymes Atg10 and Atg3 catalyse the conjugation of Atg12 to Atg5 and PE to LC3-I which causes the conversion of LC3-I into LC3-II leading ultimately to the enlargement and elongation of the autophagosome (Lee et al., 2012, Mizushima et al., 2003).

Upon completion of the autophagosome, the process of vesicle fusion and autophagosome breakdown then take place (He and Klionsky, 2009). Autophagy related protein 8 (Atg8) currently attached to the outer membrane of the autophagosome is cleaved from PE by Atg4 and released into the cytosol (He and Klionsky, 2009). The process of fusion of the lysosome to the autophagosome is reliant on the action of the membrane protein LAMP-2 and the small GTPase RAB7 (Huynh et al., 2007).

Degradation is the final step in the process of lysosomal autophagy (Yang et al., 2013). Upon completion of the fusion stage degradation of the inner vesicle occurs resulting in the breakdown of

damaged protein and organelles (Mizushima and Klionsky, 2007), this process is reliant on a number of vacuolar and acid hydrolases; cathepsin B, D and L (Tanida et al., 2005). The resulting small molecules are then transported back into the cytosol, where they can be further utilised by the cell, for example, recycled amino acids can be used in protein synthesis and cellular homeostatic maintenance, in turn ensuring healthy function of the cellular proteome (Yang et al., 2006).

1.3 Autophagy in Ageing and Disease

There has long been established a link between a decrease in proteolytic activity and the subsequent increase in damaged cellular components in all ageing mammalian tissues (Toren and Nikki, 2000). Impairment or dysfunction within the systems responsible for the removal and maintenance of these tissues therefore has a significant impact on the health and well-being of every cell (Dröge, 2005). Age induced alterations within the lysosomal system and any subsequent deficiency within the process of autophagy as a result of ageing can therefore lead to, and have been implicated within a number of age related conditions including certain cancers, muscular disorders and neurodegeneration (Shintani and Klionsky, 2004).

1.3.1 Autophagy in Cancer Pathology

The role of autophagy in cancer pathology is a dual edged sword. On the one hand it can act as a potent cancer suppressor, whilst also allowing cancer cell survival later on within the tumour growth cycle when nutrients and oxygen levels are limited (Ogier-Denis and Codogno, 2003).

The signalling pathways involved in the process of autophagy have also been implicated in the formation and promotion of cancer cell growth (Kimmelman, 2011). The class I phosphatidylinositol (PtdIns) 3-Kinase/protein-kinase (Akt/PKB) signalling pathway causes cell growth in response to mitotic signalling, however, mutations in various proteins within this pathway have been linked to a range of malignancies (Peter and Tony, 2001).

Another example of autophagy having a role in cancer is that of the protein Beclin 1, which serves to allow the formation of the autophagosome (Shintani and Klionsky, 2004). Beclin 1 has been implicated within a range of cancers including ovarian, breast and prostate where research has shown levels to have been reduced (Xiao Huan et al., 1999). Whilst on the contrary, it has also been demonstrated to have increased expression within gastric cancer cell lines, whereby a higher level of beclin 1 lead to higher survival rates (Geng et al., 2012). This is understandable as beclin-1 instigates autophagic activity and therefore aids in the prevention of tumorigenesis and inhibits proliferation,

thus any mutation which prevents the action of bectin-1 will have the opposite effect and promote cancer pathology (Qu et al., 2003).

1.3.2 Autophagy in Muscular diseases

The role of autophagy in the control and prevention of muscular diseases and it's role in the maintenance of muscle mass have been well documented (Bechet et al., 2005). Due to the very nature and role of autophagy in the turnover and maintenance of cellular function, it is not surprising that dysfunction within the processes involved in lysosomal degradation can have a negative impact on myocyte health (Henriksen et al., 2013).

The first incidence and link between dysfunctional autophagy and muscular defects was noted in a range of diseases impacting lysosomal storage, known as autophagic vacuolar myopathies (AVM) (Nishino, 2003). AVM are a range of hereditary diseases where genetic dysfunction within the lysosomal system leads to the accumulation of autophagic vesicles within skeletal myofibers due to defective clearance systems (Sugie et al., 2003). Whilst the accumulation of autophagic vesicles is evident within these disease states and potentially pathologically relevant, the mechanisms by which they act on the cell itself are not fully understood (Sandri et al., 2013). The two most prevalent AVM are Danon disease and Pompe disease (Malicdan et al., 2008).

Pompe disease, also known as glycogen storage disease type II (GSD II) is an autosomal recessive disorder, caused by a deficiency of acid alpha 1,4-glucosidase (GAA) (Remiche et al., 2014). GAA is a lysosomal enzyme utilised in the hydrolysis of glycogen to glucose, and a deficiency of this enzyme leads to the accumulation of glycogen within the lysosome leading to dysfunction (Cheng and Juan, 2014). There are multiple forms GSD II ranging from partial GAA deficiency which can lead to late onset of GSD II, to severe early-onset forms which can cause infantile death within the first year of life (Nascimbeni et al., 2012).

Danon disease or GSD type IIb, shares a similar pathology as that of pompe disease, with the eventual accumulation of glycogen within myocytes leading to loss of function (Zhongwei and Quan, 2012), it's mechanism of action however is linked to a rare x-linked mutation in the LAMP-2 protein (Perry et al., 2013). The mutation in LAMP-2 leads to a build-up of vacuolar structures containing a range of degradation associated proteins, surrounded by lysosomes. This abundance of structures eventually lead to dysfunction within the cell and ultimately cell death (Dana et al., 2011).

1.3.3 Autophagy in Neurodegeneration

A range of neurodegenerative diseases have been noted to share a similar pathology related to a potential dysfunction in the lysosomal degradation system, termed conformational disorders (Vidal et al., 2014). Conformational disorders originate from a cellular malfunction causing the build-up and accumulation of toxic misfolded or aggregated proteins within the cellular environment, resulting in widespread cellular damage and eventual apoptosis (Xilouri et al., 2016).

Autophagy or changes within the lysosomal system have been implicated in the world's most prevalent age related neurodegenerative disease; Alzheimer's disease (Martinez-Vicente and Cuervo, 2007). Alzheimer's disease, characterised by neuronal loss and the accumulation of neurofibrillary tangles has a close connection to the function and dysfunction of the lysosomal system (Cecarini et al., 2016). The implication of autophagy within Alzheimer's disease has been shown to occur prior to the accumulation of amyloid deposits, with the up-regulation of the lysosomal system resulting in the increased presence of lysosomal enzymes implicated in removal of aggregated proteins (Blandini et al., 2006). Lysosomal dysfunction eventually takes hold as the disease progresses to its latter stages, whereby the efficacy of the system can no longer effectively contribute to the clearance of misfolded and aggregated proteins, resulting in the accumulation of autophagic vacuoles within the cell that are unable to fuse with lysosomes for the degradation of their internal cargo (Li et al., 2017). One theory suggests that the accumulation of lysosomal vacuoles within the cell may impact cell trafficking or become an internal source for the generation of Amyloid β ($A\beta$) contributing to the deposition of amyloid and leading to eventual cell death (Yu et al., 2005).

Whilst autophagy is responsible for the large scale degradation and recycling of defective organelles, another important cellular degradation system is at work ensuring the maintenance of cellular homeostasis through the removal of short lived proteins; the ubiquitin proteasome system (UPS) (Dantuma and Bott, 2014).

1.4 The Ubiquitin Proteasome System

1.4.1 An introduction to the importance of the UPS

The maintenance of homeostasis within the intracellular environment through the degradation and synthesis of potentially harmful or dysfunctional proteins serves an important function in maintaining the long term viability of the cell (Alfred, 2003). Within all eukaryotic cells the ubiquitin-proteasome system (UPS) plays a major role in the destruction and synthesis of dysfunctional protein, through the action of the ubiquitin protein acting as a marker for degradation by the larger

proteolytic chamber of the proteasome system (Nath and Shadan, 2009).

The link between the process of protein ubiquitylation and degradation was awarded the 2004 Noble Prize in Chemistry affirming the importance of the role of the UPS in the maintenance of cellular function (Bassermann et al., 2014). It has since been implicated in a range of cellular functionality, including; growth, cell-cycle regulation and DNA repair (Nath and Shadan, 2009). The importance of successful function of the UPS is therefore well established and as such any dysfunction within the system could play a role within a range of diseases, such as certain cancers and neurodegeneration (Dantuma and Bott, 2014).

1.4.2 Ubiquitination

Within the intracellular environment of the eukaryote, proteins undergo a variety of post-translational modifications, allowing for the expansion and functional diversity of the proteome (Hochstrasser, 2009). These translational modifications will typically involve the attachment of small molecules, such as a phosphate or methyl groups to the protein (Pickart, 2001). Ubiquitin is one such posttranslational protein and is able to covalently conjugate a range of intracellular proteins (Zheng et al., 2016).

The process of ubiquitination requires the conserved 76-residue polypeptide forming a bond at the C terminus (G76) with the ϵ -amino acid group of a target substrate lysine residue (Hochstrasser, 2009). In order to carry out this process three specific enzymes are required to act sequentially resulting in the initiation of the ubiquitination reaction; activating enzyme (E1), a conjugating enzyme (E2) and a ligase (E3).

The process resulting in the formation of the ubiquitin chain begins with the E1 enzyme; the yeast *saccharomyces cerevisiae* (in humans), which activates ubiquitin for all downstream conjugating enzymes (Dohmen et al., 1995). The process starts with the binding of MgATP and ubiquitin, this in turn leads to the formation of ubiquitin adenylate; an intermediate that donates ubiquitin to a cysteine within the active site of E1 (Finley and Chau, 1991). The newly formed E1 molecule now carries two molecules of activated ubiquitin ready for the next stage of the cascade; one in the form of an adenylate and one as a thiol ester, a process requiring ATP binding which appears to increase the affinity of ubiquitin to E1 (Finley and Chau, 1991).

The second step involves the conjugation of the activated ubiquitin utilising the E2 enzymes of which there are 35 currently known (an increase on the number of E1 enzymes present having only just 2 forms) (Li et al., 2008). Each E2 enzyme acts on just one or a small number of E3 ligases resulting in a high degree of specificity and control over the process, however all E2 enzymes share a similar

structure of a conserved core domain of ~150 amino acids (Brazee et al., 2016).

A substrate specific E3 ligase then transfers the ubiquitin molecule to the target protein in a process involving two steps; E3 binding to the substrate and the covalent ligation of the ubiquitin to the substrate (Elton et al., 2015). Repetition of this sequence of ubiquitin transfer to a target protein can form ubiquitin chains, whereby the C-terminal glycine residue can attach the ϵ -amino group of the subsequent substrate lysine residue, acting as a signal for protein degradation by the proteasome (Zheng et al., 2016).

Whilst degradation is a major component of the ubiquitin modification, ubiquitylation has been also been linked to a range of processes outside of degradation via the proteasome, including that of protein trafficking, the assembly of protein signalling complexes and regulation of enzymes (Hoeller and Dikic, 2009).

1.4.3 The proteasome

The Lys48-linked polyubiquitin chains are regarded as the main source of ubiquitin signalling for proteasomal degradation via the 26S proteasome; although other sources and motifs have also been recognised (Bedford et al., 2011). Also called the proteasome holocomplex the 26S proteasome has been linked to a range of intracellular homeostatic controls including cell cycle related functions and DNA repair (Simon et al.). It is composed of two structures: a cylindrical structure within the centre of the proteasome; the 20S or core particle (CP) responsible for peptide hydrolysis, and the regulatory particle (RP) also known as the 19S proteasome, responsible for the recognition of substrates, removal of the ubiquitin chain and substrate unfolding (Schmidt and Finley, 2014).

The 20S proteasome is formed of a cylindrical structure which contains a number of active sites. The cylinder itself is composed of two subunits; namely an alpha (α) and beta (β) subunit (Van Way, 2004). The alpha and beta subunits are arranged in a stacked formation of heptameric rings surrounding an inner core (Collins and Goldberg, 2017). The two inner rings are composed of two beta subunits, whilst the two outer rings are made up of seven alpha subunits (Bajorek and Glickman, 2004). Important to the functionality of the proteasome system, the three beta subunits each contain an active site which cleave specific peptides. The β 1 subunit targets the caspase-like peptide cleaving acidic residues, whilst β 2 is trypsin-like, cleaving basic residues and β 5 is chymotrypsin-like, cleaving after large hydrophobic residues (Schmidt and Finley, 2014). The active sites of these β subunits face inwards, and the proteasome itself is capped by a gate which acts to regulate transport into the catalytic cavity where the process of degradation takes place (Van Way, 2004). The CP gate remains in a closed default position (Schmidt et al., 2005). In order to trigger the opening of the CP gate the N-termini need to be shifted from their axial position, resulting in the

formation of a channel allowing the free movement of the ubiquitinated proteins into the 20S proteasome (Bajorek and Glickman, 2004). The process of triggering the opening of the CP gate is regulated by proteasome activators, of which there are multiple; including the aforementioned regulatory particle or 19S proteasome, as well as the PA200 and PA28 activators (Schmidt et al., 2005). Whilst there is variety in proteasome activators, they all bind to the CP in the same way; α subunits form pockets on the outer surface of CP, which act as binding sites for the proteasome activators (Nath and Shadan, 2009). Where they differ however is in the process of N-termini displacement, a necessary step in the opening of the CP (Collins and Goldberg, 2017).

1.4.4 The 19S proteasome

The most prominent proteasome activator is the 19S proteasome, which acts through a process of ATP and ubiquitin dependent substrate turnover promotion (Roelofs et al., 2009). Activation occurs when the 19S proteasome binds to one of the two ends of the CP, forming the proteasome holocomplex (Groll and Huber, 2003). The 19S proteasome, as its name suggests is composed of 19 subunits which in turn form two subcomplexes in the process of activation; the base and the lid (Groll and Huber, 2003). The lid subunit is composed of nine non-ATPase subunits, whilst the base contains six homologous ATPases; Rpt1, Rpt2, Rpt3, Rpt4, Rpt5 and Rpt6 which form a hexameric ring located near the CP gate and have an important role in the activation of the proteasome and opening of the CP gate (Schmidt et al., 2005). The gate opening process starts with the action of three of the aforementioned ATPases; Rpt2, Rpt3 and Rpt5 binding into the α ring of the CP (Schmidt and Finley, 2014). These subunits contain a unique motif at their C-termini, known as a HbYX motif (Smith et al., 2007). The HbYX motif is made up of a hydrophobic residue, a conserved tyrosine and a variable C-terminal residue and has been implicated in the activation of gate opening through the displacement of conserved residues within the binding pockets of the CP α ring, which in turn leads to structural changes within the N-termini of the gates α subunits (Smith et al., 2007). This process then leads to the formation of a channel which allows for movement of protein into the catalytic chamber for degradation (Nath and Shadan, 2009). It should be noted however that the proteasome gate is limited to the allowance of unfolded proteins only, into the catalytic core and therefore requires the action of proteasomal ATPases to unfold target proteins in an ATP dependent process (Nath and Shadan, 2009).

1.4.5 The Ubiquitin Proteasome System in Health and Disease

The importance of the ubiquitin proteasome system is now well established in its link between healthy intracellular homeostasis and removal of unwanted protein through the process of

ubiquitylation (Dantuma and Bott, 2014). It is therefore understandable that any fluctuation in the function of the UPS could lead to cellular damage or cell death due to the accumulation of unwanted cellular components or oxidative stress and has been implicated in a range of disease states including cancer, cystic fibrosis and neurodegenerative diseases (Alfred, 2003).

1.4.6 The UPS in Cancer

Given the nature of the UPS and its impact on cellular functionality, it is understandable that it would have a role to play in a range of cancers (Hoeller and Dikic, 2009). Whilst a major functionality of the proteasome system is in the management and degradation of unwanted or damaged proteins, ubiquitylation also regulates a number of cellular process which can prevent tumour genesis, apoptosis, cell cycle progression and gene transcription, which can all play a role in cancer pathology (Bassermann et al., 2014).

The specificity of the E3 ligases makes them an interesting target for potential action in cancer pathology (Bernassola et al., 2008). E3 ligases are categorised into three types dependent on their structure, their action processes and presence of an HECT, U-box or RING domain (Hoeller and Dikic, 2009). It has been shown that a range of E3 ligases have been implicated in cancer as a result of their ability to cause degradation of tumour suppressors and oncogenes (Keiichi and Keiko, 2006) for example the RING domain E3 ligase Hdm2 has been demonstrated to have an oncogenic capacity as a negative regulator of the p53 protein (Vousden and Prives, 2005). Dysfunction therefore within the ability of the E3 ligases can have a detrimental effect on cell, resulting in the promotion of tumours or as a negative regulator of tumour suppressor proteins such as p53.

1.4.7 The UPS in Cystic Fibrosis

The UPS is also implicated within the world's most common inherited disease; cystic fibrosis, which arises as a result of a malfunction of the cystic fibrosis conductance regulator (CFTR) gene a chloride ion channel protein resulting in the thickening of secretions which can cause breathing difficulties (Rommens et al., 1989). The mutation leads to a misfolding of the CFTR protein becoming inactive (Turnbull et al., 2007). As a result of this mutation CFTR is unable to reach the epithelial plasma membrane of the lungs where it acts as a Cl^- ion channel and instead is subject to targeted degradation via the UPS (Turnbull et al., 2007). In this instance, the UPS is able to detect mutations within the CFTR protein which lead to misfolding, via the cytosolic chaperone Heat shock protein 70 (HSP70), which interacts with the E3 ligase CHIP forming a HSP70-CHIP complex diverting the misfolded protein to the proteasome for degradation (Turnbull et al., 2007).

1.4.8 The UPS in Neurodegeneration

The UPS as with all tissues plays an important role within the maintenance of neuronal function and health (Zheng et al., 2016). The Ubiquitin system may be particularly relevant however, as many neurodegenerative diseases share a similar pathology of toxic accumulation of aggregated ubiquitylated protein within the neurons of those affected (Dantuma and Bott, 2014). It has been suggested that the toxic accumulations of aggregated proteins such as α -synuclein in Parkinson's disease and β -amyloid plaques seen in Alzheimer's disease can also cause dysfunction within the UPS system itself, resulting in the delay of other compromised or damaged proteasomal substrates that have important roles in cell cycle regulation, cell signalling and apoptosis leading to further cellular dysfunction, which could play a major role within the overall pathology and progression of these diseases (Grimm et al., 2012).

1.5 Neurodegeneration

The impact of an ageing population has been linked to an increased prevalence of age-related neurodegenerative diseases (ND) such as Parkinson's disease (PD) and Alzheimer's disease (AD) (Doudet, 2007). This has been noted by the World Health Organisation as a significant and potentially catastrophic health issue, which may reach epidemic proportions if sufficient research is not undertaken to begin to understand the complex mechanisms involved in the pathology of these diseases.

1.5.1 The pathology of neurodegeneration

Neurodegenerative diseases such as Alzheimer's disease (AD), Huntington's disease (HD) and Parkinson's disease (PD) share a common age-related pathology characterised by inclusion body formation and the deposition of abnormal protein aggregates within the brain (Jomova et al. 2010).

1.5.2 Alzheimer's Disease

Alzheimer's disease is currently the world's most prolific age related neurodegenerative disease, and leading cause of senile dementia, whilst also being the fourth most common cause of death within the western world (Reinhardt, 2000). With an ever-increasing ageing population AD represents an extreme global health burden and expense that is set to increase as medical advancement leads to longer life spans (Ferri et al., 2005). Pathologically Alzheimer's is characterised by an initial brain atrophy with a loss of neurons within the CNS and other affected regions over time (Herb, 2011).

1.5.3 The Known Pathology of Alzheimer's disease

The most prevalent biological markers of Alzheimer's are the presence of amyloid plaques and neurofibrillary tangles, present within the neurons of those who suffered the disease (Ballard et al., 2011). The most prominent hypothesis for the deposition of amyloid within those affected is the 'amyloid cascade' which suggests that amyloid beta ($A\beta$) deposition is a leading cause of neuronal cellular dysfunction leading to cell death within the brain (Armstrong, 2014).

Whilst originally it was thought that the loss of cellular function within the neuron was as a result of the increased volume of amyloid within the cell, it has now become apparent that it may be within the processing of $A\beta$ into smaller peptides that the dysfunction occurs; specifically with the cleaving of the amyloid precursor protein (APP) into $A\beta$ peptides which have been shown to aggregate more readily than the APP (Weitz and Town, 2016) as well as small oligomers of $A\beta$ which have been noted to be more toxic to the intracellular neuronal environment than the mature fibrils (Gutz et al., 2012). Whilst the actual action of $A\beta$ fibril and plaque formation within AD is still hotly debated, its presence within the neurons of those affected imply that it does in fact have a significant role in the pathology of the disease.

Another implicated factor within the pathology of AD and also a consistent staple of post mortem tissue examination is the protein Tau, which has been shown to constitute the majority of the neurofibrillary tangles present within the disease state (Medina and Avila, 2014). Tau is a microtubule associated protein thought to be implicated within AD as a result of the increase in $A\beta$ levels within the intracellular environment, however pathways linking $A\beta$ and Tau are not fully understood (West and Bhugra, 2015). Under normal circumstances, intracellular tau is a soluble protein, however upon aggregation of tau monomers the subsequent β sheet formed fibrillary tangles are insoluble, and over time, and subsequent accumulation can cause damage to the neurons (West and Bhugra, 2015).

1.5.4 Huntington's Disease

Huntington's disease (HD) is an inherited autosomal-dominant genetic neurodegenerative disease which occurs as a result of a nucleic acid triplet repeat expansion (Anderson, 2011). The disorder is characterised by movement problems in the form of symptoms of dystonia and chorea, whilst patients also show impairments in cognitive and psychiatric function as the disease progresses (Watt, 1990). HD is thought to occur in approximately 5 in every 100,000 people, and unlike the

previously mentioned disorders can occur at any age within the life span, however primary symptoms usually occur between the ages of 35 and 44 years (Li et al., 2013).

1.5.5 The Known Pathology of Huntington's Disease

Huntington's disease pathology is led by a genetic mutation of the *huntingtin* protein resulting in the abnormal expansion of trinucleotide repeats which code for glutamine at the N-terminus and subsequent aggregation of the protein (Watt, 1990). Whilst the protein is expressed throughout the body and has no currently known functionality in health, it is thought that the mutant form causes neuronal damage through a combination of mitochondrial dysfunction, gene regulation defects and excitotoxicity (Porteracailliau et al., 1995). This overall neuronal decline is seen most prominently within the striatal medium spiny striatal neurons, whilst the striatum, caudate and globus pallidus have all been implicated within the disease with widespread atrophy of the brain occurring as the disease progresses (Corrochano et al., 2012).

1.6 Parkinson's Disease

Originally described by James Parkinson in 1917 as “paralysis agitans” within his work entitled “Assay on the Shaking Palsy”, Parkinson's disease, as it is now widely known is currently the second most diagnosed neurodegenerative disorder within the elderly populace, after Alzheimer's disease (Cacabelos, 2017). Parkinson's results from a cumulative loss of dopaminergic neurons within multiple brain regions but most prominently the substantia nigra pars compacta (SNpc). This in turn leads to a dopamine deficiency within the basal ganglia region resulting in the most common characteristic of PD, the motor symptoms; brady kinesia, resting tremor and gait impairment (Kalia and Lang, 2015). Whilst well known for its resulting impact on motor system functionality, Parkinson's disease is also implicated in a range of non-motor dysfunction such as sleep disorders, that are likely to occur long before motor dysfunction occurs (Kalia et al., 2013).

1.6.1 Epidemiology of Parkinson's Disease

The prevalence of Parkinson's disease has been shown to be on the rise, due in part to the steadily ageing global population. Incidence rates have been seen to range from 35.8 per 100,000 to 12,500 per 100,000 people across multiple regions of the world (Von Campenhausen et al., 2005). However, whilst the exact rates of prevalence have been shown to vary, data demonstrating the rising

prevalence of PD as an age-related condition have become conclusive. This positive correlation has been demonstrated by multiple groups via systematic review and meta-analysis on Worldwide data. Whilst early studies (Twelves et al., 2003) implicated the increased incidence of PD with age, demonstrating an overall incidence rate of the disease in 17 per 100,000 person years, according to the highest quality study methodology assessed. More recent analysis (Hirsch et al., 2016) have verified these earlier findings, demonstrating a continued trend of disease incidence in the elderly population assessed within the meta-analysis of studies conducted. Systematic review of 27 studies showed an overall incidence rate of Parkinson's in both men and women increased with age; with those 40 years and over showing an overall incidence of 61.25 (95% CI 43.57-85.00) and 37.55 (95% CI 26.20-53.83) respectively, with incidence rates increasing with age from 3.57 and 3.26 per 100,000 person years within the 40-49 year age range up to 258.47 and 103.48 at 80+ years respectively. Not only does this data demonstrate what was already believed regarding the link between PD and age but interestingly that within this data set, men had a higher incidence rate of disease throughout the age range.

1.6.2 Current Diagnosis of Parkinson's Disease

Current diagnosis of PD focuses predominately on the visible and measurable clinical data characteristics of the disease; resting tremor, rigidity, bradykinesia and postural balance issues (Katzenschlager and Lees, 2002). Non-motor dysfunction within the disease progression include psychiatric and cognitive impairment, alongside sleep disorders and chronic fatigue (Baig et al., 2015). It is often noted that presence of these non-motor symptoms onset before any noticeable tremor and therefore given what is now known about the disease in terms of its pathogenic mechanisms relating to protein degeneration leading to uncontrolled neuronal death, it is likely that the disease and its diagnosis through clinical data may be outdated and these effects may occur at a later stage of disease progression (Gershanik, 2017). As the disease state progresses both motor and non-motor symptoms worsen, with dementia being reported in a high number of long term Parkinson's disease sufferers (Cacabelos, 2017). Whilst initially the disease can be medicated to help to reduce the symptomology, towards the later stages symptoms become much more severe and are no longer manageable through conventional medicative means; due not only to a lack of uptake, but also as a result of increased dosage leading to severe side effects such as psychosis (Katzenschlager and Lees, 2002).

It is for this reason that our ability to gain a more comprehensive understanding of the cellular changes which take place during the earlier stages of PD, prior to the onset of clinical characteristics may be the key to developing a preventative medicine to combat the disease.

1.6.3 Current treatment L-DOPA

Although originally a ground-breaking finding, the use of the neurotransmitter precursor Levo (L) – DOPA as a therapeutic in the 1960's is still the most common form of treatment for the symptoms of Parkinson's. However, like most therapeutics utilised to target the symptomatic profile of PD it does not prevent the underlying neurodegenerative processes which are taking place within the cell, and itself comes with a range of severe psychological side effects (Katzenschlager and Lees, 2002).

1.6.4 The underlying pathology of Parkinson's disease

Although the underlying cause for PD onset is still unknown, it is highly likely that no single trigger causes the disease, but a range of factors are responsible for the eventual decline in neuronal function. As previously stated, it is likely based on the success of recent findings, to conclude that Parkinson like neurodegeneration is likely to occur decades before the eventual onset of clinical symptoms such as the detectable tremor.

Parkinson's disease pathology is characterised by a number of events resulting in the eventual decline in cellular homeostasis and cell death. It is thought that contributory factors of PD range from oxidative stress, mitochondrial dysfunction, excitotoxicity and aggregation, to misfolding of intracellular protein as a result of dysfunction within the UPS and lysosomal degradation pathways (Hindle, 2010), a range of these mechanisms are also present within the ageing phenotype, linking the disease progression with age.

The most notable pathological hallmark of Parkinson's disease is the overall loss of dopaminergic neurons within the substantia nigra pars compacta region of the brain, alongside the deposition of α -synuclein aggregates in the form of 'Lewy bodies' (LB) (Kalia and Lang, 2015). Loss of dopaminergic neurons within the SNpc region have been showed to be the leading cause of the motor dysfunction present within the latter stages of disease progression whilst neuronal loss has been demonstrated to be widespread throughout multiple brain regions, including; the hypothalamus and amygdala (Dickson et al., 2009).

1.6.5 Alpha Synuclein Aggregation

Accumulation of α -synuclein as the primary aggregated protein within Parkinson's disease classifies it within a range of proteinopathies known as 'synucleinopathies' (Kalia et al., 2013). Whilst it was originally speculated that the inclusion of protein aggregation induced cellular dysfunction and eventual cell death, progression within this area of investigation has led to developments which potentially challenge the role of protein inclusions as the primary factor in neurological decline (Kalia et al., 2013). Studies into the neuropathological decline in PD have demonstrated that whilst large insoluble inclusion bodies clearly play a defined role within the progression of the disease, the disease linked proteins susceptible to aggregation, including α -synuclein, have also been shown to form smaller soluble oligomers, which may also contribute to the eventual decline of neurons (Conway et al., 2000).

The structural related protein α -synuclein is a 14kDa protein, with a high level of expression within the brain (Jakes et al., 1994). Under normal intracellular physiological conditions, α -synuclein is enriched at presynaptic terminals where it plays a role in the assembly of the SNARE proteins (Jakes et al., 1994); those whose primary role is to mediate vesicle fusion (Burre et al., 2010), as well as aiding in the function and regulation of neurotransmitter release and nerve terminal health (Chandra et al., 2005). Whilst α -synuclein is believed to be an unfolded monomer within its standard form, the presence of α -synuclein rich oligomers and aggregations within the neuron imply that it is able to bind and aggregate into larger, potentially more damaging forms (Li et al., 2014). It is believed this process consists of multiple stages resulting in the eventual inclusion formation. The initial stage of the process involves the aggregation of multiple α -synuclein monomers, resulting in the formation of a soluble oligomeric form also called protofibrils (Conway et al., 2000). α -synuclein has been shown to aggregate without the need for the presence of any other protein, with increasing concentration of α -synuclein resulting in a higher affinity for aggregation, making it a potentially damaging protein and sharing common formation characteristics to that seen in A β , implicating a similar pathological role (Schieler et al., 2006).

The rate limiting nucleation step within the aggregation process involves the self-association of the oligomeric species forming a nucleus, after which further aggregation occurs through the addition of multiple oligomers binding the nucleus forming 'amyloid fibrils' (Kalia et al., 2013). Amyloid fibrils are larger protein aggregates and are common place within a range of neurodegenerative diseases, with α -synuclein aggregated fibrils being the largest component of a major hallmark of PD, the Lewy body (Cacabelos, 2017).

A number of genetic mutations within the α -synuclein gene (SNCA) have been implicated in the promotion of the aggregation of the protein and are often seen in the familial forms of the disease (Conway et al., 2000), whereby the mutation can lead to an increase in the intracellular α -synuclein

concentration, resulting in the protein having a higher affinity for aggregation (Kasten and Klein, 2013). Genetic mutation has also been implicated within the idiopathic form of Parkinson's, with SNCA mutations being shown to be known risk factors for disease progression. Whilst it is not fully understood how SNCA mutation leads to α -synuclein aggregates, it is thought that mutations within the gene could result in the increased expression of the protein and therefore lead to increased concentration within the intracellular environment, leading to further aggregation (Fuchs et al., 2008).

Increased levels of α -synuclein, and α -synuclein aggregations have also been implicated as a result of a dysfunction within the intracellular mechanisms which degrade and recycle misfolded and toxic proteins (Vidal et al., 2014). Ubiquitination of α -synuclein has been shown to reduce aggregation (Zheng et al., 2016), which also implies that any dysfunction and dysregulation within this system can lead to an increase in intracellular levels of α -synuclein resulting in an increased presence of aggregates which can ultimately lead to neuronal death. Alongside the UPS, molecular chaperones and the lysosomal degradation system have also been implicated as important factors in the cells ability to prevent intracellular aggregation via α -synuclein accumulation (Xilouri et al., 2016), and importantly have also been shown to be dysregulated as a result of an increased aggresomal presence which can in turn lead to an increased level of aggregation and damage via oxidative stress (Cacabelos, 2017).

1.6.6 Lewy bodies in Parkinson's Disease

As a general hallmark of a number of neurodegenerative diseases, the presence of aggregated protein within the neurons of those affected is also present within Parkinson's disease in the form of fibrillar aggregates containing ubiquitinated α -synuclein as a major constituent, also known as Lewy bodies (Wakabayashi et al., 2013).

The progression and accumulation of Lewy bodies within the brain have been staged by Braak (Braak et al., 2004). Stage 1 encompasses changes which are limited in their scope to the dorsal motor nucleus and olfactory bulb only, resulting in the loss of smell; an early stage marker for Parkinsonism. Stage 2 sees the spread of Lewy formation to the medulla and pons region, whilst stages 3 and 4 begin to show motor dysfunction within patients suffering the disease. Finally, during stages 5 and 6 Lewy pathology is now severe and widespread throughout multiple brain regions, including the neocortical areas resulting in the common neurological dysfunction; cognitive impairment and dementia (Braak et al., 2004).

There are two forms of Lewy body found within those effected by neurodegeneration; the brainstem or classical type and the cortical type (Wakabayashi et al., 2013). Whilst both types have a filamentous structure, expressing high levels of ubiquitin (Lennox et al., 1988). The brainstem type can be identified within post mortem tissues of PD sufferers utilising light microscopy, as single or multiple spherical or elongated accumulations harbouring a core and exterior halo. The cortical LB are less visible and prominent than their classical counter parts, having a more irregular shape and less well defined structures (Wakabayashi et al., 2013).

The formation of the brainstem form of Lewy body within neurodegeneration is a multi-step process starting with an intraneuronal accumulation of dust-like particles of neuromelanin or lipofuscin, followed by accumulation of α -synuclein and ubiquitination, the aggrasome is then condensed to ubiquitinated filamentous inclusions (Kuusisto et al., 2003). The cortical form undertakes a similar multi step process; primarily undergoing accumulation of the granular components of the LB, followed by the aggregation of filamentous components of the inclusion containing ubiquitin and α -synuclein labels, and finally spreading to dendrites where they take on their final LB form (Katsuse et al., 2003).

Alongside the LB, another form of neuronal inclusion can be seen within the most affected regions of the brain during PD progression; the SNpc and locus ceruleus, Known as 'pale bodies' (PB) (Bedford et al., 2008). LBs and PBs often coexist within the same intracellular environment, and share a similar protein composition; showing the presence of ubiquitin and aggregated α -synuclein, neurofilaments and components of the UPS, leading some to believe that the early presence of PBs within PD may be indicative of PBs being a precursor for the formation of LBs (Jellinger, 2009).

Whilst it was originally hypothesised that the LB was a major factor in the eventual irreparable cell damage and neuronal death, recent studies have proposed that the presence of LBs within the intracellular environment may serve a cytoprotective purpose in PD (Wakabayashi, 2008).

1.6.7 Risk Factors

The onset of Parkinson's disease has been linked to a number of potential risk factors; including toxin and pesticide exposure, gender, cytotoxicity, mitochondrial dysfunction and oxidative stress (Tan, 2013), whilst the most prominent and influential factor is that of age, with incidence rates seeing an almost exponential increase after the age of 80 (Hindle, 2010). PD also has a genetic link, with 10-15% of those diagnosed having a family history of the disease (Powers et al., 2007). 13 genes have been implicated within the development of PD , with a high incidence of genetic forms

occurring as the rarer form of younger on-set PD, rather than the aged form (Saiki et al., 2012). An example of a genetic defect causing the late on-set form of the disease is found in leucine-rich-repeat-kinase2 (LRRK2). Occurring in 1-2% of all PD cases, this genetic form of late on-set PD usually occurs between 50-60 years old, with a differing pathology to that of the non-genetic form of the disease (Singleton and Hardy, 2016).

1.6.8 Protein regulation in Ageing

Unlike cells of the periphery, neurons are in a constant state of homeostatic balance as they are unable to control the amount oxidative and molecular damage they sustain on a regular basis via cell division and must therefore actively rely on intracellular mechanisms to maintain a healthy and controlled environment. What makes brain tissue even more susceptible is the main component of oxidative stress within the cell; the mitochondria and their abundance within the neuron as a highly metabolically active tissue. Maintenance of this intricate balance is therefore crucial to the health of the cell and has been notably affected within ageing, as an increase in the levels of oxidised macromolecules alongside an upregulation of protein involved in the oxidative stress response are known to be present within the cellular environment (Toren and Nikki, 2000).

1.6.9 Superoxide Dismutase 1

Superoxide dismutase 1 (SOD1) is a copper-dependent anti-oxidant enzyme. It is known to catalyse the conversion of superoxide radicals into less reactive oxygen species within the intracellular environment, into hydrogen peroxide molecules which are then converted to molecular oxygen and water by the enzyme catalase (Tórsdóttir et al., 2006). The link between the accumulation of reactive oxygen species within the cell ultimately leading to cell death is well noted (Squier, 2001).

Oxidative stress through the accumulation of radical species can lead to decline in cellular function and stability and is one of the major risk factors for development of Parkinson's disease. As of yet, there is an overall lack of evidence or research into the impact or changes within SOD1 levels in human Parkinson's disease sufferers. Whilst the protein and mutations within the gene have been implicated in a range of neurodegenerative disorders; including amyotrophic lateral sclerosis (Acosta-Saltos et al., 2011) very few groups have studied the regulation of SOD1 in ageing and Parkinson's disease in humans making it a suitable target for investigation.

1.6.10 Alpha and Beta Tubulin

Increasing evidence has suggested that a dysfunction within the cytoskeleton of neuronal cells and regulation of cytoskeletal proteins could constitute a key risk factor in the prolonged development of Parkinson's and other neurodegenerative disease (Pellegrini et al., 2017). The maintained regulation of the cellular cytoskeleton plays a major role in the overall health of the cell. Accounting for a range of important factors in cellular function including; mitosis, organelle and vesicle movement, cellular structural and maintenance of homeostasis and the trafficking of a range of proteins (Pellegrini et al., 2017). Any changes in microtubule or cytoskeletal functionality would therefore lead to dysfunction within the intracellular metabolic environment as key components of the cell are unable to function due to errors within the cytoskeleton. The continued function of microtubules has been increasingly instigated in the overall development of PD and other neurodegenerative disease. The fundamental histological feature of PD, the Lewy body, have been shown to contain a range of proteins including tubulins and neurofilaments indicating a potential role for cytoskeletal protein dysfunction or the aggregation of cytoskeletal proteins within the PD pathology (Pollanen et al., 1993). For this reason, alpha and beta tubulins have been selected as a target within this study as an indicator of the protein regulation of both tubulins in PD and in ageing.

Chapter 2 Aims

Having established the link between the ubiquitin proteasome system, neurodegeneration and the ageing phenotype, as well as the continued maintenance of a range of intracellular anti-oxidant and structural proteins. This project aims to gain a more comprehensive understanding of the functionality of the UPS in PD by measuring the rate and activity of the chromatrypsin-like 20S proteasome specifically. This analysis utilising a fluorescently linked substrate to the 20S proteasome; n-succinyl-leu-leu-val-tyr-7-amido-4-methylcoumarin, will allow us to see whether UPS functionality and rate of ubiquitination is affected in those with PD and also within the ageing brain. We have selected a wide range of brain regions to undertake this study, in order to gain a more thorough understanding of which areas may be implicated within the disease state, the areas selected are; substantia nigra pars compacta (SNpc), cerebellum (Cer), caudate nucleus (CauN), hippocampus (Hipp), corpus callosum (CC) pre-frontal cortex (PFC) and thalamus (Thal). Whilst the SNpc has been implicated strongly within the disease pathology of PD, the other regions selected have had less attention focused on them within current literature and therefore make a relevant target for investigation.

Oxidative stress has also been implicated in both PD pathogenesis and ageing; we therefore aim to gain a greater understanding of the effect of ageing on multiple proteins implicated within the cellular response to oxidative clearance and homeostatic maintenance; SOD1, α -tubulin and β -tubulin.

It is therefore hypothesized that the rate of UPS activity is lowered within those suffering from PD, with the strongest reduction being noted within the SNpc brain region when compared to controls. PD pathology has been compared to that of natural ageing and as such it would be feasible for changes within the UPS to also be implicated in old age but not in lower ages. Oxidative damage has been shown to accumulate within older aged cells and therefore we expect to see a range of reduction in the oldest of brain tissue samples when compared to younger age counter parts within the same region.

Chapter 3 Materials and Methods

3.1 Ageing study brain Samples and Ethics Statement

The human postmortem tissue samples utilised within this aspect of the project belong to the brain collection of the Neuropsychopharmacology Research Group at the Department of Pharmacology of the University of Basque Country (UPV/EHU). Brain collection is registered in the national BioBank Register of the Spanish Health Department with the number C.0000035. Human brain samples were obtained at autopsy in the Basque Institute of Legal Medicine, Bilbao, Spain. All brain collection was undertaken in full compliance with the policies of research and ethical review boards for postmortem samples in research. A project submitted by the supervisor Dr Wayne Carter (University of Nottingham) has been accepted for ethical approval and any subsequent work undertaken by myself utilising these samples for the benefit of this project has therefore been approved by the relevant ethical committees.

3.1.1 Brain Tissue Data and Tissue Matching Information

In this study, all tissue samples were taken from the pre-frontal cortex region of post mortem control subjects with varying degrees of age ranging from 23-93 years. All Samples were stored at -80°C upon acquisition and until utilised within the study. The subject demographic information can be seen detailed on the following pages (**Table 1 p36**).

Sample Code	Gender (M/F)	Age (years)	PMI (h)	Cause of Death
39318	M	23	17	Accident/Work
195609	F	26	5	Accident/Traffic
182994	M	28	5	Accident/Fall from height
71697	F	22	24	Accident/Fall from height
42000	M	24	20	Accident/Traffic
134106	M	21	15	Accident/Traffic
137415	M	33	13	Accident/Traffic
70809	M	32	27	Accident/Traffic
21630	F	38	22	Accident/Traffic
78906	M	36	18	Accident/Work
136827	M	36	23	Accident/Work
142515	M	31	13	Accident/Traffic
48030	M	47	15	Accident/Traffic
171324	M	41	14	Natural/Heart Attack
34809	M	43	10	Accident/Traffic
92094	F	43	28	Accident/Train
109806	F	48	9	Accident/Fall from height
145809	F	44	9	Natural/CRF
83724	F	50	11	Natural/CRF
204015	F	57	14	Natural/CRF
144327	M	54	24	Accident/Traffic
123330	M	51	18	Accident/Traffic
85806	M	58	16	Accident/Traffic
61524	F	50	11	Natural/CRF
75606	M	61	23	Accident/Traffic
91485	F	60	27	Accident/Traffic
21288	F	66	20	Natural/CRF
171588	F	64	19	Natural/CRF
221094	M	62	19	Accident/Work
130497	M	66	18	Natural/Tumor
29397	F	74	21	Accident/Traffic
29088	M	78	29	Accident/Traffic
29997	F	75	18	Accident/Traffic
242088	M	73	19	Accident/Traffic
80109	M	71	21	Natural/CRF
156609	F	70	7	Accident/Traffic
139185	F	81	14	Accident/Traffic
242982	M	81	21	Accident/Traffic
36891	F	86	18	Natural/CRF
188994	M	85	19	Natural/CRF
55200	M	85	10	Natural/CRF
132606	F	84	18	Natural/CRF
205188	M	93	18	Natural/CRF

Table 1. Details demographic information for the Ageing study group, including sample code, gender (male/female), Age (years), PMI (hours), cause of death. *CRF=Cardio respiratory failure *PMI=Post mortem interval.

3.2. Parkinson and Control Study Brain Samples

For this study post mortem tissue was provided by the Parkinson's UK Brain Bank at the Imperial College London. The Parkinson's UK Brain Bank has approval as a Research Tissue Bank by the Wales Research Ethics Committee (REC) (ref. No 07/MRE09/72). Under conditions agreed by the REC, the Brain Bank can supply tissue or data to researchers in the UK without requirement for researchers to apply individually for REC approval. A project submitted by the supervisor Dr Wayne Carter (University of Nottingham) has been accepted for ethical approval and any subsequent work undertaken by myself utilising these samples for the benefit of this project has therefore been approved by the Parkinson's UK Brain Bank.

3.2.1 Brain Tissue Sample Data and Tissue Matching Information

In this study, all tissue samples were taken from both control and Parkinson's subjects postmortem, from seven brain regions as follows; substantia nigra pars compacta (SNpc), cerebellum (Cer), caudate nucleus (CauN), hippocampus (Hipp), corpus callosum (CC) pre-frontal cortex (PFC) and thalamus (Thal). All samples were stored at -80°C upon acquisition and until utilised within the study. The subject demographic information can be seen detailed on the following pages (**Table 2** p38 **Table 3** p39 **Table 4** p40).

Table 2. Details demographic information for the Parkinson's Disease study group, including case ID, sex (male/female), onset age (years), duration of disease (years), dementia (age), pathology, cofounding pathology (if applicable), clinical classification, post mortem interval, RNA integrity number, weight of patient (g), alpha synuclein (aSN) and Tau levels. *LBD=Lewy Body Disease *AD= Alzheimer's Disease *PD=Parkinson's Disease

Case ID	Age (years)	Sex (F/M)	Onset (age)	Duration (years)	Dementia (age)	Pathology	Confounding pathology	Clinical	PMI (hours)	RIN	Weight (g)	aSN	Tau
PD001	87	F	76	12	87	LBD	AD	PD	Unknown	7	975	6	2
PD016	85	F	67	18	82	LBD	-	PD	14	6.9	1,286	6	2
PD021	76	M	49	27	76	LBD	AD	PD	17	8.1	1,282	6	2
PD028	82	M	65	18	79	LBD	-	PD	14	7.4	1,251	6	2
PD041	77	M	67	10	76	LBD	-	PD	6	7.9	1,198	6	2
PD045	80	M	60	19	79	LBD	-	PD	16	8.3	1,314	6	2
PD067	83	M	73	9	81	LBD	-	PD	10	7.5	1,558	6	1
PD079	78	F	59	19	74	LBD	-	PD	22	7.7	1,220	6	2
PD081	73	M	65	9	Unknown	LBD	-	PD	19	7.4	1,451	6	<2
PD093	81	F	67	14	78	LBD	-	PD	22	6.9	1,116	6	2
PD099	82	M	72	11	78	LBD	-	PD	10	6	1,331	6	2

Table 3. Details demographic information for the control study group, including case ID, sex (male/female), onset age (years), post mortem interval, RNA integrity number (RIN), weight of patient (g), Sample pH and pathology at death.

Case ID	Age (years)	Sex (F/M)	PMI (hours)	RIN	Weight (g)	pH	Pathology
C034	90	M	12	7.8	1,279	5.73	Mild aging and microvascular changes
C040	61	F	15	7.3	1,167	-	Mild vascular changes
C053	89	F	22	3.7	1,068	-	Aging and microvascular pathology
C036	68	M	30	7	1,394	7.7	-
C045	77	M	22	7.5	1,381		-
C048	68	M	10	7.5	1,465	7.3	Mild vascular pathology
C054	66	M	16	5.8	1,441	7.2	Age related changes
C064	63	F	21	7.7	1,217	-	Cerebellar infarcts, basal ganglia vascular pathology
C075	88	M	8	8.5	1,167	-	Cerebellar infarct, ALBII, moderate small vessel disease
C076	87	M	31	7.6	1,303	-	Aging and infarct of right globus pallidus

Table 4. Details PD and Control subject matches utilised within the study, subjects were matched based on age, sex and post mortem interval. *PD=Parkinson's Disease *PMI=Post Mortem Interval

PD subject case ID	Matched control subject ID	Sex (F/M) of PD subject	Sex (F/M) of control subject	Age (years) of PD subject	Age (years) of control subject	PMI (hours) of PD subject	PMI (hours) of control subject
PD016	C040	F	F	85	61	14	15
PD021	C048	M	M	76	68	17	10
PD028	C076	M	M	82	87	14	31
PD041	C045	M	M	77	77	6	22
PD045	C036	M	M	80	68	16	30
PD067	C034	M	M	83	90	10	12
PD079	C064	F	F	78	63	22	21
PD081	C054	M	M	73	66	19	16
PD093	C053	F	F	81	89	22	22
PD099	C075	M	M	82	88	10	8
PD001	No match	F	No match	87	No match	Unknown	No match

3.3 Human Brain Tissue Homogenisation

Brain samples from both study groups were subject to the same homogenisation procedure.

All homogenates were initially thawed on ice post storage at -80°C . Upon reaching a suitable temperature, samples were prepared using a hand-held glass homogeniser over ice. Each sample was added to a buffer solution consisting of 1M Tris pH 7.5, 200mM ATP (pH 7), 1.5M MgCl_2 , 1M DTT and double distilled H_2O . Once homogenised, samples were moved from the glass homogeniser to pre-prepared Eppendorf tubes and centrifuged (Thermo Scientific Heraeus Fresco 21 Microcentrifuge) at $10000 \times g$ at 4°C for 10 minutes. The subsequent cytosolic fraction was then separated from the pellet and stored in fresh Eppendorf tubes at -80°C ready for protein determination.

3.4 Protein Concentration Determination Via Lowry

The protein concentration contained within samples was determined through Lowry protein determination assay. An initial standard curve was produced using a range of pre prepared standards of bovine serum albumin (Bio Rad 500-0207) at the following concentrations; $10\mu\text{g}/\mu\text{l}$, $7.5\mu\text{g}/\mu\text{l}$, $5\mu\text{g}/\mu\text{l}$, $2.5\mu\text{g}/\mu\text{l}$ and $1.25\mu\text{g}/\mu\text{l}$. Brain samples of unknown concentration were then prepared at a 1:25 dilution in a buffer composing 10mM Tris. Both protein standards and Tris buffer diluted samples at $40\mu\text{l}$, were loaded into a 96 well microplate (PerkinElmer 6005650). Finally, $20\mu\text{l}$ reagent A (Bio Rad 500-0113) and $160\mu\text{l}$ of reagent B (Bio Rad 500-0113) was added to each well. The plate was first agitated via a Spectramax plate reader for 5 seconds, to ensure the solution was mixed, and then left at room temperature covered for 15mins. Once sufficient time had past the plate was read using the SpectraMax plate reader at an absorbance value of 740nm. Values obtained from the assay were then compared to the standard curve to acquire the correct protein concentrations of each sample.

3.5 Preparation of Human Tissue Samples for Protein Separation Via SDS-PAGE

In preparation for SDS-PAGE and Western Blot, ageing study samples were made up to a $1\mu\text{g}/\mu\text{l}$ concentration, utilising the data values from the aforementioned Lowry protein concentration determination assay (see 3.4). Samples were prepared in a buffer containing 10mM TRIS-HCL, LDS sample buffer (Invitrogen NP0007) and Novex reducing agent (Invitrogen NP0009) at the acquired

volumes of each based on the Lowry determined results. Samples were then vortexed to ensure a thorough combination of solutions (Fisher Scientific FB15012). All samples were then centrifuged at 5000 x g for 10 seconds (Thermo Scientific Heraeus Fresco 21 Microcentrifuge), followed by heating at 70°C for 10 minutes. All samples were then cooled before use or stored at -20°C until ready for use.

3.5 Gel Electrophoresis

Pre-cast; 4-12% Bis-Tris Gel 1.0mm x 12 well, (NP0322BOX Invitrogen), were utilised for gel electrophoresis. Gels were prepared for use by removing the comb well inserts and washed in double distilled H₂O. The pre-cast gels were then inserted into an Xcell SureLock Mini-Cell (Invitrogen) gel tank. The inner chamber of the tank was then filled with pre-prepared gel running buffer (3-(N-morpholino) propanesulfonic acid (NP0001, Invitrogen) and an antioxidant solution was added (0.5ml NP0005 Invitrogen). The outer chamber of the SureLock Mini Cell tank was then filled to half way using the same running buffer.

A pre-stained protein molecular weight marker (10µl Invitrogen 26616) was utilised within the first well of each gel for easy identification of protein once separated. The pre-prepared human tissue samples were then added to each subsequent well at a volume of 15µl, any unused lanes were filled using a 1x Loading buffer (Invitrogen NP007) to ensure a strict linear run of each sample and prevent subsequent lane spread.

Once loaded electrophoresis was performed using a constant voltage of 125V for 100 minutes, or until the proteins had migrated to the bottom of the gel. Once completed, the gels were removed from the tank and each plate was opened using a specialised gel knife. Depending on the desired use, for direct gel imaging the gel would be subject to 15 minutes in a fixative solution (50% methanol, 10% acetic acid) and then in a Colloidal Blue protein staining kit (Invitrogen LC6025) prepared utilising a solution of double distilled H₂O, methanol and Stainer A (LC6025), placing the gel in solution on a rocking platform for 10 minutes and subsequently adding Stainer B (LC6025) directly into the solution and leaving this on the rocking platform overnight. The following day the gel could be destained by washing on a rocking platform in double distilled H₂O, replacing the water with fresh until all unwanted stain was removed from the gel leaving only the bands clear and ready for imaging. Alternatively, if the gel were to be used for Western Blot, they were washed in pre-prepared gel transfer buffer (Invitrogen NP0006).

3.6 Western Blot of Brain Tissue Samples

Prior to western blot gels were ran using the methodology described in section 3.5 Gels were washed in a transfer buffer solution (Invitrogen NP0006) and had their gel comb and bottom ridge removed with a scalpel prior to transfer. A polyvinylidene fluoride (PVDF) membrane was used for protein transfer. Initially the membrane was cut to size (~8cm x 8cm) before being submerged in 100% methanol (Fischer Scientific M/3950/17) for five minutes, then equilibrated in transfer buffer over the course of 15 minutes by removing roughly 50% of the current volume of the methanol/transfer buffer solution, until finally all the solution was removed, and fresh transfer buffer was used to cover the membrane ready for transfer.

The transfer itself was conducted using a Mini trans blot Cell (Bio Rad), in a container of ice and a frozen block added to the rear of the tank to ensure overheating did not occur during transfer. The transfer package was then constructed using the appropriate transfer 'cassette', building up on the black negative plate; a sponge, 3 pre-cut squares (8cm x 8cm) of filter paper, the prepared SDS-PAGE gel, Pre-prepared and equilibrated PVDF membrane, 3 additional squares of filter paper (8cm x 8cm) and the final sponge were then closed and sealed within the cassette ready for inclusion within the trans blot cell. The cell was then filled with pre-made cold (4°C) transfer buffer (Invitrogen NP0006), ensuring the total coverage of the transfer cassette/s and then ran at a constant voltage of 80V for 120 minutes. Upon completion of the transfer stage, PVDF membranes were removed from the trans blot cell and placed in Coomassie blue safestain (Invitrogen 46-4034) for 30 minutes to allow for protein band visualisation, after which they were left out to dry.

3.7 Addition of Antibody to Brain Tissue Samples

PVDF membranes ready for addition of primary antibodies were initially fixed in a solution of 50% methanol, 10% acetic acid (Fischer Chemical A/0400/PB17) and 40% DD H₂O on a rocking platform. After which an equilibrium of the membrane occurs over 30mins by removing 50% of the current solution every 10mins and substituting a phosphate buffered saline – Tween 20 solution (PBS-T 0.05% (v/v) plus Tween 20), with the final wash volume being 100% PBS-T.

Prior to the addition of the primary antibody, the PVDF membrane was blocked in a milk protein (5% Marvel) and PBS-T solution (w/v) for 1 hour at room temperature (~22°C). This solution aids in the prevention of non-specific binding by the primary antibody. Upon completion of this step the

membrane was now ready for the addition of the selected primary antibody (see Table 5 p42 for details of primary and corresponding secondary antibodies utilised within the study). Primary antibody was prepared at a dilution of 1:1000, at a 2ml volume and added to the PVDF membrane which was then sealed in plastic sheet and incubated at 4°C overnight on a rotating platform.

Upon completion of the incubation period, membranes were then washed in a PBS-T solution (as previous) decanting and changing the solution 3x over a 30minute period. After which the membrane was incubated at room temperature, with 2ml of a corresponding secondary horseradish peroxidase antibody complimentary to the primary utilised in the previous step (see Table 5 p42 for details of primary and corresponding secondary antibodies utilised within the study), at a 1:1000 concentration for 1 hour. The membranes were then washed once again over 30 minutes in PBS-T, decanting the solution every 10 minutes and adding fresh.

The membrane was then ready for visualisation utilising the Clarity Western Enhanced Chemiluminescence Substrate solution (Bio Rad 170-5060). The visualisation requires two solutions A and B to be mixed (1:1) and added to the PVDF membrane for five minutes. The solution is then removed by tipping the membrane forward and gently removing excess.

Membranes were then visualised via the ChemiDoc imaging system (Bio Rad) using the ImageLab 5.0 image capture software and analysed via the free hand volume tool giving a density value for each lane relevant to the arena covered by the protein band being analysed.

Table 5. (below) Details primary and secondary antibodies utilised within the study. *MW=molecular weight.

Primary Antibody Target Protein	MW(kDa)	Brand	Code	Animal Source	Secondary Antibody
Alpha Tubulin	55	Santa Cruz	SC-8035	Mouse	Goat-Anti Mouse
Beta Tubulin	55	Santa Cruz	SC-9104	Rabbit	Goat-Anti Rabbit
SOD-1	21	Santa Cruz	SC-11407	Rabbit	Goat-Anti Rabbit
GAPDH	35	Santa Cruz	SC-47724	Mouse	Goat-Anti Mouse

3.8 Fluorometric analysis of Chromotrypsin-like activity of PD vs CTRL and Ageing Study Brain Tissue Sample

The chromotrypsin-like activity of all brain tissue samples was undertaken utilising samples from both the ageing study group; pre-frontal cortex, and the PD vs Control sample set using brain tissue samples from the seven brain regions discussed in 3.1 and 3.2.

Human brain tissue samples were homogenised in a buffer composed; 1M TRIS pH 7.5, 200mM ATP pH 7.0, 1.5M MgCl₂, 1M DTT, 1ml buffer per 100µg sample and transferred to Eppendorf tubes. Upon completion of homogenisation, tubes were centrifuged at 10000G using a centrifuge at 4°C for 10mins (Thermo Scientific Heraeus Fresco 21 Microcentrifuge). After which the supernatant was extracted and added to fresh Eppendorf tubes ready for use.

Protein concentration for all samples was determined via Lowry assay in accordance with the methodology described in 3.4. Upon completion of protein concentration determination for brain tissues, samples are prepared for assay. All Samples were prepared for use at a volume of 50_{ul}, utilising the results of the protein quantification, adding sufficient homogenisation buffer (prepared earlier) to each sample to acquire the desired volume for use. Samples were prepared and analysed in pairs for accuracy.

The fluorogenic substrate n-succinyl-leu-leu-val-tyr-7-amido-4-methylcoumarin (943670-21-2 Sigma Aldrich) was prepared for use at a concentration of 1:100 in 100mM TRIS with 100ul being utilised for each individual sample. 100_{ul} of the fluorogenic substrate and 50_{ul} of prepared brain sample solution was then added to Lp3 tubes and transferred to a water bath pre-set at 37°C for 1 hour. The water bath was covered using foil as the reaction is light sensitive.

After 1 hour, the samples were removed from the water bath and 2ML of an alkaline stop solution of 80mM NaOAc was added each tube.

The Samples were then read using a fluorimeter utilising the following setup method;

Measurement: Photometry, Data Mode: Fluorescence, Ex WL: 360 nm, EM WL: 460 nm, Seen Time: 30 Sec, Delay: 2 Sec, Ex Slit: 10, Em Slit: 10, PMT voltage: 400V, Response: 2 Sec, Replicates: 1. Each LP3 tube containing sample was added to a quartz cuvette and placed into the designated slot within the spectrophotometer for analysis, a measure of fluorescence is then generated for each sample, repeating this process as required.

Chapter 4 Results

4.1 Dosage response investigation utilising purified human 20S proteasome to show how concentration of enzyme effects fluorogenic activity

In order to verify the proposed experimental design a number of pilot studies were undertaken. The first pilot study involved the analysis of the 26S proteasome using a purified 26S proteasome enzyme at known concentration (Enzo BML-PW9310-0050) utilising the same methodology as that described in 3.8 whilst substituting the brain tissue sample for the purified 26S proteasome enzyme. This is an accurate gauge of assessment of how alterations in concentration of intracellular 26S (and subsequently 20S) proteasome affect the level of fluorescent activity displayed. The experiment was repeated in two separate runs in order to demonstrate repeatability of the method and results obtained. The acquired data demonstrated a linear relationship however, did not show statistical significance when analysed via Spearmans rank; *set 1 $p=0.3305$ and set 2 $p=0.3213$.*

Pilot Dose response 20s Proteasome Substrate

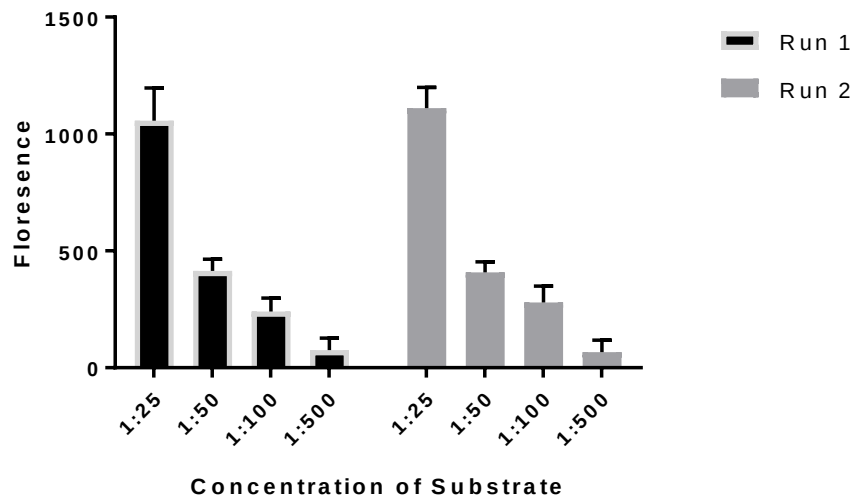


Figure. 1. Demonstrates the relationship between the change in enzyme concentration and levels of chromotrypsin-like activity, utilising a purified 26S proteasome enzyme. Spearmans r correlation analysis was undertaken on both data sets revealing no statistical significance in either trial run (run 1 $p=0.3305$ and run 2 $p=0.3213$). However, the linear relationship between concentration and 20S chromotrypsin-like activity is clearly visualised here. $n=8$ in both runs.

4.2 Time based assessment of Purified 20S proteasome Chromotrypsin-like

Chromotrypsin-like activity relative to fuorescence over the time course of 1 hour, utilising purified human 20S proteasome (1:200)

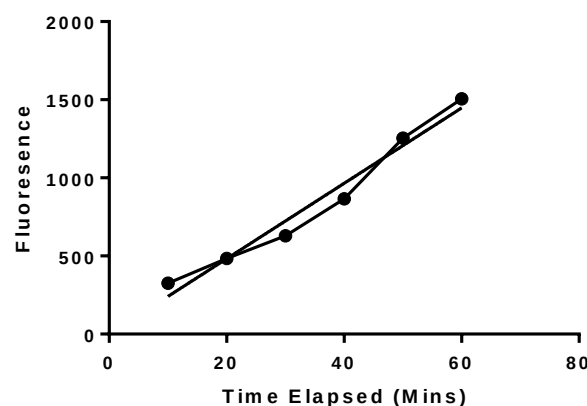


Figure. 2. shows a time course assessment of purified 26S proteasome chromotrypsin-like activity over a 1-hour period. Spearmen correlation analysis was undertaken showing statistical significance ($p<0.05$) demonstrating statistical significance of time course when measured against florescence $p=0.003$. data represented as mean where $n=12$.

The second pilot study (utilising the methodology detailed within 3.8) set to measure the time course data of the 26S proteasome to ensure the reaction occurred relative to time, rather than having a plateau affect. Purified 26S proteasome was again utilised to measure the 20S chromotrypsin-like activity via the substrate n-succinyl-leu-leu-val-tyr-7-amido-4-methylcoumarin. The 3.8 methodology was slightly altered in this instance via staggering of the addition of substrate into the sample. Substrate was added in 10-minute intervals over 1 hour, after which fluorescent activity was measured using the aforementioned protocol. Fig 2 above shows the results of this time course assessment and the fluorescence activity was subject to regression analysis; showing an R^2 value of the line to be highly linear at 0.9698, verifying that activity was relative to reaction time course, and subsequently analysis after 1 hour was a suitable representation of chromotrypsin-like activity.

To verify this relationship further a time course analysis was undertaken utilising the above methodology on a range of PD (PD028/PD041/PD021/PD79) and Control (CO76/CO36/CO48/CO64) tissues, to ensure that any data acquired utilising the purified 26S proteasome was representative of the human brain tissue samples utilised in the prior study. Figure 3 (below) demonstrates the linear relationship seen within this aspect of the investigation, whilst Spearmans rank statistical analysis was utilised to measure the power of statistical significance. P value was <0.05 in (*PD028 Vs CO76* $p=0.0027$; *PD41 Vs CO36* $p=0.0097$; *PD79 Vs CO64* $p=0.0043$) whilst p was over >0.05 in (*PD021 Vs CO48* $P=0.0510$) showing a high level of statistical significance and verifying the methodology.

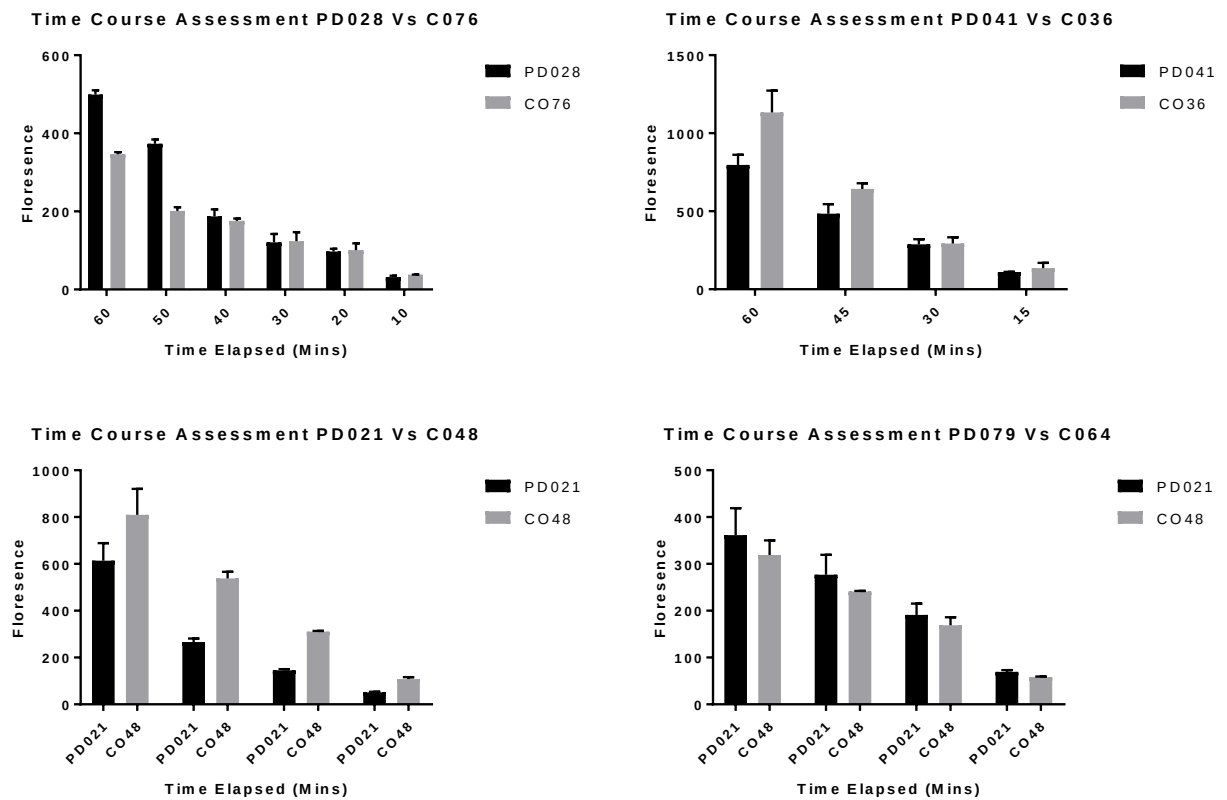


Figure. 3. shows a time course assessment of SNpc brain tissue chromotrypsin-like activity over a 1-hour period. Spearman correlation analysis was undertaken and in all cases $p < 0.05$ demonstrating statistical significance of time course when measured against florescence (PD028 Vs CO76 $p = 0.0027$; PD41 Vs CO36 $p = 0.0097$; PD021 Vs CO48 $P = 0.0510$; PD79 Vs CO64 $p = 0.0043$) $n = 6$ in PD028 Vs CO76 then $n = 4$ in remaining data sets.

4.3 Ageing study Chromotrypsin-like fluorogenic activity assay results from pre-frontal cortex brain region

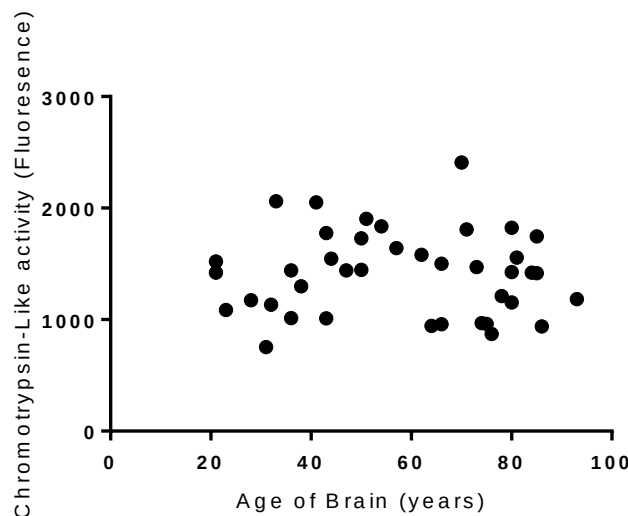


Figure. 4. Postmortem analyses of 20S proteasomal function in the Prefrontal-cortex brain region in non-diagnosed PD controls Ranging from age 21-93 years. 20S proteasomal chymotrypsin-like activities in homogenates of control brain tissues. Proteolytic activities (reaction rates) are expressed as fluorescence Units. Results, presented, were statistically analysed as not significant via Pearson's correlation coefficient giving a *P* value of **0.8790**.

The 20S proteasome chymotrypsin-like activity of the pre-frontal cortex was analysed utilising fluorogenic substrate n-succinyl-leu-leu-val-tyr-7-amido-4-methylcoumarin, which upon formation of an enzyme-substrate complex of the chymotrypsin-like 20S proteasome produced a fluorogenic signal relative to the amount of binding and subsequent activity within the cellular environment given in fluorescence units. *n*=40 ageing study samples (see Table 1. For further details) where assessed with *n* values for age groups as follows; 20-29 (*n*=4), 30-39 (*n*=6), 40-49 (*n*=5), 50-59 (*n*=5), 60-69 (*n*=4), 70-79 (*n*=7), 80-89 (*n*=8), 90-99 (*n*=1). Selected samples where then analysed for statistical significance via Person's correlation offering a non-statistically significant *p* value (*>* 0.05) of **0.8790**.

4.4 Parkinson's Vs Control brain region Chromotrypsin-like fluorogenic activity assay results from seven brain regions

Figure 5 and 6 show the 20S proteasome chymotrypsin-like activity of seven total brain regions; substantia nigra pars compacta (SNpc), cerebellum (Cer), caudate nucleus (CauN), hippocampus (Hipp), corpus callosum (CC) pre-frontal cortex (PFC) and thalamus (Thal), of age matched PD (*n*=10) and control tissues (*n*=10) (see Table 4. For age-matched pairs). Samples were analysed utilising

fluorogenic substrate n-succinyl-leu-leu-val-tyr-7-amido-4-methylcoumarin. Values were given in fluorescence units and no region showed statistically significant change ($p > 0.05$) between the PD and Control groups. Results, presented as mean, were statistically analysed via Student t-test. $P > 0.05$, in all cases when comparing PD to control in CC, Corpus Callosum ($p = .906$); PFC, Pre-Frontal Cortex ($P = .740$); Cer, Cerebellum ($p = .2321$); Thal, Thalamus ($P = .856$), in SNc, Substantia Nigra ($P = 0.4383$); Hipp, Hippocampus ($P = 0.403$); CauN, Caudate Nucleus ($P = 0.712$).

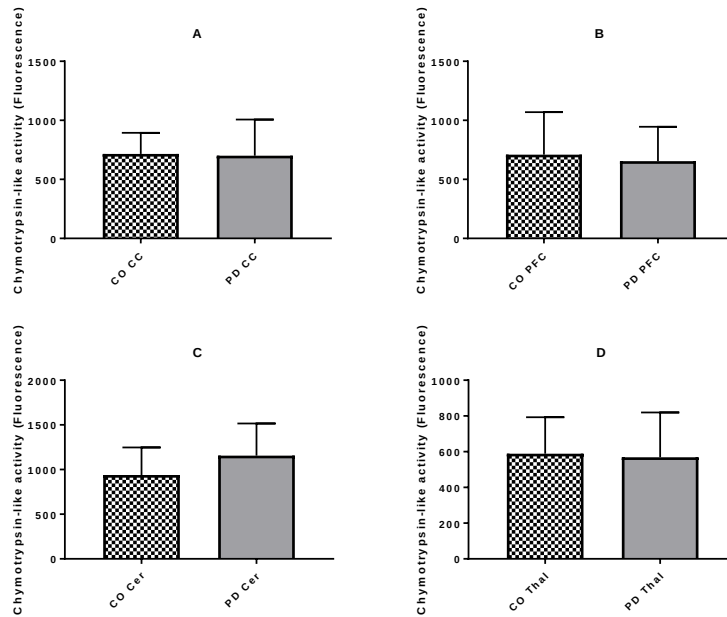


Figure. 5. Postmortem analyses of 20S proteasomal function in various brain regions in sporadic PD and age-matched controls. 20S proteasomal chymotrypsin-like activities in homogenates of control (Checked bar) and PD (grey bar) brain tissues. Proteolytic activities (reaction rates) are expressed as fluorescence Units. Results, presented as mean, were statistically analysed via Student t-test. $P > 0.05$, in all cases when comparing PD to control in CC, Corpus Callosum ($p = .906$); PFC, Pre-Frontal Cortex ($P = .740$); Cer, Cerebellum ($p = .2321$); Thal, Thalamus ($P = .856$). $n = 20$ (10 PD 10 Control).

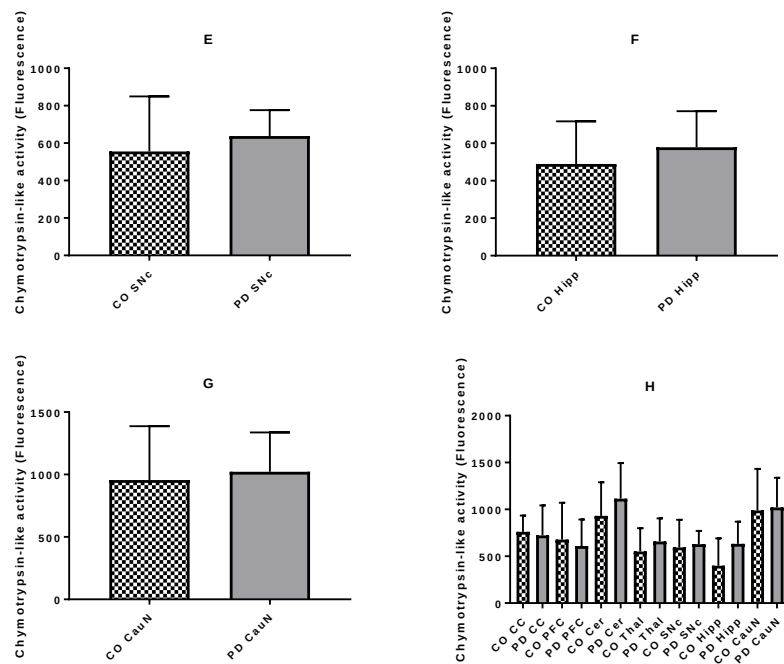


Figure. 6 Postmortem analyses of 20S proteasomal function in various brain regions in sporadic PD and age-matched controls. 20S proteasomal chymotrypsin-like activities in homogenates of control (Checked bar) and PD (grey bar) brain tissues. Proteolytic activities (reaction rates) are expressed as fluorescence Units. Results, presented as mean, were statistically analysed via Student t-test. $P > 0.05$, in all cases when comparing PD to control in SNc, Substantia Nigra ($P = 0.4383$); Hipp, Hippocampus ($P = 0.403$); CauN, Caudate Nucleus ($P = 0.712$).

4.5 Western Blot Analysis of Ageing Study Sample Pre-Frontal Cortex Brain Tissues

Western blot was undertaken on a range of proteins implicated in the oxidative stress response within the ageing study samples. The results of these western blots can be seen below, alongside statistical analysis acquired through the total protein volume of each band. All results given utilised adjusted values for the most accurate representation. Western blot procedure was undertaken in accordance with the methodology outlined within sections 3.5-3.7.

4.6.1 Analysis of SOD1 levels in Ageing Study Brain Tissues

Figure 7 (below) shows the results of the western blot analysis of SOD1 levels in the ageing brain study group. Statistical analysis has been undertaken showing no significant decrease with age ($p=0.1636$). However, although not statistically significant a visible decrease in volume can be seen within the older samples (figure 7.1), to the extent that Gel D showed very low, almost undetectable levels of SOD1 when compared to the GAPDH control.



Figure 7. Shows the relative levels of SOD1 present on the PVDF membrane of the ageing study samples after volume analysis. Samples were put into groups based on age (20-29 ($n=4$), 30-39 ($n=6$), 40-49 ($n=5$), 50-59 ($n=5$), 60-69 ($n=4$), 70-79 ($n=7$), 80-93 ($n=4$) and statistical analysis was undertaken via Spearman's rank to assess correlation in this instance SOD1 was not significantly decreased with age $p=0.1636$.



Figure. 7.1 Ageing Brain Sample images after western blot analysis, utilising the Primary SOD1 antibody. Membranes imaged based on age; Gel A shows samples – 21-36 years, Gel B 38-51 years, Gel C 54-70, Gel D 73-93 years.

4.6.2 Analysis of Alpha Tubulin levels in Ageing Study Brain Tissues

Figure 8 (below) shows the results of the western blot analysis of Alpha-tubulin levels in the ageing brain study group. Statistical analysis has been undertaken showing a significant decrease with age ($p=0.0206$) of alpha tubulin levels within the PFC region.

Figure 8. Shows the relative levels of Alpha-Tubulin present on the PVDF membrane of the ageing study samples after volume analysis. Samples were put into groups based on age (20-29 ($n=4$), 30-39 ($n=6$), 40-49 ($n=5$), 50-59 ($n=5$), 60-69 ($n=4$), 70-79 ($n=7$), 80-93 ($n=4$) and statistical analysis was undertaken via Spearman's rank to assess correlation in this instance Alpha-Tubulin was significantly decreased with age $p=0.0206$.

Western Blot Analysis of Alpha-Tubulin Levels in Ageing Study Sample

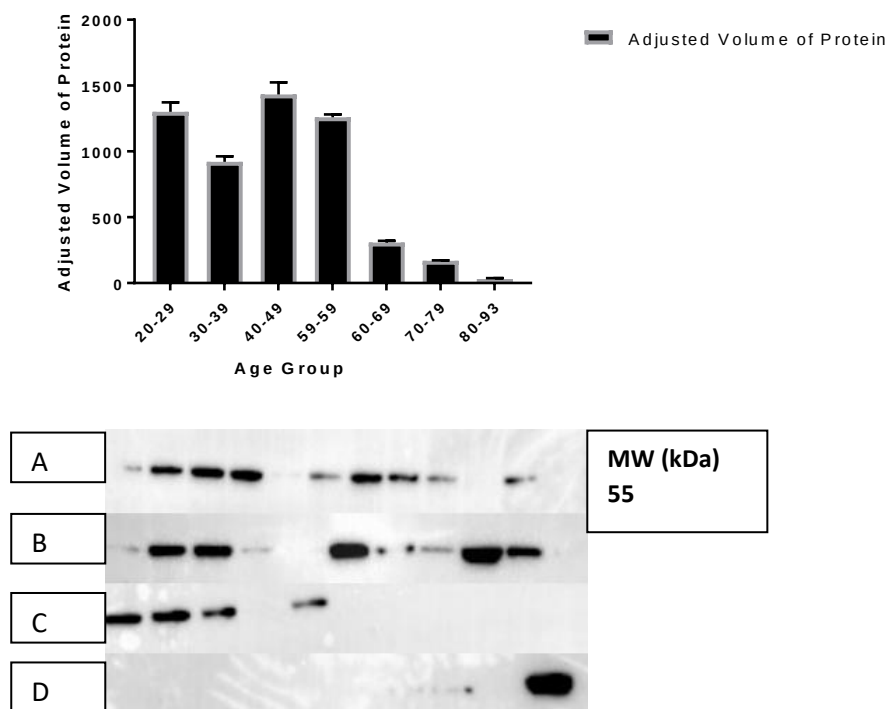


Figure. 8.1 Ageing Brain Sample images after western blot analysis, utilising the Primary Alpha-tubulin antibody. Membranes imaged based on age; Gel A shows samples – 21-36 years, Gel B 38-51 years, Gel C 54-70, Gel D 73-93 years.

4.6.3 Analysis of Beta Tubulin levels in Ageing Study Brain Tissues

Figure 9 (below) shows the results of the western blot analysis of beta-tubulin levels in the ageing brain study group. Statistical analysis has been undertaken showing no significant decrease with age ($p=0.7338$). However, although not statistically significant a visible decrease in volume can be seen within the older samples, as was seen within the SOD1 blot, to the extent that Gel C and D (Figure 10, below) show very low, almost undetectable levels of beta-tubulin when compared to the GAPDH control.

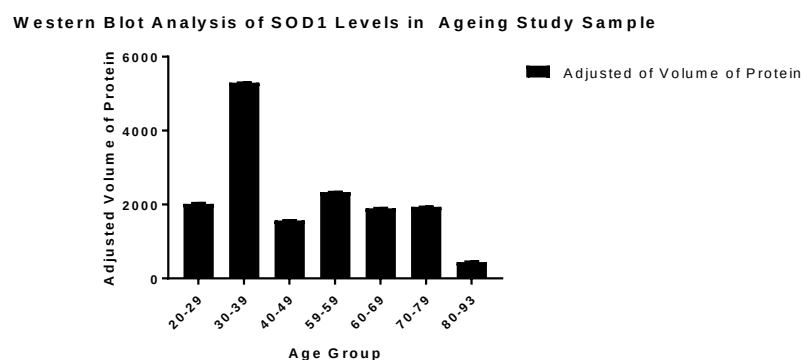


Figure. 9. Shows the relative levels of Alpha-Tubulin present on the PVDF membrane of the ageing study samples after volume analysis. Samples were put into groups based on age (20-29 ($n=4$), 30-39 ($n=6$), 40-49 ($n=5$), 50-59 ($n=5$), 60-69 ($n=4$), 70-79 ($n=7$), 80-93 ($n=4$) and statistical analysis was undertaken via Spearman's rank to assess correlation in this instance Beta-Tubulin was not significantly decreased with age $p=0.7338$.

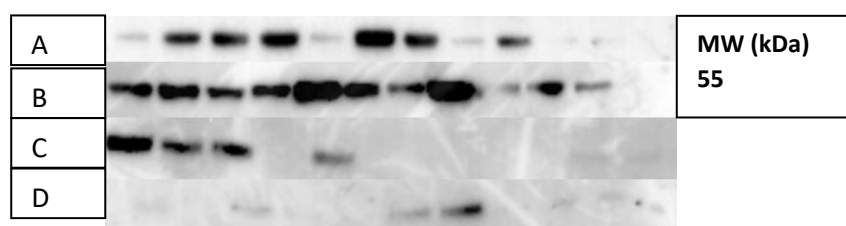


Figure 9.1 Ageing Brain Sample images after western blot analysis, utilising the Primary Alpha-tubulin antibody. Membranes imaged based on age; Gel A shows samples – 21-36 years, Gel B 38-51 years, Gel C 54-70, Gel D 73-93 years.

4.6.4 Analysis of GAPDH levels in Ageing Study Brain Tissues

Figure 10 below shows the results of the western blot analysis of GAPDH levels in the ageing brain study group. GAPDH has been utilised as a control protein within this study selected due to its consistent level of regulation within the cell throughout the life span (Yu et al., 2011).

Western Blot Analysis of GAPDH Levels in Ageing Study Sample

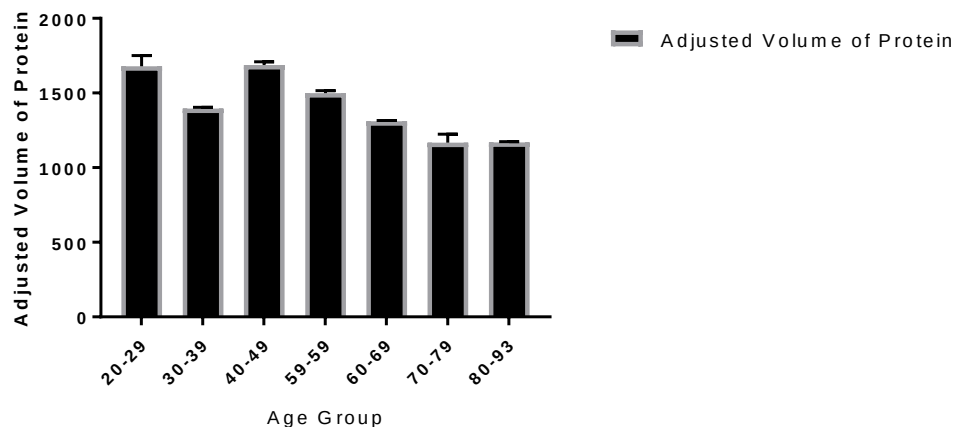


Figure 10. Shows the relative levels of GAPDH present on the PVDF membrane of the ageing study samples after volume analysis. Samples were put into groups based on age (20-29 (n=4), 30-39 (n=6), 40-49 (n=5), 50-59 (n=5), 60-69 (n=4), 70-79 (n=7), 80-93 (n=4). GAPDH was utilised as a control protein as it's regulation within the cell is maintained throughout ageing and as such makes a strong point of comparison.

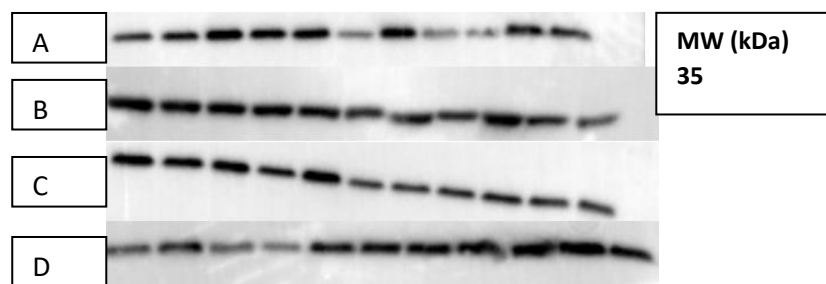


Figure 10.1 Ageing Brain Sample images after western blot analysis, utilising the Primary GAPDH antibody. Membranes imaged based on age; Gel A shows samples – 21-36 years, Gel B 38-51 years, Gel C 54-70, Gel D 73-93 years.

Chapter 5 Discussion

5.1 The role of the UPS in Parkinson's disease

Whilst our study did not reveal any significant changes within the chromotrypsin-like activity of the UPS over the seven brain regions measured or within the ageing study group ($p = >0.05$ in all cases), a number of studies undertaking similar investigations have in-fact demonstrated alterations within the UPS and brain of those affected or in animal models of PD (Bedford et al., 2008, McNaught et al., 2003).

Although no significant changes were detected within the current study, previous studies utilising animal models of 26S proteasome depletion have instigated a role for the UPS in the formation of PB and LB inclusions (Bedford et al., 2008). The study brought together a range of pathological mechanisms believed to be implicated within PD, including inhibition of the UPS and the formation of α -synuclein containing Lewy like inclusions in the form of pale bodies, as well as an accumulation of morphologically impaired mitochondria. One possible reason for a lack of change noted within the present study not seen here may have been that the Bedford et al, group utilised a functional change within the 19S regulatory particle of the proteasome to replicate the effect of UPS dysfunction rather than utilising the enzymatic activity of the 20S proteasome. The study also only utilised an animal model for UPS dysfunction which does not necessarily portray the full extent of cellular dysfunction or compensation within the human brain during neurodegeneration.

Another potential reason for the lack of significance seen in the activity of the 20S proteasome within the tissues assessed, specifically the SNpc region (which is implicated most prominently in PD), may be due to a lack of nigral neurons displaying α -synuclein positive aggregates. This was not assessed prior to undertaking the investigation, as α -synuclein aggregation is a hallmark of PD and would be assumed. However, this may be a factor as one study showed that 20S proteasome activity was significantly decreased only in those tissues containing α -synuclein aggregates, when compared to age matched PD and control counterparts which did not contain α -synuclein aggregates (Chu et al., 2009) .

Another interesting counter argument to that proposed here is that of McNaught, et al who have shown a significant change in the chromotrypsin-like activity within the SNpc region specifically which may imply a compensatory mechanism at work (McNaught et al., 2003). Within this study the group utilised a similar range of sporadic age matched PD and control samples to that of the current

study (n=16 PD and n=13 Control) whilst also following a similar methodology for measurement of proteasomal activity; measuring activity of the 20S proteasome. A potential reason for the difference being shown, outside of the range and nature of human samples, may in fact be the addition of ATP within the lysis buffer which was used for homogenisation of tissues, where the group of McNaught et al, did not substitute ATP into their buffer. This may have had a significant impact on the rate and level of chromotrypsin-like activity measured during our study as the early stages of UPS activity, including the unfolding and ubiquitin labelling of the protein is an energy dependent process requiring ATP to function (it should be noted however that some proteins can be degraded via the 20S proteasome in a process that does not require ubiquitination or ATP (Olanow, 2006)) and as such substituting ATP may have interfered with our overall results, particularly in light of new evidence that mitochondrial dysfunction can have a significant impact early on in the disease progression and has been linked to dysfunction within the UPS and Lysosomal degradation systems (Yong-Kee et al., 2012).

5.1.1 Parkin and PINK1 Loss of Function Mutation Implicate the Role of the UPS and Mitochondrial Dysfunction Within Parkinson's Disease

Whilst the vast majority of PD cases are of the sporadic form, the low number of familial forms detected have allowed us to gain an insight into the mechanisms of dysfunction within the disease state. The cells ability to prevent cellular damage and aggregation whilst maintaining a stable homeostatic environment, devoid of oxidative stress has been implicated within familial PD (Tan, 2013). Of the 15 loci, 11 genes are linked to PD and are all involved within this process in some way, be that within the UPS, autophagy, the regulation of mitochondrial function or clearance of oxidative stress (Hatano et al., 2009).

An early insight linking the UPS and mitochondrial dysfunction to PD pathology was that of the gene mutations seen in the E3 ligase protein Parkin, which has been shown to play a role in tagging toxic and unwanted protein for degradation via the proteasome (Saiki et al., 2012), as well as PTEN-induced kinase 1 (PINK1), a protein responsible for the encoding of a mitochondrially targeted Ser/Thr kinase (Whitworth and Pallanck, 2009).

Loss of function mutations in both Parkin and PINK1 are the most relevant cause of early onset PD and therefore make a high priority target for understanding the underlying mechanisms of PD and how the UPS and mitochondrial dysfunction may play a significant role within the disease (Kalia and Lang, 2015).

In terms of the distribution of these proteins within the intracellular environment, both are located based on their functional role, with PINK1 localising to the mitochondria, whilst Parkin is found

within the cytoplasm (Zhou et al., 2008). Multiple animal models have been created demonstrating the role of these proteins within disease progression.

Mouse models of Parkin and PINK1 knockouts have demonstrated the link between gene mutation and nigrostriatal defects (Kitada et al., 2009), whilst PINK1/Parkin knockouts within drosophila flies have demonstrated a link between these proteins and degeneration of dopaminergic neurons; a characteristic of PD progression (Greene et al., 2003). The mitochondria and the role it plays within PD has also been implicated through these animal models of the disease, with PINK1 and Parkin deficient drosophila demonstrating alterations within the size and shape of mitochondria (Greene et al., 2003). As a result of this, the basis for implication of Parkin and PINK1 in mitochondrial dysfunction, investigation into the shared pathway theory of the two proteins and how they may affect each other was undertaken. Interestingly, Parkin overexpression has been shown to compensate for a loss of PINK1, whilst PINK1 overexpression did not compensate for Parkin (Ira et al., 2006), this implies that in terms of cell signaling Parkin acts downstream of PINK1, further supporting the idea that PINK1 and Parkin are instigated in the regulation of mitochondrial integrity and therefore play a role in maintaining cellular homeostasis in PD.

5.1.2 The role and Mechanism of PINK1 in Parkinson's disease

As a protein kinase PINK1 joins a host of protein kinases within the intracellular environment responsible for signal transduction, leading to changes in enzyme activity and ultimately downstream signalling events causing changes in cell fate (Philip, 2002).

Under normal homeostatic conditions the action of PINK1 involves incorporation into the mitochondrial interior through the action of a multi subunit complex including TOM40 and TIM 23 core containing complexes located on the outer membrane of the mitochondria, after which PINK1 is subject to cleaving through the action of multiple proteases, namely; MPP and PARL (Li et al., 2008). The newly formed PINK1 cleavages trigger rapid degradation via the N-end rule pathway resulting in the maintenance of low levels of PINK1 in healthy cells (Matsuda, 2016). Whilst under conditions of cellular damage or maintenance PINK1 on the outer mitochondrial membrane is able to induce Parkin recruitment as a means of mitochondrial damage mitigation, through mitophagy; (Matsuda et al., 2010).

5.1.3 The Activation of the E3 ligase Parkin by PINK1 Results in Mitophagy

Whilst the link between PINK1 and Parkin may lead to mitochondrial dysfunction, the E3 ligase Parkin plays an important role within the intracellular environment as a means of ubiquitinating a range of substrate proteins (Hideki et al., 2000). Much like other ubiquitin ligases Parkin has a structure composed of a ubiquitin-like domain at the N-terminus, a RING finger domain at the C-terminus and a centralised linker region (Olanow et al., 2006). Parkin functions through an action process utilising a range of E2 enzymes, resulting in the ubiquitination of a variety of substrates implicated within the progression of PD, including; O-glycosylated isoform of α -synuclein (aSp22) and cyclin e α/β tubulin (Imai et al., 2000). Parkin's role within the UPS utilises its ubiquitin-like domain to interact with the 26S proteasome, specifically subunit Rpn10, which plays a key role in the recognition of ubiquitinated substrates by the regulatory cap (Pickart, 2001). The overall result in Parkin mutation could lead to a significantly compromised UPS leading to dysfunction throughout the cell, including in mitochondrial activity, which is so important in the sustain of the cells maintenance machinery (UPS, CMA, MA), thus leading to the aggregation and cellular dysfunction seen within PD pathology.

5.2 Age Induced Protein Changes Could Lead to Cellular Dysfunction

Whilst no detectable changes were noted in the levels of proteasomal activity within the ageing brain study group, a number of protein level changes were noted in our most aged samples that may have a significant impact on the health of the neurons within the pre-frontal cortex, of which the samples were taken. The ageing phenotype, or the accumulation of cellular damage, through the dysfunction of important cellular processes, cellular maintenance mechanisms (UPS, AP, CMA), and mitochondria leading to an increase in oxidative stress and eventual cell death is well established within many aged cells (Toren and Nikki, 2000) and the health of these cells may be affected in the long term by this subsequent alteration in protein regulation.

5.2.1 SOD1 Down Regulation in Ageing

The cellular antioxidant Superoxide dismutase 1 (SOD1) is an enzyme notably implicated in the cells ability to ward off the damage of oxidative stress. One such study implicated the importance of intracellular SOD1 levels and its impact on the health of the cell, utilising SOD1 knockout mice (Pérez

et al., 2009). The study showed that SOD1 lacking mice suffered a significantly reduced life span as a result of a host of symptoms including; hearing and sight loss, muscle atrophy and development of neoplasms as a result of ageing. Thus, in a very focused environment where there is no SOD1 present, damage to cells is a significant factor of cell death and eventual morbidity. Whilst this may be an unrealistic example in human tissues it does indicate the importance of SOD1 as a means of preventing oxidative damage of tissues, and subsequently would imply that even with low levels of reduction of SOD1, the impact on the intracellular environment could be quite severe.

Whilst the current study did not show a significant reduction in SOD1 levels, they were notably reduced within the oldest of the population groups studied (80-93). Parkinson's and neurodegenerative risk is known to be increased as we age and is particularly common within the population in this older age group (Niccoli and Partridge, 2012). SOD1 reduction at this age could therefore pose a significant risk to the health and function of neurons as they begin to accumulate oxidative radicals, and a deficiency of cellular defense mechanisms such as SOD1 could indicate a causation for this increased susceptibility in later life.

Reduced levels of SOD1 have been implicated in a number of neurodegenerative diseases such as PD, whereby a decrease of SOD1 regulation has noted in a range the plasma of patients who suffered the disease (Tórsdóttir et al., 2006). Whilst elevated levels of SOD1 may be beneficial to combat the ever-increasing levels of oxidative radicals present within the intracellular environment, it may also contribute to the aggregation process through accumulation of SOD1 into protein aggregates. Over expression of SOD1 through increased transcription or a mutation within the transcription factor regulating SOD1 could eventually lead to an over accumulation of SOD1 within the intracellular environment, which may undergo posttranslational modification as a result of the increased levels of ROS within the cell, and eventually aggregate leading to further cellular dysfunction. In this instance the increase in SOD1 would be visualised as an overall loss as it would not show during western blotting, as a modification to the enzyme via oxidative damage or the aggregated form of the enzyme would not be visualised. This may be unlikely however as animal studies of PD; which replicate an exaggerated cellular ageing process in neurons have shown over expression of SOD1 to be protective resulting in an increased chance of neuronal survival (Botella et al., 2008).

5.2.2 Regulation of the Cytoskeletal Proteins Alpha Tubulin and Beta Tubulin in Ageing, With Implications to Neurodegeneration

In this study both α and β tubulin were shown to be down regulated in the older aged subjects when compared to both the younger counter parts and the control protein (GAPDH) (although not statistically significant). This down regulation suggests a dysfunctional cellular mechanism at work and may be indicative of neurodegeneration.

Within the cellular environment, microtubules are responsible for a range of diverse functionality. The action of polymerization of α and β tubulin heterodimers forming microtubules is an important functional process driven and influenced by a range of mediators, including and most importantly the cellular concentration of free tubulin heterodimers (Ren et al., 2003). This is a tightly controlled and maintained process as α and β tubulin over expression can have a toxic effect on the cell (Weinstein and Solomon, 1990). As a result of the complexity and reversible nature of the regulation and folding of α and β tubulin within the cell, misfolded monomers are common place and need to be degraded rapidly in order to prevent toxic accumulation leading to cytotoxicity. Over expression has been linked to PD pathology as ubiquitinated tubulins are present within LB aggregates (Ren et al., 2003). There have also been studies showing the binding capacity of the Parkin gene, responsible for a number of intracellular homeostatic functionality to microtubules resulting in a potential increase in ubiquitination of α and β tubulin (Ren et al., 2003). This study also demonstrated that the mutant form of Parkin, seen in a range of familial PD did not ubiquitinate α and β tubulins, inferring that dysfunction within the Parkin gene can result in an over accumulation of cytotoxic tubulins which will not be targeted for degradation via the UPS. The study also demonstrated an increased 26S proteasome functionality for microtubule degradation in non- mutant Parkin overexpression, further enforcing the idea that Parkin is an E3 ligase for α and β tubulin (Ren et al., 2003).

Dysfunction within the cytoskeleton has also been linked to problems in the process of autophagy (He and Klionsky, 2009). The cytoskeleton plays an important role in protein trafficking within the processing of the autophagosome and in the process of autophagy, as demonstrated by a study which utilised the microtubule depolymerising drug Nocodazole, resulting in the inhibition of the autophagosome (Köchl et al., 2006). This is a significant implication, particularly in those most susceptible to neurodegeneration; the elderly, who were most affected by a loss of tubulins within

the current study. Any alteration which may impact the efficiency or ability of the autophagy-lysosomal pathway can have a serious impact on the maintenance of cellular function. A dysfunction within this system can result in a toxic accumulation of protein aggregates and increased levels of oxidative stress within the cell and eventual cell death.

Chapter 6 Conclusions

Although this data did not demonstrate a change in 20S proteasome function within PD or within ageing, multiple studies have implicated its role within the disease state. The UPS is being shown to play an ever-important role within the progression of PD in an effort to reduce levels of cellular damage through ubiquitination and subsequent degradation of damaged or misfolded proteins as a result of cellular oxidative damage. The implication of the Parkin gene mutation and the link between Parkin and PINK1 could have a major impact on the way in which we investigate the disease state, with mitochondrial dysfunction seemingly playing an ever-increasing role within the pathology of PD and in the dysfunction of mechanisms which defend against oxidative damage and eventual neuronal cell death.

The visible decrease in proteins responsible for the maintained functionality of the cell within this study and others implicate and offer further evidence for the increase in oxidative damage and cumulative stress within ageing and in neurodegeneration. It will be more important over the coming years as we begin to gain a more comprehensive understanding of neurodegeneration and ageing to target the underlying mechanisms which maintain cellular viability, particularly within the neurons. With the global population increasing at a steady rate with no indication of reduction, it will be increasingly important that we gain a thorough understanding of how age impacts the overall health of the brain at a cellular level.

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