A study of α-synuclein, parkin and TDP-43; proteins implicated in neurodegenerative disease

by

Penelope Foulds B.Sc Hons, PGCE

August 2008

Thesis submitted in part fulfilment of the requirements for the degree of Doctor of Philosophy at The University of Lancaster

I declare that this thesis is my own work and it has not been submitted for a higher degree elsewhere.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

A study of α-synuclein, parkin and TDP-43; proteins implicated in neurodegenerative disease

Penelope Foulds B.Sc Hon, PGCE

August 2008

Abstract

α-Synuclein (αS) is a 14.5 kDa neuronal protein associated with Parkinson's disease (PD) and related disorders. Aggregated protein inclusions called Lewy bodies found in the brain in PD contain αS. Rare inherited forms of early-onset PD are caused by autosomal dominant mutations in αS or by autosomal recessive mutations in parkin, a 53kDa E3 ubiquitin ligase. αS and parkin have been reported to interact functionally, with parkin ubiquitinating a glycosylated, heavier 22-24 KDa form of αS (αSp22).

ELISA assays were developed to investigate if parkin interacts with α-synuclein in human blood plasma. Immunoprecipitation followed by immunoblotting techniques were utilised to first capture and then assess the molecular weight of the complex. Ion exchange chromatography and gel-filtration chromatography allowed the complex to be further isolated and characterised.

ELISA assays detected the αS/parkin complex in plasma from patients with PD and controls. The αS/parkin complex was isolated from plasma by immunoprecipitation, and immunoblotting revealed the αS to be the normal 14.5 kDa form. Application of plasma to an ion exchange column allowed isolation of the complex.

Autopsy studies have shown that about 55% of patients with frontotemporal lobar degeneration (FTLD) and 25% of patients with Alzheimer's disease (AD) harbour TDP-43 immunoreactive pathological changes in their brains. Again, ELISAs were developed to investigate whether TDP-43 was present, or indeed increased amounts detected, in plasma. Elevated levels of TDP-43 protein were detected in plasma of 46% patients with FTLD with clinical frontotemporal dementia (FTD) and 22%

patients with AD, compared to 8% of control subjects. The proportions of patients with FTD and AD showing raised plasma TDP-43 levels correspond closely to those proportions known from autopsy studies to contain TDP-43 pathological changes in their brains. Raised TDP-43 plasma levels may thereby reflect TDP-43 pathology within the brain.

This study has confirmed that αS and parkin do interact in biological samples. However, the target for parkin does not appear to be the reported 22-24 kDa glycosylated isoform of αS, but the unmodified protein. For the first time, the existence of parkin and the complex has been demonstrated in human plasma. Plasma TDP-43 levels may be a biomarker that can provide a laboratory test capable of identifying the presence of TDP-43 brain pathology in neurodegenerative disease during life. These findings may prove to be useful in the diagnosis of neurodegenerative diseases.

Acknowledgements

I would like to acknowledge my supervisors, Professor David Allsop and Dr Gavin Brown, extending my thanks to Gavin for giving me the opportunity to study towards a PhD, and to David for introducing me to the field of neuroscience.

Secondly, I would like to extend my gratitude to Professor David Mann for his generous help and support throughout, Dr Bob Lauder for his assistance with the chromatography, Dr Sultan Salem and Dr Sheelan Talibani who introduced me to the lab environment, and Mrs Kath Lamb for general help around the lab.

May I express my gratitude to The University of Lancaster for funding this project. In addition, I would like to aknowledge the patients who contributed samples that were critically important, not only to this study, but to past, present and future research.

And finally, may I acknowledge my family, especially my husband Doug, for his continuous support and encouragement, without which this period of study would not have been possible.

This thesis is dedicated to my grandparents, Win and Harry Herbert, who both suffered the consequences of neurodegenerative disease.

Publications

Penelope Foulds, Erica McAuley, Linda Gibbons, Yvonne Davidson, Stuart M. Pickering-Brown, David Neary, Julie S. Snowden, David Allsop, David M.A. Mann (2008) TDP-43 protein in plasma may index TDP-43 brain pathology in Alzheimer's disease and frontotemporal dementia. *Acta Neuropathol* **116**(2): 141-146.

Presentations

Penelope Foulds, McAuley E, Allsop D, Mann D. TDP-43 protein in plasma may index TDP-43 brain pathology in Alzheimer's disease and frontotemporal dementia. Presented at the 109th Meeting of the British Neuropathological Society, 10th January 2008, Institute of Child Health, London.

Abbreviations

Aβ	Beta amyloid
AD	Alzheimer's disease
AEBSF	Aminoethyl-benzene sulfonyl fluoride hydrochloride
ALS	Amyotrophic lateral sclerosis
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ARJP	Autosomal recessive Juvenile Parkinson's
BSA	Bovine serum albumin
CBD	Corticobasal degeneration
CNS	Central nervous system
CSF	Cerebrospinal fluid
COMT	Catchol-O-methyltransferase
DA	Dopamine
DAQ	Dopamine-*O*-quinone
DAT	Dopamine transporter
DLB	Dementia with Lewy bodies
DLDH	Dementia that lacks distinctive histology
DMP	Dimethyl pimelimidate
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-related kinase
FABP	Fatty acid binding protein
FPLC	Fast protein liquid chromatography
FTD	Frontal temporal dementia
FTD+MND	Frontal temporal dementia with Motor neurone disease
FTLD	Frontotemporal Lobe Degeneration
FTDP-17T	Frontotemporal Dementia and Parkinsonism linked to chromosome 17 with tau pathology
FTLD	Frontotemporal Lobar Degeneration
FTLD-U	Frontotemporal Lobar Degeneration with ubiquitin histology

HD	Huntington's disease
HRP	Horse radish peroxidase
IP	Immunoprecipitation
LB	Lewy body
LN	Lewy neurite
L-DOPA	Dihydroxyphenylalanine (Levodopa)
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with 0.05% Tween 20
PD	Parkinson's disease
pI	Isoelectric point
PGRN	Progranulin gene
PNFA	Progressive non-fluent aphasia
PSP	Progressive supranuclear palsy
MAPT	Tau gene
MAO	Monoamine oxidase
MND	Motor Neurone Disease
MPC	Magnetic particle concentrator
MSA	Multiple system atrophy
Mw	Molecular weight
SD	Semantic dementia
SN	Substantia nigra
SNCA	α-synuclein gene
SNCB	β-synuclein gene
SOD1	Superoxide dismutase
SSRI's	Selective serotonin reuptake inhibitors
(SUMO)-1	Small ubiquitin-like modifier
αSp22	Glycosylated α-synuclein
α-syn	α-synuclein
TARDBP	TAR DNA Binging protein gene
TDP-43	TAR DNA Binding Protein 43
TH	Tyrosine hydroxylase
Ub	Ubiquitin
UCH	Ubiquitin Carboxyl-terminal Hydolases

UCHL-1	Ubiquitin C-terminal hydrolase L1
UPP	Ubiquitin-proteosome pathway
VMAT-2	Vesicle monoamine transporter 2

Table of contents

Table of figures

Table of tables

CHAPTER ONE

1 Introduction

1.1 Abnormal protein aggregation and neurodegenerative disorders

The reasoning around neurodegenerative disorders has changed in recent years, as it became clear that most of them feature a degree of protein aggregation or abnormal protein misfolding (Ingelsson et al. 2005). For example, the protein tau is important both in the number of diseases in which it is implicated, and also by the number of individuals that it affects worldwide. Tau is an important component in cell cytoskeletal physiology forming the neurofibrillary tangles of Alzheimer's disease (AD), Pick's disease, progressive supranuclear palsy, frontotemporal dementia (FTD), corticobasal degeneration, postencephalitic parkinsonism, and others. The next most common protein in epidemiological terms is said to be β-amyloid (Aβ), which forms the amyloid plaques of AD and then α-synuclein which forms the aggregates in Parkinson's disease (PD), dementia with Lewy bodies (DLB), multiple system atrophy (MSA) and a few others (see table 1.1). α-synuclein and tau may interact to produce an increased risk of PD (Golbe et al. 2001). There is only a partial overlap between the cells in which the protein aggregates, and the cells that degenerate. The correlation between the amount of protein misfolding/aggregation and the severity of disease has not been established. Moreover, it is not known whether the protein aggregation actually results in neurodegeneration, or conversely results from it. However, it is most likely that inclusion bodies and other visible protein aggregates represent an end stage of a series of molecular mechanisms, and that earlier steps in the cascade may be more directly tied to pathogenesis than the inclusions themselves (Ross and Poirier 2004). Variations in genetics in some diseases can help explain the pathogenesis of the more common sporadic forms.

Huntingtins disease (HD), a progressive neurodegenerative disorder, is caused by such a variation in genetics, a repeat CAG coding for polyglutamate in the amino terminus of the huntingtin protein. Polyglutamate lengths of 36 or more are responsible for

causing the disease, and those of less than 36 do not (Scherzinger et al. 1999). There is a direct correlation between the number required for aggregation *in vitro* and those required for disease in humans and the longer the repeats, the earlier the onset of disease. HD is a progressive neurodegenerative disorder which is caused by a mutation in one gene. However, the picture is far from clear, as neurons with inclusions do not correlate exactly with the regions of the brain that degenerate (Vonsattel et al. 1985), although there is a good relationship between the CAG repeat length and the density of inclusions.

AD is a late-onset dementia with progressive loss of memory, task performance, speech and the ability to recognise people and objects. There is degeneration of neurons in the basal forebrain and hippocampus, along with altered neuronal connections and synaptic plasticity (Hardy and Selkoe 2002). AD aggregates come in mainly two forms. Extracellular aggregates known as neuritic plaques are mainly composed of βA, which has its origin from the proteolytic processing of the amyloid precursor protein (APP). Also, there are intracellular aggregates of micro-tubule-associated protein tau, named neurofibrillary tangles. AD pathogenesis has been correlated with genetic mutations responsible for familial forms of the disease (Ingram and Spillantini 2002; Goedert 2004). These rare mutations are within presenilins, proteins which are involved in APP cleavage, and also in APP itself. Furthermore, tauopathies such as FTD can be caused by mutations in the tau protein.

Amytrophic lateral sclerosis (ALS) is caused by degeneration of lower motor neurones in the lateral horn of the spinal cord and upper motor neurons of the cerebral cortex. This fatal disease results in progressive motor weakness (Cleveland and Rothstein 2001). Mutations in the superoxide dismutase (*SOD1*) gene can cause rare early-onset familial forms of the disease. Disease pathology does not appear to be due to any alteration in SOD1 enzyme activity, although transgenic mice over expressing the mutated enzyme have cytoplasmic inclusions containing aggregates of the SOD1 protein (Rakhit et al. 2002). SOD1 ubiquitinated aggregates are found in the brains of individuals with the mutation, although it is not usually detected in sporadic forms of the disease. SOD1 does not usually form fibrillar structures *in vitro*. Recently, TDP-43, an RNA-binding and DNA-binding protein encoded by the TAR DNA-binding protein gene (*TARDBP*) has revealed that mutations in this gene might cause

neurodegeneration through both gains and losses of function. The finding of pathogenic mutations in *TARDBP* implicates TDP-43 as an active mediator of neurodegeneration in ALS and other TDP-43 proteinopathies (Van Deerlin et al. 2008).

Prion disease can either be sporadic, be caused by environmental transmission or by genetic mutations (Prusiner 2001). Environmentally, the disease can be passed on by eating prion particles derived from infected brain material, or surgically via unsterilized surgical instruments. Genetically, prion disease can be caused by point mutations in the prion gene, and therefore an altered prion protein. Protein aggregation is caused by the abnormally folded prion protein, and is both intracellular and extracellular (Ma and Lindquist 2002; Ma et al. 2002).

Aggregates usually consist of fibres containing misfolded protein with β-sheet conformation, termed amyloid. The classical definition of amyloid is an extracellular, proteinaceous deposit, exhibiting cross-beta structure due to misfolding of unstable proteins. Recently this definition has come into question as some classic, amyloid species have been observed in distinctly intracellular locations, which may be termed "amyloid-like" (Sunde et al. 1997).

The proteins implicated in neurodegenerative diseases often appear to be natively unfolded (Uversky 2003). Amyloid fibrils are the most characteristic aggregates, existing as filamentous structures with a width of around 10 nm and a length of 0.1-10 μm. The initiation of the aggregation process in a cell may be an event of "seeded polymerisation" (Dobson 2004). It is possible that an increase in protein concentration could promote aggregation. Familial PD can be caused by duplication or triplication of the α-synuclein locus which causes altered dosage (Singleton and Gwinn-Hardy 2004). It is known that individuals with Down's syndrome, who carry an extra copy of the APP locus on chromosome 21, often have early deposition of Aβ plaques. A mutation in a protein coding sequence may result in a primary structure which is more prone to aggregation, or changes in the promoter sites of disease-associated genes may increase transcription and therefore protein concentrations.

Table 1.1 Neurodegenerative diseases, proteins and pathology

Disorder	Aetiology	Pathology	Disease protein
Huntington's disease (HD)	Huntingtin (dominant)	Intranuclear inclusions, cytoplasmic aggregates	Huntingtin with polyglutamate expansion
Alzheimer's disease (AD)	Sporadic (Apiloprotein E risk factor) Amyloid precursor protein (APP) (dominant) Presenilin 1,2 (dominant)	Neuritic plaques and neurofibrillary tangles As sporadic As sporadic	Aβ peptide (from APP) and hyperphosphorylated protein tau As sporadic As sporadic
Parkinson's disease (PD)	Sporadic α-synuclein Parkin (also DJ-1, PINK1)	Lewy bodies, Lewy neurites in the substantia nigra Similar to sporadic Lewy bodies absent (or rare)	α-synuclein α-synuclein α-synuclein (when present)
Frontal temporal dementia (FTD)	Progranulin	Inclusions in neurones and glial cells Pick bodies	TDP-43
Amyotrophic lateral sclerosis	Sporadic Superoxide dismutase-1 (SOD-1) (dominant)	Bonina bodies and axonal spheroids As sporadic	TDP-43, SOD-1
Prion diseases (kuru, CJD, new varient CJD, GSS varient)	Sporadic, genetic and infectious	Spongiform degeneration, amyloid and other aggregates	Prion protein
Frontal temporal dementia with Parkinsonism	Tau mutations (dominant)	Pick bodies, neurofibrillary tangles	Tau

In general, sporadic neurodegenerative diseases are associated with aging, which is often coupled to oxidative protein modifications. These covalent protein modifications may promote aggregation, for example, in the case of α-synuclein which can be subject to oxidative modification via dopamine adducts (Conway et al. 2001) and where oxidative stresses are involved in mechanisms underlying the pathogenesis of glial cell inclusions in MSA.

The α-synuclein from Lewy bodies (LB's), associated with PD pathology, is extensively phosphorylated at Ser 129 (Okochi et al. 2000; Iwatsubo 2003). Investigations using cell culture suggest this phosphorylation strongly modulates interactions between synphilin-1 and α-synuclein, and subsequently inclusion formation. Therefore, it is possible that phosphorylation may have a role in LB formation. Phosphorylation is also implicated in AD as a high proportion of neurofibrillary tangles contain hyperphosphorylated tau protein. Also, it is suggested that nitrative stresses are involved in mechanisms underlying the pathogenesis of LB's in PD, as well as α-synuclein pathologies in other synucleinopathies (Giasson et al. 2000; Souza et al. 2000).

There may be other covalent protein modifications involved in protein aggregation. HD is characterised by the accumulation of a huntingtin protein, which contains an abnormal polyglutamine expansion. A pathogenic fragment of huntingtin can be modified either by small ubiquitin-like modifier (SUMO)-1, or by ubiquitin, on identical lysine residues (Steffan et al. 2004). The role of ubiquitin will be described in more detail later (see section 1.2).

Some neurodegenerative diseases involve proteolytic cleavage, as with Aβ, the main component of the cerebral plaques found in the brains of patients with AD. This insoluble 40 to 42 amino acid peptide is formed by the cleavage of APP, although this can also be cleaved normally into a non-amyloidogenic peptide. When APP is intact it shows little tendency to aggregate, but the small Aβ peptide has a high tendency to do so (with $A\beta_{40}$ being less toxic than $A\beta_{42}$) (Esler and Wolfe 2001). As previously mentioned earlier, in HD, proteolytic cleavage may be involved in protein aggregation. Short N-terminal fragments of huntingtin containing the expanded polyglutamine repeats are a good deal more toxic to cells than the longer or full length

huntingtin (de Almeida et al. 2002). These N-terminal huntingtin fragments can conformationally change to form polyglutamate aggregates (Poirier et al. 2002). Regarding PD, the role of proteolytic cleavage is less well established, in as much as it is known that LB's contain both N and C terminal fragments, as well as other truncated fragments, although the aggregation process can only be surmised (Lee et al. 2004).

And so, it is known that abnormal deposition of insoluble protein aggregates or inclusion bodies within CNS cells are commonly associated with many neurodegenerative diseases. Most of these protein deposits contain ubiquitinated proteins. These protein aggregates are sometimes believed to result from structural changes in the protein substrates which may in itself promote aggregation, or alternatively, it may prevent recognition and degradation by the ubiquitin-proteasome pathway (UPP). The UPP is the main non-lysosomal degradation pathway for intracellular proteins. Impaired proteolysis from malfunction or overload of the UPP might contribute to the synaptic dysfunction seen early in neurodegenerative diseases.

1.2 The Ubiquitin-Proteasome Pathway

Proteolysis by the UPP involves two main steps: ubiquitination followed by degradation. A de-ubiquitination step also plays an important role in this pathway as it removes the ubiquitin flag for recycling.

The small 76 amino acid protein ubiquitin (Ub) can form polyubiquitin chains. These chains are attached to target proteins for proteasomal degradation and this is achieved by the successive attachment of Ub monomers, by an isopeptide bond formed between the side chain of Lys48 in one ubiquitin and the carboxyl group of the C-terminal Gly76 of a neighbouring Ub. Attachment of polyubiquitin chains to lysine residues on a protein results in at least a 10-fold increase in its degradation rate (Beal et al. 1996).

The polyubiquitination of proteins is a complex progression of four steps (see figure 1.1).

1. A high-energy thioester bond is formed between Ub and an ubiquitin-activating enzyme (E1) in a reaction that requires ATP hydrolysis.
2. A thioester bond is formed between the activated ubiquitin and ubiquitin-conjugating enzymes (E2).
3. The carboxyl terminal of Ub is covalently attached to the protein substrate, by ubiquitin ligases (E3), which confer substrate specificity to the UPP. Generally, the first ubiquitin conjugated to a protein is attached to the ε-amino group of substrate lysines via an isopeptide bond. (In some cases, ubiquitin can be transferred directly to the protein substrate by ubiquitin-conjugating enzymes (E2).)
4. Multiubiquitin chains are assembled by a family of ubiquitination factors (E4) that produces longer Ub-chains (Koegl et al. 1999).

While the number of E1 enzymes is only small, there are many E2 and E3 enzymes, (around 50 and 1,000 respectively, in humans) possibly indicating that this pathway operates through selective proteolysis. E3 proteins, e.g. parkin implicated in PD, recognise specific protein substrates for ubiquitination and then ligate Ub to the substrate.

The covalent binding of Ub to proteins flags them for degradation by the 26S proteasome, a multicomponent enzymatic complex with a molecular mass of approximately 2,000 kDa (DeMartino and Slaughter 1999) (see figure 1.2). The 26S proteasome includes two main units. The 20S particle, known as the 20S proteasome, is the catalytic core, and the 19S particle, known as PA700, is the regulatory component.

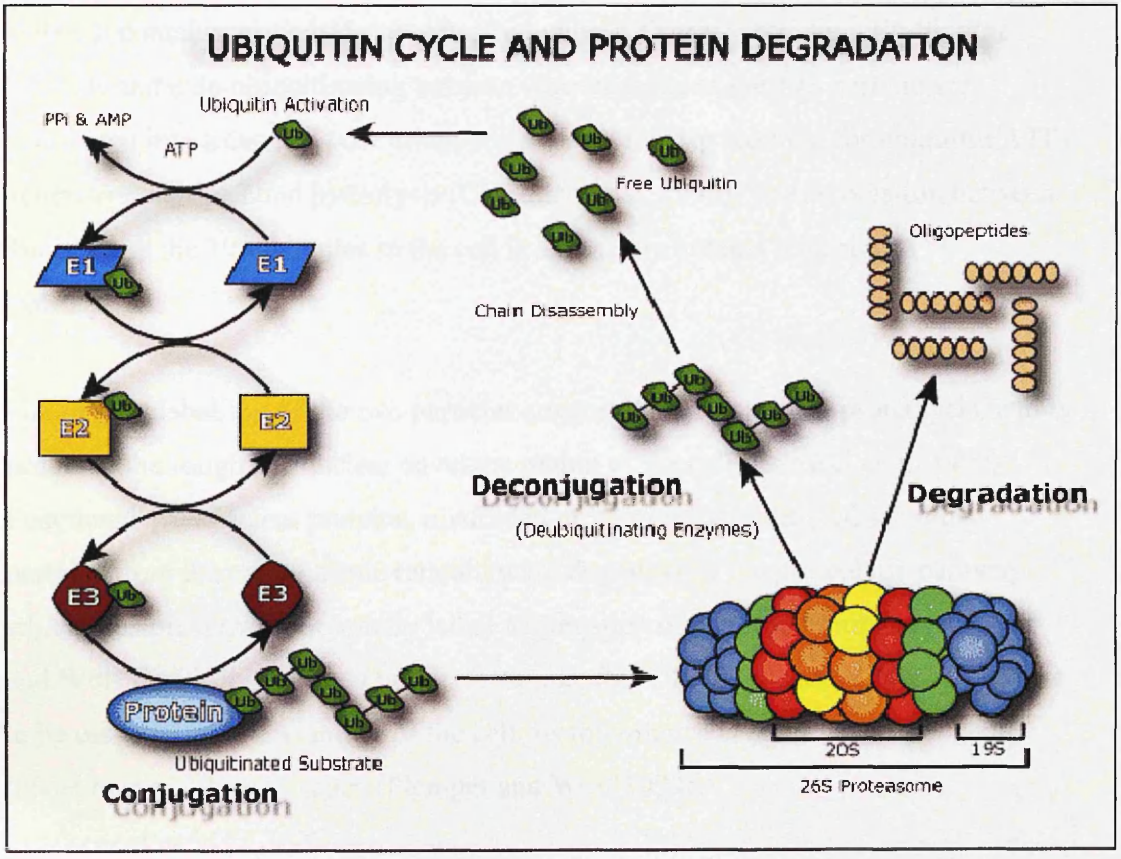


Figure 1.1 The Ubiquitin-Proteasome Pathway

This pathway functions widely in intracellular protein turnover. It plays a central role in degradation of short-lived and regulatory proteins important in a variety of basic cellular processes. (Taken from www.bostonbiochem.com/upp.php)

The 20S proteasome hydrolyses most of the peptide bonds in the protein to be degraded (Orlowski et al. 1993). The particle is composed of 28 subunits arranged in four heptameric-stacked rings, forming a cylindrical structure with a hollow centre where proteolysis occurs (see figure 1.2). The smaller 19S particle gives ubiquitin/ATP-dependency to proteolysis by the 26S proteasome (Orlowski et al. 1993). It contains around 15 subunits, including ATPases, polyubiquitin-binding subunits and a de-ubiquitinating enzyme. The subunits in the 19S particle are distributed into a cap and core arrangement, with the cap required for ubiquitin/ATP-dependent peptide bond hydrolysis (Glickman et al. 1998). The association between the 20S and the 19S particles in the cell is a dynamic process requiring ATP-hydrolysis.

Fluorescent labelling of the two particles suggest that proteasomal proteolysis mainly occurs at the rough ER/nuclear envelope region of the cell (Enenkel et al. 1999). Functionally inefficient proteins, misfolded proteins or unassembled secretory proteins from the endoplasmic reticulum are degraded by the proteolytic pathway, which thus prevents a potentially lethal aggregation of abnormal proteins (Plemper and Wolf 1999). It is pointed out that although this UPP degradation does not appear to be essential for the viability of the cell, its importance is underlined by it being linked to many disease states (Plemper and Wolf 1999).

The function of inclusion bodies in neuronal cells and their role in neurodegenerative disease is not clear. The formation of these inclusions may be an attempt by the cell to sequester these ubiquitinated, abnormal proteins out of harms way, as they have not been successfully degraded by the UPP. And it could be surmised that these inclusions will eventually impede the normal functioning of the cell, and could contribute to cell death. It would seem logical that the size of the inclusions, and their abundance, would be a factor regarding their toxicity to the cell.

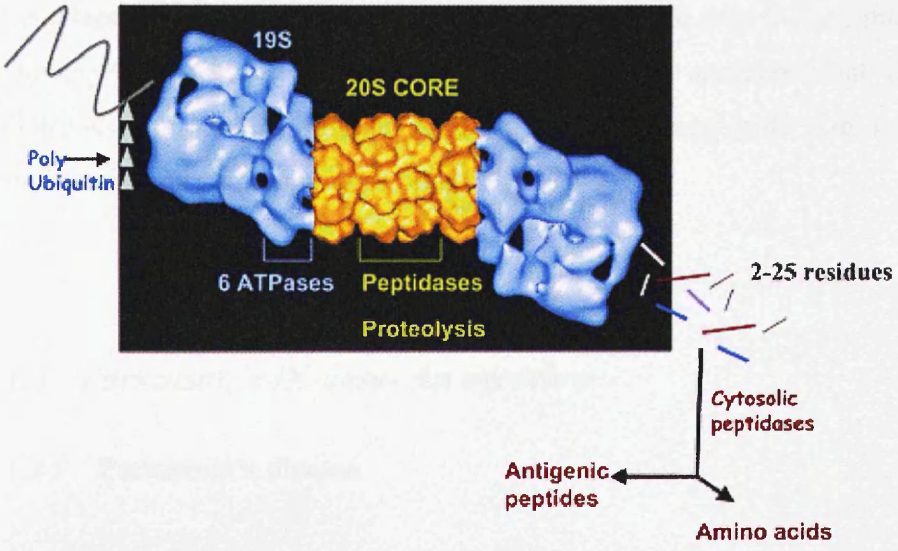


Figure 1.2 The 26S proteasome

The 26S proteasome binds the ubiquitin chain, using ATP to unfold and translocate the substrate into the 20S core particle for degradation (taken from (Goldberg 2005)). A chain of at least four ubiquitin moieties is required for substrate recognition by the 26S proteosome complex. The complex consists of a 19S 'cap' and a 20S proteolytic core. The 'cap' cleaves the ubiquitins from the substrate, unfolds the protein and channels it through to the proteolytic core. Here, three peptidases actively cleave the polypeptide into short peptides. As well as this 26S proteosome, there are many different proteins that associate transiently with this unit, further increasing the complexity of the system (Glickman and Ciechanover 2002).

Inclusion bodies can be found in different areas within the cells, with some located in the cytoplasm of neurones in certain diseases such as PD and others being within the nucleus, such as in FTLD. It is known that molecules of a molecular weight over 70kDa cannot diffuse freely through the cell's nuclear envelope, including neuronal cells (Trushina et al. 2003). As most ubiquitinated proteins are likely to have a MW in excess of this amount, it is unlikely that the complexes will passively diffuse from the cytoplasm into the nucleus, or visa versa. This would only be possible if the nuclear envelope was disrupted in some way. It can be surmised that the sub-cellular distribution of protein aggregates is determined by aggregate size, nuclear diffusion limits and nuclear membrane integrity.

1.3 Parkinson's Disease- An overview

1.3.1 Parkinson's disease

Parkinson's disease (PD) is defined as a disorder that mainly affects individuals of middle to old age. PD is the second most common neurodegenerative disorder after AD, with a worldwide distribution and no gender preference (Wood 1997). The prevalence is 0.1-0.2% overall, but this increases to 1.4% amongst 55 year olds and to 3.4% amongst 75 year olds. The disease is characterised by severe motor symptoms including resting tremor, muscle rigidity and slowness of voluntary movement. A number of patients exhibit further symptoms of postural imbalance, gait disturbance and a mask-like facial expression. Others can show non-motor symptoms including, anxiety, depression, dementia and psychosis, in advanced stages of the disease.

Friedrich Lewy was the first to describe the main pathological feature of PD, the spherical intracytoplasmic inclusions. Tretiakoff named them Lewy bodies, and showed that they are particularly numerous in the substantia nigra pars compacta. This region of the brain is concentrated with dopaminergic neurones that project into the striatum, the major area of input from the basal ganglia. Death of the dopaminergic neurones of the substantia nigra (SN) leads to loss of dopamine (DA), 3,4-dihydroxyphenyl acetate, homovanillic acid, tyrosine hydroxylase and the dopamine

transporter, resulting in severe DA deficiency and the clinical symptoms of PD (Forno 1996).

Electron microscopy in the 1960's revealed that LB's contained abnormal filaments, although the biochemical composition of these inclusions remained unknown until 1997, when Goedert and collegues showed that they were immunoreactive for the protein α-synuclein (Spillantini et al. 1997). LB's (see figure 1.3) also contain neurofilaments, components of the UPP, heat-shock proteins, lipids and carbohydrates (Olanow et al. 2004).

α-synuclein immunoreactive inclusions are found in other neurodegenerative diseases and together are referred to as the "synucleinopathies". These disorders include DLB, MSA, neurodegeneration with brain iron accumulation type 1 and the parkinsonism-dementia complex of Guam (Goedert 2001).

Clinically normal individuals are known to have LBs in their brains. As many as 8-12% of normal people over 60 years of age have LBs in brainstem nuclei, which is often termed incidental Lewy Body disease (iLBD) (Gibb and Lees 1988; Mikolaenko et al. 2005). When the sample group includes individuals with dementia, then the frequency increases to over 30% (Jellinger 2004). The areas of the brain that are affected are the same as in PD. It is suggested that iLBD represents preclinical PD rather than non-specific changes related to aging (Dickson et al. 2008).

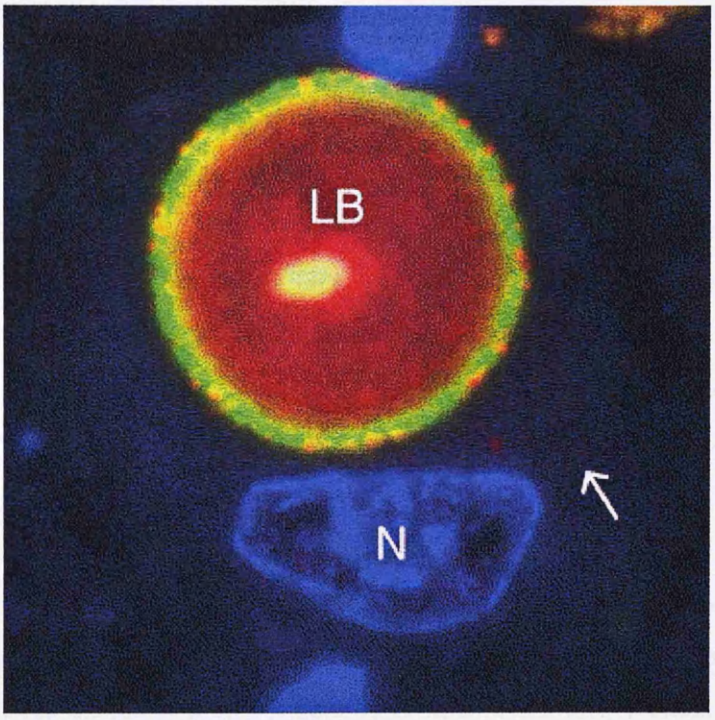


Figure 1.3 The Lewy body

A spherical Lewy body (LB) in a dopaminergic neuron, in the substantia nigra from an individual who died having sporadic PD, was shown using a fluorescent-based multiple immunostaining protocol. Red fluorescence (Alexa Fluor 594), green fluorescence (Alexa Fluor 488) and blue fluorescence (Hoescht 3358) represent antibodies to ubiquitinated proteins, α-synuclein and cell nuclei (N) respectively. The predominance of ubiquitinated proteins was noted and that α-synuclein is largely present in the periphery of the Lewy body. Note also the presence of a trail and possibly transport of small aggregates of ubiquitinated proteins (arrow) to form the LB (taken from (McNaught and Olanow 2006).

1.3.2 The Aetiology of Parkinson's disease

The relative contributions of genetic versus environmental factors in the aetiology of PD have been keenly debated. The cause of the disease is considered to be multifactorial because the majority of cases are sporadic, with no obvious family history. The first evidence for an environmental causative agent became known in 1983, when a group of Californian drug addicts injected themselves with heroin that was contaminated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston et al. 1999). The individuals exposed to MPTP showed dopaminergic neuronal loss similar to that observed in sporadic PD, and developed Parkinsonism, but with the absence of LB pathology. The discovery of this, and other toxins, strengthened the hypothesis that PD may have an environmental aetiology.

The hypothesis that heredity is the basis for the aetiology of PD was initially controversial and traditionally had little support from epidemiological data. However, even though agreement between monozygotic and dizygotic twins in longitudinal twin studies have indicated that PD has a genetic contribution, larger cross sectional twin studies have argued against heritability (Piccini et al. 1999; Tanner et al. 1999). And so, although 10-30% of PD patients report a first degree relative with the same disease, it is thought that the familial association should be attributed to sharing the same environment rather than sharing genes.

Over the years, epidemiological studies have indicated that a number of environmental factors may increase the risk of developing PD. These include exposure to pesticides, certain metals, rural living, head trauma and infection (Olanow and Tatton 1999; Lai et al. 2002). On the other hand, smoking (Quik 2004), caffeine and dietary antioxidants may hold some protective effect against neurodegenerative disease (Esposito et al. 2002).

However, the discovery of inherited forms of the disease shifted the emphasis back over to genetic factors. Studies of familial PD have documented several PD-associated genes. These include; α-synuclein (Polymeropoulos et al. 1997), α-synuclein locus

duplication (Chartier-Harlin et al. 2004) and triplication (Singleton et al. 2003), parkin (Kitada et al. 1998), ubiquitin-C-terminal hydrolase-L1 (UCHL-1) (Leroy et al. 1998), DJ-1 (Bonifati et al. 2003) and others listed in table 1.2.

The first familial form of PD was identified in a Greek family as a missense mutation in the α-synuclein gene (*SNCA*), resulting in an alanine to threonine substitution at point 53 (A53T) (Golbe et al. 1999). In families with an A53T substitution, the disease is fully penetrant at an average age of onset of 46 years, 10 years younger than sporadic PD (Polymeropoulos et al. 1997), and dementia appears to be more frequent (Spira et al. 2001). Another *SNCA* mutation resulting in an alanine to proline substitution at point 30 (A30P) was identified in a German family, but this time the disease was less penetrant with a slightly later age of onset (Kruger 2004). A more recently identified mutation results in a glutamate to lysine substitution at point 46 (E46K) with dominant parkinsonism and DLB (Zarranz et al. 2004), is associated with an age of onset between 50 and 65 years, but with dementia presenting within around two years.

Recent findings suggest that the *SNCA* gene dosage and the corresponding protein levels might go some way as to determine the age of onset of PD and also disease progression (Eriksen et al. 2005; Mizuta et al. 2006; Mukaetova-Ladinska and McKeith 2006). Triplication of *SNCA*, which results in four normal copies of the gene, induces a severe form of early onset PD with rapid disease progression (Singleton et al. 2003). Duplication of the gene has been identified as having a similar clinical presentation and disease progression as sporadic PD (Chartier-Harlin et al. 2004; Ibanez et al. 2004). Also, polymorphisms in the *SNCA* promoter associated with higher α-synuclein expression have been found to increase the risk of developing PD (Holzmann et al. 2003).

Table 1.2 Genetic causes of parkinsonism (adapted from (Farrer 2006)

Gene	Locus	Age of onset	Mutations	Pathology
PINK1 (PTEN-induced kinase 1)	PARK6	20-40 years	Recessive missense and exon-deletion mutations	Undetermined
DJ-1	PARK7	20-40 years	Recessively inherited, homozygous missense and deletion mutations	Undetermined
UCHL-1 (ubiquitin carboxyl-terminal hydroxylase L1)	PARK5	55-58 years Late onset	Linkage for the Ile 93 Met substitution Susceptibility for sporadic PD with Ser18Tyr polymorphism	Undetermined UCHL-1 protein prominent component of LB's
SNCA (gene that encodes α-synuclein)	PARK1 and PARK4	46-65 years (mutations) 38-65 years (duplications) 24-48 years (triplications)	Dominant Ala30Pro, Glu46Lys ad Ala53Thr substitutions Genomic duplications and triplications	Diffuse Lewy body disease, with prominent nigral and hippocampal neuronal loss
PARK2 (gene that encodes parkin)	PARK2	30 years on average	Recessive homozygous, compound heterozygous and exonic deletion / duplication / triplication mutations	Predominantly nigral neuronal loss, although compound heterozygotes with LB's or tau pathology are described
LRRK2 (leucine rich repeat kinase 2)	PARK8	Between 50 and 70 years (range 32-79)	Many dominant substitutions	Predominantly LB's, rare cases with neurofibrillary tangles and/or nigral neuronal loss

Mutations in another gene implicated in PD, *parkin,* were first identified in Japanese patients with autosomal recessive Parkinson's disease (ARPD) (Matsumine et al. 1997; Kitada et al. 1998). Subsequently, many other *parkin* mutations have been described associated with both early and late onset PD. Parkin is a member of the E3 ubiquitin ligase family (Shimura et al. 2001). This protein is primarily expressed in the central nervous system (CNS) and co-localises with α-synuclein in normal brain. Parkin is found in LB's in sporadic PD (Schlossmacher et al. 2002). A loss of function of this protein has been proposed to cause an accumulation of a modified, glycosylated form of α-synuclein, termed αSp22, which is said to build up to pathological levels in parkin-deficient PD brains (Shimura et al. 2001). This process will be discussed later in Chapter 3. Pathological hallmarks of ARPD due to parkin mutations including loss of nigral and locus coeruleus neurones and the apparent absence of LB formation (Metman et al. 2001; Mizuno et al. 2001; Junn et al. 2002). The lack of LB's could be consistent with the inability of the UPP to form ubiquitinated aggregates of α-synuclein.

It is worth mentioning mutation in another gene involved in the UPP, *ubiquitin C-terminal hydrolase (UCHL-1).* This gene was identified in a family with autosomal dominant PD (Leroy et al. 1998). However, this data has recently been challenged, based on evidence the UCHL-1 gene does not exhibit a protective effect in PD (Healy et al. 2006). Further investigations, of both sporadic and inherited PD cases over the last few years, have failed to identify any further mutations in this gene.

1.3.3 The Synuclein family

The synuclein group consists of three known proteins, α-, β-, and γ-synuclein. They are a family of small, soluble proteins (ranging from 123 to 140 amino acids in length) and have 55-62% homology in sequence, with a similar domain organisation (Lavedan 1998). The α-, β- and γ-synuclein proteins are primarily expressed in the brain, with low levels in other tissues (see table 1.3). The γ-synuclein protein is also found in the

cell bodies and axons of the peripheral nervous system and retina (Buchman et al. 1998), but its expression in breast tumours is a marker for tumour progression.

Table 1.3 Tissue expression of the human synuclein genes

	Pancreas	Kidney	Skeletal muscle	Liver	Lung	Placenta	Brain	Heart	Breast cancer
α	+	+	++	-	+	+	++++	++	-
β	-	-	+	-	-	-	++++	-	-
γ	+	+	++	+	-	-	++++	++	++

The relative expression, determined by Northern blot analysis of different tissues, is indicated for a particular gene but cannot be compared here between genes: α-synuclein, β-synuclein (Jakes et al. 1994), γ -synuclein (Lavedan 1998) (adapted from http://www.genome.org/cgi/content/full/8/9/871/T1).

The human α-synuclein gene encodes a highly conserved 140 amino-acid protein that is extensively expressed throughout the brain, predominantly in the presynaptic terminals of neuronal cells. It is also expressed to a much lesser extent in the cells of non-neuronal tissues such as the heart and pancreas (see table 1.3). Conformational analysis of α-synuclein has revealed that it belongs to a group of unfolded proteins, which are characterised by a unique combination of low hydrophobicity and large net charge (Weinreb et al. 1996). α- and γ-synuclein are slightly more compact and structured than β-synuclein, which displays a random coil, but all are natively unfolded under physiological conditions (Uversky 2003). It has been shown that α-synuclein displays conformational plasticity, adopting a range of different conformations, depending in the surrounding environment.

The α-synuclein sequence can be divided into three separate regions, or domains (see figure 1.4). An amino-terminal domain occupies amino acid residues 1-65, a central hydrophobic region at position 61-95 and then an acidic carboxy-terminal domain at residues 96-140. Both the amino-terminal and the hydrophobic domain contains 6

repeats of an 11 amino acid sequence containing variations of the consensus sequence KTKEGV (Nonaka et al. 2005), and a recent study shows that several of the lysine residues within these repeats are subject to ubiquitination. The amino-terminal domain of the protein binds negatively charged phospholipids and adopts an α-helical conformation upon binding. This conformation is similar to the lipid-binding domain of some apolipoproteins, suggesting that the protein may be normally be membrane associated (Davidson et al. 1998; Eliezer et al. 2001). Furthermore, lipid rich environments have been shown to promote aggregation in α-synuclein (Welch and Yuan 2003). The central protein domain is highly amyloidogenic which enables α-synuclein to undergo a conformational transformation, changing it from a random coil to a β-sheet structure (el-Agnaf and Irvine 2002). For α-synuclein to assemble into filaments, a stretch of 12 hydrophobic amino acid residues (71-82) is essential (Giasson et al. 2001). The acidic carboxy-terminal domain of the protein inhibits aggregation (Murray et al. 2003) and has been shown to reveal chaperone activity (Kim et al. 2002). α-synuclein has sites for glycosylation (McLean and Hyman 2002), nitration (Takahashi et al. 2002) and phosphorylation (Negro et al. 2002) in the carboxy-terminal domain.

Although β- and γ-synuclein show similar structural properties to α-synuclein, they vary in their ability to aggregate. On the contrary, they have been shown to actually inhibit the tendency of α-synuclein to aggregate (Uversky et al. 2002; Park and Lansbury 2003; Hashimoto et al. 2004). β- and γ-synuclein have been shown to accumulate in hippocampus axon terminals in PD and DLB (Galvin et al. 1999). Also, mutations in the β-synuclein gene (*SNCB*) that have resulted in amino acid substitutions V70M and also P123H have been identified in DLB (Ohtake et al. 2004). Therefore, β- and γ-synuclein are now also implicated in the pathogenesis of these diseases.

Figure 1.4 Sequence comparison of human α-, β- and γ-synucleins

Amino acid identities between at least two of the three sequences are boxed in blue. The positions of the α-synuclein mutations implicated in familial PD (A30P and A53T) are indicated in red. The amino-terminal half of the synucleins is taken up by 11 amino acid repeats with the consensus sequence KTKEGV, and α-synuclein assembles into filaments through these repeats. (Taken from (Goedert 2001))

1.3.4 Suggested functions of α-synuclein

A few of the functions suggested for α-synuclein are it being involved in synaptic plasticity, dopamine metabolism and signalling (Clayton and George 1999; Yu et al. 2005; Kim et al. 2006). The role in synaptic plasticity is supported by studies showing that protein expression is up regulated, and the synaptic terminals rearranged, during a critical period of song learning in zebra finches (Clayton and George 1998). It is thought that in mammals synaptic vesicle function might involve α-synuclein, as knockout mice display faulty synaptic transmission. It is thought that this is as a result of vesicle depletion and impaired vesicle mobilisation (Cabin et al. 2002). α-synuclein has been found to bind phospholipid vesicles, containing acidic phospholipid molecules in *in vitro* studies (Davidson et al. 1998). The protein has also been shown to interact with, and inhibit, phospholipase D, a key enzyme of the phospholipid signalling pathway (Ahn et al. 2002). As mentioned earlier, α-synuclein with selective phosphorylation at serine 129 is evident in LB's. This phosphorylation by G protein-coupled receptors, results in a reduced ability of the protein to interact with phospholipids and phospholipase D (Fujiwara et al. 2002).

Activated Fyn specifically phosphorylates tyrosine α-synuclein residue 125, so might be involved in the Fyn-mediated signalling pathway (Nakamura et al. 2001). It has also been suggested that α-synuclein modulated the extracellular signal-related kinase (ERK) signalling pathway by down regulation of ERK activity (Subramaniam et al. 2003). So therefore, it is possible that α-synuclein is a regulatory component in various signalling pathways in neuronal cell differentiation and plasticity.

A further role for α-synuclein involved in dopamine metabolism is supported by several studies. Over expression of the protein down regulates the transcription of several enzymes involved in the synthesis of dopamine, including tyrosine hydroxylase and dopa decarboxylase (Baptista et al. 2003) (figure 1.5). α-synuclein has also been shown to down-regulate the dopamine transporter (DAT)-mediated uptake of extracellular dopamine (Sidhu et al. 2004). This supports previous suggestions that a loss of normal α-synuclein activity in PD may disrupt dopamine homeostasis and have a negative affect on dopaminergic neuronal survival (Perez and Hastings 2004).

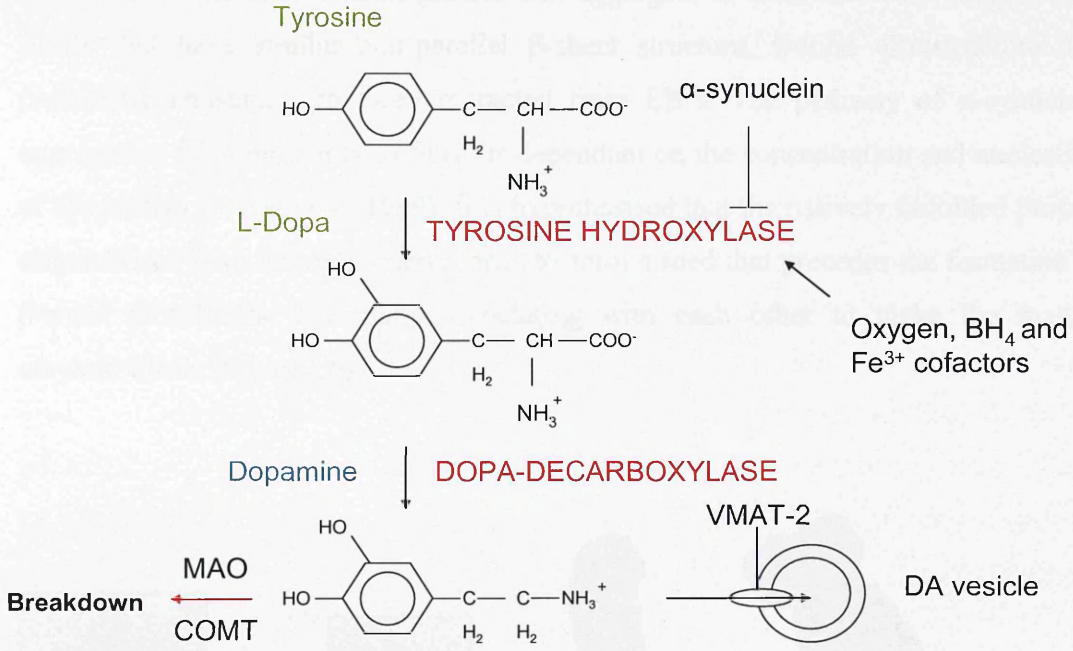


Figure 1.5 Dopamine synthesis and metabolism

DA synthesis occurs in the presynaptic terminals of dopaminergic neurones. The rate limiting tyrosine hydroxylase (TH) uses oxygen, iron and tetrahydrobiopterin (BH_4) cofactors to convert tyrosine to dihydroxyphenylalanine (L-DOPA) (reviewed in (Lotharius and Brundin 2002)). Decarboxylation of L-DOPA yields DA within the cytosol which is then sequestered into vesicles via the vesicle monoamine transporter 2 (VMAT-2). Monoamine oxidase (MAO) can deaminate DA to form 3,4-dihydroxyphenylacetic acid and hydrogen peroxide. Catchol-O-methyltransferase (COMT) can also metabolise DA to 3-methoxytyramine. Interestingly, synuclein mutants can inhibit TH activity (reviewed in (Takeuchi and Takahashi 2007)). Inhibition by α-synuclein provides a possible mechanism for selective loss of DA-producing neurones in PD.

1.3.5 Aggregation and α-synuclein

Under physiological conditions α-synuclein is prone to aggregation *in vitro* (Conway et al. 2000). The recombinant protein will aggregate to form insoluble amyloid-like fibrils that have similar anti-parallel β-sheet structure, wound ultrastructure and proteolytic resistance, to those extracted from LB's. The pathway of α-synuclein aggregation from monomer to fibril, is dependant on the concentration and nucleation of the protein (Wood et al. 1999). It is hypothesised that the natively unfolded protein oligomerises from its' monomeric form, to form a seed that precedes the formation of β-sheet protofibrils, eventually associating with each other to make the mature amyloid fibrils (figure 1.6).

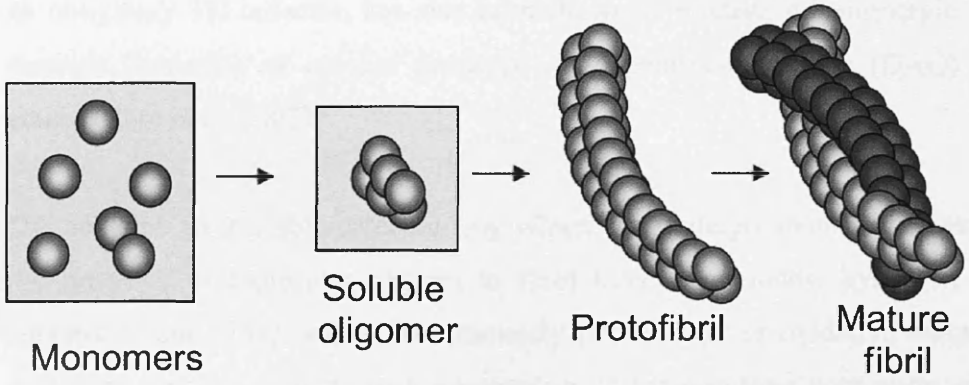


Figure 1.6 The formation of mature fibrils from α-synuclein monomers

The hypothetical scheme depicts various proposed pathways of natively unfolded α-synuclein monomers converting into partially folded and/or aggregated forms. (Taken from (Paleologou et al. 2005))

The amino acid substitutions associated with familial PD, A30P, A53T and E46K, have been shown to increase the propensity for α-synuclein to aggregate (Conway et al. 2000). However, there are differences in the aggregation behaviour of the separate substitutions and the wild-type protein. Although the formation of soluble oligomers is accelerated in the A30P and A53T mutants, the fibril formation is lower in A30P and higher in A53T (Conway et al. 2000). The filament formation rate of E46K is similar to A53T (Choi et al. 2004).

Other factors have been shown to accelerate the propensity of the protein to aggregate. These include low pH and high temperatures (Uversky et al. 2001), metal ions (Uversky et al. 2001), pesticides (Uversky et al. 2001), glycosaminoglycans (Cohlberg et al. 2002), cellular polyamines (Antony et al. 2003), double stranded DNA (Cherny et al. 2004) and dopamine (Cappai et al. 2005). The mechanisms that lead to the aggregation of α-synuclein in the brain remain unknown.

Protofibrillar α-synuclein, in contrast to monomeric and fibrillar forms, can bind synthetic vesicles causing transient permeability (Volles et al. 2001). If this were true for DA vesicles, cytosolic DA concentrations would increase. Obviously, this has implications for PD pathogenesis as DA induces oxidative damage both *in vitro* and *in vivo*. For example, MPTP used to model PD in animals causes production of free radicals by DA oxidation (Lotharius and O'Malley 2000). Tetrahydrobiopterin (BH_4), an obligatory TH cofactor, has also been shown to mediate dopaminergic cell death through formation of quinine products via dopamine-*o*-quinone (DAQ) oxidative action (Choi et al. 2003).

DA contains an unstable catechol ring which can undergo spontaneous oxidation in the presence of molecular oxygen to form three compounds: hydrogen peroxide, superoxide and DAQ, which are ultimately responsible for oxidative damage. DAQ can form covalent adducts with α-synuclein which stabilises potentially pathogenic protofibrils (Conway et al. 2001). As mentioned previously, monoamine oxidase (MAO) can deaminate DA to form 3,4-dihydroxyphenylacetic acid and hydrogen peroxide (reviewed in (Lotharius and Brundin 2002)). Toxic hydrogen peroxide in the presence of transition metal ions such as Cu^{2+} and Fe^{3+} can form hydroxyl radicals via Fenton's reaction (Choi et al. 2003):

$$M^{n+} + H_2O_2 \longrightarrow M^{(n+1)+} + {}^{\cdot}OH + {}^{-}OH$$

Superoxide can interconvert with hydrogen peroxide (Tabner et al. 2002):

$$O_2^{\cdot -} + e^- + 2H^+ \rightleftharpoons H_2O_2$$

Hydroxyl radicals can react and add readily to C=C bonds and abstract hydrogen atoms from many organic compounds to yield a variety of products which may themselves be toxic, or cause depletion of critical cellular proteins (Tabner et al. 2002).

1.3.6 Diagnosis and treatment of Parkinson's disease

PD is just one of several neurologic movement disorders that produce similar symptoms. The clinical examination must rule out all of the different disorders that can masquerade as PD. PD must be considered if the person exhibits more than one of the cardinal symptoms of the disease, including tremor at rest, bradykinesia, rigidity, and in more advanced cases, postural instability (Savitt et al. 2006). The presence of resting tremor supports the pathology of PD more than the other symptoms, although around 20% of patients fail to develop this symptom (Suchowersky et al. 2006). Non-motor symptoms may include disrupted sleep, depression and fatigue. Because progression of neurologic movement disorders and their treatment varies greatly, proper clinical diagnosis is crucial.

The neurological examination evaluates a patient with a movement disorder, the doctor taking a medical history and performing a physical examination. The doctor asks the patient and the family members or friends about symptoms and observes the patient, asking him or her to walk around the room, to sit down, stand up, turn around, and so on, observing aspects of the patient's movement, coordination and balance. Although genetic testing for *parkin* and *SNCA* is available, it is only offered when there is a strong family history, or when symptoms present themselves at an unusually young age. Unfortunately, there is no diagnostic test that can confirm PD, although research into levels of α-synuclein in plasma and CSF is currently being investigated as a potential diagnostic biomarker for PD.

Electroencephalograms (EEGs) record some aspects of brain electrical activity, but they are not effective in spotting PD. The MRI and CAT scans of the brain produce detailed anatomic pictures, but unfortunately scans of the brains of people with PD

appear normal. So, with no diagnostic tests to provide specific answers, clinicians must base their diagnosis of PD on judgment.

The matter is further confused in that an early PD patient may complain about suffering the cardinal signs of the disease, but the neurological examination may appear normal (de Lau et al. 2006). The fact that the cardinal symptoms of PD may be absent or shared with other movement disorders complicates matters further. An early study found that only 69-70% of people with autopsy-confirmed PD had at least two of the cardinal signs of PD, and 20-25% of people with two of the symptoms had a pathological diagnosis other than PD. Moreover, 13-19% of people who demonstrated all three of the cardinal features had a pathological diagnosis other than PD (Hughes et al. 1992; Hughes et al. 2001).

Dopamine replacement therapy remains the most effective medication to date for treating the symptoms of PD. As dopamine itself cannot cross the blood-brain barrier and is toxic to peripheral tissues, a dopamine precursor, levodopa (L-DOPA), is administered in conjunction with a dopa-decarboxylase inhibitor such as carbidopa or benzeraside, which slows the peripheral conversion of dopamine and enables more L-DOPA to reach the brain. Once in the brain, the L-DOPA is converted into DA that is then released physiologically. Most PD patients have a good initial response to this treatment, although there are limitations. The L-DOPA does not stem any further neuronal loss, and consequently, the symptoms of the disease continue to worsen and the response to treatment reduces over time. Chronic treatment is associated with severe side effects including dyskinesias, motor fluctuations and dementias. DA agonists, ropinirole or pramipexole can be used to enhance efficacy and reduce motor symptoms. Inhibitors of COMT can be used to prevent the breakdown of DA and extend its viability (Marsh and Dawson 2000; Tanner 2000).

Identification of mutations responsible for the familial forms of PD have created opportunities to address the pathological changes seen in the brain. One powerful strategy that has emerged is the overexpression of parkin. After it was shown that parkin was an E3 ubiquitin ligase, many studies focussed on how parkin polyubiquitination activity could clear aggregation prone substrates (Imai et al. 2001). Recently, it has been suggested that the proteosome-independent ubiquitination by a

lysine 63-linked process could also play a major role in neuroprotection by intracellular stress-related pathway regulation. There is compelling evidence that two of the loss of function parkin mutations cause c-Jun N-terminal kinase (JNK) activation, which is a mediator of stress-induced apoptosis, leading towards selective dopaminergic cell death (Hasegawa et al. 2008). The wild type parkin protects against oxidative stress by inhibiting the JNK signalling pathway. It has been reported that parkin expression increases after stress induced by kainite and rotenone in cultured cells resulting in neuroprotection (Henn et al. 2007). Moreover, parkin has also been associated with mitochondrial morphology along with PINK1 in Drosophila, possibly by increasing mitochondrial fission (Poole et al. 2008). And so as PD has been previously associated with mitochondrial pathology, these findings open new mechanistic possibilities and explanations of PD pathology, and present the question as to whether overexpression of parkin could provide a novel strategy for neuroprotection in PD (Ulusoy and Kirik 2008).

1.4 Frontotemporal Lobe Degeneration – An overview

1.4.1 Frontotemporal Lobe Degeneration

The term Frontotemporal Lobe Degeneration (FTLD) is a general term describing a group of clinical syndromes associated with dementia of non-Alzheimer type and degeneration of the frontal and temporal lobes. It is the third most common cause of dementia, after AD and DLB (Neary et al. 1998), with onset between the ages of 35 and 75 years, with equal prevalence in males and females (Mott et al. 2005). Fortyfive percent of patients with this disorder have at least one family member with a similar disorder, and 89% of these latter individuals show an inheritance pattern consistent with an autosomal dominant mode of transmission.

Immunohistochemical investigations in FTLD have concentrated on the identification of pathological aggregations of proteins such as tau and ubiquitin, within neurones and glial cells (Chow et al. 1999). These investigations have led to a pathological classification of FTLD, as follows (Cairns et al. 2007);

1. One class are cases characterised by pathological aggregations of tau protein (the "tauopathies") which include FTLD with Pick bodies, Corticobasal degeneration (CBD), Frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), as well as Progressive supranuclear palsy (PSP).
2. Another group, Frontotemporal lobe degeneration with ubiquitin positive inclusions (FTLD-U), represents most FTLD cases, and includes cases with ubiquitin-positive, tau and α-synuclein negative inclusions (McKhann et al. 2001; Josephs et al. 2004).
3. A third class covers the cases of dementia that lack distinctive histology (DLDH) (Shi et al. 2005) and any cases of neuronal intermediate filament inclusions containing neurofilament protein (Cairns et al. 2007).
4. Tau positive intraneuronal neurofibrillary tangles or Pick-like bodies, associated with mutations in the tau gene (*MAPT*).

In the study by Shi *et al,* (2005) the single most common cause of FTLD was FTLD-U, with 34% patients being affected. DLDH was the next most common with 22% patients being affected, followed by tau Pick-type histology with 20% and FTDP-17T histology with 16%. 4% (3 cases) with clinical FTD were due to cerebrovascular changes, not FTLD.

To summarise, it can be broadly said that FTLD is associated with either a tau-based pathology (tauopathy) or a ubiquitin based pathology (FTLD-U or FTD/MND when clinical MND is also present).

1.4.2 Aetiology of FTLD-U

The diverse pathology that exists across the FTLD-U group could imply that different pathological processes are at work. It could be the case that there are different proteins involved, or different modifications of the same protein which underlie the pathogenesis of each FTLD-U class. Studies on familial cases of FTDP-17 showing FTLD-U pathology have led to the identification of causal mutations in the

progranulin gene, *PRGN (Baker et al. 2006; Cruts et al. 2006)*. Progranulin is a 593 amino acid multifunctional growth factor (Daniel et al. 2000). The protein is mainly expressed in epithelial, haematopoietic and some fibroblastic cell lines, and is found in a range of tissues throughout the body, from the epididymis, to the mammary glands, GI tract, and to specific parts of the brain. Compared to other tissues, progranulin expression in the brain is actually very low, but high expression is seen in certain subsets of cells, specifically cortical neurons, Purkinje cells of the cerebellum and granule cells of the hippocampus (Daniel et al. 2000). Granulins are peptides derived from progranulin, that can alter cell growth in culture (Daniel et al. 2000). The rate of epithelial cell proliferation is directly proportional to the *PGRN* gene expression (He and Bateman 1999). Increased progranulin has been associated with tumorigenesis.

Although progranulin, and derived peptides regulate cell growth in culture, their roles *in vivo* are not fully understood (Daniel et al. 2000). However, recent findings suggest that it is essential for neuronal survival (Baker et al. 2006), and this supports the hypothesis that neurodegeneration in FTLD results from loss of growth factor support (Capsoni et al. 2000), (although recently cases of FTD and MND with TDP-43 pathology have been found, without the associated progranulin mutation (Seelaar et al. 2007)). Very recently, it has been suggested that progranulin mediates proteolytic cleavage of TDP-43, generating ~35 and ~25 kDa species. It is possible that abnormal metabolism mediated by progranulin may play a pivitol role in neurodegeneration (Zhang et al. 2007).

Examination of cases with *PRGN* mutations, all show a characteristic pathological picture with ubiquitin-positive neurites, neuronal cytoplasmic inclusions and neuronal intranuclear inclusions consistent with certain types of FTLD-U. However, the ubiquitinated inclusions in FTLD-U do not contain the progranulin protein. Disease is as a result of a loss of function in the protein, and not from the accumulation of the mutated protein. This is consistent with the opinion that mutations in *PRGN* result in the production of prematurely terminated transcripts that are immediately destroyed by nonsense-mediated degradation (Baker et al. 2006; Cruts et al. 2006).

Despite exhaustive immunohistochemical investigations over the past 15 years, the protein present in the ubiquitin-positive FTLD-U inclusions remained unknown until

recently. The ubiquitinated target protein was identified as the TAR DNA-binding protein, TDP-43 (Arai et al. 2006; Neumann et al. 2006).

1.4.3 The TDP-43 protein

TDP-43 is a 41443 Da transcription factor with two functions; transcription repression and activation of exon skipping. It contains two RNA-recognition motifs as well as a glycine-rich C-terminal sequence (Buratti and Baralle 2001). The protein can therefore be said to have regulatory roles in multiple transcription and splicing, or interact with survival motor neurone protein, playing a role in the arrangement of nuclear bodies in eukaryotic cells (Buratti et al. 2004; Wang et al. 2004; Mercado et al. 2005; Arai et al. 2006; Neumann et al. 2006). So the physiological functions of TDP-43 may be diverse and these functions may involve binding to single stranded DNA, RNA, and proteins to regulate biological processes by multiple mechanisms.

TDP-43 immunohistochemistry in unaffected neurones produces the expected nuclear staining, but in FTLD-U, the same pathological changes previously seen with anti-ubiquitin immunostaining are also replicated with anti-TDP-43 antibodies (Neumann et al. 2006; Davidson et al. 2007).

TDP-43 pathology has been demonstrated in a number of clinical entities and this has led to the classification of these disorders under the term of 'TDP-43 proteinopathies'. This includes cases of sporadic and familial FTLD-U, FTD/MND and sporadic MND. TDP-43 pathology was found in familial cases of FTLD-U, other than those associated with the progranulin mutations (Neumann et al. 2006). TDP-43 immunoreactive changes are observed in the brains of 24-26% patients with AD (Amador-Ortiz et al. 2007; Uryu et al. 2008). Recent studies have shown TDP-43 pathological changes to be present in Paget's disease and FTD associated with mutations in VCP gene (Guinto et al. 2007; Neumann et al. 2007), in a proportion of patients with DLB (Nakashima-Yasuda et al. 2007), in Guam parkinsonism dementia complex (Hasegawa et al. 2007; Geser et al. 2008) and in some cases of CBD (Uryu et al. 2008). TDP-43 has not however been associated with other neurodegenerative

diseases such as, MSA, PSP, FTDP-17, tangle-only dementia nor basophilic inclusion body disease (Skibinski et al. 2005; Arai et al. 2006; Cairns et al. 2007; Davidson et al. 2007), in tau-containing Pick bodies in FTLD (Davidson et al. 2007; Freeman et al. 2008; Uryu et al. 2008), or in those ubiquitinated inclusions of certain other cases of FTLD-U called atypical FTLD-U (Mackenzie et al. 2008; Roeber et al. 2008).

In FTLD-U, examination of the distribution and morphology of the TDP-43 pathology confirmed previous classifications of FTLD-U (Davidson et al. 2007). However, although the pathogenic mechanisms of sporadic and familial cases of FTLD-U all involve the TDP-43 protein, they must differ in some way as they produce different histological phenotype and different neurodegenerative disorders.

TDP-43 pathological changes have been reported in a variety of disorders including low grade tumours, indicating alterations in the structure or function of this protein may occur in a wide range of neurodegenerative diseases and other disorders (Lee et al. 2008).

Mutations in *TARDBP* have been reported in some cases of both familial and sporadic forms of MND (Gitcho et al. 2008; Kabashi et al. 2008; Sreedharan et al. 2008; Van Deerlin et al. 2008; Yokoseki et al. 2008), although none have been found in cases of familial FTLD-U (Van Deerlin et al. 2008). Moreover, there has been no genetic association between any *TARDBP* polymorphisms and FTLD-U in case control studies (Neary et al. 1998; Nakashima-Yasuda et al. 2007). However, it is possible that variations within *TARDBP* may predispose the deposition of TDP-43 in some disorders, although this has not been investigated to date. Therefore the underlying reason as to why only some individuals with AD, DLB, CBD and so on, show TDP-43 pathological changes, while most do not, is unclear.

1.4.4 Aggregation and TDP-43

Investigations to give an insight into possible pathogenic mechanisms relating to TDP-43 have been made. Immunoblotting of urea extracts of protein from affected regions in cases of both sporadic and familial cases of FTLD-U have identified a 45kDa band, plus 24 and 26kDa bands which correspond with C-terminal TDP-43 fragments. As with tau proteins in AD and other tauopathies, the 45kDa band collapses to a 43kDa band after alkaline phosphatase treatment, which implies that the TDP-43 protein may be abnormally phosphorylated (Arai et al. 2006; Cairns et al. 2007; Neumann et al. 2007). It also suggests that the biochemical profile of the TDP-43 protein in both sporadic and familial cases of FTLD-U is similar.

Electron microscopy of TDP-43 inclusions shows them to be composed of granular material that is not fibrillar like the proteins in other groups of diseases such as tauopathies and synucleinopathies. Moreover, the inclusions do not stain with amyloid dyes such as congo red and riboflavin S (Cairns et al. 2007).

The dysfunction or dysregulation of the progranulin protein, as seen in FTLD-U, may be a factor in the abnormal compartmentalisation of TDP-43. As protein phosphorylation is an important event in cellular signalling, it is feasible that hyperphosphorylation of TDP-43 may cause disruption of important signalling pathways or might even directly affect the trafficking of TDP-43 itself, resulting in neuronal dysfunction. However, the mechanistic aspects of the aggregation of pathologic TDP-43 in either cytoplasmic, neuritic, or nuclear inclusions, or the role in the pathogenesis of neurodegeneration, are still being investigated.

1.4.5 Diagnosis and treatment of FTLD

The clinical diagnostic consensus criteria for FTLD describes the syndrome as changes in personality, behaviour and social conduct such as disinhibition, passivity, inertia, over activity and stereotyped behaviour (Neary et al. 1998). The condition

may last from 3 to 17 years before death, with an average duration of eight years after diagnosis.

FTLD includes three principle clinical disorders. About 70% of patients present with the clinical syndrome FTD, whilst the other clinical conditions, namely Semantic dementia (SD) and Progressive non-fluent aphasia (PNFA) have been recognised. Some patients may develop motor disorders such as parkinsonism during their disease (Foster et al. 1997). Importantly, it is recognised that there is a considerable overlap between FTD cases and those with Motor neurone disease (MND), and a related clinical disorder FTD with MND (FTD/MND) (Lomen-Hoerth et al. 2002). FTLD can be considered as a heterogeneous term, encompassing international criteria in an attempt to identify and categorise distinct clinical and pathological syndromes (Neary et al. 1998).

FTD usually presents between 45-65 years of age. Unlike AD, there is some preservation of perception, spatial orientation and memory, but the cognitive impairment (i.e. poor organisation and attention) that exists, may compromise these functions. Speech is reduced, sometime progressing to mutism (Neary et al. 1998).

SD has a mean age of onset of 58 years, with a mean length of illness of 9 years (Davies et al. 2005). SD is characterised by a progressive loss of conceptual knowledge and impaired comprehension. This results in the loss of understanding and the meaning of words. Speech is fluent, but is empty of content and conveys little meaning (Bozeat et al. 2000). Some patients may present with a predominant verbal deficit and this is associated with a more left sided pathology, whilst others with visual problems have a more right –sided atrophy (Neary et al. 1998; Snowden et al. 2004). In PNFA the patient suffers from progressive difficulties with speech production. There can be problems in different parts of speech production, hence patients can present with articulatory breakdown (hesitant speech or speech apraxia), agrammatism (difficulties with syntax e.g. use of the wrong tense or word order), anomia or phonemic breakdown (difficulties with sounds). However, it is rare for patients to have just one of these problems and most people will present with several symptoms. As the disease develops speech quantity decreases and many patients will become mute (Neary et al. 1998). Cognitive domains other than language are rarely

affected early on. However, as the disease progresses other domains can be affected. Problems with writing, reading and speech comprehension can occur as can behavioural features similar to FTD, however comprehension is relatively preserved (Neary et al. 1998). The majority of patients with SD will have ubiquitin-positive, tau-negative inclusions although like all of the FTLD syndromes other pathologies have been described including Pick's disease and other tau positive pathology (Davies et al. 2005).

Some patients present with the clinical features of FTD but with the motor weakness and features of Motor neurone disease (FTD/MND). The pathological changes associated with MND are seen in the spinal cord (Neary et al. 1990). In other forms of familial FTLD, parkinsonism may be present in addition to the FTD. Genetic linkage studies in these cases found association with mutation in the *MAPT* on chromosome 17, and therefore the hereditary disorder Frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) was identified (Foster et al. 1997).

International criteria have been developed to assist clinical diagnosis of the disorders mentioned here. Despite attempts to simplify these criteria and aid diagnosis, the heterogeneity that exists in clinical presentation, and the overlap that exists between disorders, makes clinical diagnosis a challenge (Neary et al. 1998; McKhann et al. 2001). The only way to ascertain a confident diagnosis of a distinct disease entity is on neuropathological examination of the brain at autopsy (Cairns et al. 2007).

At present there is no cure for FTD and no effective way to slow its progression. Treatment relies on managing the symptoms. Anti-psychotics that can block the effects of dopamine may be of value in managing aggressive, irrational and compulsive behaviours that can sometimes develop with FTD. Tranquilizers may be of help if agitation or other types of hyperactivity become problems. Speech therapy may be offered to people with primary progressive aphasia, who may benefit from therapy to help them adjust to their language difficulties and learn alternate ways to communicate. Antidepressants called selective serotonin reuptake inhibitors (SSRIs) may offer some relief from apathy and depression and help reduce food cravings, loss of impulse control and compulsive activity. The main effect of the disease is circumscribed loss of pyramidal neurones, and it is suggested that as a single

pharmacological approach, 5-HT_{1A} receptor antagonist treatment, may be appropriate (Bowen et al. 2008).

As the present clinical management of FTD patients depends mainly on the construction of a network of carer and family support (Neary et al. 2005), perhaps one important area of research relating to the management issue of these disorders relates to the determining the underlying pathological process (FTLD-U or FTLD-Pick histology).

1.5 Biomarkers and neurodegenerative diseases

Generally, a biomarker is defined as a measure of a biological process that can be correlated with the disease process, or the response of that process to treatment. As neurodegenerative diseases are complex conditions that may potentially have many underlying causes that differ between individuals, a biomarker (or set of biomarkers) will most probably require several methods of measurement. Research in the scientific community aims to identify several types of biomarkers;

- Disease trait biomarkers predict the genetic predisposition that an individual will get neurodegenerative disease.
- Disease state biomarkers would distinguish individuals with a particular disease, for example PD, from other disorders that mimic the disease, or would identify individuals in the earliest stages of disease.
- Disease rate biomarkers would record disease progression, which would be particularly important for developing treatments that could slow or stop a disease.
- Translational biomarkers may predict whether or not an individual would respond to a specific treatment, or whether the drug itself is actually reaching its target and acting upon it. A biomarker of this type would be critical for drug development although not directly related to the disease process itself.

The development of new treatments for neurodegenerative diseases is hindered by the fact that, at present, there is a lack of reliable biomarkers that can accurately identify

individuals with a particular type of neurodegenerative disorder, measure the disease progression and monitor treatment effects. Intervention with protective drugs and therapies would be possible (once these become available) as soon as a biomarker that can identify people with, or at risk, for these diseases before significant neuronal loss has occurred.

Clinical tests lack the sensitivity and objectivity to definitively establish the presence of a particular disease or to track disease progression. However, the greatest obstacle to the identification and validation of biomarkers is the requirement of longitudinal studies that can ensure disease is well-characterised in a population of individuals. These studies involve recruiting a group of patients who exhibit the clinical symptoms, then observing them at regular intervals over an extended period of time. The extent of the symptoms, or any changes in the individuals' status could be measured and correlated with the change in the biomarker.

Research to identify a biomarker that accurately measures the underlying disease process include markers taken from blood, urine and cerebral spinal fluid. These tissues/fluids can be used to measure/identify genetic, protein or other chemical biomarkers that may be a molecular 'signature' of the disease.

Previously, methods were developed in our laboratory to detect oiligomeric forms of α-synuclein, which led to an extensive study of blood plasma and CSF (El-Agnaf et al. 2003; El-Agnaf et al. 2006). There was a highly statistically significant difference between the concentration of these oligomers between PD samples and controls, with most of the PD samples giving high signals, whereas only a few control samples gave a high signal. Following on from these studies, techniques and methods were developed for the study of the α-synuclein-parkin complex, and consequently the newly discovered protein TDP-43, to investigate whether these proteins/protein complexes were useful in the development of biomarkers for neurodegenerative disease.

1.6 Aims of the thesis

The initial aim of the work carried out for this thesis was to test the hypothesis that an interaction between α-synuclein and parkin exists, and that the binding partner for parkin is in fact the 22-24kDa glycosylated form of α-synuclein (αSp22) reported previously (Shimura et al. 2001). The proposal was to then attempt to isolate this glycosylated α-synuclein species, fully characterise it, and further investigate its role in disease pathogenesis.

With this in mind, methods were developed for the detection and assay of the α-synuclein-parkin complex, and these methods were applied to extracts of human post-mortem brain tissue, and also to samples of human blood plasma. The latter studies arose from previous work in our laboratory, demonstrating that α-synuclein is present in human CSF and blood plasma (El-Agnaf et al. 2003; El-Agnaf et al. 2006) and might be useful as a biomarker for PD and related disorders (El-Agnaf et al. 2006; Tokuda et al. 2006). The presence of parkin, or a complex between α-synuclein and parkin in human body fluids, in addition to α-synuclein, would not only shed light on this interaction but would also open up new opportunities for the development of biomarkers for disease.

During the course of these investigations, a new protein (TDP-43) was discovered to be involved in neurodegenerative disease (Arai et al. 2006; Neumann et al. 2006). For the second part of this thesis, the presence of this protein was investigated in human blood plasma, and the levels of this protein measured in a large number of blood plasma samples from patients with various neurodegenerative diseases. The aim was to investigate the utility of this protein as a biomarker for those brain diseases with TDP-43 pathology.

CHAPTER TWO

2 Materials and Methods

2.1 Solutions, buffers and antibodies

All reagents were of analytical grade and obtained from Sigma-Aldrich or Merck unless otherwise stated. Solutions are detailed in Table 2.1, in alphabetical order, and antibodies are listed in Tables 2.2 and 2.3.

Table 2.1 Summary of solutions and buffers

Solution	Details
Dithiothreitol (DTT)	1 M in dH_2O, stored at -20°C
SDS-PAGE sample loading buffer	3x stock solution: 0.125 M Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue. Before use, 100 mM DTT was added.
SDS-PAGE running buffer	10 x stock solution: 30.3 g Tris base, 144 g glycine, 50 ml 20% (w/v) SDS, dH_2O to 1 litre.
	1 x working solution, pH ≈ 8.6:25 mM Tris base, 192 mM glycine, 0.05% (w/v) SDS.
De-staining solution	0.25% (v/v) ethanol, 10% glacial acetic acid in dH_2O.
PBS	1 x PBS: 137 mM NaCl, 7 mM KCl, 3 mM $NaHPO_4$, 1.4 mM KH_2PO_4

Coomassie blue staining solution	0.25% (w/v) Coomassie blue R50, 40% (v/v) ethanol, 10% (v/v) acetic acid in dH_2O.
Blocking solutions for immunoblotting	5% (w/v) Marvel dried milk in PBS-T
Anion exchange buffers	A- 50 mM Tris HCl, pH7.5 B- 50 mM Tris HCl, 2 M NaCl, pH7.5, filtered
Anion exchange buffers with non-ionic detergent	A- 50 mM Tris HCl, 1% NP-40, pH8 B- 50 mM Tris HCl, 2 M NaCl, 1% NP-40, pH8
Running buffer for Superose 6 column (dissociative conditions)	50 mM Tris HCl, 0.15 M NaCl, 4 M Guanidine hydrochloride, pH7, filtered
Running buffer for Superose 6 column	50 mM Tris HCl, 0.15 M NaCl, pH7, filtered
Running buffer for Superdex 200 column	0.15 M Na Cl 50 mM Tris HCl, pH7, filtered
Blocking solution for ELISA	2.5% gelatine in PBS-T, made fresh
0.5 M Sodium phosphate buffer	Add 2.65 ml of 0.5 M $NaH_2PO_4.H_2O$ to 47.35 ml of 0.5 M $Na_2HPO_4.H_2O$
20 mM DMP buffer	Dissolve 5 mg of dimethyl pimelamidate in 1 ml of 0.2 M triethanolamine

Table 2.2 Summary of primary antibodies

Primary Antibody	Epitopes
Anti-α,β,γ-synuclein (FL-140) rabbit polyclonal (200 μg/ml), Santa Cruz Biotechnology, CA.	Amino acids 1-140 (full length of human α-syn)
Anti-α-synuclein (211) mouse monoclonal (200 μg/ml), Santa Cruz Biotechnology, CA.	Amino acids 121-125 of human α-syn
Anti-human parkin (N-18) goat polyclonal (200 μg/ml), Santa Cruz, CA.	Amino terminus of human parkin
Anti-human parkin (PARK 11-S) rabbit polyclonal (100 μg/100 μl), A Diagnostic International, CA.	Carboxy terminus of human parkin
Anti TDP-43 rabbit polyclonal antibody (10782-2-AP) (30μg/150μl), Proteintech Europe Ltd., UK	Full length recombinant human TDP-43
Anti-TDP-43 mouse monoclonal antibody (H00023435-M01, clone 2E2-D3), Abnova Corporation, Taiwan	Full length recombinant human TDP-43 with GST tag

Table 2.3 Summary of secondary antibodies

Rabbit IgG (purified immunoglobulin), Sigma (Cat. No. A-0418)

Mouse IgG (purified immunoglobulin), Sigma (Cat. No. A-2478)

Peroxidase- conjugated goat anti-rabbit (HRP), DAKO

Rabbit IgG (purified immunoglobulin), Sigma (Cat. No. A-0418)

Peroxidase-conjugated rabbit anti-goat (HRP), (DAKO)

Peroxidase-conjugated goat anti-mouse (HRP), DAKO

2.2 Human plasma samples for α-synuclein/parkin study

The controlled experiment compares the results obtained from the experimental blood plasma samples from individuals with a neurodegenerative disease against a control sample group, which is practically identical to the experimental sample except for the one aspect whose effect is being tested. Control blood samples were obtained from the Haematology Department at Blackpool Victoria Hospital with ethical approval. Around 3 ml of blood was collected in tubes containing sodium citrate, and the plasma was separated by centrifuging the blood at 3,000 rpm at 4°C for 20 min. The plasma was transferred to plastic tubes containing protease inhibitors including AEBSF, aprotinin, E-64, EDTA and leupeptin (Calbiochem-Novabiochem Corporation, San Diego, CA), and stored at -80°C. Appropriate care was taken as to not contaminate the plasma samples with cells or components of the pellet obtained from the centrifugation. The samples were thawed at room temperature directly before analysis. Repeated freeze/thaw cycles were avoided. Blood samples were obtained from patients that had been clinically diagnosed with a neurodegenerative disease by a Consultant Neurologist within the out-patients clinic at the Cerebral Function Unit, Greater Manchester Neurosciences Centre at Hope Hospital, Salford, UK. Patients had undergone historical interview in the presence of a family caregiver and comprehensive cognitive assessment to obtain information regarding the nature and progression of functional and cognitive decline. Patients fulfilled the National Institute of Neurological and Communicative Disorders and Stroke – Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria for probable AD (McKhann et al. 1984), and International criteria for FTD (Neary et al. 1998). Again, all blood samples were collected with the approval of the local ethics research committee.

2.3 *Human plasma samples for TDP-43 study*

For this study patients were recruited as above, although control subjects comprised of either spouses of patients attending the CFU clinic, Greater Manchester Neurosciences Centre (n=13), or a sample of healthy volunteers (n=72) drawn randomly from a cohort of 767 mentally normal people aged over 50 years, resident within the same Greater Manchester region from which the patients suffering from neurodegenerative diseases were recruited (Payton et al. 2003). These latter control subjects belonged to a longitudinal ageing study group who had been screened psychologically for the absence of any cognitive impairment consistent with a dementia. All blood samples had been collected with the approval of the local ethics research committee.

2.4 *Human brain lysate*

Post-mortem brain samples were supplied by Professor David Mann, Greater Manchester Neurosciences Centre, Hope Hospital, Salford, U.K. Ethical approval was obtained for all samples. A frozen post-mortem sample (3 g) of frontal cortex was homogenised in 3 ml CellLytic buffer, (Sigma-Aldrich Company Ltd., Dorset, U.K., Product number: C3228) consisting of a mild detergent, bicine, 150 mM NaCl and containing protease inhibitors (as before), and centrifuged at 3,000 x g for 30 min. The supernatant was collected and stored at -80°C prior to analysis.

2.5 *Methods*

2.5.1 Recombinant α-synuclein

Recombinant α-synuclein was prepared from *E. coli* using a previously described protocol (King 2005). α-synuclein pJEK2 was used to transform FB850, a *rec* A^- derivative of BL21 (DES) pLysS. FB850 carrying this plasmid was grown in an 800 ml batch culture. At OD_{600} of 0.4-0.5 (T=0), protein expression was induced through

addition of isopropyl-β-D-thiogalactopyranoside (IPTG). A protein with a molecular weight of approximately 17 kDa started to accumulate in the cells 30 min after induction and reached maximum levels after 150 min. Immunoblot analysis identified this protein as α-synuclein using mouse monoclonal antibody (211). After a 3 h induction, the suspension was centrifuged, and the cells resuspended in buffer. The cells were lysed by sonication, then cell debris and insoluble material was removed by centrifugation at 4°C for 1 h at 30,000 rpm. α-synuclein was extracted from the supernatant by ammonium sulphate precipitation, then purified using a series of chromatography columns; phenyl sepharose, heparin, hydroxyapatite and mono Q. After purification, 5μg of protein ran as a single band when observed on a Coomassie blue-stained SDS gel, corresponding to monomeric α-synuclein.

2.5.2 Bradford Assay

Protein standards were prepared from a stock solution of bovine serum albumin (BSA) (Sigma-Aldrich, UK) of concentration 5 mg/ml. The stock solution was then used to prepare a range of concentrations by serial dilution from 5 mg/ml down to 0.05 mg/ml in Phosphate buffered saline (PBS) (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4). 0.5 ml of distilled water was added to 0.5 ml of Bradford reagent (8.5% phosphoric acid, 5% ethanol, 0.001% Coomassie Blue G-250) in an Eppendorf tube and 5 μl of the known protein sample was added and mixed well. The liquid was then transferred to a plastic cuvette and the absorbance was measured at 595 nm. A control was prepared by adding 5 μl of distilled water to the 1 ml solution of water and Bradford reagent. Each concentration was prepared in triplicate, an average calculated, and the results used to generate a standard curve (figures 2.1 and 2.2).

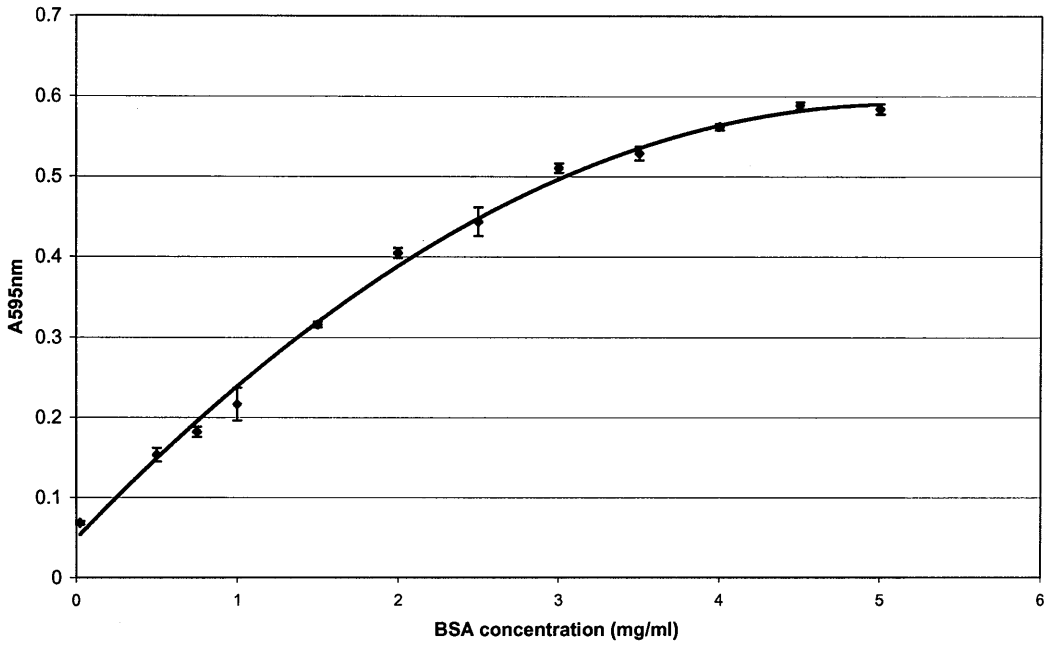


Figure 2.1 Bradford assay to estimate protein content

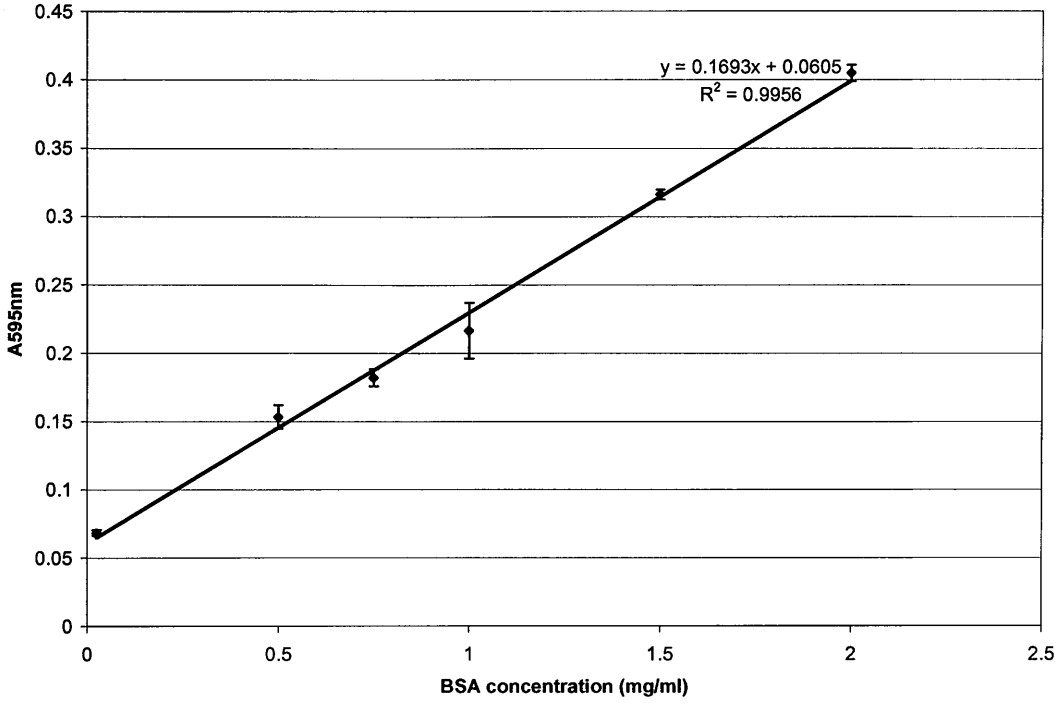


Figure 2.2 Bradford assay effective range between 0.1 – 2 mg/ml BSA

2.5.3 Determining total protein concentration of plasma samples for α-synuclein/parkin study

Each individual plasma sample was diluted by adding 2 µl plasma sample to 400 µl of distilled water. 5 µl of the previously diluted sample was then added to 0.5 ml Bradford reagent and 0.5 ml of distilled water. The solution was mixed, transferred to a cuvette, and absorbance measured at 595 nm. Each plasma sample was measured in triplicate and the previously generated standard curve equation (x = (y - 0.0605)/0.1693) was used to estimate the total protein concentration of each sample. The plasma samples were then standardised to an equal protein concentration (60 mg/ml), making the volume up to 600 µl using filtered PBS (table 2.4).

Table 2.4 Standardisation of plasma protein concentrations

Plasma sample	Average A595nm	1:200 Protein concentration (mg/ml)	Actual protein concentration (mg/ml)	Volume plasma required (µl)	Volume PBS required (µl)
AD(225)	0.119	0.35	69.10	521	79
AD(228)	0.115	0.32	64.38	559	41
AD(230)	0.118	0.34	67.93	530	70
AD(232)	0.123	0.36	73.83	488	112
AD(233)	0.115	0.32	64.38	559	41
AD(234)	0.122	0.36	72.65	496	104
AD(99)	0.123	0.36	73.83	488	112
CON(236)	0.116	0.33	65.56	549	51
CON(237)	0.118	0.34	67.93	530	70
CON(238)	0.121	0.36	71.47	484	116
CON(239)	0.125	0.38	76.19	473	127
CON(240)	0.119	0.34	67.93	530	30
CON(241)	0.124	0.38	75.01	480	120
CON(242)	0.120	0.35	70.29	512	88
CON(243)	0.116	0.33	65.56	549	51
CON(244)	0.113	0.31	62.02	580	20
DLB(226)	0.112	0.31	60.84	600	0
DLB(227)	0.125	0.38	76.19	472	128
DLB(81)	0.113	0.31	62.02	581	19
FTD(100)	0.120	0.35	70.29	512	88
FTD(224)	0.125	0.38	76.19	472	128
FTD(72)	0.111	0.30	59.70	600	0
PD(119)	0.115	0.32	64.38	559	41
PD(13)	0.119	0.34	67.93	530	70
PD(14)	0.128	0.40	79.74	452	148
PD(163)	0.125	0.38	76.19	472	128
PD(166)	0.118	0.34	67.93	530	70
PD(26)	0.112	0.31	60.84	600	0
PD(64)	0.129	0.40	79.74	452	148
SD(142)	0.114	0.32	63.20	570	30
SD(231)	0.117	0.33	66.74	540	60

2.5.4 Immunoassay for measuring total α-synuclein

This assay was used to measure the total α-synuclein in plasma samples, as previously described (Salem 2004). Salem performed experiments initially to optimise the conditions required, and the specificity of the antibodies were checked. These conditions were verified to ensure that the quoted descriptions were correct.

An ELISA plate was coated with 100 µl/well of anti-α-synuclein (211) i.e. 0.1 µg per well, diluted in 200 mM $NaHCO_3$, pH 9.6, and incubated at 4°C overnight (i.e. 0.1 µg of antibody per well). The wells were then washed 4 times with PBS 0.05% Tween-20, and incubated for 2 h at 37°C with 200 µl/well of freshly prepared blocking buffer. The plate was washed again 4 times with PBS-T. 100 µl of the plasma samples were added to each well, (each sample was diluted 1:1 with PBS), and the assays were performed in triplicate to calculate a standard error. Following this, the plate was incubated at 37°C for 2 h. After a repeat washing with PBS-T, 100 µl/well of the detection antibody, anti-α/β/γ-synuclein (FL-140) , dilution 1:1000 in blocking buffer was added (i.e. 0.02 µg per well of antibody), and the plate incubated at 37°C for 2 h.. After another wash with PBS-T, the plate was incubated with 100 µl/well of secondary antibody (goat anti-rabbit HRP, dilution 1:10 000 in blocking buffer at 37°C for 2 h. The plate was then washed again with PBS-T before adding 100 µl/well Sure Blue TMB Microwell Peroxidase Substrate (KPL, USA) and leaving the colour to develop for 30 min at room temperature. Finally 100 µl/well of stop solution (0.3 M H_2SO_4) was added and absorbance at 450 nm determined.

2.5.5 Preparation of biotinylated antibody

200g Sulfo-NHS-LC-Biotin (Pierce) was reacted with the antibody to be biotinylated (1 ml at 200 µg/ml) in PBS and then placed on ice for 2 h. The mixture was desalted on Bio-Spin-6 columns to remove excess uncoupled biotin. The biotinylated antibodies were stored at 4°C until use.

2.5.6 Immunoassay for measuring oligomeric α-synuclein

This assay was used to measure the total oligomeric α-synuclein, as previously described (Salem 2004). This author performed experiments initially to optimise the conditions required, and the specificity of the antibodies were checked. These conditions were verified to ensure that the quoted descriptions were correct.

An ELISA plate was coated by overnight incubation with 1 μg/ml of non-biotinylated anti-α-synuclein (211), diluted in 200 mM $NaHCO_3$, pH 9.6, containing 0.02% (w/v) sodium azide, at 4°C overnight. The wells were then washed 4 times with PBS 0.05% Tween-20, and incubated for 2 h at 37°C with 200 μl/well of freshly prepared blocking buffer. The plate was washed again 4 times with PBS-T, and 100 μl of the sample was added to each well (the assays were performed in triplicate to calculate a standard error). Following this, the plate was incubated at 37°C for 2 h. After a repeat washing with PBS-T, 100 μl/well of biotinylated anti-α-synuclein (211) diluted to 1 μg/ml in blocking buffer was added and the plate incubated at 37°C for 2 h. The plate was washed 4 times with PBS-T and incubated with 100 μl/well of ExtrAvidin-alkaline phosphatase diluted to 3:5000 in blocking buffer and incubated for 1 h at 37°C. After another 4 washes with PBS-T, the enzyme substrate para-nitrophenylphosphate (pNPP) was added (100 μl/well) and the colour left to develop for 30 min at room temperature. Finally 100 μl/well of stop solution (3 M NaOH) was added and absorbance at 405 nm determined.

2.5.7 Immunoassay for measuring total parkin

The ELISA plate was coated with 100 μl/well of the capture antibody, anti-parkin (N-18) diluted in 200mM $NaHCO_3$, pH 9.6, at a ratio of 1:1000 (0.02 μg per well of antibody). Detection was via 100 μl/well of the anti-parkin antibody (PARK 11-S), dilution 1:10 000 in blocking buffer (i.e.0.01μg of antibody per well). Following this, the plate was incubated with 100 μl/well of secondary antibody, goat anti-rabbit HRP, dilution 1:10 000 in blocking buffer. All other parameters remained the same as the previously described α-synuclein assay (section 2.1.4).

2.5.8 Immunoassay protocol for measuring the α-synuclein/parkin complex

Capturing α-synuclein and detecting parkin

This immunoassay protocol was based on the method described in section 2.1.4. An ELISA plate was coated with 100 µl/well of anti-α-synuclein (211) as before and detection was via 100 µl/well of the anti-parkin antibody (PARK 11-S), dilution 1:10,000 in blocking buffer (i.e. 0.01µg of antibody per well). Following this, the plate was incubated with 100 µl/well of secondary antibody, goat anti-rabbit HRP, dilution 1:10,000 in blocking buffer. All other parameters remained the same.

Capturing parkin and detecting α-synuclein

This immunoassay protocol was based on the method described in section 2.1.4. An ELISA plate was coated with 100 µl/well of the capture antibody, anti-parkin (N-18) and detection was via 100 µl/well of the anti-α-synuclein antibody (211). Following this, the plate was incubated with 100 µl/well of secondary antibody, goat anti-rabbit HRP, dilution 1:10,000 in blocking buffer. All other parameters remained the same.

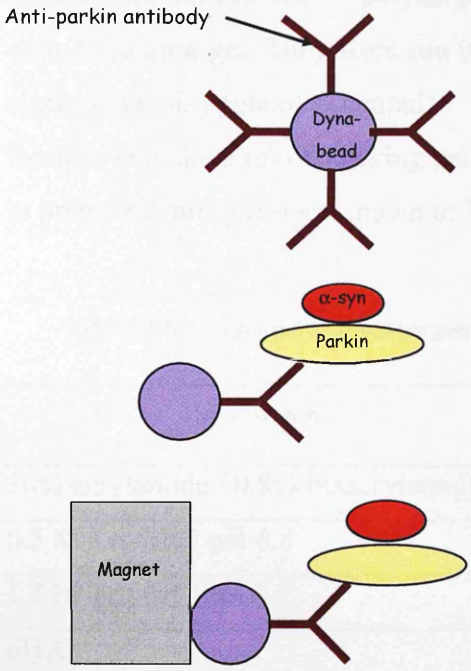
2.5.9 Crosslinking of antibody to Dynabeads

Dynabeads covalently coupled with recombinant protein A, were vortexed for 1-2 min before 100 µl was transferred to a plastic Eppendorf tube. The tube was then placed in the Dynal MPC (Magnetic Particle Concentrator) for 1 min before the liquid was removed. The Dynabeads were then washed 3 times with 0.1 M sodium phosphate buffer pH 8.2, using the MPC holder each time to separate the beads from the solution. 100 µl of 0.5 M sodium phosphate buffer pH 8.2, was mixed with 400 µl of antibody. The antibody solution was then added to the tube containing the Dynabeads and incubated for 30 min at room temperature, mixing by rotation. After this period of time, the Dynabeads were washed 3 times with 0.5 ml sodium phosphate buffer pH

8.2, and then again washed twice with 1 ml of 0.2 M triethanolamine pH 8.2. 1 ml of 20 mM DMP buffer was added to the tube containing the Dynabeads and incubated for 30 min, mixing by rotation at room temperature. The reaction was stopped by placing the tube in the MPC holder, removing the DMP buffer, and then adding 1 ml of 50 mM Tris buffer pH 7.5. The tube was then incubated for 30 min by rotation at room temperature. Finally the beads were washed 3 times in PBS then stored at 4°C in PBS containing 0.02% NaN_3.

2.5.10 Immunoprecipitation

Dynabeads are previously crosslinked to antibody, as previously described. 400 µl of plasma was added to the beads and incubated overnight at 4°C overnight. The beads were then washed 3 times with 0.1 M phosphate buffer pH 8.2. Captured proteins were eluted from the beads by boiling for 10 min in NUPAGE LDS sample (see figure 2.3). As a control, an equal amount of beads were coated with non-specific IgG and incubated with the same samples.



The magnetic Dynabeads are crosslinked to anti-parkin antibody.

The beads are incubated with the plasma sample. The complex will bind to the antibody

The Dynabeads and α-syn/parkin complex are separated from the sample (using a magnet).

The proteins are released from the Dynabeads by boiling in SDS sample buffer. Identification of the proteins present is performed by SDS PAGE, followed by immunoblotting (IB).

Figure 2.3 Immunoprecipitation of human plasma with Dynabeads crosslinked with anti-parkin antibody

2.5.11 Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS – PAGE)

One dimensional gel electophoresis under denaturing conditions separates proteins on the basis of molecular size as they migrate through a polyacrylamide gel matrix towards the anode. SDS – polyacrylamide gels were cast as a resolving gel topped with a stacking gel. They were run under a constant voltage in a Mini-PROTEAN®3 electrophoresis apparatus (Biorad).

Recipes to make 3 ml of stacking gel and 10 ml of resolving gel (the volumes required to prepare 2 mini gels) are shown in Table 2.5.

Table 2.5 Recipe for polyacrylamide gels

Stock solution	Stacking gel (ml)	12.5 % Resolving gel (ml)
30% acrylamide / 0.8% bisacrylamide	0.51	4.17
0.5 M Tris-HCl pH 6.8	0.75	
1.5 M Tris-HCl pH 8.8		2.5
dH_2O	1.68	3.13
10 % (w/v) SDS	0.03	0.1
10 % (w/v) ammonium persulphate	0.03	0.1
TEMED	0.003	0.01

Samples to be analysed by SDS-PAGE were solubilised by diluting with SDS sample buffer and then heated to 98°C for 3 min. The gels then underwent electrophoresis in SDS-PAGE running buffer at a constant 150 V. Protein samples to be analysed by the NUPAGE pre-cast gel system (Invitrogen) were solubilised by diluting with 4x LDS sample loading buffer (Invitrogen) and then heated to 98°C for 3 min. NuPAGE 4-12% gradient gels (Invitrigen) were electrophoresed in 1xMES, or 1xMOPS buffer (Invitrogen) at 200V. Both SDS-PAGE and NuPAGE gels were run until the tracking dye reached the bottom of the gel. Proteins in the gel were visualised either by Coomassie blue staining or immunoblotting.

2.5.12 Coomassie Blue Staining

Polyacrylamide gels were covered with Coomassie staining solution and gently agitated for at least 4 h. The detection of protein bands in the gels is dependant on the non-specific binding of the dye to the protein. The staining solution was then replaced by a destaining solution, which was continued until the gel itself achieves a clear background, with the proteins visible as blue bands. Gels were stored in 7% (v/v) acetic acid.

2.5.13 Immunoblotting

Immunoblotting (Western blotting) was used to identify specific proteins recognised by either monoclonal or polyclonal antibodies. Immunoblots were carried out using the blotting module. Nitrocellulose membrane, (0.45μm, Invitrogen) was prepared by briefly soaking it in the transfer buffer. The filter paper is soaked for 10 min in the same buffer. The transfer stacks were assembled and transfer of proteins achieved by applying a constant voltage of 30 V for 1 h.

Membranes were blocked with 5% Marvel dried skimmed milk, dissolved in PBS-T for 1 h on a rocking platform. The blocking buffer was then poured off and replaced with the primary antibody, diluted with PBS-T. The membranes were probed with the following antibodies:- anti-α-synuclein (211) mouse monoclonal antibody, 1 : 1000; anti-α-synuclein (FL-140) rabbit polyclonal antibody, 1 : 1000; anti-parkin (N-18) goat polyclonal antibody, 1:1000; anti-TDP-43 rabbit polyclonal antibody, 1:1000; or, anti-TDP-43 mouse monoclonal antibody, 1:1000. The membrane was incubated for 1 h at room temperature, or left at 4°C overnight, on a rocking platform. The primary antibody was then removed and the membrane rinsed for 2 X 5 min then 1 X 15 min in PBS-T. The secondary antibody, horseradish peroxidase (HRP) conjugated to either goat anti-rabbit, rabbit anti-goat or goat anti-mouse as appropriate, was diluted in PBS-T to the correct ratio and applied to the membrane for 1 h at room temperature, on a rocking platform. Then the secondary antibody solution was poured off and the membrane washed as before in PBS-T. Finally, Super Signal West-Pico

chemoiluminescent substrate (Piearce) was applied for 5 min, and the membrane then exposed to film in the darkroom.

2.5.14 Dialysis of samples

Samples were dialysed in Snakeskin pleated dialysis tubing MWCO 3500 (Piearce) against 3 X 2 l of the appropriate buffer.

2.5.15 Size exclusion chromatography

Superose 6 was packed into a 44cm long column with an internal diameter of 1 cm. Samples (0.5 ml) were loaded onto the column connected to a fast protein liquid chromatography (FPLC) system (Atka Purifier, GE Healthcare) and eluted with running buffer (0.15 M NaCl, 50mM Tris HCl) at a flow rate of 0.5 ml/min. The column was equilibrated with 20^{th} of a column of buffer and eluted with 1.5 columns of buffer. Absorbance of the eluate was monitored on line at 280 nm. Fractions of 1 ml were collected.

2.5.16 Anion, and cation, exchange chromatography

The Q sepharose 1 ml Fast Flow HiTrap XL strong anion column (GE Healthcare) (or SP sepharose strong cation column) was loaded with 25 µl of human plasma (approximately 2 mg total protein). The column was connected to an FPLC system (Atka purifier, GE Healthcare) and equilibrated with the binding buffer (50mM Tris HCl, pH7.4) at 0.5 ml/min for five mins before the sample was injected. Bound proteins were eluted by a salt gradient of 0-1 M NaCl over 20 min. 0.5 ml fractions were collected and the fractions containing protein were identified from the UV chromatogram.

3 Optimisation of an ELISA method for detection of parkin and for any α-synuclein / parkin complex

3.1 Introduction

Parkinson's disease (PD) is characterised pathologically by selective loss of dopaminergic neurons from the substantia nigra (SN), and also with the presence of aggregated intraneuronal protein deposits called Lewy bodies (LB's), and neuronal processes called Lewy neurites (LN's) (Dauer and Przedborski 2003). LB's and LN's contain several proteins, including α-synuclein (Spillantini et al. 1998) and parkin (Schlossmacher et al. 2002). α-Synuclein has also been linked to other neurodegenerative diseases such as multiple system atrophy (MSA) and DLB, disorders collectively known as α-synucleinopathies (Goedert 2001). The molecular pathogenesis of PD remains elusive, although several genes are thought to play a part in some cases. Along with mutations in the α-synuclein gene, mutations in the parkin and UCHL-1 genes have also been implicated in familial forms of the disease (Kitada et al. 1998). Autosomal dominant PD arises from mutations in the α-synuclein gene (Polymeropoulos et al. 1997), and an α-synuclein gene triplication has been discovered in two families (Singleton et al. 2003) that results in both a doubling of α-synuclein concentration in the blood and clinical signs of Lewy body disease (Miller et al. 2004). Mutations in parkin cause an autosomal recessive juvenile form of PD (ARJP) (Kitada et al. 1998) but LB's are not usually present. ARJP patients suffer midbrain dopaminergic neurone loss and develop typical Parkinsonian symptoms.

As previously discussed, intracellular protein degradation within eukaryotic cells can be signalled by the covalent linkage of ubiquitin to proteins (Glickman and Ciechanover 2002). An ubiquitin-activating enzyme (E1) initially activates the ubiquitin molecule which is transferred to a ubiquitin-conjugating protein (E2) and ubiquitin-protein isopeptide ligase (E3) enzymes (Tanaka et al. 2001). Substrates with

four or more conjugated ubiquitin molecules are targeted for degradation by the 26S proteasome. Studies have revealed that parkin has E3 ubiquitin-protein ligase activity (Shimura et al. 2001). It is suggested that loss of parkin function may lead to the accumulation of abnormal parkin substrates, which could promote neurotoxicity and neuronal cell death. Several protein substrates have been identified, through protein-protein interaction studies, for their ubiquitin-ligase activity. These include CDCrel-1, a protein involved in cytokinesis that may influence synaptic vesicle function (Kubo et al. 2001), synphilin-1, an α-synuclein interacting protein (Chung et al. 2001) and a 22-kD O-glycosylated isoform of α-synuclein, termed αSp22 (Shimura et al. 2001). There are many uncertainties regarding the biochemical relationship between parkin and α-synuclein and their relevance to PD. It may be of consequence that both proteins interact with vesicles (Lee et al. 2001) and that α-synuclein can bind and negatively regulate the activity of human dopamine transporter (hDAT) (Lee et al. 2001).

As mentioned previously, it has been proposed that α-synuclein and parkin interact functionally, with parkin ubiquitinating α-synuclein normally and with this process being altered in ARJP (Shimura et al. 2001). A protein complex was identified in human brain that included parkin as an E3 ubiquitin-ligase, UbcH7 as its E2 ubiquitin conjugating enzyme, and a novel 22 kDa O-glycosylated form of α-synuclein (αSp22) as its substrate. In contrast to wild type parkin, the mutant parkin that is associated with ARJP failed to bind αSp22. αSp22 was modified by normal but not mutant parkin, in an *in vitro* ubiquitination assay, into a polyubiquitinated, higher molecular weight species. The authors surmised that this would lead to the accumulation of non-ubiquitinated αSp22 in parkin-deficient ARJP disease brains.

As α-synuclein is present in extracellular biological fluids, including human plasma (El-Agnaf et al. 2003), this thesis investigates whether the α-synuclein/parkin interaction could also be detected in human blood plasma using antibody-sandwich ELISA assays. This work was undertaken with the view that any interaction between the two proteins would be of great interest in understanding the molecular pathology of PD. If it is only the glycosylated form of α-synuclein that interacts with parkin, then what could be the significance of this? Also, it would be useful to investigate the level of the α-synuclein/parkin complex as a potential biomarker for PD and ARJP. In addition, other diseases were studied that sometimes mimic PD, such as DLB and

FTD, to explore whether there might be differences between the levels of any α-synuclein/parkin interaction in the synucleinopathies and the non-synucleinopathies.

An ELISA that detects soluble forms of α-synuclein has been developed in this laboratory (Salem 2004). Initially, Salem (2004) identified which antibodies were useful in detecting the protein in biological samples, and then the concentrations of the antibodies were optimised to give the most sensitive signal with the lowest background. Different concentrations of both the capture antibody and the detection antibody were applied to known concentrations of the recombinant protein, ranging from 0.0001 μg/ml to 10 μg/ml. The best combination of antibodies was found to be anti-α-synuclein antibody (211) to capture the protein, and anti-α-synuclein (FL-140) to detect it. The results established the most effective concentration of the anti-α-synuclein capture antibody (211) was 1:200, and the anti-α-synuclein detection antibody (FL-140) concentration at 1:1000. Investigations confirmed that these concentrations were also found to give the best "signal to noise" ratio in the current study.

3.2 ELISA α-synuclein detection levels in PBS, and BSA

To determine whether the other proteins in biological samples, for example blood albumins, effect the sensitivity of the ELISA signal/absorbance, known concentrations of the recombinant α-synuclein protein ranging from 100 μg/ml down to 0.001 ng/ml were dissolved in PBS and assayed for α-synuclein as previously described (section 2.1.4). Each concentration was prepared in triplicate to calculate a standard error. Subsequently, the assay was repeated using α-synuclein dissolved in PBS containing 1 mg/ml bovine serum albumin (BSA). The two sets of data can be compared to see if the high concentrations of other proteins affect the sensitivity of the assay.

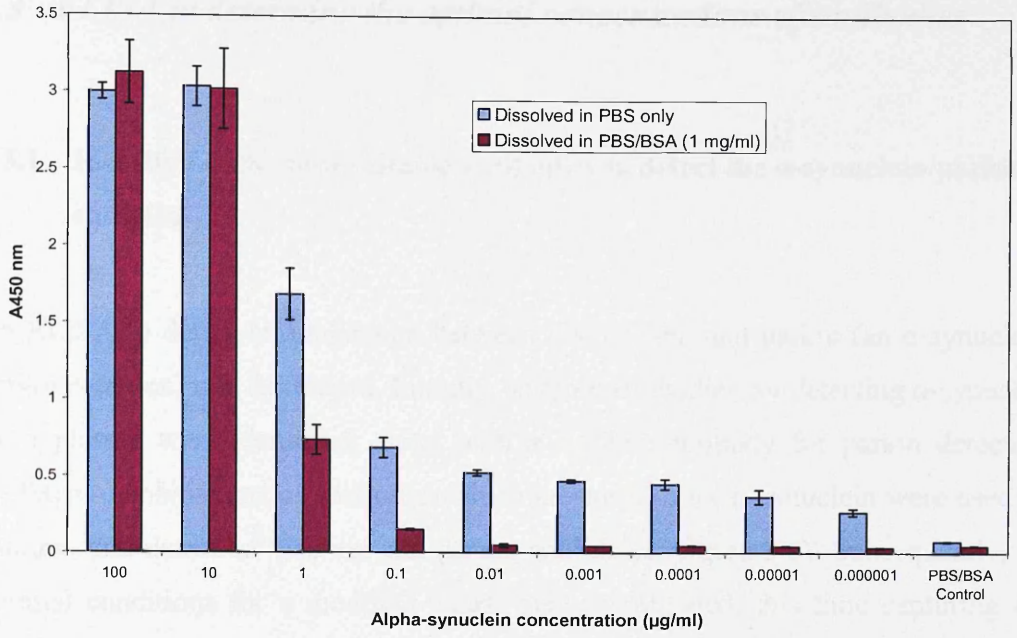


Figure 3.1 ELISA to detect recombinant α-synuclein when dissolved in PBS only

The ELISA can detect protein levels as low as 0.001 ng/ml when the recombinant α-synuclein is dissolved in PBS alone (figure 3.1). However, the sensitivity of the assay is greatly reduced when the recombinant protein is mixed with BSA. The detection level is now reduced to 0.1 µg/ml, approximately 10^5 less sensitive. It is possible that the detection antibody experiences a "masking" effect due to the presence of the BSA in the solution. With this in mind, it was decided that all the plasma samples should be assayed for protein content and the concentration adjusted to a standard, to ensure an equal inhibitory effect for all samples analysed (see Section 2.1.3).

3.3 ELISA to determine the optimal concentrations of antibodies

3.3.1 Identifying the most suitable antibodies to detect the α-synuclein/parkin complex

An ELISA to detect an interaction between α-synuclein and parkin (an α-synuclein-parkin complex) was developed. Initially, suitable antibodies for detecting α-synuclein from plasma were identified, along with a suitable antibody for parkin detection. Different combinations of antibodies which are specific for α-synuclein were used for capture, and detection was via anti-parkin antibodies (figure 3.2). Subsequently, the optimal conditions for a modified assay were investigated, this time capturing any parkin present with an anti-parkin antibody, and detecting α-synuclein with an anti-α-synuclein antibody (figure 3.3). The antibody concentrations used were the manufacturers' recommended starting dilution for ELISA analysis. Anti-α-synuclein (211) was diluted to 1:100, anti-α-synuclein (FL-140) diluted to 1:200, and anti-parkin (N-18) was diluted to 1:100, with anti-parkin (PARK-11S) to 1:10 000. Two blood plasma samples (1:1, plasma : PBS) were used in this investigation. One gave a high ELISA absorbance for the previous α-synuclein assay, and the other a low reading. As there is a reported link between α-synuclein and parkin, it was hypothesised that if a plasma sample with a relatively high α-synuclein level may also have a higher level of the associating parkin protein. The combinations of both capture and detection antibodies are shown in figures 3.2 and 3.3. The PBS, which was used as a negative control, gave background absorbance that was deducted from the absorbance values from the plasma samples, before they were plotted. Recombinant α-synuclein, 4μg/ml, was ultilised as a further control. This protein gave absorbance signals comparable to the background absorbance from the PBS controls. This suggests that the anti-parkin detection antibody does not interact with the α-synuclein.

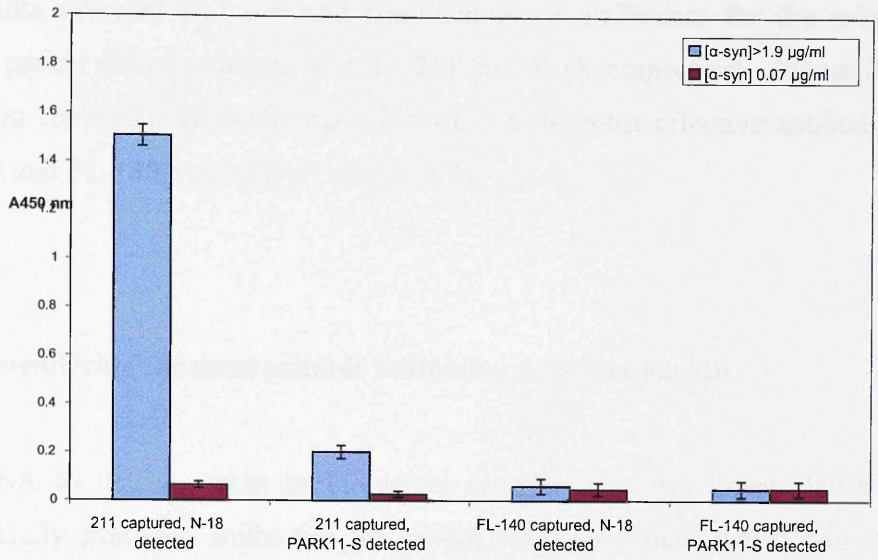


Figure 3.2 Optimisation of antibodies for ELISA, α-synuclein capture, parkin detection, for two different plasma samples (high and low levels of α-synuclein)

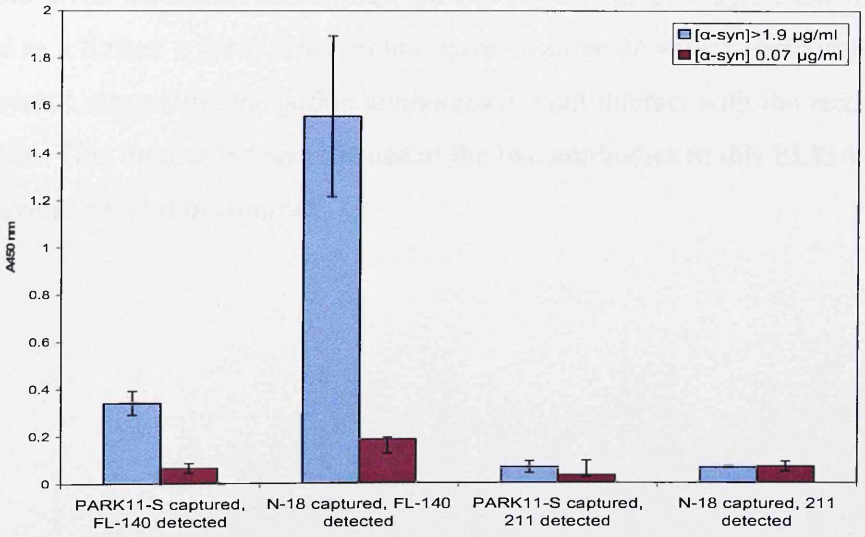


Figure 3.3 Optimisation of antibodies for ELISA, α-synuclein capture, parkin detection, for two different plasma samples (high and low levels of α-synuclein)

The results revealed that the best combination of antibodies for the α-synuclein capture, parkin detection assay, was the 211 and N-18, respectively (figure 3.2). For the parkin capture, α-synuclein detection ELISA the most effective antibodies were the N-18 and FL-140, respectively (figure 3.3).

3.3.2 Identifying the most suitable antibodies to detect parkin

An ELISA to detect parkin in biological samples was developed. Initially, two commercially available antibodies for parkin were identified, which had different epitopes. The antibody concentrations used were the manufacturer's recommended starting dilution for ELISA analysis. The different combinations of these two different parkin antibodies were used for capture or detection (figure 3.4). The same blood plasma samples used in the previous ELISA (in section 3.3.1) were assayed with these anti-parkin antibodies, as explained in the previous section. Again, PBS values were deducted from measured absorbance values. Recombinant α-synuclein 4μg/ml was utilised as a further control. This protein gave absorbance values comparable with the PBS control, suggesting the parkin antibodies did not interact with the recombinant α-synuclein. This implies the specific use of the two antibodies in this ELISA will detect any α-synuclein/parkin complex.

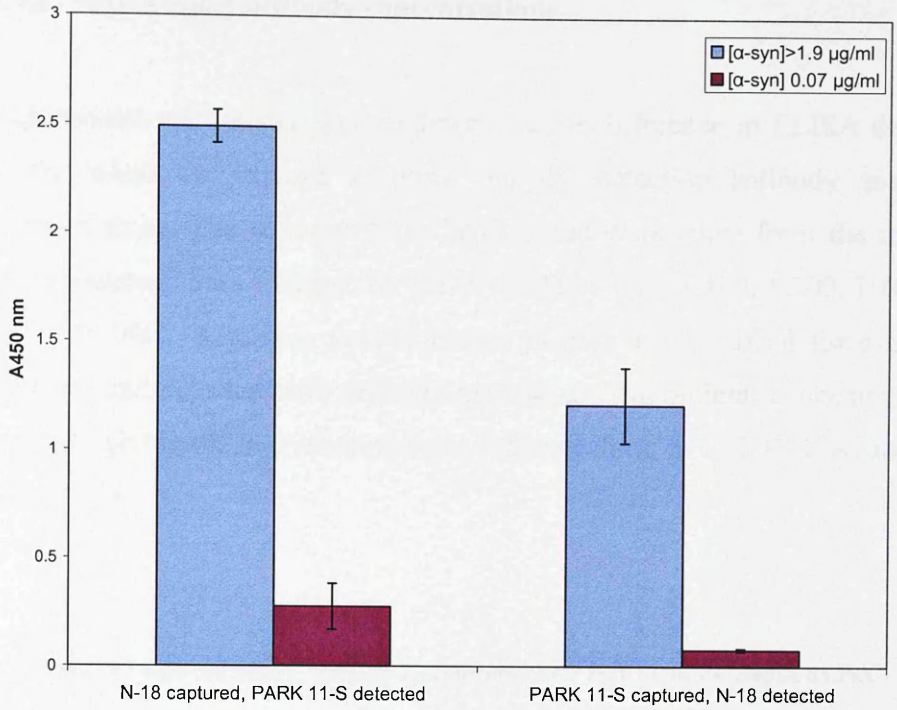


Figure 3.4 Optimisation of antibodies for ELISA to detect parkin, for two different plasma samples (high and low levels of α-synuclein)

The results revealed that the best combination of antibodies for the parkin capture, parkin detection assay, was the N-18 for capture followed by detection with the PARK 11-S (figure 3.4). The negative control plasma sample and the "blank" PBS gave a measured absorbance value of less than 0.1.

This ELISA would benefit from a standard curve constructed through the assay of known concentrations of the parkin protein. Parkin was not commercially available and so attempts were made to recombinantly express the protein using E. coli. Unfortunately, after several months and many attempts, these efforts were unsuccessful. Therefore, it was not possible to validate this assay using a positive control.

3.3.3 Optimising antibody concentrations

Experiments were undertaken to determine the difference in ELISA detection/signal levels, when the capture antibody and the detection antibody are at different concentrations. The concentrations investigated were taken from the manufacturers' recommended dilution ranges for ELISA's. They were 1:100, 1:200, 1:1000, 1:10 000 and 1:20 000. A plasma sample known to give a high signal for α-synuclein was captured and detected with different antibodies. The optimal concentrations for each antibody giving the best measured absorbance values, in each ELISA, are summarised in table 3.1.

Table 3.1 Optimal ELISA antibody concentrations for each of the developed ELISA's

ELISA	Antibody	Concentration (Dilution)
Detection of the α-synuclein/parkin complex	Anti-α-synuclein (211)	0.1 µg/100 µl (1:200)
	Anti-parkin (N-18)	0.1 µg/100 µl (1:1000)
Detection of the parkin/α-synuclein complex	Anti-parkin (N-18)	0.1 µg/10 µ l (1:1000)
	Anti-α-synuclein (211)	0.02 µg/100 µl (1:1000)
Detection of parkin	Anti-parkin (N-18)	0.02 µg/100 µl (1:1000)
	Anti-parkin (PARK 11-S)	0.01 µg/100 µl (1:20000)

3.3.4 Standard curve for measuring total α-synuclein

The ELISA method to measure total α-synuclein, i.e. all forms of α-synuclein including monomeric, oligomeric and complexed forms, (section 2.1.4) was used to measure absorbance values at different concentrations of recombinant α-synuclein dissolved in PBS, ranging between 0.001 µg/ml and 2 µg/ml. The recombinant α-synuclein was captured with anti-α-synuclein (211) and detected with anti-α-synuclein (FL-140) antibody. These data were used to create a standard curve (figure 3.5 and 3.6).

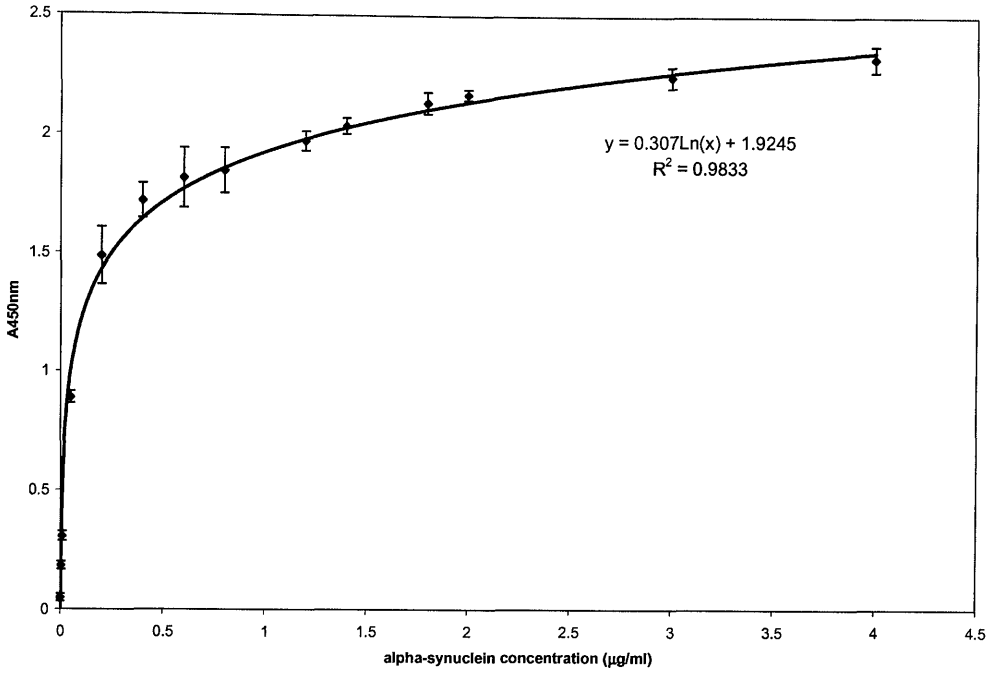


Figure 3.5 Standard curve for ELISA measuring all forms of α-synuclein, between 0-4.0 µg/ml

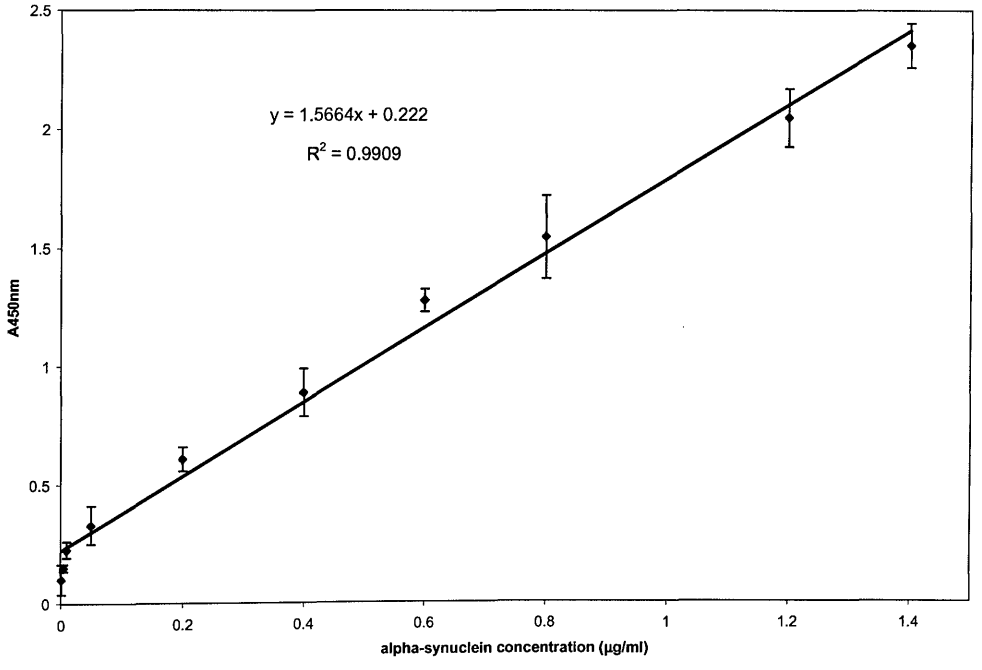


Figure 3.6 Standard curve for ELISA measuring all forms of α-synuclein, between 0-1.4 µg/ml

The ELISA is linear between the α-synuclein concentration ranges of 0.01 – 1.4 μg/ml. At very low concentrations the assay is not accurate, and at values above 1.4 μg/ml the ELISA plateaus off as the assay starts to saturate (see figure 3.5). This standard curve enables an estimation of the α-synuclein concentration in each blood plasma sample to be calculated (figure 3.6).

It should be noted however, that these values may be an under estimation of α-synuclein concentration, as the recombinant α-synuclein is dissolved in PBS alone, i.e. not in the presence of other proteins. As discussed previously (section 3.2), other plasma proteins may have an inhibitory effect on the assay. A more biologically representative standard curve would be gained if the recombinant α-synuclein was assayed dissolved in a plasma sample that had previously been determined as lacking in α-synuclein. This was not carried out as larger volumes of plasma samples were not available.

These preliminary results provide, for the first time, some evidence that a complex of some description may exist between α-synuclein and parkin, and it might be present in human blood plasma. Moreover, the parkin ELISA suggests that parkin is present in blood plasma, and this finding has not been documented before either. However, these initial findings need further validation and to this extent, a more detailed investigation follows.

4 Detection of parkin and α-synuclein/parkin complex in human plasma by ELISA

4.1 Introduction

Because α-synuclein is present in extracellular biological fluids, including human plasma (El-Agnaf et al. 2003), in this study investigations were carried out to explore whether the α-synuclein/parkin interaction could also be detected in human blood plasma, using the previously developed antibody-sandwich ELISA assays. If detection of the complex was possible, attempts could be made as to further characterise the protein interaction, and confirm that the complex does indeed include a glycosylated form of α-synuclein, as previously reported (Shimura et al. 2001). If so, blood plasma could be utilised as a source to purify and characterise the glycosylated α-synuclein species. In addition to this, investigations into the relative levels of this complex, between individuals with neurodegenerative disease and control subjects, may potentially be of use as a biomarker for PD and ARJP. And so with this in mind, other diseases were studied as well, that can clinically mimic PD, such as DLB, AD and FTD, in order to explore any differences between the levels of any putative α-synuclein/parkin interaction in the synucleinopathies and the non-synucleinopathies.

An ELISA that detects soluble forms of parkin and the α-synuclein/parkin complex has been developed. Initially, antibodies that were useful in detecting the proteins in biological samples were identified, and then the concentrations of the antibodies were optimised to give the most sensitive signal with the lowest background. Different concentrations of both the capture antibody and the detection antibody were applied to human plasma samples. The best combination of antibodies was found to be anti-α-synuclein antibody (211) to capture the protein complex, and anti-parkin (N-18) to detect it. These results established that the most effective concentration of the anti-α-synuclein capture antibody (211) was 1:200, and the anti-parkin detection antibody

(N-18) concentration at 1:1000. Investigations confirmed that these concentrations were also found to give the best measured absorbance values in the current study.

4.2 Measuring total α-synuclein in plasma from patients with synucleinopathies and control samples

The total concentration of α-synuclein protein from 31 plasma samples was determined by ELISA. Nineteen of the samples were from patients with a neurodegenerative synucleinopathy (46% male, 54% female, age range 53-85 years, mean age 63.5), and 3 were of patients from a similar age range with frontal temporal dementia (FTD), a non-synucleinopathy (table 2.4). The blood plasma α-synuclein concentration can be estimated by assaying the samples with the α-synuclein ELISA (figure 4.1), and different disease groups can be compared to levels in control plasma samples from patients with no pathological disorder. The α-synuclein standard curve (figure 3.6) was then used to calculate the α-synuclein concentration in individual samples (figure 4.2).

It should be noted that the data for figure 3.6, the α-synuclein standard curve to estimate protein concentration, was acquired at the same time as the data for figure 4.1, the detection of α-synuclein in plasma samples. The experiments were carried out at the same time in order to ensure consistency and comparability.

Also of note is that the plasma absorbance readings above 2.0 are above the calibrated range and were saturated. In order to achieve a more reliable estimation of α-synuclein concentration, those plasma samples in question would have had to be diluted down with PBS and assayed again. This was not carried out as the availability of plasma samples was limited.

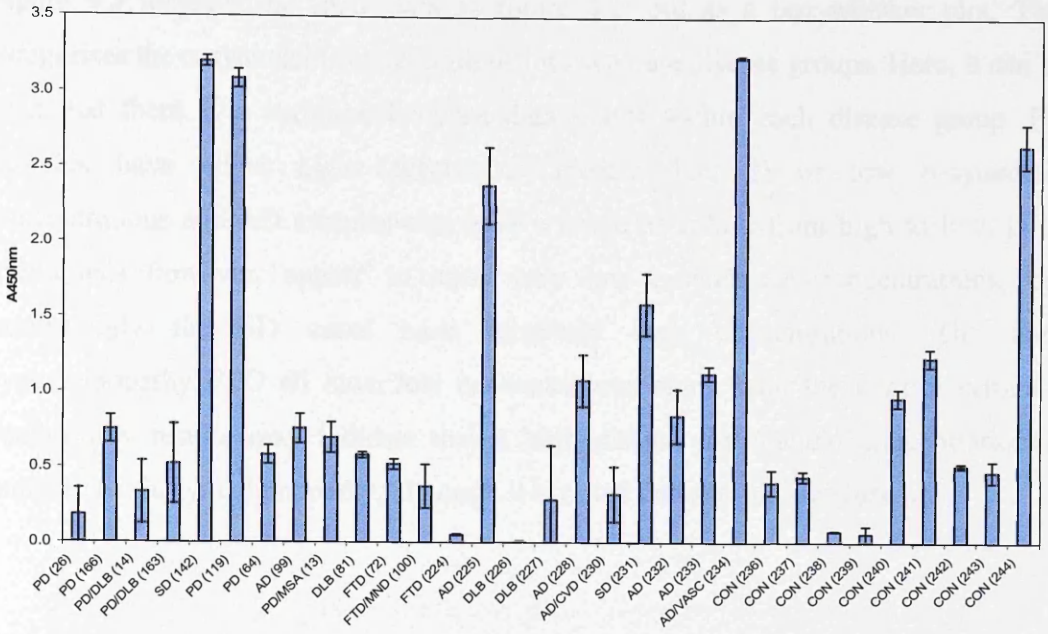


Figure 4.1 Detection of α-synuclein in human plasma samples

Measured ELISA absorbance values from plasma samples (diluted 1:1 in PBS). The assay is done in triplicate in order to calculate a standard error. The α-synuclein standard curve is used to estimate the protein concentration (figure 3.6).

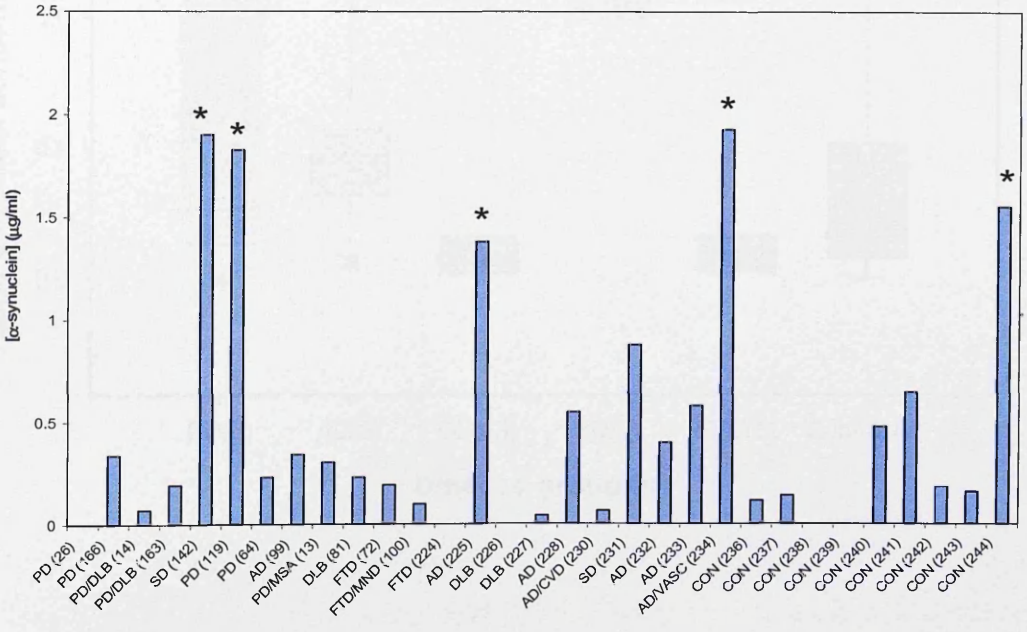


Figure 4.2 Concentration of α-synuclein in human plasma samples

(* indicates values which may be saturated and therefore potentially under-represents the α-synuclein concentration). As the plasma samples are diluted 1:1 in PBS, the actual α-synuclein concentration should be doubled for the true estimation of plasma concentration.

Figure 4.3 displays the same data as figure 4.2, but as a box-whisker plot. This categorises the α-synuclein concentrations into separate disease groups. Here, it can be seen that there is a variation between data points within each disease group. PD samples have either high (absorbance greater than 2) or low α-synuclein concentrations and AD samples also have a range of values from high to low. DLB individuals however, appear to have very low α-synuclein concentrations, but interestingly the SD cases have relatively high concentrations. The non-synucleinopathy FTD all have low concentrations along with the control samples. Preliminary results may indicate that a high plasma α-synuclein concentration is indicative of a synucleinopathy, although it is noted that sample sizes are low.

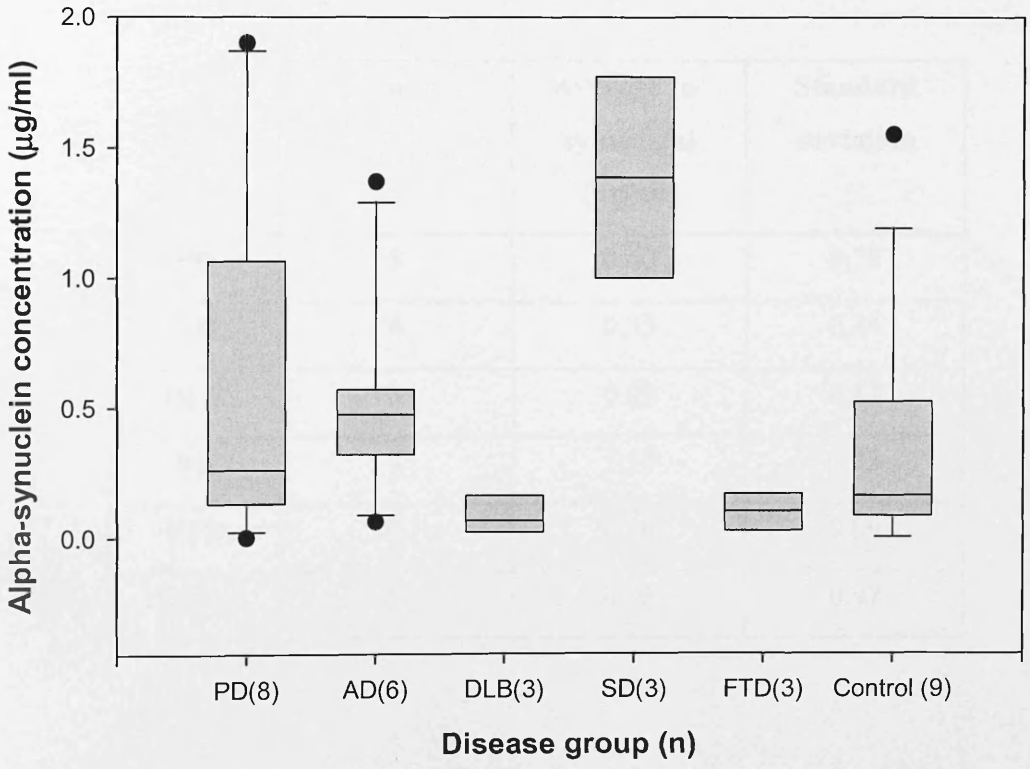


Figure 4.3 Box-whisker plots for the plasma ELISA results

The length of the box represents the interquartile range (25-75%) of the sample, the line drawn across the box the median and outliers being denoted by dots.

It might be expected that PD patients for example, have a higher than average level of α-synuclein in the blood, but on the other hand, if the protein is sequestered into Lewy bodies they might have a lower than average concentration. Either way, one may expect to see a difference in α-synuclein levels in synucleinopathies and control cases, even with this small group of samples. Figures 4.3 reveals the SD plasma samples have the highest mean α-synuclein concentration, and the non-synucleinopathy FTD group the lowest concentration.

Table 4.1 reveals the mean α-synuclein concentration within different disease groups and the standard deviation.

Table 4.1 Average plasma α-synuclein concentrations for different disease groups

	n	Average [α-synuclein] (μg/ml)	Standard deviation
PD	8	0.60	0.78
AD	6	0.55	0.44
DLB	3	0.09	0.12
SD	3	1.39	0.73
FTD	3	0.10	0.09
Control	9	0.36	0.47

The validity of any statistical analysis would have been undermined by the small sample sizes, and was therefore not carried out. Initially, as the study was focussing on the characterisation of the α-synuclein/parkin complex in plasma, further investigations to validate these results (i.e. to extend them to larger numbers of samples) were postponed for potential work in the future.

4.3 Parkin interacts with α-synuclein in human plasma

To determine whether a parkin/α-synuclein complex could be detected in human blood plasma, antibody-sandwich ELISA assays were developed (Chapter 3). The plasma samples (that were previously assayed in the α-synuclein ELISA) were assayed again, this time to detect any parkin that was complexed with the α-synuclein protein. Anti-parkin antibody N-18 (1:1000 dilution) coated the wells as the parkin capture antibody, and detection was via the anti-α-synuclein antibody FL-140 (1:1000), followed by goat anti-rabbit HRP (1:10,000). There was no apparent correlation between the levels of the protein complex in the different disease groups, with 1 out of 7 PD samples giving a very high signal (A 450 nm $\geq$ 1.0), 6 out of 19 AD samples giving a similarly high reading, 1 out of 5 dementia with DLB, 1 out of 7 FTD samples and 1 out of 6 control samples giving the same high absorbance (figure 4.4). The medians of the groups are all similar (figure 4.5). However, analysis of more samples may reveal genuine differences between disease groups.

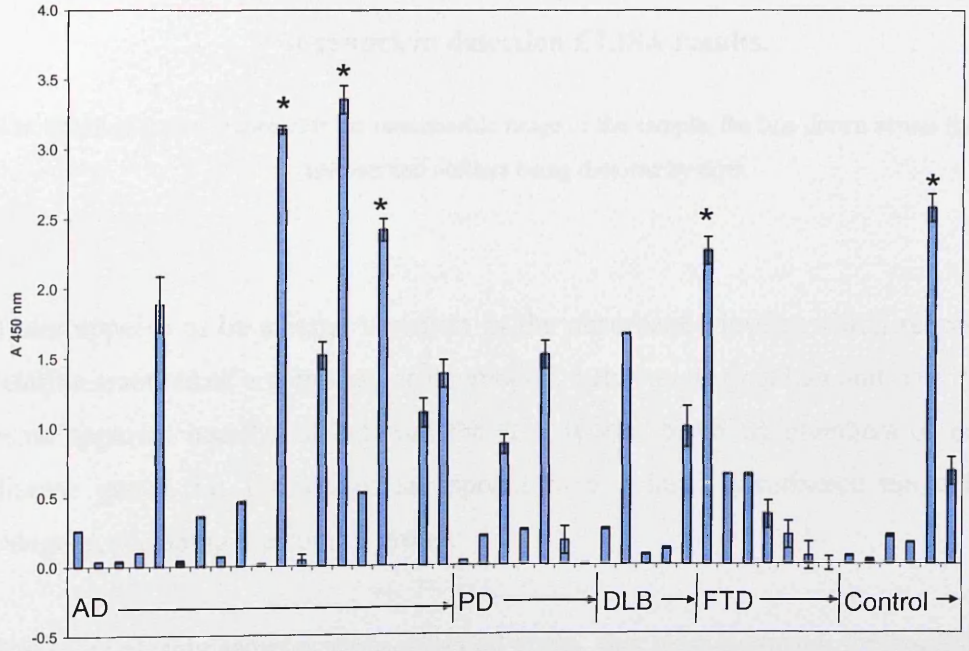


Figure 4.4 The levels of association between parkin and α-synuclein in human plasma

(* indicates measured absorbance values above 2.0 which may be saturated and therefore under-represent the true level.)

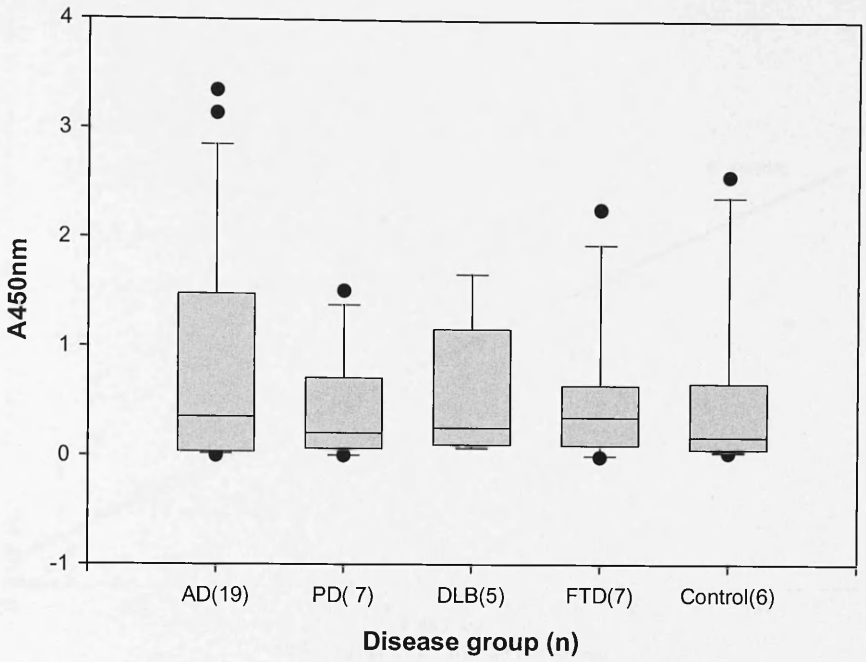


Figure 4.5 Box-whisker plots for the same plasma samples - parkin capture, α-synuclein detection ELISA results.

The length of the box represents the interquartile range of the sample, the line drawn across the box the median and outliers being denoted by dots.

There appears to be a large variation in the absorbance levels, which represent the relative amounts of a complex, or interaction, between α-synuclein and parkin. There is no apparent correlation between the A_{450} levels shared by members of the same disease group, i.e. the values are spread over a large absorbance range in each category, including the control group.

The same plasma samples were screened again, this time capturing the complex with anti-α-synuclein monoclonal antibody (211) (1:200) and detecting with anti-parkin antibody N-18 (1:1000). For each plasma sample, the average absorbance at 450nm for the α-synuclein capture, parkin detection ELISA was plotted against the A_{450} for the parkin capture, α-synuclein detection ELISA.

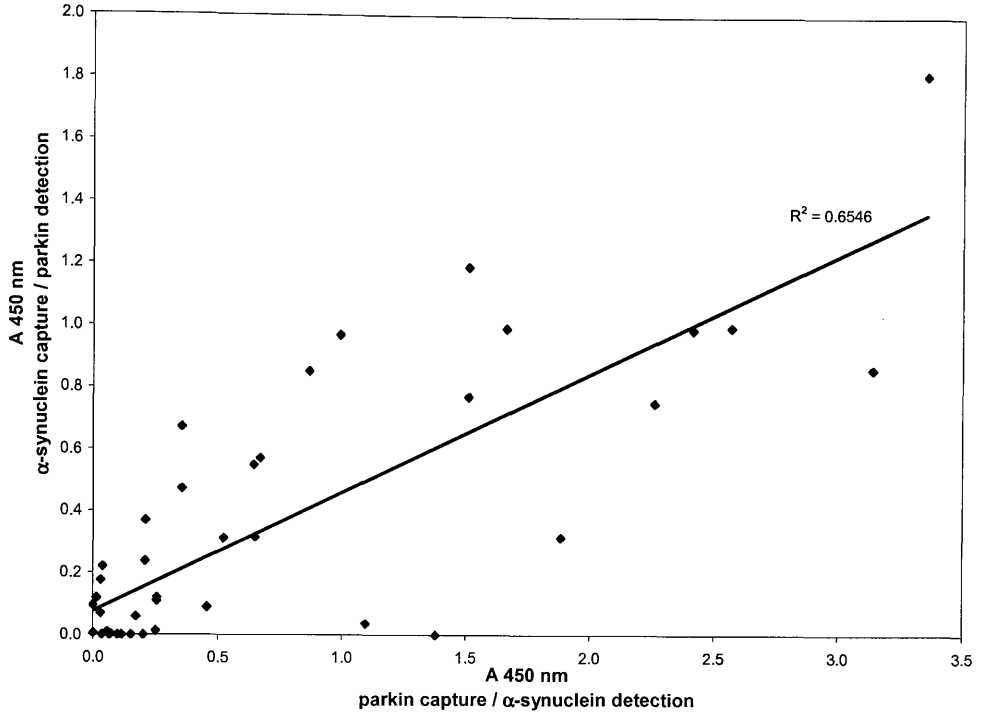


Figure 4.6 Confirmation of the association of parkin and α-synuclein in human plasma

As expected, the two sets of data showed some agreement, with R^2 value of 0.65 (figure 4.6) showing that there is an association between the data from the two assays. It is to be expected that when the sandwich ELISA captured α-synuclein, then followed by detection of parkin, then that data would be mirrored when undertaking the assay the other way round, i.e. capturing the parkin protein and detecting any α-synuclein, if the assay was detecting a complex between α-synuclein and parkin.

The data were analysed using SPSS v 14.0 and Spearman's Rank employed to test whether the ranks of the two sets of measurements are linearly associated. As expected, the two variables show a highly significant correlation ($p<0.001$). There is a strong association between the two sets of data.

4.3.2 Comparison of the plasma levels of parkin with the levels of the parkin/α-synuclein complex

The same plasma samples were screened in a further assay, this time to detect the levels of parkin. This ELISA detects parkin levels by capturing the protein with anti-parkin N-18 (1:1000), and detecting it with anti-parkin PARK 11-S (1:20 000). Again, the average absorbance at 450nm for each plasma sample in the ELISA to measure total parkin (y axis) versus the average absorbance values measured in the ELISA to detect any protein complex, i.e. the parkin capture, α-synuclein detection, is shown in figure 4.8.

.

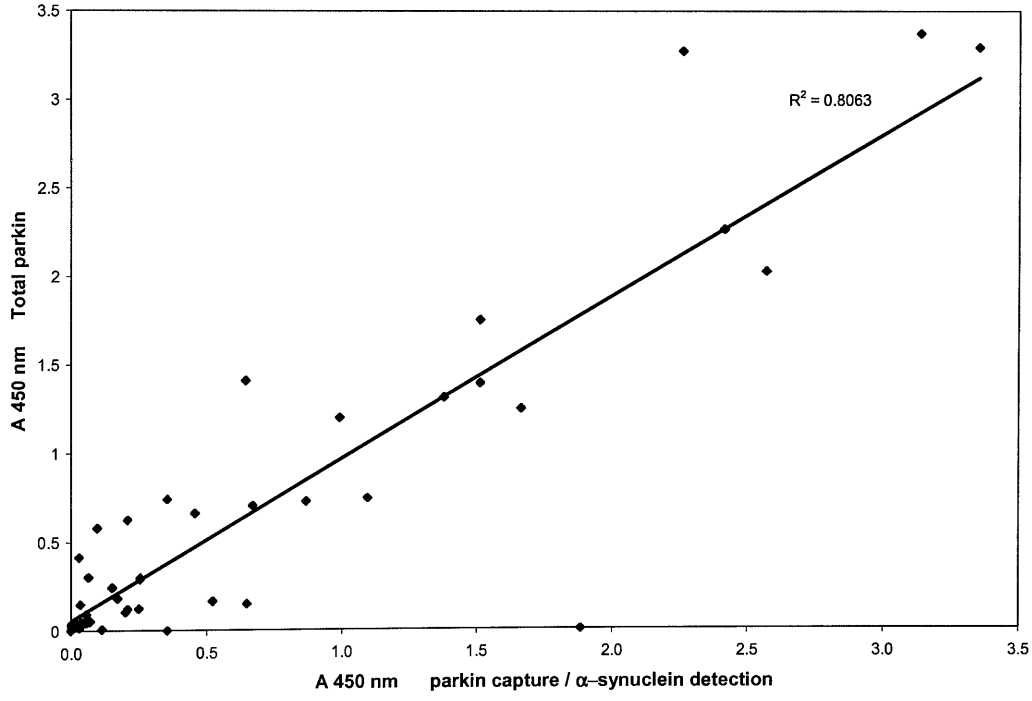


Figure 4.8 Parkin levels in plasma compared to the parkin/α-synuclein complex levels

The two sets of data are plotted to reveal an overall direct correlation between the two sets of data. Once again data were analysed using SPSS v 14.0 and Spearman's Rank employed to test whether the ranks of the two sets of measurements are linearly

associated. SPSS shows that the probability significance (2-tailed) of getting such test statistics is <0.001. There is a strong association between the amount of parkin and the concentration of α-synuclein/parkin complex. When the levels of parkin are high in a particular sample, the levels of the complex are also high. The R^2 value for this data comparison is 0.81, whereas previously when comparing the levels of the complex to levels of α-synuclein, the R^2 value is only 0.54. This suggests the degree of association between these two groups of data is more significant then the previous comparison (the α-synuclein versus α-synuclein/parkin complex). It may be the case that the level of parkin most directly influences the amount of complex present in the plasma.

4.4 Discussion

The α-synuclein protein was linked to Parkinson's disease with the discovery of mutations (Polymeropoulos et al. 1997), duplication (Chartier-Harlin et al. 2004) and triplication (Singleton et al. 2003) of the *SNCA* gene, encoding the α-synuclein protein, in familial cases of PD and DLB. Lewy bodies which are the main pathological feature of PD and DLB brains are formed by the conversion of soluble monomers of the α-synuclein protein into insoluble aggregates (Spillantini et al. 1998). Studies on mouse models that either overexpress *SNCA* or lack the *SNCA* gene (*SNCA* knock-out mice) suggest a central role for α-synuclein in PD. It has also been suggested that parkin molecules are a factor when considering the formation of Lewy bodies (Shimura et al. 2001).

In this study, ELISA methods were used to measure the amounts of α-synuclein, parkin, and some form of complex between these two proteins, in plasma from control cases and from individuals with a neurodegenerative disease.

4.4.1 Possible methods of secretion of α-synuclein

Because α-synuclein lacks a target sequence for the ER, it has been thought to exist as an intracellular protein (Jakes et al. 1994). However, the results presented here support more recent thinking that the protein may also exist as an extracellular form in plasma (El-Agnaf et al. 2003).

The mechanisms whereby proteins are sorted to the major organelles and compartments of cells are well understood. The initial 'sorting' occurs during the first growth of polypeptide chains on cytosolic ribosomes. Some proteins contain a specific signal targeting sequence, the endoplasic reticulum (ER) signal sequence that directs the ribosomes synthesising them to the ER. This sequence is usually found on the N-terminus and contains a run of hydrophobic amino acids. Protein synthesis is completed on ribosomes attached to the ER membrane. The completed polypeptide chains are then moved to the Golgi complex and are subsequently secreted to various destinations. Synthesis of proteins that lack the ER targeting sequence is completed on free ribosomes and the proteins released into the cytosol, where some of them are directed to other organelles, e.g. the mitochondria, peroxisomes or the nucleus, depending on the other specific signal sequences they may contain.

Although the precise mechanism by which α-synuclein is released extracellularly is not known, it may be significant that the C-terminal tail of the protein contains two di-acidic motifs in the form of Asp-X-Glu. This motif is known to be an ER to Golgi directing signal (Nishimura et al. 1999) and it interacts with the ER coat protein II complex (COPII) (Barlowe 2002). COPII consists of three proteins (Sar1, Sec23-Sec24 and Sec13-Sec31 complex) that are sequentially recruited to the ER membrane surface. Sar1 is a small 21 kDa protein GTPase, whereas Sec23-Sec24 and Sec13-Sec31 are large protein complexes. COPII forms transport vesicles to carry newly synthesised proteins from the ER to the Golgi and collect the appropriate cargo proteins into these vesicles (Barlowe 2002; Barlowe 2003). Although α-synuclein does not have an ER directing signal, and so is thought to be cytosolic in its distribution, it is enriched in synaptosomal fractions of mouse and human brain, and similarities between the N-terminus of α-synuclein and the lipid-binding domains of some apolipoproteins suggest a role for α-synuclein interacting with lipid membranes

(Davidson et al. 1998). This data is supported by other findings that α-synuclein has a motif at the N- and the C-terminal that is homologous to the fatty acid binding protein (FABP) signature motif (Sharon et al. 2001). Other studies have suggested that the N-terminal region of the protein may reversibly interact with membranes via 7 repeated motifs of 11 amino acids each that are shared with the class A2 helices of apolipoproteins (Clayton and George 1999). α-synuclein appears to associate with membranes, not only via this N-terminal domain, but also via its C-terminal domain, in which no such lipophilic sequence exists (McLean et al. 2000). Additionally, the amphipathic α-helical, lipid-binding domain of α-synuclein has been reported to strongly bind to membranes (Sharon et al. 2001; Volles and Lansbury 2002) and even make them permeable. It could be suggested that these membrane binding properties might allow α-synuclein to associate with the ER membrane. There, the di-acidic motifs on the protein could interact with the COPII complex, transporting proteins from the ER to the Golgi, and then via the default pathway of secretion from the cell.

Work on vimentin, a large 53kDa intermediate filament protein, has provided evidence that large proteins without ER leader sequence, can be secreted from cells. Studies have shown that , despite lacking an ER sequence, in some circumstances vimenitin can cross into the ER and be secreted from the cell through the ER-Golgi pathway (Mor-Vaknin et al. 2003). It may be of significance that, like α-synuclein, vimentin also has a di-acidic Asp-X-Glu motif on its C-terminus.

Synucleins (α- and γ-synuclein) are expressed in blood platelets. Investigations into the synucleins contained in platelets from PD and normal control individuals showed that neither of these proteins were secreted upon thrombin activation (Li et al. 2002), and therefore it was deemed unlikely that the α-synuclein in human plasma originated from platelets. There was no significant difference between the levels of platelet synucleins in PD and normal samples. Using AD as an analogy, it has been reported that in a transgenic mouse model of AD, after peripheral administration of a monoclonal antibody to Aβ, a rapid increase in plasma Aβ was observed, and the magnitude of this increase was highly correlated with the amyloid concentration in the hippocampus and cortex (DeMattos et al. 2002). As this data suggests that Aβ can efflux from the brain to the plasma, it is possible that an equivalent mechanism could do the same for other neuronal proteins, including α-synuclein. Glial cytoplasmic

inclusions in oligodendrocytes, in MSA brains, contain α-synuclein fibrils (Gai et al. 1998; Spillantini et al. 1998), although the protein is not expressed in these cells (Solano et al. 2000). This could suggest that these α-synuclein deposits found in the brains of patients with MSA may also originate from neuronal secretion.

It has also been demonstrated that, as a part of the normal life cycle of the protein, some of the intracellular α-synuclein is released from neuronal cells to the culture medium, via vesicular entry and exocytosis (Lee et al. 2005). It is speculated that this might represent a novel mechanism by which cells expel damaged or misfolded α-synuclein. Furthermore, the study (Lee et al. 2005) shows that α-synuclein is highly prone to aggregation within the vesicles. The aggregates are secreted from cells, and mitochondrial and proteasomal dysfunctions (conditions that are associated with intracellular aggregation and PD pathogenesis; (Ciechanover 2001)) cause elevated secretion of α-synuclein aggregates. This release of α-synuclein may explain the presence of the protein in human blood and also CSF (El-Agnaf et al. 2003). Moreover, it has been established that over 30% of newly synthesised cellular proteins are discarded without being properly folded, even though they are normally synthesised without mutations in their genes or errors in translation (Schubert et al. 2000).

Taking all this evidence into account, it could be speculated that full length α-synuclein is released by neurons as part of normal cellular processing, rather than as a result of neuronal degeneration. The protein could be secreted into the surrounding media in the brain, and this could circulate to the CSF and on to the blood.

Investigations into the existence of an unbroken neuronal chain, from the enteric to the central nervous system, lends support to the hypothesis that a putative environmental pathogen capable of passing the gastric epithelial lining may induce alpha-synuclein misfolding and aggregation in specific cell types of the submucosal plexus (Braak et al. 2006). This could reach the brain via a consecutive series of projection neurons. If this were the case, then it is feasible that plasma α-synuclein may originate from the neurones of the enteric system and not within the brain itself.

One of the more interesting possibilities to arise from this is the potential use of α-synuclein, or other proteins implicated in neurodegenerative disorders, in biological fluids as a biomarker for PD or related diseases.

4.4.2 Detection of total α-synuclein in human blood plasma

Using different anti-α-synuclein antibodies for capture and detection, one type of ELISA can detect all forms of α-synuclein. This assay was used to measure the total amount of α-synuclein in plasma samples from patients with FTD, DLB, PD, AD and control cases. Present results show that there was a wide variation within the disease groups and the control group, with no obvious links to specific disease. The comparatively high levels of α-synuclein in plasma are not confined to patients with PD or other synucleinopathies. If the presence of α-synuclein in blood plasma was to be used as a diagnostic marker for PD, the levels would have to be significantly different from those diseases that were not associated with α-synuclein, i.e. the non-synucleinopathies.

The exact function of α-synuclein remains unclear, although it has been linked to many cellular processes, including the possibility that it may be a signalling molecule. Other possible functions are lipid binding and synaptic plasticity, i.e. the moulding and re-moulding of the synapse to accommodate synaptic transmission. Involvement with vesicle formation and dopamine transport has also been suggested. Consequently, because the precise function of α-synuclein remains unknown, its role in plasma can only be speculated. As the protein is only 14.4kDa in size, one would expect it to be readily excreted by the kidneys. In order to become large enough to avoid excretion, α -synuclein might need to be complexed with either itself or other proteins.

4.4.3 Detection of an α-synuclein/parkin interaction

An ELISA capturing parkin and detecting α-synuclein was developed to assay human plasma for the presence of any protein complex that contains the two proteins. This assay suggested that parkin was present in plasma complexed with α-synuclein. To corroborate these results, another ELISA was developed that captured α-synuclein and detected parkin. The absorbance values recorded from this assay correlated with the absorbance levels from the first ELISA (figure 4.7). One would predict that the relative absorbance levels measured for an individual plasma sample would be similar, e.g. if the α-synuclein capture, parkin detection absorbance was very low, the parkin capture, α-synuclein detection readings would also be low. There is not a 100% correlation when comparing the two sets of results, possibly due to the nature of the assays. It is recognised that as ELISA experiments are so sensitive, very slight variations in parameters such as incubation times and temperature may have effects on the results obtained. It is also possible, regarding these two experiments, that epitopes on the proteins within the complex were partially shielded, resulting in a difference in the measured absorbance at 450nm.

α-synuclein is reported to be a substrate for parkin (Shimura et al. 2001) and a novel, glycosylated form of α-synuclein, αSp22, has been found complexed with parkin. It has been suggested that parkin, an E3 ubiquitin ligase, is a factor in the formation of Lewy bodies (Shimura et al. 2001). Lewy bodies are the characteristic inclusions that form the basis of the post-mortem diagnosis for PD patients. However, Lewy bodies are usually absent in parkin-deficient ARJP brains (van de Warrenburg et al. 2001). It is not known whether Lewy bodies are pathological, or protective in nature. It is possible that their influence changes as the disease progresses. For example, in the early stages of PD, excess α-synuclein may be "mopped up" by parkin into Lewy bodies as a protective mechanism, only for these inclusions to interfere with normal cellular processes in the later stages of disease, leading to neuronal death. The α-synuclein identified in Lewy bodies is poly-ubiquitinated (Trojanowski et al. 1998). One popular hypothetical model suggests that the loss of parkin in the brains of ARJP patients, and therefore the loss of E3 ubiquitin ligase activity, could initiate an accumulation of parkin substrates, including non-ubiquitinated αSp22. This build up may generate accelerated neuronal loss and a younger age of onset of disease (Kitada

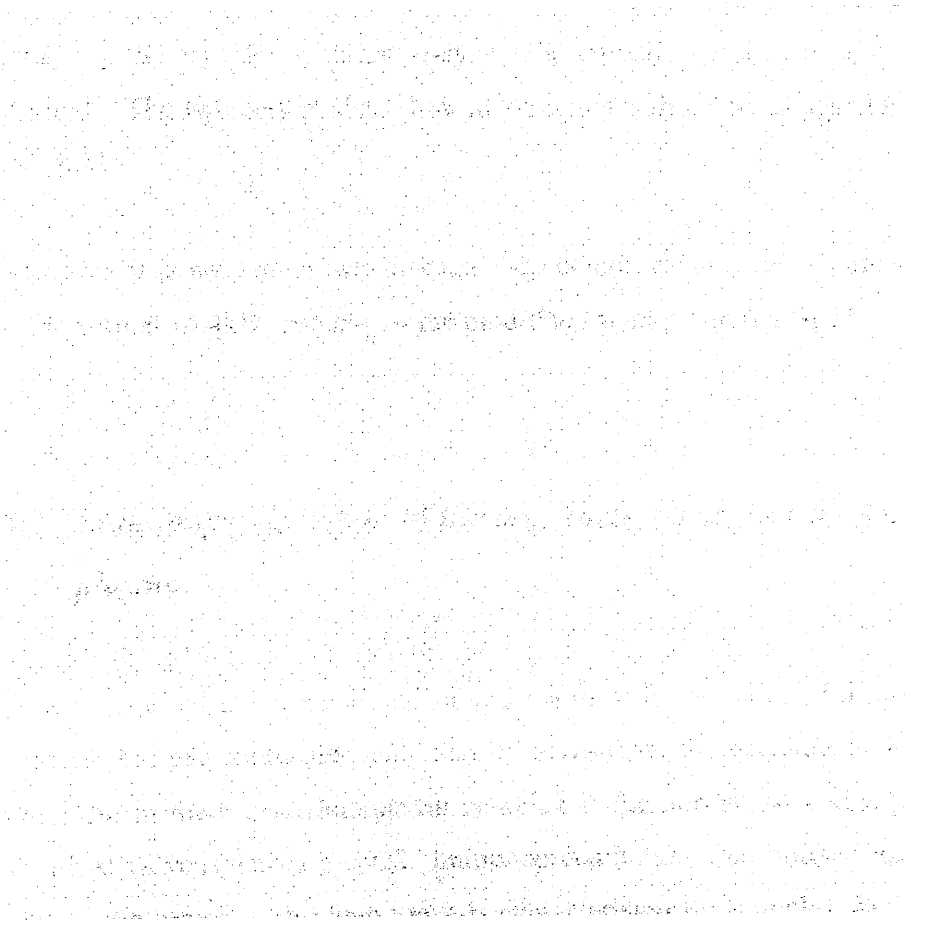
et al. 1998). However, if this is the case, why has αSp22 not been identified in Lewy bodies or elsewhere in the brain? On the contrary in PD, parkin is thought to ubiquitinate this modified α-synuclein and target it for degradation by the proteasome. This would continue up until a time when the process is not efficient enough to prevent a build up of substrate, and results in a later onset of the disease. However, very little is known about this O-glycosylated form of α-synuclein, and even its existence has not been confirmed by other groups (Giasson and Lee 2001). The results of the present study support the theory that α-synuclein is bound by parkin and may be a substrate. However, there is ambiguity as to which form of α-synuclein is complexed to the parkin.

The present study reports that the α-synuclein/parkin complex is detected in blood plasma. α-synuclein, as mentioned previously, is believed to be secreted from the cell through exocytosed vesicles. Also, like α-synuclein, parkin has been found to be associated with cellular vesicles (Kubo et al. 2001). Therefore, it seems feasible that parkin could be secreted from the cell already complexed to α-synuclein. Once it is secreted from the neuronal cells, the complex may cross the blood-brain barrier, where it would enter the plasma.

Why the parkin is complexed to α-synuclein in the first place is open to speculation. Parkin possibly targets misfolded/mutated/excess α-synuclein for ubiquitination and consequently proteosomal degradation. It may be possible that as well as degradation, the proteins are sequestered into cytoplasmic inclusions, as both α-synuclein and parkin are present in Lewy bodies. Maybe the vesicular exocytosis is another mechanism by which the proteins are regulated within the cell. As mentioned previously, parkin may be responsible for the formation of Lewy bodies, as these are lacking in ARJP patients (who lack the functional parkin protein). Perhaps the non-ubiquitinated α-synuclein accumulates, thus attributing to cell death. It is also possible that one of the other physiological targets of parkin may contribute to the pathogenesis of PD.

One type of ELISA can detect monomeric and complexed forms of parkin, using different parkin antibodies for capture and detection. This assay was used to measure the comparative levels of parkin in plasma samples from patients with FTD, DLB, PD,

AD and control cases (figure 4.6). The assay suggested that parkin was indeed present in the plasma samples. The absorbance values recorded from this assay can be seen to correlate with the absorbance levels from the ELISA that detects the α-synuclein/parkin complex (figure 4.8). When the level of parkin is high in a particular sample, generally the levels of the complex are also elevated. The results suggest that the level of parkin is the factor that determines the level of complex.



5 Detection of α-synuclein and parkin in human brain and plasma by immunoprecipitation and immunoblotting

5.1 Introduction

Previously, an ELISA has been developed that suggests that parkin interacts with α-synuclein in human plasma. Initially the sandwich ELISA captured the parkin (anti-parkin antibody N-18, 1:1000 dilution) and detection was via an anti-α-synuclein antibody (FL-140, 1:1000). The same plasma samples were screened again, this time capturing the complex with anti-α-synuclein antibody and detecting with anti-parkin antibody. The two sets of data showed agreement, therefore suggesting these results are valid.

However, it is not known whether the α-synuclein in the α-synuclein/parkin complex is the normal 14.4kDa protein, or the modified (glycosylated) α-Sp22.

5.2 Immunoprecipitation of the α-synuclein/parkin complex in human plasma

Data is required to further establish and confirm the presence of a protein complex between the two molecules, and also, to investigate the question of whether the α-synuclein in the α-synuclein/parkin complex is the normal 14.4 kDa protein, or the modified (glycosylated) α-Sp22. Immunoprecipitation was utilised as a method to capture the proteins, and help towards characterising the complex further. A plasma sample with a high measured absorbance value of over 2.5 at 450nm for the synuclein/parkin complex ELSA, and a control sample with low reading of near zero, were incubated for immunoprecipitation (IP) using magnetic Dynabeads coupled to

anti-parkin antibody (N-18), and also control beads coupled to rabbit IgG. The bound proteins were eluted from the beads, separated on SDS gels before transfer to nitrocellulose for analysis by immunoblotting. The blots were probed with anti-parkin (PARK 11-S), and also with anti-α-synuclein antibodies (FL-140 and 211).

Immunoblots with anti-parkin (PARK 11-S) revealed the 53kDa parkin protein as expected (figure 5.1). This would suggest that the parkin immunoprecipitation had been successful. The immunoblots on the low reading plasma sample gave no bands/data. The control beads coated with the IgG showed no non-specific binding.

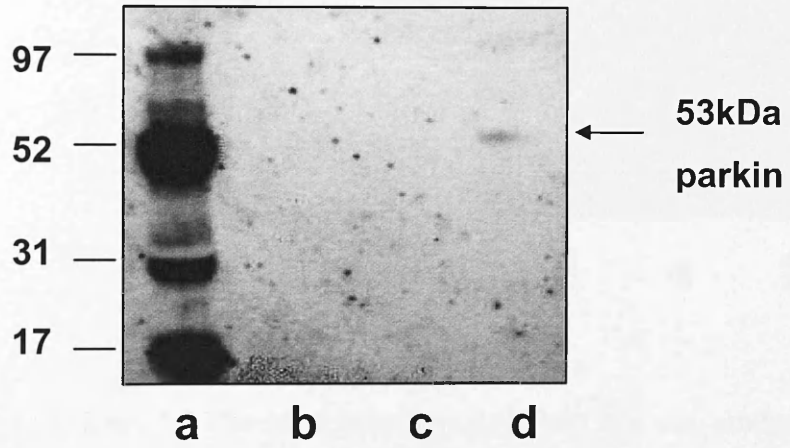


Figure 5.1 Plasma that gave a high α-synuclein/parkin ELISA absorbance value of over 2.5 was immunoprecipitated with anti-parkin antibody (N-18)

The precipitate was analysed by immunoblotting with anti-parkin antibody (PARK 11-S). The 53kDa parkin protein was specifically captured by the anti-parkin antibody that was crosslinked to the Dynabeads. 12.5% SDS-PAGE. Lane a, Multimark molecular weight markers (Invitrogen); lane b, control beads coated with rabbit IgG and incubated with plasma that gave a high ELISA absorbance reading when assayed for the complex; lane c, beads coated with N-18 and incubated with plasma with a low complex onxentration; and lane d, beads coated with anti-parkin antibody N-18 and incubated with a high complex concentration plasma.

Immunoblots with both primary anti-α-synuclein antibodies FL-140 and 211, to detect any α-synuclein present, both revealed the reported binding partner of parkin, α-synuclein (figure 5.2). This confirms that α-synuclein does co-precipitate with parkin. However, the α-synuclein is not the reported 22-24 kDa form αSp22, but an approximately 17 kDa form that co-migrates with the recombinant α-synuclein, and so appears to be non-glycosylated.

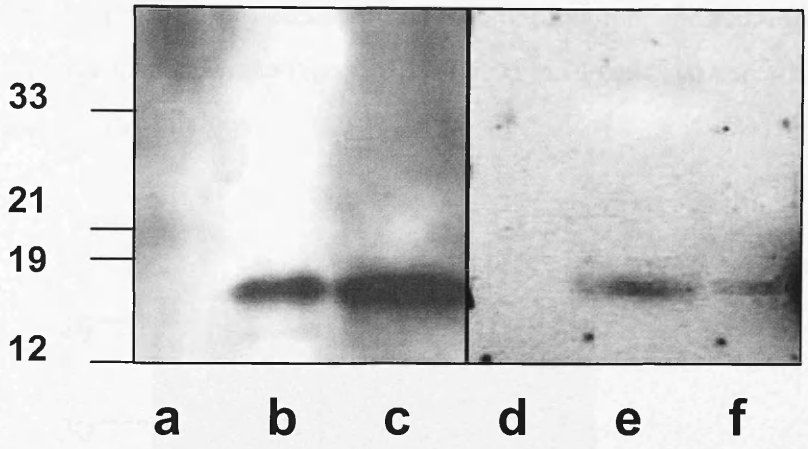


Figure 5.2 The same immunoprecipitate was also analysed by immunoblotting with anti-α-synuclein antibodies FL-140 (figure 5.2 a-c) and 211 (figure 5.2 c-d)

Results show that both antibodies reveal a band corresponding to α-synuclein, suggesting that this protein co-precipitates with parkin. However, as the protein co-migrates with the recombinant α-synuclein, it may be deduced that this synuclein is not the reported 22-24 kDa form αSp22, but the non-glycosylated protein. 12.5% SDS-PAGE. Lanes a and d, control beads coated with IgG and incubated with a plasma sample that gave a high absorbence reading when assayed for the complex by ELISA; lanes b and e, beads coated with anti-α-synuclein antibody and incubated with the same high complex concentration plasma sample and lanes c and f, recombinant α-synuclein.

A second immunoprecipitation experiment was then performed. Again, the same plasma sample with a high absorbance of over 2.5 at 450nm for the synuclein/parkin complex ELISA, and the control sample with a low reading of near zero, were incubated for immunoprecipitation using magnetic Dynabeads, this time coupled to monoclonal anti-α-synuclein antibody (211), along with control beads coupled to rabbit IgG. The bound proteins were eluted from the beads, and then separated on SDS gels before transfer to nitrocellulose for analysis by immunoblotting. The blots were probed with anti-α-synuclein antibody (FL-140), which revealed the 17kDa form of α-synuclein protein (figure 5.3). This would suggest that the α-synuclein immunoprecipitation had been successful. The immunoblots on the low reading plasma sample gave no bands/data (figure 5.3). The control beads coated with the IgG showed no non-specific binding.

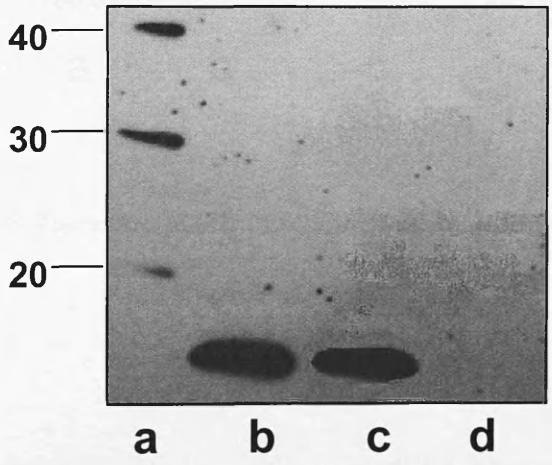


Figure 5.3 A plasma sample that gave a relatively high ELISA absorbance at 450 nm of 2.5, was immunoprecipitated with anti-α-synuclein antibody (211)

The precipitate was analysed by immunoblotting with anti-α-synuclein antibody (FL-140). The 14.4kDa α-synuclein protein was specifically captured by the anti-α-synuclein antibody that was crosslinked to the Dynabeads. 12.5% SDS-PAGE. Lane a, Magicmark (Invitrogen); lane b, beads coated with anti-α-synuclein antibody (211) and incubated with plasma that gave a high absorbence when assayed for the complex; lane c, recombinant α-synuclein, lane d, beads incubated with a low complex concentration plasma sample.

Immunoblots with primary anti-parkin antibody N-18, to detect any parkin present in the precipitate, revealed the reported binding partner of α-synuclein, the 53 kDa parkin (figure 5.4). This confirms that parkin does co-precipitate with α-synuclein. Experiments on the low reading plasma gave no data. The control beads coated with the IgG showed no non-specific binding to any protein.

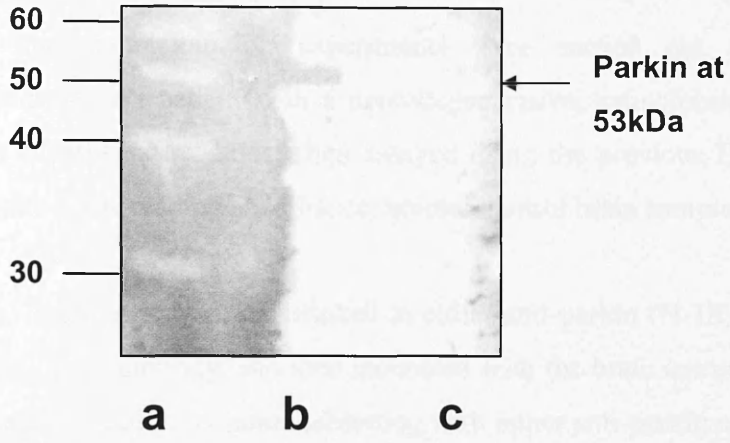


Figure 5.4 The same precipitate analysed by immunoblotting with parkin antibody PARK-11S

Results show that the antibody reveals a band corresponding to parkin, suggesting that this protein co-precipitates with α-synuclein. 12.5% SDS-PAGE. Lane a, Magicmark (Invitrogen); lane b, beads coated with anti-α-synuclein antibody (211) and incubated with a plasma sample that gave a high reading by ELISA when assayed for the protein complex; lane c, beads incubated with a plasma sample that contained a low complex concentration.

5.3 *Immunoprecipitation of the α-synuclein/parkin complex in human brain*

If parkin has been found to bind non-glycosylated α-synuclein in human plasma from patients with neurodegenerative diseases and also normal control subjects, the question arises as to whether the α-synuclein in the brain is also bound to parkin, and if so, is it the normal 14.4 kDa form or the modified, heavier form.

Identical immunoprecipitation experiments were carried out on human brain homogenates from a patient with a neurodegenerative, synucleinopathy (DLB) that gave high absorbance readings when assayed using the previous ELISA techniques, and also with a low reading/absorbance, normal control brain sample.

As before, Dynabeads were crosslinked to either anti-parkin (N-18) antibody or anti-α-synuclein (211) antibody, and then incubated with the brain extract. This was again followed with detection by immunoblotting with either anti-parkin antibody (N-18) or anti-synuclein antibody (FL-140).

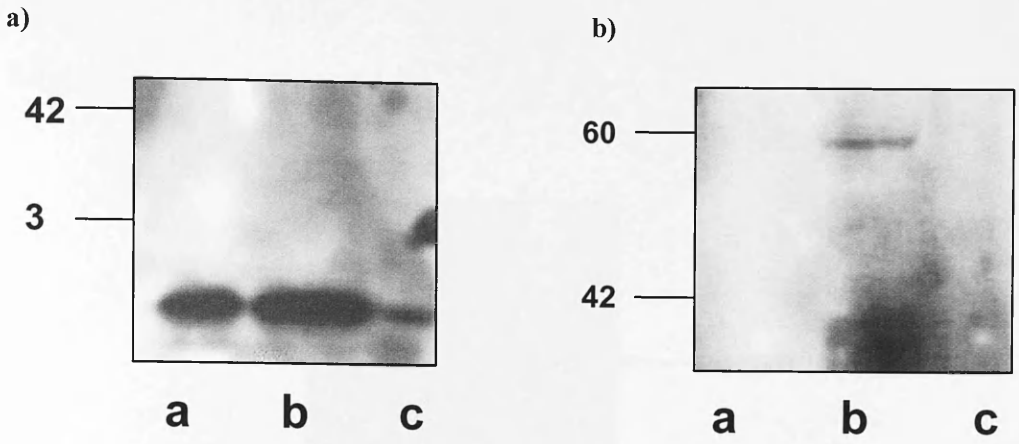


Figure 5.5 α-synuclein immunoprecipitation experiments carried out on human brain tissue

Brain tissue from a patient with DLB that gave a high absorbance measurement when assayed for the α-synuclein/parkin complex, and a normal, low reading control brain. Dynabeads were crosslinked to anti-synuclein (211) antibody, then incubated with the human brain homogenates. This was followed with detection by immunoblotting, either with anti-synuclein (FL-140) (figure a) or anti-parkin (N-18) antibody (figure b). 12.5% SDS-PAGE. Lane a, beads incubated with a normal brain sample that had a low concentration of complex when assayed b y ELISA; lane b, sample beads coated with anti-α-synuclein antibody (211) and incubated with a DLB brain sample that had a high concentration of complex; and lane c, recombinant α-synuclein.

The synuclein immunoblot displayed three bands (figure 5.5a). The protein was present in the normal brain tissue, and in the DLB brain sample. As these bands migrated at the same rate as the recombinant protein, it could be surmised that this was the normal α-synuclein, not the larger αSp22. Parkin was not detected in normal, control brain tissue (figure 5.5b, lane a), but the band was present in the DLB sample tissue. The parkin must have co-precipitated with the α-synuclein. This data largely mirrored the data obtained from the plasma sample that gave a relatively high measured absorbance of over 2.5 for the α-synuclein/parkin complex ELISA (figure 5.5b).

5.6a)

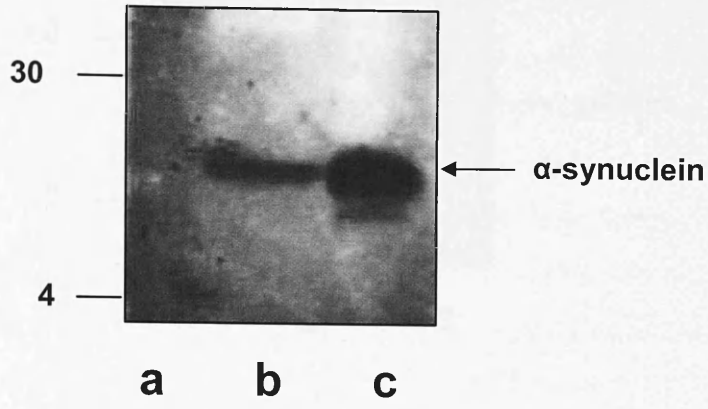


Figure 5.6 Further immunoprecipitation experiments carried out on human brain tissue

Brain tissue was obtained from a patient with DLB that gave a high A450nm reading of over 2.5 when assayed for the α-synuclein/parkin complex, and a normal, low reading (A450 nm of near zero) control brain. Dynabeads were crosslinked to anti-parkin (N-18) antibody, and then incubated with the human brain homogenates. This was followed with detection by immunoblotting, either with anti-α-synuclein antibody (FL-140) (figure 5.6a) or anti-parkin antibody (PARK 11-S) (figure 5.6b).

Figure 5.6a

12.5% SDS-PAGE followed by immunoblotting with anti-α-synuclein antibody (FL-140). Lane a, beads coated with anti-parkin antibody (N-18) and incubated with a low complex concentration, normal brain sample; lane b, beads incubated with a high complex concentration DLB brain sample; and lane c, recombinant α-synuclein.

5.6b)

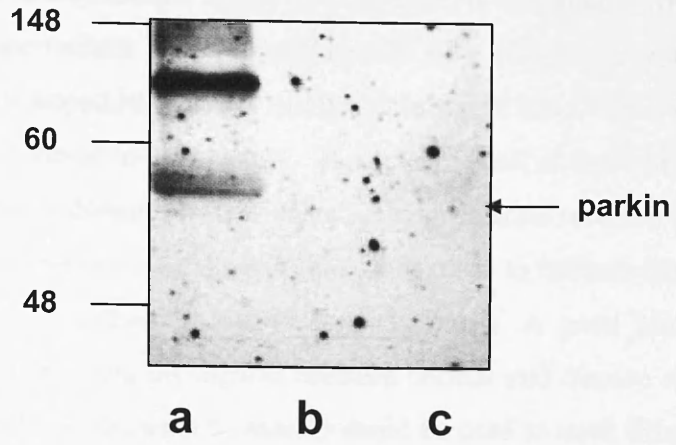


Figure 5.6b

12.5% SDS-PAGE followed by immunoblotting with anti-parkin antibody (PARK 11-S). Lane a, beads coated with anti-parkin antibody (N-18) and incubated with a high complex concentration DLB brain sample (A450 nm over 2.5); lane b, beads incubated with a low complex concentration normal brain sample (A450 nm of near zero); and lane c, recombinant α-synuclein.

The data reveals a band corresponding to parkin at 52 kDa, and a further immunoreactive band at a higher molecular weight was detected.

5.4 Discussion

5.4.1 Confirmation of a functional relationship between parkin and α-synuclein

This study has confirmed a direct relationship between two proteins associated with PD, parkin and one of its substrates, α-synuclein. The novel data identifies parkin in human blood plasma, and also an interaction in human plasma and brain that involves parkin and α-synuclein. However, the target for parkin does not appear to be the reported 22-24 KDa O-glycosylated isoform of α-synuclein, αSp22 (Shimura et al. 2001), but a form of the protein that co-migrates with recombinant α-synuclein.

5.4.2 In conclusion

The detection of α-synuclein, and/or of complexed or modified forms in plasma, could offer new opportunities for the development of a diagnostic test for PD or other related synucleinopathies. In this study, parkin was discovered in human plasma, apparently complexed to α-synuclein. The development of a good biomarker for these neurodegenerative diseases would dramatically accelerate research on these disorders. Biomarkers are biological substances that can be used to indicate the presence or onset of a disorder, in contrast to post-mortem diagnosis. A good biomarker should be precise and reliable, and distinguish between normal and disease states and different diseases. In addition to this, a biomarker could be used to track disease progression in diagnosed patients. However, although samples numbers are very small, this study suggests that the quantification of plasma α-synuclein, parkin, or α-synuclein/parkin complex is not a useful diagnostic biomarker for PD, or other synucleinopathies, given the variable amounts of the proteins in question found in both patient and control samples.

This study could suggest that a relatively high level of α-synuclein accompanies a high level of parkin and the α-synuclein/parkin complex results. It is possible that parkin is responsible for either (1) the ubiquitination of excess, damaged or misfolded α-synuclein to ensure it is degraded by the proteasome; (2) to ensure that the proteins are incorporated into inclusion bodies, or equally (3) to excrete the proteins from the cell by vesicular exocytosis. What role Lewy bodies play in the pathogenesis of PD is not yet understood. An interesting analogy can be drawn here. The absence of the E6-AP ubiquitin ligase in the neurological disorder spinocerebellar ataxia type 1 leads to a decrease in the amount of ataxin containing inclusions, and the clinical symptoms and neuronal injury are increased considerably (Cummings et al. 1999). It is possible that in PD, it is parkin that is responsible for the formation of Lewy bodies, a process that is impaired in ARJP where the parkin protein is mutated. It might be unconjugated (to parkin) α-synuclein, that would otherwise have been ubiquitinated, degraded or sequestered into Lewy bodies, that accumulates and leads to cell death.

Interestingly, it has also been shown that the human mutants of α-synuclein, A30P and A53T, demonstrate a decrease in the α-helical structure of the molecule (Li et al. 2002). If this is the case, it may potentially affect α-synuclein/parkin binding. It is

known that patients with these mutations have an earlier onset of PD than sporadic cases (Polymeropoulos et al. 1997). This could be partially be due to a reduction in α-synuclein ubiquitination, leading to a reduction in its degradation by the proteasome and a decrease in the amount sequestered into inclusion bodies or secreted in vesicles. Also, the same may apply to the A46K α-synuclein mutation that has been illustrated to have effects on phospholipid binding capacity and filament formation when compared to wild-type protein (Choi et al. 2004).

The present study confirms previous work (Salem 2004) that has identified α-synuclein as a component of human blood plasma. Also, the data identifies parkin in plasma, and also a protein complex in human plasma that involves parkin and α-synuclein. Further to this, the levels of both proteins, both individually and complexed together, were investigated in blood plasma from patients with Parkinson's and other neurodegenerative diseases. The results suggest that there is a correlation between the levels of expression of α-synuclein, parkin and the complex in individual plasma samples, but no clear relationship when comparing disease and control groups (although the number of samples analysed are small). At present, levels of these proteins would have to be investigated further, within much larger sample groups, to find if they were to be useful as diagnostic indicators. However, the ability to track this complex, using the methods described, will be essential in future work to characterise α-synuclein and the α-synuclein/parkin complex in plasma.

6 Isolation of the parkin/α-synuclein complex from human plasma by chromatography

6.1 Introduction

This study obtained substantial evidence to suggest that α-synuclein and parkin interact in human brain and in human plasma. The ELISA methods previously described give us the ability to track this complex, and would be essential in work to characterise α-synuclein and the α-synuclein/parkin complex in plasma.

This chapter describes the development of protocols for attempting the purification of the α-synuclein/parkin complex, as well as the two individual proteins, by utilising various chromatography techniques.

To determine the molecular weight (M_w) of the α-synuclein/parkin complex, and also the size of the individual proteins themselves, size exclusion chromatography is utilised.

6.2 Size exclusion chromatography

Size exclusion chromatography was used to determine the M_w of the immunoreactive parkin and α-synuclein, as well as any α-synuclein/parkin complex present in brain tissue. A Superdex 200 column was initially used in this study and was calibrated using a set of gel filtration molecular weight protein standards with M_w's of 181 Da to 669 kDa (Sigma), which gave a linear calibration on plot (figure 6.1).

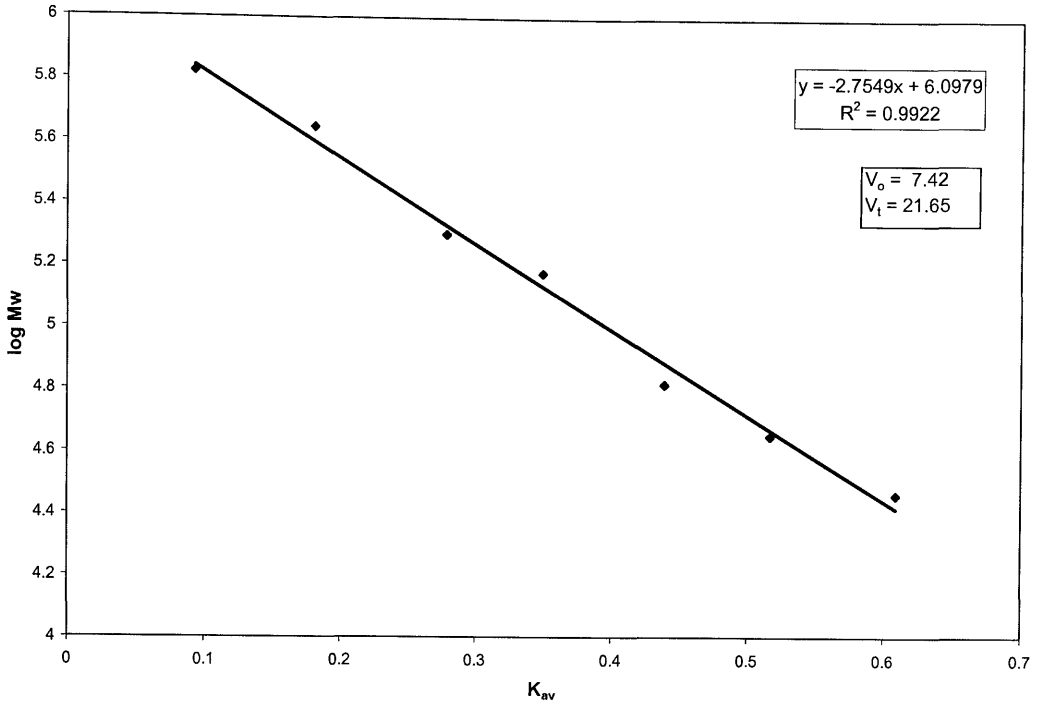


Figure 6.1 Calibration curve for Superdex 200

Graph presented as K_{av} to log MW ($K_{av}=V_e-V_o/V_t-V_o$, where V_e = elution volume, V_o = void volume, V_t = total column volume).

Initially, a 0.1mg sample of recombinant α-synuclein was injected onto the column. This recombinant protein was frozen at -80°C immediately after purification from the *E.coli* lysate, and freeze thaw processes were avoided, to ensure that the protein was in its monomeric form. The peak at 13.77 ml (figure 6.2) gave a K_{av} of 0.45 and a M_w of around 72 kDa. It has previously been reported that synuclein runs at a high molecular weight through size exclusion columns (Lashuel et al. 2002), suggesting a non-globular conformation.

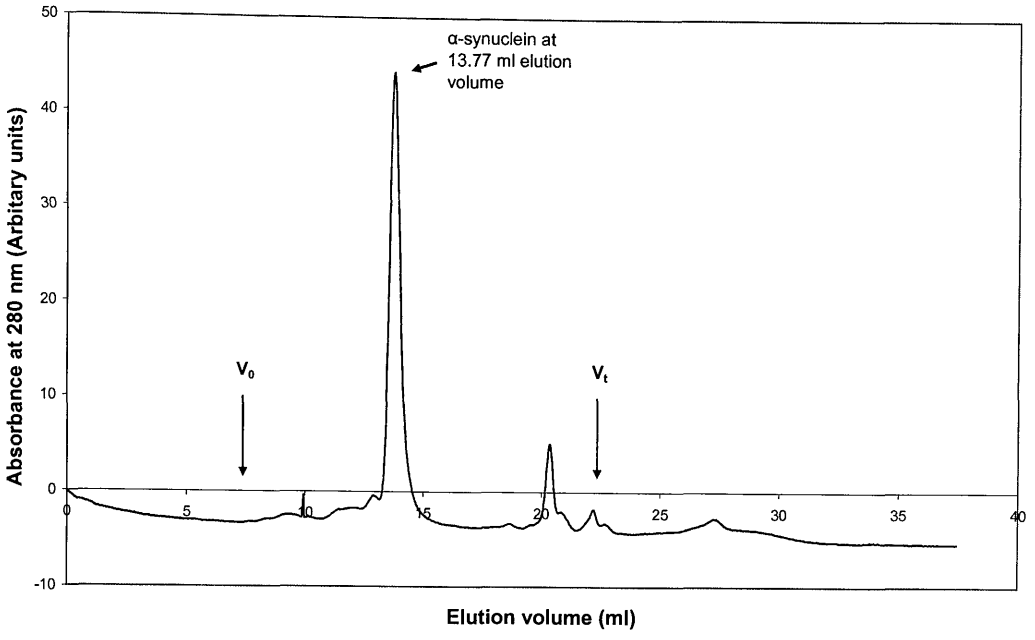


Figure 6.2 UV chromatogram showing application of recombinant α-synuclein and elution of proteins, from the Superdex 200 column

To find the M_w of the complex present in the brain of a DLB patient, we applied a 200µl sample of filtered protein extract from brain tissue to the column, and the resulting fractions were collected. The UV chromatogram revealed a large peak as the tissue extract was applied, from elution volume A10-D10 (figure 6.3). Analysis of these fractions by ELISA, would reveal if the complex could be detected in the eluate from the column. This would give us the V_e for the complex, which would enable the M_w to be calculated.

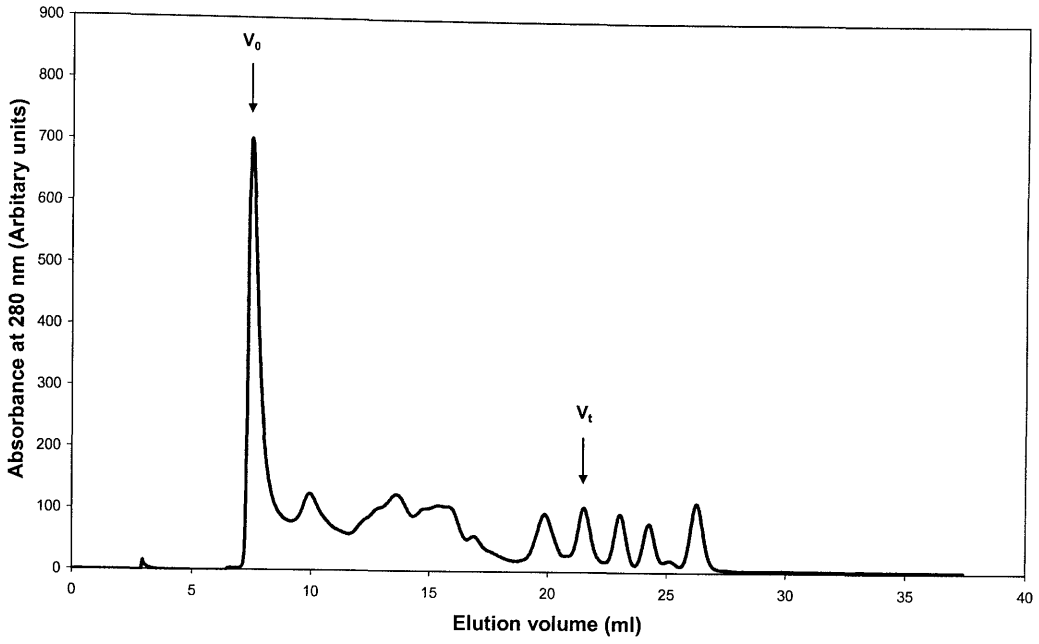


Figure 6.3 UV chromatogram showing application of filtered brain tissue extract and elution of proteins, from the Superdex 200 column

The fractions eluted from the size exclusion column were assayed using the previously developed ELISA's, to track where the α-synuclein, parkin and the complex elute from the column. The results showed that the proteins of interest had voided, i.e. they eluted with the void volume (data not shown). The M_w must have been larger than 600 kDa for this to have happened. It is possible that the proteins of interest were interacting with the many other proteins and/or lipids within the brain tissue sample, which may not be representative of true interactions within the brain. It is also likely that the separation of proteins within blood plasma will be more successful, as there is a lower concentration of lipids present that may interact with α-synuclein.

An AD plasma sample (DA01) that gave a high absorbance reading for the α-synuclein/parkin ELISA's, was centrifuged at 13000 rpm for 30 mins in a 0.45 μm filter, to remove any insoluble material. 250 μl of the plasma was applied to a Superdex 200 column using the previous method. The emerging 0.5 ml fractions were collected and assayed for α-synuclein and for the α-synuclein/parkin complex. Elution

volumes 2-3 ml, which had no protein content (according to the UV chromatogram), were used for the control/background wells on the ELISA plate. As can be seen on the UV chromatogram (figure 6.3), there are several large peaks as proteins are eluted from the column. Elution volume 5-26 ml were assayed by ELISA and the results show that, as with the brain tissue sample, both the α-synuclein and the α-synuclein/parkin complex elute in the same fractions (figure 6.4).

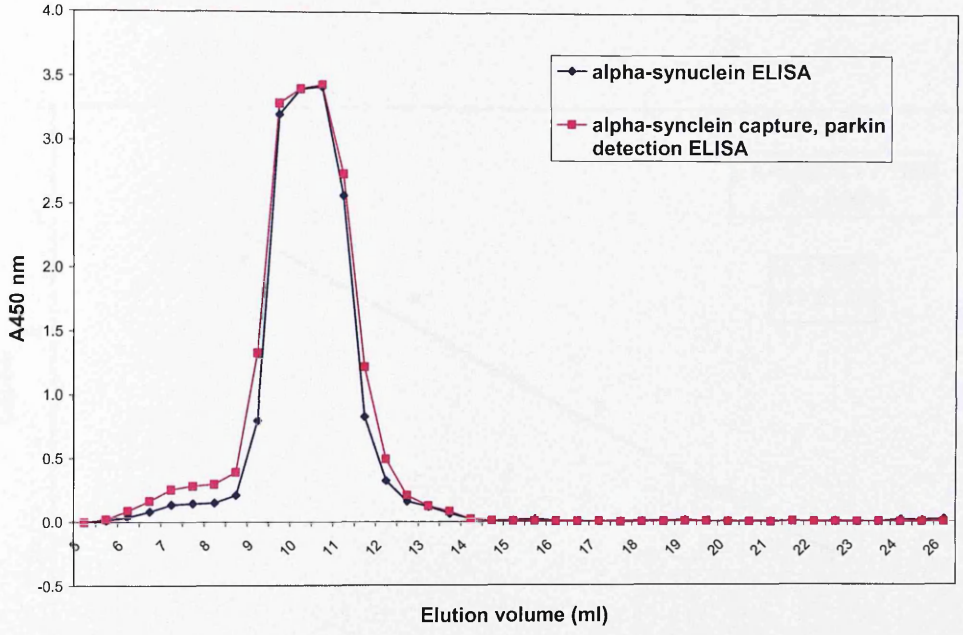


Figure 6.4 To determine the molecular weight of the α-synuclein/parkin complex

Plasma was applied to a Superdex 200 column and the fractions eluted were assayed by ELISA to determine the elution positions of the proteins. Both sets of data reveal that the proteins elute together in the same fractions.

This could suggest several different scenarios. Either; a) all the α-synuclein is interacting with the parkin, b) the α-synuclein and the α-synuclein/parkin complex are both interacting with a separate molecular entity, or c) the protein and the complex

elute independently in the same fractions. The proteins were eluted at approximately 12 ml, which gives a K_{av} of 0.322 and a M_w of 162 kDa.

It was decided to attempt to confirm the M_w of α-synuclein using an alternative size exclusion column. Further chromatography under dissociative conditions, would then be attempted to separate and partially purify the two proteins of interest. The Superose 6 size exclusion column was initially calibrated under the same method and associative running conditions as the Superdex 200 (figure 6.5).

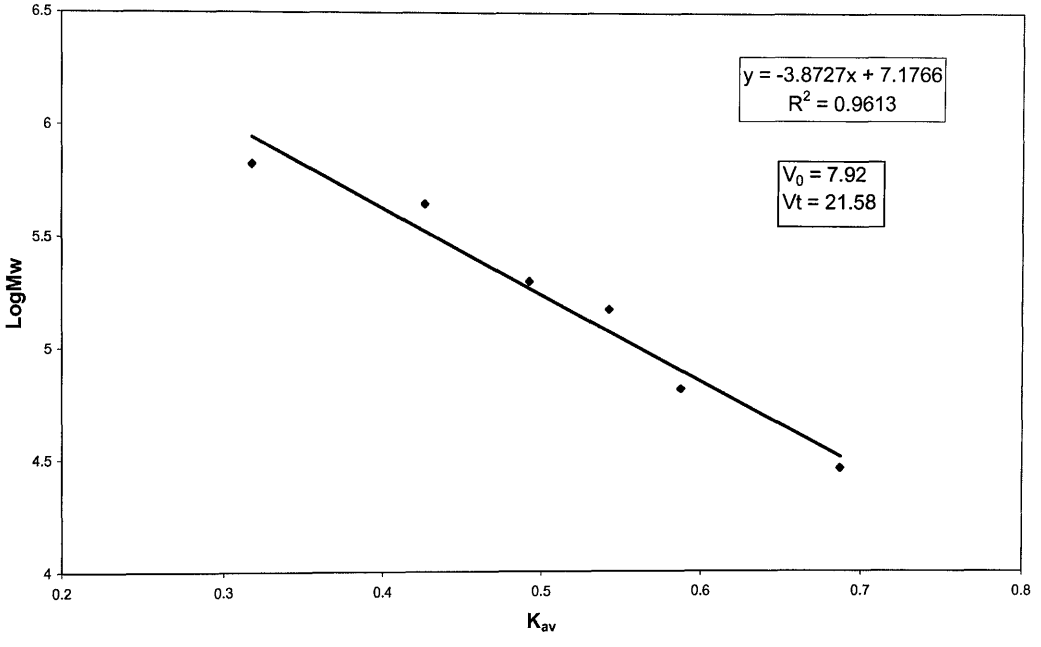


Figure 6.5 Calibration curve for the Superose 6 size exclusion column

The recombinant α-synuclein protein was run through the Superose 6 column, and the K_{av} calculated as 0.551, giving a M_w of 82 kDa (as opposed to the M_w of 72 kDa on the Superdex 200).

Again, the AD plasma sample that gave the high reading absorbance of over 2.5 for the α-synuclein/parkin ELISA's, was centrifuged at 13000 rpm for 30 mins in a 0.45

μm filter, to remove any insoluble material. 250μl of the plasma was applied to the Superose 6 column using the previous method. The emerging fractions were collected and assayed for α-synuclein and for the synuclein/parkin complex. Fractions 2-3 ml, that had no protein content according to the UV detector, were used for the control/background wells on the ELISA plate. As can be seen on the UV chromatogram (figure 6.6), there are several large peaks as proteins are eluted from the column.

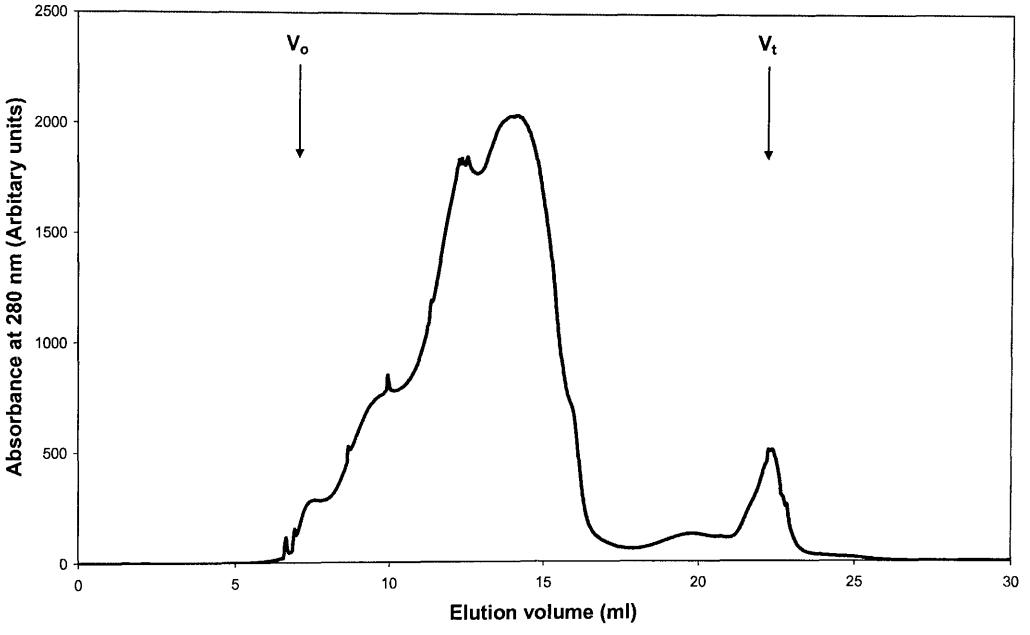


Figure 6.6 UV chromatogram showing flow-through of plasma sample and elution of proteins from the Superose 6 column

Once again fractions 5.0 ml through to 25.0 ml were assayed by ELISA. The results (figure 6.7) show that, as with the brain tissue sample, both the α-synuclein and the synuclein/parkin complex eluted in the same fractions, although this time there are differences in the elution pattern.

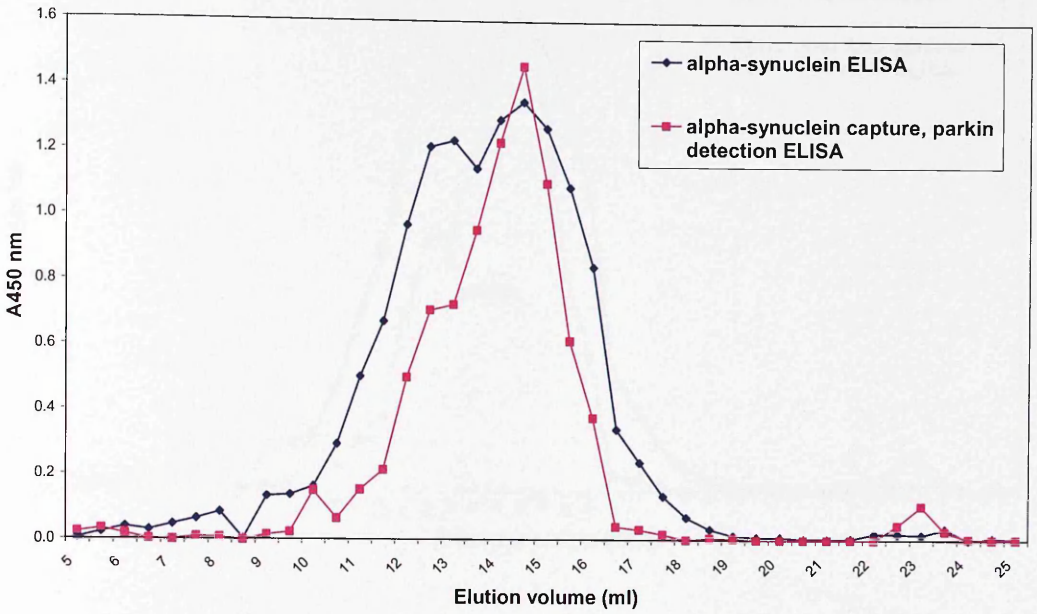


Figure 6.7 To determine the molecular weight of the α-synuclein/parkin complex plasma was applied to the Superose 6 column and the eluted fractions assayed by ELISA

Using the calibration curve for this column, we can estimate the M_w of the "peaks" of the α-synuclein/parkin complex. The main peak at fractions 15.5-16 ml (figure 6.7) suggest that most of the complex is eluting at this position. We can use this to estimate a M_w of approximately 42 – 67 kDa. Figure 6.8 shows the calculated molecular weights for the corresponding peaks on the previous graph.

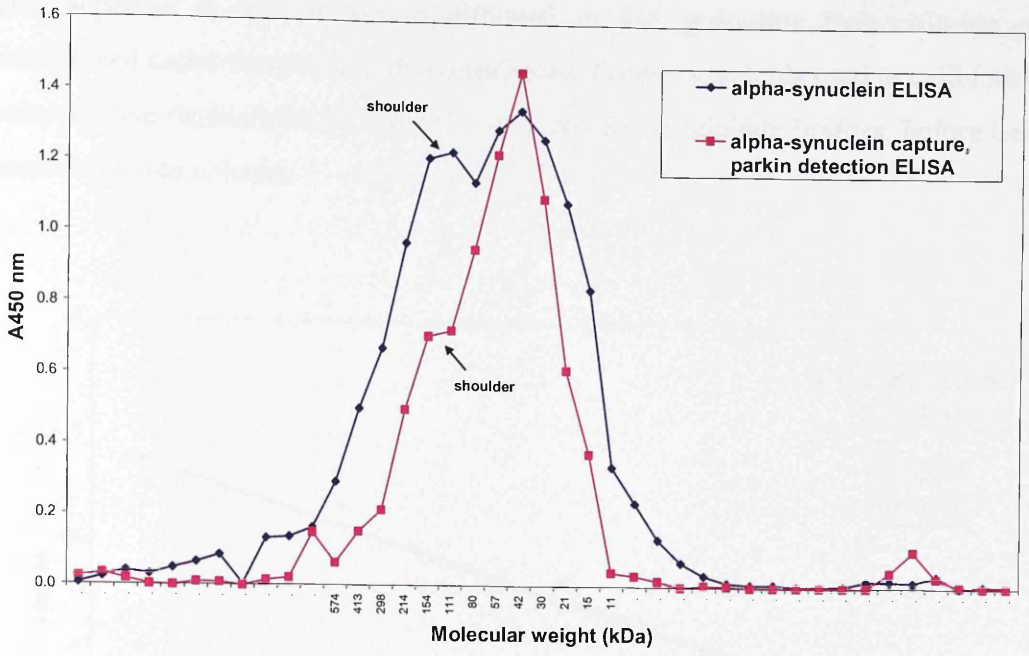


Figure 6.8 The previous graph is replicated along with the molecular weights of the main peak fractions

The peak representing the α-synuclein/parkin complex (figure 6.8 in pink) is the "narrower" of the two peaks. The peak representing the α-synuclein (in blue) is much broader, and encompasses molecules of MW from around 15 kDa to 298 kDa. This is a very large range in M_w values. There is a "shoulder" in this trace (in blue) that follows the α-synuclein peak (in pink), which poses the question whether the right hand, lower M_w peak is the α-synuclein that is together in a complex with parkin. The MW of around 67kDa may point to that possibility. However, the left hand peak is at a larger MW so could not be α-synuclein, unless it was in an oligomeric form or if it was interacting with other molecules. The main complication to this of course, is that the recombinant α-synuclein ran at 82 kDa on this column under these elution conditions. With no explanation to why this is the case, it was decided that further investigations should be continued under dissociative conditions to attempt to minimise or eliminate any protein-protein interactions.

The Superose 6 column was equilibrated in 4M guanidine hydrochloride and recalibrated under these dissociative conditions. Protein standards used to calibrate the column were denatured with 1M DTT to break any disulphide bridges, before being loaded onto the column.

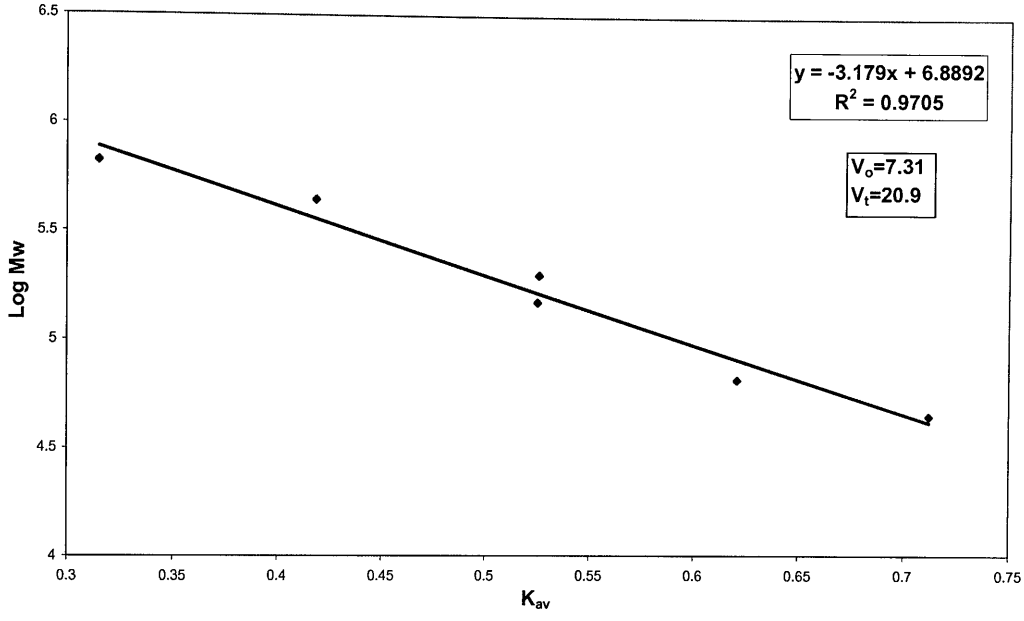


Figure 6.9 Calibration curve for the Superose 6 size exclusion column under dissociative conditions

Again, the recombinant α-synuclein protein was run through the Superose 6 column, this time under dissociative conditions, and the K_{av} calculated as 0.591, giving a larger M_w of 102.5 kDa (as opposed to the M_w of 82kDa on the Superdose 6 under associative conditions).

The same AD plasma sample that gave the high reading absorbance for the α-synuclein/parkin ELISA's, was once again prepared as before, and 250 µl of the filtered plasma applied to the Superose 6 column using the same method. The flow-through fractions were again collected and assayed for α-synuclein and for the synuclein/parkin complex. As can be seen on the UV chromatogram (figure 6.10),

there are several large peaks as proteins are eluted from the column. Once again fractions 5-25 ml were assayed by ELISA. On this occasion, the fractions were diluted three fold in PBS, as the running buffer conditions would otherwise have denatured the ELISA coating antibody, but unfortunately, only the α-synuclein capture, α-synuclein detection ELISA gave data. The results are presented in figure 6.11. This α-synuclein ELISA data revealed that the sensitivity of the assay was much reduced (see figure 6.11, y axis).

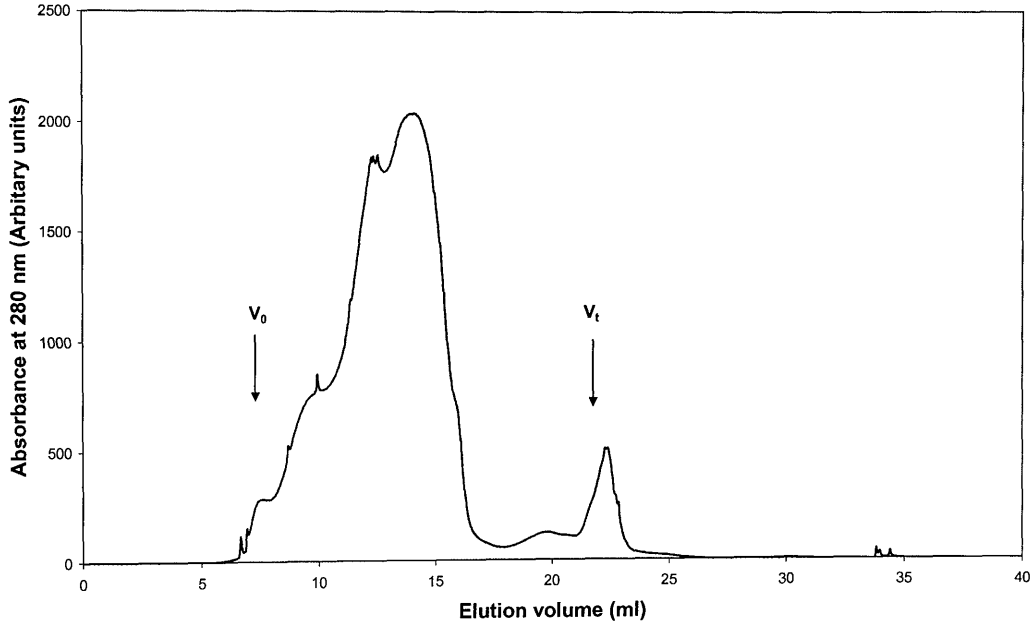


Figure 6.10 UV chromatogram showing flow-through from the plasma sample and elution of proteins from the Superose 6 column under dissociative conditions

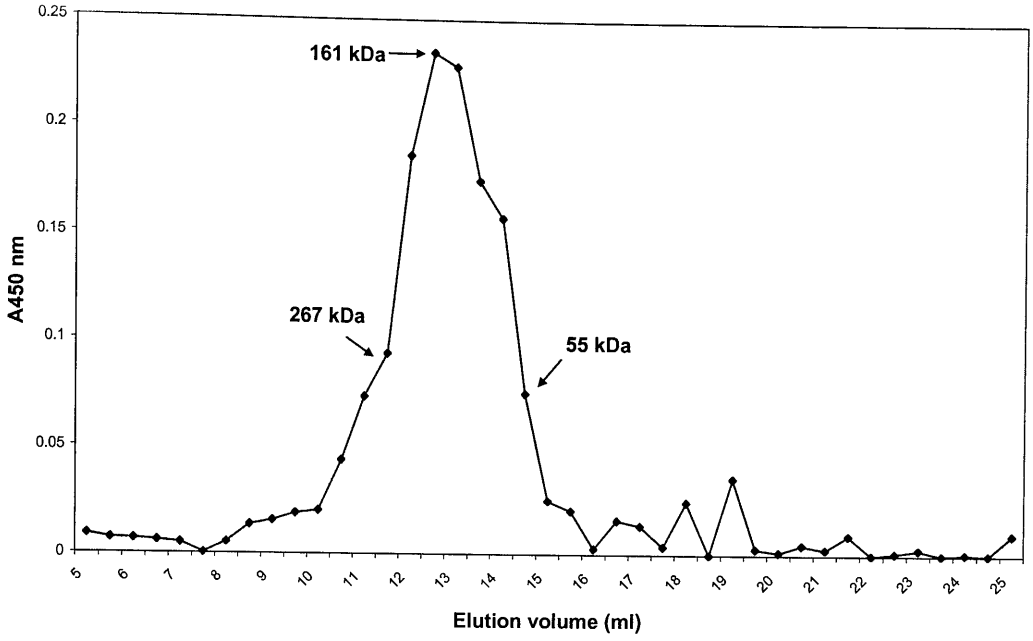


Figure 6.11 To determine the molecular weight of the α-synuclein in plasma, the tissue was applied to the Superose 6 column and the eluted fractions assayed by ELISA

It was noted that there is hardly any difference between the elution properties of the proteins under associative or dissociative conditions (4M guanidine hydrochloride). The parkin ELISA's did not give any data, probably due to the guanidine denaturing the coating antibody, and the actual protein being in such a low concentration within the diluted fractions.

After these investigations, the data was considered. The size exclusion columns provided estimations of the M_w of both recombinant α-synuclein and plasma α-synuclein, under both physiological and dissociative conditions (table 6.1).

Table 6.1 Estimating the molecular weight of both plasma, and recombinant, α-synuclein using two size exclusion columns

	MW recombinant α-syuclein (kDa)	MW plasma α-synuclein (kDa)
Superdex 200	72	162
Superose 6	82	42
Superose 6 (dissociative)	102	161

It was difficult to draw any conclusions from these data. Possible explanations could be such that the α-synuclein is interacting with itself, or with other protein molecules in the plasma, or with lipids in the fluid. It was decided at this point that other chromatographic techniques should be utilised to attempt to partially purify the proteins of interest from the plasma, before attempting to estimate their molecular weights. Other techniques that are not based on separation by molecular size might give useful information on the nature of any 'complex'.

6.3 Ion exchange chromatography

Ion exchange chromatography is based on the binding of charged sample molecules to oppositely charged groups attached to an insoluble matrix. Substances bind when they carry a net charge opposite to that of the ion exchanger (the binding is electrostatic and reversible). When exposed to a pH below its isoelectric point, the molecule will carry a positive charge and would bind to a cation exchanger. At a pH above its pI, the molecule will be negatively charged and would bind to an anion exchanger.

Anion exchange chromatography with a Q Sepharose Fast Flow HiTrap column was chosen for the first round of the purification of α-synuclein, parkin and the α-synuclein complex, from the human plasma sample. This step was selected because of

the isoelectric points of the proteins of interest. The isoelectric point (pI) is the point at which a particular molecule or surface carries no net charge. Amphoteric molecules called zwitterions contain both positive and negative charges depending on the functional groups present in the molecule, in this case the amino acids. They are affected by pH of their surrounding environment and can become more positively or negatively charged due to the loss or gain of protons (H+). Parkin has a pI of 6.71 and α-synuclein a pI of 4.67. Theoretically, both should adhere to an anion exchange column, by electrostatic interactions, run in a buffer of pH 8, and would be eluted with an increasing salt gradient. A continuous salt gradient is usually used. The Q Sepharose column was set with a flow rate of 1 ml/min, injecting 30 µl of high complex concentration plasma sample (approximately 2 mg total protein) diluted with an equal volume of buffer A.

The UV chromatogram at 280 nm showed a peak where proteins voided/did not adhere to the column, and a peak of proteins that were eluted by the salt gradient. The fractions from this run were analysed by ELISA to track where, in the progressing salt gradient, each protein of interest and the complex were eluted from the column.
Analysis of the fractions showed that the proteins did not adhere to the column as expected (figure 6.12). However, 0.5 mg of recombinant α-synuclein, run under the same conditions, did interact with the column, eluting at a salt concentration of 0.35 M NaCl (11.7 ml).

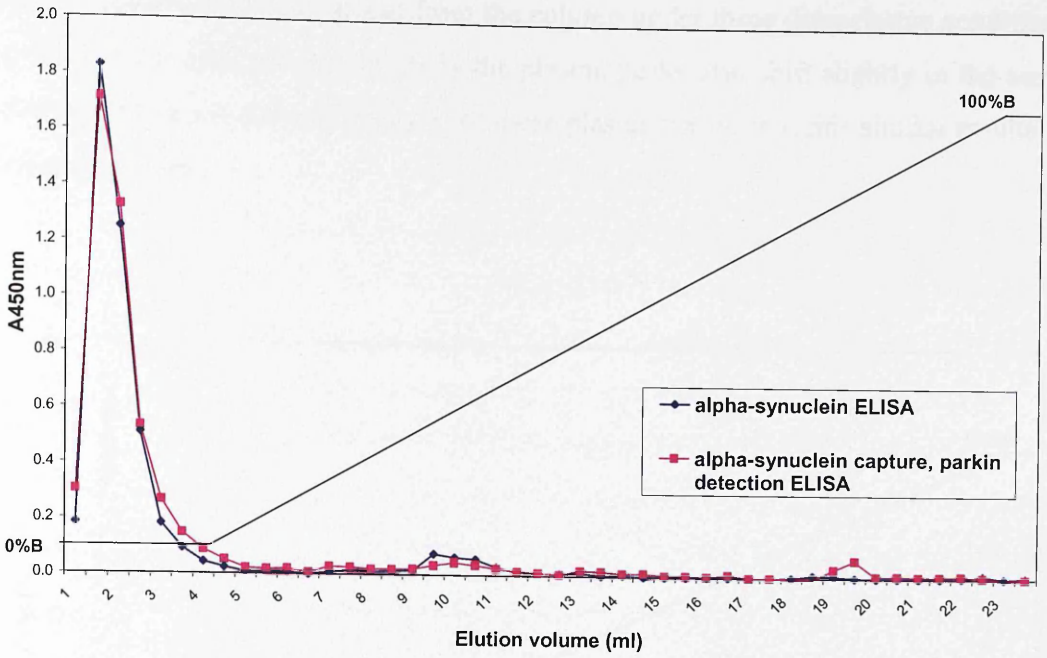


Figure 6.12 Q Sepharose FF HiTrap fractions from a plasma sample that assayed as containing a relatively high α-synuclein/parkin complex

The column was run under physiological conditions. Fractions were assayed in two separate ELISA's to track where the α-synuclein and the α-synuclein/parkin complex elute from the column.

The α-synuclein ELISA (figure 6.12, in pink) reveals that most of our protein is not binding to the column, although there is a small amount eluting around 10 ml, at around 0.25 M NaCl. The α-synuclein complex is also eluting with the void volume of the column, although there is also a small amount eluting at 10 ml, there is also a small amount released from the column at 20 ml, around 0.75 M NaCl.

One explanation as to why the plasma α-synuclein and parkin did not adhere to the anion exchange column is that they are interacting with other plasma proteins or lipids which are altering the pI of the whole protein complex to such an extent that it was not bound by the column. Therefore, it was decided to run the same anion column under the dissociative conditions of 7M urea, in an attempt to separate any protein complex interactions.

Recombinant α-synuclein eluted from the column under these dissociative conditions at 0.3M NaCl (10.2ml) and similarly the plasma peaks also shift slightly in the same direction (data not shown). Analysis of these plasma fractions yields similar results to the previous run.

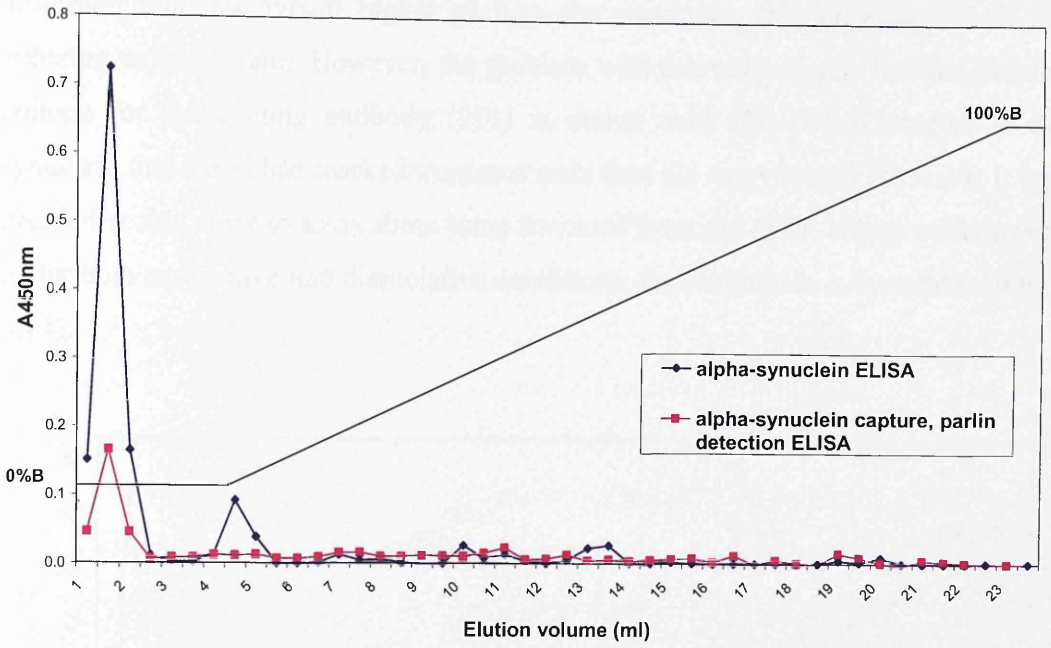


Figure 6.13 Q Sepharose FF HiTrap fractions from a plasma sample that assayed as containing a relatively high α-synuclein/parkin complex

The fractions were assayed in two separate ELISA's to track where the α-synuclein and the α-synuclein/parkin complex elute from the column.

It was noted immediately that the strength of the absorbance readings is reduced for these assays, probably due to the urea denaturing the ELISA coating antibody.

Although the urea should dissociate the protein interactions this does not seem to be the case. The α-synuclein is not binding to the column (Figure 6.13, in blue). If the α-synuclein was monomeric, it should all bind to the column and then elute at the same salt concentration as the recombinant protein. This is not the case. Also, the α-synuclein/parkin complex has not been dissociated, and it is not binding the column. It

is possible that the complex has a pI greater than 7, and therefore would not bind an anionic column. One possible explanation to this scenario could be that as the N or C terminal of the protein is heavily negatively charged with aspartates and glutamates, and as this terminus may be involved in the multimerisation, it may therefore be "masked". The overall pI would increase and the protein would not bind to the column. If the protein was truncated at this end, or if the α-synuclein was a very stable multimer, with an overall higher pI than the monomer, this may explain it not adhering to the column. However, the problem with this reasoning is the fact that the epitope for the coating antibody (211) is amino acid 120-125. Therefore if the synuclein molecules had masked/truncated ends then the assay would not work. It was decided at this point to assay these same fractions from the Q FF Hitrap column, run under both associative and dissociative conditions, for multimeric α-synuclein (figure 6.14).

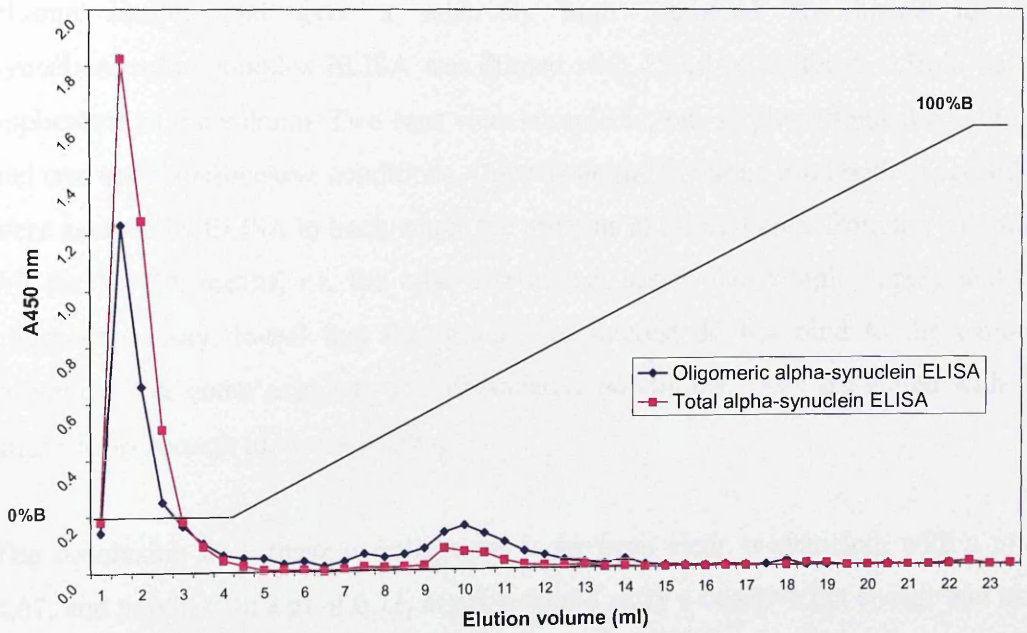


Figure 6.14 Q Sepharose FF HiTrap fractions from a plasma sample that assayed as containing a relatively high α-synuclein/parkin complex

The fractions were assayed in another separate ELISA to determine whether the α-synuclein was in a monomeric or an oligomeric form.

The two assays mirror each other. This suggests that the total synuclein is in an oligomeric form. It may be the case that the "multimer" may be composed of just two α-synuclein molecules, together with many other proteins. It may not necessarily be aggregating with itself. However, the main question that arises here is why the α-synuclein is not binding the column. Is it urea stable? The protein complex is not SDS stable, as treatment allowed separation of the complex on analysis by SDS PAGE (Chapter 5). (The ELISA's measuring oligomeric and monomeric α-synuclein, in the flow-through fractions, when the column is run under dissociative conditions gave no data. This is presumably due to the buffer denaturing the coating antibody.)

It follows that if the proteins of interest do not adhere to an anionic exchange column as expected, then they would be attracted to a cationic column, at the same pH. A cation exchange column, SP Sepharose FF HiTrap column was utilised. The same method was employed as before, with the identical buffers. Again, 150 µl of the plasma sample that gave a relatively high measured absorbance for the synuclein/parkin complex ELISA was diluted with 150µl of buffer A, 15min before application to the column. Two runs were completed, one in physiological conditions and one under dissociative conditions. Once again the fractions from both experiments were assayed by ELISA to track where the proteins of interest elute from the column. All the ELISA results, i.e. the total α-synuclein assay, the complex assay and the oligomeric assay, reveal that the proteins of interest do not bind to the cationic column, either under associative or dissociative conditions. They are eluted with the initial flow-through (data not shown).

The conclusion from these investigations is far from clear. α-synuclein with a pI of 4.67, and parkin with a pI of 6.71, at pH 8 should carry a negative net charge and bind to an anion exchanger. This is not happening. Neither are they binding to a cation exchanger. An explanation as to why this may be the case could be the proteins are "shielded" by other molecules, for example in lipid micelles present in the blood plasma. With this scenario in mind, it was decided to repeat the experiments, but this time including the non-ionic detergent, Nonidet P-40, in the buffers, at a concentration of 1.5%. NP-40 is used in many extraction protocols to solubilise membranes. It has a polar polyethylenglycol chain and a non-polar end, leading to the formation of lipids trapped in water-soluble micelles. The 150 µl plasma sample was incubated in 150 µl

running buffer A, for 2 h at room temperature before the chromatography, to ensure any protein/lipid interactions had been dissociated. The fractions from this chromatography were once again assayed by ELISA to detect the total α-synuclein, total parkin and the α-synuclein/parkin complex (figure 6.15).

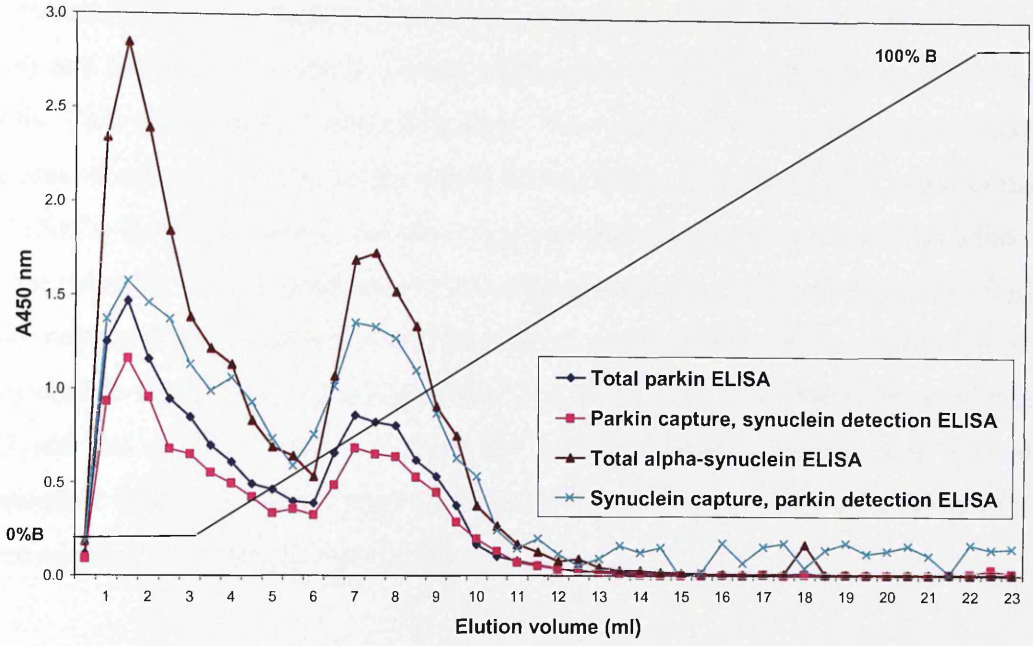


Figure 6.15 Q Sepharose FF HiTrap fractions from the plasma sample run under associative conditions, with a non-ionic detergent, 1.5% NP-40

The fractions were assayed in four separate ELISA's to track where the α-synuclein, the parkin and the α-synuclein/parkin complex elute from the anionic column.

For the first time, it appears that the proteins of interest have bound to the anion exchange column. A proportion of the proteins still did not interact with the anion exchanger, eluting straight through the column in elution volume 0-5ml, but then a significant amount have bound, eluting at around 0.25 M NaCl, in elution volume 6-10 ml. The α-synuclein, the parkin and the α-synucelin/parkin complex have eluted

from the column in the same fractions. This could mean that that the proteins/protein complexes have a similar pI. Alternatively, it is possible that they are still interacting with each other. With the latter scenario in mind, it was decided that the fractions that contained the proteins that interacted with the column should be pooled and applied to the same anionic column, this time under the dissociative conditions of urea.

The previous run was repeated, and this time the fractions that contained the proteins of interest were pooled together (elution fractions from 6-9 ml, around 4 ml sample in total) and dialysed overnight in a Slide-a-lyser, M_w cut off of 3500 Da, in 4 M urea buffer. This was to achieve three objectives. The volume of the sample was reduced, the urea introduced into the sample was to initiate the protein dissociation and also the salt (NaCl) from the previous run was removed (thus allowing the proteins to adhere to the column). The dialysed sample was loaded onto the anionic Q Sepharose Fast Flow column using the previous method, and once again, the fractions collected were subjected to the tracking ELISA's. Before the fractions were assayed, the urea was dialysed out so that it did not affect the ELISA coating antibody. The 1.5 ml Eppendorf tubes that were utilised to collect the fractions from the chromatography were adapted for overnight dialysis (figure 6.16).

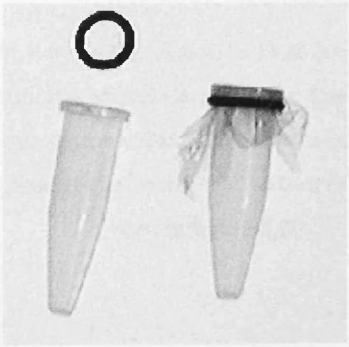


Figure 6.16 The 1.5 ml Eppendorf tubes were adapted for dialysis

The caps were removed. A small piece of dialysis membrane (M_w cut off 3.5 kDa) that had been previously soaked in water, was placed in between the tube and the O-ring, and the tubes then suspended upside down in a large 3 l beaker of distilled water, checking that the sample was in contact with the membrane.

The 46 tubes were suspended upside down in 3 l of water, which was changed 3 times over the course of 24 hours. Following this procedure, the fractions were assayed. The results for each assay are showed in figures 6.17 to 6.19, with figure 6.20 representing a comparison of all three ELISA's.

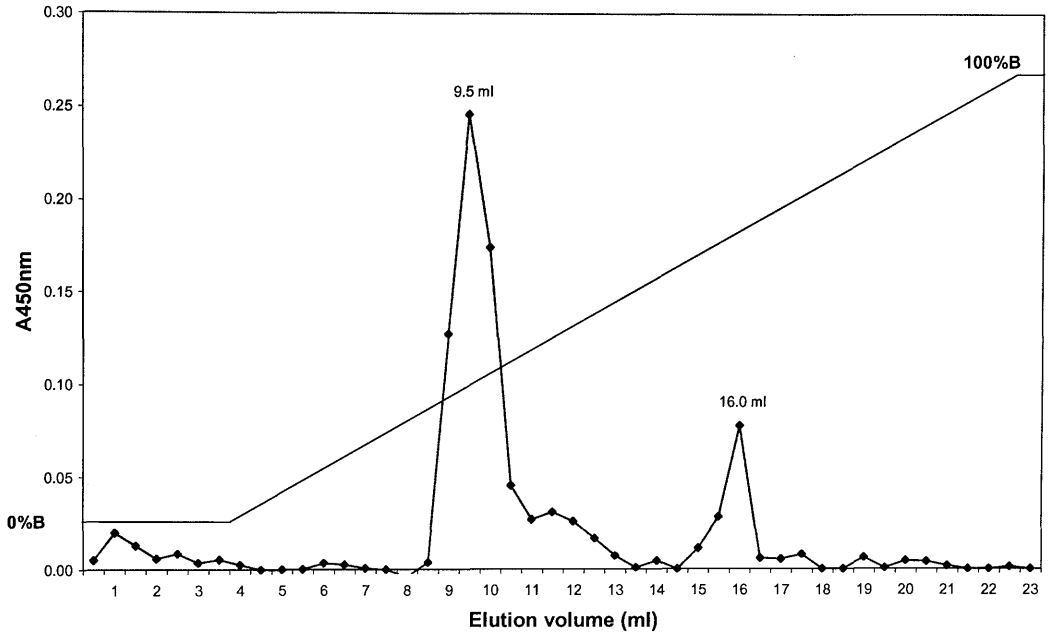


Figure 6.17 ELISA data for total α-synuclein

Analysis by α-synuclein capture, α-synuclein detection ELISA of the flow through fractions from the Q Sepharose FF HiTrap column, run initially in non-ionic detergent, followed by the dissociative conditions of 7 M urea. The peak elution volumes are shown and the NaCl gradient is represented. (It should be noted here that the peak at elution volume 16.0ml is a 'real' peak, as this cannot be easily seen in figure 6.20.)

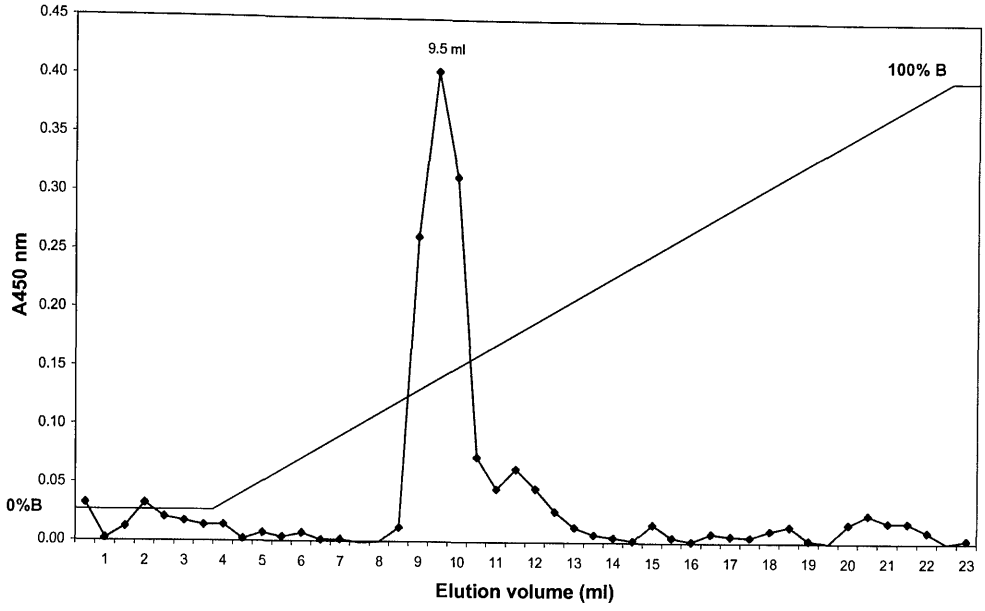


Figure 6.18 ELISA data for α-synuclein/parkin complex

Analysis by α-synuclein capture, parkin detection ELISA of the flow through fractions from the Q Sepharose FF HiTrap column, run initially in non-ionic detergent, followed by the dissociative conditions of 7 M urea. The peak elution volume is shown and the NaCl gradient is represented.

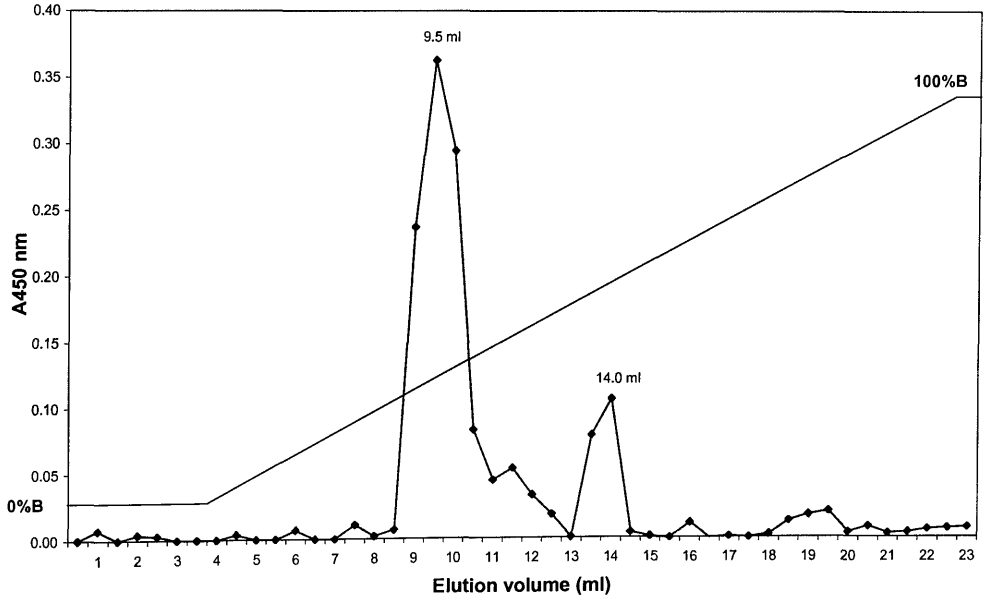


Figure 6.19 ELISA data for total parkin

Analysis by parkin capture, parkin detection ELISA of the flow through fractions from the Q Sepharose FF HiTrap column, run initially in non-ionic detergent, followed by the dissociative conditions of 7 M urea. The peak elution volumes are shown and the NaCl gradient is represented.

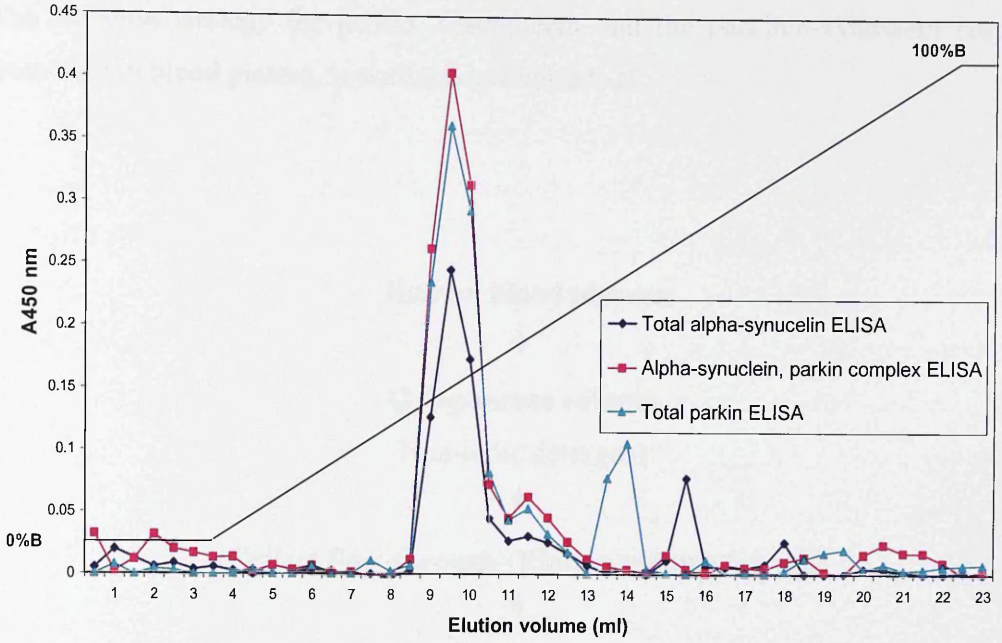


Figure 6.20 Comparison of ELISA data

The previous three individual plots (figures 6.17-6.19) are included to highlight the fact that although α-synuclein, parkin and the complex are eluting together in the same elution volume, they also are eluting from the column separately.

As can be seen from the ELISA data, the proteins all interacted with the anion exchange column. The peak at fraction 9.5 ml represents the α-synuclein/parkin complex eluting from the column at around 0.35 M NaCl. It appears that the urea has dissociated some of the protein complex into the individual proteins. Parkin elutes at 14.0 ml (0.6 M NaCl) and α-synuclein at the higher salt molarity at fraction 16.0 ml (0.7 M NaCl). By increasing the salt concentration, the molecule with the weakest ionic interactions (parkin) was disrupted first and elutes earlier in the salt gradient. The molecules that have a stronger ionic interaction (α-synuclein) require a higher salt concentration and elute later in the gradient.

6.4 *Overview of the isolation strategy*

The isolation strategy for parkin, α-synuclein and the parkin/α-synuclein complex from human blood plasma, is outlined in Figure 6.21.

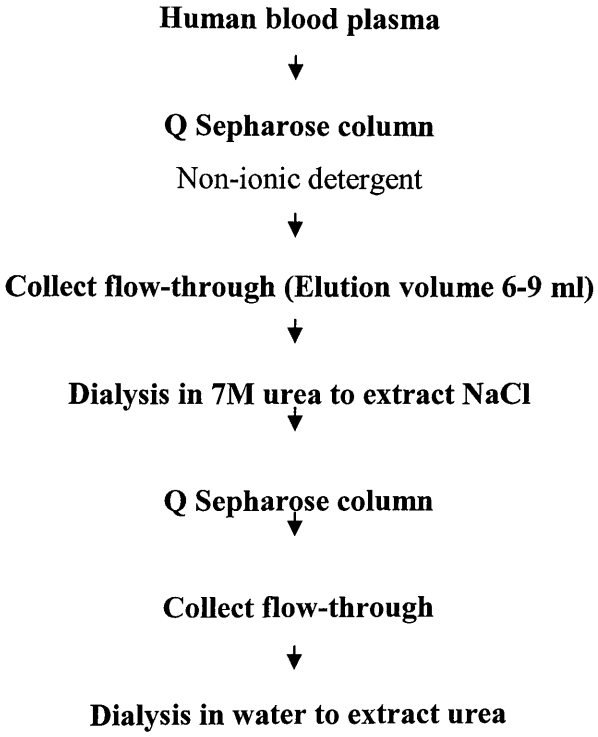


Figure 6.21 Flow-diagram outlining the strategy for isolating parkin, α-synuclein and the parkin/α-synuclein complex from human blood plasma

6.5 Gel filtration chromatography repeated

The whole strategy was repeated. The peak from the last anion exchange column containing the α-synuclein/parkin complex, at elution volume 9-10.5 ml, was loaded on the Superpose 6 gel filtration column, under both associative and dissociative conditions. The information gained from doing this may suggest whether it was probable that the two proteins are; a) migrating together because they are interacting with lipid, or b) if they are interacting with each other. The above process was once more repeated and the resulting dialysed fractions were reduced in volume by vacuum centrifugation to around 50 µl/tube (total 150 µl). This sample was loaded onto the Superose 6 column, using the same method as before. However, before the sample was run, the Superose 6 gel filtration column is recalibrated for accuracy, giving an R^2 value of 0.9552, and an equation to calculate the M_w of the unknown as $y = -3.4393x + 6.7419$.

The sample was loaded onto the Superose 6 column. The UV chromatogram (at high resolution) reveals a small peak at 14.57 ml and another at 16.01 ml. The 0.5 ml fractions are assayed by ELISA to track when our proteins of interest are eluting from the column. Only two assays gave data; the total α-synuclein (figure 6.22) and the α-synuclein capture, parkin detection assays (figure 6.23).

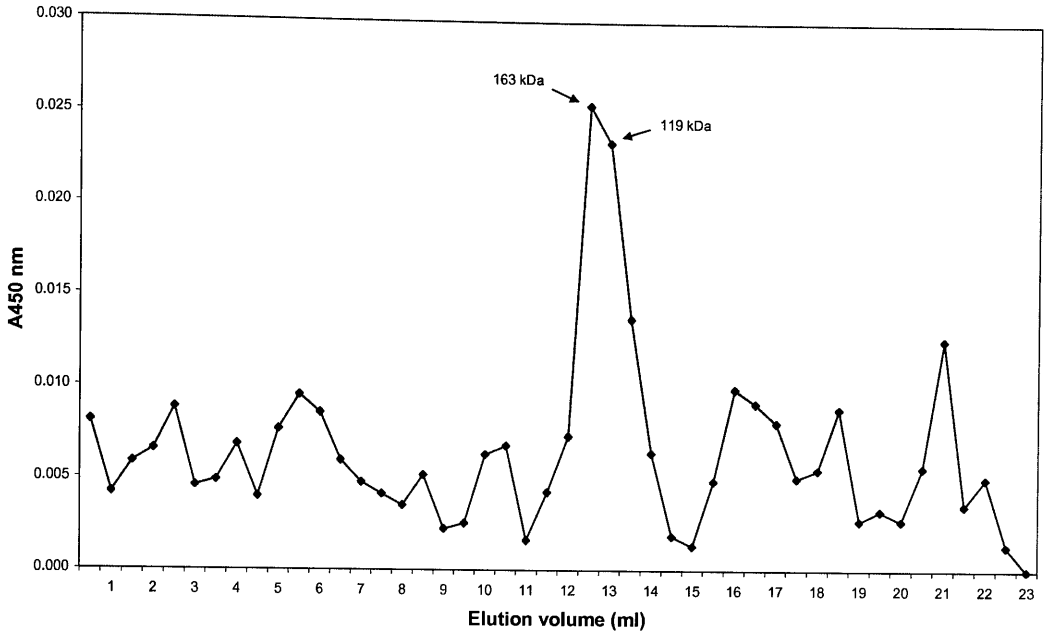


Figure 6.22 Total α-synuclein ELISA data from the protein isolation process and subsequent Superose 6 gel filtration chromatography

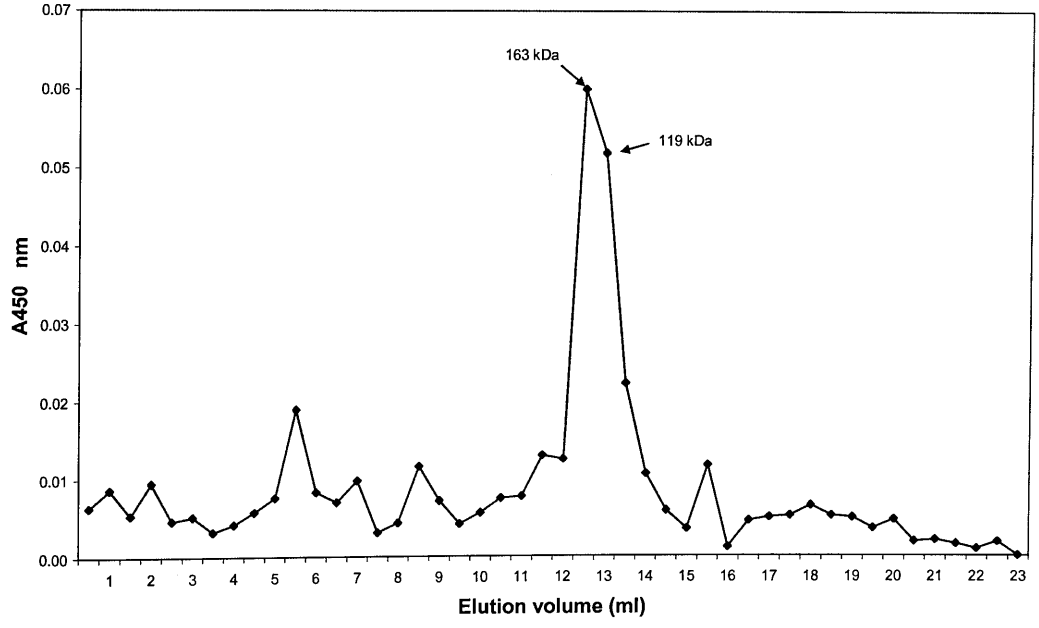


Figure 6.23 Total α-synuclein/parkin complex ELISA data for from the protein isolation process and subsequent Superose 6 gel filtration chromatography

The α-synuclein is detectable although the measured absorbtion value is much reduced (<0.025), the data revealing that the protein is eluting at 12.5-13 ml. The complex is also just detectable, and also eluted in these fractions. These fractions correspond to a peak on the chromatogram at 14.57 ml, and the calibration curve equation gives the molecular weight of the protein complex to be 156 kDa.

Recombinant α-synuclein was run through the column again, eluting at 15.69 ml, which corresponds to 77 kDa.

The M_w of the complex at 156 kDa is small and does not suggest a lipid interaction, rather a small number of units.

6.6 Discussion

Application of plasma to an ion-exchange chromatography column allows the separation of the complex and the individual proteins. The previously developed ELISA's track the constituent proteins and the complex as they are eluted from the column. The large peak on figure 6.20 contains both α-synuclein and parkin, the proteins co-eluting as they interact to form a complex. The smaller peaks to the right are the elution positions of the individual proteins themselves.

The molecular weight of the complex was investigated using gel-filtration chromatography, and was estimated at **156 kDa**. Parkin has a molecular weight of 53 kDa and recombinant α-synuclein runs at 77 kDa, (although the protein is only 14.4 kDa, it is known to run larger, as previously discussed). This data suggests that the complex is a single species with a simple molecular composition, for example a 1:1 complex of α-synuclein and parkin.

Very recently, Kawahara *et al.* investigated whether α-synuclein accumulation might be involved in PD pathogenesis by interfering with the solubility of parkin (Kawahara

et al. 2008). They concluded that α-synuclein and parkin co-immunoprecipitate in neuronal cell lines, and confirmed the association in neuronal cells by performing co-immunoprecipitation experiments. This was achieved by α-synuclein being immunoprecipitated from a neuronal cell extract with an anti-α-synculein antibody, followed by immunoblotting with an anti-parkin antibody, and then performing the experiment the other way around. The authors suggest that this evidence supports the possibility that interactions between α-synuclein and parkin lead to the formation of insoluble aggregates that could possibly damage the neuronal cytoskeleton, rather than impair the function of parkin. Unfortunately, the nature/size of the α-synuclein that interacts with parkin was not investigated.

To conclude, this work has confirmed that α-synuclein and parkin do interact in biological samples. For the first time, the existence of parkin and the complex has also been demonstrated in human plasma. These latter findings may prove to be useful as a method to attempt purification and full analysis of the nature and composition of the complex. It is of interest that these two molecules interact, and it is important to understand the roles of α-synuclein and parkin, and their interactions, in health and disease. It is possible these findings may be useful in the diagnosis of PD and other neurodegenerative diseases.

7 An ELISA method for detection for TDP-43

7.1 Introduction

In 2006 it was discovered that the TAR DNA binding protein (TARDBP), TDP-43, was the target protein in the ubiquitinated cytoplasmic inclusions in neurones of certain histological forms of frontotemporal lobar degeneration (FTLD), known as FTLD-ubiquitinated (FTLD-U), and also in motor neurone disease (MND) (Arai et al. 2006; Neumann et al. 2006). Prior to this discovery, little was known of the function of this protein apart from it having a role in nuclear transcription in relationship to alternative splicing or exon skipping (Buratti et al. 2004; Wang et al. 2004). Now, the involvement of TDP-43 in the ubiquitinated lesions of FTLD-U and MND has been fully confirmed (Cairns et al. 2007; Cairns et al. 2007; Davidson et al. 2007; Mackenzie et al. 2007; Tan et al. 2007). Importantly, TDP-43 lesions are characteristically seen in patients who have inherited forms of FTLD, for example those with mutations in the progranulin gene (*PGRN*) (Boeve et al. 2006; Pickering-Brown et al. 2006; Snowden et al. 2006; Mackenzie 2007). Conversely, in those patients with other inherited forms of FTLD, such as those with mutations in the tau gene (*MAPT*), or those with sporadic illness associated with Pick body type histology (where the underlying histology is based on aggregated tau proteins), no such TDP-43 pathology is seen (Davidson et al. 2007). It is also of note that other studies have shown that TDP-43 pathological changes can also be present in around a quarter of patients with AD (Amador-Ortiz et al. 2007), and also prevalent in patients with DLB (Nakashima-Yasuda et al. 2007), or with parkinsonism-dementia complex of Guam (Hasegawa et al. 2007).

Therefore, it is conceivable that a pathological involvement of TDP-43 in neurodegenerative disease could far outstretch the confines of FTLD-U and MND. Clinically, around 70% of FTLD patients show behavioural and personality changes, which together are known as frontotemporal dementia (FTD) (Neary et al. 2005).

Pathological studies, both prospective and retrospective, have suggested that around half of all FTD cases are either tau-positve, TDP-43-negative histology, the other half being ubiquitin-positive, TDP-43-positive (Lipton et al. 2004; Mott et al. 2005; Shi et al. 2005; Mackenzie et al. 2006; Davidson et al. 2007). At present, it is not possible to differentiate which group a patient with FTD would belong to, based on clinical symptoms alone. Only when a patient with FTD has inherited either a *MAPT* or *PGRN* mutation can tau or ubiquitin/TDP-43 based histology can be inferred. Theoretically, blood levels of TDP-43 may serve as a biomarker that could distinguish between histological subtypes of FTLD. This would be important in the future development of drugs aimed at preventing neuronal lesions in these diseases. To that extent, an ELISA-based assay has been developed to test whether TDP-43 can be detected in the blood of patients with FTD or AD.

7.2 Identifying suitable antibodies to detect TDP-43

An ELISA to detect TDP-43 was developed. Initially, suitable antibodies for detecting the protein in plasma were identified. The two available antibodies that were specific for TDP-43 were investigated in the roles of both capture and detection. Subsequently, the optimal conditions for a TDP-43 ELISA assay were investigated. The antibody concentrations used were the manufacturers' recommended starting dilution for ELISA analysis. Anti-TDP rabbit polyclonal antibody was diluted to 1:100, and anti-TDP-43 mouse monoclonal diluted to 1:200. Recombinant TDP-43 protein was diluted to 0.05 μg/ml and 0.005μg/ml, and utilised in the investigation, along with PBS as a blank control. The combinations of the two antibodies, both in capture and detection, are shown in figure 7.1. (The PBS, which was used as a negative control, gave background absorbance that was deducted from the absorbance values from the plasma samples, before they were plotted.)

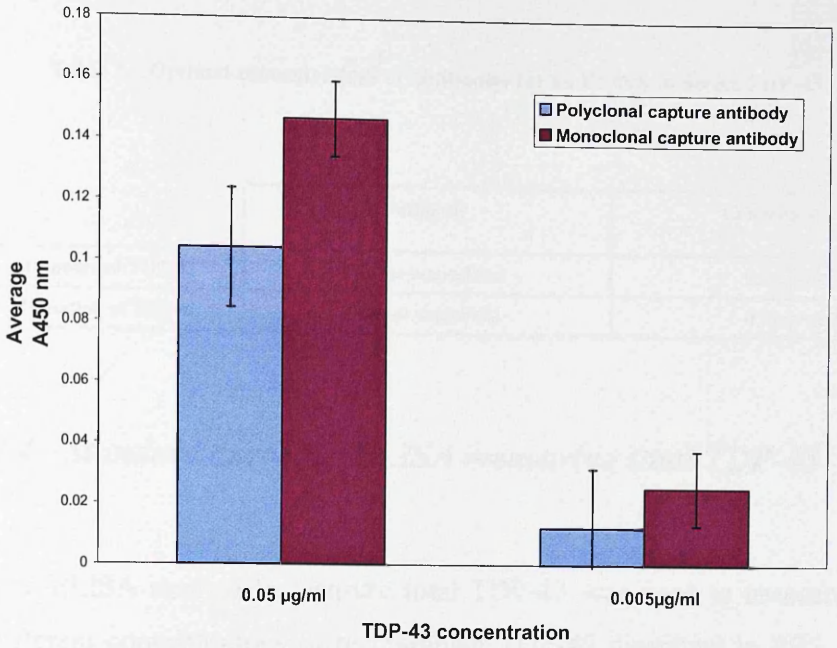


Figure 7.1 Optimisation of antibodies for TDP-43 ELISA

The results revealed that the best combination of antibodies for the TDP-43 detection assay was the monoclonal antibody for capture, and the rabbit polyclonal antibody as the primary antibody for detection (figure 7.1).

7.3 Optimising the antibody concentrations

Experiments were undertaken to determine the difference in ELISA detection/signal levels, when the capture antibody and the detection antibody are at different concentrations. The concentrations investigated were taken from the manufacturers' recommended dilution ranges for ELISA's. They were 1:100, 1:200, 1:1000, 1:10 000 and 1:20 000. Recombinant TDP-43 at a concentration of 0.05µg/ml was captured then detected with the different combinations of the different antibodies. The optimal concentrations for each antibody giving the best signal to noise ratio, in both ELISA's, are summarised in table 7.1.

Table 7.1 Optimal concentrations of antibodies for an ELISA to detect TDP-43

	Antibody	Concentration (Dilution)
Capture of TDP-43	Mouse monoclonal	0.2 µg/well (1:1000)
Detection of TDP-43	Rabbit polyclonal	0.2 µg/well (1:1000)

7.4 Standard curve for ELISA measuring total TDP-43

The ELISA method to measure total TDP-43 was used to measure absorbencies at different concentrations of recombinant TDP-43 dissolved in PBS, ranging between 0.001 µg/ml and 2µg/ml. The recombinant α-synuclein was captured with mouse monoclonal anti-TDP-43 and detected with rabbit anti-TDP-43 antibody. This data was used to create a standard curve (figure 7.2).

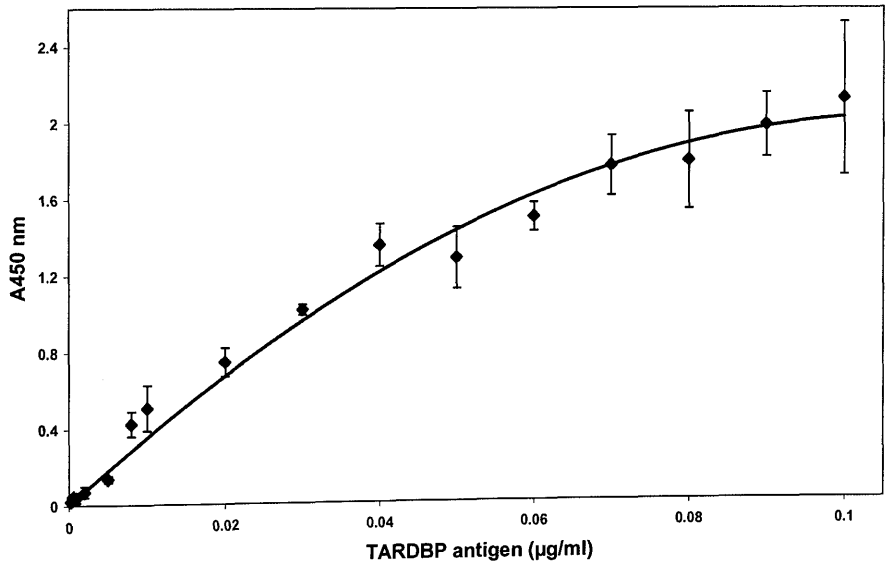


Figure 7.2 Standard curve for the TDP-43 ELISA using recombinant TARDBP antigen (µg/ml) at known concentrations from 0.0003 µg/ml to 0.1 µg/ml

The ELISA standard curve, polynomial (order 2), is relatively linear between the TARDBP antigen concentration ranges of 0.0003–0.1 μg/ml. At very low concentrations the assay is not accurate, and at values above 0.1 μg/ml the ELISA plateaus off as the assay starts to saturate. This standard curve will enable an estimation of the TDP-43 concentration in each blood plasma sample.

It was noted however, that these values may not be a true estimation of TDP-43 concentration, as the recombinant TDP-43 is dissolved in PBS alone, i.e. not in the presence of other proteins. The other proteins present in plasma may have an inhibitory effect on the assay. A more biologically representative standard curve would be gained if the recombinant TDP-43 was assayed, dissolved in a plasma sample that had previously been determined as lacking TDP-43.

7.4.1 ELISA TDP-43 detection levels in PBS, and human plasma

To determine whether the other proteins in biological samples, for example blood albumins, effect the sensitivity of the measured ELISA absorbance value, known concentrations of the recombinant TDP-43 protein ranging from 10 μg/ml down to 0.1 ng/ml were dissolved in PBS and assayed for TDP-43 as previously described. Each concentration was prepared in triplicate to calculate a standard error. Along side this experiment, the assay was repeated using TDP-43 dissolved in human plasma. The two sets of data can be compared to see if the high concentrations of other proteins affect the sensitivity of the assay.

The ELISA can detect protein levels as low as 0.001 μg/ml when the recombinant TDP-43 is dissolved in PBS alone. However, the sensitivity of the assay is slightly reduced when the recombinant protein is mixed with plasma (figure 7.3). It is possible that the detection antibody experiences a "masking" effect due to the presence of the other proteins present in the plasma. However, the sensitivity of the assay at higher concentrations above 0.01 μg/ml, the measured absorbance values are very similar. Therefore, it was decided that this time the plasma samples would not be assayed for

protein content and concentration adjusted to a standard (as was the case when the α-synuclein/parkin ELISA's were carried out).

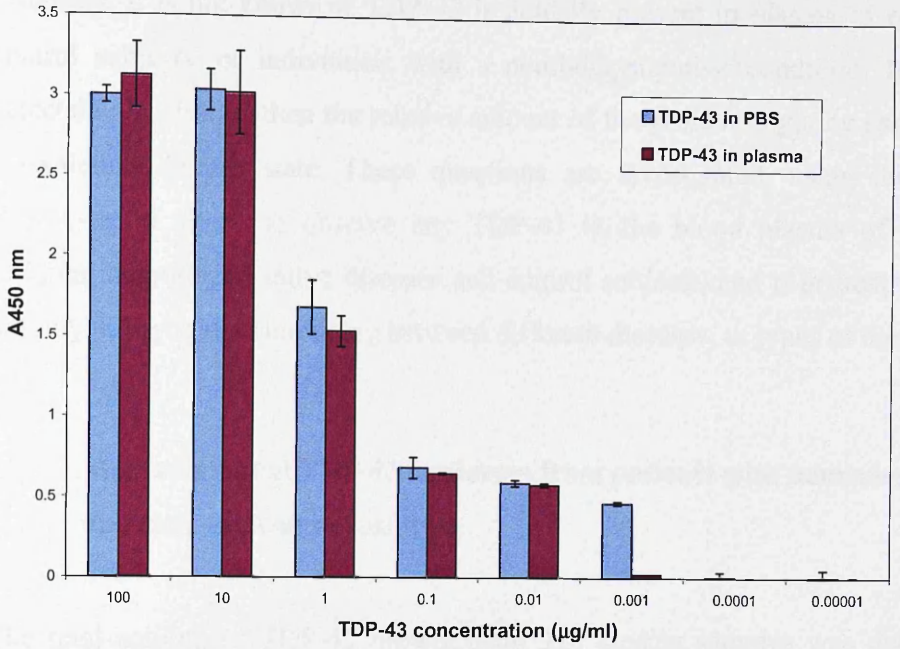


Figure 7.3 ELISA to detect recombinant TDP-43 when dissolved in PBS, and also in a previously assayed plasma sample known to have very low TDP-43 concentration

7.5 Detection of TDP-43 in human plasma

At present, there are no procedures, blood analyses or other *in vivo* tests, which can definitely specify a diagnosis of any dementia syndrome. Diagnosis of dementia is made by clinical, neuropsychological and radiological testing, and various levels of probability are ascribed (eg possible/probable AD) according to consensus guidelines. A biochemical assay that could detect differences between neurodegenerative diseases, including AD, FTLD, and the various subtypes of FTD would be of immense diagnostic value. A diagnostic test could be highly significant in the future development of therapeutic and preventative drugs aimed at removing or averting the

intraneuronal inclusions of tau or TDP-43 proteins. Blood levels of TDP-43 could theoretically be such a biomarker.

Presently, it is not known if TDP-43 is actually present in plasma in either normal control subjects, or individuals with a neurodegenerative condition. If TDP-43 is detectable in plasma, then the relative amount of the protein might be associated with a particular disease state. These questions are investigated, using the developed ELISA-based assay, to observe any TDP-43 in the blood plasma of people with different neurodegenerative diseases and control subjects, and if present whether this has any utility in discriminating between different diseases, or types of disease.

7.5.1 Measuring total TDP-43 in plasma from patients with neurodegenerative diseases, and control samples

The total amount of TDP-43 protein from 328 plasma samples was determined by ELISA (see table 8.2). 102 of the samples were from patients with a AD, 35 were from patients with FTD, 34 with LBD, 16 with MND, 12 with FTD + MND, 11 with PA, 11 with SD, 10 with PD, 7 with CBD, and 85 control subjects. The levels of absorbance when patient plasma was assayed, using the previously designed TDP-43 ELISA, for patients in the different disease groups, can be compared to levels in control plasma samples from individuals with no neurological disease.

The TDP-43 standard curve could then have been utilised to estimate the TDP-43 concentration in individual samples. However, it was decided not to calculate this, but rather use the "raw" absorbance data. There were two reasons for this. Firstly, it was known that the ELISA may not be very accurate at very low concentrations of protein, and secondly, the plasma absorbance readings above 2.1 are above the calibrated range and were saturated. Any calculation using a measured absorbance value of over 2.1 and the standard curve equation would have produced an inaccurate protein concentration. In order to achieve a more reliable estimation of TDP-43 concentration, those plasma samples with a high absorbance would have to be diluted down with PBS and assayed again. This was not carried out as the available residue plasma volumes were too small.

Table 7.2 Demographics of neurological plasma and control samples examined in this study (n/a = not applicable, na* = data not available)

Dementia Groups	Patients (n)	Disease Duration (years)	Age of Onset (years)	Gender; M:F % : (n)
AD	102	2.90 ± 1.82	61.53 ± 9.41	51.9:48.1 (53:49)
FTD	35	2.74 ± 2.06	69.66 ± 8.42	48.6:51.4 (17:18)
MND	16	3.00 ± 3.25	61.21 ± 10.12	80.0:20.0 (12:03)
FTD + MND	12	4.81 ± 1.60	61.81 ± 9.15	50.0:50.0 (06:06)
PA	11	5.72 ± 2.57	64.00 ± 9.31	54.5:45.5 (06:05)
SD	11	5.81 ± 2.31	58.36 ± 4.84	45.5:54.5 (05:06)
LBD/DLB	34	3.09 ± 2.42	66.93 ± 8.59	73.5:26.5 (25:09)
VASC	3	3.66 ± 2.08	61.66 ± 2.51	66.7:33.3 (02:01)
CBD	7	3.71 ± 1.79	61.00 ± 9.89	42.9:57.1 (03:04)
PD	10	na*	na*	70.0:30.0 (07:03)
HD	2	3.50 ± 0.70	59.50 ± 0.70	50.0:50.0 (01:01)
Controls	85	n/a	n/a	43.5:56.5 (37:48)

The ELISA data for all groups (table 7.3) is shown in a chart in figure 7.4 a) and b), again as a box-whisker plot in figure 7.5.

Table 7.3 ELISA data for all groups

Group	Patients (n)	Mean Absorbance	Mean plasma Absorbance, compared to control	Median Absorbance	Standard Deviation	Percentage of samples over the 0.11 "normal"
Controls	85	0.02	-	0.00	0.32	8.8%
AD	102	0.13	6.5 X	0.02	0.33	23%
FTD	35	0.260	13 X	0.11	0.52	46%
MND	16	0.090	4.5 X	0.03	0.15	31%
FTD+MND	12	0.24	12 X	0.03	0.43	33%
PD	10	0.06	3 X	0.01	0.12	20%
SD	11	0.09	4.5 X	0.09	0.12	36%
LBD/DLB	34	0.07	3.5 X	0.05	0.07	25%
VASC	3	0.04	2 X	0.01	0.07	n/a
CBD	7	0.08	4 X	0.05	0.08	n/a
HD	2	0.06	3 X	-	-	n/a

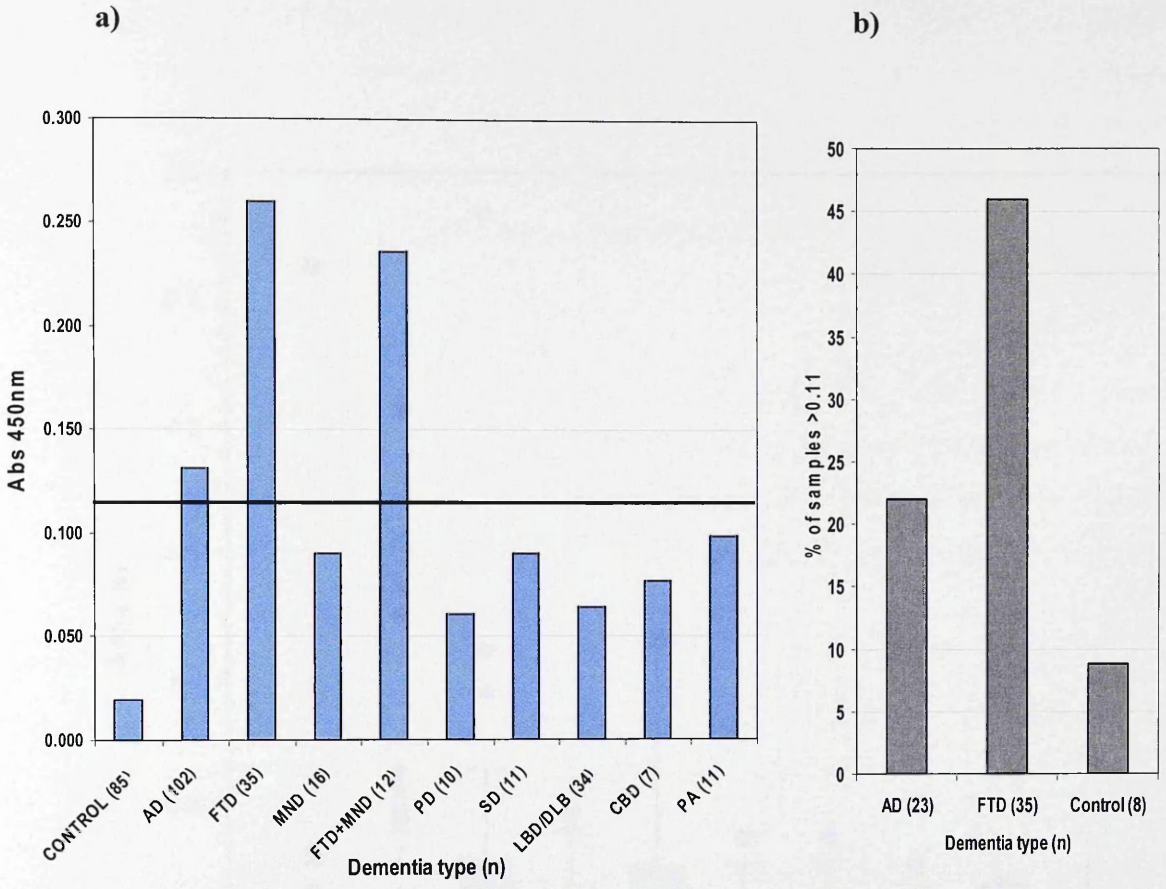


Figure 7.4 a) Dementia Group mean absorbance values. The line represents the 0.11 'cut off' point of 'normal' TDP-43 values; b) The percentage of patients with absorbance values greater than 0.11 for the three main groups

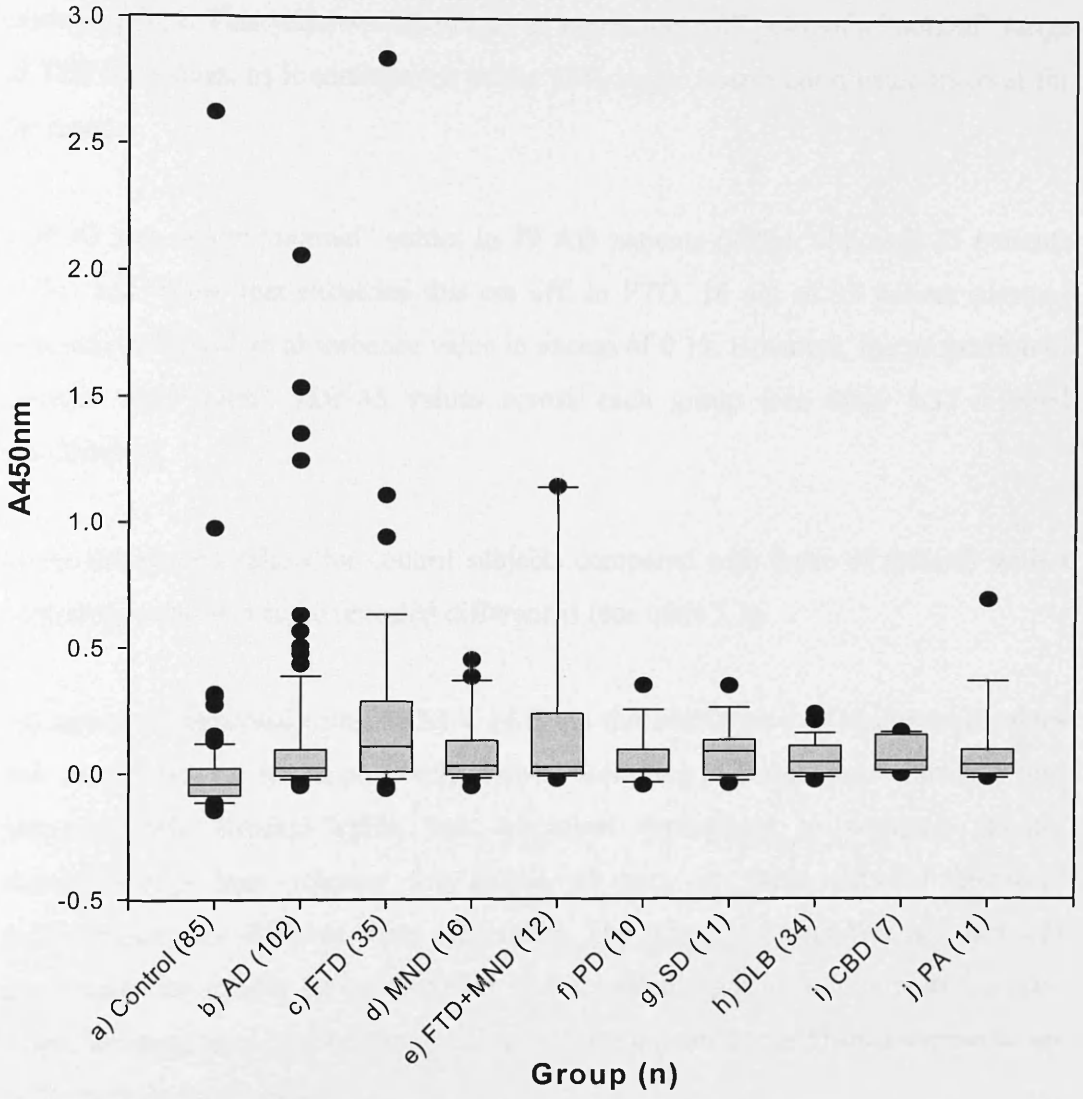


Figure 7.5 Box-whisker plots for the plasma ELISA results

The length of the box represents the interquartile range of the sample, the line drawn across the box the median and outliers being denoted by dots.

Regarding the ELISA data from the control plasma samples (see table 7.3 and figures 7.4 and 7.5), on most occasions, TDP-43 is barely detectable with an average absorbance (OD) value of 0.02 (±0.32), with only 8 subjects (8.8%) having values exceeding 0.11. This value was chosen as an upper "cut off" point of a "normal" range of TDP-43 values, as it corresponds to the 99% upper bound confidence interval for the mean.

TDP-43 was below "normal" values in 79 AD patients (78%), although 23 patients (22%) had values that exceeded this cut off. In FTD, 16 out of 35 patient plasmas assayed (46%) had an absorbance value in excess of 0.11. However, the proportion of patients with "high" TDP-43 values across each group (see table 8.3) differed significantly.

Mean absorbance values for control subjects compared with those of patients with a neurodegenerative disease revealed differences (see table 7.3).

All data were analysed using SPSS v 14.0. As the distribution of absorbance values was not normal, i.e. it was positively skewed according to Kolgomorov-Smirnov test, non-parametric Kruskal-Wallis was employed throughout to compare groups. Kruskal-Wallis tests whether the means of two, or more, sets of unrelated measurements are different from each other. The post-hoc Mann-Whitney test was used when the results of the previous test revealed significant group differences. Mann-Whitney tests whether the medians of two unpaired sets of measurements are different from each other.

Table 7.4 Krustal-Wallis test for control subjects with other groups

Disease groups tested against controls	χ^2	p value
All disease groups	66.1	<0.001
AD and FTD	53.2	<0.001
AD and (FTD, FTD+MND, PA,SD)	57.8	<0.001

Controls and patients with FTD and AD were significantly different by Kruskal-Wallis test (χ^2 = 53.2; $p<0.001$). The differences were also significant when comparing the control group to both AD and FTD groups (χ^2 = 53.2; $p<0.001$), and also when comparing AD with the disease groups pathologically associated with TDP-43.

Table 7.5 Post-hoc Mann-Whitney test

Disease group	p value
Controls and AD	<0.001
Controls and FTD	<0.001
FTD and AD	0.037
Controls and FTD+MND	0.001
Controls and PD	0.008
Controls and SD	0.001
Controls and LBD/DLB	<0.001

Post-hoc Mann-Whitney tests reveal that the median values for the AD, FTD, FTD+MND, SD and DLB groups are significantly different from controls ($p\leq0.001$). Differences between controls and the PD group are still significant ($p = 0.008$), with the median absorbance in FTD group being significantly greater than that in AD (0.037). However, if no correction was applied to these comparisons there would be a chance of 0.3017 (30.17%) of finding one or more significant differences in 07 tests. Bonferroni's adjustment of lowering the alpha (p value) for each test to 0.007 (at a mean correlation of zero giving the full Bonferroni adjustment), still allows five out of seven of the above tests to be significant.

Further statistical analysis was undertaken to investigate whether or not there was an association between the duration of disease and TDP-43 levels. Spearman's Rank was employed to reveal any rank correlation between the two variables.

Only the main groups were considered. Each disease group was analysed twice. First, all the cases were taken into account in the calculation, and secondly, only the values within each group that exceeded the 0.11 cut off were used.

Table 7.6 Spearman's Rank Correlation data

To test whether there is a relationship between TDP-43 levels and duration of disease, for all samples, and then for only those values over the "normal" 0.11 "cut off"

Disease group	All patients Sig.(2-tailed)	High cases Sig.(2 tailed)
AD	0.227	0.997
FTD	0.378	0.987

Table 7.7 Spearman's Rank Correlation data

To test whether there is a relationship between TDP-43 levels and age on onset of the disease, for all samples, and then only for those with a value exceeding the "normal" 0.11 "cut off".

Disease group	All patients Sig.(2-tailed)	High cases Sig.(2 tailed)
AD	0.660	0.090
FTD	0.113	0.922

There is no clear association between duration of disease and TDP-43 levels, but there was a trend towards higher TDP levels with later age of onset in AD (Sig. = 0.090). This is interesting as it goes with tendencies for age of onset to be later in AD cases with TDP pathology compared to those without. Although this figure is not significant at the 0.05 cut off, it does suggest that there may be a chance of finding a significant effect with a larger sample size, which may be followed up on in the future.

7.5.2 Immunoprecipitation of TDP-43 from human plasma

A human plasma sample that gave a high absorbance reading when assayed with the TDP-43 ELISA, and also a sample that gave a very low signal, were added to beads cross-linked with rabbit polyclonal anti-TDP-43 antibody and incubated at 4°C overnight. Any captured protein was eluted from the beads by boiling in sample loading buffer. The eluted proteins were separated on SDS-NuPAGE 4–12% gradient gels and transferred to nitrocellulose membranes. The membrane was probed with mouse monoclonal anti-TDP-43 antibody. Figure 8.6 shows the detection of a band at approximately 43 kDa in lane b that corresponds to full length TDP-43. This band is present in the elute from the high absorbance plasma immunoprecipitate, but is absent form the elute originating from the low value sample (figure 7.6, lane d).

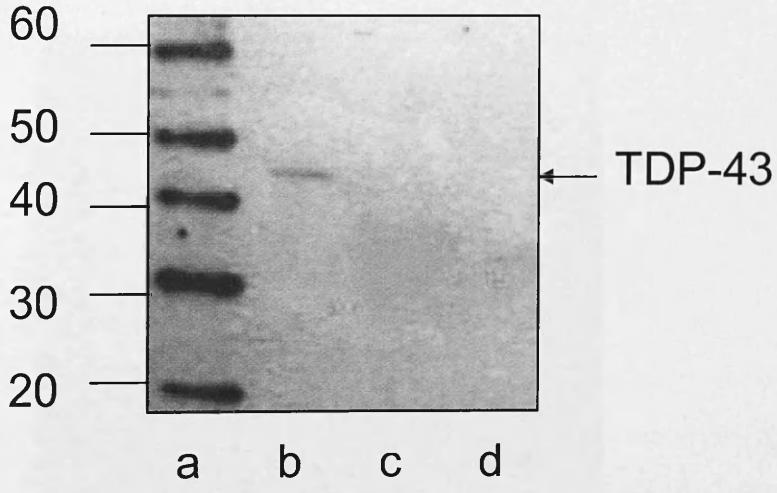


Figure 7.6 Detection of TDP-43 in human plasma

Human blood plasma samples were incubated with magnetic Dynabeads crosslinked to anti-TDP-43 polyclonal antibody and the resulting immunoprecipitates were fractionated on SDS-NuPAGE gels, transferred to nitrocellulose and probed with the monoclonal anti-TDP-43 antibody. Lane a, molecular weight markers (kDa); lane b, a high TDP-43 concentration plasma sample; lane c, empty lane; and lane d, a low TDP-43 concentration plasma sample.

7.5.3 Detection of TDP-43 from human plasma by Immunoblotting

Plasma samples from 3 patients with a neurodegenerative disease that gave a high absorbance reading when assayed with the TDP-43 ELISA, and also 3 samples that gave a very low signal, were prepared for immunoblotting. 3µl of plasma was boiled for 3 min with 20µl LDS sample buffer and 2µl DTT, of which 8µl was loaded onto SDS Nu-PAGE 4-12% gel. Recombinant TDP-43 was run as a positive control (the recombinant protein is a protein fragment with a GST tag, MW = 53.4kD).

A membrane was probed with mouse monoclonal anti-TDP-43 antibody. Figures 8.7 shows the detection of a band at approximately 43 kDa that corresponds to full length TDP-43 and also bands at approximately 25 kDa that corresponds to TDP-43 fragments. However, it should be noted that these bands are present in both the high absorbance plasma immunoprecipitate (figure 7.7, lane d,e,f), and also originating from the low value sample (figure 7.7, lanes h,i,j).

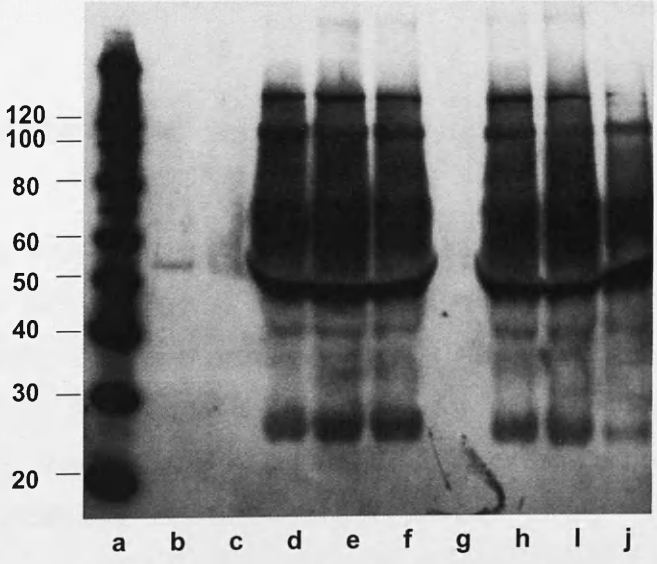


Figure 7.7 Detection of TDP-43 in human plasma

4-12% NuPAGE gradient gel showing analysis of plasma proteins by immunoblotting using anti-TDP-43 monoclonal antibody. Lane a, Magicmark molecular weight markers (Invitrogen); lane b, TDP-43 antigen at MW = 53.4kDa as a positive control; lane c, empty; lanes d-f, human plasma samples that were high TDP-43 content; lane g, empty; lanes h-j, human plasma samples that were low TDP-43 content.

This banding pattern is consistent with the immunoblot analysis performed on the brains of patients with FTLD-U (Arai et al. 2006; Neumann et al. 2006), revealing pathological TDP-43 immunoreacive bands at approximately 24, 26, 45 and high MW bands. From figure 8.7 it is clear that a band at 43 kDa is common to all plasma samples, although the 24/26 kDa bands are more prominent in the "high" TDP-43 samples (lanes d – f) than in the "low" samples (lanes h-j). The 24/26 kDa molecular weight bands may represent proteolytically cleaved fragments, or alternatively spliced isoforms of TDP-43. If the former, it is not clear whether these are generated from wild-type TDP-43 or from the hyperphosphorylated pathological 45 kDa protein (Arai et al. 2006; Neumann et al. 2006).

This immunoprecipitation experiments and plasma immunoblots identifiy and confirm the presence of TDP-43 in human blood plasma and further support and validate the ELISA data.

7.6 Discussion

In this study we have shown for the first time that the TAR DNA-binding protein can be detected within plasma of normal control subjects, as well as individuals with neurodegenerative diseases such as AD, FTD, DLB, MND and SD. The ELISA data indicates that TDP-43 is present at extremely low concentrations, or is absent, in the vast majority of normal people, and in these diseases there are substantial numbers of patients in whom TDP-43 is likewise barely present.

7.6.1 Possible methods of secretion of TDP-43

TDP-43 is a nuclear transcription factor, and so would be expected to normally be present within cell nuclei. Therefore its presence in neuronal nuclei in FTLD-U, like in other disorders is not surprising. However, it has been shown to be absent from

glial cell nuclei (Cairns et al. 2007) suggesting that this protein only operates in neurones.

It has been suggested that pathological TDP-43 redistributes from the nucleus to the cytoplasm, producing the characteristic neuronal cytoplasmic inclusions (Neumann et al. 2006). However, it may be the case that the TDP-43 cannot cross the nuclear membrane, and therefore aggregates within the cytoplasm. The latter argument is consistent with the results of immunoblotting (Arai et al. 2006; Neumann et al. 2006), demonstrating that pathological TDP-43 is hyperphosphorylated, which could prevent it being able to cross through the nuclear envelope.

The mechanism(s) by which TDP-43 is secreted from the cell is unknown, but the reasoning as to why it is detected in plasma could be similar to the discussions presented in Chapter 4 with the case of α-synuclein.

7.6.2 Detection of total TDP-43 in human blood plasma

Group comparisons of ELISA absorbance levels reveal a highly significant increase (approximately 13-fold) in mean plasma TDP-43 levels in FTD compared to controls, whereas in AD the mean TDP-43 plasma level was about 6.5-fold increased (table 7.3). The validity of the ELISA method is supported by the immunoblotting results (figure 7.7). In accord with the ELISA data, a single immunoreactive band migrating at 43 kDa, which corresponds precisely to the molecular weight of full-length TDP-43, was detected only in the high-reading plasma sample and not in the low-reading sample. This result not only confirms the specificity of the antibody pair employed for the sandwich ELISA, but also shows that the readout from the ELISA accurately reflects the levels of TDP-43 present in plasma.

Histological studies show that around 50% of patients with FTD have TDP-43/ubiquitin–based histology (Cairns et al. 2007; Davidson et al. 2007). Therefore, it could be assumed that a similar proportion of patients would be present in the FTD sample group. If this histology was present in the brain, and there was secretion of TDP-43 into the plasma, then again, it may be expected that a similar proportion of

patients would have "high" TDP-43 levels (i.e. readings above the 0.11 absorbance level corresponding to the 99% upper confidence interval for the control group). The proportion of FTD patients with "high" TDP-43 levels in plasma is 46% (table 7.3) which is comparable to the ≈50% seen at autopsy. Likewise in AD, it is identified that around 20-25% of patients with this form of dementia have TDP-43-based pathology (Amador-Ortiz et al. 2007), and therefore again it may be assumed that a similar proportion of AD patients in our group sample would have high plasma TDP-43 levels. The proportion of such patients in our AD group was actually 23% which again mirrors what would be predicted from histological studies. Therefore, the proportion of patients showing high plasma TDP-43 levels in both FTD and AD closely match the proportions that would be predicted from histological studies.

Regarding the control group, the few elderly subjects with high TDP-43 levels (figures 7.3 and 7.5) may have high incipient neurodegenerative disease, most likely AD, since it has been shown that a very high proportion of mentally normally elderly subjects show at autopsy some degree of Alzheimer-type pathological changes in the brain, for example senile plaques and neurofibrillary tangles.

7.6.3 In conclusion

The proportions of patients with FTD and AD showing "high" TDP-43 plasma levels, and those displaying TDP-43 pathological changes at autopsy are statistically similar. Therefore it could be implied that plasma measurement of this protein could infer TDP-43 within the brain of that FTD or AD patient. However, it may be that these numerical associations are simply by chance. This relationship would be confirmed if autopsy follow-up of patients with AD or FTD with the high plasma levels were available. However, this may not be possible for several years, if at all, as the patients investigated here are mostly in the early stages of the disease. A different approach would be to compare plasma TDP-43 levels of FTD patients with the *MAPT* of *PGRN* mutations. It is known from histological studies that FTD patients bearing the *MAPT* mutation and tauopathy do not show TDP-43 pathological changes in their brains (Davidson et al. 2007), whereas those with *PGRN* mutations do (Baker et al. 2006;

Boeve et al. 2006; Mackenzie et al. 2006; Pickering-Brown et al. 2006; Snowden et al. 2006; Davidson et al. 2007). Unfortunately, there was only one bearer of the *PRGN* mutation within the present patient groups, and not one with the *MAPT* mutation, so this comparison was not possible.

The functional significance of the presence of TDP-43 pathology in the brains of patients with neurodegenerative diseases is not known. However, because brain TDP-43 levels could be predicted in such patients by this ELISA test, such knowledge may be of benefit in the future regarding the management or prevention of these diseases. This data suggests that measurement of TDP-43 protein levels in plasma may indicate those patients with TDP-43 pathology within the brain, as opposed to those with tau-based histological changes, even if it does not discriminate between clinical neurodegenerative diseases. With the advent of either tau-based or TDP-43-based therapies in the future, knowing who could potentially benefit from which treatment would be of great value.

CHAPTER EIGHT

8 Discussion

Neurodegenerative disorders pose a significant public health burden which will only increase as medical and technological advances ensure that the average life expectancy continues to increase. For example, the incidence and prevalence of PD increases with advancing years, occurring in about 1% of people over the age of 65 years. Future demographic predictions project that the population aged over 65 will approximately double by the middle of the century, with the corresponding increase in individuals suffering from PD, not to mention all the other neurodegenerative disorders. The World Health Organisation expect that at this time, the number of individuals suffering from neurodegenerative disorders will exceed cancer as the most common cause of death (www.who.int/en/). Of course, the situation will be made worse if there are no treatments available to address the problem of progressive neurodegeneration, as the existing drugs are symptomatic at best with surgical therapies being only transiently effective. As of yet, the biochemical processes that underlie these diseases, and result in the formation of protein aggregates, are not fully understood. Therefore it is clear that the normal and the pathological processes of the brain should be fully investigated, and the functions of the implicated proteins identified, as these may provide potential therapeutic agents, or be useful as biomarkers for the disease.

The α-synuclein "self-aggregation hypothesis", proposes that the soluble, unfolded protein oligomerises to form various structured intermediates, seeding aggregation to form insoluble, amyloid-like fibrils. These small intermediates, the soluble oligomers in the aggregation process, can confer synaptic dysfunction, whereas large, insoluble deposits may function as "reservoirs" of the bioactive oligomers. The distinct protein aggregates that are found in AD, PD, HD and prion diseases are implicated in these disorders. The "self-aggregation hypothesis" was exemplified on the discovery that a fibrillar form of the presynaptic protein is the major component of LB's, in the insoluble inclusions characteristic of PD. This emerging concept may also apply to AD where Aβ oligomers adversely affect synaptic structure and plasticity. The same

hypothesis could be proposed for TDP-43, which is the major protein present in the insoluble inclusions seen in FTLD-U. An array of environmental factors, plus three gene mutations associated with familial PD that result in amino-acid substitutions in α-synuclein (A30P, A53T and E46K), have been shown to accelerate the process *in vitro* and in transgenic animal models of PD. It is therefore widely accepted that aggregation of α-synuclein is central to the pathogenesis of PD, even though the toxic form of α-synuclein and its mechanism of neurotoxicity remain unknown. In the TDP-43 proteinopathies (including cases of sporadic and familial FTLD-U, FTD/MND and sporadic MND) the scenario is different in that it is a mutation in the progranulin gene that is, on the whole, thought to be responsible for the disease, although the protein that aggregates is not the progranulin protein, but TDP-43. It is possible that abnormal metabolism mediated by progranulin may play a pivotal role in neurodegeneration. Again, the toxic form of TDP-43, the method of aggregation and the mechanism of toxicity are unknown. Research into other neurodegenerative diseases indicates that a broadly similar process of neuronal dysfunction is induced by diffusible oligomers of misfolded proteins.

The self-aggregation hypothesis does not explain why ubiquitous proteins such as α-synuclein aggregate specifically in dopaminergic neurones of the substantia nigra in PD, or why TDP-43 aggregates in either cytoplasmic, neuritic, or nuclear inclusions in FTLD-U. Why age is a risk factor for these disorders is not clear, although there are several lines of evidence that suggest elevated oxidative stress is an issue, and oxidative damage to biomolecules in the SN in PD is involved. This has led to the proposal that oxidative stress may contribute to the death of neuronal cells in PD, with dopaminergic neurones being particularly susceptible due to high levels of pro-oxidants such as dopamine. Oxidative stress in the brain increases with age as a result of the accumulation of redox-active metals such as Fe and Cu (Morita et al. 1994; Takahashi et al. 2001; Maynard et al. 2002). This indicates that a breakdown in metal homeostasis could predispose to oxidative stress and age-related neurodegenerative diseases.

ELISA's were developed using different antibodies for capture and detection, to detect α-synuclein, the α-synuclein/parkin complex, the parkin protein and TDP-43. More

extensive clinical studies will be required to investigate whether the ELISA's would be useful as a diagnostic tool or whether it could be utilised to track the disease progression, e.g. in drugs trails. It would be interesting to determine if there was a correlation between detection of oligomers and the severity and/or stage of the disease. Studies on blood samples from familial cases or from transgenic mouse models would be useful for validating the ELISA as an early diagnostic method. It is possible that medication taken by patients, especially dopaminergic medication taken by PD patients, could influence the data (Conway et al. 2001). Studies on newly diagnosed patients who are not yet taking medication could indicate whether the drugs had any confounding effects. Moreover, the principle of the ELISA could be developed for other sensitive diagnostic tests, for the presence of other forms of protein aggregates, such as ubiquitinated TDP-43 or α-synuclein. Diagnosis of AD has been based on detection of phosphorylated tau protein (Vandermeeren et al. 1993) and Aβ peptides (Seubert et al. 1992) in biological fluids, especially plasma and CSF. This has led to development of a commercial assay for tau protein (INNOTEST hTAU Ag by Innogenetics, Belgium) and also the CSF-A42 test from the same company. The combined use of CSF-tau and CSF-A42 markers results in some sensitivity and specificity for AD versus normal aging, and other neurodegenerative diseases.

Research is still in the early stages of understanding the processes of how neurodegenerative diseases develop, but it is apparent that a new classification of neurodegenerative disorders, the TDP-43 proteinopathies, has emerged, and this will have significant implications for the diagnosis and treatment of FTDs and ALS. This is understandable based on similar earlier advances in recognising mechanisms of tau, Aβ- and α-synuclein-mediated neurodegeneration in AD and other tauopathies, as well as in PD and related α-synucleinopathies. In this thesis, an assay was developed to monitor the levels of normal TDP-43 in plasma. Further assays could also be developed to monitor levels of pathological TDP-43 and could lead to the development of diagnostic tests to distinguish TDP-43 proteinopathies from other clinically similar neurodegenerative disorders. Moreover, the development of imaging ligands, that facilitate the detection of TDP-43 neuropathology in living individuals, would provide a powerful tool not only for diagnosis but also for following the response of patients with a neurodegenerative TDP-43 proteinopathy to disease-modifying therapies. However, more importantly, the recognition that TDP-43

pathology underlies and links several neurodegenerative disorders will be a significant driving force towards efforts to develop more effective therapies for these diseases.

Efforts have been underway over the past few years to find biochemical, neuropathological and genetic biomarkers for neurodegenerative diseases. This would enable the disease to be diagnosed at earlier stages of the disorder, often when the currently available treatment is more effective. The neuropathological diagnosis is currently the only firm diagnosis, but of course is done after autopsy. It would be much more preferable to have a firm diagnosis at early stages. Patients may then have the opportunity to receive early treatment which may slow down the progression of the disease. Moreover, a biomarker could not only be used to help in predicting the onset, or diagnosing the diseases, but also to help in overseeing the rate of progression, or in responding to treatment. A biomarker would be required to distinguish normal aging individuals experience with advancing years from dementia, one disorder from another with dementia, as well as to help in identifying the exact cause of a dementia. Therefore, the biomarkers for PD, AD, FTD and other neurodegenerative diseases such as ALS and HD, must be reliable and specific, and they should be useful in guiding clinicians to make more accurate diagnosis and better treatment of the disease in question.

Two biochemical markers have been very helpful in detecting the onset of PD. The loss of the dopamine transporter detected by PET imaging and the presence of the α-synuclein protein located in the Lewy body lesions, are both characteristic of PD. Several other biochemical markers in blood and CSF have also been proposed. A recent study in our laboratory has recognised decreased levels of α-synuclein in the CSF of aged individuals and subjects with PD (Tokuda et al. 2006). The validity of these markers in clinical applications is currently under investigation. The present study confirms previous work (Salem, 2004) that has identified α-synuclein as a component of human blood plasma. We investigated whether α-synuclein can be used as a biomarker for PD. Other work in our laboratory has been to develop a novel ELISA method that detects only oligomeric "soluble aggregates" of alpha-synuclein (El-Agnaf et al. 2006).

The data presented in this thesis identifies parkin in plasma, and also a protein complex in human plasma that involves parkin and α-synuclein. In this thesis, the levels of two proteins implicated in PD, α-synuclein and parkin, both individually and complexed together, were investigated in blood plasma from patients with PD and other neurodegenerative diseases. The results suggest that there is a correlation between the levels of expression of α-synuclein, parkin and the complex in individual plasma samples, but no clear relationship when comparing disease and control groups (although the number of samples analysed is small at present). At present, levels of these proteins would have to be investigated further, within much larger sample groups, to find if they were to be useful as diagnostic indicators. However, much larger sample groups were assayed when investigating TDP-43 levels across control and disease groups. The proportions of patients with FTD and AD showing "high" TDP-43 plasma levels, and those displaying TDP-43 pathological changes at autopsy are statistically similar. Therefore it could be implied that plasma measurement of this protein could infer TDP-43 within the brain of that FTD or AD patient. Although the functional significance of the presence of TDP-43 pathology in the brains of patients with neurodegenerative diseases is not yet known, brain TDP-43 levels could be predicted in such patients by this ELISA test, and such knowledge may be of benefit in the future regarding the management or prevention of these diseases. The data in this thesis suggest that measurement of TDP-43 protein levels in plasma may indicate those patients with TDP-43 pathology within the brain, as opposed to those with tau-based histological changes, even if it does not discriminate between clinical neurodegenerative diseases. With the advent of either tau-based of TDP-43-based therapies in the future, knowing who could potentially benefit from which treatment could potentially be of importance.

Rapid progress towards understanding the molecular mechanisms of neurodegenerative disorders, such as PD and FTLD, will revolutionize drug discovery for these conditions. The development of models for these disorders is accelerating efforts to translate insights from neurodegenerative mechanisms into disease-modifying therapies. However, there is an urgent need for biomarkers to accurately diagnose neurodegenerative disorders early in their course, when therapy is likely to be more effective, and to monitor responses of patients to new therapies.

8.1 Future work

8.1.1 α-synuclein/parkin

Further work could be focused on the full characterisation of α-synuclein in plasma, as well as investigating the molecular composition of the α-synuclein/parkin complex and determining the precise nature of the interaction. Initially, the presence of parkin in blood plasma could be confirmed by mass spectrometry, and concentrations of the parkin protein could be determined when the recombinant protein becomes available, to enable the construction of a standard calibration curve.

The pathogenic mechanisms underlying α-synuclein/parkin pathology in the various neurodegenerative diseases remains elusive. The normal and pathological functions of the interaction between α-synuclein and parkin should be investigated, along with histopathological studies, and also studies as to whether the targeted disruption/promotion of this interaction could provide a novel approach to the treatment of PD.

Further biomarker studies could focus on oligomers of α-synuclein, the α-synuclein/parkin complex, as well as phosphorylated and ubiquitinated forms of the proteins.

8.1.2 FTLD-U

The pathogenic mechanisms underlying TDP-43 pathology in the various FTLD-U cases remain elusive; with questions regarding its presence both in the cytoplasm and the blood plasma remain unanswered. Previous studies of granular glial inclusions which were ubiquitin negative but TDP-43 positive (Cairns et al. 2007) suggest that the pathogenic mechanisms initially involve the modification of the TDP-43 protein, followed by ubiquitination. Developing an ELISA method to detect ubiquinated TDP-43 in plasma, and/or brain tissue and comparing this data with the TDP-43 results, may give a better insight into the pathological processes involved in FTLD-U.

As mentioned previously, the relationship between individuals who have "high" TDP-43 plasma levels, and those who show TDP-43 in the brain at autopsy could be followed up; however, this may not be possible for several years, as the patients investigated here are mostly in the early stages of the disease. A different approach would be to compare plasma TDP-43 levels of FTD patients with the *MAPT* of *PGRN* mutations, draw comparisons with the frequency of APO E ε4 allele, and compare the age of onset of disease with plasma levels, the duration and severity of disease.

The presence of high TDP-43 levels in a good proportion of AD cases (~20%), (which matches the FTLD-U type TDP-43 pathology in AD), has raised the possibility of concurrent FTLD-U in these cases, which extrapolates into a large number of cases of AD worldwide being unrecognised mixed AD/FTLD-U cases (Amador-Ortiz et al. 2007). Demographic parameters such as duration of disease or the APO E genotype may account for these differences. However, it may be the case that the large number of cases diagnosed with AD could possibly have the mixed pathology, which in turn may account for differences in the age of onset of disease, duration, and so on. Such a possibility has important diagnostic implications and can be the focus of future studies on AD pathology.

9 References

Ahn, B.H., Rhim, H., Kim, S.Y., Sung, Y.M., Lee, M.Y., Choi, J.Y., Wolozin, B., Chang, J.S., Lee, Y.H., Kwon, T.K., Chung, K.C., Yoon, S.H., Hahn, S.J., Kim, M.S., Jo, Y.H., and Min, D.S. 2002. alpha-Synuclein interacts with phospholipase D isozymes and inhibits pervanadate-induced phospholipase D activation in human embryonic kidney-293 cells. *J Biol Chem* **277**(14): 12334-12342.

Amador-Ortiz, C., Lin, W.L., Ahmed, Z., Personett, D., Davies, P., Duara, R., Graff-Radford, N.R., Hutton, M.L., and Dickson, D.W. 2007. TDP-43 immunoreactivity in hippocampal sclerosis and Alzheimer's disease. *Ann Neurol* **61**(5): 435-445.

Antony, T., Hoyer, W., Cherny, D., Heim, G., Jovin, T.M., and Subramaniam, V. 2003. Cellular polyamines promote the aggregation of alpha-synuclein. *J Biol Chem* **278**(5): 3235-3240.

Arai, T., Hasegawa, M., Akiyama, H., Ikeda, K., Nonaka, T., Mori, H., Mann, D., Tsuchiya, K., Yoshida, M., Hashizume, Y., and Oda, T. 2006. TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochem Biophys Res Commun* **351**(3): 602-611.

Baker, M., Mackenzie, I.R., Pickering-Brown, S.M., Gass, J., Rademakers, R., Lindholm, C., Snowden, J., Adamson, J., Sadovnick, A.D., Rollinson, S., Cannon, A., Dwosh, E., Neary, D., Melquist, S., Richardson, A., Dickson, D., Berger, Z., Eriksen, J., Robinson, T., Zehr, C., Dickey, C.A., Crook, R., McGowan, E., Mann, D., Boeve, B., Feldman, H., and Hutton, M. 2006. Mutations in progranulin cause tau-negative frontotemporal dementia linked to chromosome 17. *Nature* **442**(7105): 916-919.

Baptista, M.J., O'Farrell, C., Daya, S., Ahmad, R., Miller, D.W., Hardy, J., Farrer, M.J., and Cookson, M.R. 2003. Co-ordinate transcriptional regulation of

dopamine synthesis genes by alpha-synuclein in human neuroblastoma cell lines. *J Neurochem* **85**(4): 957-968.

Barlowe, C. 2002. COPII-dependent transport from the endoplasmic reticulum. *Curr Opin Cell Biol* **14**(4): 417-422.

-. 2003. Signals for COPII-dependent export from the ER: what's the ticket out? *Trends Cell Biol* **13**(6): 295-300.

Beal, R., Deveraux, Q., Xia, G., Rechsteiner, M., and Pickart, C. 1996. Surface hydrophobic residues of multiubiquitin chains essential for proteolytic targeting. *Proc Natl Acad Sci U S A* **93**(2): 861-866.

Boeve, B.F., Baker, M., Dickson, D.W., Parisi, J.E., Giannini, C., Josephs, K.A., Hutton, M., Pickering-Brown, S.M., Rademakers, R., Tang-Wai, D., Jack, C.R., Jr., Kantarci, K., Shiung, M.M., Golde, T., Smith, G.E., Geda, Y.E., Knopman, D.S., and Petersen, R.C. 2006. Frontotemporal dementia and parkinsonism associated with the IVS1+1G->A mutation in progranulin: a clinicopathologic study. *Brain* **129**(Pt 11): 3103-3114.

Bonifati, V., Rizzu, P., van Baren, M.J., Schaap, O., Breedveld, G.J., Krieger, E., Dekker, M.C., Squitieri, F., Ibanez, P., Joosse, M., van Dongen, J.W., Vanacore, N., van Swieten, J.C., Brice, A., Meco, G., van Duijn, C.M., Oostra, B.A., and Heutink, P. 2003. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science* **299**(5604): 256-259.

Bowen, D.M., Procter, A.W., Mann, D.M., Snowden, J.S., Esiri, M.M., Neary, D., and Francis, P.T. 2008. Imbalance of a serotonergic system in frontotemporal dementia: implication for pharmacotherapy. *Psychopharmacology (Berl)* **196**(4): 603-610.

Bozeat, S., Lambon Ralph, M.A., Patterson, K., Garrard, P., and Hodges, J.R. 2000. Non-verbal semantic impairment in semantic dementia. *Neuropsychologia* **38**(9): 1207-1215.

Braak, H., de Vos, R.A., Bohl, J., and Del Tredici, K. 2006. Gastric alpha-synuclein immunoreactive inclusions in Meissner's and Auerbach's plexuses in cases

staged for Parkinson's disease-related brain pathology. *Neurosci Lett* **396**(1): 67-72.

Buchman, V.L., Hunter, H.J., Pinon, L.G., Thompson, J., Privalova, E.M., Ninkina, N.N., and Davies, A.M. 1998. Persyn, a member of the synuclein family, has a distinct pattern of expression in the developing nervous system. *J Neurosci* **18**(22): 9335-9341.

Buratti, E. and Baralle, F.E. 2001. Characterization and functional implications of the RNA binding properties of nuclear factor TDP-43, a novel splicing regulator of CFTR exon 9. *J Biol Chem* **276**(39): 36337-36343.

Buratti, E., Brindisi, A., Pagani, F., and Baralle, F.E. 2004. Nuclear factor TDP-43 binds to the polymorphic TG repeats in CFTR intron 8 and causes skipping of exon 9: a functional link with disease penetrance. *Am J Hum Genet* **74**(6): 1322-1325.

Cabin, D.E., Shimazu, K., Murphy, D., Cole, N.B., Gottschalk, W., McIlwain, K.L., Orrison, B., Chen, A., Ellis, C.E., Paylor, R., Lu, B., and Nussbaum, R.L. 2002. Synaptic vesicle depletion correlates with attenuated synaptic responses to prolonged repetitive stimulation in mice lacking alpha-synuclein. *J Neurosci* **22**(20): 8797-8807.

Cairns, N.J., Bigio, E.H., Mackenzie, I.R., Neumann, M., Lee, V.M., Hatanpaa, K.J., White, C.L., 3rd, Schneider, J.A., Grinberg, L.T., Halliday, G., Duyckaerts, C., Lowe, J.S., Holm, I.E., Tolnay, M., Okamoto, K., Yokoo, H., Murayama, S., Woulfe, J., Munoz, D.G., Dickson, D.W., Ince, P.G., Trojanowski, J.Q., and Mann, D.M. 2007. Neuropathologic diagnostic and nosologic criteria for frontotemporal lobar degeneration: consensus of the Consortium for Frontotemporal Lobar Degeneration. *Acta Neuropathol (Berl)* **114**(1): 5-22.

Cairns, N.J., Neumann, M., Bigio, E.H., Holm, I.E., Troost, D., Hatanpaa, K.J., Foong, C., White, C.L., 3rd, Schneider, J.A., Kretzschmar, H.A., Carter, D., Taylor-Reinwald, L., Paulsmeyer, K., Strider, J., Gitcho, M., Goate, A.M., Morris, J.C., Mishra, M., Kwong, L.K., Stieber, A., Xu, Y., Forman, M.S., Trojanowski, J.Q., Lee, V.M., and Mackenzie, I.R. 2007. TDP-43 in familial

and sporadic frontotemporal lobar degeneration with ubiquitin inclusions. *Am J Pathol* **171**(1): 227-240.

-. 2007. TDP-43 in Familial and Sporadic Frontotemporal Lobar Degeneration with Ubiquitin Inclusions. *Am J Pathol.*

Cappai, R., Leck, S.L., Tew, D.J., Williamson, N.A., Smith, D.P., Galatis, D., Sharples, R.A., Curtain, C.C., Ali, F.E., Cherny, R.A., Culvenor, J.G., Bottomley, S.P., Masters, C.L., Barnham, K.J., and Hill, A.F. 2005. Dopamine promotes alpha-synuclein aggregation into SDS-resistant soluble oligomers via a distinct folding pathway. *Faseb J* **19**(10): 1377-1379.

Capsoni, S., Ugolini, G., Comparini, A., Ruberti, F., Berardi, N., and Cattaneo, A. 2000. Alzheimer-like neurodegeneration in aged antinerve growth factor transgenic mice. *Proc Natl Acad Sci U S A* **97**(12): 6826-6831.

Chartier-Harlin, M.C., Kachergus, J., Roumier, C., Mouroux, V., Douay, X., Lincoln, S., Levecque, C., Larvor, L., Andrieux, J., Hulihan, M., Waucquier, N., Defebvre, L., Amouyel, P., Farrer, M., and Destee, A. 2004. Alpha-synuclein locus duplication as a cause of familial Parkinson's disease. *Lancet* **364**(9440): 1167-1169.

Cherny, D., Hoyer, W., Subramaniam, V., and Jovin, T.M. 2004. Double-stranded DNA stimulates the fibrillation of alpha-synuclein in vitro and is associated with the mature fibrils: an electron microscopy study. *J Mol Biol* **344**(4): 929-938.

Choi, H.J., Kim, S.W., Lee, S.Y., and Hwang, O. 2003. Dopamine-dependent cytotoxicity of tetrahydrobiopterin: a possible mechanism for selective neurodegeneration in Parkinson's disease. *J Neurochem* **86**(1): 143-152.

Choi, W., Zibaee, S., Jakes, R., Serpell, L.C., Davletov, B., Crowther, R.A., and Goedert, M. 2004. Mutation E46K increases phospholipid binding and assembly into filaments of human alpha-synuclein. *FEBS Lett* **576**(3): 363-368.

Chow, T.W., Miller, B.L., Hayashi, V.N., and Geschwind, D.H. 1999. Inheritance of frontotemporal dementia. *Arch Neurol* **56**(7): 817-822.

Chung, K.K., Zhang, Y., Lim, K.L., Tanaka, Y., Huang, H., Gao, J., Ross, C.A., Dawson, V.L., and Dawson, T.M. 2001. Parkin ubiquitinates the alpha-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease. *Nat Med* **7**(10): 1144-1150.

Ciechanover, A. 2001. Linking ubiquitin, parkin and synphilin-1. *Nat Med* **7**(10): 1108-1109.

Clayton, D.F. and George, J.M. 1998. The synucleins: a family of proteins involved in synaptic function, plasticity, neurodegeneration and disease. *Trends Neurosci* **21**(6): 249-254.

-. 1999. Synucleins in synaptic plasticity and neurodegenerative disorders. *J Neurosci Res* **58**(1): 120-129.

Cleveland, D.W. and Rothstein, J.D. 2001. From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS. *Nat Rev Neurosci* **2**(11): 806-819.

Cohlberg, J.A., Li, J., Uversky, V.N., and Fink, A.L. 2002. Heparin and other glycosaminoglycans stimulate the formation of amyloid fibrils from alpha-synuclein in vitro. *Biochemistry* **41**(5): 1502-1511.

Conway, K.A., Lee, S.J., Rochet, J.C., Ding, T.T., Harper, J.D., Williamson, R.E., and Lansbury, P.T., Jr. 2000. Accelerated oligomerization by Parkinson's disease linked alpha-synuclein mutants. *Ann N Y Acad Sci* **920**: 42-45.

Conway, K.A., Rochet, J.C., Bieganski, R.M., and Lansbury, P.T., Jr. 2001. Kinetic stabilization of the alpha-synuclein protofibril by a dopamine-alpha-synuclein adduct. *Science* **294**(5545): 1346-1349.

Cruts, M., Gijselinck, I., van der Zee, J., Engelborghs, S., Wils, H., Pirici, D., Rademakers, R., Vandenberghe, R., Dermaut, B., Martin, J.J., van Duijn, C., Peeters, K., Sciot, R., Santens, P., De Pooter, T., Mattheijssens, M., Van den Broeck, M., Cuijt, I., Vennekens, K., De Deyn, P.P., Kumar-Singh, S., and

Van Broeckhoven, C. 2006. Null mutations in progranulin cause ubiquitin-positive frontotemporal dementia linked to chromosome 17q21. *Nature* **442**(7105): 920-924.

Cummings, C.J., Reinstein, E., Sun, Y., Antalffy, B., Jiang, Y., Ciechanover, A., Orr, H.T., Beaudet, A.L., and Zoghbi, H.Y. 1999. Mutation of the E6-AP ubiquitin ligase reduces nuclear inclusion frequency while accelerating polyglutamine-induced pathology in SCA1 mice. *Neuron* **24**(4): 879-892.

Daniel, R., He, Z., Carmichael, K.P., Halper, J., and Bateman, A. 2000. Cellular localization of gene expression for progranulin. *J Histochem Cytochem* **48**(7): 999-1009.

Dauer, W. and Przedborski, S. 2003. Parkinson's disease: mechanisms and models. *Neuron* **39**(6): 889-909.

Davidson, W.S., Jonas, A., Clayton, D.F., and George, J.M. 1998. Stabilization of alpha-synuclein secondary structure upon binding to synthetic membranes. *J Biol Chem* **273**(16): 9443-9449.

Davidson, Y., Kelley, T., Mackenzie, I.R., Pickering-Brown, S., Du Plessis, D., Neary, D., Snowden, J.S., and Mann, D.M. 2007. Ubiquitinated pathological lesions in frontotemporal lobar degeneration contain the TAR DNA-binding protein, TDP-43. *Acta Neuropathol (Berl)* **113**(5): 521-533.

Davies, R.R., Hodges, J.R., Kril, J.J., Patterson, K., Halliday, G.M., and Xuereb, J.H. 2005. The pathological basis of semantic dementia. *Brain* **128**(Pt 9): 1984-1995.

de Almeida, L.P., Ross, C.A., Zala, D., Aebischer, P., and Deglon, N. 2002. Lentiviral-mediated delivery of mutant huntingtin in the striatum of rats induces a selective neuropathology modulated by polyglutamine repeat size, huntingtin expression levels, and protein length. *J Neurosci* **22**(9): 3473-3483.

de Lau, L.M., Koudstaal, P.J., Hofman, A., and Breteler, M.M. 2006. Subjective complaints precede Parkinson disease: the rotterdam study. *Arch Neurol* **63**(3): 362-365.

DeMartino, G.N. and Slaughter, C.A. 1999. The proteasome, a novel protease regulated by multiple mechanisms. *J Biol Chem* **274**(32): 22123-22126.

DeMattos, R.B., Bales, K.R., Cummins, D.J., Paul, S.M., and Holtzman, D.M. 2002. Brain to plasma amyloid-beta efflux: a measure of brain amyloid burden in a mouse model of Alzheimer's disease. *Science* **295**(5563): 2264-2267.

Dickson, D.W., Fujishiro, H., DelleDonne, A., Menke, J., Ahmed, Z., Klos, K.J., Josephs, K.A., Frigerio, R., Burnett, M., Parisi, J.E., and Ahlskog, J.E. 2008. Evidence that incidental Lewy body disease is pre-symptomatic Parkinson's disease. *Acta Neuropathol* **115**(4): 437-444.

Dobson, C.M. 2004. Principles of protein folding, misfolding and aggregation. *Semin Cell Dev Biol* **15**(1): 3-16.

el-Agnaf, O.M. and Irvine, G.B. 2002. Aggregation and neurotoxicity of alpha-synuclein and related peptides. *Biochem Soc Trans* **30**(4): 559-565.

El-Agnaf, O.M., Salem, S.A., Paleologou, K.E., Cooper, L.J., Fullwood, N.J., Gibson, M.J., Curran, M.D., Court, J.A., Mann, D.M., Ikeda, S., Cookson, M.R., Hardy, J., and Allsop, D. 2003. Alpha-synuclein implicated in Parkinson's disease is present in extracellular biological fluids, including human plasma. *Faseb J* **17**(13): 1945-1947.

El-Agnaf, O.M., Salem, S.A., Paleologou, K.E., Curran, M.D., Gibson, M.J., Court, J.A., Schlossmacher, M.G., and Allsop, D. 2006. Detection of oligomeric forms of alpha-synuclein protein in human plasma as a potential biomarker for Parkinson's disease. *Faseb J* **20**(3): 419-425.

El-Agnaf, O.M., Walsh, D.M., and Allsop, D. 2003. Soluble oligomers for the diagnosis of neurodegenerative diseases. *Lancet Neurol* **2**(8): 461-462.

Eliezer, D., Kutluay, E., Bussell, R., Jr., and Browne, G. 2001. Conformational properties of alpha-synuclein in its free and lipid-associated states. *J Mol Biol* **307**(4): 1061-1073.

Enenkel, C., Lehmann, A., and Kloetzel, P.M. 1999. GFP-labelling of 26S proteasomes in living yeast: insight into proteasomal functions at the nuclear envelope/rough ER. *Mol Biol Rep* **26**(1-2): 131-135.

Eriksen, J.L., Przedborski, S., and Petrucelli, L. 2005. Gene dosage and pathogenesis of Parkinson's disease. *Trends Mol Med* **11**(3): 91-96.

Esler, W.P. and Wolfe, M.S. 2001. A portrait of Alzheimer secretases--new features and familiar faces. *Science* **293**(5534): 1449-1454.

Esposito, E., Rotilio, D., Di Matteo, V., Di Giulio, C., Cacchio, M., and Algeri, S. 2002. A review of specific dietary antioxidants and the effects on biochemical mechanisms related to neurodegenerative processes. *Neurobiol Aging* **23**(5): 719-735.

Forno, L.S. 1996. Neuropathology of Parkinson's disease. *J Neuropathol Exp Neurol* **55**(3): 259-272.

Foster, N.L., Wilhelmsen, K., Sima, A.A., Jones, M.Z., D'Amato, C.J., and Gilman, S. 1997. Frontotemporal dementia and parkinsonism linked to chromosome 17: a consensus conference. Conference Participants. *Ann Neurol* **41**(6): 706-715.

Freeman, S.H., Spires-Jones, T., Hyman, B.T., Growdon, J.H., and Frosch, M.P. 2008. TAR-DNA binding protein 43 in Pick disease. *J Neuropathol Exp Neurol* **67**(1): 62-67.

Fujiwara, H., Hasegawa, M., Dohmae, N., Kawashima, A., Masliah, E., Goldberg, M.S., Shen, J., Takio, K., and Iwatsubo, T. 2002. alpha-Synuclein is phosphorylated in synucleinopathy lesions. *Nat Cell Biol* **4**(2): 160-164.

Gai, W.P., Power, J.H., Blumbergs, P.C., and Blessing, W.W. 1998. Multiple-system atrophy: a new alpha-synuclein disease? *Lancet* **352**(9127): 547-548.

Galvin, J.E., Uryu, K., Lee, V.M., and Trojanowski, J.Q. 1999. Axon pathology in Parkinson's disease and Lewy body dementia hippocampus contains alpha-, beta-, and gamma-synuclein. *Proc Natl Acad Sci U S A* **96**(23): 13450-13455.

Geser, F., Winton, M.J., Kwong, L.K., Xu, Y., Xie, S.X., Igaz, L.M., Garruto, R.M., Perl, D.P., Galasko, D., Lee, V.M., and Trojanowski, J.Q. 2008. Pathological TDP-43 in parkinsonism-dementia complex and amyotrophic lateral sclerosis of Guam. *Acta Neuropathol* **115**(1): 133-145.

Giasson, B.I., Duda, J.E., Murray, I.V., Chen, Q., Souza, J.M., Hurtig, H.I., Ischiropoulos, H., Trojanowski, J.Q., and Lee, V.M. 2000. Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. *Science* **290**(5493): 985-989.

Giasson, B.I. and Lee, V.M. 2001. Parkin and the molecular pathways of Parkinson's disease. *Neuron* **31**(6): 885-888.

Giasson, B.I., Murray, I.V., Trojanowski, J.Q., and Lee, V.M. 2001. A hydrophobic stretch of 12 amino acid residues in the middle of alpha-synuclein is essential for filament assembly. *J Biol Chem* **276**(4): 2380-2386.

Gibb, W.R. and Lees, A.J. 1988. The relevance of the Lewy body to the pathogenesis of idiopathic Parkinson's disease. *J Neurol Neurosurg Psychiatry* **51**(6): 745-752.

Gitcho, M.A., Baloh, R.H., Chakraverty, S., Mayo, K., Norton, J.B., Levitch, D., Hatanpaa, K.J., White, C.L., 3rd, Bigio, E.H., Caselli, R., Baker, M., Al-Lozi, M.T., Morris, J.C., Pestronk, A., Rademakers, R., Goate, A.M., and Cairns, N.J. 2008. TDP-43 A315T mutation in familial motor neuron disease. *Ann Neurol* **63**(4): 535-538.

Glickman, M.H. and Ciechanover, A. 2002. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* **82**(2): 373-428.

Glickman, M.H., Rubin, D.M., Coux, O., Wefes, I., Pfeifer, G., Cjeka, Z., Baumeister, W., Fried, V.A., and Finley, D. 1998. A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. *Cell* **94**(5): 615-623.

Goedert, M. 2001. Alpha-synuclein and neurodegenerative diseases. *Nat Rev Neurosci* **2**(7): 492-501.

-. 2004. Tau protein and neurodegeneration. *Semin Cell Dev Biol* **15**(1): 45-49.

Golbe, L.I., Di Iorio, G., Lazzarini, A., Vieregge, P., Gershanik, O.S., Bonavita, V., and Duvoisin, R.C. 1999. The Contursi kindred, a large family with autosomal dominant Parkinson's disease: implications of clinical and molecular studies. *Adv Neurol* **80**: 165-170.

Golbe, L.I., Lazzarini, A.M., Spychala, J.R., Johnson, W.G., Stenroos, E.S., Mark, M.H., and Sage, J.I. 2001. The tau A0 allele in Parkinson's disease. *Mov Disord* **16**(3): 442-447.

Goldberg, A.L. 2005. Nobel committee tags ubiquitin for distinction. *Neuron* **45**(3): 339-344.

Guinto, J.B., Ritson, G.P., Taylor, J.P., and Forman, M.S. 2007. Valosin-containing protein and the pathogenesis of frontotemporal dementia associated with inclusion body myopathy. *Acta Neuropathol* **114**(1): 55-61.

Hardy, J. and Selkoe, D.J. 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* **297**(5580): 353-356.

Hasegawa, M., Arai, T., Akiyama, H., Nonaka, T., Mori, H., Hashimoto, T., Yamazaki, M., and Oyanagi, K. 2007. TDP-43 is deposited in the Guam parkinsonism-dementia complex brains. *Brain* **130**(Pt 5): 1386-1394.

Hasegawa, T., Treis, A., Patenge, N., Fiesel, F.C., Springer, W., and Kahle, P.J. 2008. Parkin protects against tyrosinase-mediated dopamine neurotoxicity by suppressing stress-activated protein kinase pathways. *J Neurochem* **105**(5): 1700-1715.

Hashimoto, M., Rockenstein, E., Mante, M., Crews, L., Bar-On, P., Gage, F.H., Marr, R., and Masliah, E. 2004. An antiaggregation gene therapy strategy for Lewy body disease utilizing beta-synuclein lentivirus in a transgenic model. *Gene Ther* **11**(23): 1713-1723.

He, Z. and Bateman, A. 1999. Progranulin gene expression regulates epithelial cell growth and promotes tumor growth in vivo. *Cancer Res* **59**(13): 3222-3229.

Healy, D.G., Abou-Sleiman, P.M., Casas, J.P., Ahmadi, K.R., Lynch, T., Gandhi, S., Muqit, M.M., Foltynie, T., Barker, R., Bhatia, K.P., Quinn, N.P., Lees, A.J., Gibson, J.M., Holton, J.L., Revesz, T., Goldstein, D.B., and Wood, N.W. 2006. UCHL-1 is not a Parkinson's disease susceptibility gene. *Ann Neurol* **59**(4): 627-633.

Henn, I.H., Bouman, L., Schlehe, J.S., Schlierf, A., Schramm, J.E., Wegener, E., Nakaso, K., Culmsee, C., Berninger, B., Krappmann, D., Tatzelt, J., and Winklhofer, K.F. 2007. Parkin mediates neuroprotection through activation of IkappaB kinase/nuclear factor-kappaB signaling. *J Neurosci* **27**(8): 1868-1878.

Holzmann, C., Kruger, R., Saecker, A.M., Schmitt, I., Schols, L., Berger, K., and Riess, O. 2003. Polymorphisms of the alpha-synuclein promoter: expression analyses and association studies in Parkinson's disease. *J Neural Transm* **110**(1): 67-76.

Hughes, A.J., Daniel, S.E., Kilford, L., and Lees, A.J. 1992. Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. *J Neurol Neurosurg Psychiatry* **55**(3): 181-184.

Hughes, A.J., Daniel, S.E., and Lees, A.J. 2001. Improved accuracy of clinical diagnosis of Lewy body Parkinson's disease. *Neurology* **57**(8): 1497-1499.

Ibanez, P., Bonnet, A.M., Debarges, B., Lohmann, E., Tison, F., Pollak, P., Agid, Y., Durr, A., and Brice, A. 2004. Causal relation between alpha-synuclein gene duplication and familial Parkinson's disease. *Lancet* **364**(9440): 1169-1171.

Imai, Y., Soda, M., Inoue, H., Hattori, N., Mizuno, Y., and Takahashi, R. 2001. An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. *Cell* **105**(7): 891-902.

Ingelsson, M., Nilsson, L., Basun, H., Aquilonius, S.M., and Lannfelt, L. 2005. [Conformationally altered proteins cause neurodegenerative diseases]. *Lakartidningen* **102**(47): 3542-3543, 3545-3546, 3549 passim.

Ingram, E.M. and Spillantini, M.G. 2002. Tau gene mutations: dissecting the pathogenesis of FTDP-17. *Trends Mol Med* **8**(12): 555-562.

Iwatsubo, T. 2003. Aggregation of alpha-synuclein in the pathogenesis of Parkinson's disease. *J Neurol* **250 Suppl 3**: III11-14.

Jakes, R., Spillantini, M.G., and Goedert, M. 1994. Identification of two distinct synucleins from human brain. *FEBS Lett* **345**(1): 27-32.

Jellinger, K.A. 2004. Lewy body-related alpha-synucleinopathy in the aged human brain. *J Neural Transm* **111**(10-11): 1219-1235.

Josephs, K.A., Holton, J.L., Rossor, M.N., Godbolt, A.K., Ozawa, T., Strand, K., Khan, N., Al-Sarraj, S., and Revesz, T. 2004. Frontotemporal lobar degeneration and ubiquitin immunohistochemistry. *Neuropathol Appl Neurobiol* **30**(4): 369-373.

Junn, E., Lee, S.S., Suhr, U.T., and Mouradian, M.M. 2002. Parkin accumulation in aggresomes due to proteasome impairment. *J Biol Chem* **277**(49): 47870-47877.

Kabashi, E., Valdmanis, P.N., Dion, P., Spiegelman, D., McConkey, B.J., Vande Velde, C., Bouchard, J.P., Lacomblez, L., Pochigaeva, K., Salachas, F., Pradat, P.F., Camu, W., Meininger, V., Dupre, N., and Rouleau, G.A. 2008. TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis. *Nat Genet* **40**(5): 572-574.

Kawahara, K., Hashimoto, M., Bar-On, P., Ho, G.J., Crews, L., Mizuno, H., Rockenstein, E., Imam, S.Z., and Masliah, E. 2008. alpha-Synuclein aggregates interfere with Parkin solubility and distribution: role in the pathogenesis of Parkinson disease. *J Biol Chem* **283**(11): 6979-6987.

Kim, K.S., Choi, S.Y., Kwon, H.Y., Won, M.H., Kang, T.C., and Kang, J.H. 2002. Aggregation of alpha-synuclein induced by the Cu,Zn-superoxide dismutase and hydrogen peroxide system. *Free Radic Biol Med* **32**(6): 544-550.

Kim, S.J., Kim, S.Y., Na, Y.S., Lee, H.J., Chung, K.C., and Baik, J.H. 2006. Alpha-synuclein enhances dopamine D2 receptor signaling. *Brain Res* **1124**(1): 5-9.

King, J. 2005. Expression, purification and metal-binding properties of alpha-synuclein, a protein implicated in Parkinson's disease. In. Lancaster University.

Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y., and Shimizu, N. 1998. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* **392**(6676): 605-608.

Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H.D., Mayer, T.U., and Jentsch, S. 1999. A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell* **96**(5): 635-644.

Kruger, R. 2004. Genes in familial parkinsonism and their role in sporadic Parkinson's disease. *J Neurol* **251 Suppl 6**: VI/2-6.

Kubo, S.I., Kitami, T., Noda, S., Shimura, H., Uchiyama, Y., Asakawa, S., Minoshima, S., Shimizu, N., Mizuno, Y., and Hattori, N. 2001. Parkin is associated with cellular vesicles. *J Neurochem* **78**(1): 42-54.

Lai, B.C., Marion, S.A., Teschke, K., and Tsui, J.K. 2002. Occupational and environmental risk factors for Parkinson's disease. *Parkinsonism Relat Disord* **8**(5): 297-309.

Langston, J.W., Forno, L.S., Tetrud, J., Reeves, A.G., Kaplan, J.A., and Karluk, D. 1999. Evidence of active nerve cell degeneration in the substantia nigra of humans years after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine exposure. *Ann Neurol* **46**(4): 598-605.

Lashuel, H.A., Petre, B.M., Wall, J., Simon, M., Nowak, R.J., Walz, T., and Lansbury, P.T., Jr. 2002. Alpha-synuclein, especially the Parkinson's disease-associated mutants, forms pore-like annular and tubular protofibrils. *J Mol Biol* **322**(5): 1089-1102.

Lavedan, C. 1998. The synuclein family. *Genome Res* **8**(9): 871-880.

Lee, E.B., Lee, V.M., Trojanowski, J.Q., and Neumann, M. 2008. TDP-43 immunoreactivity in anoxic, ischemic and neoplastic lesions of the central nervous system. *Acta Neuropathol* **115**(3): 305-311.

Lee, E.N., Cho, H.J., Lee, C.H., Lee, D., Chung, K.C., and Paik, S.R. 2004. Phthalocyanine tetrasulfonates affect the amyloid formation and cytotoxicity of alpha-synuclein. *Biochemistry* **43**(12): 3704-3715.

Lee, F.J., Liu, F., Pristupa, Z.B., and Niznik, H.B. 2001. Direct binding and functional coupling of alpha-synuclein to the dopamine transporters accelerate dopamine-induced apoptosis. *Faseb J* **15**(6): 916-926.

Lee, H.J., Patel, S., and Lee, S.J. 2005. Intravesicular localization and exocytosis of alpha-synuclein and its aggregates. *J Neurosci* **25**(25): 6016-6024.

Leroy, E., Boyer, R., Auburger, G., Leube, B., Ulm, G., Mezey, E., Harta, G., Brownstein, M.J., Jonnalagada, S., Chernova, T., Dehejia, A., Lavedan, C., Gasser, T., Steinbach, P.J., Wilkinson, K.D., and Polymeropoulos, M.H. 1998. The ubiquitin pathway in Parkinson's disease. *Nature* **395**(6701): 451-452.

Li, J., Uversky, V.N., and Fink, A.L. 2002. Conformational behavior of human alpha-synuclein is modulated by familial Parkinson's disease point mutations A30P and A53T. *Neurotoxicology* **23**(4-5): 553-567.

Li, Q.X., Campbell, B.C., McLean, C.A., Thyagarajan, D., Gai, W.P., Kapsa, R.M., Beyreuther, K., Masters, C.L., and Culvenor, J.G. 2002. Platelet alpha- and gamma-synucleins in Parkinson's disease and normal control subjects. *J Alzheimers Dis* **4**(4): 309-315.

Lipton, A.M., White, C.L., 3rd, and Bigio, E.H. 2004. Frontotemporal lobar degeneration with motor neuron disease-type inclusions predominates in 76 cases of frontotemporal degeneration. *Acta Neuropathol (Berl)* **108**(5): 379-385.

Lomen-Hoerth, C., Anderson, T., and Miller, B. 2002. The overlap of amyotrophic lateral sclerosis and frontotemporal dementia. *Neurology* **59**(7): 1077-1079.

Lotharius, J. and Brundin, P. 2002. Pathogenesis of Parkinson's disease: dopamine, vesicles and alpha-synuclein. *Nat Rev Neurosci* **3**(12): 932-942.

Lotharius, J. and O'Malley, K.L. 2000. The parkinsonism-inducing drug 1-methyl-4-phenylpyridinium triggers intracellular dopamine oxidation. A novel mechanism of toxicity. *J Biol Chem* **275**(49): 38581-38588.

Ma, J. and Lindquist, S. 2002. Conversion of PrP to a self-perpetuating PrPSc-like conformation in the cytosol. *Science* **298**(5599): 1785-1788.

Ma, J., Wollmann, R., and Lindquist, S. 2002. Neurotoxicity and neurodegeneration when PrP accumulates in the cytosol. *Science* **298**(5599): 1781-1785.

Mackenzie, I.R. 2007. The neuropathology and clinical phenotype of FTD with progranulin mutations. *Acta Neuropathol (Berl)* **114**(1): 49-54.

Mackenzie, I.R., Baborie, A., Pickering-Brown, S., Du Plessis, D., Jaros, E., Perry, R.H., Neary, D., Snowden, J.S., and Mann, D.M. 2006. Heterogeneity of ubiquitin pathology in frontotemporal lobar degeneration: classification and relation to clinical phenotype. *Acta Neuropathol (Berl)* **112**(5): 539-549.

Mackenzie, I.R., Baker, M., Pickering-Brown, S., Hsiung, G.Y., Lindholm, C., Dwosh, E., Gass, J., Cannon, A., Rademakers, R., Hutton, M., and Feldman, H.H. 2006. The neuropathology of frontotemporal lobar degeneration caused by mutations in the progranulin gene. *Brain* **129**(Pt 11): 3081-3090.

Mackenzie, I.R., Bigio, E.H., Ince, P.G., Geser, F., Neumann, M., Cairns, N.J., Kwong, L.K., Forman, M.S., Ravits, J., Stewart, H., Eisen, A., McClusky, L., Kretzschmar, H.A., Monoranu, C.M., Highley, J.R., Kirby, J., Siddique, T., Shaw, P.J., Lee, V.M., and Trojanowski, J.Q. 2007. Pathological TDP-43 distinguishes sporadic amyotrophic lateral sclerosis from amyotrophic lateral sclerosis with SOD1 mutations. *Ann Neurol* **61**(5): 427-434.

Mackenzie, I.R., Foti, D., Woulfe, J., and Hurwitz, T.A. 2008. Atypical frontotemporal lobar degeneration with ubiquitin-positive, TDP-43-negative neuronal inclusions. *Brain* **131**(Pt 5): 1282-1293.

Marsh, L. and Dawson, T.M. 2000. Treatment of early Parkinson's disease. *Bmj* **321**(7252): 1-2.

Matsumine, H., Saito, M., Shimoda-Matsubayashi, S., Tanaka, H., Ishikawa, A., Nakagawa-Hattori, Y., Yokochi, M., Kobayashi, T., Igarashi, S., Takano, H., Sanpei, K., Koike, R., Mori, H., Kondo, T., Mizutani, Y., Schaffer, A.A., Yamamura, Y., Nakamura, S., Kuzuhara, S., Tsuji, S., and Mizuno, Y. 1997. Localization of a gene for an autosomal recessive form of juvenile Parkinsonism to chromosome 6q25.2-27. *Am J Hum Genet* **60**(3): 588-596.

Maynard, C.J., Cappai, R., Volitakis, I., Cherny, R.A., White, A.R., Beyreuther, K., Masters, C.L., Bush, A.I., and Li, Q.X. 2002. Overexpression of Alzheimer's disease amyloid-beta opposes the age-dependent elevations of brain copper and iron. *J Biol Chem* **277**(47): 44670-44676.

McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D., and Stadlan, E.M. 1984. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* **34**(7): 939-944.

McKhann, G.M., Albert, M.S., Grossman, M., Miller, B., Dickson, D., and Trojanowski, J.Q. 2001. Clinical and pathological diagnosis of frontotemporal dementia: report of the Work Group on Frontotemporal Dementia and Pick's Disease. *Arch Neurol* **58**(11): 1803-1809.

McLean, P.J. and Hyman, B.T. 2002. An alternatively spliced form of rodent alpha-synuclein forms intracellular inclusions in vitro: role of the carboxy-terminus in alpha-synuclein aggregation. *Neurosci Lett* **323**(3): 219-223.

McLean, P.J., Kawamata, H., Ribich, S., and Hyman, B.T. 2000. Membrane association and protein conformation of alpha-synuclein in intact neurons. Effect of Parkinson's disease-linked mutations. *J Biol Chem* **275**(12): 8812-8816.

McNaught, K.S. and Olanow, C.W. 2006. Protein aggregation in the pathogenesis of familial and sporadic Parkinson's disease. *Neurobiol Aging* **27**(4): 530-545.

Mercado, P.A., Ayala, Y.M., Romano, M., Buratti, E., and Baralle, F.E. 2005. Depletion of TDP 43 overrides the need for exonic and intronic splicing enhancers in the human apoA-II gene. *Nucleic Acids Res* **33**(18): 6000-6010.

Metman, L.V., Gillespie, M., Farmer, C., Bibbiani, F., Konitsiotis, S., Morris, M., Shill, H., Bara-Jimenez, W., Mouradian, M.M., and Chase, T.N. 2001. Continuous transdermal dopaminergic stimulation in advanced Parkinson's disease. *Clin Neuropharmacol* **24**(3): 163-169.

Mikolaenko, I., Pletnikova, O., Kawas, C.H., O'Brien, R., Resnick, S.M., Crain, B., and Troncoso, J.C. 2005. Alpha-synuclein lesions in normal aging, Parkinson disease, and Alzheimer disease: evidence from the Baltimore Longitudinal Study of Aging (BLSA). *J Neuropathol Exp Neurol* **64**(2): 156-162.

Miller, D.W., Hague, S.M., Clarimon, J., Baptista, M., Gwinn-Hardy, K., Cookson, M.R., and Singleton, A.B. 2004. Alpha-synuclein in blood and brain from familial Parkinson disease with SNCA locus triplication. *Neurology* **62**(10): 1835-1838.

Mizuno, Y., Hattori, N., Kitada, T., Matsumine, H., Mori, H., Shimura, H., Kubo, S., Kobayashi, H., Asakawa, S., Minoshima, S., and Shimizu, N. 2001. Familial Parkinson's disease. Alpha-synuclein and parkin. *Adv Neurol* **86**: 13-21.

Mizuta, I., Satake, W., Nakabayashi, Y., Ito, C., Suzuki, S., Momose, Y., Nagai, Y., Oka, A., Inoko, H., Fukae, J., Saito, Y., Sawabe, M., Murayama, S., Yamamoto, M., Hattori, N., Murata, M., and Toda, T. 2006. Multiple candidate gene analysis identifies alpha-synuclein as a susceptibility gene for sporadic Parkinson's disease. *Hum Mol Genet* **15**(7): 1151-1158.

Mor-Vaknin, N., Punturieri, A., Sitwala, K., and Markovitz, D.M. 2003. Vimentin is secreted by activated macrophages. *Nat Cell Biol* **5**(1): 59-63.

Morita, A., Kimura, M., and Itokawa, Y. 1994. The effect of aging on the mineral status of female mice. *Biol Trace Elem Res* **42**(2): 165-177.

Mott, R.T., Dickson, D.W., Trojanowski, J.Q., Zhukareva, V., Lee, V.M., Forman, M., Van Deerlin, V., Ervin, J.F., Wang, D.S., Schmechel, D.E., and Hulette,

C.M. 2005. Neuropathologic, biochemical, and molecular characterization of the frontotemporal dementias. *J Neuropathol Exp Neurol* **64**(5): 420-428.

Mukaetova-Ladinska, E.B. and McKeith, I.G. 2006. Pathophysiology of synuclein aggregation in Lewy body disease. *Mech Ageing Dev* **127**(2): 188-202.

Murray, I.V., Giasson, B.I., Quinn, S.M., Koppaka, V., Axelsen, P.H., Ischiropoulos, H., Trojanowski, J.Q., and Lee, V.M. 2003. Role of alpha-synuclein carboxy-terminus on fibril formation in vitro. *Biochemistry* **42**(28): 8530-8540.

Nakamura, T., Yamashita, H., Takahashi, T., and Nakamura, S. 2001. Activated Fyn phosphorylates alpha-synuclein at tyrosine residue 125. *Biochem Biophys Res Commun* **280**(4): 1085-1092.

Nakashima-Yasuda, H., Uryu, K., Robinson, J., Xie, S.X., Hurtig, H., Duda, J.E., Arnold, S.E., Siderowf, A., Grossman, M., Leverenz, J.B., Woltjer, R., Lopez, O.L., Hamilton, R., Tsuang, D.W., Galasko, D., Masliah, E., Kaye, J., Clark, C.M., Montine, T.J., Lee, V.M., and Trojanowski, J.Q. 2007. Co-morbidity of TDP-43 proteinopathy in Lewy body related diseases. *Acta Neuropathol* **114**(3): 221-229.

-. 2007. Co-morbidity of TDP-43 proteinopathy in Lewy body related diseases. *Acta Neuropathol (Berl).*

Neary, D., Snowden, J., and Mann, D. 2005. Frontotemporal dementia. *Lancet Neurol* **4**(11): 771-780.

Neary, D., Snowden, J.S., Gustafson, L., Passant, U., Stuss, D., Black, S., Freedman, M., Kertesz, A., Robert, P.H., Albert, M., Boone, K., Miller, B.L., Cummings, J., and Benson, D.F. 1998. Frontotemporal lobar degeneration: a consensus on clinical diagnostic criteria. *Neurology* **51**(6): 1546-1554.

Neary, D., Snowden, J.S., Mann, D.M., Northen, B., Goulding, P.J., and Macdermott, N. 1990. Frontal lobe dementia and motor neuron disease. *J Neurol Neurosurg Psychiatry* **53**(1): 23-32.

Negro, A., Brunati, A.M., Donella-Deana, A., Massimino, M.L., and Pinna, L.A. 2002. Multiple phosphorylation of alpha-synuclein by protein tyrosine kinase Syk prevents eosin-induced aggregation. *Faseb J* **16**(2): 210-212.

Neumann, M., Kwong, L.K., Truax, A.C., Vanmassenhove, B., Kretzschmar, H.A., Van Deerlin, V.M., Clark, C.M., Grossman, M., Miller, B.L., Trojanowski, J.Q., and Lee, V.M. 2007. TDP-43-positive white matter pathology in frontotemporal lobar degeneration with ubiquitin-positive inclusions. *J Neuropathol Exp Neurol* **66**(3): 177-183.

Neumann, M., Mackenzie, I.R., Cairns, N.J., Boyer, P.J., Markesbery, W.R., Smith, C.D., Taylor, J.P., Kretzschmar, H.A., Kimonis, V.E., and Forman, M.S. 2007. TDP-43 in the ubiquitin pathology of frontotemporal dementia with VCP gene mutations. *J Neuropathol Exp Neurol* **66**(2): 152-157.

Neumann, M., Sampathu, D.M., Kwong, L.K., Truax, A.C., Micsenyi, M.C., Chou, T.T., Bruce, J., Schuck, T., Grossman, M., Clark, C.M., McCluskey, L.F., Miller, B.L., Masliah, E., Mackenzie, I.R., Feldman, H., Feiden, W., Kretzschmar, H.A., Trojanowski, J.Q., and Lee, V.M. 2006. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* **314**(5796): 130-133.

Nishimura, N., Bannykh, S., Slabough, S., Matteson, J., Altschuler, Y., Hahn, K., and Balch, W.E. 1999. A di-acidic (DXE) code directs concentration of cargo during export from the endoplasmic reticulum. *J Biol Chem* **274**(22): 15937-15946.

Nonaka, T., Iwatsubo, T., and Hasegawa, M. 2005. Ubiquitination of alpha-synuclein. *Biochemistry* **44**(1): 361-368.

Ohtake, H., Limprasert, P., Fan, Y., Onodera, O., Kakita, A., Takahashi, H., Bonner, L.T., Tsuang, D.W., Murray, I.V., Lee, V.M., Trojanowski, J.Q., Ishikawa, A., Idezuka, J., Murata, M., Toda, T., Bird, T.D., Leverenz, J.B., Tsuji, S., and La Spada, A.R. 2004. Beta-synuclein gene alterations in dementia with Lewy bodies. *Neurology* **63**(5): 805-811.

Okochi, M., Walter, J., Koyama, A., Nakajo, S., Baba, M., Iwatsubo, T., Meijer, L., Kahle, P.J., and Haass, C. 2000. Constitutive phosphorylation of the Parkinson's disease associated alpha-synuclein. *J Biol Chem* **275**(1): 390-397.

Olanow, C.W., Perl, D.P., DeMartino, G.N., and McNaught, K.S. 2004. Lewy-body formation is an aggresome-related process: a hypothesis. *Lancet Neurol* **3**(8): 496-503.

Olanow, C.W. and Tatton, W.G. 1999. Etiology and pathogenesis of Parkinson's disease. *Annu Rev Neurosci* **22**: 123-144.

Orlowski, M., Cardozo, C., and Michaud, C. 1993. Evidence for the presence of five distinct proteolytic components in the pituitary multicatalytic proteinase complex. Properties of two components cleaving bonds on the carboxyl side of branched chain and small neutral amino acids. *Biochemistry* **32**(6): 1563-1572.

Paleologou, K.E., Irvine, G.B., and El-Agnaf, O.M. 2005. Alpha-synuclein aggregation in neurodegenerative diseases and its inhibition as a potential therapeutic strategy. *Biochem Soc Trans* **33**(Pt 5): 1106-1110.

Park, J.Y. and Lansbury, P.T., Jr. 2003. Beta-synuclein inhibits formation of alpha-synuclein protofibrils: a possible therapeutic strategy against Parkinson's disease. *Biochemistry* **42**(13): 3696-3700.

Payton, A., Holland, F., Diggle, P., Rabbitt, P., Horan, M., Davidson, Y., Gibbons, L., Worthington, J., Ollier, W.E., and Pendleton, N. 2003. Cathepsin D exon 2 polymorphism associated with general intelligence in a healthy older population. *Mol Psychiatry* **8**(1): 14-18.

Perez, R.G. and Hastings, T.G. 2004. Could a loss of alpha-synuclein function put dopaminergic neurons at risk? *J Neurochem* **89**(6): 1318-1324.

Piccini, P., Burn, D.J., Ceravolo, R., Maraganore, D., and Brooks, D.J. 1999. The role of inheritance in sporadic Parkinson's disease: evidence from a longitudinal study of dopaminergic function in twins. *Ann Neurol* **45**(5): 577-582.

Pickering-Brown, S.M., Baker, M., Gass, J., Boeve, B.F., Loy, C.T., Brooks, W.S., Mackenzie, I.R., Martins, R.N., Kwok, J.B., Halliday, G.M., Kril, J., Schofield, P.R., Mann, D.M., and Hutton, M. 2006. Mutations in progranulin explain atypical phenotypes with variants in MAPT. *Brain* **129**(Pt 11): 3124-3126.

Plemper, R.K. and Wolf, D.H. 1999. Retrograde protein translocation: ERADication of secretory proteins in health and disease. *Trends Biochem Sci* **24**(7): 266-270.

Poirier, M.A., Li, H., Macosko, J., Cai, S., Amzel, M., and Ross, C.A. 2002. Huntingtin spheroids and protofibrils as precursors in polyglutamine fibrilization. *J Biol Chem* **277**(43): 41032-41037.

Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E.S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W.G., Lazzarini, A.M., Duvoisin, R.C., Di Iorio, G., Golbe, L.I., and Nussbaum, R.L. 1997. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* **276**(5321): 2045-2047.

Poole, A.C., Thomas, R.E., Andrews, L.A., McBride, H.M., Whitworth, A.J., and Pallanck, L.J. 2008. The PINK1/Parkin pathway regulates mitochondrial morphology. *Proc Natl Acad Sci U S A* **105**(5): 1638-1643.

Prusiner, S.B. 2001. Shattuck lecture--neurodegenerative diseases and prions. *N Engl J Med* **344**(20): 1516-1526.

Quik, M. 2004. Smoking, nicotine and Parkinson's disease. *Trends Neurosci* **27**(9): 561-568.

Rakhit, R., Cunningham, P., Furtos-Matei, A., Dahan, S., Qi, X.F., Crow, J.P., Cashman, N.R., Kondejewski, L.H., and Chakrabartty, A. 2002. Oxidation-induced misfolding and aggregation of superoxide dismutase and its implications for amyotrophic lateral sclerosis. *J Biol Chem* **277**(49): 47551-47556.

Roeber, S., Mackenzie, I.R., Kretzschmar, H.A., and Neumann, M. 2008. TDP-43-negative FTLD-U is a significant new clinico-pathological subtype of FTLD. *Acta Neuropathol.*

Ross, C.A. and Poirier, M.A. 2004. Protein aggregation and neurodegenerative disease. *Nat Med* **10 Suppl**: S10-17.

Salem. 2004. Study of α-synuclein, implicated in Parkinson□s disease, in human brain, cerebrospinal fluid and blood plasma. In *Biological Sciences*. University of lancaster, Lancaster.

Salem, S. 2004. Study of alpha-synuclein, implicated in Parkinson's disease, in human brain, cerebrospinal fluid and blood plasma. In. Lancaster University.

Savitt, J.M., Dawson, V.L., and Dawson, T.M. 2006. Diagnosis and treatment of Parkinson disease: molecules to medicine. *J Clin Invest* **116**(7): 1744-1754.

Scherzinger, E., Sittler, A., Schweiger, K., Heiser, V., Lurz, R., Hasenbank, R., Bates, G.P., Lehrach, H., and Wanker, E.E. 1999. Self-assembly of polyglutamine-containing huntingtin fragments into amyloid-like fibrils: implications for Huntington's disease pathology. *Proc Natl Acad Sci U S A* **96**(8): 4604-4609.

Schlossmacher, M.G., Frosch, M.P., Gai, W.P., Medina, M., Sharma, N., Forno, L., Ochiishi, T., Shimura, H., Sharon, R., Hattori, N., Langston, J.W., Mizuno, Y., Hyman, B.T., Selkoe, D.J., and Kosik, K.S. 2002. Parkin localizes to the Lewy bodies of Parkinson disease and dementia with Lewy bodies. *Am J Pathol* **160**(5): 1655-1667.

Schubert, U., Anton, L.C., Gibbs, J., Norbury, C.C., Yewdell, J.W., and Bennink, J.R. 2000. Rapid degradation of a large fraction of newly synthesized proteins by proteasomes. *Nature* **404**(6779): 770-774.

Seelaar, H., Schelhaas, H.J., Azmani, A., Kusters, B., Rosso, S., Majoor-Krakauer, D., de Rijik, M.C., Rizzu, P., ten Brummelhuis, M., van Doorn, P.A., Kamphorst, W., Willemsen, R., and van Swieten, J.C. 2007. TDP-43 pathology in familial frontotemporal dementia and motor neuron disease without Progranulin mutations. *Brain* **130**(Pt 5): 1375-1385.

Seubert, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Davis, D., Sinha, S., Schlossmacher, M., Whaley, J., Swindlehurst, C., and et al. 1992. Isolation and quantification of soluble Alzheimer's beta-peptide from biological fluids. *Nature* **359**(6393): 325-327.

Sharon, R., Goldberg, M.S., Bar-Josef, I., Betensky, R.A., Shen, J., and Selkoe, D.J. 2001. alpha-Synuclein occurs in lipid-rich high molecular weight complexes, binds fatty acids, and shows homology to the fatty acid-binding proteins. *Proc Natl Acad Sci U S A* **98**(16): 9110-9115.

Shi, J., Shaw, C.L., Du Plessis, D., Richardson, A.M., Bailey, K.L., Julien, C., Stopford, C., Thompson, J., Varma, A., Craufurd, D., Tian, J., Pickering-Brown, S., Neary, D., Snowden, J.S., and Mann, D.M. 2005. Histopathological changes underlying frontotemporal lobar degeneration with clinicopathological correlation. *Acta Neuropathol (Berl)* **110**(5): 501-512.

Shimura, H., Schlossmacher, M.G., Hattori, N., Frosch, M.P., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosik, K.S., and Selkoe, D.J. 2001. Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. *Science* **293**(5528): 263-269.

-. 2001. Ubiquitinination of a New Form of alpha-synuclein by Parkin form Human Brain: Implications for Parkinson's Disease. *Science* **293**: 263-269.

Sidhu, A., Wersinger, C., and Vernier, P. 2004. alpha-Synuclein regulation of the dopaminergic transporter: a possible role in the pathogenesis of Parkinson's disease. *FEBS Lett* **565**(1-3): 1-5.

Singleton, A. and Gwinn-Hardy, K. 2004. Parkinson's disease and dementia with Lewy bodies: a difference in dose? *Lancet* **364**(9440): 1105-1107.

Singleton, A.B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J., Hulihan, M., Peuralinna, T., Dutra, A., Nussbaum, R., Lincoln, S., Crawley, A., Hanson, M., Maraganore, D., Adler, C., Cookson, M.R., Muenter, M., Baptista, M., Miller, D., Blancato, J., Hardy, J., and Gwinn-Hardy, K. 2003. alpha-Synuclein locus triplication causes Parkinson's disease. *Science* **302**(5646): 841.

Skibinski, G., Parkinson, N.J., Brown, J.M., Chakrabarti, L., Lloyd, S.L., Hummerich, H., Nielsen, J.E., Hodges, J.R., Spillantini, M.G., Thusgaard, T., Brandner, S., Brun, A., Rossor, M.N., Gade, A., Johannsen, P., Sorensen, S.A., Gydesen, S., Fisher, E.M., and Collinge, J. 2005. Mutations in the endosomal ESCRTIII-complex subunit CHMP2B in frontotemporal dementia. *Nat Genet* **37**(8): 806-808.

Snowden, J.S., Neary, D., and Mann, D.M. 2004. Autopsy proven sporadic frontotemporal dementia due to microvacuolar-type histology, with onset at 21 years of age. *J Neurol Neurosurg Psychiatry* **75**(9): 1337-1339.

Snowden, J.S., Pickering-Brown, S.M., Mackenzie, I.R., Richardson, A.M., Varma, A., Neary, D., and Mann, D.M. 2006. Progranulin gene mutations associated with frontotemporal dementia and progressive non-fluent aphasia. *Brain* **129**(Pt 11): 3091-3102.

Solano, S.M., Miller, D.W., Augood, S.J., Young, A.B., and Penney, J.B., Jr. 2000. Expression of alpha-synuclein, parkin, and ubiquitin carboxy-terminal hydrolase L1 mRNA in human brain: genes associated with familial Parkinson's disease. *Ann Neurol* **47**(2): 201-210.

Souza, J.M., Giasson, B.I., Chen, Q., Lee, V.M., and Ischiropoulos, H. 2000. Dityrosine cross-linking promotes formation of stable alpha -synuclein polymers. Implication of nitrative and oxidative stress in the pathogenesis of neurodegenerative synucleinopathies. *J Biol Chem* **275**(24): 18344-18349.

Spillantini, M.G., Crowther, R.A., Jakes, R., Cairns, N.J., Lantos, P.L., and Goedert, M. 1998. Filamentous alpha-synuclein inclusions link multiple system atrophy with Parkinson's disease and dementia with Lewy bodies. *Neurosci Lett* **251**(3): 205-208.

Spillantini, M.G., Crowther, R.A., Jakes, R., Hasegawa, M., and Goedert, M. 1998. alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. *Proc Natl Acad Sci U S A* **95**(11): 6469-6473.

Spillantini, M.G., Schmidt, M.L., Lee, V.M., Trojanowski, J.Q., Jakes, R., and Goedert, M. 1997. Alpha-synuclein in Lewy bodies. *Nature* **388**(6645): 839-840.

Spira, P.J., Sharpe, D.M., Halliday, G., Cavanagh, J., and Nicholson, G.A. 2001. Clinical and pathological features of a Parkinsonian syndrome in a family with an Ala53Thr alpha-synuclein mutation. *Ann Neurol* **49**(3): 313-319.

Sreedharan, J., Blair, I.P., Tripathi, V.B., Hu, X., Vance, C., Rogelj, B., Ackerley, S., Durnall, J.C., Williams, K.L., Buratti, E., Baralle, F., de Belleroche, J., Mitchell, J.D., Leigh, P.N., Al-Chalabi, A., Miller, C.C., Nicholson, G., and Shaw, C.E. 2008. TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. *Science* **319**(5870): 1668-1672.

Steffan, J.S., Agrawal, N., Pallos, J., Rockabrand, E., Trotman, L.C., Slepko, N., Illes, K., Lukacsovich, T., Zhu, Y.Z., Cattaneo, E., Pandolfi, P.P., Thompson, L.M., and Marsh, J.L. 2004. SUMO modification of Huntingtin and Huntington's disease pathology. *Science* **304**(5667): 100-104.

Subramaniam, S., Strelau, J., and Unsicker, K. 2003. Growth differentiation factor-15 prevents low potassium-induced cell death of cerebellar granule neurons by differential regulation of Akt and ERK pathways. *J Biol Chem* **278**(11): 8904-8912.

Suchowersky, O., Reich, S., Perlmutter, J., Zesiewicz, T., Gronseth, G., and Weiner, W.J. 2006. Practice Parameter: Diagnosis and prognosis of new onset Parkinson disease (an evidence-based review). Report of the Quality Standards Subcommittee of the American Academy of Neurology. *Neurology*.

Sunde, M., Serpell, L.C., Bartlam, M., Fraser, P.E., Pepys, M.B., and Blake, C.C. 1997. Common core structure of amyloid fibrils by synchrotron X-ray diffraction. *J Mol Biol* **273**(3): 729-739.

Tabner, B.J., Turnbull, S., El-Agnaf, O.M., and Allsop, D. 2002. Formation of hydrogen peroxide and hydroxyl radicals from A(beta) and alpha-synuclein as a possible mechanism of cell death in Alzheimer's disease and Parkinson's disease. *Free Radic Biol Med* **32**(11): 1076-1083.

Takahashi, S., Takahashi, I., Sato, H., Kubota, Y., Yoshida, S., and Muramatsu, Y. 2001. Age-related changes in the concentrations of major and trace elements in the brain of rats and mice. *Biol Trace Elem Res* **80**(2): 145-158.

Takahashi, T., Yamashita, H., Nakamura, T., Nagano, Y., and Nakamura, S. 2002. Tyrosine 125 of alpha-synuclein plays a critical role for dimerization following nitrative stress. *Brain Res* **938**(1-2): 73-80.

Takeuchi, H. and Takahashi, R. 2007. [Pathogenesis of Parkinson disease]. *Nippon Ronen Igakkai Zasshi* **44**(4): 415-421.

Tan, C.F., Eguchi, H., Tagawa, A., Onodera, O., Iwasaki, T., Tsujino, A., Nishizawa, M., Kakita, A., and Takahashi, H. 2007. TDP-43 immunoreactivity in neuronal inclusions in familial amyotrophic lateral sclerosis with or without SOD1 gene mutation. *Acta Neuropathol (Berl)* **113**(5): 535-542.

Tanaka, K., Suzuki, T., Chiba, T., Shimura, H., Hattori, N., and Mizuno, Y. 2001. Parkin is linked to the ubiquitin pathway. *J Mol Med* **79**(9): 482-494.

Tanner, C.M. 2000. Dopamine agonists in early therapy for Parkinson disease: promise and problems. *Jama* **284**(15): 1971-1973.

Tanner, C.M., Ottman, R., Goldman, S.M., Ellenberg, J., Chan, P., Mayeux, R., and Langston, J.W. 1999. Parkinson disease in twins: an etiologic study. *Jama* **281**(4): 341-346.

Tokuda, T., Salem, S.A., Allsop, D., Mizuno, T., Nakagawa, M., Qureshi, M.M., Locascio, J.J., Schlossmacher, M.G., and El-Agnaf, O.M. 2006. Decreased alpha-synuclein in cerebrospinal fluid of aged individuals and subjects with Parkinson's disease. *Biochem Biophys Res Commun* **349**(1): 162-166.

Trojanowski, J.Q., Goedert, M., Iwatsubo, T., and Lee, V.M. 1998. Fatal attractions: abnormal protein aggregation and neuron death in Parkinson's disease and Lewy body dementia. *Cell Death Differ* **5**(10): 832-837.

Trushina, E., Heldebrant, M.P., Perez-Terzic, C.M., Bortolon, R., Kovtun, I.V., Badger, J.D., 2nd, Terzic, A., Estevez, A., Windebank, A.J., Dyer, R.B., Yao,

J., and McMurray, C.T. 2003. Microtubule destabilization and nuclear entry are sequential steps leading to toxicity in Huntington's disease. *Proc Natl Acad Sci U S A* **100**(21): 12171-12176.

Ulusoy, A. and Kirik, D. 2008. Can overexpression of parkin provide a novel strategy for neuroprotection in Parkinson's disease? *Exp Neurol.*

Uryu, K., Nakashima-Yasuda, H., Forman, M.S., Kwong, L.K., Clark, C.M., Grossman, M., Miller, B.L., Kretzschmar, H.A., Lee, V.M., Trojanowski, J.Q., and Neumann, M. 2008. Concomitant TAR-DNA-binding protein 43 pathology is present in Alzheimer disease and corticobasal degeneration but not in other tauopathies. *J Neuropathol Exp Neurol* **67**(6): 555-564.

Uversky, V.N. 2003. Protein folding revisited. A polypeptide chain at the folding-misfolding-nonfolding cross-roads: which way to go? *Cell Mol Life Sci* **60**(9): 1852-1871.

Uversky, V.N., Lee, H.J., Li, J., Fink, A.L., and Lee, S.J. 2001. Stabilization of partially folded conformation during alpha-synuclein oligomerization in both purified and cytosolic preparations. *J Biol Chem* **276**(47): 43495-43498.

Uversky, V.N., Li, J., and Fink, A.L. 2001. Metal-triggered structural transformations, aggregation, and fibrillation of human alpha-synuclein. A possible molecular NK between Parkinson's disease and heavy metal exposure. *J Biol Chem* **276**(47): 44284-44296.

-. 2001. Pesticides directly accelerate the rate of alpha-synuclein fibril formation: a possible factor in Parkinson's disease. *FEBS Lett* **500**(3): 105-108.

Uversky, V.N., Li, J., Souillac, P., Millett, I.S., Doniach, S., Jakes, R., Goedert, M., and Fink, A.L. 2002. Biophysical properties of the synucleins and their propensities to fibrillate: inhibition of alpha-synuclein assembly by beta- and gamma-synucleins. *J Biol Chem* **277**(14): 11970-11978.

van de Warrenburg, B.P., Lammens, M., Lucking, C.B., Denefle, P., Wesseling, P., Booij, J., Praamstra, P., Quinn, N., Brice, A., and Horstink, M.W. 2001.

Clinical and pathologic abnormalities in a family with parkinsonism and parkin gene mutations. *Neurology* **56**(4): 555-557.

Van Deerlin, V.M., Leverenz, J.B., Bekris, L.M., Bird, T.D., Yuan, W., Elman, L.B., Clay, D., Wood, E.M., Chen-Plotkin, A.S., Martinez-Lage, M., Steinbart, E., McCluskey, L., Grossman, M., Neumann, M., Wu, I.L., Yang, W.S., Kalb, R., Galasko, D.R., Montine, T.J., Trojanowski, J.Q., Lee, V.M., Schellenberg, G.D., and Yu, C.E. 2008. TARDBP mutations in amyotrophic lateral sclerosis with TDP-43 neuropathology: a genetic and histopathological analysis. *Lancet Neurol* **7**(5): 409-416.

Vandermeeren, M., Mercken, M., Vanmechelen, E., Six, J., van de Voorde, A., Martin, J.J., and Cras, P. 1993. Detection of tau proteins in normal and Alzheimer's disease cerebrospinal fluid with a sensitive sandwich enzyme-linked immunosorbent assay. *J Neurochem* **61**(5): 1828-1834.

Volles, M.J. and Lansbury, P.T., Jr. 2002. Vesicle permeabilization by protofibrillar alpha-synuclein is sensitive to Parkinson's disease-linked mutations and occurs by a pore-like mechanism. *Biochemistry* **41**(14): 4595-4602.

Volles, M.J., Lee, S.J., Rochet, J.C., Shtilerman, M.D., Ding, T.T., Kessler, J.C., and Lansbury, P.T., Jr. 2001. Vesicle permeabilization by protofibrillar alpha-synuclein: implications for the pathogenesis and treatment of Parkinson's disease. *Biochemistry* **40**(26): 7812-7819.

Vonsattel, J.P., Myers, R.H., Stevens, T.J., Ferrante, R.J., Bird, E.D., and Richardson, E.P., Jr. 1985. Neuropathological classification of Huntington's disease. *J Neuropathol Exp Neurol* **44**(6): 559-577.

Wang, H.Y., Wang, I.F., Bose, J., and Shen, C.K. 2004. Structural diversity and functional implications of the eukaryotic TDP gene family. *Genomics* **83**(1): 130-139.

Weinreb, P.H., Zhen, W., Poon, A.W., Conway, K.A., and Lansbury, P.T., Jr. 1996. NACP, a protein implicated in Alzheimer's disease and learning, is natively unfolded. *Biochemistry* **35**(43): 13709-13715.

Welch, K. and Yuan, J. 2003. Alpha-synuclein oligomerization: a role for lipids? *Trends Neurosci* **26**(10): 517-519.

Wood, N. 1997. Genes and parkinsonism. *J Neurol Neurosurg Psychiatry* **62**(4): 305-309.

Wood, S.J., Wypych, J., Steavenson, S., Louis, J.C., Citron, M., and Biere, A.L. 1999. alpha-synuclein fibrillogenesis is nucleation-dependent. Implications for the pathogenesis of Parkinson's disease. *J Biol Chem* **274**(28): 19509-19512.

Yokoseki, A., Shiga, A., Tan, C.F., Tagawa, A., Kaneko, H., Koyama, A., Eguchi, H., Tsujino, A., Ikeuchi, T., Kakita, A., Okamoto, K., Nishizawa, M., Takahashi, H., and Onodera, O. 2008. TDP-43 mutation in familial amyotrophic lateral sclerosis. *Ann Neurol* **63**(4): 538-542.

Yu, S., Ueda, K., and Chan, P. 2005. Alpha-synuclein and dopamine metabolism. *Mol Neurobiol* **31**(1-3): 243-254.

Zarranz, J.J., Alegre, J., Gomez-Esteban, J.C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atares, B., Llorens, V., Gomez Tortosa, E., del Ser, T., Munoz, D.G., and de Yebenes, J.G. 2004. The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. *Ann Neurol* **55**(2): 164-173.

Zhang, Y.J., Xu, Y.F., Dickey, C.A., Buratti, E., Baralle, F., Bailey, R., Pickering-Brown, S., Dickson, D., and Petrucelli, L. 2007. Progranulin mediates caspase-dependent cleavage of TAR DNA binding protein-43. *J Neurosci* **27**(39): 10530-10534.