Ubiquitin proteasome system impairment and its relationships to cognitive and non-cognitive symptoms, pathology and synaptic dysfunction, in the Lewy body dementias.

Alghamdi, Amani Ahmed

Awarding institution:
King's College London

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Amani Ahmed Alghamdi

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King's College London
Wolfson Centre for Age-Related Diseases
School of Biomedical Sciences
King's College London

ABSTRACT

Lewy body dementia, which includes dementia with Lewy bodies (DLB) and Parkinson's disease dementia (PDD), is collectively the second most common neurodegenerative dementia and is pathologically characterized by α-synuclein positive cytoplasmic inclusions, with varying amounts of Aβ and tau aggregates in addition to synaptic loss. Clinical hallmarks include fluctuating and deteriorating cognition, hallucinations and parkinsonism. A dysfunctional ubiquitin proteasome system (UPS) may be a mediating factor of disease progression and of the development of α-synuclein aggregates. In the present study, protein expression of some key component subunits of the UPS and two of the three main proteolytic-like (chymotrypsin- and PGPH-) activities have been determined in the frontal cortex (Brodmann, BA9), the parietal cortex (BA40) and the anterior cingulate gyrus (BA24) of DLB, PDD, Alzheimer's disease (AD) and matched controls. Clinical and pathological data were available for the cases studied, with regard to the duration of dementia and parkinsonism, the Mini-Mental State Examination (MMSE) score, the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) plaque, the Braak stage and the α-synuclein score. We hypothesised that cognitive decline and non-cognitive symptoms were associated with the proteasome impairment as a consequence of synaptic dysfunction and increased protein aggregation in LBD and AD. To address the link between proteasome impairment, AD and LBD pathology, cognition decline and non-cognitive symptoms, and the synaptic dysfunction, alteration of the proteasome components and activities have been investigated to identify clinical-pathological correlations. Our aim was to investigate possible relationships between a) decreased level of proteasome components and semi-quantitative scores of AD and LBD pathology in the

selected brain areas, b) proteasome dysfunction and the cognition function and non-cognitive symptoms in LBD and AD and c) reduction of proteasome components and synaptic dysfunction. Due to the importance of the protein degradation pathways in the development of Lewy bodies and the evidence from our studies indicative of proteasome dysfunction, the lysosomal pathway was also examined. Two lysosomal markers were chosen for investigation: cathepsin D and lysosomal-associated membrane protein 1 (LAMP1) and the same clinico-pathological correlations were applied for the lysosomal markers. The major finding of this project was the reduction in the RPT6 ATPase 19S regulatory subunit in DLB and AD; this reduction was associated with the decrease in proteasome activity and synaptic markers (PSD-95, ZnT3, synaptophysin and beta-III-tubulin). Both reductions of RPT6 and decreases in proteasome activity predicted cognitive decline, depression and severity of amyloid-beta and tau pathology in the examined brain regions.

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LIST OF ABBREVIATIONS

AAA family	ATPases associated with a variety of cellular activities
AC	Amygdala
AD	Alzheimer's disease
ALP	Autophagy lysosome pathway
APOE	Apolipoprotein E
APP	amyloid precursor protein
ATP	adenosine triphosphate
ATPase	Adenosine Triphosphatase
Aβ	Amyloid β peptide
Aβ40	Amyloid β peptide 40 amino acid
Aβ42	Amyloid β peptide 42 amino acid
BA	Brodmann area
BACE1	Beta-site APP cleaving enzyme 1
bLB	brainstem Lewy body
CA2	Cornu Ammonis region 2
CaMKII	Calcium/calmodulin-dependent protein kinase II
CBB	Coomassie Brilliant Blue
CDT	Clock drawing test
CERAD	Consortium to Establish a Registry for Alzheimer's Disease
ChAT	Choline Acetyltransferase

ChE-I	Cholin Esterase-Inhibitors
cLB	cortical Lewy bodies
CMA	Cohen-Mansfield Agitation Inventory
CP	Core particle
DLB	Dementia with Lewy bodies
dLBD	diffuse Lewy body disease
DMV	dorsal motor nucleus of the vagus nerve
DNA	Deoxyribonucleic acid
EPS	Extrapyramidal Motor Symptoms
FC	Fluctuation in cognition
GABA	Gamma-Aminobutyric acid
GBA	Glucocerebrosidase
GDS	Geriatric Depression Scale scores
HbYX	C-terminal hydrophobic-tyrosine-X
HD	Huntington's disease
IFN-γ	Interferon-gamma
IHC	Immunohistochemistry
irx	Intermediate reticular zone
KO	Knock-out
LBD	Lewy body dementias
LBs	Lewy Bodies
LC	Locus coeruleus
LMP2	Low molecular weight protein 2

LMP7	Low molecular weight protein 7
LNs	Lewy Neurites
LRRK2	leucine-rich repeat kinase 2
MCI	mild cognitive impairment
MECL1	Multicatalytic endopeptidase complex-like 1
MHC	Major histocompatibility complex
MMSE	Mini Mental State Examination
MWM	molecular weight marker
nbM	Nucleus basalis of Meynert
NFT	Neurofibrillary tangles
NIA	National Institute of Aging
NMDA	N-methyl-D-aspartate
NPI	Neuropsychiatric Inventory
Ntn	N-terminal nucleophile
NTs	neuropil threads
PB1	Phox and Bem1p
PD	Parkinson's disease
PDD	Parkinson's disease dementia
PET	Positron Emission Tomography
PGPH	Peptidyl-glutamyl peptide-hydrolyzing
PiB	11C] 6-OH-BTA-1 [Pittsburgh Compound-B
PIGD	postural-instability gait difficulty
PSMC1-6	(proteasome (prosome, macropain) 26S subunit, ATPase, 1-6)

PTM	post-translational modification
R	Raphe
RCT	randomised clinical trial
REM	Rapid eye movement
RP	regulatory particle
RPN	Regulatory Particle Non-ATPase
RPT1 – 6	Regulatory Particle Triphosphatases/ RP Triple-A ATPase
sAPPα	secreted (Amyloid Precursor Peptide)α
sAPPβ	secreted (Amyloid Precursor Peptide)β
SDS	sodium dodecyl-sulphate
SDS-PAGE	sodium dodecyl-sulphate polyacrylamide gel electrophoresis
SN	Substantia nigra
SNARE	soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors
SNCA	α-synuclein gene
SPP	Synaptophysin
TOex	Temporo-occipital cortex
Ub	Ubiquitin
UBA	Ub-associated
UBA	Ubiquitin associate domain
UbL	Ubiquitin-like domain
UBL	Ub-like
UIM	Ubiquitin interacting motif
UPDRS	Unified Parkinson's Disease Rating Scale

UPS	Ubiquitin proteasome pathway
VaD	Vascular Dementia
VH	Visual hallucination
WT	Wild-type

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WHITFIELD, D. R., VALLORTIGARA, J., **ALGHAMDI, A**., HORTOBAGYI, T., BALLARD, C., THOMAS, A. J., O'BRIEN, J. T., AARSLAND, D. & FRANCIS, P. T. 2014a. Depression and Synaptic Zinc Regulation in Alzheimer Disease, Dementia with Lewy Bodies, and Parkinson Disease Dementia. The American journal of geriatric psychiatry.

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CHAPTER 1 INTRODUCTION 1

Chapter 1 Introduction

1.1 General overview:

Lewy body diseases (LBD), which include Parkinson's disease (PD), Parkinson's disease dementia (PDD) and dementia with Lewy bodies (DLB), are pathologically characterized by the presence of abnormal inclusions called Lewy bodies (LBs), and the development of thread-like and thicker Lewy neurites (LNs) within the neurons. LBs and LNs are intraneuronal inclusion bodies with pathological aggregated and hyperphosphorylated α-synuclein as their main component (Spillantini et al., 1997). This was determined after the discovery of a mutation in the α-synuclein gene (SNCA), which is a rare cause of familial PD (Polymeropoulos et al., 1997). The aggregation of α-synuclein inside the neurons is thought to be a key event in LB formation. The presence of LBs can occur in LBD alone or in combination with Alzheimer's disease (AD) pathology (Howlett et al., 2014, Tsuboi and Dickson, 2005). In the case of PD, the presence of LBs throughout the brainstem accompanies the loss of dopaminergic neurons from the substantia nigra, which is considered to underlie the motor symptoms (Braak et al., 2004, Dauer and Przedborski, 2003, Jellinger, 2009). On the other hand, the widespread distribution of LBs in virtually every brain region in cases of LBD is likely to contribute to the variety of cognitive and behavioural symptoms present in these conditions, such as visual hallucinations, delusions, delusional misidentifications, anxiety, apathy, cognitive decline and fluctuating cognition, agitation and aggressive behaviours (Jellinger, 2009).

In considering Lewy body dementia, cognitive impairment is the key presenting feature of DLB, while parkinsonism is the first feature of PDD (McKeith et al., 2004). As the disease progresses, it becomes clinically difficult to differentiate between DLB and PDD patients, both of whom will be characterised by cognitive impairment, psychiatric symptoms and

parkinsonian symptoms. They are also similar in their neuropathological characteristics: abundance of LN and LB in most brain regions and cortical extracellular Aβ plaques and neurofibrillary tangles (NFT) (McKeith, 2007). This is why most researchers use the "one year rule" to distinguish between PDD and LBD. According to the "one year rule", when the motor symptoms have been present for one year or more before the onset of dementia, the diagnosis should be PDD (McKeith et al., 2005, McKeith et al., 1996). However, if the dementia and motor symptoms begin in the same year, or the cognitive symptoms start before the motor symptoms, then the diagnosis should be DLB (McKeith et al., 2005).

The clinical features of LBD are associated with the affected areas of the brain. Most patients with LBD experience at least one or two behavioural symptoms during the course of their illness, which are a major cause of personal distress to the patients and those who care for them. In a study using a large number of PDD patients, 90% of these 537 PDD patients had at least one neuropsychiatric symptom, and 77% had two or more, with depression, apathy, anxiety and hallucinations being the most common (Aarsland et al., 2007). Another study evaluated the behavioural and psychological symptoms in different stages of DLB, and it was found that anxiety, depression, apathy, agitation and sleep disorders were the most common symptoms and that these symptoms tended to progressively worsen over time (Borroni et al., 2008). Furthermore, patients with LBD experienced very high sensitivity to antipsychotic and anti-agitation medications (McKeith et al., 1992); therefore, an important un-met medical need in DLB is the management of the behavioural symptoms.

The clinical characteristics of DLB can be classified as the central, core, suggestive and supportive features with regard to the consequence criteria for the clinical and pathological

diagnosis of dementia with Lewy bodies (McKeith et al., 2005, McKeith et al., 1996). The central feature of DLB is 'Dementia defined as progressive cognitive decline of sufficient magnitude to interfere with normal social or occupational function'; this can be accompanied by an increasing degree of memory loss and worsening attention, executive function and visuospatial function (McKeith et al., 2005). Cognitive decline with one of the three core features, 'fluctuating cognition with pronounced variations in attention and alertness, recurrent visual hallucinations that are typically well formed and detailed, and spontaneous features of parkinsonism' leads to the diagnosis of possible DLB (McKeith et al., 2005). If the patients experience two or more core features, the diagnosis of DLB is probable. The presence of one or more suggestive symptoms (REM sleep behaviour disorder, neuroleptic sensitivity and low dopamine transporter uptake in the basal ganglia) and 'supportive' features (see subsequent section on other symptoms) with the dementia and the core symptoms will also lead to the diagnosis of probable DLB (McKeith et al., 2005). One or more suggestive symptoms without any of core features, the diagnosis of DLB is possible; however, the diagnosis of probable DLB cannot be made with the suggestive symptoms alone (McKeith et al., 2005).

1.2 Epidemiology

DLB is the 2nd most common neurodegenerative dementia after AD. Clinically diagnosed DLB accounts for 10-20% of elderly dementia patients; this proportion is consistent with autopsy studies, which suggest that DLB accounts for approximately 15-30% of dementias (Barker et al., 2002, Byrne et al., 1989, Perry et al., 1990d). In an epidemiological clinical study by Zaccai and colleagues, an estimate of the prevalence of DLB, depending on the case criteria, falls within the range of 0 to 5% in the general population, and between 0 and 30.5% of all dementia cases (Zaccai et al., 2005). The only estimate for DLB incidence is 0.1% per year for the general population and 3.2% per year for all new dementia cases (Zaccai et al., 2005). There are an estimated 100,000 cases in the UK (McKeith et al., 1999), and the prevalence of DLB is estimated to occur at 1% in the overall population of people over the age of 65 (McKeith et al., 1996). This percentage is reported to rise with age thus in those over 85 it represents approximately 22% of all dementia cases (Rahkonen et al., 2003, Shergill et al., 1994, Stevens et al., 2002).

The difficulty in determining clear prevalence statistics for LBD is primarily the Lewy bodies - the hallmark of this disorder - due to their linkage with several forms of neurodegenerative disease, can make it very difficult to distinguish LBD from other conditions. One population-based autopsy study found that Lewy bodies were equally common in demented and non-demented individuals (Esiri et al., 2001). Another autopsy study investigated 248 cases (50-90 years of age), and Lewy bodies were found in 22 of those cases (7.7%), with 9 of them having cortical Lewy bodies. None of those 9 cases had dementia (Lindboe and Hansen, 1998). No classical epidemiological studies to investigate age and sex variation, or potential risk factors for DLB, have as of yet been reported

1.3 Clinical features of Lewy body dementia

1.3.1 Cognitive impairment

Progressive cognitive decline is the central clinical diagnostic feature of DLB. The defect in cognitive dysfunction in DLB/PDD is normally characterized as impaired visuospatial skills, substantial attentional deficits and executive impairment (Aarsland et al., 2003, Dubois and Pillon, 1997, McKeith et al., 1996).

Patients with DLB/PDD exhibit less memory impairment when compared to those with AD, but as the disease progresses, the memory impairment will become more developed (McKeith et al., 2005). These features are severe enough to impact normal social or occupational functions.

The following characteristics can be used to differentiate between LBD and AD (Ballard et al., 2002). A person with DLB/PDD is likely to perform well on tests of verbal memory, but worse on visuospatial performance tasks and tests of attention than those with AD (Gnanalingham et al., 1997, Hansen et al., 1990, Noe et al., 2004). Visual-constructional deficits may play an important role as a sensitive indicator for DLB, and in differentiating between different dementias. By comparing a pentagon drawing done by DLB and AD patients, it was found that the test had a sensitivity of 88% and a specificity of 59% in the diagnosis of DLB (Ala et al., 2001). A more recent study shows that DLB patients were significantly worse at pentagon drawing when compared with PD and AD individuals, but were not worse than patients with PDD (Cormack et al., 2004). The same results were found when the patients were asked to draw the face of a clock. The patients

with DLB/PDD did poorly on both the copy and draw part of the test, while AD patients did well on the copying part, but poor on the drawing part (Gnanalingham et al., 1996).

Fluctuation in cognition (FC) is one of the core features of DLB (McKeith et al., 2005), and has been described as an unprompted alteration in the level of arousal, cognition and attention (Ferman et al., 2004, McKeith et al., 2005). Irresistible daytime drowsiness, a decrease in the level of awareness, illogical or disorganized thinking and staring spells appear to be important features of arousal disturbance (Ferman et al., 2004, Walker et al., 2000b).

FC symptoms are usually evident on a day to day basis and may fluctuate within much shorter periods of time, from moments to hours (Walker et al., 2000a). Approximately 20% of people with AD (Escandon et al., 2010, Kolbeinsson and Jonsson, 1993) and 35%-50% of people with vascular dementia (VaD) (Roman et al., 1993) exhibit a FC. In DLB, this symptom is increased up to 90%. It is associated with rapid changes between alertness and normal cognition, followed by confusion and an inability to pay attention or make decisions (Byrne et al., 1989, McKeith et al., 2005).

1.3.2 Visual hallucination (VH)

Visual hallucination (VH) is the second core feature for the diagnostic criteria of DLB/PDD, occurring in 60-80% of DLB patients (Aarsland et al., 2001a, Ballard et al., 1999, Hirono et al., 1999), 54% of PDD cases (Aarsland et al., 2001a, Aarsland et al., 2007) and 25% of PD patients (Aarsland et al., 1999, Aarsland et al., 2001c). It is generally

present from the early stages of illness and persists throughout the course of the disease (McKeith et al., 2005). VH is one of the prominent symptoms and represents an important tool for diagnosis and differentiating between DLB from AD (Auning et al., 2011, McKeith et al., 2005, Tiraboschi et al., 2006, Troster, 2008).

VH usually occurs most days of the week in a complex form, typically manifesting in brightly coloured, three-dimensional images of animals and people (Ballard et al., 1996, McKeith et al., 1996). Hallucinations involving children, inanimate objects, fire, insects and birds can occur, but less frequently (Ballard et al., 1996, Ballard et al., 1997, McKeith et al., 1996). Visual hallucinations can be accompanied by auditory hallucinations such as speech or noise (Ballard et al., 1997).

Deficits in function related to the visual system are normal in the elderly population; however, the coexistence of visual impairment may increase the susceptibility of DLB patients to VH. A recent study demonstrated poor visual attention to be an independent predictor of VH (Cagnin et al., 2013). Furthermore, patients with visual hallucinations complain of poor vision more often than patients without visual hallucinations (McShane et al., 1995).

In LBD, the main factor that may contribute to visual hallucinations is the presence of LB pathology. Several studies linked the development of LB in the temporal lobe,

amygdala, transentorhinal region and frontal lobe with the presence of visual hallucinations (Gallagher et al., 2011, Harding et al., 2002, Papapetropoulos et al., 2006). Earlier onsets of visual hallucinations are also associated with limbic Lewy body in DLB (Ferman et al., 2013). The presence of VH in DLB is suggested to be associated with deficits in choline acetyltransferase (ChAT). It has been found that there is a relationship between reduced levels of ChAT in the part of the visual association area (parahippocampal gyrus - BA36) and VH (Ballard et al., 2000b, Perry et al., 1990a). There is also a link between the induction of hallucinations and the start of anti-cholinergic therapies (McKeith et al., 2000).

1.3.3 Parkinsonism:

Parkinsonism has been classified as one of the three core features of DLB (McKeith et al., 2005, McKeith et al., 1996), and 40%-100% of DLB patients experience extrapyramidal signs at some stage of the disease (Gnanalingham et al., 1997, Louis et al., 1997). These signs are similar to those seen in PD patients, such as bradykinesia, rigidity, postural instability and gait disturbance. The first consensus guidelines for the clinical and pathological diagnosis of DLB assumes that the extrapyramidal signs tend to be mild and symmetrical in DLB when compared to PD (McKeith et al., 1996). Many studies did not support this view, and found that extrapyramidal features in DLB are similar in severity in some aspects, such as resting tremors and abnormal posture, and more severe in other features, such as body bradykinesia, gait and rigidity, than in PD (Aarsland et al., 2001b).

Louis et al. had the same findings, but with smaller sample sizes (Louis et al., 1997). Another study showed that DLB patients had higher rigidity and a reduced tapping speed than PD patients, but less tremor dominance when compared to PD (Gnanalingham et al., 1997). There was no difference presenting with resting tremors between PDD and DLB patients (Noe et al., 2004); although, in one study, resting tremors did not occur early in the disease, and were more common in PD (85%) than DLB (55.5%) (Louis et al., 1997). Another motor comparative study had the same view with respect to the resting tremor, and found it to be more evident in PD then DLB patients (82% vs. 67%, respectively) (Gnanalingham et al., 1997).

In a more detailed comparative study done by Burn et al., it was found that postural-instability gait difficulty was more common in PDD and DLB, then in PD (Burn et al., 2003). The study also showed that the opposite was true with tremor dominance (Burn et al., 2003). The differences in the results between studies may depend on the cases used in the study, the stage of the disease, age and gender. In the end, parkinsonism is one of the core features, and DLB can be diagnosed without the presence of the extrapyramidal signs.

1.3.4 Depression

Consensus criteria for the clinical diagnosis of DLB and PDD listed depression as one of the supportive features in 2005 (McKeith et al., 2005). Studies show that the prevalence of depressive symptoms in DLB is higher than AD (73% vs. 56%), and similar to PD (Fritze et al., 2011b). A one-year follow-up study found that cognitive decline was associated with depression, and of those with depression at the time of the follow-up,

cognitive decline was higher in DLB than in AD patients (Fritze et al., 2011a). Another comprehensive study demonstrated that depression scores in DLB patients were twice as high as those for AD patients using the Geriatric Depression Scale scores (GDS) (Yamane et al., 2011). However, Caputo et al. failed to show consistent results with a large study involving 921 patients and found no difference in depression between AD and DLB (Caputo et al., 2008).

Depression has been shown to be a risk factor for incidences of dementia including AD, VD and LBD (Andersen et al., 2005, Chen et al., 2008, Gatz et al., 2005, Hebert et al., 2000, Saczynski et al., 2010). Two meta-analysis of the world literature found that a history of depression increases the risk of developing AD (Ownby et al., 2006) or dementia in general (Jorm, 2001). In a population-based cohort, the relationship of depressive symptoms and dementia over long and short follow-up periods was monitored and the author concluded that late-life depressive symptoms are part of a dementia prodromal rather than an independent risk factor of dementia (Brommelhoff et al., 2009). Thus, depressive symptoms may be an early sign of dementia rather than a separate condition or a risk factor.

The mechanism that links depression to dementia is not fully understood. It has been shown by a neuropathological study that the development of hippocampal plaque and tangle is associated with a history of depression compared to those without such a history (Rapp et al., 2006). Additional evidence showed that a high ratio of plasma Aβ 40 to Aβ 42 was associated with depression and reduced cognition in a subset of depressed individuals (Sun et al., 2008). However, these results are inconclusive. Another study found that low Aβ42 was associated with depression (Sun et al., 2011), and no relationship Aβ42 and

depression has been also reported (Moon et al., 2011). A more recent study reported low Aβ42/Aβ40 and an APOE e4 allele had an increased risk for depression (Metti et al., 2013).

Another factor that overlaps with dementia is related to the hormone cortisol. Hypercortisolemia is associated with depression as a result of stress (Byers and Yaffe, 2011). A recent study in 80 AD patients indicated a significant association between high levels of plasma cortisol and AD (Zverova et al., 2013). The study supported the use of high plasma cortisol as biomarkers for AD with depressive symptoms as well as AD in the early stage of dementia development.

In a positron emission tomography (PET) scan study, the gradual accumulation of β-amyloid (Aβ) peptides was observed with [11C] 6-OH-BTA-1 [Pittsburgh Compound-B] (PiB) in the brain of nine participants with remitted late-onset depression and mild cognitive impairment. Approximately 50% of participants showed PiB retention consistent with that found in AD (Butters et al., 2008). These findings also support the notion that depression is a prodromal marker for dementia.

1.3.5 Delusions

Delusions are also common in LBD and commonly reported as a supportive symptom of a diagnosis of DLB and PDD (McKeith et al., 2005). The amount of delusions was found to be higher in patients with DLB (57%-76%) than in patients with AD (45%) (Gauthier et al., 2010), PDD (29%-54%) and PD (7%-14%) (Aarsland et al., 2001a). The content of these delusions is normally fixed, false and complex. Delusional mis-

identification is the most common symptom of this type in people with dementia, followed by persecutory or paranoid delusions (Aarsland et al., 2001a, Simard et al., 2000). Delusional mis-identifications occur in 33% of DLB/PDD patients (Simard et al., 2000). Mistaking fictional events on television for reality occurs in 19%, Capgras syndrome (the belief that a family member has been substituted by an impostor) occurs in 10%, mis-identification of the patient's own self occurs in 9.5% and the delusion that the house is not one's home occurs in 2.4% (Ballard et al., 1996).

1.3.6 Rapid eye movement (REM) sleep behaviour disorder:

Rapid eye movement (REM) sleep behaviour disorder (RBD) is termed a 'suggestive feature of DLB' according to the 3rd report of the DLB consortium (McKeith et al., 2005). REM Sleep Behaviour Disorder (RBD) is characterized by a lack of normal muscle atonia during rapid eye movement sleep, and manifests as dream-enacting behaviour (Olson et al., 2000). The body movement during periods of REM sleep will be increased, depending on what the patient sees in their dreams, and the person will move, cry out, speak, push, kick or throw themselves out of the bed (Olson et al., 2000). There may be more pronounced confusion between the dream and waking reality when the person awakens.

REM Sleep Behaviour Disorder is often associated in patients with LBD, DLB, PDD and PD or, in general, with synucleinopathies (Boeve et al., 2003, Boeve et al., 2001), and seems to be less frequent with non-synucleinopathies, such as AD or fronto-temporal dementia (Arnulf et al., 2005). It may also be present for years preceding the onset of

dementia and parkinsonism (Boeve et al., 2003). A recent study of 27 non-demented individuals with REM sleep behaviour disorder found that around 63% of patients developed DLB or PDD (Claassen et al., 2010)

1.3.7 Apathy

Apathy is a lack of interest, motivation, or interpersonal involvement in daily activities (Dujardin et al., 2007), and is one of the most common psychiatric symptoms in people with DLB, occurring in 56.1% of the patients compared to only 32.5% of those with AD (Bjoerke-Bertheussen et al., 2012). Recognising and directing treatment for such symptoms will decrease the risk of life disturbances for these patients, and stress for their caregivers.

Many studies indicate that anxiety is one of the most prominent psychiatric symptoms featured in LBD. Up to 84% of DLB patients feel anxious (Rockwell et al., 2000a), and 38% have anxiety at the onset of the disease (Ballard et al., 1999).

1.3.8 Agitation and aggressive behaviour

Agitation is defined as the “inappropriate verbal, vocal, or motor activity that is not explained by the needs or confusion of the individual’’ (Cohen-Mansfield et al., 1989). Agitated behaviours can manifest as physical vs. vocal/verbal and aggressive vs. Non-aggressive. Examples of verbal aggression include cursing, making strange noises, screaming and verbal abuse, while verbal non-aggressive behaviour involves complaining, negativism, repeating words, sentences or questions and requests for attention or help.

Physical non-aggressive behaviours include wandering, hiding things, general restlessness, repetitive movements, trying to get to a different place and sometimes eating inappropriate things. Examples of physical aggressive behaviours include pushing, hurting oneself or others, kicking, biting, throwing things, scratching and hitting.

Agitation and aggression is commonly seen in people with advanced dementia (Mega et al., 1996). Estimates of the prevalence of agitation and aggression in dementia vary immensely from 10% to 100%, probably due to bias introduced by the setting of the study and caregiver interviews (a common source of information on psychiatric symptoms) (Sachs, 2006). In a study of 408 nursing home residents, the occurrence of behavioural problems was reported in 93% of patients (Cohen-Mansfield et al., 1989). Furthermore, the prevalence of behavioural disturbance was reported to be 82% in a study of 647 nursing home residents in Australia (Brodaty et al., 2001). Jost and Grossberg reported agitation and aggression in 81% of 100 patients with autopsy-confirmed AD (Jost and Grossberg, 1996). Burns et al. found that behavioural abnormalities were greater in those with more severe dementia. Out of a sample of 178 patients with AD, aggression was observed in 20% and wandering in 19% (Burns et al., 1990).

Behavioural disturbances significantly impact the quality of life for patients and their caregivers, and are a major source of stress, discomfort and disquiet - more so than cognitive impairment (Ballard et al., 2000a, Coen et al., 1997, Schulz et al., 1995). Agitation and aggressive behaviour increase the risk of institutionalization and hospitalization (O'Donnell et al., 1992, Suh, 2004), or placement of a family member in a nursing home and, therefore, increase the cost of care. Over time, family members and

friends decrease their frequency of visits because they become embarrassed and do not know how to respond to these aggressive behaviours. Aggression is normally associated with delirium, depression and psychosis. Managing behavioural disturbances in LBD is problematic due to the high sensitivity to neuroleptics or antipsychotic agents. Severe neuroleptic sensitivity has now become one of the suggestive features.

The assessment of agitated behaviour can be done using either Cohen-Mansfield Agitation Inventory (CMAI) (Cohen-Mansfield et al., 1989) or the Neuropsychiatric Inventory (NPI). The CMAI characterizes four different categories of unusual agitated behaviour, which can be verbally and physically aggressive or verbally and physically non-aggressive (Cohen-Mansfield et al., 1989). In a comparison between these two scales, CMAI ratings of behavioural changes were shown to be more reliable than NPI (Zuidema et al., 2011).

Anatomical and biochemical changes within the brain could be significant contributors to agitation and aggression. Studies of brain metabolism in AD patients suggest a relationship between agitation in AD and frontal and temporal lobe hypofunction rather than parietal hypometabolism (Sultzer et al., 1995). This relationship was supported by another study, which demonstrated the association of frontal lobe dysfunction in AD patients and the likelihood of these patients becoming agitated (Senanarong et al., 2004). A similar link was reported earlier in 2001 between frontal lobe dysfunction and agitation and aggression (Norton et al., 2001). A neuropathological study demonstrated a correlation between a high score of agitation and neurofibrillary tangle in orbitofrontal cortex and anterior cingulate cortex (Tekin et al., 2001). Neurochemistry studies indicated that a

reduction in the number of α1-adrenoceptors in the prefrontal cortex is associated with increased aggression in AD patients (Sharp et al., 2007). Animal studies have shown a reduction in gamma-aminobutyric acid (GABA) is associated with aggregation (Kalueff and Nutt, 1996). Little is known about the effect of a decrease in GABA activity in agitation and aggression in AD patients, but the treatment with anti-convulsants, such as carbamazepine and valproate, supports the relationship between GABA dysfunction and agitation (Gleason and Schneider, 1990, Lemke, 1995, Tariot et al., 1994). Furthermore, a decrease in cholinergic function contributes to aggression (Garcia-Alloza et al., 2005) and the defect in cholinergic neurotransmission may lead to agitation in AD patients (Cummings and Kaufer, 1996).

1.4 Genetic factors in Lewy body dementia

DLB is typically considered to be a sporadic disorder. However, given that the clinical and pathological features of DLB and PDD overlap with each other and with other neurodegenerative disorders (PD and AD). This suggests that although DLB, PDD, PD and AD are distinct disorders, they could share genetic factors. Due to the similarity in the pathological and clinical features between Lewy body disorders, including DLB, PDD, and PD, many of the genes that may be involved in DLB and PDD are determined from studies of genes involved in PD. Indeed, genetics familial cases of DLB have been reported (Galvin et al., 2002, Tsuang et al., 2002, Tsuang et al., 2004). Kurz and colleagues conducted a systematic review into familial PDD and DLB and reported 24 families with a history of PD and dementia occurring in the same individuals, most had mutations in the SNCA gene, some in the gene encoding β-syn (Kurz et al., 2006). Small number of reports suggesting the involvement of genetic factors known to be implicated in AD as risk factors for the development of DLB (Kobayashi et al., 2011, Pickering-Brown et al., 1994). In this section, several genes that have been found to be directly linked to PD and AD, and have a critical role in LBD, will be discussed.

During the past decade, the discovery of a mutation in the α-synuclein (SNCA) gene added new knowledge to our understanding of the genetic factors influencing the pathogenesis of Lewy body disorders (Polymeropoulos et al., 1997), providing evidence for the genetic associations in both PD and DLB. Three point mutations have been identified. The first mutation, A53T, was identified in a large Italian-Greek family with autosomal

dominant familial Parkinson's disease with LBs (Polymeropoulos et al., 1997). The second mutation, A30P, was later found in a small German family with Parkinson's disease and cognitive impairment (Kruger et al., 1998). The third mutation, E46K, was found in a Spanish family, and linked with Lewy body dementia (Zarranz et al., 2004). Additional cases with the A53T mutation in DLB have been found with a family history of PD (Morfis and Cordato, 2006, Yamaguchi et al., 2005). After this discovery, the interest in α-synuclein has increased, and it has been found to be the major component in Lewy bodies (Spillantini et al., 1997).

Studies show that α-synuclein can increase membrane curvature causing smaller vesicles and tubules to form (Varkey et al., 2010). A30P and E46K mutations in α-synuclein reduce membrane curvature and so, by this mechanism, may behave as a brake on vesicle binding and neurotransmitter release and cause an accumulation of vesicles in synaptic terminal reserve pool (Auluck et al., 2010, Perlmutter et al., 2009).

The duplication and triplication of the α-synuclein (SNCA) gene have been identified in several families (Fuchs et al., 2007, Singleton et al., 2003), and these multiplications cause the over-expression of mRNA and increases in the protein levels of α-synuclein. It is believed that there is a gene dosage effect in the progression of the disease, and the families with SNCA gene duplications have two copies of the gene in one allele, with a 50% dose increase. They are affected in their fifties with slow clinical progression, compared to the families with triplication in the SNCA gene. The families with triplication have three copies in one allele, with a 100% dose increase, and these families are affected in their thirties with a severe clinical course, and more likely to exhibit dementia (reviewed

in Hardy et al., 2009) (Chartier-Harlin et al., 2004, Fuchs et al., 2007). Since the gene is dominant and the disease can be caused by duplication, it has been suggested that the mechanism of α-synuclein toxicity is related to its normal propensity to self-aggregate (reviewed in (Hardy et al., 2009). In addition, the point mutation may also increase the propensity of α-synuclein to aggregate by a factor of two, as the age onset of individuals with a point mutation in SNCA is similar to those with duplication (Hardy et al., 2009).

A mutation in the genes encoding leucine-rich repeat kinase 2 (LRRK2) was discovered in 2004 in a series of families from the Basque Country in Spain, and has been linked to autosomal dominant PD (Paisan-Ruiz et al., 2004). Since the discovery, a number of mutations in the LRRK2 gene have been found (Paisan-Ruiz et al., 2004, Zimprich et al., 2004). Mutations in LRRK2 are the greatest known genetic contributors to PD, with an estimate of 1-5% of PD cases having defects in this gene overall (Kumari and Tan, 2009), but up to 29% in the Ashkenazi Jewish and 37% (Ozelius et al., 2006) in the North African Arab populations (Lesage et al., 2006). LRRK2 encodes a large protein consisting of 2527 amino acids with GTPase and kinase activity. The most common mutation in LRRK2 is the G2019S located in the kinase domain, suggesting the importance of the enzymatic activity of this protein in the disease process (Greggio et al., 2006). The occurrence of these mutations in Lewy body dementia is unclear, but pathologically, the G2019S mutation is associated with Lewy bodies and Lewy neurites (Wider et al., 2010), although other study do not support this observation (Gaig et al., 2009). In contrast, three other mutations (R1441C, Y1699C and I2020T) are associated with nigral degeneration but not Lewy bodies (Taylor et al., 2006, Zimprich et al., 2004).

Glucocerebrosidase (GBA) is the gene mutated in Gaucher's disease, which is the most common lysosomal storage disorder, and also the most common inherited disorder in the Ashkenazi Jewish population (Beutler et al., 1993). It is caused by a deficiency in glucocerebrosidase, which is the enzyme that cleaves glucose from glucocerebroside to form acylsphingosine. The association of the GBA gene with PD was first recognised with the clinical finding that a small group of patients worldwide diagnosed with Gaucher's disease had developed Parkinsonism (Rosenbloom et al., 2011). Furthermore, relatives of the patients with Gaucher's disease had been reported to be diagnosed with Parkinsonism (Goker-Alpan et al., 2004, Halperin et al., 2006). These findings suggest that the mutation in the GBA gene may be a high risk factor for PD and associated Lewy body disorders. Indeed a large number of studies show that the mutation in the GBA gene contributes to the development of both PD and DLB (Goker-Alpan et al., 2006). GBA-associated Parkinsonism is characterised clinically by Parkinsonism, a greater association with cognitive decline and dementia, and it tends to have an earlier onset of PD. The occurrence of the GBA mutation was found to be greater in DLB cases (present in 24% of cases) when compared to PD cases (present in 4% of cases) (Goker-Alpan et al., 2006). It is possible that this mutation in GBA may affect the processing of α-synuclein and thereby increase the susceptibility to DLB (Cullen et al., 2011).

1.5 LB pathology

The pathology of LBD is characterised by the presence of intraneuronal proteinaceous inclusions called Lewy bodies (LB), Lewy neurites (LN), and pale bodies (PB) (Figure 1-1) (McKeith et al., 2005, McKeith et al., 1996).

LBs are spherical insoluble cytoplasmic inclusion bodies composed of a central aggregated mass of proteins surrounded by a halo of radiating fibrils that are approximately 10 nm wide. LNs are elongated thread-like dystrophic axons and dendrites. (McKeith et al., 2005).

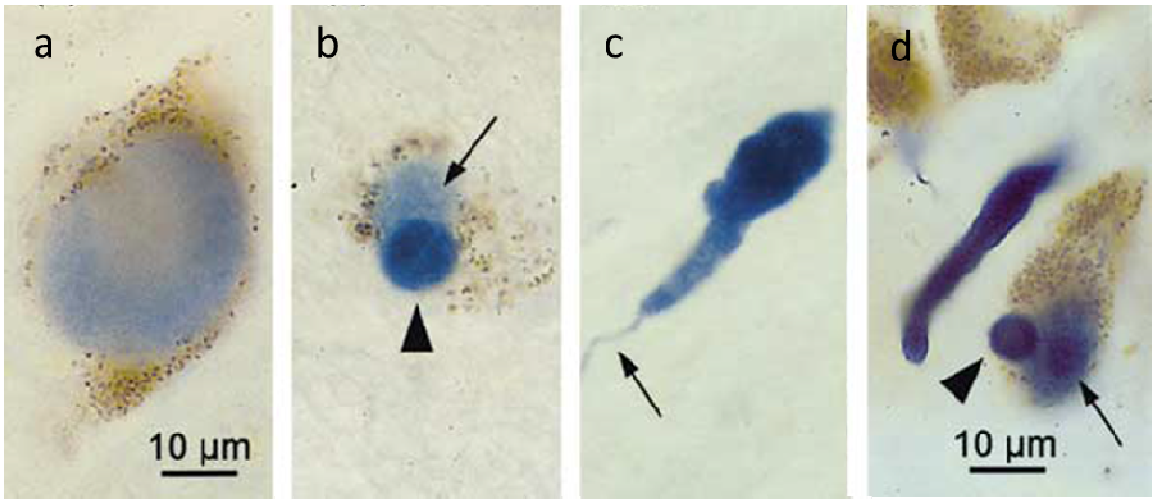


Figure 1-1: Neuropathology of LBD

Neuropathology of LBD characterised by Lewy body, Lewy neurite aggregate and pale body: (a) large pale body; (b and d) a combination of pale body (arrows) and a small LB (arrowheads); (c) A thread-like LN. (Modified from (Braak et al., 2003))

Lewy bodies were named after their discovery by Frederick Lewy in 1912 in a patient with paralysis agitans, a condition that is now called Parkinson's disease (PD) (Goedert et al., 2013, McKeith et al., 2005, McKeith et al., 1996). In 1961, Okazaki et al. provided the first description of cortical Lewy bodies and suggested their relationship to dementia (Okazaki et al., 1961). In 1984, Kosaka et al. reported abundant Lewy bodies in the cerebral cortical neurons of some patients with dementia. Proposing that these cases should be considered a new disease entity, they adopted the term "diffuse Lewy body disease" (Kosaka et al., 1984). Since then, many autopsied cases with dLBD have also been reported (Dickson et al., 1987, Hansen et al., 1990). Before the first international workshop and consortium on dementia with Lewy bodies, several researchers had proposed different terms to describe Lewy body dementia (McKeith et al., 1996). These include "diffuse Lewy body disease", "Lewy-body dementia", "senile dementia of the Lewy body type", and "dementia associated with cortical Lewy bodies" (McKeith et al., 2004). DLB is now considered under the umbrella of Lewy body dementias (LBD), which include PD, PDD and DLB (McKeith, 2007, McKeith et al., 2005).

LBs are composed of over 250 proteins the main component of which is the presynaptic protein α-synuclein (Kuusisto et al., 2003, Spillantini et al., 1997). α-synuclein is a member of the synuclein family of proteins (α, β, and γ) and is a soluble natively unfolded protein containing 140 amino acids located in the presynaptic terminal. The precise physiological function of α-synuclein remains equivocal. However, α-synuclein plays an important role in regulating synaptic plasticity, binding fatty acids, regulating

dopaminergic and glutamatergic neurotransmission, in addition to functioning as a molecular chaperone (Abeliovich et al., 2000, Cabin et al., 2002, Liu et al., 2004, Yavich et al., 2004). The location of α-synuclein in the presynaptic terminal is thought to have a role in the complex assembly of soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNARE) (Burre et al., 2010). α- synuclein drives the formation of the SNARE complex through a chaperone-like activity that involves binding to phospholipids and synaptobrevin-2.(Burre et al., 2010).

Various factors are responsible for the mechanism of the toxic function of α-synuclein, including the overexpression of α-synuclein and α-synuclein mutation, oxidative stress, mitochondrial dysfunction, excitotoxicity, impairment of the ubiquitin-proteasomal system, and autophagy dysfunction (Dauer and Przedborski, 2003) (Figure 1-2). These factors may cause a modification of α-synuclein that will result in its misfolding into pathogenic species of α‑synuclein (dimers, trimers and oligomers) that further assembly into insoluble fiber aggregates (protofibrils, other intermediates and amyloid fibrils) which serve as a building blocks for Lewy bodies and Lewy neurites (Lee and Trojanowski, 2006).

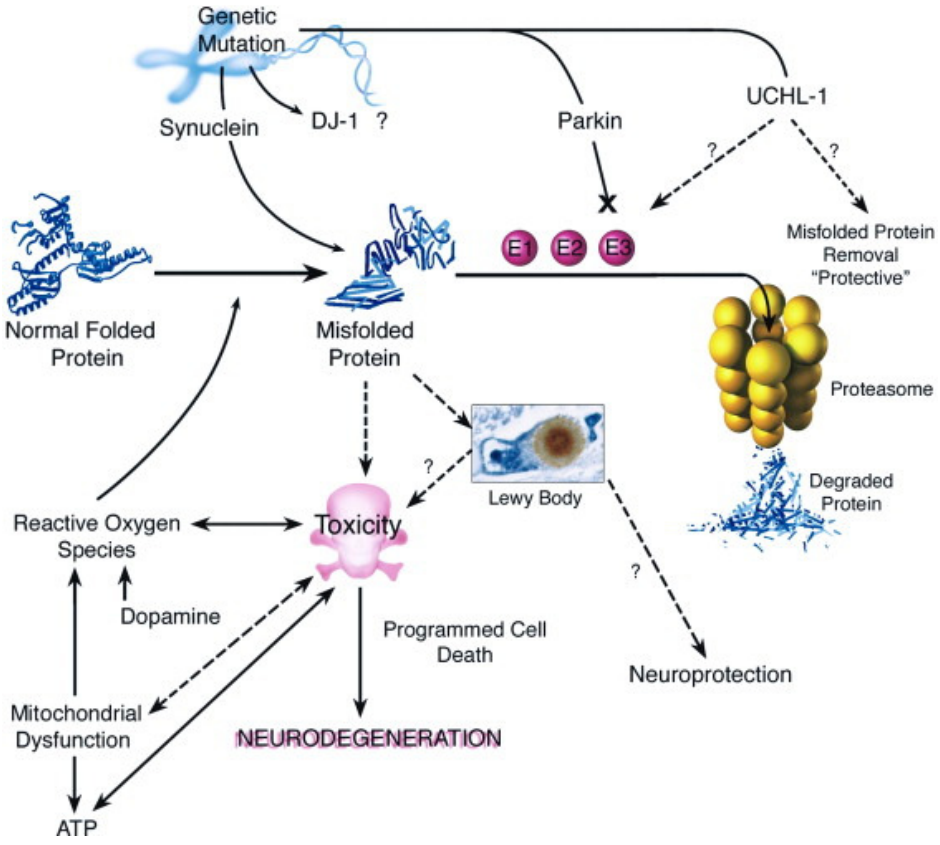


Figure 1-2: Factors responsible for the mechanism of α-synuclein modification

Various factors responsible for the mechanism of the toxic function of α-synuclein: α-synuclein mutation, oxidative stress, mitochondrial dysfunction, excitotoxicity, impairment of the ubiquitin-proteasomal system, and autophagy dysfunction. Figure taken from (Dauer and Przedborski, 2003).

LB pathology progresses in PD, it ascends from the brainstem in the early stages of the disease, moves through the limbic regions, and culminates in the cortex in the later stages of the disease (Braak et al., 2003). Braak developed the staging system of PD from a study of 110 cases (69 incidental and 41 clinically diagnosed PD patients). The staging system consisted of six stages. The first two pre-symptomatic stages referred to incidental LB disease, with the LB pathology process commencing in the lower brainstem in the dorsal motor nucleus of the vagus nerve (DMV), as well as in the anterior olfactory structures. Braak et al. proposed that the motor signs of the parkinsonian system appear when the synuclein pathology reaches Stage 3 (midbrain) and Stage 4 (limbic). It then eventually reaches the cerebral cortex in the last two stages, where it is associated with the cognitive impairment (Figure 1-3) (Braak et al., 2003). Some studies supported the Braak staging of α-synuclein pathology (Dickson et al., 2010, Jellinger, 2004, Parkkinen et al., 2008), whereas other studies reported that the topographical spread of Lewy pathology in a number of PD cases did not follow the typical caudo-rostral (Attems and Jellinger, 2008, Beach et al., 2009, Kalaitzakis et al., 2008, Parkkinen et al., 2008), bringing the Braak Lewy body stages under increasing criticism. Furthermore, some of these studies described the presence of LB pathology in the substantia nigra without involvement of the medulla, which raised questions because in a subset of cases, there was an absence of pathology in this nucleus (Dickson et al., 2008).

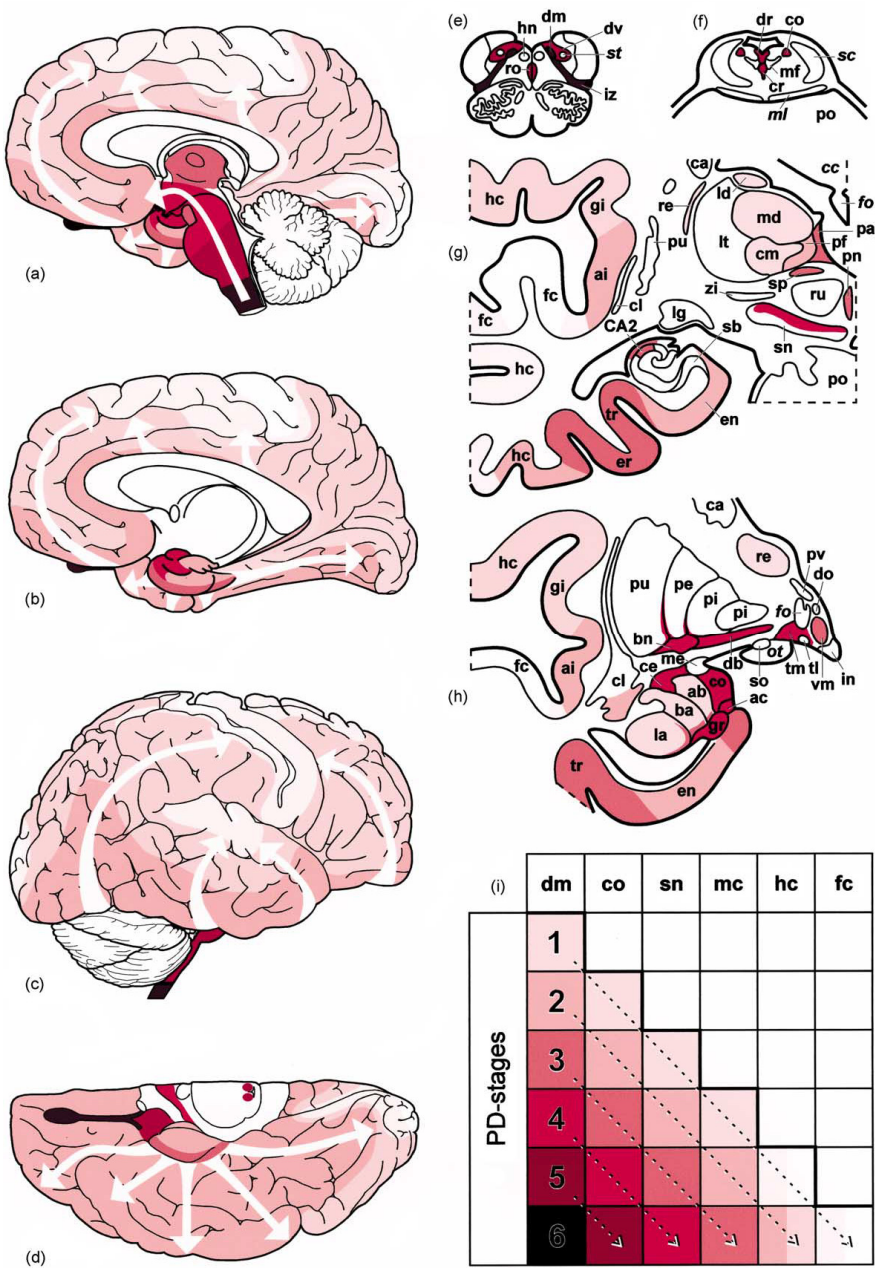


Figure 1-3: Braak staging of Parkinson's disease.

Diagrams showing the progression of PD-related intraneuronal pathology through the brain according to the different Braak stages. The depth of colour correlates with the density of Lewy pathology. The white arrow indicates the gradual involvement of related structures.

Relevant abbr; dm, dorsal motor nucleus of the glossopharyngeal and vagal nerves; co, coeruleus–subcoeruleus complex; sn, substantia nigra; mc, anteromedial temporal mesocortex; fc, first order sensory association areas, premotor areas, as well as primary sensory and motor fields; hc, high order sensory association areas and prefrontal fields. Taken from Braak et al (Braak et al., 2003).

According to the Braak stages of the α-synuclein pathology, topographic staging is the concept that with the increasing distribution and progression pattern of LBs pathology, there is a worsening of disease severity and a progression from motor symptoms to cognitive symptoms (Jellinger, 2008). This classification has been accepted and supported by several studies that reported a correlation between pathological findings and both clinical data and disease severity in PDD (Jellinger, 2008, Kovari et al., 2003, Mattila et al., 2000, van den Berge et al., 2012). This is generally accepted to be the case with PD and PDD patients. Classic parkinsonism does not appear until synuclein pathology reaches stage 3-4, when the substantia nigra is affected. Later, PD patients will develop dementia in Braak stages 5 or 6, with the involvement of the cerebral cortex (Aarsland et al., 2005a, Apaydin et al., 2002, Emre et al., 2007), In contrast, other studies reported no correlation between Braak LBs and the severity of the disease and dementia in PD (Beach et al., 2009, Halliday et al., 2008, Jellinger, 2008, Kalaitzakis et al., 2008). In fact, some studies reported that AD pathology, rather than the distribution of LBs, plays a significant role in the progression of dementia (Aarsland et al., 2004). Additionally, 30–55% of autopsies in elderly subjects without any neuropsychiatric symptoms showed the development of LB

pathology across multiple brain regions, which casts further doubt on the applicability of the original Braak staging to all cases of Lewy pathology (Jellinger, 2004, Leverenz et al., 2008, Parkkinen et al., 2008).

Until now, it has not been clear whether PDD and DLB are the same disease or different entities. In later stages of the disease, both PDD and DLB will have the same pathological features, especially in DLB patients who develop parkinsonism (Aarsland et al., 2004; Tsuboi and Dickson, 2005). Cortical Lewy pathology cannot be used to distinguish PDD from DLB (Harding and Halliday, 2001), but evidence has been reported to show subtle differences, particularly in AD pathology. Some studies reported that AD pathology is more likely to play a significant role in determining disease progression in DLB (Aarsland et al., 2004). In fact, Weisman et al. went so far as to suggest that the presence of AD pathology determined diagnostic success, in that when AD pathology was high, it became much harder to distinguish DLB accurately (Weisman et al., 2007).

The presence of cortical Lewy pathology in elderly patients without parkinsonism or dementia suggests a compensatory mechanism and that the formation of LB may in fact be neuroprotective instead of a primary cause of the disease. In addition, in an intense debate, many studies reported the presence of cortical Lewy bodies in non-demented PD patients, which revealed the relationship between LB pathology and cognitive impairment (Jellinger, 2009). Jellinger *et al*. reported cortical Lewy pathology in non-demented PD patients and in elderly control subjects (Jellinger, 2009). Colosimo *et al*. reported multiple cases of clinical PD patients without dementia who, upon autopsy, were found to have extensive neocortical Lewy pathology consistent with that typically encountered in DLB

(Colosimo et al., 2003). Aho et al. found the presence of LB pathology in individuals who were cognitively unimpaired, (Aho et al., 2008), which was repeated by other studies (Parkkinen et al., 2008). The implication of these findings is that neocortical Lewy bodies are not necessarily the pathological correlate of dementia in PDD and DLB. Other factors may contribute to LB pathology and affect the development of symptoms. For example, glial and neuronal loss and other pathologies, such as tau and Aβ, play an important role (Jellinger, 2008, Parkkinen et al., 2008).

The revised consensus of pathologic guidelines of DLB proposed two major aspects that should be considered in the pathology of DLB (McKeith et al., 2005). The first distinguishes DLB as having three phenotypes (brainstem, transitional/limbic and diffuse neocortical) by the semi-quantitative scoring of synuclein pathology in specific regions of the brain. Second, based on the various degrees of Alzheimer-related pathology, the clinical symptoms of DLB depend on the severity of LB pathology and are inversely related to AD pathology. Although the complex interaction between LB and AD pathology is not yet clear, some studies indicated that AD pathology triggers the formation of LB (Iseki et al., 2003, Saito et al., 2004), and others reported that only Aβ enhanced the formation of LB (Pletnikova et al., 2005). The BrainNet Europe Consortium made recommendations regarding the immunohistochemical protocol and assessment criteria related to α-synuclein pathology, based on the assessment of α-synuclein-immunoreactivity in 13 defined neuroanatomical regions including: medulla with dorsal motor nucleus of vagus (dmV) and intermediate reticular zone (irx), pons with locus coeruleus (LC) and raphe (R), midbrain with substantia nigra (SN), basal forebrain with nucleus basalis of Meynert (nbM) and amygdala (AC), hippocampus with cornu Ammonis region 2 and temporo-occipital cortex

(CA2 and TOex), cingulate gyrus, temporal cortex, frontal cortex and parietal cortex (Alafuzoff et al., 2009a, Alafuzoff et al., 2008b). α-synuclein-immunoreactivity is assessed as being present or absent in conjunction with the type of lesions (i.e. LB or LN), (Alafuzoff et al., 2009a, Alafuzoff et al., 2008b) whereas Braak and McKeith staging protocols are semi-quantitavive (Braak et al., 2003, McKeith et al., 2005)

1.6 AD pathology

AD is the most common neurodegenerative disease. It is characterised clinically by progressive memory loss and cognitive impairment, leading to a gradual loss of all basic functions prior to death. These clinical features result from the gross atrophy of neurons and synapses in the cerebral cortex and certain subcortical regions (Masliah et al., 1990, Masliah et al., 1991, Scheff et al., 1990, Terry et al., 1991). Furthermore, a deficit in neurotransmitter systems, particularly the cholinergic system, and the loss of cortical and hippocampal choline acetyltransferase (ChAT) activity has been identified (Bowen et al., 1976, Davies and Maloney, 1976), and correlated with dementia and cognitive impairment (Francis et al., 1999).

The classical pathological hallmarks of AD are plaques formed principally of amyloid-beta (Aβ), and intracellular neurofibrillary tangles (NFT) composed of hyperphosphorylated tau (Crews and Masliah, 2010, Serrano-Pozo et al., 2011). Alzheimer's type pathology is common in both DLB and PDD (Hansen et al., 1989, Kosaka et al., 1988, McKeith et al., 2005, Mrak and Griffin, 2007, Perry et al., 1990d), particularly Aβ plaque (Tsuboi and Dickson, 2005), Some studies suggested a possible

interaction between Aβ peptide and α-synuclein (Jensen et al., 1997). The percentage of patients with high plaque counts is greater in DLB then in PDD (Harding and Halliday, 2001). In addition, it has been reported that cognitive impairment in DLB is more likely to be related to Aβ (Aarsland et al., 2004). As previously mentioned, one aspect that should be considered in the pathological features of DLB, according to the revised consensus criteria, is the concurrent AD-related pathology (McKeith et al., 2005).

A number of different pathological criteria have been developed in attempts to correlate pathological changes with disease progression in AD (Braak et al., 2006, Braak and Braak, 1991, Mirra et al., 1991, Montine et al., 2012). Braak and Braak described six stages based upon the occurrence and distribution of neurofibrillary tangle (NFT) and neuropil threads (NT) (Braak et al., 2006, Braak and Braak, 1991, Braak and Braak, 1997). NFTs and NPs develop slowly and symmetrically in both hemispheres, starting in the transentorhinal and entorhinal area before spreading to the hippocampus, the association cortices, and the rest of the cortex. Stage I is characterised by few NFTs and NPs in the transentorhinal region. In Stage II, the number of NFTs increases more prominently than in stage I, with beginning of entorhinal region involvement. Braak Stages I and II are both called the transentorhinal stage, but this stage is clinically silent. Stage III is characterized by the severe involvement of both the transentorhinal and the entorhinal regions and changes are now detectable in the hippocampus, proneocortex and some subcortical nuclei. In Stage IV, large numbers of "ghost tangles ", the remnants of intracellular NFTs where the neuron has disappeared (Ikeda et al., 1992), are present in both the transentorhinal and entorhinal regions. Moderate alterations occur in the hippocampal formation, in the temporal and pro-neocortical areas, and in a few subcortical nuclei. The mature neocortex

remains virtually free of neurofibrillary changes. The lesions that characterize stages III and IV are capable of producing the first clinically detectable functional deficits because the transfer of information between the sensory association fields, the higher-order components of the limbic system, and the prefrontal cortex is affected. The hallmark of stage V is the widespread devastation of the neocortex. All parts of the hippocampal formation are now involved: only the acoustic system, the primary motor field, primary sensory areas, and unimodal secondary fields remain uninvolved. In stage VI, lesions are visible even in the border areas of the primary regions. The end stages of AD are accompanied by cortical atrophy, a notable loss in brain weight, and severe dementia.

The Consortium to Establish a Registry for Alzheimer's Disease (CERAD) assessment is based on a combination of age-related NP scores in three neocortical regions (middle frontal gyrus, superior and middle temporal gyri, and inferior parietal lobule) and clinical history (Mirra et al., 1991). Plaque pathology is rated as sparse, moderate, or frequent. The highest value of the evolution is compared with the age of the patient in three age categories: less than 50, 50 to 75, and over 75. These scores are then integrated with clinical information regarding the presence or absence of dementia to reach the final diagnosis of AD, i.e., definite, probable or possible AD.

A comparison of staining techniques and assessment criteria across the BrainNet Europe centres was conducted and concluded that use of antibodies against Aβ and hyperphosphorylated tau gave the highest reliability (Alafuzoff et al., 2008a, Alafuzoff et al., 2012, Alafuzoff et al., 2006, Alafuzoff et al., 2009b). Other schema proposed by the working group of the National Institutes of Health, the Reagan Institute of the Alzheimer

Association, and the National Institute of Aging (NIA–Reagan criteria), require the presence of both senile plaque and NFTs for recognition as a heterogeneous clinicopathological entity. Post-mortem examination alone can yield statements of the probability of an Alzheimer's diagnosis (Hyman and Trojanowski, 1997). The likelihood of AD diagnosis increases with the increased frequency of both neuritic plaques (using the scoring system currently used by CERAD) and NFTs in specified brain regions (using the staging system devised by Braak and Braak). The NIA–Reagan criteria are summarized in Table 1-1.

Table 1-1: National Institute of Aging (NIA)-Reagan criteria for Alzheimer's disease

CERAD[a] senile plaque score	Braak neurofibrillary tangle (NFTs)			
	NO NFT	**I-II**	**III-IV**	**V-VI**
Frequent senile plaque	NOT AD	Low	Intermediate	High
Moderate senile plaque	NOT AD	Low	Intermediate	Intermediate
Spare senile plaque	NOT AD	Low	Low	Low
No Plaque	NOT AD	NOT AD	NOT AD	NOT AD

[a] Consortium to Establish a Registry for Alzheimer's Disease

In 2012, the National Institute on Aging and the Alzheimer's Association (NIA-AA) updated the 1997 guidelines to focus primarily on neuropathologic changes instead of clinical criteria (Montine et al., 2012). The guidelines recommend the "ABC" staging protocol for the neuropathologic changes of AD, based on three morphologic characteristics of the disease: A—the amyloid described by (Thal et al., 2002), B—the Braak neurofibrillary tangle (NFT) staging protocol (Braak and Braak, 1991), and C—the Consortium to Establish a Registry for AD neuritic plaque scoring system (Mirra et al., 1991) (Table 1-2 and 1-3).

Table 1-2: AD Neuropathologic Change. Modified from (Montine et al., 2012)

AD neuropathologic change should be ranked along three parameters (Amyloid, Braak, CERAD) to obtain an "ABC score"
A. Aβ plaque score (modified from Thal et al., 2002)
A0: no Aβ or amyloid plaques
A1: Thal phase 1 or 2
A2: Thal phase 3
A3: Thal phase 4 or 5
B. NFT stage (modified from (Braak and Braak, 1991)
B0: no NFTs
B1: Braak stage I or II
B2: Braak stage III or IV
B3: Braak stage V or VI
C. Neuritic plaque score (modified from Mirra et al., 1991)
C0: no neuritic plaques
C1: CERAD score sparse
C2: CERAD score moderate
C3: CERAD score frequent
Reporting
"Alzheimer Disease Neuropathologic Changes: A1, B0, C0" or
"Alzheimer Disease Neuropathologic Changes: A3, B3, C3"
Using the system shown in Table 3, the ABC scores are transformed into one of four levels of AD neuropathologic change**: None, Low, Intermediate or High**.

Table 1-3: Level of AD neuropathologic change according to the revised National Institute of Aging (NIA)-Reagan criteria for Alzheimer's disease (Montine et al., 2012)

A:Aβ/amyloid plaque score (Thal phases)	**C: Neuritic plaque score (CERAD)**	**Braak neurofibrillary tangle (NFTs)**		
		B0 or B1 (none or I/II)	**B3 (III-IV)**	**B3 (V-VI)**
A0 (0)	C0 (none)	NOT	NOT	NOT
A1 (1/2)	C0 or C1 (none to sparse)	Low	Low	Low
	C2 or C3 (moderate. To frequent)	Low	Intermediate	Intermediate
A2 (3)	Any C	Low	Intermediate	Intermediate
A3 (4/5)	C0 or C1 (none to sparse)	Low	Intermediate	Intermediate
	C2 or C3 (moderate. To frequent)	Low	Intermediate	High

CERAD, Consortium to Establish a Registry for Alzheimer's disease.

1.6.1 Aβ and Senile plaque

Histological examinations of AD brain revealed extracellular spherical deposits of insoluble aggregated peptide β-amyloid fibrils (Aβ), which are referred to as senile or neuritic plaques (NP). Neuritic plaques are usually 10 and 200 mM in diameter. They have a dense core of Aβ, which was originally demonstrated by using the Bielschowsky silver impregnation technique by Alois Alzheimer in 1907 [reviewed by (Castellani et al., 2008, Castellani et al., 2010)]. This finding was confirmed by Divry in 1922 with Congo red staining [reviewed by (Castellani et al., 2010)]. The discovery that Congo red had a strong affinity for amyloid deposit that had enhanced birefringence after staining suggested that the amyloid deposit had an organised structure [reviewed by (Boller et al., 2007)]. However, the cross-β- β-pleated sheet was not discovered until Glenner et al. isolated Aβ from senile plaques (Glenner et al., 1971a, Glenner et al., 1971b) and later Kang et al. found Aβ to be a result of proteolytic cleavage of the amyloid precursor protein (APP) (Kang et al., 1987). Neuritic plaques are frequently associated with astrocyte, microglia and degenerated dystrophic neuritic processes (Mandybur and Chuirazzi, 1990).

Multiple plaque subtypes have been described based on morphology: e.g. diffuse, focal or stellate. Plaques in AD are often accompanied by a corona of dystrophic cell processes, which are termed “neuritic” plaques [reviewed by (Duyckaerts et al., 2009)]. Diffuse plaques are morphologically diverse across different regions of the brain. They lack the compact fibrillar appearance of classical NPs, and they are not associated with glial responses or synaptic loss, neuritic dystrophy, or the pathogenesis of AD. They are

commonly found in elderly patients with intact cognitive function, suggesting that diffuse plaques may not be overtly toxic [reviewed by (Serrano-Pozo et al., 2011)]. Diffuse plaques are usually detected together with NPs, which suggested that they could mature from the diffuse type to the neuritic type (Joachim et al., 1989).

Plaque is formed when glycoprotein amyloid precursor protein (APP) cleavage products, Aβ, accumulates and is deposited as extracellular senile plaques in AD brain. Mutations in the gene encoding of APP indicated that Aβ might be central to the pathogenesis of AD (Selkoe, 2000). All known genetic mutations linked to familial AD and genetic risk factors for sporadic AD are associated with increased Aβ42 and Aβ40 (Tanzi and Bertram, 2005).

APP undergoes proteolytic processing by one of two pathways: the amyloidogenic pathway, which leads to Aβ generation; and the anti-amyloidogenic pathway, which prevents Aβ generation (Haass et al., 2012). APP is cleaved within the amino terminus of the Aβ by membrane-associated metalloprotease α-secretase, resulting in the release of extracellular N-terminal fragment (APPsα) from the cell and preventing the formation of Aβ (Esch et al., 1990, Sisodia et al., 1990) and reviewed by (Findeis, 2007, Haass et al., 2012, Selkoe, 2001). The remaining membrane-bound C-terminal fragment C83 might undergo further processing by γ-secretase, which liberates a γ-stub into the cytosol and leads to the secretion of p3 (Haass et al., 1993). In the amyloidogenic pathway, APP is first cleaved at the N-terminal region of the Aβ sequence by β-secretase, which is the enzyme known as BACE1 (β-site APP-cleaving enzyme), leading to the secretion of slightly shorter soluble N-terminus APPsβ and leaving C99 in the membrane (Haass, 2004). Subsequently,

γ-secretase cleavage of C99 produces C-terminal 50 residues of APP and Aβ peptide. γ-Secretase is a multiprotein complex consisting of four individual proteins: presenilin (PSEN), nicastrin, Aph-1, and Pen-2 (Kaether et al., 2006).

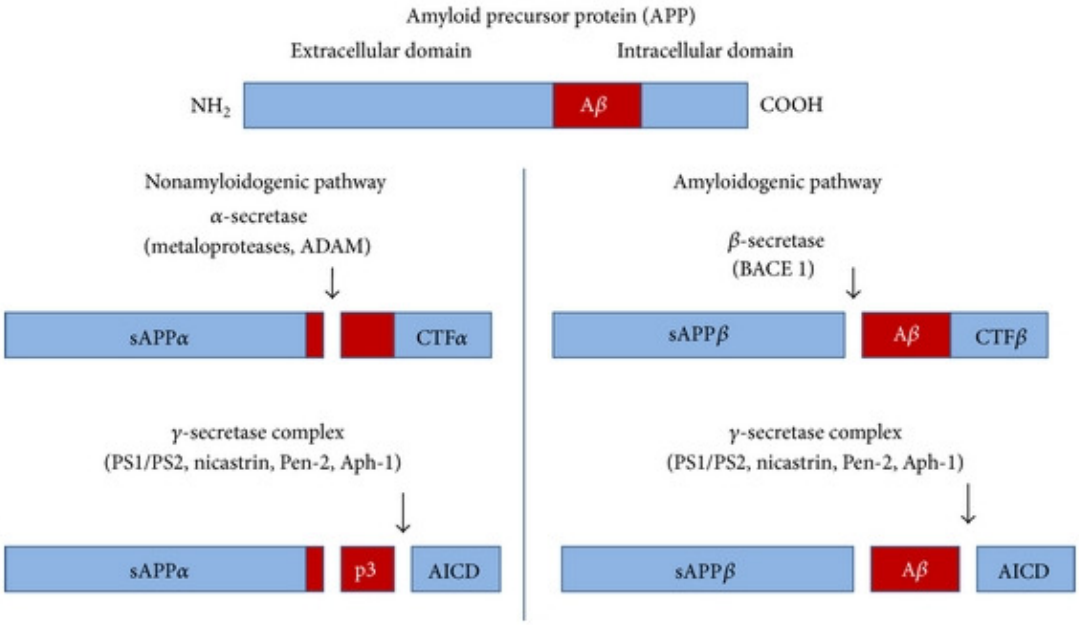


Figure 1-4: A diagram of amyloid precursor protein (APP) processing pathways (Carrillo-Mora et al., 2014).

Aβ is a 4kD peptide consisting of 40–42 amino acid residues (Masters et al., 1985). Cleavage by β-secretase and γ-secretase produces a mix of peptide fragments comprised of 39 to 43 amino acids, the common forms of which are 40 (Aβ40) and 42 (Aβ42) amino acids in length (Dong et al., 2012, Selkoe, 1994, Selkoe, 2001). Although both Aβ42 and Aβ40 are amyloidogenic and neurotoxic, Aβ42 is more prone to aggregation into protofibrils and fibril than Aβ40, this is because of the presence of two additional

hydrophobic amino acid residues (Dahlgren et al., 2002). Aβ42 is the major component of amyloid plaques in AD (Bernstein et al., 2009, Chen and Glabe, 2006). An elevated level of the Aβ42/Aβ40 ratio in the plasma was shown to correlate with familial forms of Alzheimer's disease (Johnson et al., 2013). However, it is not known whether soluble Aβ or Aβ plaque mediate the activation of astrocytes and microglia and the injury to neurites and cell bodies in AD brain.

The "amyloid cascade hypothesis" has been the focus of AD researchers during the last two decades (Dong et al., 2012). According to this hypothesis, aggregated Aβ leads to NFT formation, which in turn causes neuronal death. This progressive process manifests clinically as memory loss and other symptoms associated with dementia (Hardy and Allsop, 1991, Selkoe, 1991). Many studies supported the hypothesis and that the actual Aβ is pathogenic: first, that neuritic plaques containing β-amyloid (Aβ) are its main component (Ghiso and Frangione, 2002); second, familial Alzheimer's mutations have a connection to the overexpression of Aβ (Tanzi and Bertram, 2005); and third, both in vivo and in vitro studies have shown that Aβ is toxic to neurons and reproduces the neuropathologic and behavioural alterations observed in patients with AD (Atwood et al., 2003). However, this hypothesis was recently challenged by several studies that demonstrated that Aβ not only exhibits neurotoxic properties but also has neuroprotective effects (Lee et al., 2007). Increased Aβ42 fraction, memory deficit, decreased spine density, and deficit in hippocampal neurotransmission were observed in Tg2576 mice expressing human APP before plaque deposition (Jacobsen et al., 2006). Neuronal loss within the hippocampus was reported in areas with and without amyloid aggregation in APP/PS-1 double-transgenic mice (Schmitz et al., 2004). The presence of substantial Aβ pathology in non-demented

controls also shed doubt upon Aβ pathology involvement in causing AD (Dickson et al., 1992, Jellinger and Attems, 2012, Knopman et al., 2003). The poor correlation of plaques to clinical severity and neuronal loss has also been reported (Neve and Robakis, 1998).

A recent version of the amyloid hypothesis stated that soluble Aβ oligomers contribute to the pathology of AD (Sheng et al., 2012). Several studies indicated that Aβ accumulated inside the cells and may cause neurotoxicity in AD (LaFerla et al., 2007). Intracellular Aβ was found to accumulate before plaque formation in different mouse models of AD and in human brain. Intracellular Aβ accumulation was reported in a PS1 AD mouse model, which exhibit AD-like neurodegeneration without plaques (Chui et al., 1999). All these evidence suggested that soluble Aβ initiates AD pathology.

1.6.2 Neurofibrillary tangles

NFTs are a major histopathological hallmark of AD. NFTs do not appear to be specific to AD as they are also seen in several other human neurodegenerative diseases, such as Parkinson's disease, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), frontotemporal lobar degeneration (FTLD) (Apaydin et al., 2002, Braak et al., 2005). Although NFTs appear in many neurodegenerative diseases, in AD, they are a very important marker because of the strong correlation with cognitive dysfunction, synaptic dysfunction, and brain atrophy. Moreover, NFT density parallels the duration of AD and the severity of dementia (Arriagada et al., 1992a, Eckermann et al., 2007, Takashima, 2009).

NFTs form when tau protein becomes hyperphosphorylated and accumulates in the form of soluble tau aggregates and insoluble paired helical filaments (PHF) that often

occupy the cell body and extend into the apical dendrite [for review, see (Ballatore et al., 2007)]. Other proteins in NFTs include ubiquitin (Love et al., 1988, Perry et al., 1987), cholinesterases (Mesulam and Asuncion Moran, 1987) and Aβ (Hyman et al., 1989), but tau is considered its critical constituent (Dong et al., 2012, Duan et al., 2012, Perl, 2010). Tau is a microtubule-associated protein. Under normal physiological conditions, because of its phosphorylation state, tau is involved in the assembly and stabilization of microtubules through the action of many kinases and phosphatases on the tau molecule (Kosik et al., 1986, Mandelkow and Mandelkow, 1995, Mandelkow et al., 1995). In AD, abnormal hyperphosphorylated tau decreases its tubulin binding capacity, which leads to the detachment of tau from microtubules. Cytosolic concentration is then increased, promoting the disorganisation of microtubules, which then self-polymerize and aggregate in the form of PHFs and NFTs (Dong et al., 2012, Duan et al., 2012, Perl, 2010).

Neurofibrillary lesions found in AD brains include NFTs in neuronal cell bodies, threads and dystrophic neurites in processes (Duyckaerts et al., 2009). However, NFTs survive the degeneration of tangle-bearing neurons and may be released into the extracellular space of the AD brain. These extracellular NFTs are referred to as “ghost tangles” (Ikeda et al., 1992). Consequently, tau pathology shows a regular, highly area-specific spread throughout the brain during the progression of the disease, allowing its classification into the six Braak stages mentioned previously, based on tau pathology and its correlation with clinical severity (Braak and Braak, 1991).

An increasing amount of evidence has suggested that tau pathology is induced by Aβ (Duyckaerts et al., 2009). In fact, a substantial accumulation of NFTs has been observed

in neurodegenerative diseases, such as fronto-temporal lobar degeneration (FTLD), without Aβ peptide accumulation (Hutton et al., 1998), whereas mutations of APP causing an increase in Aβ production initiated a pathway terminating in production of tau pathology (Goate, 2006, Goate et al., 1991, Goate and Hardy, 2012). The addition of Aβ to transgenic mice expressing a human isoform of tau was reported to increase the rate of tau phosphorylation and accumulation of NFTs (Gotz et al., 2001). Aβ oligomers promoted the phosphorylation of tau in primary cultures of hippocampus or in neuroblastoma cells (De Felice et al., 2008). Aβ was shown to increase phosphorylation and decrease its ability to bind microtubules (Busciglio et al., 1995). Ittner and Gotz indicated that tau hyperphosphorylation occurred in APP transgenic mice, and in tau transgenic mice there was no Aβ pathology (Ittner and Gotz, 2011). Although these studies suggested that tau pathology is downstream of Aβ plaque, Roberson et al., 2007, demonstrated that tau knockout mice was less susceptible to Aβ and experimentally induced seizures (Roberson et al., 2007). Based upon these studies still the link between Aβ and tau pathologies remains one of the significant outstanding questions of AD research.

1.7 Neurotransmitter abnormalities

1.7.1 The cholinergic system

Accumulating evidence now indicates that cholinergic dysfunction is involved in the pathogenesis of Lewy body dementia. It has been reported that in DLB brains, there was a significant reduction in ChAT activity, particularly in the cerebral cortex, which was associated with cognitive impairment and hallucination (Ballard et al., 2000b, Perry et al., 1990a, Perry et al., 1990b, Tiraboschi et al., 2002). Furthermore, ChAT activity was also reduced in the thalamus in cases with PDD (Ziabreva et al., 2006). In the neocortex, reduced choline acetyltransferase levels occurred earlier in the disease course in DLB than in AD, and it was independent of coexistent AD changes (Davis et al., 1999, Perry et al., 1994, Tiraboschi et al., 2002). In addition, the significant loss of cholinergic pedunculopontine tegmental nuclei/laterodorsal tegmental nuclei neurons was reported in DLB brains (Schmeichel et al., 2008). Furthermore, elevated levels of both cholinergic nicotinic and muscarinic receptors were identified in DLB brain. Investigations of nicotinic receptor binding showed a reduction in nicotinic acetylcholine receptor (nAChRs) binding containing β2 and α4, in common with AD (Colloby et al., 2010, Court et al., 2000, Gotti et al., 2006, Perry et al., 1995, Perry et al., 1990c). The nAChRs deficit in DLB was found to be correlated with ChAT reduction in DLB (Reid et al., 2000). Increased nAChRs binding was also reported as occipital in DLB and was linked to hallucinations (O'Brien et al., 2008). Recently, an imaging study showed that nicotinic α4 and β2 receptors correlated with cognitive progression in DLB and PDD (Colloby et al., 2010). Muscarinic acetylcholine receptors (mAChRs) and the muscarinic receptor subtypes (m1-m4) were also implicated in the pathophysiology of both DLB and PDD (Ballard et al., 2000b, Shiozaki et

al., 2001, Shiozaki et al., 1999). The expression levels of mAChRs were varied in different brain regions, with a reduced total amount of mAChRs in DLB compared to the control (Ballard et al., 2000b, Shiozaki et al., 2001, Shiozaki et al., 1999). The muscarinic receptor subtypes of the m3 in the frontal cortex were significantly increased when the m4 receptor was significantly decreased in the temporal cortex, compared with the control specimens (Shiozaki et al., 1999).

1.7.2 The dopaminergic system

The involvement of the dopaminergic system in DLB and PDD has been considered as the main neuropathological hallmark in Parkinson's disease is the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). In DLB post-mortem studies reported reductions in dopamine levels in the rostral caudate nucleus (Perry et al., 1990b) and also in substantia nigra (SN) neurons in DLB (Perry et al., 1993). The reduction in dopamine D2 receptors was correlated with cognitive decline in the temporal cortex of DLB patients (Piggott et al., 2007). The densities of dopamine D1 and D2 receptors densities were reduced in DLB caudate putamen by 57% and 20%, respectively, compared to the controls, and dopamine levels were reduced by 72% in these patients (Piggott et al., 1999).

1.7.3 Other neurotransmitter systems

The significance of other neurotransmitter systems, such as the glutamatergic, serotonergic, and noradrenergic systems, have not been extensively studied in DLB. In addition, they have not been linked to the pathology of the disease or to clinical symptoms and progression. One study demonstrated that there was no change in the expression of the glutamate transporter, excitatory amino acid transporter 1 (EAAT1) in DLB, in contrast abnormal expression of EAAT1 was observed in cases showing Alzheimer-type neuropathology (Scott et al., 2002). However other studies have reported impairment in metabotropic glutamate receptors (mGluRs) transduction pathway with significant reduction of the expression levels of mGluR1 in cerebral cortex in DLB (Albasanz et al., 2005). Furthermore reduction in glutamate receptor immunoreactivity in the hippocampus and entorhinal cortex of patient with Lewy body variant of AD has been also observed (Thorns et al., 1997). More research is needed to determine the possible role of glutamatergic systems in DLB using a range of markers (Francis, 2003).

1.8 Treatment of LBD

Currently, no specific drug has been identified for the treatment of Lewy body dementia. Neuroleptic or antipsychotic medications, which are often used to treat hallucinations, are known to exacerbate the extrapyramidal features of DLB. Approximately 50% of individuals with DLB elicit sensitivity to neuroleptic agents (Aarsland et al., 2005b, McKeith et al., 1992), and high frequencies were reported in both Parkinson's disease (27%) and PDD (39%) (Aarsland et al., 2005b). In contrast, severe neuroleptic sensitivity was not seen in patients with AD (Aarsland et al., 2005b). Quetiapine, which is often used for the treatment of agitation and other symptoms of psychosis, showed no effect on agitation or other psychosis and did not worsen parkinsonism (Kurlan et al., 2007). Randomized controlled trials comparing the effects of citalopram and risperidone in DLB and AD reported worsening psychotic symptoms in DLB patients (Culo et al., 2010). This finding suggests that antipsychotic medication for the treatment of Lewy body dementia cannot be considered the first choice in treating and managing psychotic symptoms, especially when they are not severe.

Dopaminergic drugs, such as L-dopa, which is the main medication used in the treatment of motor symptoms in PD, are also often used to treat the motor symptoms in DLB. L-dopa has been shown to improve parkinsonian symptoms in DLB and PDD, but was less successful compared to the benefits in PD (Molloy et al., 2005). Molloy et al. demonstrated an improvement in neuropsychiatric score in PD and PDD (Molloy et al., 2005). The use of L-dopa is problematic because it has a deleterious effect on psychotic symptoms, such as hallucinations, hypersomnolence, and orthostatic hypotension (Mosimann and McKeith, 2003).

The N-methyl-D-aspartate (NMDA) antagonist memantine is approved for the treatment of cognitive symptoms in AD (McShane et al., 2006). There have been conflicting reports regarding memantine and psychiatric symptoms in DLB and PDD. Studies have reported both worsening and improvement in equal measures (Ridha et al., 2005, Sabbagh et al., 2005). Ridha et al. described worsening psychotic symptoms, including visual hallucinations and delusions in three LBD patients, caused by memantine treatment (Ridha et al., 2005), whereas Sabbagh et al. reported the reduction of visual hallucinations and improvement in cognition (Sabbagh et al., 2005). Recently, three randomized control trials suggested that memantine might be potentially effective for the treatment of DLB but not PDD (Aarsland et al., 2009, Emre et al., 2010, Leroi et al., 2009). The first study, by Leroi et al., was a randomized, placebo-controlled trial of 25 patients with PDD over 22 weeks (Leroi et al., 2009). At the end of the drug treatment (week 16), there was no significant difference between the placebo-controlled and the memantine-drug groups according to an efficiency parameter: Mini-mental State Exam (MMSE), Dementia Rating Scale (DRS), Neuropsychiatric Inventory (NPI), and Unified Parkinson's Disease Rating Scale (UPDRS). However, six weeks following the withdrawal of the drug, a greater number of participants treated with memantine deteriorated ($p = 0.04$), compared to patients treated with the placebo (Leroi et al., 2009). Second, Aarsland et al. conducted a double blind, placebo-controlled study over 22 weeks with 72 patients with mild to moderately severe DLB and PDD (Aarsland et al., 2009). The results demonstrated a moderate benefit to patients using the Clinical Global Impression of Change (CGIC) scale, with greater effects shown in the PDD group (Aarsland et al., 2009). The third and largest study was double blind, placebo-controlled, 22-week duration, with 199 (121 with PDD

and 78 with DLB) patients with mild to moderately severe PDD and DLB (Emre et al., 2010). Emre's study revealed that DLB group treated with memantine showed greater improvement according to the CGIC analysis, compared to the DLB placebo group. However, the difference was not significant in PDD (Emre et al., 2010). Furthermore, more patients on placebo group were deteriorated. Similarly there was a significant improvement in neuropsychiatric outcomes (specifically in delusions, hallucinations, sleeping/night-time behaviour and appetite/eating disorder) in the memantine DLB group, compared to the placebo DLB group although the difference was not significant in the PDD group (Emre et al., 2010). The results of a cognitive test showed that none of the DLB or PDD patients showed any consistent effect with memantine treatment. The findings by Emre were contrasted with those by Aarsland et al., who demonstrated that memantine improved attention, executive function and global cognition, but no improvement with NPI score. In addition, Aarsland et al. found a more pronounced global benefit of memantine in PDD compared to DLB (Aarsland et al., 2009, Emre et al., 2010). These contrary findings make it very difficult to determine whether memantine is beneficial in DLB and/or PDD or not.

Cholinesterase inhibitors, such as Exelon (rivastigmine), Aricept (donepezil) and Razadyne (galantamine), have been approved and licensed for the symptomatic treatment of patients with mild to moderate AD (Ballard et al., 2011, Farrimond et al., 2012, Pettenati et al., 2003). These drugs work by increasing the level of ACh in the brain by inhibiting or blocking the enzyme in charge of its breakdown, which is the job of acetylcholinesterase (AChE) (Francis et al., 1999). Several randomized, placebo controlled studies of rivastigmine, donepezil, and galantamine in AD showed a modest benefit in mild to moderate cases, and a few studies showed benefit in severe AD (Ballard et al., 2011).

Cholinesterase inhibitors showed moderate improved cognition and stabilized function, improved behavioral symptoms, improvements in mood (particularly apathy), delayed nursing home placement, maintained daily function and quality of life (Ballard et al., 2011, Birks, 2006, Birks et al., 2009, Wilkinson et al., 2009). However, possible side effects, such as vomiting and diarrhoea, proved difficult for the patient (Potyk, 2005, Standridge, 2004).

In DLB, evidence of the involvement of cholinergic deficits prompted researchers to study the effects of cholinesterase inhibitors in DLB and PDD. However, there are still relatively few data on their use in both DLB and PDD. There have been only two placebo-controlled randomised controlled trials of rivastigmine: one for DLB and one for PDD. Only one trial of donepezil has been conducted on PDD (Dubois et al., 2012, Emre et al., 2004, McKeith et al., 2000). McKeith et al. showed a statistically and clinically significant improvement in neuropsychiatric symptoms in DLB patients, which was the primary outcome of the study, in addition to benefits in cognition and function (McKeith et al., 2000). Following McKeith et al., a few open open-label studies were conducted to study the effects of cholinesterase inhibitors on PDD. Reading et al. studied the efficacy of rivastigmine in 12 PDD patients and reported an improvement in cognition measured by MMSE, as well as improvement in the neuropsychiatric symptoms measured by NPI (Reading et al., 2001). No changes were reported in motor symptoms. In contrast, Giladi et al. reported an increase in Unified Parkinson Disease Rating Scale (UDPRS) scores and a non-significant improvement after eight weeks of rivastigmine, and the results of an MMSE test showed significant improvement to attention. In addition, worsening cognitive measurement according to the Alzheimer's Disease Assessment Scale (ADAS-cog) was

found throughout the study period (Giladi et al., 2003). Following these open label studies, a large randomized, placebo-controlled clinical trial with 410 PDD patients showed that rivastigmine significantly improved cognition function and neuropsychiatric symptoms, compared to the placebo group (Emre et al., 2004). However, patients treated with rivastigmine exhibited side effects, including parkinsonian symptoms, in addition to worsening of tremor and dizziness (Emre et al., 2004). The Emre study indicated that hallucinations and orthostatic hypotension were more common in the placebo group (Emre et al., 2004). Although the efficacy was modest, the results of the Emre study have led to the use of rivastigmine in PDD patients. The study was followed up by an open-label study, which reported that the beneficial effects observed during the first six months of the trial were largely maintained, and the safety profile was similar to that in the Emre study (Poewe et al., 2006). Wesnes et al. reported the benefits of rivastigmine in attention in PDD patients enrolled in Emre et al. (Wesnes et al., 2005).

Only one large randomized, placebo-controlled clinical trial of donepezil has been conducted for PDD in 550 mild to moderate PDD patients over 24 weeks. This study found no significant differences in cognitive function measured by ADAS-cog in the activity of daily living scale and in the behavioural scale (Dubois et al., 2012). Adverse effects were more common in PDD patients treated with donepezil compared to patients treated with placebo, but they were mostly mild to moderate in severity.

Based on the clinical trials conducted with AChEIs in PDD and DLB patients, the Cochrane review has identified rivastigmine as beneficial in the treatment of cognitive impairment in PDD (Maidment et al., 2006, Rolinski et al., 2012). Although there is a still a

debate regarding the efficacy of cholinesterase inhibitors in DLB and PDD, it is the most frequently used drug in the treatment of neuropsychiatric symptoms in these patients. In fact, rivastigmine has been approved and licensed for the treatment of PDD in Europe, the US, and elsewhere (Reingold et al., 2007).

1.9 The ubiquitin proteasome system

The ubiquitin-proteasome system (UPS) is the major proteolytic pathway responsible for the clearance of short lived proteins, and is found in all eukaryotes and archaea and some bacteria (Dahlmann et al., 1989, Gille et al., 2003). UPS regulate most cellular processes, including: protein ‘‘quality control’’(Brodsky and McCracken, 1999), stress (Bregere et al., 2006, Mathew et al., 1998), immune responses (Borissenko and Groll, 2007b, Kloetzel et al., 1999), cell cycle regulation (Takeuchi and Toh-e, 1997), deoxyribonucleic acid (DNA) repair (Bergink et al., 2006, Walters et al., 2003) and gene expression (Blagosklonny et al., 1996, Wu et al., 2000, Zimmermann et al., 2001). Proteasomes are also responsible for the degradation of misfolded and damaged proteins, which is essential for maintaining cellular homeostasis. The most common form of proteasome in eukaryotic cells is the 26S (S=Svedberg sedimentation coefficient) proteasome, consisting of two subcomplexes: the 20S ~700 kDa core particle (CP) sandwiched between two 19S regulatory particles (RP) (Glickman and Ciechanover, 2002). The 26S is a part of the main pathway, which is the ubiquitin proteasome system (UPS). Protein degradation by the UPS is a process that occurs in two discrete and successive steps: first, the covalent attachment of the poly-ubiquitin chain to the protein substrate is recognised by the 26S, and then it is degraded by the 26S proteasome complex (Glickman and Ciechanover, 2002).

1.9.1 Structure and components of the 26S proteasome

1.9.1.1 The 20S core particle

The 20S sub-complexes are cylinder-like structures, composed of 28 α and β subunits organized in four stacked rings, ~150 Å X ~120 Å in dimension (Groll et al., 1997). Each of the two outer rings contains seven different α subunits, and each of the inner two rings contains seven different β subunits, resulting in a symmetrical structure: (α1 – 7)-(β1 – 7)-(β1 – 7)-(α1 – 7) (Lowe et al., 1995) (Figure 1-4).

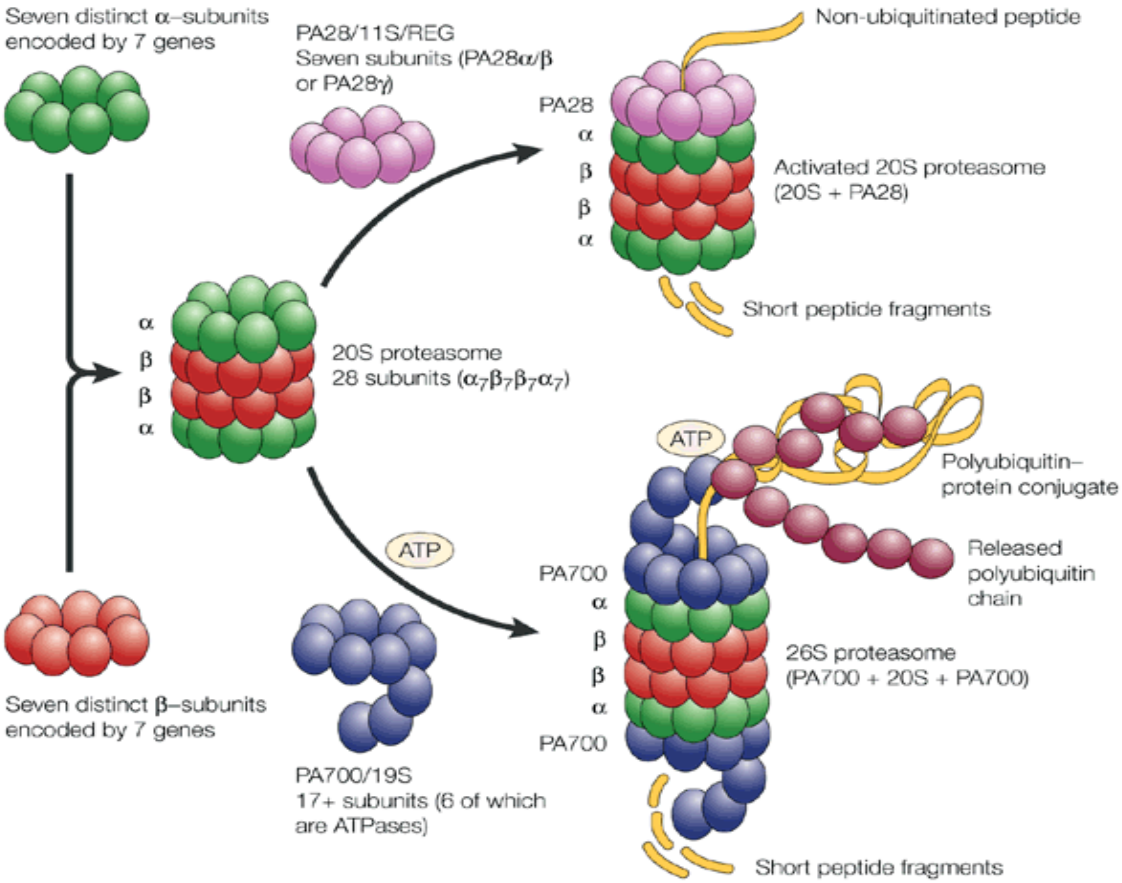


Figure 1-5: Model structure of the 26S and 20S proteasome.

The 26S proteasome consists of a catalytic core, 20S, and a regulatory particle, 19S. The 20S consists of 28 α and β subunits, organized in four stacked rings. Each of the inner two rings contains seven different β subunits. Three of the β subunits (β1, β2, and β5) in each β ring contain the proteolytic active sites where the proteolysis of the protein occurs. The two outer rings contain seven different α subunits that serve as anchors for the 19S (PA 700) that bind to form 26S. The 11S (PA 28) can also bind the 20S and activate the proteasome to degrade non-ubiquitinated protein in an ATP-independent manner. Figure taken from (McNaught et al., 2001).

The two outer α-rings provide a binding site for the 19S regulatory particle (RP) and a narrow channel for the translocation of the unfolded and extended polypeptides to the inner proteolytic chamber. The inside of the 20S CP is divided into two "ante chambers" found between the α-ring and a β-ring, which store the substrate in an unfolded conformation (Ruschak et al., 2010, Sharon et al., 2006), and one single "main chamber", found between the two β rings (Fig. 1-5).

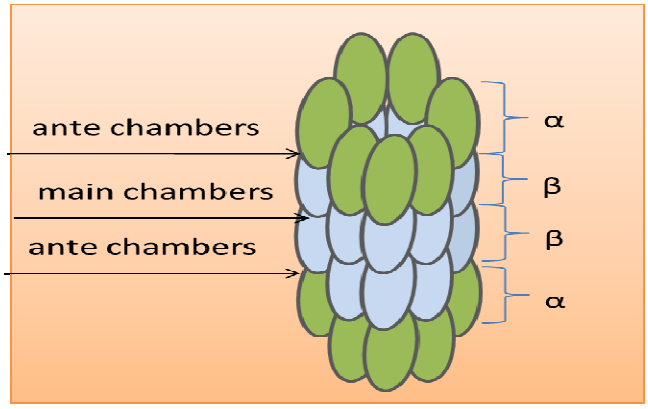


Figure 1-6: Inner structure of the 20S proteasome.

The inside of 20S CP, divided into two "ante chambers" found between α ring and a β ring, and one single "main chamber", found between two β rings. Figure adapted from (Jung and Grune, 2012).

Three of the β subunits (β1, β2, and β5) in each β-ring contain the proteolytic active sites where proteolysis occurs. These β subunits belong to the superfamily of N-terminal nucleophile (Ntn) hydrolases (Seemuller et al., 1995); these enzymes use the side chain of the amino-terminal residue as the nucleophile in the catalytic attack at the carbonyl carbon. In the proteasome, the nucleophile is threonine (Seemuller et al., 1995). The active sites of the 20S proteasome face the inner wall of the core particle, termed proteolytic chamber (Groll et al., 1997). Each of these three subunits has its own specificity: the β1 subunit has a peptidyl-glutamyl peptide-hydrolyzing (PGPH)-like activity site and cuts preferentially after acidic residues; the β2 subunit has a trypsin-like activity site and cuts mainly after basic residues; and the β5 subunit has a chymotrypsin-like activity site and cuts mainly after hydrophobic residues (Borissenko and Groll, 2007a, Dick et al., 1998). In response to Interferon-gamma (IFN-γ) induction, cells are able to produce immunoproteasomes, which replace the active β subunits β1, β2, and β5, with β1i also named low molecular weight protein 2 (LMP2), β2i multicatalytic endopeptidase complex-like 1 (MECL-1), and β5i low molecular weight protein 7 (LMP7), respectively (Yewdell, 2005). Immunoproteasomes play a critical role in immune defence, due to their ability to generate specific antigenic peptides produced following degradation displayed on major histocompatibility complex (MHC) class I molecules (Yewdell, 2005).

The average size of the peptides generated by the proteasome are 8–12 residues (Kohler et al., 2001), but they could vary as much as 2–35 (Luciani et al., 2005). The vast majority of these products are further degraded into individual amino acids by cytosolic aminopeptidases, such as amino peptidases or tripeptidyl peptidase (TPPII) (Reits et al.,

2004). However, a small portion of the peptides produced following degradation are used for antigen presentation by MHC class I molecules (Reits et al., 2004, York et al., 2003).

In order for the target protein to be hydrolysed by the 20S proteolytic core, protein should enter the core particles, which are closed by the N-terminal tails of several α subunits (α-2, α-3, and α-4) to obstruct the entrance (Groll et al., 2000). The N-termini of the α-3 subunit play a critical role in the central channel sealing formation, as shown in the crystal structure of the CP (Groll et al., 2000). Deleting the N-terminal tail of the α-3 subunit increases the peptidase activity of the CP by opening the gate of the catalytic core (Groll et al., 2000). Mild chemical treatment of the purified 20S proteasome with low levels of sodium dodecyl sulphate (SDS) induce a slight unfolding of the proteasome subunits, resulted in a significant increase of proteolytic activity causes by gate opening in the α-ring.(Coux et al., 1996). In eukaryotes, entry of the substrate to the catalytic chamber can be controlled by binding to the 19S regulatory complex forming the 26S proteasome complex, which increases activity up to ten-fold (Adams et al., 1998, Glickman et al., 1998b). 19S regulatory complex induces unfolding of the ubiquitinated substrate; and opens the gate, allowing entry of the substrate to the proteolytic core (Stadtmueller and Hill, 2011). The 19S activator can bind to one, or both ends of a 20S proteasome (Stadtmueller and Hill, 2011, Voges et al., 1999), resulting in rearrangement of the N-terminal tails of the α subunits and opening the gate (Groll et al., 1997). There are other activators of the proteasome, such as interferon-induced 11S complex (PA28) (Stadtmueller and Hill, 2011).

1.9.1.2 The 19S regulatory particle

The hydrolysis of peptide bonds and the proteolytic activity of the 26S is performed by the 20S CP, while substrate recognition and translocation is regulated by the 19S RP. The 19S RP has several roles in regulating ubiquitin-dependent proteasomal activity. The 26S is ATPase dependent, and the 19S RP provides the ATP for the proteasome function. The 19S RP is responsible for the recognition of polyubiquitinated protein substrates, unfolding of the protein substrate, and translocation of the substrate to the PC (Figure 1-6) (Tomko and Hochstrasser, 2013). The 19S RP bind to the 20S PC to activate the proteasome and open the gate of the pore, which is normally closed by α subunits (Glickman and Ciechanover, 2002, Tanaka, 2009). 19S also generate monomeric ubiquitin in the deubiquitination step by cleaving the polyubiquitinated chain (Tanaka, 2009).

The 19S can be subdivided into the “base” and the “lid”, which together consist of at least 17 subunits (Glickman et al., 1998a, Glickman et al., 1998b). The base of the 19S RP binds directly to the α subunits of 20S CP; and responsible for gate opening, activating the proteasome, and substrate unfolding, whereas the “lid” is involved in substrate recognition (Tomko and Hochstrasser, 2013). The base consists of nine subunits, including six ATPases, referred to as RP Triphosphatases (RPT) that form a heterohexameric ring (Tomko et al., 2010), and four RP Non-ATPases, these including Rpn1 and Rpn2, and the ubiquitin receptors Rpn13and Rpn10 (Tomko and Hochstrasser, 2013). The lid consists of nine subunits (Rpn3, 5–9, 11, 12, and 15), of which just one, the deubiquitylase Rpn11, displays enzyme activity.

The ATPases belong to a family of chaperone-like ATPases known as the AAA family, which is an abbreviation for "**A**TPases **a**ssociated with a variety of cellular **a**ctivities" (Soto, 2003). The six ATPases are encoded by different genes; RPT2 (*PSMC1*), RPT1 (*PSMC2*), RPT5 (*PSMC3*), RPT3 (*PSMC4*), RPT6 (*PSMC5*), and RPT4 (*PSMC6*) genes (Takalo et al., 2013). These subunits share substantial sequence similarity; they all contain conserved 200 amino acid ATP-binding domains (Djuranovic et al., 2009, Lander et al., 2012, Zhang et al., 2009a). Each AAA domain contains Walker A and Walker B motifs required for ATP binding and hydrolysis respectively (Rubin et al., 1998). At the N-terminus, RPT subunits also contain N-terminal coiled-coil (CC) domains, where the RPT-RPT subunits hold each other, and a central oligonucleotide/oligosaccharide binding (OB) domain (Djuranovic et al., 2009, Lander et al., 2012, Zhang et al., 2009a).

The six ATPases bind to the core particle (CP) through an interaction between C-terminal hydrophobic-tyrosine-X (HbYX) motif of RPT2, RPT3, and RPT5 and the α subunits of the CP (Kohler et al., 2001, Rabl et al., 2008, Smith et al., 2007). During the regulatory particle (RP) assembly, RPT6 binds with high specificity to the α2α3 CP pocket through its HbYX motif (Park et al., 2013). When the lid complex joins the proteasome, the RPT6–CP bond is broken to accommodate stable binding of RPT2/3/5 in mature proteasomes (Park et al., 2013).

There are about 14 non-ATPase subunits, each of which has a different structure and function. The functions of most of the non-ATPase subunits are still largely unknown (Ehlinger and Walters, 2013). The four non-ATPase subunits within the base subcomplex are the scaffolding proteins Rpn1 and Rpn2 and the ubiquitin receptors Rpn10 and Rpn13

(Glickman et al., 1998). Rpn1 and Rpn2 are the largest subunits at the proteasome; both contain proteasome/cyclosome (PC) repeats (Kajava, 2002). The PC repeats have been shown to interact with the N-terminal end of the Rpt6/Rpt3 coiled-coil (CC) domains, while the Rpt1/Rpt2 CC pair appears to interact with Rpn1 (Beck et al., 2012). Both Rpn1 and Rpn2 properly function as scaffolds for the assembly of the ATPase subunits (Pedersen and Heegaard, 2013). One of the most important functions of the 19S RP is recognition of the substrate by binding the poly-ubiquitin chain. Rpn10 was the first proteasome subunit found to function as a ubiquitin-binding protein (Fu et al., 1998).

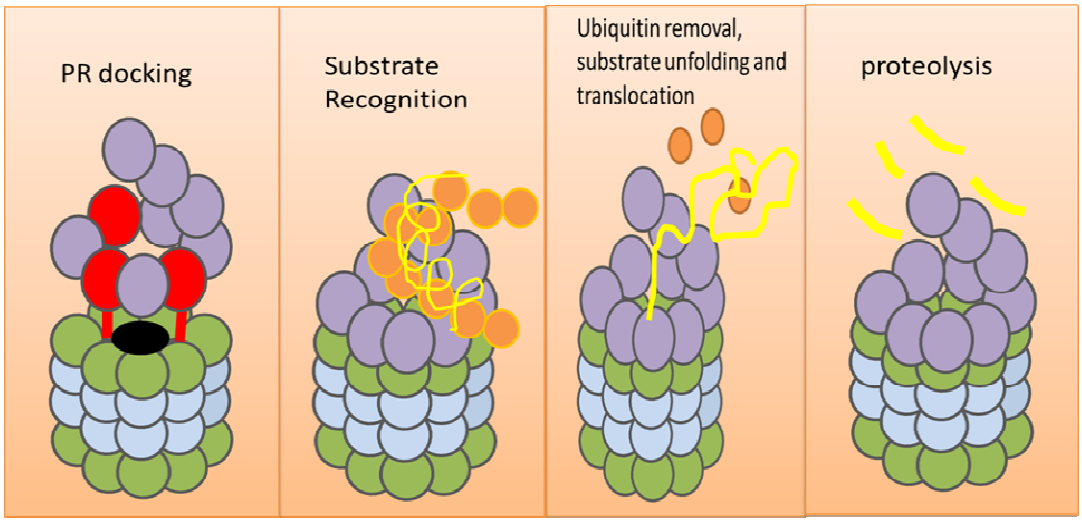


Figure 1-7: 19S proteasome functions.

Docking of the C-terminal HbYX motifs of the ATPase subunits (Rpt2, Rpt3, and Rpt5) with the α-ring of the 20S PR opens the gate of the pore to allow entry of the substrate. Ubiquitin chains on substrates are recognized by the ubiquitin-binding domains (UBDs) found in Rpn10 and Rpn13. Ubiquitin is then removed and recycled by deubiquitinating enzymes. ATP hydrolysis produces conformational changes in the ATPases that induce unfolding and translocation of the substrate into the CP, where it is proteolysed into short peptides. (The idea of the figure adapted from (Ehlinger and Walters, 2013)).

1.9.2 Proteasome degradation pathway

For recent reviews on the proteasome function, structure and pathway see (Jung and Grune, 2012, Tanaka, 2009, Tomko and Hochstrasser, 2013). Ubiquitin (Ub) is a highly evolutionarily conserved, 76-amino-acid residue polypeptide (Weissman, 2001). Protein ubiquitination is an enzymatic, protein post-translational modification (PTM) that forms an isopeptide bond between the terminal glycine residue of the ubiquitin and lysine in the target protein. Ubiquitination of a protein is carried out by a set of three different enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase in an ATP-dependent manner (E3) (Pickart, 2001). In the first step, Ub becomes activated by the E1 enzyme producing a high-energy (Ub-E1) thiolester intermediate (Groettrup et al., 2008). The E2 enzyme then carries the activated Ub from E1 and catalyses its transfer to the next destination which is either E3 HECT (homologous to E6-AP terminus) ligase by forming a covalent (thiolester) bond between the ubiquitin and E3 before transferring it to the substrate, or by the direct transfer of Ub from the Ub-E2 complex without the addition of thiolester to a specific substrate protein via E3 RING (Really Interesting New Gene) as shown in (Figure 1-7) (Michelle et al., 2009). The E3 ligase sequentially elongates the Ub chain by attaching other Ub molecules on the Lys residue of the first Ub molecule and creating Ub-Ub isopeptide bond (Deshaies and Joazeiro, 2009). In some substrates, an additional enzyme, E4 (also called E4 ligases), a type of E3-like enzyme, catalyses chain extension (Hoppe, 2005, Koegl et al., 1999). There are seven Lys residues in Ub (K6, K11, K27, K29, K33, K48, and K63) (Pickart and Fushman, 2004). The polyubiquitin chain at K48 is the most common signal recognised by the 26S proteasome for protein degradation (Chau et al., 1989, Finley et al., 1994). Following the ubiquitination step, substrates are

delivered to the proteasome for degradation step (Hochstrasser, 1996). Rpn10 and Rpn13 are the proteasome components that contain Ub binding domain and function as Ub receptors (Deveraux et al., 1994, Husnjak et al., 2008, Schreiner et al., 2008). Three other proteins, Rad23 (radiation gene 23), Dsk2 (dominant suppressor of Kar2) and Ddi1 (DNA damage molecule-1) contain both a UBL domain and a polyubiquitin-interacting ubiquitin-associated (UBA) domain. These three proteins function as "shuttling" proteins that transfer polyubiquitylated protein substrates to the 26S proteasome (Welchman et al., 2005). After attachment of the substrate to the proteasome, a deubiquitination reaction takes place before substrate unfolding and degradation by the proteasome component Rpn11 (Verma et al., 2002). Some substrates, however, do not require the attachment of Ub in order for degradation degraded by the proteasome (Shringarpure et al., 2003). Several substrates have recently been found to be Ub-independent, where the targeted role of Ub can be replaced by other protein or by signal in the sequence of the protein undergoing degradation (Finley, 2009, Orlowski and Wilk, 2003).

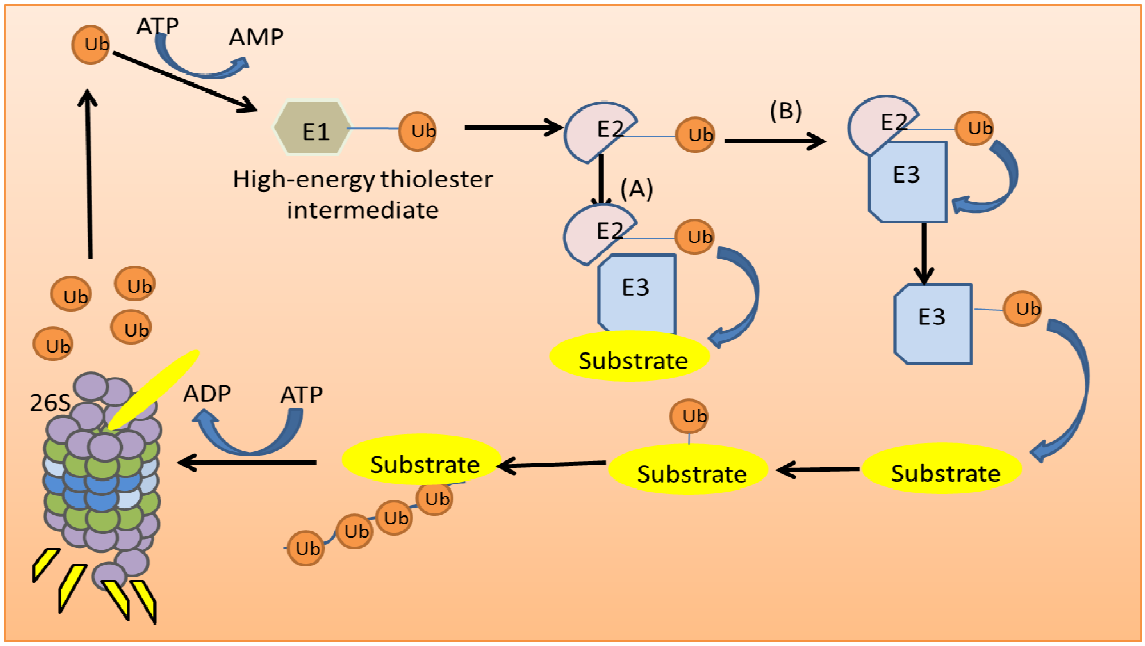


Figure 1-8: Overview of protein degradation by the UPS.

Free ubiquitin (Ub) monomers in the cytoplasm are first activated at the C-terminal domain in an ATP-dependent manner by the ubiquitin-activating enzyme, E1, to a high-energy thiolester intermediate. It is then transferred, still as a high-energy intermediate, to the ubiquitin-conjugating enzyme (E2). From E2 it can be transferred directly to the substrate that is specifically bound to a member of the ubiquitin-protein ligase family. This occurs when the E3 belongs to the RING finger family of ligases (A). In the case of HECT 'Homologous to the E6-AP Carboxyl Terminus' (B), the activated ubiquitin is transferred first to the E3, to generate yet another high-energy thiolester intermediate, before it is transferred to the target substrate. This process is repeated until a polyubiquitin chain forms that targets the substrate to the proteasome for degradation. The poly-ubiquitinated substrate is bound specifically to the 26S proteasome complex, and the substrate is degraded to short peptides. Deubiquitinating enzymes (DUBS) remove ubiquitin from the

substrate, which can be reused for another process. (The idea of the figure was taken from (Pagan et al., 2013) with adding both RING (A) and HECT (B) ligases not only RING as shown in the original figure)

1.9.3 Role of UPS in regulating synaptic function

In the central nervous system, communications between neurons take place at the synapse by a process called synaptic transmission reviewed by (Sudhof, 2004). The synapse consists of a presynaptic terminal, a synaptic cleft and a postsynaptic terminal. Synaptic transmission begins when the action potential travels down the presynaptic cell to the synapse. The depolarization causes the voltage gated calcium channel to open. Calcium diffuses into the presynaptic neurons and triggers synaptic vesicle exocytosis, enabling the vesicle to release neurotransmitters into the synaptic space. The released neurotransmitters bind to receptors on the postsynaptic neurons, thus enabling signalling to be transmitted towards the cell body of the postsynaptic neuron (Sudhof, 2004). In the last few years, a considerable amount of literature has been published on the role of UPS in regulating synaptic function. In synaptosomes fraction, acute depolarization in the presence of Ca^{2+} results in a global decrease in ubiquitin positive proteins as a specific result of deubiquitination, rather than of protein degradation, suggesting that UPS modifying presynaptic function through ubiquitination and protein turnover takes place as a result of synaptic activity (Chen et al., 2003).

Evidence of the role of UPS in regulating synaptic function at the presynaptic terminal also comes from the fact that proteins involved in the synaptic vesicle cycle are

targeted at the proteasome for degradation. Examples of these proteins include synaptophysin, syntaxin and Rab3-interacting molecule 1α (RIM1α) (Chin et al., 2002, Wheeler et al., 2002, Yao et al., 2007). Synaptophysin is an integral membrane protein in synaptic transmission, implicated in neurotransmitter release. (Alder et al., 1995, Mullany and Lynch, 1998). Syntaxin is a presynaptic protein that plays a role in synaptic vesicle exocytosis. RIM1α functions to form a presynaptic scaffold that links synaptic vesicles with fusion machinery, priming vesicles for release. This function of RIM1α is controlled by a ubiquitin ligase F-box protein, SCRAPPER, which regulates the amount of RIM1α (Yao et al., 2007). SCRAPPER knockout mice exhibit impaired short-term synaptic plasticity due to an increase in the expression level of RIM1, suggesting that SCRAPPER regulates synaptic transmitter release (Yao et al., 2007).

Inhibition of the UPS in drosophila neuro-muscular junction NMJ increases the level of Drosophila Uncoordinated Protein 13 (DUNC-13), leading to a strengthening of neurotransmission, compared to controls (Aravamudan et al., 1999). Increased synaptic transmission after inhibition of the proteasome may be due to DUNC-13 accumulation, as DUNC-13 is found to be ubiquitinated and accumulated at the presynaptic terminal, suggesting that DUNC-13, which is a critical protein for priming the synaptic vesicles, is a substrate for UPS (Aravamudan and Broadie, 2003).

The regulatory role of the proteasome is also possible by controlling the size of the recycling pool of the synaptic vesicles. Thus, Willeumier et al. have found that, in cultured hippocampal neurons, acute inhibition of the proteasome results in an increase in the size of the recycling vesicle pool, whereas the rate of release is unaffected (Willeumier et al.,

2006). Willeumier showed that this mechanism was independent of protein synthesis. These observations suggest that proteasomal degradation at the presynaptic terminal may act as a negative mechanism to perturb exocytotoxic activity (Willeumier et al., 2006).

Many studies have indicated that the UPS regulates a variety of proteins that are involved in the postsynaptic response, including neurotransmitter receptors and postsynaptic density proteins (Hegde, 2010, Tai and Schuman, 2008, Yi and Ehlers, 2007). For example, PSD-95 is a postsynaptic protein involved in cellular scaffolding and regulating the localization of AMPA receptors (AMPARs) and NMDA receptors (NMDARs) (Schluter et al., 2006). PSD-95 provides a docking site for the AMPA receptor. In response to AMPA activity, PSD-95 undergoes ubiquitination by the E3 ligase Mdm2, and its removal from the synapse is mediated by the UPS in response to NMDARs activation. PSD-95 degradation leads to AMPA receptor internalisation and mutation or inhibition of the proteasome that blocks PSD-95 ubiquitination. Blocking the internalization of AMPARs can be induced by direct stimulation of NMDARs (Colledge et al., 2003). The stimulation of AMPA receptors leads to a decrease in dendritic PSD-95 in a proteasome-dependent manner, while the over-expression of PSD-95 inhibits AMPAR endocytosis (Bingol and Schuman, 2004). Several other proteins including both receptors and receptor-associated PSD proteins, in addition to PSD-95, such as glutamate receptor interacting protein 1 (GRIP1), protein interacting with C-kinase 1 (PICK1) and Spine-associated RapGAP (SPAR), appear to be regulated by UPS (Lin and Man, 2013).

Taken together, these findings suggest that UPS is an essential component for the regulation of synaptic proteins. Impairment of the proteasome may play an important role in synaptic defects in adult brains under normal and diseased conditions.

1.9.4 The UPS in neurodegenerative disorders

Most neurodegenerative diseases are characterised by intracellular inclusion or extracellular aggregate in specific brain areas (Ross and Poirier, 2004, Soto, 2003). The pathological hallmarks of each neurodegenerative disorder consist of misfolded disease-specific proteins within their aggregate, and they all share the common feature that these misfolded proteins yield a β-sheet structure that promotes the formation of amyloid fibril (Ross and Poirier, 2004, Soto, 2003). Amyloid fibrils are an assembly of soluble proteins misfolded into insoluble fibres which are normally resistant to proteolysis (Ross and Poirier, 2004, Soto, 2003). As a result of inefficient clearance of the misfolded proteins, more proteins aggregate and the size of the fibril grows, thereby promoting the development of an inclusion body around or inside the degenerating neurons (Ross and Poirier, 2004, Soto, 2003). The pathological hallmarks of Alzheimer's disease AD include extracellular senile plaques (SP), mainly consisting of β-amyloid (Aβ) (Glenner and Wong, 1984, Glenner et al., 1984), and intracellular neurofibrillary tangles (NFTs), containing aggregated, hyperphosphorylated tau protein (Grundke-Iqbal et al., 1986). The typical hallmark of Lewy body dementia is the presence of Lewy bodies and Lewy neurites containing the small presynaptic protein α-synuclein (Spillantini et al., 1997). A further example of protein inclusions within neurodegenerative diseases are the formation of TAR DNA-binding protein (TDP-43) inclusions, observed in frontotemporal dementia (Goedert et al., 2012). Protein misfolding and its accumulation in these diseases might be the result

of different genetic mutations or environmental factors. Once aggregated protein develops in one cell, the misfolded protein might transfer between cells, functioning as a seed and spreading to other cells, or from one brain area to another (Guo and Lee, 2014, Masuda-Suzukake et al., 2013, Polymenidou and Cleveland, 2011). Indeed, such a hypothesis may account for the spreading nature of these disorders, where typically pathology is seen early on in during the disease process in certain brain regions, eventually spreading and encompassing many other regions. Neurodegenerative disorders are now known under the umbrella category of "proteinopathies" of the central nervous system (CNS) (Golde and Miller, 2009). In many of these disorders, immunostaining in post-mortem brains revealed positive staining with anti-ubiquitin antibodies, which are signals for protein degradation by the proteasome (Leigh et al., 1991, Lennox et al., 1988, Lowe et al., 1988). These proteins were mostly tagged with the Ub for degradation, but for some reason were not efficiently removed, leading to their accumulation. UPS components have also been found to accumulate with LBs and other inclusions (Kwak et al., 1991, Lennox et al., 1989, Schlossmacher et al., 2002, Zhou et al., 2004). Proteasome activity decreases in post-mortem brain tissue in both AD and DLB (Dahlmann, 2007). Several mutations in UPS genes are linked to neurodegenerative disorders (Yi and Ehlers, 2007). In the next section post-mortem brain studies, genetic studies and experimental studies support the involvement of UPS in pathogenesis of α-synucleinopathies will be discussed.

1.9.4.1 The UPS in pathogenesis of α-synucleinopathies

1.9.4.1.1 The UPS in pathogenesis of α-synucleinopathies (studies on post-mortem brains)

The majority of the studies presented in the next three sections investigate the impairment of the UPS in PD. Lewy body dementia is closely related to PD; both are currently demonstrated with the same α-synuclein pathology and diagnosed by the detection and quantification of Lewy bodies. Although there are not a lot of studies investigating the role of UPS dysfunction in LBD, studies in PD could conclude with supportive evidence to the role of UPS dysfunction in the pathogenesis of α-synucleinopathies and Lewy body formation. In this section, the role of the UPS in α-synucleinopathies in post-mortem brain studies will be discussed.

Evidence from both post-mortem brain and experimental studies has indicated that the UPS has a pivotal role in the pathology of Lewy body dementia. As mentioned above, the degradation by UPS requires the formation of polyubiquitinated protein. Post-mortem studies have indicated the presence of ubiquitinated protein (Kuzuhara et al., 1988, Lennox et al., 1989) and UPS components, including proteasomal subunits, ubiquitin, parkin, ubiquitination and deubiquitination enzymes, and proteasome activators, within Lewy body inclusions (Kwak et al., 1991, Lennox et al., 1989, Schlossmacher et al., 2002, Zhou et al., 2004). The presence of ubiquitinated protein and proteasome components indicates the failure of the UPS to clear unwanted protein, as the ubiquitin serves as a signal for proteasomal degradation. Co-localization of ubiquitin carboxy-terminal hydrolase L1 (UCHL1), a deubiquitinating enzyme, has been detected in Lewy bodies (Yasuda et al., 2009). UCHL1 mRNA and the expression level of UCHL1 protein have been found to be

reduced in the cerebral cortex and substantia nigra in DLB (Barrachina et al., 2006a). UCHL1 catalyze the hydrolysis of polymeric ubiqutin chains and its reduction may affect the level of free ubiqutin. The E3 ubiquitin-ligase SIAH-1 is also a component of Lewy body inclusions (Liani et al., 2004). SIAH-1 interacts with synphilin-1 and promotes its polyubiquitination and degradation by the proteasome. Synphilin-1 is not a UPS component, but it can interact with the RPT5 subunit of the 19S RP and decrease proteasome function (Marx et al., 2007). Both RPT5 and synphilin-1 co-localize in LBs in PD (Marx et al., 2007).

Significant reduction in all proteolytic active sites of the 20S subunit in the substantia nigra pars compacta (SNc) of patients with sporadic PD was first reported in 2001, and subsequently further investigated (McNaught et al., 2003, McNaught and Jenner, 2001, Tofaris et al., 2003). One year later, the same group reported a selective loss of the α-subunit of the 20S proteasome in post-mortem studies in the substantia nigra in PD (McNaught et al., 2002c). A significant reduction was also found in the expression level of both 20S alpha-6 and alpha-4 in the substantia nigra in PD (Bukhatwa et al., 2010a). The loss of the 20S α-subunit causes the 20S/26S to become unstable, reduces the assemblage and binding with 19S and impairs proteolytic activity (Voges et al., 1999). Analysis of post-mortem cortical tissue indicated alterations in the level of α-subunit in the cingulate gyrus of DLB patients, which correlated with the duration and severity of cognitive impairment (MacInnes et al., 2008). 19S RP levels were found to be reduced in the SNc while expression was upregulated in other brain area (McNaught et al., 2003). Recent studies have also reported a reduction in the α-subunit of the 20S proteasome in the cingulate gyrus of patients with LBD (MacInnes et al., 2008). In the brains of PD patients,

perinuclear aggresome-like structures rich in 20S, 19S, and 11S subunits of the 26S proteasome subunits have been reported in relatively unaffected areas only, such as the ventral tegmental area and dorsal midbrain (Johnston et al., 1998, McNaught et al., 2003, Wigley et al., 1999).

1.9.4.1.2 The UPS in pathogenesis of α-synucleinopathies (mutations of genes)

Several genetic studies support the involvement of the UPS in the pathogenesis of α-synucleinopathies especially in PD by the discovery of mutations in two proteins (parkin and UCH-L1) directly involved in the UPS. Two of the PD genes; synucleins and DJ-1 have been found to be candidate biomarker for Lewy body dementia. Mutation in Parkin and UCH-L1 are associated with familial early-onset PD and until now none of these mutations have been associated with LBD, although both proteins have been found to be components of cortical Lewy bodies (Ross and Poirier, 2004, Schlossmacher et al., 2002).

PARK2 or parkin is the gene most commonly known to cause early-onset PD, encoding a 52kDa protein, 465 amino acids in length (Dale et al., 1992, Voges et al., 1999). Mutations in PARK2 account for approximately 50% of all autosomal recessive juvenile Parkinson's disease (Dale et al., 1992, Lucking et al., 2000). To date, several mutations in parkin have been identified in early-onset Parkinsonism, including numerous point mutations and exonic rearrangements such as deletion, duplication and triplication reviewed in (Johnston et al., 1998). However mutations in this gene have been found in late-onset of PD. Parkin function as an E3 ubiquitin ligase protein (Shimura et al., 2000). Parkin can modulate the activity of the 26S proteasome by ubiquitination of the target protein for degradation. It has been shown that disruption of the ubiquitin–protein ligase

function of Parkin leads to a reduction in the degradation of the target protein by UPS, and resulted in protein accumulation that was selectively toxic to dopaminergic neurons (Hattori and Mizuno, 2004, Dale et al., 1992, Lucking et al., 2000). Parkin is involved in its own proteasome-dependent degradation as it has the ability to self-ubiquitinate. Furthermore, it has also been shown to promote the degradation of synaptic proteins such as synphillin-1(Chung et al., 2001). Synphillin-1 is a protein known to interact with α-synuclein. It has been implicated in the pathogenesis of both PD and DLB and was found to be present in LBs in PD and DLB patients (Iseki et al., 2002, Wakabayashi et al., 2000).

PARK5 or *UCH-L1* is a gene encoding ubiquitin carboxy-terminal hydrolase L1 (UCH-L1). UCH-L1 is a neuronal protein consisting of 223 amino acids that processes the ubiquitin by its deubiquitinating activity (Wilkinson et al., 1989). The hydrolase activity of UCH-L1 is believed to facilitate the activity of the UPS by increasing the UB monomers in order to target the substrate to be degraded (Wilkinson, 2000). In addition to its hydrolase activity, UCH-L1 also has a ligase activity, which in turn has a role in stabilizing Ub monomers (Osaka et al., 2003). The link between mutations in *UCH-L1*and Parkinson's disease was identified by the discovery of an I93M missense detected in two siblings of a German family with autosomal dominant familial PD (Leroy et al., 1998). The I93M mutation was found to reduce the activity of the proteasome by 50% (Leroy et al., 1998). Although it is not clear whether the I93M mutation is a pathogenic mutation or a rare polymorphism, UCHL1 knock-out mice did not demonstrate any neurodegeneration in the substantia nigra although they did suffer from gracile axonal dystrophy (*gad*) (Liu et al., 2002).

1.9.4.1.3 Role of UPS dysfunction in the pathogenesis of α-synucleinopathies (experimental studies)

Experimentally, a range of experimental studies support the role of UPS dysfunction in α-synucleinopathies. Indeed many studies have documented the use of proteasome inhibitors to produce a model that displays many pathological features of α-synucleinopathies including; formation of ubiquitinated cytoplasmic inclusions and apoptotic cell death (Ardley et al., 2003, McNaught et al., 2002a, Rideout et al., 2005, Rideout et al., 2001).

Inhibition of proteasomal function using proteasome inhibitors, such as the naturally occurring compound lactacystin, in foetal rat ventral mesencephalic cultures (McNaught et al., 2002b, Rideout et al., 2005) and PC12 cells (Rideout et al., 2001), induced cell death of dopaminergic neurons, and the formation of inclusion bodies that stained positive with α-synuclein and ubiquitin antibodies. A similar finding was observed when foetal rat ventral mesencephalic cultures were treated with ubiquitin aldehyde to inactivate ubiquitin hydrolases (McNaught et al., 2002b). As these studies used high doses of lactacystin (5–10 μM for 24–48 hours), increased efforts to further understand what occurs in these cells during PD pathogenesis have been made, using lower doses of proteasome inhibitors. In those studies, chronic low-level proteasome inhibition using a different inhibitor (MG115 ;100nM) was induced for several weeks in neural SH-SY5Y cells that allows for the analysis of more subtle cellular and molecular alterations (Ding et al., 2003, Sullivan et al., 2004). These studies found increased cytosolic protein oxidation and protein aggregation, decreases in the activity of complex I and complex II and increases in the production of

oxygen free radicals indicating alterations in mitochondrial homeostasis (Ding et al., 2003, Sullivan et al., 2004).

The possibility of producing an *in vivo* model of PD using proteasome inhibitors is controversial; this is maybe due to the reproducibility of this model across different laboratories (Cook and Petrucelli, 2009, Lim and Tan, 2007). Stereotactic injections of the proteasome inhibitor lactacystin into the SNc of rats causes a degeneration of dopaminergic neurons and cytoplasmic accumulation of α-synuclein and progressive bradykinesia (McNaught et al., 2002a). McNaught et al extended their study and produced a rat model by systematic injections with either the naturally occurring proteasome inhibitor epoxomicin or the synthetic proteasome inhibitor (Z-lle-Glu(OtBu)-Ala-Leu-al [PSI]) over a period of 2 weeks (McNaught and Olanow, 2004, McNaught and Olanow, 2006). The activity of the proteasome was reported to be reduced in the ventral midbrain, and lower brainstem. However, elevated proteasome activity was observed in the cerebral cortex, striatum, cerebellum, and spinal cord (McNaught and Olanow, 2004). These results suggest that the up-regulation of the proteasome activity was due to the compensatory mechanisms against proteasome interference in some, but not all, brain areas. Treated animals developed a progressive parkinsonism symptom with bradykinesia, rigidity, tremor (McNaught and Olanow, 2004). Furthermore, neurodegeneration was accompanied by the appearance of α-synuclein and ubiquitin positive intracytoplasmic inclusions observed in locus coeruleus, dorsal motor nucleus of the vagus and substantia nigra (McNaught and Olanow, 2004). These data provide a novel PD model based on the inhibition of the proteasome system. Unfortunately, other studies have failed to replicate the technique they used to induce the neurodegeneration PD model (Bove et al., 2006, Kordower et al., 2006, Manning-Bog et

al., 2006) Only two groups so far have managed to reproduce the nigral pathology (Schapira et al., 2006, Zeng et al., 2006a), and only one detected any α-synuclein aggregates (Zeng et al., 2006a). Although Parkinson's disease has been a major focus of most studies, the contribution of UPS to the neuronal loss and α-synuclein pathology has been applied to DLB. A recent experimental observation has shown that direct injection of proteasome inhibitors into the rodent cholinergic forebrain neurons leads to loss of those neurons, accompanied by the development of cortical intra-neuronal α-synuclein aggregates (MacInnes et al., 2008).

Bedford et al. reported that genetically disrupting the 26S proteasome subunit in mouse brain neurones causes neurodegeneration and development of Lewy body-like inclusions in these animals, providing a greater understanding of the link between 26S proteasome dysfunction and the development of α-synuclein neuropathology (Bedford et al., 2008). In their study, they generated knock-out mouse using the Cre/loxP system which genetically ablated one of the ATPase subunits from the 19S regulatory particle (RPT2) subunit only in forebrain, and thus, prevented the formation of the 26S proteasome, leaving the 20S proteasome subunit, which is ubiquitin-independent, unaffected (Bedford et al., 2008). Interestingly, the same group recently generated the same mouse model lacking α-synuclein and reported that α-synuclein was not essential for the formation of pale body-like inclusions and neurodegeneration caused by 26S proteasomal depletion (Paine et al., 2013). PB-like inclusions have been considered precursors of LBs (Dale et al., 1992). These data indicate that other additional factors may lead to proteasome impairment, which in turn may leads to the formation of α-synuclein inclusion. These data suggest a strong direct link between proteasome dysfunction and neuronal death. Our group examined the

impact of the proteasome inhibitor in primary neuronal cell cultures and found that a significant loss of synaptic proteins (β-III-tubulin, synaptophysin, and drebrin) occurred prior to neuronal death (Bajic et al., 2013). These data indicate an important role of the proteasome in synaptic function which may represent an early event in LBD.

From all the evidence discussed so far, it is clear that UPS dysfunction plays a key role in neuronal degeneration and the formation of neuronal inclusion. Further study is required to investigate how the changes in the expression levels of different proteasome subunits can contribute in the impairment in motor and cognitive function.

1.9.4.2 The Ubiquitin proteasome system in Alzheimer's disease

Growing evidence suggests an involvement of the UPS in the pathogenesis of AD. As with Lewy body aggregates, accumulation of Ub has been detected in both plaques and tangles (Lennox et al., 1988, Lowe et al., 1988, Mori et al., 1987). In addition to Ub, a mutant ubiquitin carrying a 19-amino acid C-terminal extension, ubiquitin-B+1 (UBB+1) has also been observed in AD lesions (van Leeuwen et al., 1998). UBB+1 arises from the molecular misreading of the Ub^{+1} gene, resulting in a transcriptional dinucleotide deletion (van Leeuwen et al., 1998). UBB+1 has been reported to inhibit the proteasome activity in a dose-dependent manner (van Tijn et al., 2007). It also blocks degradation by proteasome in the cell line (Lindsten et al., 2002), and has been suggested to contribute in mediating -Aβ neurotoxicity (Song et al., 2003).

Significant reduction in proteasome activity was reported in AD post-mortem brain tissue in the hippocampus, parahippocampal and middle temporal gyri, and in the inferior parietal lobule. The reduction in proteasome activity was observed to be associated with an

increased UBB conjugation and correlated with the reduction in synaptic proteins (Keck et al., 2003, Keller et al., 2000). Lopez and colleagues investigated the proteasome activity and also the enzymes involved in the ubiquitin pathway (E1 and E2) (Lopez Salon et al., 2000). Lopez et al. indicated a significant reduction in the proteasome activity, particularly trypsin-like activity in the cytosol of AD brain tissue and a reduction in both E1 and E2 enzymes (Lopez Salon et al., 2000). Furthermore, post-mortem expression profiling of proteasome subunits 20S α5 and 19S non-ATPase S1/Rpn2 was found to be down-regulation in the amygdala and cingulate cortex, two brain regions affected early in AD (Loring et al., 2001). Proteasome post-mortem brain studies also revealed that the 19S regulatory subunit S6b/RPT3 was detected in neurofibrillary tangles in AD-affected brain areas and other taupathies (Fergusson et al., 1996).

Further evidence indicating the dysfunction of the UPS is the accumulation of oxidized proteins (Forero et al., 2006, Zhu et al., 2007); these aggregates may further impair proteasome activity (Bence et al., 2001). Of these proteins UCH-L1, a de-ubiquitinated enzyme, is oxidized in AD and is down-regulated in specific brain regions of early AD cases (Choi et al., 2004). Moreover, UCH-L1 concentration was found to be inversely proportional to the number of tangles and its immunostaining associated with NFTs (Choi et al., 2004). An experimental study in transgenic mice overexpressing APP indicated that UCH-L1 rescues synaptic dysfunction in these mice and also in hippocampal slices treated with Aβ oligomers (Gong et al., 2006).

1.9.4.2.1 Association of UPS with Aβ pathology

Several studies in the past few years have indicated a link between proteasome impairment, Aβ oligomers and the accumulation of Aβ (Almeida et al., 2006, Barelli et al., 1997, Checler et al., 2000, Gregori et al., 1995, Gregori et al., 1997, Lopez Salon et al., 2003, Marambaud et al., 1997, Oh et al., 2005, Tseng et al., 2008, Zhao and Yang, 2010). Previous in vitro studies revealed that binding of the Aβ protein to the 20S proteasome inhibits ubiquitin-dependent protein degradation and selectively inhibits the chymotrypsin-like activity of the 20S proteasome (Gregori et al., 1995, Gregori et al., 1997). Furthermore, inhibition of the proteasome in cell culture induced Aβ production, exacerbated Aβ-neurotoxicity (Barelli et al., 1997, Checler et al., 2000, Marambaud et al., 1997) and also caused a marked decrease in Aβ42 degradation (Lopez Salon et al., 2003). Accumulation of Aβ in APP mutant neurons has also been shown to inhibit the activities of the proteasome and de-ubiquitinating enzymes (Almeida et al., 2006). In addition, in these primary neurons, it has been shown that extracellular Aβ could enter the neurons and inhibit the proteasome (Oh et al., 2005). In the 3xTg-AD mice, dysfunction of proteasome activity was found to be correlated with Aβ oligomers, and inhibition of the proteasome in the pre-pathological 3xTg-AD mice increased the Aβ oligomers level, which lead to Aβ accumulation (Tseng et al., 2008). Furthermore, Tseng et al. reported that Aβ immunotherapy in the 3xTg-AD mice decreased the Aβ oligomers level and reactivated the UPS (Tseng et al., 2008). Moreover, aggregated forms of $A\beta_{42}$ are subject to proteasomal degradation and competitive substrates for the chymotrypsin-like activity of the human 20S proteasome (Zhao and Yang, 2010). All these evidences suggest a relationship between

proteasome dysfunction and Aβ accumulation and the pathogenesis of AD [reviewed by (Hong et al., 2014)], which, in turn, suggest the important role of the UPS in Aβ pathology.

1.9.4.2.2 The association of UPS with tau pathology

Extensive evidence from in vivo and in vitro studies suggest a role of UPS in the tau degradation and aggregation (for review see (Lee et al., 2013). The first clue was derived from the fact that UB and ubiquitinated tau are components of NFTs and PHFs. PHFs were purified AD brain and post-translation modification was identified by mass spectrometry, which tau found to be ubiquitinated at lys-254, lys-311 and lys-353, as an early event in AD (Cripps et al., 2006) and the polyubiquitin chains was linked through lys-48, lys-11, and lys-6 of the ubiquitin molecules (Cripps et al., 2006). Ubiquitination through lys-48 is a signal for targeting the protein to be degraded by the proteasome (Glickman and Ciechanover, 2002). While Polyubiquitin chains linked by Lys-6 inhibit ubiquitin-dependent proteolysis (Shang et al., 2005), and may favour formation of aggregates instead of tau clearance. Tau has been shown to be a substrate for both 20S and 26S proteasome (Lee et al., 2013). Incubation of recombinant tau with 20S proteasome in the presence and absence of proteasome inhibitors indicated a decrease in the length of tau protein. The degradation occurs in the absence of proteasome inhibitors (David et al., 2002). Furthermore, ubiquitinated tau has been found to be a substrate of the 26S proteasome in the presence of MgCl2 and ATP, and has also been found to inhibit the proteasome and lead to its accumulation (Zhang et al., 2005). Additionally, rat brain cortex extract, which contains all the component of the UPS and endogenous tau, was incubated with the proteasome activators MgCl2 and ATP. This resulted in the degradation not only of tau but also AD P-tau in vitro by 26S, which was blocked by applying proteasome inhibitors, such

as lactacystin. This provides further evidence that tau was degraded by proteasome (Zhang et al., 2005). Although there are a large number of reports claiming that tau degradation is catalysed by the proteasome, many other studies show that UPS may not be the initial pathway for tau degradation (Brown et al., 2005, Feuillette et al., 2005).

In contrast, numerous studies of the contribution of the lysosomal pathway to tau degradation have suggested that tau is a lysosomal substrate. Inhibition of the lysosomal pathway delays the degradation of tau protein and enhances the formation of higher molecular species of tau (Wang et al., 2009, Zhang et al., 2009b). In contrast, lysosomal inducers enhance the clearance of abnormal tau and protect against its toxicity in Drosophila. Furthermore, the human tauopathy mouse model study showed that the lysosomal activators reduced the level of aggregated tau and demonstrated an effect in neuronal survival (Kruger et al., 2012, Schaeffer et al., 2012). Methylene blue has been observed to induce autophagy in tau transgenic mice and reduce the total phospho-tau level. It also has the ability to improve the cognitive performance in these mice (Congdon et al., 2012, Hosokawa et al., 2012).

The above studies indicated that tau is degraded by the UPS and the lysosomal pathway. Targeting the UPS for degradation may occur when tau is in excess and still soluble. Mono-ubiquitination tau and tau aggregates are likely to be degraded by the lysosomal pathway [review by (Lee et al., 2013)].

1.10 Autophagy

1.10.1 Overview

Eukaryotic cells contain multiple proteolytic systems for protein degradation: the main two are the ubiquitin-proteasome system (UPS) and autophagy-lysosome pathway (ALP). Both pathways are responsible for regulating cellular protein turnover. UPS mainly degrades short-lived proteins while the lysosome pathway is involved in the degradation of long-lived protein and cellular organelles, which are too large for degradation via the UPS. Dysfunction of both pathways might contribute to the pathogenesis of a variety of neurodegenerative disorders. The lysosomal degradation pathway appears to play a fundamental role in neurodegenerative disorders such as Parkinson's disease (Anglade et al., 1997, Cuervo et al., 2004, Spencer et al., 2009, Winslow et al., 2010, Xilouri et al., 2009), Alzheimer's disease (Boland et al., 2008, Cataldo et al., 2004, Nixon, 2007, Nixon et al., 2005, Pickford et al., 2008) and Huntington's disease (Atwal and Truant, 2008, Martinez-Vicente et al., 2010, Petersen et al., 2001, Ravikumar et al., 2004). In Parkinson's disease and related synucleinopathies, α-synuclein—the main component of Lewy bodies—was degraded by both the UPS and ALP systems (Ebrahimi-Fakhari et al., 2011, Rott et al., 2011, Rott et al., 2008, Webb et al., 2003). The mechanism recruited to maintain protein homeostasis depends on the protein burden (Ebrahimi-Fakhari et al., 2012). Under a normal turnover, monomeric α-synuclein can be degraded by both UPS and chaperone-mediated autophagy (CMA) (Vogiatzi et al., 2008, Webb et al., 2003). At early disease stages, excess levels of α-synuclein and aggregate species block both the UPS and CMA, at these stages, macroautophagy is up-regulated to degrade α-synuclein (Ebrahimi-Fakhari et al., 2011, Ebrahimi-Fakhari et al., 2012). In the late stage of the disease, α-synuclein inhibits

macroautophagy and lysosomal degradation (Ebrahimi-Fakhari et al., 2012). While the ALP and its relevance in neurodegeneration is presented in this section, the role of UPS in neurodegenerative disorders is discussed in detail in Section 1.9 of this chapter.

1.10.2 The autophagy lysosomal pathway

Autophagy is a catabolic process by which intracellular components are delivered to lysosomes for degradation by their resident hydrolases (Cuervo, 2004, Klionsky, 2005). The Autophagy Lysosomal Pathway is comprised of three distinct pathways based on the way the substrates reach the lysosomal lumen: macroautophagy, microautophagy and CMA. These are described below and summarised in (Figure 1-9). Also, for more details, the autophagy lysosomal pathway has been extensively described and reviewed by (Komatsu and Ichimura, 2008, Nixon, 2013, Ravikumar et al., 2010, Son et al., 2012, Yue et al., 2009, Zhang et al., 2009b).

1.10.2.1 Macroautophagy

Macroautophagy is a multi-step process commonly referred as autophagy or ‘‘self-eating’’. It is a vacuolar degradation pathway in which cellular components, including proteins, membrane fragments and whole organelles such as mitochondria, are engulfed or sequestered by a double-membrane structure, which fuses around the substrate, producing autophagic vesicles referred as the autophagosome (Feng et al., 2014). These vesicles do not contain hydrolyses enzymes but receive them after fusing with the lysosome that contains hydrolytic enzyme-forming autophagolysosome, which is considered to be the characteristic component of autophagy. The hydrolytic enzymes then degrade the contents of the autophagolysosome, including the inner membrane (Feng et al., 2014). The

components are broken down into their constituent amino acids and fatty acids, which are then released into the cytoplasm and recycled.

1.10.2.2 Microautophogy

Microautophogy refers to a process in which a region of the cytosol-containing proteins to be degraded is captured through an invagination in the surface of the lysosomal membrane.

1.10.2.3 Chaperone-Mediated Autophagy (CMA)

CMA is a process in which the substrate proteins containing a specific motif are selectively recognized by a specific cytosolic chaperone protein, also known as a heatshock protein (Hsp), which plays an important role in CMA during substrate recognition, targeting, unfolding, and transporting (Arias and Cuervo, 2011, Cuervo, 2010, Dice, 2007). Hsp70 chaperones recognize the CMA-targeting motif (KFERQ motif) in the protein substrates and deliver them to the lysosomal surface (Chiang et al., 1989, Dice, 1990). At the lysosomal membrane, the substrate interacts with the cytosolic tail of lysosome-associated membrane protein type 2A (LAMP-2A), which is a CMA receptor. LAMP-2A facilitates the transportation of the protein into the lysosomal lumen for degradation by the hydrolases (Cuervo and Dice, 1996). The resulting amino acid could be used for the synthesis of essential proteins.

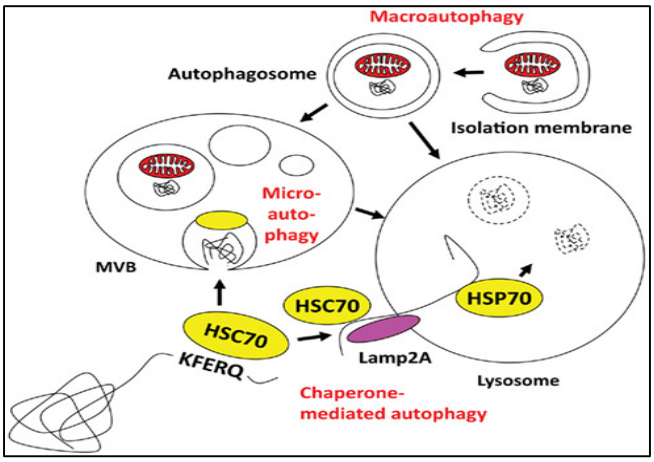


Figure 1-9: Autophagy - the lysosomal system.

The Autophagy Lysosomal Pathway is comprised of the following three fundamentally different types of autophagy processes, based on the way substrates reach the lysosomal lumen: **Macroautophagy** is a double-membrane engulfed substrate, which forms the autophagosome, and is destined for degradation. The autophagosome fuses with the lysosome that contains the hydrolytic enzyme, forming autophagolysosome. The hydrolytic enzymes in the lysosome then degrade the content inside the autophagolysosome in addition to the double membrane. The components are broken down into their constituent amino acids and fatty acids, which are then released into the cytoplasm and recycled. **Microautophagy** refers to a process in which a region of the cytosol-containing proteins are degraded and captured through an invagination in the surface of the lysosomal membrane. **Chaperone-Mediated Autophagy (CMA)** is a process which involves the recognition of the cytosolic protein and its translocation across the lysosome membrane through the action of the cytosolic and lysosomal chaperone hsp70, and the lysosomal membrane protein LAMP-2A. Figure Taken from (Munz, 2011).

1.10.3 Dysfunction of the autophagy-lysosomal pathway in neurodegenerative disorders

Several reports indicate the role of ALP in LBD. The first evidence supporting the involvement of lysosomal dysfunction in LBD is that macroautophagy and CMA degrade α-synuclein (Ebrahimi-Fakhari et al., 2011, Mak et al., 2010, Vogiatzi et al., 2008). Inhibition of CMA leads to an accumulation of soluble high molecular weight and the detergent-insoluble species of α-synuclein (Vogiatzi et al., 2008). This suggests that degradation of α-synuclein by CMA is crucial and its dysfunction may be one of the factors involved in synucleinopathies, such as Lewy body dementia. Importantly, it has been found that the A53T and A30P mutants of α-synuclein cannot be degraded by CMA. Instead, impaired CMA functions via a higher-binding affinity to the lysosomal receptor LAMP2a, preventing the movement of α-synuclein or other substrates into the lysosome (Cuervo et al., 2004). In addition to mutant α-synuclein, post-translational modifications of the wild-type α-synuclein, including monoubiquitination of a-synuclein, also impact CMA degradation without affecting degradation of other substrates (Cuervo et al., 2004, Engelender, 2008).

Overexpression of both wild-type and mutated α-synuclein also contributes to neuronal death through the inhibition of CMA-mediated degradation of myocyte specific enhancer factor 2D (MEF2D), a neuronal survival factor. MEF2D levels were increased in the brains of α-synuclein transgenic mice and patients with Parkinson's disease (Yang et al., 2009).

Further evidence for the involvement of lysosomal dysfunction in LBD is that the degradation of α-synuclein by autophagy occurs via the lysosomal enzyme cathepsin D (Sevlever et al., 2008). Cathepsin D is an aspartyl lysosomal protease, which mediates the degradation of aggregated and damaged proteins (Hasilik and Neufeld, 1980). Cathepsin D-deficient mice grow normally for up to two weeks, but die at around 26 days of age from a combination of pathologies including intestinal necrosis and neurodegeneration, indicated the important role of cathepsin D in maintaining the function of the autophagy-lysosomal pathway (Koike et al., 2000). Deficiency of cathepsin D in transgenic mice also leads to the accumulation of a high molecular weight of α-synuclein species, but not monomeric, in neurons, despite the compensatory up-regulation of other lysosomal proteases, without the increase of α-synuclein mRNA expression (Qiao et al., 2008). In these mice, the proteasome activity decreased without affecting key factors of UPS. This suggests a link between these two pathways at the level of activity rather than a reduction of protein levels. Furthermore, cathepsin D overexpression reduced α-synuclein aggregation and shows a neuroprotective effect in dopaminergic cell lines and in Caenorhabditis elegans (Qiao et al., 2008).

It was also found that aggregated α-synuclein induced by phosphorylation of Ser129 led to an increase in the activity of cathepsin D, which, when inhibited, caused a reduction in the formation of truncated species of α-synuclein, including oligomers and inclusions. This suggests that cathepsin D may play a role in generating toxic truncated species of α-synuclein (Takahashi et al., 2007).

1.11 Hypothesis

Neurodegenerative disorders share a common feature, which is the accumulation of misfolded proteins in the form of insoluble protein aggregates (both intra and extracellular) or intracellular inclusion bodies. Ubiquitin has been identified as a component of protein aggregates and inclusion bodies in many neurodegenerative disorders, suggesting that the impairment of the UPS is involved in the formation of these structures. As reviewed, LBD features Lewy bodies and aggregates of α-synuclein, tau and Aβ and therefore a role for the UPS has been widely suggested in pathogenesis.

Defects in the 26/20S components of the UPS and synaptic dysfunction have been implicated in the pathogensis of several neurodegenerative disorders. Furthermore, previous studies by our research group have identified specific synaptic changes that appear to underly cognitive and non-cognitive symptoms of LBD. It was therefore hypothesized that cognitive decline and non-cognitive symptoms in LBD were associated with synaptic dysfunction consequent upon alterations of proteasome subunit expression, proteasome activity and increased protein aggregation.

In order to evaluate the involvement of proteasome dysfunction, the aims of the study were as follows:

- To investigate the expression level of the proteasome subunits (α3, α6 and RPT6), together with the proteasome activity in discrete brain regions of human post-mortem brains from LBD and AD patients, and in controls using western blot and immunohistochemistry.

- To evaluate the relationship between alterations of the proteasome subunit and protease activities of the proteasome in LBD and AD.
- To investigate whether alteration of the expression level of the proteasome subunit in different brain areas correlates with semi-quantitative scores of AD and LBD pathology.
- To evaluate the relationship between proteasome dysfunction and the cognitive decline and non-cognitive symptoms in LBD and AD.
- To determine if alteration in the expression level of the proteasome subunit correlates with pre- and/or postsynaptic markers.

To further our knowledge on the second important proteolytic pathway that mediates protein degradation — the lysosomal pathway — also aimed to investigate the following:

- Investigate the expression level of two lysosomal markers, cathepsin D and lysosomal-associated membrane protein 1 (LAMP1), in discrete brain regions of the human post-mortem brain from LBD and AD patients, and in controls using western blot.
- Determine if there is a correlative relationship between the two major proteolytic pathways—the ubiquitin-proteasome pathway and the lysosomal pathway.
- Investigate whether alteration of the expression level of the lysosomal markers in different brain areas correlated with semi-quantitative scores of AD and LBD pathology.
- Evaluate the relationship between lysosomal markers and the cognitive decline and non-cognitive symptoms in LBD and AD.

- Determine if alteration in the expression level of the lysosomal markers correlated with either pre- or postsynaptic markers.

Overall it was hoped that the results of thesae studies will provide greater insight into the mechanisms of pathogenesis of LBD, particularly in relation to emergence of synaptic pathology. Furthermore, such investigations may also identify novel targets for pharmacological intervention in LBD.

Chapter 2 Material & Methods

2.1 Subjects

Post-mortem brain tissue was obtained from 130 cases: 55 with DLB, 34 with PDD, 16 with AD and 25 normal controls matched for age, gender and post-mortem delay. Post-mortem brain tissue was obtained from several sources, including University Hospital Stavanger (Norway), MRC Brain Bank at the Institute of Psychiatry, King's College London, Newcastle Brain Tissue Resource and the Thomas Willis Oxford Brain Collection. From each case one brain hemisphere was frozen and one hemisphere was fixed in formaldehyde. After the cerebrum was sectioned and isolated by an expert, a 500 mg of frozen tissue from each brain area was placed individually into plastic bags, then labeled and sealed appropriately. All samples were stored at -70˚C for further analysis. Three brain areas were used for this study: the anterior cingulate gyrus (Brodmann area 24), the pre-frontal cortex (Brodmann area 9) and the parietal cortex (Brodmann area 40).

A variety of different clinical assessment scales were used on a regular basis for these cases before death, including evolution of cognitive defect and behavioural nature. The Mini-Mental State Examination (MMSE) (Folstein et al., 1975) was used to measure the severity of cognitive impairment several times upon the first assessment, upon the last interview before death and also to measure average decline/year. The MMSE scores ranged from 0-30. In this project, four categories of cognitive impairment were used for classification purposes as previously described (Whitfield et al., 2014b): 'unimpaired cognition' for clinical control cases, 'mildly impaired cognition without dementia' (score of 25-30), 'mildly impaired cognition with dementia' (score of 17-24), 'moderately impaired cognition' (score of 10-16) and 'severely impaired cognition' (score of 9 or less). The classification into these categories is based on placing a third of the cases in each group

according to the MMSE score using the 'cutoff' scores of 9 and 16. But because there were a few cases with scores of 29 and 30, it was not preferable to add them to a category containing cases with a score of 17. This is why the category 'mildly impaired cognition without dementia' was included to accommodate any case with a score of 24 or above. Professors Clive Ballard and Dag Aarsland recommended using the same 'cutoff' levels found in published criteria (Boller et al., 2002, Reisberg et al., 1994).

Individuals were categorised according to the duration and severity of each behavioural symptom: agitation, depression, hallucinations and persecution on a scale of 0 to 3, where 0 was none (agitation, depression, hallucinations or persecution), 1 was intermittent and mild, 2 was moderate (intermittent but significant) and 3 was persistent and/or severe. For all individuals with dementia, scores from standardised tests or semi-structured interviews were used to derive each behavioural symptom score; principally, this was the Neuropsychiatric Inventory (NPI) mood item (n = 41-58% of dementia cases). In some cases (n = 15), depression was measured with the Montgomery-Asberg Depression Rating Scale (MADRS) (Montgomery and Asberg, 1979) (n = 15-20% of dementia cases). The thresholds for MADRS were 15 and higher for a score of severe/persistent, 7-14 for a score of moderate and 6 or lower for a score of mild. For NPI, the thresholds were 7 or higher for a score of severe/persistent, 4-6 for a score of moderate and 3 or lower for a score of mild. However, in some instances, only Cambridge Mental Disorders of the Elderly Examination (CAMDEX) (Roth et al., 1986) scores were available (n = 15), which rate depression as absent, mild/moderate and severe. Summary of clinical assessment used in the prospective study of patients during the course of LBD and AD are described in

Table 2-1. I would like to express my thanks to Professors Clive Ballard and Dag Aarsland and Dr Julie Vallortigara for their significant contributions to the compilation and standardisation of this clinical data.

Neuropathological assessment was performed according to standardised neuropathological scoring/grading systems, including Braak staging, Consortium to Establish a Registry for Alzheimer's Disease (CERAD) scores, Newcastle/McKeith Criteria for Lewy body disease, National Institute on Aging - Alzheimer's Association (NIA-AA) guidelines and phases of amyloid-β (Aβ) deposition (Aβ-phases) (Braak et al., 2006, McKeith et al., 2005, Mirra et al., 1991, Montine et al., 2012, Thal et al., 2002) For Braak staging were divided into 1 (mild) (Braak stages 0-II), 2 (moderate) (Braak stages III or IV) and 3 (severe) (Braak stages V or VI). CERAD guidelines involve a four-tiered semi-quantitative scale representing cortical neuritic plaque (NP) density: 0 (none), 1 (mild), 2 (moderate) and 3 (severe/frequent).

Semi-quantitative assessments of Aβ, tau and α-synuclein pathology were conducted by neuropathologists blind to clinical diagnosis using a four-tiered scale: 0 (none), 1 (mild), 2 (moderate) and 3 (severe/frequent) to score sections from each brain area according to published criteria (Alafuzoff et al., 2008a, Alafuzoff et al., 2009a). Labelling of Aβ plaques, phosphorylated-tau positive neurofibrillary tangles and α-synuclein positive inclusions was undertaken by Dr David Howlett using standard protocols (Howlett et al., 2014). Primary antibodies added for detection of senile Aβ plaques, tau and α-synuclein sections were (Aβ, 1000 DAKO M0872; phosphorylated tau, AT8 1:4000 Thermo Scientific MN1020; α-synuclein, NCL-SYN 1:30 Novacastra Laboratories). Semi-quantitative assessments of Aβ, tau and α-synuclein pathology were

conducted blind to clinical diagnosis, by neuropathologists (Dr Johannes Attems and Dr Tibor Hortobágyi).

Control subjects were obtained from an autopsy series of cases without a history of psychiatric disorders and had never suffered from any neurological problems. The controls cases were cognitively normal. Neuropathological examinations were undertaken in all cases and none of the cases met CERAD criteria for AD, only mild age-associated neuropathological changes in some cases (e.g. neurofibrillary tangle, Braak stage < II).

Information on medication history such as cholinesterase inhibitors, antidepressants, anti-manics, memantine, anxiolytics, hypnotics and anti-parkinsonians (L-DOPA) was available for some of the cases. Unfortunately, data was not available on the medication taken by the AD cases and certain DLB and PDD cases, making it impossible to reliably elucidate any effect on the proteins of interest by medication. The medication appendix table (Appendix IV) shows what data was available, from which it can be seen that medication was broadly similar across DLB and across PDD patients.

Table 2-1: Brief summary of clinical assessment used in the prospective study of patients during the course of LBD and AD.

Assessment	Describtion
Mini Mental State Examination (MMSE)	MMSE was developed in 1975 by Folstein (Folstein et al., 1975), as a screening tool to test cognitive function of older people. MMSE assesses function including attention, recall, language use, orientation and basic motor skills. It provides a total score that can place each individual on a scale cognitive function. Thirty is the maximum score that can be obtained with lower score indicating a greater degree of cognitive impairment. MMSE cannot be used for diagnosis different type of dementia in its own. MMSE does not test all areas of cognitive function for example; fluctuating cognition and visual hallucinations (McKeith et al., 2005)(McKeith et al., 2005) and other factors may affect the MMSE score such as the education level and sensory deficit such as vision and hearing. However, despite development of more complex and sensitive tests, the MMSE is still appropriate and accurate for screening cognitive function and cited almost in every research project that attempts to report cognitive function.
Neuropsychiatric Inventory (NPI)	The Neuropsychiatric Inventory (NPI) is one of the most commonly used assessments of behavioural symptoms and disturbances in dementia. It is questionnaire that assesses twelve different behavioral and psychological disturbances including (hallucinations, delusions, agitation/aggression, dysphoria/ depressed mood, anxiety, irritability, disinhibition, euphoria, apathy, aberrant motor behaviour, sleep and night-time behaviour changes and appetite and eating changes symptoms). NPI also evaluates the impact of these symptoms on the caregiver. The

	score is a multiplication of the intensity of the symptom by the frequency. Carers and caregivers represent a superior source of information on these symptoms to patients as patients are prone to forgetting symptoms; an issue that can be compounded by patients with severe dementia having difficulty in understanding relevant questions. Thus it is the carer who is asked to rate the severity and frequency of any of the listed behaviours which has occurred in the given time-frame, in addition to the degree of distress caused to the carer by the behaviour in question (Cummings, 1997, Cummings et al., 1994).
Unified Parkinson's Disease Rating scale	The Unified Parkinson's Disease Rating (UPDRS) scale is a widely used instrument for measuring severity of parkinsonian symptoms in clinical research and in practice. This scale is subdivided in four separate parts 1) mentation, behaviour and mood; 2) activities of daily living; 3) motor symptom; 4) complications of therapy. These are evaluated by interview. A total of 199 points are possible. 199 represents the worst (total) disability), and 0 corresponding no disability (Movement Disorder Society Task Force on Rating Scales for Parkinson's, 2003).
Montgomery-Asberg Depression Rating Scale (MADRS)	Montgomery –Asberg Depression Rating Scale (MADRS) was developed in the late 1970sby Montgomery and Asberg (Montgomery and Asberg, 1979) to measure the degree of severity of depressive symptoms among patient who have a diagnosis of depression, and particularly as a sensitive to the effects of antidepressant medications, primarily tricyclic antidepressants (TCAs). The MADRS has 10 items and uses a 0 to 6 severity scale, that is completed during a clinical interview. The following items are included in the MADRS: 1) Apparent sadness; 2) Reported sadness; 3) Inner tension; 4) Reduced sleep; 5) Reduced appetite;

	6) Concentration difficulties; 7) Lassitude; 8) Inability to feel; 9) Pessimistic thoughts; 10) Suicidal thoughts. Higher scores reflecting more severe depressive symptoms. Ratings can be added to form an overall score (from 0 to 60). Snaith et al. proposed the following cut-offs: scores of 0-6 indicate an absence of symptoms; 7-19 represent mild depression; 20-34 moderate; 35-60 indicate severe depression (Snaith et al., 1986).
Cambridge Mental Disorders of the Elderly Examination (CAMDEX)	CAMDEX is an instrument for the diagnosis of mental disorder in the elderly with special reference to the early detection of dementia. The CAMDEX includes three components: A structured clinical interview with the patient to obtain systematic information about the present state, past history and family history; a range of objective cognitive tests which constitute a mini-neuropsychological battery; a structured interview with a relative or other informant to obtain independent information about the respondent's present state, past history and family history. The three components of the CAMDEX can be divided into eight sections, A to I. After the clinical interview, the psychiatrist makes a diagnosis based on operational criteria that are described in the manual. The severity of dementia and depression is graded on a five-point scale. Scores can be derived to indicate organicity, 14 from relative interview, and two from interview observations (Roth et al., 1986)

2.2 Tissue pH determination

It is well know that the pH may vary as a consequence of agonal state which significantly influences the biochemistry of brain tissue leading to hypoxia and an increase in brain tissue lactate (Paschen et al., 1987). Therefore, after thawing of each case of frozen tissue pH was determined at room temperature using the Orion 3-star Benchtop pH Meter (Leicestershire, UK) previously calibrated with two standards (pH 4.00 and pH 7.00). Briefly, brain homogenates were prepared from 100 mg of brain tissue from the grey matter of one of the three selected regions in 2 ml ddH2O and placed in Falcon tubes. The pH measurement was determined in triplicate for each individual sample. The pH is stable during storage (Alafuzoff and Winblad, 1993, Ravid et al., 1992), therefore the measurement were done once for each case.

2.3 Western Blotting using human samples

2.3.1 Preparation of human brain tissue homogenate

Preparation of tissue for Western blotting was performed as previously described (Kirvell et al., 2006). Briefly, for each sample, cortical grey matter was dissected from white matter and meninges at 4°C. Approximately 300 mg of grey matter was homogenised in 6 ml ice cold buffer containing 50mM tris-HCL, 5mM EGTA, 10mM EDTA, 'complete protease inhibitor cocktail tablets' (Roche, 1 tablet per 50ml of buffer), and 2µg/ml pepstatin A dissolved in ethanol:DMSO 2:1 (Sigma). Homogenisation was performed using an IKA Ultra-Turrax mechanical probe (KIA Werke, Germany) until the liquid appeared homogenous resulting in a crude homogenate. The crude homogenates were aliquoted and immediately frozen on dry ice and stored at -70 °C until processed for immunoblotting.

2.3.2 Determining the protein concentration

The total protein concentration in the crude homogenate was determined using the Bradford method assay (Bradford, 1976) using Coomassie Plus protein assay reagent (Thermo Scientific, USA) and measuring absorbance at 595 nm. Briefly, aliquots containing 1 ml of the crude homogenate was thawed to be prepared for Western blotting. 10µl from each brain homogenate was then mixed with 490µl deionised H_2O to be diluted 1:50. A standard curve was obtained using bovine serum albumin (BSA) protein standard (Sigma-Aldrich, USA), with a final concentration of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 mg/ml. The protein content for both standards and brain samples were assessed in triplicate. 10 µl of both BSA standards and diluted brain samples were loaded on a 96-well plate (Nunc A/S, Denmark), and then 300 µl of Coomassie Blue reagent was pipetted to each well on the plate. A spectrophotometer reading was taken at 595 nm using a FlexStation 3 (Molecular Devices LTD, UK). The standard curve was generated as a linear regression between the protein concentration and the absorbance reading at 595 nm. The unknown protein concentration of samples was determined from the standard curve using Graph Pad Prism software version 5.0 (GraphPad Software Inc, USA).

2.3.3 Semi-quantitative Western blotting

Crude brain homogenate after protein determination was diluted 1:4 with 5x sample buffer (Genscript MB01015), vortexed for 2 minutes, boiled for 5 minutes in a heat block and then stored at -20°C to be used for Western blotting (Kirvell et al., 2006).

TV200 twin-plate 20.5 x 10 cm (W x H) format mini-gel units were used (Scis-Plas, Cambridge, UK) to separate the proteins bands from PDD, DLB, AD and control brain homogenates. A twelve percent resolving gel mixture (9.87 ml ddH_2O, 12 ml protogel, 7.8 ml protogel buffer, 300 µl 10% ammonium persulfate (APS) and 30 µl Tetramethylethylenediamine (TEMED) (TEMED was added last because it tends to solidify the gel very quickly) was carefully poured in-between glass plates using 10 ml disposable pipettes.

A stacking gel (12.2 ml ddH_2O, 2.6 ml protogel, 5 ml stacking buffer, 200 µl 10% APS and 20 µl TEMED) mixture was added at the top of the resolving solid gel. A separation comb was inserted into the stacking gel and it was allowed to set for about 20 min. The comb was then removed and the glass gasket containing the gels was placed in the tank and covered with a running buffer (0.025 M Tris–HCl, 0.2 M glycine and 0.05% SDS).

Western blots were run in duplicate. On each gel, 20 µl of each human sample were loaded once onto each of the tandem gels. Brain homogenate with a unified protein concentration of 20µg from either a rat cortex or a human cortex was also used as an internal standard in each gel in triplicate. Human cortex was used only when detecting Proteasome 20S α6 subunit and cathepsin D due to the specificity of antibodies to human protein only. On each gel there were four lanes containing rat cortex, (these were always the second lane, 12^{th}, 14^{th} and the last lane) and 1.5µl of full range molecular weight marker (MWM, sigma) in the first and 13^{th} lane.

Gels were run at 160 V for about 90 min, the time at which the blue dye was noted as almost leaking out of the gel. The contents of the gel were transferred onto nitrocellulose

membranes (Hydrobond-C, Amersham) using electroblotting for mini and maxi gels (Scis-Plas, Cambridge, UK) in a final transfer buffer concentration of 0.025 M Tris–HCl, 0.2 M glycine and 20% methanol at 60 V for 1.5 hrs. Non-specific binding sites were blocked by incubating the membrane for 1 h at 25 °C in 5% (w/v) dried skimmed milk (Marvel) dissolved in phosphate-buffered saline and tween (PBST), final concentrations were as follows: 0.14 M NaCl, 2.7 mM KCl, 6.5 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4, 1% tween). After blocking, the membranes were probed overnight at 4 °C with the appropriate primary antibody in 5% (w/v) dried skimmed milk (Marvel) PBST (see table for dilutions and more details on primary antibody). The membranes were normally washed three times for 5 min with PBST. After washing, the membranes were incubated with the relevant secondary antibodies, either a IRDye 680LT goat anti-Mouse IgG (Licor Biosciences), visible under the red channel, or a IRDye 800CW Donkey anti-Rabbit, visible under the green channel (Licor Biosciences) at dilutions of 1:5000 in 5% milk PBST for 1 hr at room temperature on the shaker. After incubation with secondary antibodies, the membranes were washed three times for 5 min each with PBST.

All antibodies were optimized prior to use by loading gels with incremental amounts of protein (5 – 40µg) prepared from human grey matter of BA9 region. Signals from 20µg loaded protein fell within a linear range of detection at the recommended antibody dilution suggested by the suppliers (Table 2-2). Thus, 20µg total protein was loaded on the gel in all cases.

Table 2-2: List of primary and secondary antibodies used for Western blotting.

Antibody	**Species**	**Dilution**	**Supplier**	**Secondary antibody**
Proteasome 20S α3 subunit, (MCP257)	Mouse monoclonal	1:2000	Enzo Life Sciences	IRDye 680LT goat anti-Mouse IgG
Proteasome 20S α6 subunit, (MCP20)	Mouse monoclonal	1:2000	Enzo Life Sciences	IRDye 680LT goat anti-Mouse IgG
Proteasome 19S ATPase subunit Rpt6, (p45-110)	Mouse monoclonal	1:2000	Enzo Life Sciences	IRDye 680LT goat anti-Mouse IgG
LAMP1	Rabbit polyclonal	1:2000	Abcam	IRDye 680LT goat anti-Mouse IgG
LAMP2	Rabbit polyclonal	1:2000	Abcam	IRDye 800CW Donkey anti-Rabbit
Cathepsin D	Goat polyclonal	1:2000	Santa Cruz	IRDye 680LT rabbit anti-Goat IgG

2.3.4 Quantification of blots

The density of the lanes and bands was quantified using LI-COR® Biosciences' Odyssey® Infrared Imaging System, software version 3.0. The quantification values were expressed as a ratio of the integral of band density in a sample to standard rat or human brain homogenate, which was run on each blot as a positive control and for standardization purposes for each sample. An example of Western blot for α-6 and RPT6 are shown in (Figure 2-2 and 2-3)

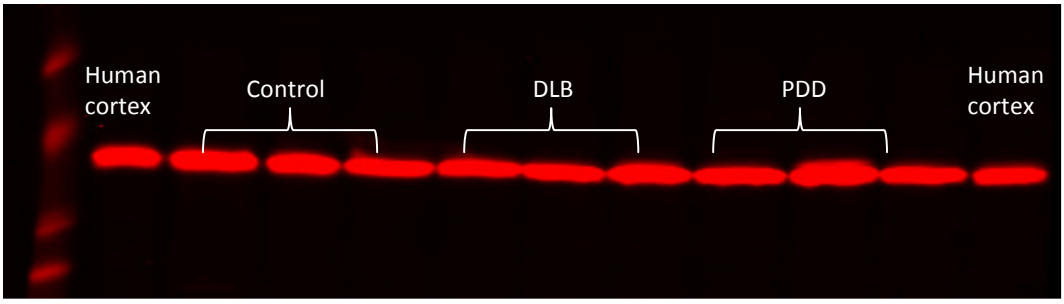


Figure 2-1: An example of a Western blot for α-6.

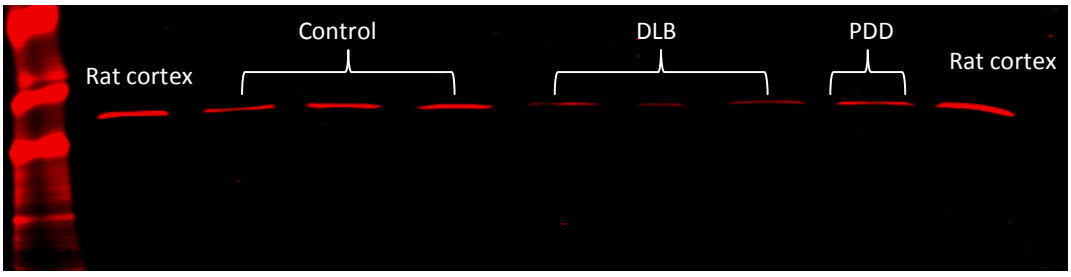


Figure 2-2: An example of a Western blot for RPT6.

2.4 Immunohistochemistry

Paraffin wax-embedded tissue sections (7 μm thickness) selected from the anterior cingulate cortex (Brodmann area (BA) 24), the prefrontal cortex (BA 9) and the parietal cortex (BA40) were de-paraffinised using xylene and rehydrated in a graded ethanol series 100%, 90% and 70%, and then washed in distilled water. The samples were then subjected to antigen retrieval by microwaving sections in 0.01 M citrate buffer, pH 6.0. Endogenous peroxidase activity was quenched by treating the sections with 0.3% hydrogen peroxide (from Sigma) in a PBS buffer for 30 min to avoid any non-specific reaction with di-amino-benzidine (DAB) (Vector Laboratories). After washing with PBS (three times for 5 min), the sections were incubated with 20% normal goat or rabbit serum diluted in blocking solution containing 2% BSA, 0.3% Triton X-100 and 0.01 sodium azide for 30 min at room temperature to block non-specific binding. The sections then were incubated with the following primary antibodies in the blocking solutions overnight at 4 °C α-3 1:200 (Enzolife), α-6 1:200 (Enzolife) or RPT6 1:200 (Enzolife). After washing three times with PBS (5 min each), the sections were incubated with biotinylated anti-rabbit or anti-mouse secondary antibody as appropriate for 60 min at room temperature. The sections were then rinsed three times with PBS (5 min each) and treated with avidin biotin-peroxidase complex (ABC) for 60 min. The immunoreactive signal was revealed by using the chromogen 3, 3'-diaminobenzidine tetrachloride (DAB). After washing in distilled water and then counter-staining with haematoxylin for 40 sec, sections were rinsed with PBS (three times for 5 min), dehydrated with graded concentrations of alcohol (70%, 90%, 100%) and cover-slipped using depex (DPX; BDH) (Dorest, UK) as a mounting medium.

The optimal antibody concentration was chosen after a titration experiment for each antibody via IHC to choose the antibody dilution that gives the best staining with minimum background/non-specific binding.

2.5 Measurement of proteasomal activity

Chymotrypsin- and PGPH-like proteasomal enzyme activities were assayed in the anterior cingulate cortex (Brodmann area 24), the prefrontal cortex (Brodmann area 9) and the parietal cortex (BA40) using synthetic peptide substrates linked to the fluorometric aminomethylcoumarin (AMC).

2.5.1 Preparation of brain tissue homogenate for proteasome assay

The DLB, PDD, AD and control brain tissues were retrieved from –70 °C storage and immediately homogenised in an ice-cold proteolysis buffer (50 mM Tris-HCl, pH 7.4 (containing 5 mM $MgCl_2$, 5 mM ATP and 1 mM dithiothreitol) by ultra turrax for 30 sec. Homogenates were then centrifuged (allegra 64R centrifuge, Beckman) at 19,000 rpm for 15 min at 4 ˚C. The resulting supernatants were placed on ice and used immediately to determine proteasome activity. Determination of total protein content in supernatants was assessed by the Bradford methods (as explained above). According to the protein concentration, each sample was diluted to 1 mg/ml protein concentration with 50 mM of Tris-HCl pH 7.4 proteasome assay buffer containing 5 mM $MgCl_2$, 5 mM ATP, 1 mM dithiothreitol, 10% glycerol, 2 mM phenylmethylsulphonyl fluoride (PMSF), 5% protease inhibitor cocktail set III.

2.5.2 Proteasome enzyme activity assay

Proteasome activity was assessed in post-mortem brain tissue using fluorogenic synthetic peptide substrates [for chymotrypsin-like activity, Suc-Leu-Leu-Val-Tyr-AMC; for PGPH-like activity, Z-Leu-Leu-Glu-AMC] as described previously (Zeng et al., 2005) The two catalytic activities were assessed by their ability to hydrolyse the fluorogenic substrates into highly fluorescent end-products 7-amino-4-methyl-coumarin (AMC). The resulting fluorescence by AMC cleaved from the substrate by the proteasome was then detected by a Devices plate reader at an emission wavelength of 355 nm and an excitation wavelength of 460 nm (Molecular Devices). Measurements were performed in 96 well plates (total volume 100 μl per well) and all samples were assayed in triplicate for both activities.

In brief, brain lysates (100 μl per well) containing 1 mg/ml of protein were incubated with 5 mM chymotrypsin or 1 mM PGPH substrates for 60 min at 37 °C (Table 2-3; (Zeng et al., 2005). In a separate well, lysates were also pre-incubated with 5 mM carbobenzoxyl-leucinyl-leucinyl-leucinal (MG-132; a final concentration of 50 μM for chymotrypsin-like activity) (Zeng et al., 2005) and 150 μM for PGPH-like activity, or 100% DMSO for 30 min at room temperature (Table 2-3). The background fluorescence values obtained by incubating the lysates with the proteasome inhibitors were subtracted from activity values. Proteasomal activity rates are expressed as fluorescence units (FU)/mg protein/hour. The substrate hydrolysis was determined by measuring the fluorescence intensity of the AMC released using a FlexStation 3 (Molecular Devices LTD, UK). The specificity of the proteasomal assay was confirmed by the ability of the proteasome

inhibitor to nearly totally inhibit chymotrypsin-like and peptidylglutamyl-peptide hydrolase (PGPH)-like activities.

Table 2-3: Proteasome inhibitor, substrate volumes and excitation/emission used for proteasome activity assay.

	Chymotrypsin-like activity		**PGPH-like activity**	
	Blank	*Test*	*Blank*	*Test*
Sample solution (1mg/ml)	100 µl	100 µl	100 µl	100 µl
Proteasome inhibitor	1 µl (5 mM MG-132)	1 µl DMSO	3 µl (5 mM MG-132)	3 µl DMSO
Incubation for 60 min at room temperature				
Proteasome substrate	1 µl (5 mM substrate III)	1 µl (5 mM substrate III)	10 µl (1 mM substrate II)	10 µl (1 mM substrate II)
Incubation for 60 min at 37 °C				
Excitation/Emission	380/460	380/460	380/460	380/460

2.5.3 Measurement of proteasome inhibition and dose-inhibition curve

To measure proteasome inhibition in the presence of proteasome inhibitors, stock solutions of MG132 (5 mM dissolved in DMSO; Biomol International, Exeter, UK), AdaAhx3L3VS (0.5 mM dissolved in DMSO) and 1 mM lactacystin (Sigma-Aldrich, UK) were prepared and diluted with DMSO into different concentrations. Inhibition tests contained 100 µL standard human brain tissue (1 mg/ml protein content) and a range of different proteasome inhibitor concentrations (final concentrations are given in the result section for each inhibitor).

2.5.4 Analysis of proteasomal enzyme activity

Proteasomal enzyme activity was determined as an increase of fluorescence reaction products. Chymotrypsin-, trypsin- and PGPH-like proteasomal activity was inhibited by MG-132, and this reading was used as background fluorescence. The difference in the fluorescence intensity of AMC between the presence and absence of inhibitors was taken as proteasomal enzyme activity.

$$Protesomal\ enzyme\ acivity = test\ (without\ inhibitors) - blank\ (with\ inhibitors)$$

2.6 Statistical Analysis

The quantification values were expressed as a ratio of integral of band density in a sample to standard rat brain homogenate, which was run on each blot for standardization purposes. Analysis of the data was carried out using SPSS version 20 (SPSS, Chicago, Illinois, USA). For each brain region, comparisons of the average expression levels of proteins were made between controls and subjects with AD, DLB and PDD. Initially, the normality of the data for each protein in each brain region was determined using the Shapiro–Wilk (SW) test; this test is the most appropriate for data size up to n-2000. Next, the relationships between the protein value and the demographic data (age at death, gender, post-mortem delay (PMD), brain tissue pH and years in storage) were determined using Spearman's rank correlation.

If there was any significant correlation with the demographic data, the protein values were subsequently expressed as residuals (unstandardized) created from the multivariable regression analysis to eliminate the confounding effect of the demographic variables (gender, post mortem delay, age at death and length of brain storage) on the protein values. Briefly, any demographic data found to be correlated with the protein value were entered into a regression analysis using the enter method, and the protein values were entered as dependent variables with the confounding demographic data variables as independent factors. If the demographic data variables were significant predictors for the protein value, then the unstandardized residual from this regression was saved and used as a dependent variable. If any of the demographic data variables were not significant predictors, they were removed from the analysis and the regression was redone without non-significant predictors. The residual variables can be either positive or negative values;

it was necessary to shift all values above 0, usually by adding 1 to all values from the proteins measured by semi-quantitative Western blot analysis. For the proteasome activity, only PGPH-like activity in BA40 was correlated with PMD. The negative values from the residual were up to about -4000; to shift all values above zero, 5000 was added to all values measured for PGPH-like activity in BA40. Multiple linear regressions were followed by transforming the residual protein values into a normal distribution. Because there were so many transformations, log10 and square root were attempted in the same order, but whenever possible, the same operation was used for all data sets in a brain. The differences in protein levels between groups were determined using one-way ANOVA and a Bonferroni post-hoc test or a Kruskal–Wallis ANOVA, followed by a Mann Whitney U test as appropriate, with a significance level of $p < 0.05$. Additional comparisons between protein levels and pathological or clinical scores were undertaken using a one-way ANOVA, a Kruskal-Wallis ANOVA or a Mann-Whitney U test and multiple linear regressions as appropriate. Relationships between the proteasome sub-unit and the synaptic proteins were determined by Pearson product moment (r) and Spearman rank correlation (Rs) with a significance level of $p < 0.01$.

Chapter 3 DEVELOPMENT OF PROTEASOME ASSAY

3.1 Introduction

The proteasome is characterized by three main catalytic activities that differ in their specificities against peptide substrate (Orlowski, 1990, Rivett, 1989). The catalytic activities of the proteasome are located within the two-heptameric β rings. Three of the β subunits (β1, β2, and β5) in each β-ring contain the proteolytic active sites where the proteolysis of the protein occurs. β1 subunit has the PGPH-like activity site cuts preferentially after acidic residues, β2 subunit has the Trypsin-like activity site cuts mainly after basic residues and β5 subunit has chymotrypsin-like activity site responsible for cleaves after hydrophobic residues. Since the proteasome is involved in many important cellular processes, it is not surprising that alteration in the proteasome activity have been implicated in the pathogenesis of a number of diseases. These including: PD, AD diabetes, and a variety of cancer. In the last decade, the proteasome inhibitor Bortezomib (Velcade) was approved in 2003 for the treatment of multiple myeloma and mantle cell lymphoma and since open the way to for the validation of the ubiquitin-proteasome pathway as a potential therapeutic target for the treatment of human disease.

In order to measure the proteolytic activity of the proteasome in cells or tissues, cell lysates or tissue homogenate are often incubated with fluorescently tagged substrates specific for the main components of the proteasome activity. These substrates are composed of three to four amino acid residues peptide with a fluorogenic reporter at the C terminus, most commonly, 7-amino-4methycoumarin (AMC), 2-naphtylamine (NA) and 4-methoxy-2-naphtylamine (MNA). After incubation with tissues homogenate or cell lysates, the proteasome cleave the amide bond between the last amino acid and the fluorogenic

reporter releasing the fluorescence molecule. Therefore, the increase in fluorescence is proportional to the proteasome activity. Proteasome inhibitors such as lactacystin or MG132 are normally used to confirm the specificity of the proteasomal assay, by the ability of the proteasome inhibitor to inhibit the catalytic activities of the proteasome.

As a part of this study to assess the activity of the proteasome in DLB, PDD and AD cases compare to controls, different methods were examined aimed at measuring the main catalytic activities of the proteasome. To choose which proteasome inhibitors could be used for the assay, the effect of different proteasome inhibitors on chymotrypsin-, trypsin- and PGPH-like proteasomal activity was examined.

3.2 Development of the proteasome assay

Fluorogenic peptide substrates were used to measure the proteasome activity. Initially, the crude homogenates prepared for the western blot (see above), stored at -70°C were used for the proteasome assay. Three samples only were used to test the assay. The crude homogenate of these three samples were centrifuged at 19,000 rpm for 15 minutes at 4°C and diluted to 1 mg/ml protein concentration with proteasome assay buffer containing (5 mM $MgCl_2$, 5 mM ATP, and 1 mM dithiothreitol, 2mM phenylmethylsulphonyl fluoride (PMSF), 5% protease inhibitor cocktail set III). Measurements were performed in 96 well plates (total volume 100 µl per well). Brain lysates (100 µl per well) containing 1mg/ml of protein were incubated with 2 µl (5mM trypsin substrate), 1µl (5mM chymotrypsin substrate) or 10 µl (1mM PGPH substrate) for 60min at 37°C. In a separate well brain lysates were pre-incubated with a range of different concentrations of PSI, 5mM

carbobenzoxyl-leucinyl-leucinyl-leucinal (MG-132; a final concentration of 50μM; (Zeng et al., 2005), 20μl (2.5mM adamantine-acetyl-(6-aminohexanoyl)3-(leucinyl)3-vinyl-(methyl)-sulfone (AdaAhx3L3VS;), 10μl 1mM lactacystin or 100% DMSO for 30min at room. The resulting fluorescence by AMC cleaved from the substrate by the proteasome was then detected by a Devices plate reader at an emission wavelength of 355 nm and an excitation wavelength of 460 nm (Molecular Devices). The activities values did not show any different between the blank well (sample + proteasome inhibitor + substrate) and the test well (sample +substrate), also these values were very low, which suggested that the fluorogenic peptide substrates did not undergo the hydrolysis.

The second attempt was to prepare the crude homogenate using 50mM Tris base lysis buffer (pH8.0) containing 1% Triton-X-100, 150mM sodium chloride, 5mM ethylenediaminetetraacetic acid (EDTA), 2mM phenylmethylsulphonyl fluoride (PMSF) and 5% protease inhibitor cocktail set III, followed by exactly the same method as above. This experiment was repeated several times with changing the condition of the samples, for example: using fresh and stored crude homogenate. Again the activity values were very low.

Then ice-cold proteolysis buffer; 50 mM Tris-HCl, pH 7.4 containing (5 mM MgCl2, 5 mM ATP, and 1 mM dithiothreitol, 2mM phenylmethylsulphonyl fluoride (PMSF), 5% protease inhibitor cocktail set III) was used to homogenise the brain tissue. Initially, samples were stored after homogenizations. In this condition, the activity values for both blank and test were low. Then the samples were homogenized, centrifuged, and stored at –20°C for further use. Again the activity values were low. The findings of this process concluded that the crude homogenate should be prepared on the same day of the

experiment, using freshly prepared ice-cold proteolysis buffer (the buffer should prepared at the same day of the experiment).

3.3 Inhibition of the proteasome activities by MG-132

To investigate the effect of MG-132 on chymotrypsin-, trypsin-, and PGPH-like proteasomal activities, a range of MG-132 concentrations (5,000, 1,000, 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.5 nM) were investigated and a concentration-response curve for determining inhibitory concentration (IC50) values was generated.

MG-132 reduced chymotrypsin-, trypsin- and PHPG-like proteasomal activity in a concentration-dependent manner in human brain tissue. According to Figures 3-1, 3-2 and 3-3, the inhibitory concentration 50 (IC50) for MG-123 on chymotrypsin-, trypsin- and PGPA-like activity were 23, 177 and 241 nM, respectively. Efficient inhibition by 90% was achieved by an MG-132 inhibitor concentration of 50 μM and 200 μM on chymotrypsin- and PGPH-like activity, respectively. Only partial inhibition of trypsin-like activity could be achieved using this protocol (Fig 3-2) and therefore only chymotrypsin-like and PGPH-like proteasomal activities were measured using MG-123.

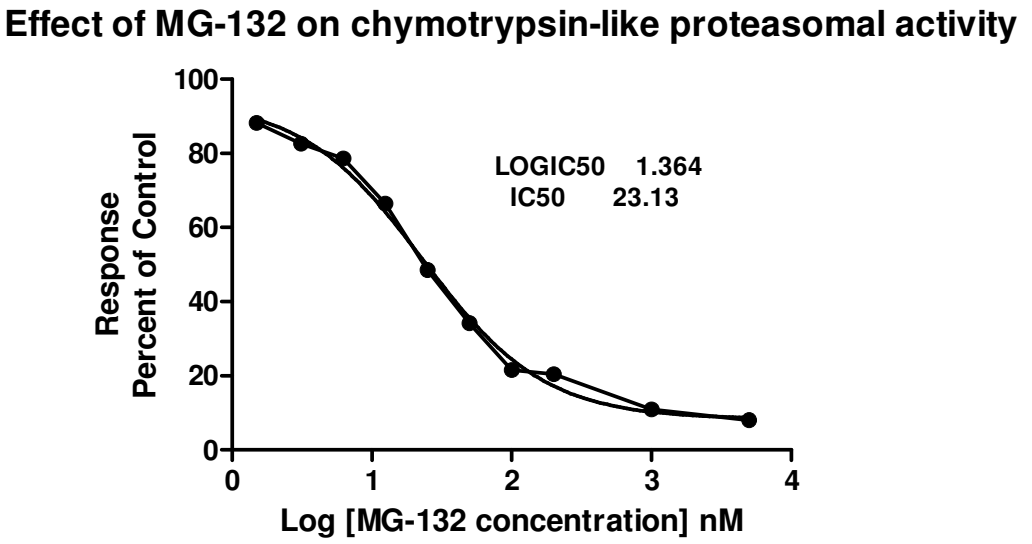


Figure 3-1: Concentration-response curves for the determination of IC50 values on chymotrypsin-like activity in standard human tissue.

A range of MG-132 concentrations (5,000, 1,000, 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.5 nM) were used in standard human tissue to investigate the effect of MG-123 on chymotrypsin-like proteasomal activity. Levels of proteasome inhibition (%) were plotted against the logarithmic concentrations of the proteasome inhibitors to determine the IC50. The IC50 for MG-132 was 23 nM.

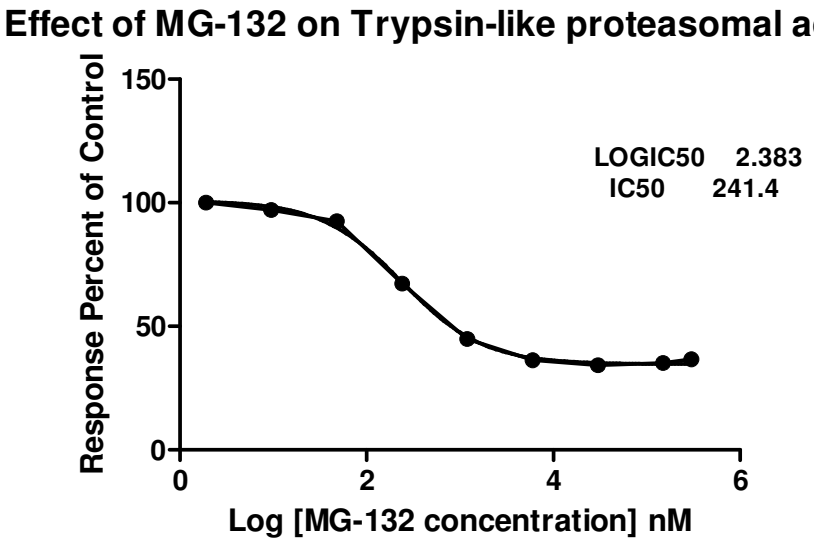


Figure 3-2: Concentration-response curves for the determination of IC50 values on trypsin-like activity in standard human tissue.

A range of MG-132 concentrations (300, 150, 30, 6, 1.2, 0.240, 0.048, μM) were used in standard human tissue to investigate the effect of MG-132 on trypsin-like proteasomal activity. Levels of proteasome inhibition (%) were plotted against the logarithmic concentrations of the proteasome inhibitors to determine the IC50. The IC50 for MG-123 was 241 nM.

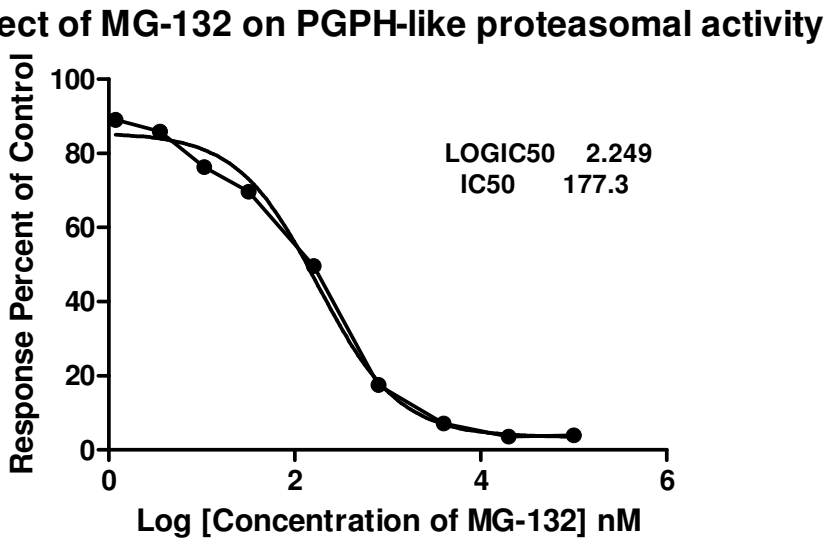


Figure 3-3: Concentration-response curves for the determination of IC50 values on PGPH-like activity in standard human tissue.

A range of MG-132 concentrations (100, 20, 4, 0.8, 0.160, 0.032, 0.0106 μM) were used in standard human tissue to investigate the effect of MG-132 on PGPH-like proteasomal activity. Levels of proteasome inhibition (%) were plotted against the logarithmic concentrations of the proteasome inhibitors to determine the IC50. The IC50 for MG-123 was 177 nM.

3.4 Inhibition of the proteasome activities by proteasome inhibitor AdaAhx3L3VS

In a second approach, the effect of the proteasome inhibitor AdaAhx3L3VS on both trypsin-like and PGPH-like activities was determined. The effect of MG-132 was more pronounced than the effect of AdaAhx3L3VS on both trypsin- and PHPG-like activity. For this reason, MG-132 was used to inhibit both chymotrypsin- and PHPG-like activity to measure the proteasome activity. AdaAhx3L3VS reduced the PGPH-like activity in human brain tissue by 83% when used at 100 μM concentration, compared to 96.1% when using the same amount of MG-132. The IC50 of PGPH-like activities was obtained at 31.6 μM AdaAhx3L3VS, compared to 177 nM MG-132 (Figure 3-4).

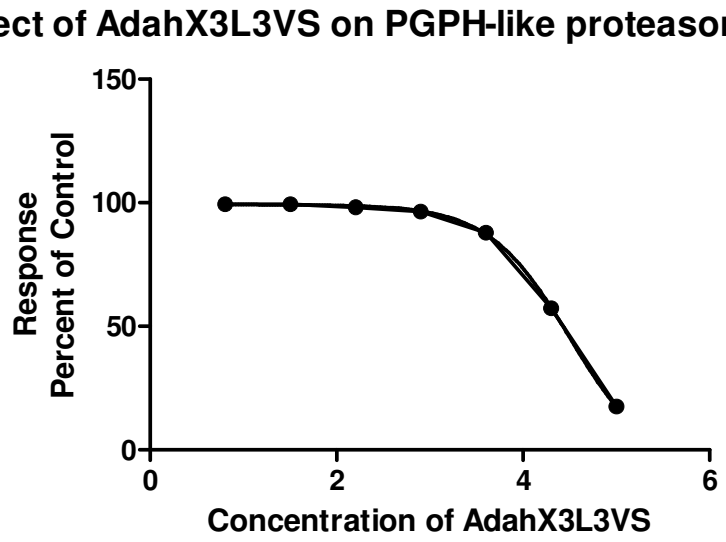


Figure 3-4: Concentration-response curves for the determination of IC50 values on PGPH-like proteasomal activities in standard human tissue.

A range of AdaAhx3L3VS concentrations (100, 20, 4, 0.8, 0.160, 0.032, 0.0064, μM) were used in standard human tissue to investigate the effect of AdaAhx3L3VS on PGPH-like proteasomal activities. Levels of proteasome inhibition (%) were plotted against the logarithmic concentrations of the proteasome inhibitors to determine the IC50. The IC50 for AdaAhx3L3VS was 31 μM.

3.5 Inhibition of the proteasome activities by lactacystin

Addition of lactacystin, a specific inhibitor of the 26S proteasome, at various concentrations in the standard human tissue was significantly less affected in both chymotrypsin-like activity and PGPA-like activity compared to trypsin-like activity. Lactacystin inhibited trypsin-like activity with an of IC50 4.5 μM. In contrast, much lower

doses of MG-132 were required to obtain 50% inhibition of the trypsin-like activity with IC50 241 nM (Figure 3-5)

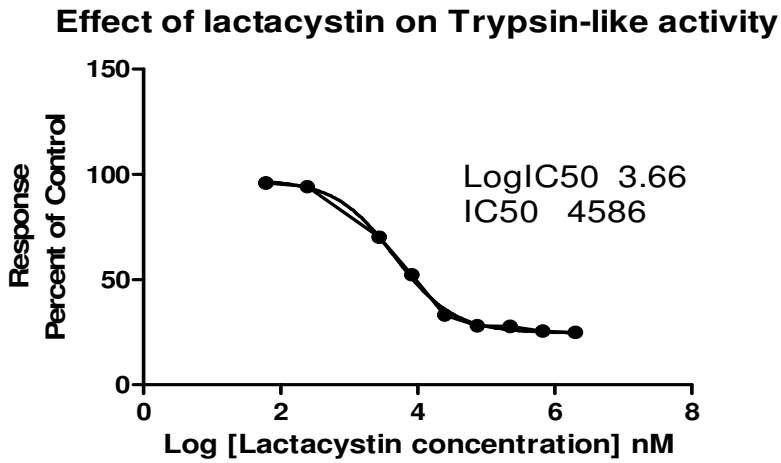


Figure 3-5: Dose-response curves for the determination of IC50 values on trypsin-like proteasomal activities in standard human tissue.

A range of lactacystin concentrations (2,000, 666, 222, 74.07, 24.69, 8.23, 2.74, 0.244, 0.061 μM) were used in standard human tissue to investigate the effect of lactacystin on trypsin-like proteasomal activities. Levels of proteasome inhibition (%) were plotted against the logarithmic concentrations of the proteasome inhibitors to determine the IC50. The IC50 for lactacystin was 4.5 μM.

3.6 Discussion

The results illustrated some of the difficulties of measuring the activities of the proteasome and suggest that preparation of the tissue homogenate and the assay buffer should be carried out at the same day of the experiment.

From the three selective inhibitors (MG-132, lactacystin and AdaAhx3L3VS), MG-132 was able to maximally inhibit more than 90% of the chymotrypsin- and PGPA-like activity and was selected as background reading for the proteasome assay in this study. Two different concentrations were selected for each activity based on the concentration that inhibited more than 90% of each proteasomal activity and the most frequent used in published studies. The maximum inhibition for the trypsin-like activity was 70%, even when adding a very high concentration of MG-132. Therefore, the concentrations of MG-132 were 5 μM, and 20 μM in the proteasomal enzyme activity assay for chymotrypsin- and PGPH-like proteasomal activities in this thesis.

The inhibitory concentration 50 (IC50) of MG-132 for each enzyme activity was used as a standard in each plate for normalisation. This provides confidence as to the standardisation of the assay: any day-to-day variability could be controlled.

Proteasomal enzyme activity was determined as an increase of fluorescence reaction products. Chymotrypsin- and PGPH-like proteasomal activity was inhibited by MG-132

and this reading was used as background fluorescence. The difference in the fluorescence intensity of AMC between the presence and absence of inhibitors was taken as proteasomal enzyme activity. Results illustrated that none of the three inhibitors used could completely inhibit the trypsin-like activity of the proteasome. For this reason, only two activities of the 20S proteasomes [chymotrypsin-like, and peptidylglutamyl-peptide hydrolase (PGPH)-like activities] were analysed in this thesis.

Chapter 4 Characterisation of patient samples group and transformation of statistical data from semi-quantitative Western blotting protein values and proteasome activity measurement

4.1 Introduction

As discussed previously in Chapter 2 Section 2.1, post-mortem brain tissue was obtained from several sources. It is very important for post-mortem human brain specimens to be of high quality and well characterised. Each autopsy sample is affected by a combination of demographic (age at death and gender) and post/ante -mortem factors (agonal state and PMD). Aging is associated with many changes in brain structure and function (Raz and Rodrigue, 2006). Gender-specific changes can be related to sex hormones (Kelly et al., 1999); such as testosterone or oestrogen, or to the expression of sex-linked genes (Carruth et al., 2002).

Tissue-specific information such as brain pH and PMI are as important as patient demographics. For post-mortem human brain research, brain pH is an important indicator for tissue quality (Monoranu et al., 2009, Stan et al., 2006), it can have serious consequence for genetic and biochemical measurement. For example some enzymes such as phosphate-activated glutaminase and glutamic acid decarboxylase were correlated with tissue pH in agonal control cases and also the activity of these enzymes were reduced by in vitro acidification (Yates et al., 1990). In contrast, brain pH does not appear to significantly affect brain proteins or receptor binding (Harrison et al., 1995, Kornhuber et al., 1988). However, brain pH is a major factor for mRNA integrity (Kingsbury et al., 1995, Li et al., 2004). Modification of brain pH may be the result of prolonged agonal states, leading to hypoxia and an increase in brain tissue lactate (Hardy et al., 1985).

PMD can be defined as the time course between death of the patient and removal of tissue for study at autopsy. It is an important factor in studying post-mortem brain: although

many studies reported no relationship between PMD and mRNA quality and mRNA was reported to be stable for up to 24 hours (Cummings et al., 2001, Heinrich et al., 2007, Miller et al., 2004, Schramm et al., 1999), Lipska showed a relationship between PMD and mRNA quality (Lipska et al., 2006).

Since demographic and post-/ante-mortem factors may affect protein levels, controls, DLB, PDD and AD were matched as closely as possible for age, PMD and pH. In addition, any relationships found between protein measurements and demographics/post-mortem factors were controlled for via the creation of unstandardized residuals.

Clinicians generally use various tools and tests to assess and diagnose each dementia case. Diagnosis of DLB was made when cognitive impairment or hallucinations were present before or within one year of onset of parkinsonism. Classification of PDD was made when parkinsonism preceded dementia by more than a year (McKeith et al., 2005). AD cases were selected on the basis of meeting the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria for a diagnosis of probable or definite AD, DLB according to international consensus criteria (McKeith et al., 2005) and PDD according to Movement Disorders Society criteria (Emre et al., 2007).

Assessment and measurement of cognitive function, behavioural changes and histopathological analysis are all important to help understand the disease process. The availability of all this information, in addition to the demographic nature and post-/ante-mortem factors allowed the development of a large database that could be used for further

analysis (see Appendix I, II, III for demographic nature, post/ante -mortem factors, clinical and pathological semi-quantitative scores).

4.2 Patient demographics

Demographic variables for the study cohort are summarised in Table 4-1, and fully detailed on a case-by-case basis in the (Appendix I). There were no significant differences in PMD, tissue pH or gender between diagnostic groups. AD patients were significantly older at death (one-way ANOVA $F(3;126) = 6.044$, $p = 0.001$) than controls ($p = 0.001$) or patients with DLB ($p = 0.008$) or PDD ($p = 0.001$).

Table 4-1: Patient demographic data.

Diagnosis	Gender (M/F) %	Age at death (mean)	PMD (mean hours)	pH (mean)
Control (25)	60/40	79.7 ± 7.6	39.1 ± 22.9	6.47 ± 0.28
PDD (34)	53/47	79.9 ± 6.0	33.5 ± 15.6	6.44 ± 0.34
DLB (55)	58/42	81.7 ± 6.5	41.3 ± 28.0	6.37 ± 0.41
AD (16)	31/69	88.0 ± 7.8	34.9 ± 23.9	6.30 ± 0.33

Data are means ± SD age in years; PMD = post-mortem delay; DLB = dementia with Lewy bodies; PDD = Parkinson's disease dementia; AD = Alzheimer's Disease. PMD and pH were not significantly different between the groups in the one-way analysis of variance (ANOVA) ($P < 0.05$)

4.3 Pathological and Clinical data

4.3.1 Pathological data

The average scores from semi-quantitative analysis of Aβ, tau and α-synuclein in BA9, BA24 and BA40 for PDD, DLB, AD and controls are shown in Table 4-2 and Figure 4-1. The data are presented as means ± standard deviations. The control cases showed an absence of α-synuclein pathology. There was little, if any, tau pathology detected in some cases, while Aβ pathology was more common in all three cortical regions studied. PDD cases were characterised by both α-synuclein and Aβ pathology and by less tau pathology across all three brain regions. In DLB cases, the mean values for all three pathology scores were greater than PDD in all three cortical regions studied. AD cases showed severe plaque and tangle pathology in BA9 and BA40 but not in BA24, while α-synuclein pathology was sparse.

Table 4-2: Mean pathology scores for senile plaques, neurofibrillary tangles and α-synuclein inclusions in three cortical areas in PDD, DLB, AD and control cases.

	Plaque BA9	**n**	**Plaque BA24**	**n**	**Plaque BA40**	**n**
Control	0.35 ± 0.65	23	0.29 ± 0.78	21	0.39 ± 0.78	23
PDD	1.39 ± 1.10	33	0.97 ± 0.83	34	1.23 ± 0.91	31
DLB	1.68 ± 1.07	53	1.28 ± 1.07	50	1.47 ± 1.09	55
AD	2.81 ± 0.54	16	1.47 ± 1.06	15	2.63 ± 0.72	16
	Tangles BA9	**n**	**Tangles BA24**	**n**	**Tangles BA40**	**n**
Control	0.17 ± 0.39	23	0.10 ± 0.30	21	0.04 ± 0.21	23
PDD	0.53 ± 0.61	32	0.53 ±0.63	34	0.48 ± 0.72	33
DLB	0.93 ± 0.84	55	1.23 ± 1.02	52	0.98 ± 0.89	55
AD	2.56 ± 0.51	16	1.44 ± 1.26	16	2.81 ± 0.40	16
	α-synuclein BA9	**n**	**α-synuclein BA24**	**n**	**α-synuclein BA40**	**n**
Control	0.00	23	0.00	21	0.00	20
PDD	0.79 ± 1.06	34	1.85 ± 1.04	34	0.59 ± 1.77	34
DLB	1.62 ± 1.05	52	2.28 ± 0.92	54	1.39 ± 0.98	54
AD	0.13 ± 0.34	16	0.31 ± 0.70	16	0.13 ± 0.34	16

Plaque, tangle and α-synuclein pathology were assessed on a semi-quantitative scale as described in Materials and Methods. Data are presented as means ± standard deviations from patient scores. Data taken from (Howlett et al., 2014).

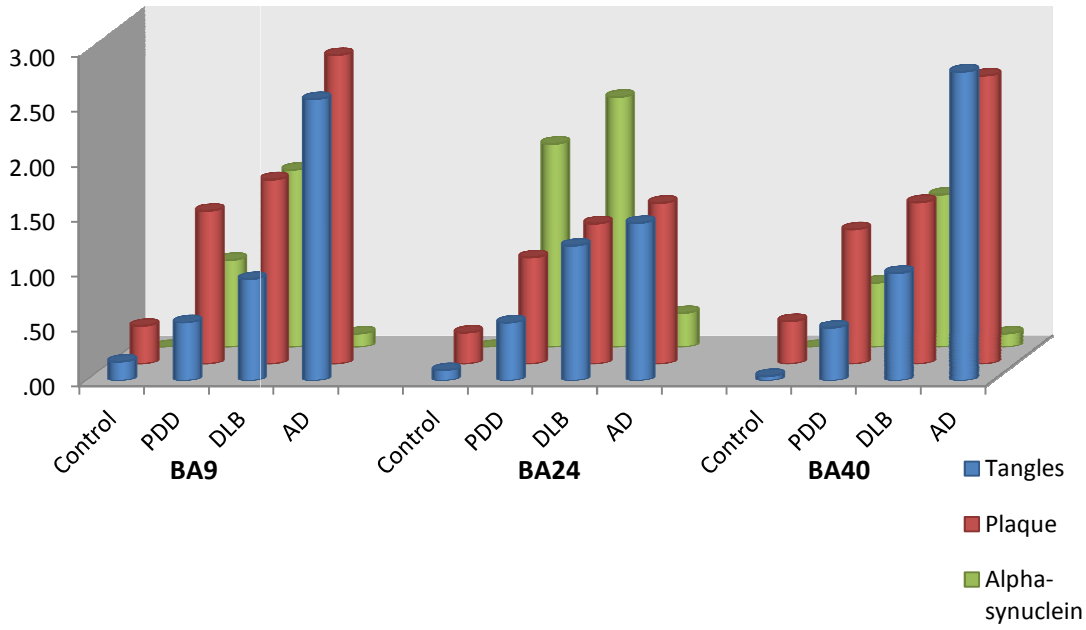


Figure 4-1: Average scores of semi quantitative analysis of pathology scores in control, PDD, DLB and AD brain

Average scores of semi quantitative analysis of pathology scores Aβ, tau and α-synuclein in BA9, BA24, and BA40 for PDD, DLB, AD (Graphical presentation of the values in Table 4-2).

4.3.2 Clinical data

The Mini-Mental State Examination (MMSE) (Folstein et al., 1975) was used to measure the severity of cognitive impairment several times from the time of dementia diagnosis, usually over the 8 to 10 years prior to death (MMSE-decline). The MMSE scores ranged from 0-30. In this project, four categories of cognitive impairment were used for classification purposes: 'unimpaired cognition' for clinical control cases, 'mildly impaired cognition without dementia' (score of 25-30), 'mildly impaired cognition with dementia' (score of 17-24), 'moderately impaired cognition' (score of 10-16) and 'severely impaired cognition' (score of 9 or less). More details on classification into these categories can be seen on (Chapter 2, Section 2.1).

Data were available for most of the dementia cases, but not for the controls. However clinical notes from all controls were examined in detail by the brain banks supplying tissue and confirmed that there was no evidence of cognitive impairment. As discussed previously in Chapter 2, Section 2.1, MMSE scores were divided into five categories (see also Whitfield et al 2014), and the control cases were deemed to have 'unimpaired cognition' (See Chapter 2, Section 2.1 for further details). Table 4-3 and Figure 4-2 shows the distribution of cases among these cognitive impairment categories according to clinical diagnosis. More than 50% of the AD cases were classified as having severe cognitive impairment, whereas none had MCI. The rest of the AD cases were distributed between mild and moderate impairment. Among the PDD cases, 34% were classified with moderate and 37.5% with severe cognitive impairment. The majority of DLB cases were categorised as moderate, with a few cases of MCI (Figure 4-2).

Semi-quantitative analysis of behavioural data for agitation, depression, hallucinations and persecution are summarised in Table 4-4 and Figure 4-3 as means ± standard deviations. For all individuals with dementia, scores from standardised tests or semi-structured interviews were used to derive each behavioural symptom score; principally, this was the Neuropsychiatric Inventory (NPI) mood item on a scale of 0 to 3, where 0 was none, 1 was intermittent and mild, 2 was moderate (intermittent but significant) and 3 was persistent and/or severe. For depression, different tests were used as explained in Chapter 2 Section 2.1. Figure 4-3 illustrates that AD had relatively high mean scores for agitation compared to both DLB and PDD. Mean scores for depression were very low in DLB cases, as the majority of the cases were classified as mild, with only one case each of moderate and severe impairment. PDD and AD cases were distributed between different depression scoring groups, and the mean values for both PDD and AD differed significantly from those of DLB cases. Hallucination was more common in DLB and PDD cases than in the AD group. Mean scores for persecution did not differ significantly between the various dementia diagnostic groups. All dementias had significantly higher scores (depression, persecution, hallucination, and agitation) than control subjects.

Table 4-3: Percentage of cases according to cognitive impairment category.

	control	MCI	mild	moderate	severe
control	100	0	0	0.0	0.0
PDD	0	44.4	29.4	37.9	40.0
DLB	0	55.6	52.9	48.3	33.3
AD	0	0	17.6	13.8	26.7
Total	100	100	100	100	100

The Mini-Mental State Examination (MMSE) was used to measure the severity of cognitive impairment. (Control) 'Unimpaired cognition', (MCI) 'mildly impaired cognition without dementia', (mild) 'mildly impaired cognition with dementia', moderate 'moderately impaired cognition' (severe) 'severely impaired cognition'

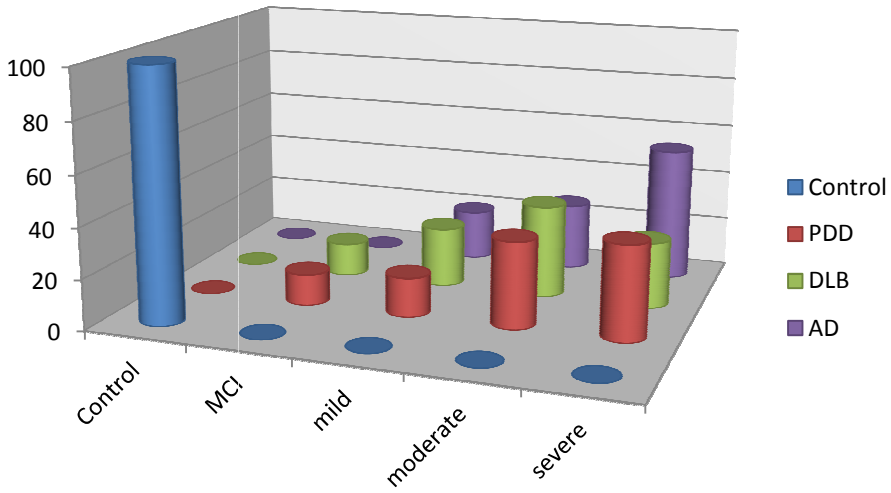


Figure 4-2: Percentage of cases according to cognitive impairment category.

The frequency of each cognitive impairment category within each clinical diagnostic group was calculated and represented graphically. For MMSE classification purposes (see section x) (Graphical presentation of the values in Table 4-3).

Table 4-4: Mean clinical scores for agitation, depression, persecution and hallucination in PDD, DLB, AD and control cases.

	Agitation	n	Depression	n	Persecution	n	Hallucination	n
control	0.0 ± 0.0	25	0.0 ± 0.0	25	0.0 ± 0.0	25	0.0 ± 0.0	25
PDD	1.06 ± 1.11	33	1.30 ± 1.32	33	0.85 ± 0.93	33	1.24 ± 1.03	33
DLB	0.88 ± 1.07	24	0.55 ± 0.73	29	1.04 ± 1.08	24	1.23 ± 1.19	30
AD	1.6 ± 1.18	15	1.60 ± 1.18	15	1.27 ± 1.28	15	0.4 ± 0.73	15

Agitation, depression, persecution and hallucination were assessed via a semi-quantitative scale as described in Materials and Methods. Data are presented as means ± standard deviations.

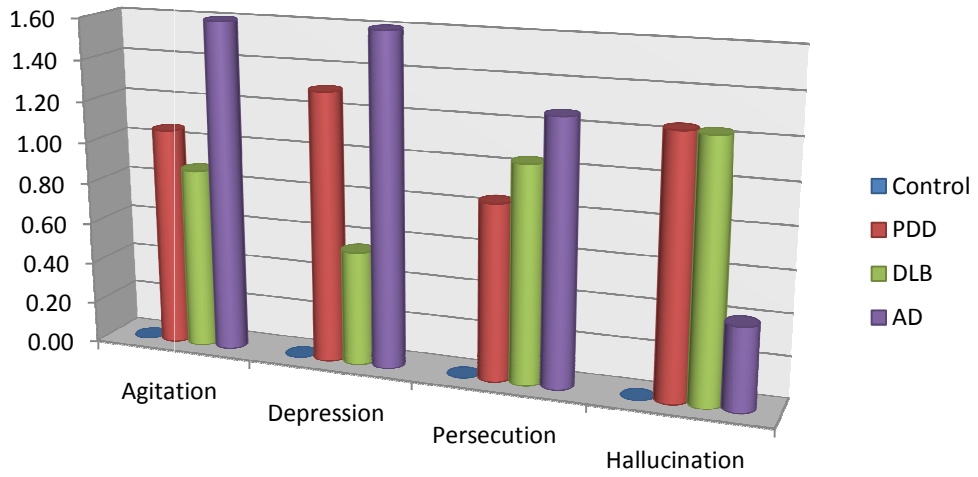


Figure 4-3: Mean scores for semi-quantitative assessment of behavioural symptoms in control, PDD, DLB and AD brain.

Agitation, depression, persecution and hallucination were assessed via a semi-quantitative scoring scale (as described in Materials and Methods) and differed significantly between diagnostic groups (Kruskal–Wallis $\chi^2(3) = 26.179, 34.945, 21.665, 31.081$, $p < 0.001$). The Mann–Whitney U-test showed that individuals with DLB (n = 29) were characterised by significantly lower depression scores than those with PDD (n = 33, p = 0.007) or AD (n =

15, p = 0.004). PDD and DLB subjects had significantly higher mean scores for hallucination compared to the AD group (Mann–Whitney U: $p < 0.05$). The mean values for agitation and persecution were not significantly different between the dementia groups (PDD, DLB and AD), whilst, as expected, all dementias had significantly higher agitation, depression, persecution, and hallucination scores than control subjects (n = 24, Mann–Whitney U, $p < 0.001$) (Graphical presentation of the values in Table 4-4).

4.4 Transformation of data from semi-quantitative Western blotting protein values and proteasome activity measurement

Prior to comparative analysis, all semi-quantitative Western blot scores were analysed for normality and (unstandardised) residuals calculated using linear regression. This had the effect of removing any effect of demographic data analysis (as explained in detail in Chapter 2 Section 2.6). All the values used in the comparative analysis and figures represent the final output after normality and the residual creation (see Appendix V, VI, VII, VIII for the initial protein quantification values, ratio and the final output). Some of these final residual variables contained negative values. To allow easy visual comparison of the data when depicted as scatter plots, the decision was taken to translate any variables that contained negative values by an amount sufficient to ensure the largest negative value became positive. An advantage of this is that the data points do not risk being obscured by their original spread across the x axis, and the mean bars do not fall on the X axis. To avoid any repetitive and complexity in the figure caption, the statistical transformation of semi-quantitative Western blotting values for each protein and the proteasome activity in each brain region is explained in section 4.4.1.

4.4.1 Transformation of data from semi-quantitative Western blotting protein values for RPT6, α-3, α-6, LAMP1, cathepsin-D, and proteasome activity measurement in BA9

Age at death and brain tissue pH significantly predicted RPT6 and α-3 values in BA9 (R^2 = 0.032, beta = -0.178, p = 0.049 and R^2 = 0.068, beta = -0.261, p = 0.003, respectively). Therefore, regression analysis was used to create a residual value (unstandardised) for RPT6 and α-3 to remove the effects of age at death and brain tissue pH. This was applied to all RPT6 and α-3 values from all cases.

Only RPT6, α-3, LAMP1 and cathepsin D protein values in BA9 were not normally distributed. The protein values for RPT6, α-3, LAMP1 and cathepsin D were transformed by taking logarithms.

LAMP1, α-6, cathepsin-D values, and chymotrypsin-like and PGPH-like activity were not correlated with any of the demographic data (age at death, gender, PMD, brain tissue pH and years in storage) and therefore no residuals were calculated.

Table 4-5: Correlations between biochemical and demographic data in BA9.

BA9	Age at death	Gender	Brain tissue pH	Post-mortem delay (PMD)
RPT6	**p<0.05**	None (p>0.05)	None (p>0.05)	None (p>0.05)
α-3	None (p>0.05)	None (p>0.05)	**p<0.05**	None (p>0.05)
α-6	None (p>0.05)	None (p>0.05)	None (p>0.05)	None (p>0.05)
LAMP1	None (p>0.05)	None (p>0.05)	None (p>0.05)	None (p>0.05)
CathepsinD	None (p>0.05)	None (p>0.05)	None (p>0.05)	None (p>0.05)
Chymotrypsin-like activity	None (p>0.05)	None (p>0.05)	None (p>0.05)	None (p>0.05)
PGPH-like activity	None (p>0.05)	None (p>0.05)	None (p>0.05)	None (p>0.05)

Spearman's rank correlation was used to determine the effect of demographic factors (age at death and gender) and variables associated with the tissue donation process (pH, PMD and years in storage) on the protein values obtained from semi-quantitative Western blotting.

4.4.2 Transformation of data from semi-quantitative Western blotting protein values for RPT6, α-3, α-6, LAMP1, cathepsin-D, and proteasome activity measurement in BA40

RPT6 values in BA40 were significantly correlated with gender, PMD and age at death. Only PMD and age at death significantly predicted RPT6 values in BA40 (R^2 = 0.141, beta = -0.201/-0.202, p = 0.049 and R^2 = 0.068, beta = -0.261, p = 0.016/0.026). Therefore, regression analysis was used to create a residual value (unstandardised) for RPT6 in order to remove the effects of both PMD and age at death.

Age at death significantly predicted α-6 values in BA40 (R^2 = 0.037, beta = -0.192, p = 0.032); regression analysis was used to create a residual value (unstandardised) for α-6 in order to remove the effect of age at death. This was applied to all α-6 values from all cases.

PMDs were also significantly predicted by PGPH-like activity values measured using a synthetic peptide substrate in BA40 (R^2 = 0.137, beta = -0.37, p = 0.015). Regression analysis was used to create a residual value (unstandardised) for PGPH-like activity in order to remove the effect of PMD. This was applied to all PGPH-like activity values from all cases. The negative values from the residual were as low as -4000; to shift all data above zero, a value of 5000 was added to all measurements representing PGPH-like activity.

LAMP1, α-3, cathepsin-D values and chymotrypsin-like activities did not correlate with any of the demographic data (age at death, gender, PMD, brain tissue pH and years in storage).

The protein values for RPT6, α-3 and LAMP1 and both activities (chymotrypsin- and PGPH-like activity) were transformed by taking logarithms. After taking the logarithms for chymotrypsin-like activity values, it was necessary to multiply all values by 500 to allow easy visual comparison of the data when comparing with other brain region BA9 and BA24. The range of the chymotrysin-like activity in BA9 and BA24 was around 500-1500 and in BA40 3.1-3.9 after the logarithms. By multiplying all the values from BA40 by 500 we will get the same range to BA9 and BA24.

Table 4-6: Correlations between biochemical and demographic data in BA40.

BA40	Age at death	Gender	Brain tissue pH	Post-mortem delay (PMD)
RPT6	**p<0.05**	**p<0.05**	None (p>0.05)	**p<0.05**
α-3	None (p>0.05)	None (p>0.05)	None (p>0.05)	None (p>0.05)
α-6	**p<0.05**	None (p>0.05)	None (p>0.05)	None (p>0.05)
LAMP1	None (p>0.05)	None (p>0.05)	None (p>0.05)	None (p>0.05)
Cathepsin D	None (p>0.05)	None (p>0.05)	None (p>0.05)	None (p>0.05)
Chymotrypsin-like activity	None (p>0.05)	None (p>0.05)	None (p>0.05)	None (p>0.05)
PGPH-like activity	None (p>0.05)	None (p>0.05)	None (p>0.05)	**p<0.05**

Spearman's rank correlation was used to determine the effect of demographic factors (age at death and gender) and variables associated with the tissue donation process (pH, PMD and years in storage) on the protein values obtained from semi-quantitative Western blotting.

4.4.3 Transformation of data from semi-quantitative Western blotting protein values for RPT6, α-3, α-6, LAMP1, cathepsin-D, and proteasome activity measurement in BA24

PMDs significantly predicted RPT6 values in BA24 (R^2 = 0.035, beta = -0.186, p = 0.044). Therefore, regression analysis was used to create a residual value (unstandardised) for RPT6 in order to remove the effect of PMD. This was applied to all RPT6 values from all cases. Brain tissue pH significantly predicted α-3 values in BA24 and in BA9 (R^2 = 0.035, beta = -0.186, p = 0.044). Thus, regression analysis was used to create a residual value (unstandardised) for α-3 in order to remove the effect of brain tissue pH. α-6, LAMP1, cathepsin-D values and both chymotrypsin-like and PGPH-like activity did not correlate with any of the demographic data (age at death, gender, PMD, brain tissue pH and years in storage).

RPT6 and α-6 were normalised by taking logarithms. Square root transformation was used for α-3 protein values because transforming using logarithm did not normalise the data. LAMP1, cathepsin-D and proteasome activity were normally distributed

Table 4-7: Correlations between biochemical and demographic data in BA24.

BA24	Age at death	Gender	Brain tissue pH	Post-mortem delay (PMD)
RPT6	None (p>0.05)	None (p>0.05)	None (p>0.05)	**p<0.05**
α-3	None (p>0.05)	None (p>0.05)	**p<0.05**	None (p>0.05)
α-6	None (p>0.05)	None (p>0.05)	None (p>0.05)	None (p>0.05)
LAMP1	None (p>0.05)	None (p>0.05)	None (p>0.05)	None (p>0.05)
Cathepsin D	None (p>0.05)	None (p>0.05)	None (p>0.05)	None (p>0.05)
Chymotrypsin-like activity	None (p>0.05)	None (p>0.05)	None (p>0.05)	None (p>0.05)
PGPH-like activity	None (p>0.05)	None (p>0.05)	None (p>0.05)	None (p>0.05)

Spearman's rank correlation was used to determine the effect of demographic factors (age at death and gender) and variables associated with the tissue donation process (pH, PMD and years in storage) on the protein values obtained from semi-quantitative Western blotting.

4.5 Discussion

The use of post-mortem tissue has yielded many important insights into the pathology of different types of dementia. However the quality and handling of samples is a key consideration and can be a limiting factor in the success and value of biochemical research. To ensure the reliability of obtained data, all factors related directly or indirectly to the disease, to demography and to the quality of the tissue, such as post-mortem factors, should be documented analysed and taken into account at interpretation of datasets.

All cases were prospectively assessed by experienced clinicians using validated clinical rating instrument. Diagnosis of DLB was made when the dementia and motor symptoms begin in the same year, or the cognitive symptoms started before the motor symptoms. Diagnosis of PDD was made when the motor symptoms had been present for one year or more before the onset of dementia (McKeith et al., 2005). AD cases were selected based on the Braak stage to which they were assigned by V/VI and the Consortium to Establish Registry criteria (CERAD) for Alzheimer's disease diagnosis of probable or definite (Braak et al., 1998, Mirra et al., 1991), DLB according to international criteria (McKeith et al., 2005), PDD according Movement Disorder Society criteria (Emre et al., 2007). Neuropathological assessment was performed according to standardised neuropathological scoring/grading systems, including Braak staging, Consortium to Establish a Registry for Alzheimer's Disease (CERAD) scores, Newcastle/McKeith Criteria for Lewy body disease, National Institute on Aging - Alzheimer's Association (NIA-AA) guidelines and phases of amyloid-β (Aβ) deposition (Aβ-phases) (Braak et al., 2006, McKeith et al., 2005, Mirra et al., 1991, Montine et al., 2012, Thal et al., 2002). For more details about the cohort see (Chapter 2 Section 2.1).

There were no significant differences in PMD, tissue pH or gender between diagnostic groups. AD patients were significantly older at death than controls or patients with DLB and PDD, which was due to the selection of AD cases with pure AD pathology in order to avoid overlap with the Lewy body pathology. Prior to comparative analysis, all values obtained from semi-quantitative Western blot were subjected to further transformation to remove any effect of demographic and post-mortem factors.

As expected, the data indicated no significant tau or α-synuclein pathology among the control cases. However, some of the control cases were categorised as having predominantly non-AD type diffuse plaques (without accompanying significant tau-pathology), which could be related to the pathological effects of aging and did not justify the diagnosis of AD. Several previous studies reported pathological changes related to AD, PD or cerebrovascular disease (CVD) in post-mortem brain tissue from normal controls without any signs of cognitive impairment (Duyckaerts et al., 2009, Jellinger and Attems, 2012, O'Brien et al., 2009). Although NFT (Nelson et al., 2012, Nelson et al., 2009, Nelson et al., 2007) and synapse loss (Scheff and Price, 1993, Scheff et al., 2007, Scheff et al., 2006) are the best correlates for cognitive impairment, AD-related pathology has been reported in non-demented controls (Arriagada et al., 1992b, SantaCruz et al., 2011). Aβ pathology was found to be frequently with only minimal to mild neuritic changes corresponding to Braak tau stages 0–IV (SantaCruz et al., 2011).

The distribution of α-synuclein inclusions and Aβ plaques in PDD cases were lower than DLB, which is consistent with previous reports of greater Lewy body pathology and Aβ plaques in the temporal lobe in DLB compared to PDD (Harding and Halliday, 2001).

In the latter study, NFTs were rare in both DLB and PDD Studies have also suggested that DLB cases showed greater tendency than PDD for cortical Lewy body pathology (Iseki, 2004, Richard et al., 2002), although an earlier study (employing small group numbers) reported the same degree of LB pathology in DLB and PDD (Harding and Halliday, 2001).

In agreement with previous studies, DLB cases in the present study showed a degree of AD-related pathology in addition to α-synuclein pathologies. The level of AD pathology in DLB was found to be greater than that in PDD cases, which is in accordance with previous comparisons of AD-related pathology between DLB and PDD (Halliday et al., 2011b, Jellinger and Attems, 2006). Dementia and cognitive decline in Lewy body dementia were found to be better correlates for AD-related pathology in comparison to α-synuclein pathologies (Halliday et al., 2011a, Merdes et al., 2003). However, other studies found that cognitive function was associated with Lewy body pathology and was independent of amyloid and tau pathology in DLB (Horvath et al., 2013).

The AD patients likewise showed severe plaque and tangle pathology, but a fairly sparse occurrence of Lewy bodies. This is due to the selection criteria, only AD cases with Braak V/VI where included to the study for comparative purposes.

According to clinical criteria for diagnosis of dementia with Lewy bodies, hallucination is one of the core features. Individuals with DLB/PDD are much more likely than those with AD to experience visual hallucination (Tiraboschi et al., 2006, Bjoerke-Bertheussen et al., 2012, McKeith et al., 2005) and, hallucination was more common in DLB/PDD compared to AD groups in the present study.

There is a debate as to the relative prevalence of depression in these dementias; studies show that the prevalence of depressive symptoms in DLB is higher than in AD (73% compared with 56%) and is similar to that of PD (Fritze et al., 2011b). The present results indicate that depression was more frequent in AD and PDD groups compared to DLB. This may reflect the small number of cases in each group compared to clinical studies. However, another comprehensive study on autopsy-confirmed diagnosis failed to show differences in depressive symptomatology between DLB and AD (Rockwell et al., 2000b).

With the exception of the AD group, most of the cases in each group were classified as having moderate and severe cognitive impairment, and all groups included some proportion of cases with mild cognitive impairment and MCI. In contrast, most cases in the AD group were categorised as having severe impairment and none were diagnosed with MCI.

Chapter 5 Expression levels of proteasome sub-units and proteasome activity in human post-mortem brain from people with DLB, PDD, AD and age-matched controls

5.1 Introduction

The UPS is a cellular system responsible for removing and degradation unwanted proteins (a detailed overview of the UPS is presented in chapter 1 section 1.9). Dysfunction of the 26S proteasome has been increasingly recognised as playing a fundamental role in the pathogenesis of many neurodegenerative disorders (for review see (Dennissen et al., 2012, Paul, 2008) and Chapter 1 Section 1.9.4 for more details on the implication of the UPS in neurodegenerative disorders). Neurodegenerative disorders share a common feature which is the accumulation of misfolded proteins in the form of insoluble protein aggregates or inclusion bodies. Each of these aggregates has a specific protein component depending on the disease, such as α-synuclein in Lewy bodies, the pathological hallmark of LBD. In addition, ubiquitin has been identified as a component of these inclusion bodies in many neurodegenerative disorders (Alves-Rodrigues et al., 1998), suggesting that the impairment of the UPS is involved in inclusion body formation, including Lewy bodies.

Several lines of evidence support the involvement of the UPS in Lewy body diseases. Post-mortem studies of subjects with PD have shown a reduction in proteasomal activity in the substantia nigra (McNaught et al., 2003). Preliminary studies have also identified proteasomal abnormalities in PDD/DLB (MacInnes et al., 2008) however this requires confirmation in a larger group. Furthermore, experimentally, it is possible to inhibit proteasome activity, which leads to the accumulation of ubiquitinated proteins (Figueiredo-Pereira et al., 1994) and α-synuclein aggregation (Dyllick-Brenzinger et al., 2010, Paxinou et al., 2001). Finally, aggregated α-synuclein may inhibit the UPS directly (Snyder et al., 2003).

Considering the current evidence, we aimed to investigate the expression level of the proteasome subunits and relevant proteasome activities in LBD and AD patients in comparison to controls. For this study, we had more than 130 available cases (DLB, PDD, AD, and controls), for which clinical and pathological data were available. Synaptic biochemistry data on zinc transporter 3, synaptophysin and synphilin were also available from other projects, undertaken by Dr Julie Vallortigara and Dr David Whitfield (both Wolfson CARD), who studied the same cases. Such data is included to explore the potential relationship between changes in proteasome function and components of the synaptic biochemistry (the role of the UPS in synaptic function is presented in Chapter 1 Section 1.9.3.3).

In order to establish a general overview of the proteasome in LBD, sub-units of different functions were chosen for investigation. This included two α sub-units (α3 and α6) of the 20S proteasome and one sub-unit from the 19S regulatory complex (RPT6). The α-subunits define a gated channel leading into the proteolytic chamber (Groll et al., 2000, Groll et al., 1997). The centre of the 20S proteasome is closed by several α-subunits including (α2, α3 and α4) which block the access to the proteolytic chamber (Groll et al., 2000). α-3 is one of the main subunits involved in gating of the proteasome (Groll et al., 2000). The gates of the proteasome remain closed without an activator (such as 19S regulator complex) and block the free access.

RPT6 expression levels were considered important for investigation, since ATPase sub-units are essential for cellular survival; furthermore, the ATPase sub-units are hypothesized to recognize the polyubiquitin degradation signal and to unfold the protein substrates for their degradation by the 20S core thereby controlling the access of substrates

to the proteolytic core (Braun et al., 1999, Lam et al., 2002). The 20S CP alone has a closed gate and requires an activator to regulate its protease activity.

The key aspect of the present study was to find out if there was any alteration in proteasome subunits and activity between diagnostic groups by determining the level of α3, α6 and RPT6 together with the chymotrypsin- and PGPH-like activities of the proteasome. Expression levels of the proteasome sub-units in discrete brain regions of post-mortem human tissue from DLB, PDD and AD patients, compared to normal control subjects, were determined using semi-quantitative Western blotting. Analysis involved the use of primary antibodies to the 19S ATPase RPT6 proteasome sub-unit: 20S proteasome α6 sub-unit and 20S proteasome α3 sub-unit. Immunohistochemistry was also performed to compare the anatomical distribution with the semi-quantitative Western blot results.

The catalytic activities of the 20S proteasome were measured using fluorogenic substrates assay. Only two of three activities of the 20S proteasome were measured [chymotrypsin-like, and peptidylglutamyl-peptide hydrolase (PGPH)-like activities]. The trypsin-like activity was excluded from this study because none of the three inhibitors used could completely inhibit its activity. For this reason, only two activities of the 20S proteasomes were analysed in this thesis.

Chymotrypsin-like, and PGPH-like activities were analyzed using appropriate artificial substrates Suc-Leu-Leu-Val-Tyr-AMC and Z-Leu-Leu-Glu-AMC. Chymotrypsin- and PGPH- like activities of the 20S proteasome cleave proteins at hydrophobic and acidic residues, respectively. The specificity of the assay was indicated by the ability of MG-132,

a selective inhibitor of proteasomal function, to almost completely inhibit fluorescence change.

Three brain regions were chosen for the study were the prefrontal cortex (Brodmann area 9), the anterior cingulate cortex (Brodmann area 24), and the parietal cortex (Brodmann area 40). BA9 was selected due to its proposed role in executive function and cognition decline (Fuster, 2001), which is a cardinal symptom of DLB and PDD (McKeith et al., 2005). BA24 was selected for the early development of pathology encountered in this region in DLB and PDD (Alafuzoff et al., 2009a) whilst BA40 was selected because of its pathological predominance in AD as opposed to DLB and PDD (Alafuzoff et al., 2008a). Thus this region acted as a comparison between the relative contributions of AD pathology and Lewy body pathology.

5.2 Results

5.2.1 Expression levels of proteasome sub-units in human post mortem brains of DLB, PDD, AD, and age-matched control

5.2.1.1 Frontal cortex – Brodmann area 9

5.2.1.1.1 Regulatory particles composed of ATPase (RPT6) proteasome sub-unit in BA9

The expression levels of RPT6 in discrete brain regions of post-mortem human tissue from DLB, PDD and AD patients, compared to normal control subjects were determined using semi-quantitative Western blotting. Western blotting was perfomed using a specific antibody to RPT6. The antibody was a mouse monoclonal IgG (p45-110) recognised very distinct band at approximatly 48 kDa which is consisent with published size (Myeku and Figueiredo-Pereira, 2011) and data sheet from the supplier.

A significant reduction in the regulatory particles (RPT6) proteasome sub-unit was seen in DLB (17%, p = 0.001), PDD (21%, p = 0.001) and AD (22%, p = 0.001) compared to the controls (one-way ANOVA, F = 24.303, d.f. = 3, 119; p = 0.001; Bonferroni post hoc test) (Figure 5-1). To provide additional confirmation and information regarding the spatial change of RPT6, representative selections of five cases from each diagnostic group were stained with anti-RPT6 (Figure 5-2: a, b, c and d). The high-magnification photomicrographs demonstrate a strong nuclear staining in control cases compared to those from PDD, DLB, and AD.

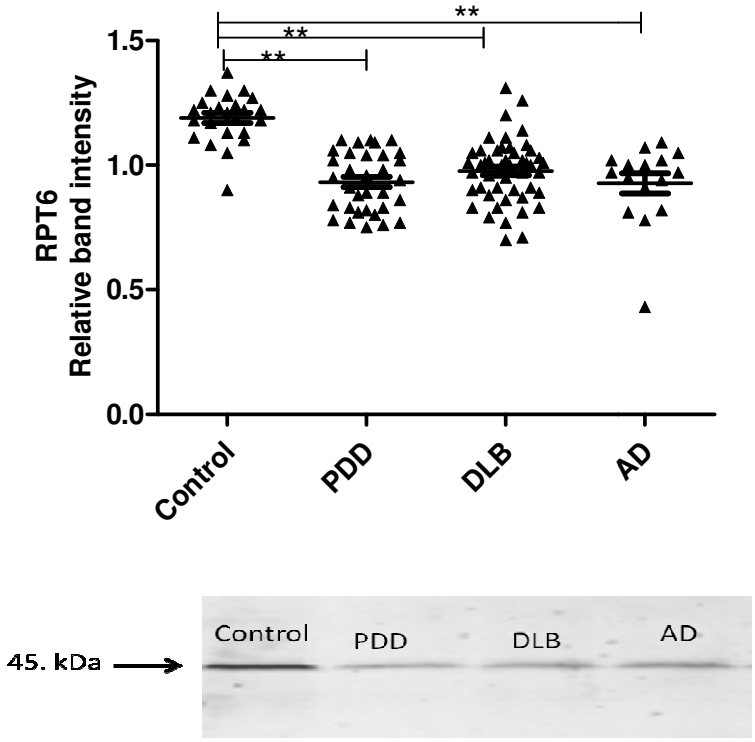


Figure 5-1: 19S ATPase RPT6 proteasome sub-unit values, from semi-quantitative Western blotting in PDD, DLB, AD, and control in frontal cortex (BA9).

A) Scatter plot of 19S ATPase RPT6 proteasome sub-unit relative intensity determined by semi-quantitative Western blot analysis in DLB, PDD, AD, and age-matched control samples. Statistical analysis was performed using One-way ANOVA ($F = 24.303$, d.f. = 3 , 119, $p = 0.001$ followed by Bonferroni post hoc test): mean RPT6 values for the control group ($n = 24$) were significantly higher than those for PDD ($p = 0.001$, $n = 33$), DLB ($p = 0.001$, $n = 50$) and AD ($p = 0.001$, $n = 16$) groups. The horizontal bars within the data points in the graphs represent the mean values. **B)** Representative Western blot showing levels of the 19S ATPase RPT6 proteasome sub-unit in DLB, PDD, AD, and control.

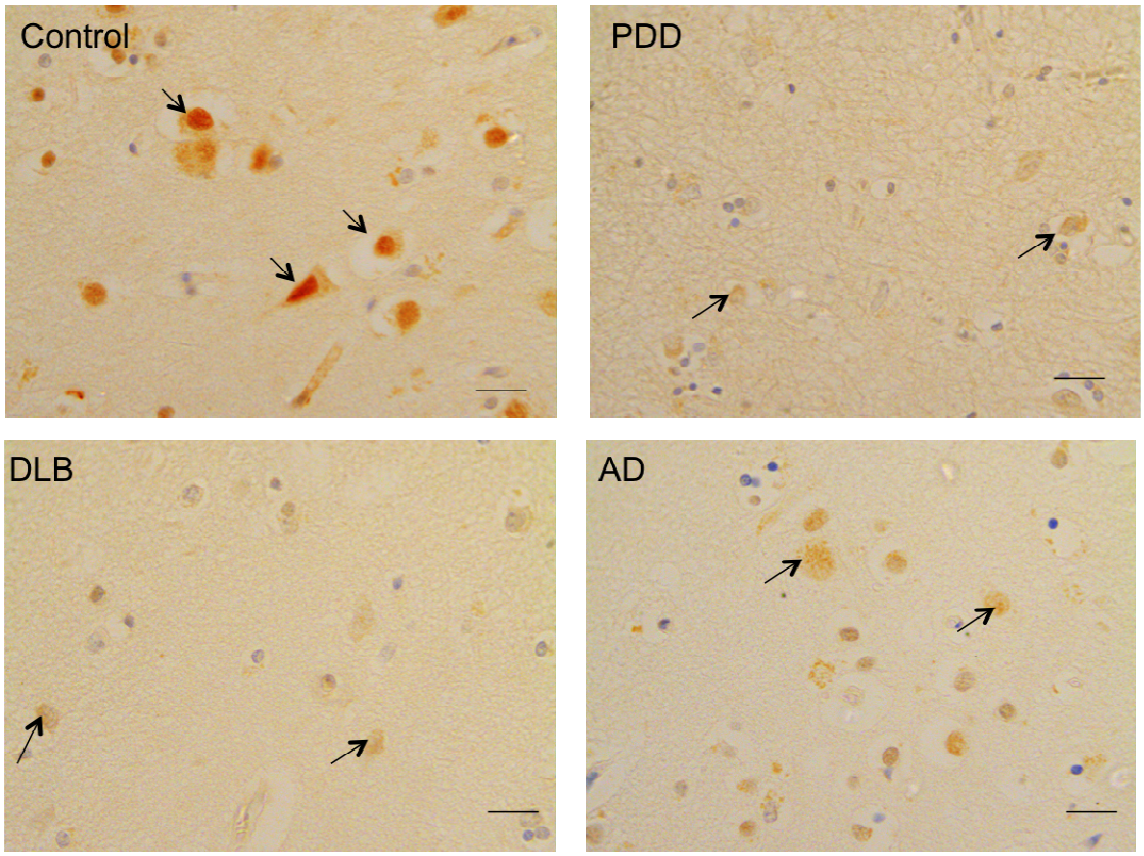


Figure 5-2: Photomicrographs of RPT6 staining in the frontal cortex region of the post-mortem human brain from control and dementia cases.

The photomicrograph illustrates immunohistochemistry for RPT6 in the frontal cortex of post-mortem brain from control, PDD, DLB and AD. The photomicrographs demonstrate a strong nuclear stain for the control cases compared to PDD, DLB, and AD. Scale bar represents 15 microns.

5.2.1.1.2 Proteasome α-6 sub-unit

The expression levels of proteasome 20S α6 subunit in discrete brain regions of post-mortem human tissue from DLB, PDD and AD patients, compared to normal control subjects were determined using semi-quantitative Western blotting. Western blotting was perfomed using specific antibody to the proteasome 20S α6 subunit. The antibody was a mouse monoclonal IgG (MCP20) that recognised a very distinct band at approximately 28 kDa which is consisent with published size (Camargo et al., 2014) and data sheet from the supplier.

There was no significant alteration in the level of 20S α-6 sub-unit in patients with DLB (1.24 ± 0.02, n = 49) or PDD (1.21 ± 0.03, n = 33) compared with the control. Likewise, there was no significant difference between PDD and DLB. Mean 20S α-6 sub-unit levels were lower in AD compared to PDD (23%, p = 0.001), DLB (25%, p = 0.001) and controls (28%, p = 0.001) (Figure 5-3). 20S α-6 sub-unit immunostaining also confirms the Western blotting analysis, as the level of staining of the cytoplasm and nucleus was similar for all groups with the exception of the AD group (Figure 5-4).

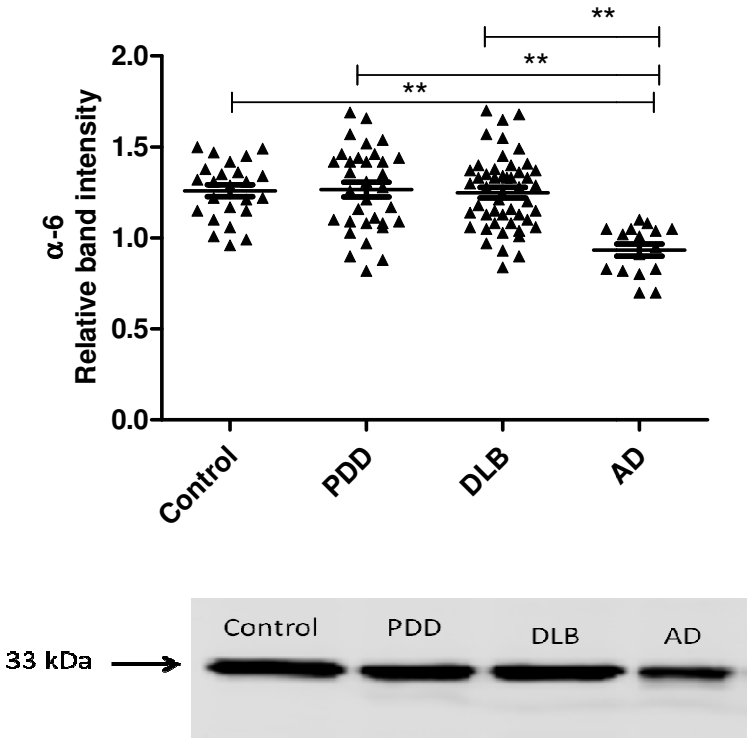


Figure 5-3: 20S α-6 proteasome sub-unit values, from semi-quantitative Western blotting in PDD, DLB, AD, and control in frontal cortex (BA9).

A) Scatter plot of 20S α-6 proteasome sub-unit relative intensity determined by semi-quantitative Western blot analysis in DLB, PDD, AD, and age-matched control samples. Statistical analysis was performed using One-way ANOVA (F = 14.781, d.f. = 3 and 118, p = 0.001; Bonferroni post hoc test): mean α-6 proteasome sub-unit values for AD (n = 16) group were significantly lower than those for control (p = 0.001, n = 24), DLB (p = 0.001, n = 49) and PDD (p = 0.001, n = 33) groups. The horizontal bars within the data points in the

graphs represent the mean values. B) Representative Western blot showing levels of the 20S α-6 proteasome sub-unit in DLB, PDD, AD, and control.

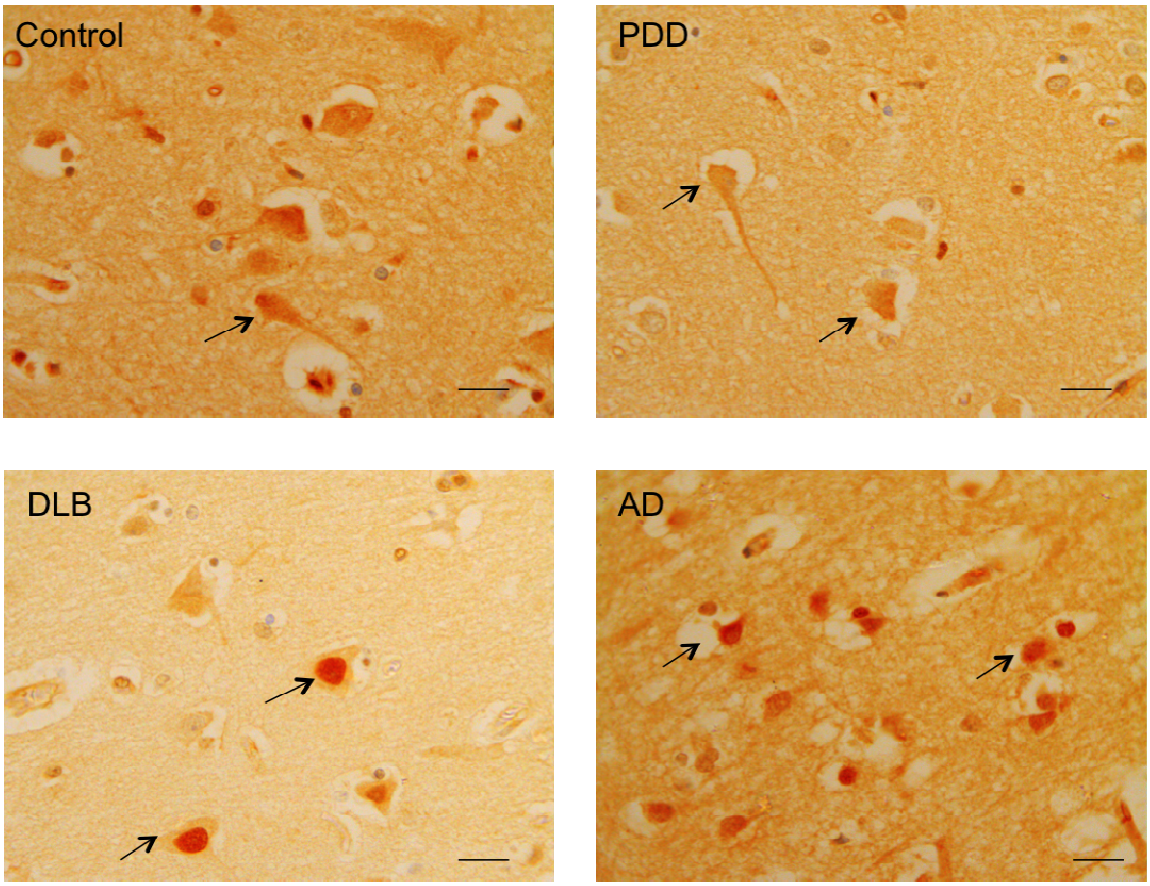


Figure 5-4: Photomicrographs of α-6 staining in the frontal cortex region of the post-mortem human brain from control and dementia cases.

The photomicrograph illustrates immunohistochemistry for α-6 in the frontal cortex of post-mortem brain from control, PDD, DLB and AD. The photomicrographs demonstrate the level of cytoplasm and nucleus staining was the same for all for groups and less for the AD group. Scale bar represents 15 microns.

5.2.1.1.3 Proteasome α-3 sub-unit

The expression levels of proteasome 20S α3 subunit in discrete brain regions of post-mortem human tissue from DLB, PDD and AD patients, compared to normal control subjects were determined using semi-quantitative Western blotting. Western blotting was perfomed using specific antibody to the proteasome 20S α3 subunit. The antibody was a mouse monoclonal IgG IgG (MCP257) recognised a very distinct band at approximately 29 kDa which is consisent with published size (Wang et al., 2014) and data sheet from the supplier.

There were no significant differences in the mean levels of 20S α-3 sub-unit between the patient groups compared to the controls according to the post hoc test (Figure 5-5, $p>0.05$). However, mean 20S α-3 sub-unit levels in patients with DLB were significantly elevated by 11% compared to PDD ($p = 0.001$).

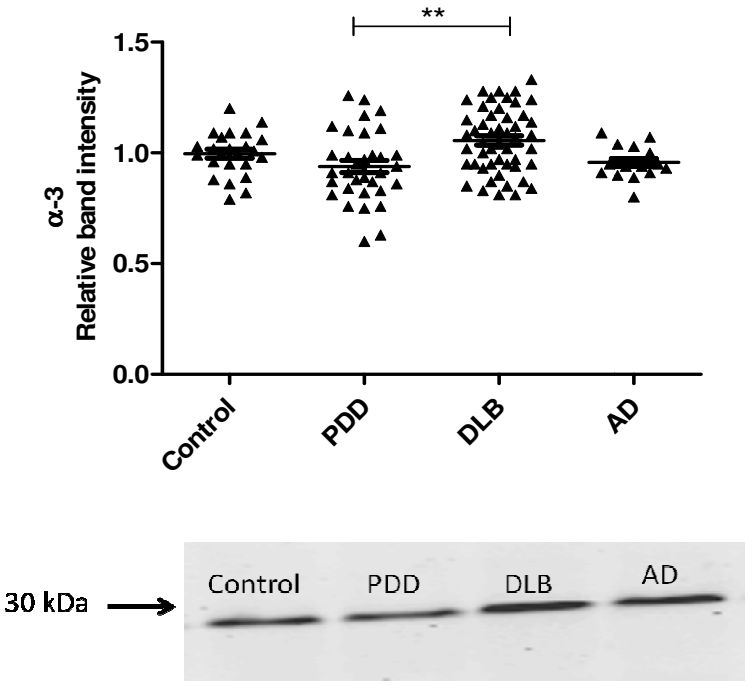


Figure 5-5: 20S α-3 proteasome sub-unit values, from semi-quantitative Western blotting in PDD, DLB, AD, and control in frontal cortex (BA9).

A) Scatter plot of 20S α-3 proteasome sub-unit relative intensity determined by semi-quantitative Western blot analysis in DLB, PDD, AD, and age-matched control samples. Statistical analysis was performed using One-way ANOVA (F = 5.81, d.f. = 3 and 119, p = 0.001; Bonferroni post hoc test): mean α-3 proteasome sub-unit values for the DLB (n = 49) group were significantly higher than the PDD group (p = 0.001, n = 16) and did not reach the significant compare to the AD (p = 0.07, n = 33) group. There was no difference between control group in comparison to the dementia groups (DLB, PDD, AD) according to the post hoc test. The horizontal bars within the data points in the graphs represent the mean values. **B)** Representative Western blot showing levels of the 20S α-3 proteasome sub-unit in DLB, PDD, AD, and control.

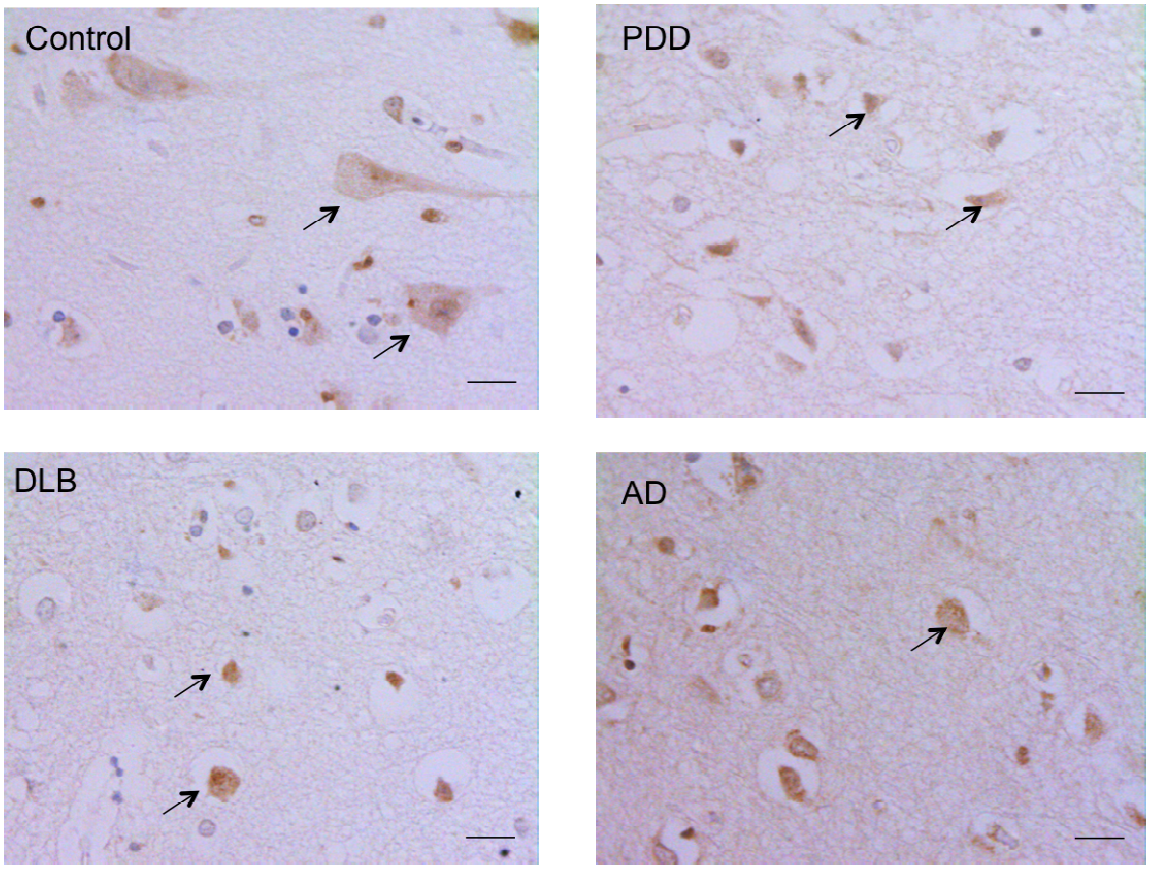


Figure 5-6: Photomicrographs of α-3 staining in the frontal cortex region of the post-mortem human brain from control and dementia cases.

The photomicrograph illustrates immunohistochemistry for α-3 in the frontal cortex of post-mortem brain from control, PDD, DLB and AD. The photomicrographs demonstrate the level of cytoplasm and nucleus staining was the same for all for groups. Scale bar represents 15 microns.

5.2.1.2 Parietal cortex—Brodmann area 40

5.2.1.2.1 Regulatory particles composed of ATPase (RPT6) proteasome sub-units in BA40

Significant reductions in regulatory particles (RPT6) proteasome sub-unit mean levels were seen in DLB (14%, p = 0.001, n = 52) and AD (23%, p = 0.001, n = 16) compared to the controls (n = 25) (one-way ANOVA, F = 16.33, d.f. = 3 and 121, p = 0.001; Bonferroni post hoc test). The post hoc test revealed that there was no difference between the control and PDD groups, but both control and PDD groups were significantly higher than DLB and AD groups. To provide additional confirmation and information regarding the spatial change of RPT6 representative sections of five cases from each diagnostic group were stained with anti-RPT6 (Figure 5-7: a, b, c and d). The high-magnification photomicrographs demonstrate a strong nuclear stain for the control cases compared to PDD, DLB, and AD. The photomicrographs also illustrate that the weakest stain was in the AD group, which was consistent with the Western blotting analysis (Figure 5-6).

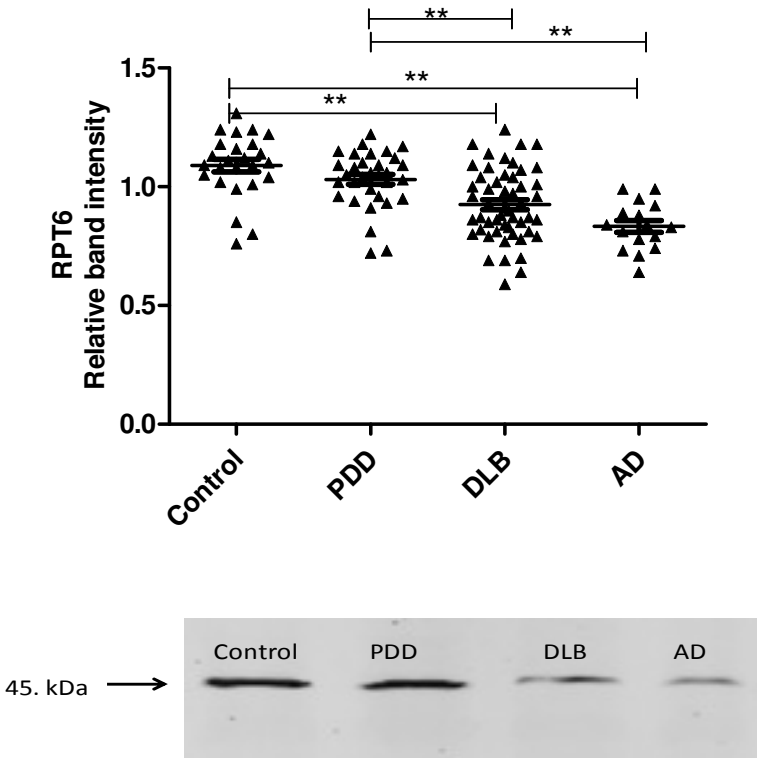


Figure 5-7: 19S ATPase RPT6 proteasome sub-unit values, from semi-quantitative Western blotting in PDD, DLB, AD, and control in parietal cortex (BA40).

A) Scatter plot of 19S ATPase RPT6 proteasome sub-unit relative intensity determined by semi-quantitative Western blot analysis in DLB, PDD, AD, and age-matched control samples. Statistical analysis was performed using One-way ANOVA ($F = 16.333$, d.f. = 3 and 121, $p = 0.001$; Bonferroni post hoc test): mean RPT6 values for the control group ($n = 24$) and PDD ($n = 33$) groups were significantly higher than DLB ($p < 0.05$, $n = 52$) and AD ($p = 0.001$, $n = 16$) groups. The horizontal bars within the data points in the graphs represent the mean values. **B)** Representative Western blot showing levels of the 19S ATPase RPT6 proteasome sub-unit in DLB, PDD, AD, and control.

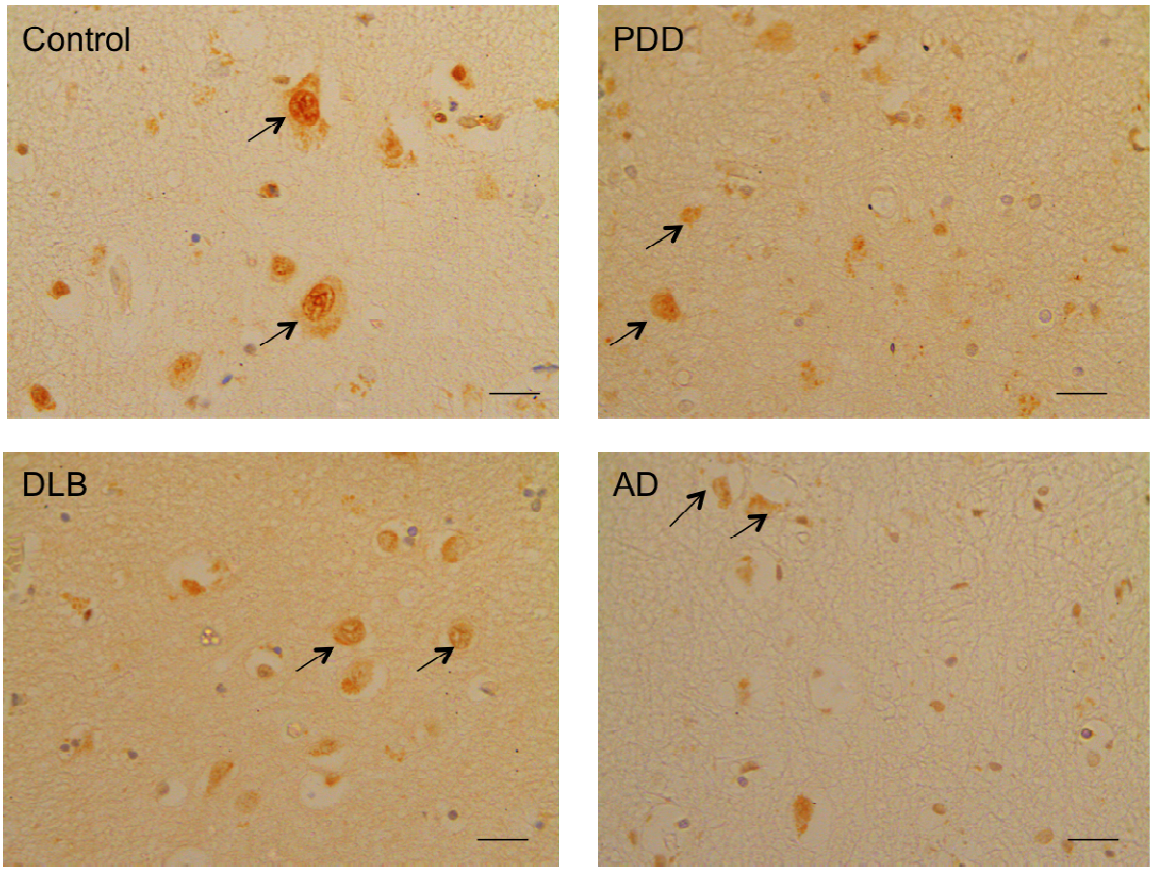


Figure 5-8: Photomicrographs of RPT6 staining in the parietal cortex (BA40) region of the post-mortem human brain from control and dementia cases.

The photomicrograph illustrates immunohistochemistry for RPT6 in the parietal cortex (BA40) of post-mortem brain from control, PDD, DLB and AD. The photomicrographs demonstrate a strong nuclear stain for the control and PDD cases compared to DLB, and AD. Scale bar represents 15 microns.

5.2.1.2.2 Proteasome α-6 sub-unit

No difference in expression level of α-6 was observed in the PDD, DLB, or AD groups compared with control subjects with mean (± SEM) relative intensity (1.04 ± 0.02, 0.99 ± 0.02, 0.98 ± 0.01, and 0.98 ± 0.02 for the control, PDD, DLB, and AD, respectively) (Figure 5-8 and 5-9).

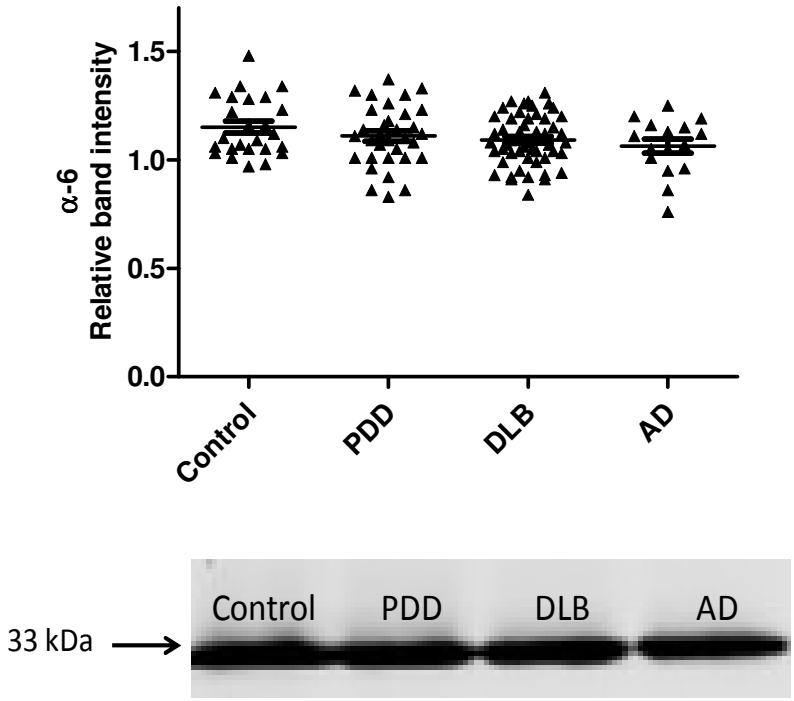


Figure 5-9: 20S α-6 proteasome sub-unit values, from semi-quantitative Western blotting in PDD, DLB, AD, and control in parietal cortex (BA40).

A) Scatter plot of 20S α-6 proteasome sub-unit relative intensity determined by semi-quantitative Western blot analysis in DLB, PDD, AD, and age-matched control samples. Statistical analysis was performed using One-way ANOVA (F = 1.138, d.f. = 3 and 122, p = 0.336; Bonferroni post hoc test): Statistical analysis confirmed there was no significant difference between diagnostic groups for α-6 values. The horizontal bars within the data points in the graphs represent the mean values. **B)** Representative Western blot showing levels of the 20S α-6 proteasome sub-unit in DLB, PDD, AD, and control

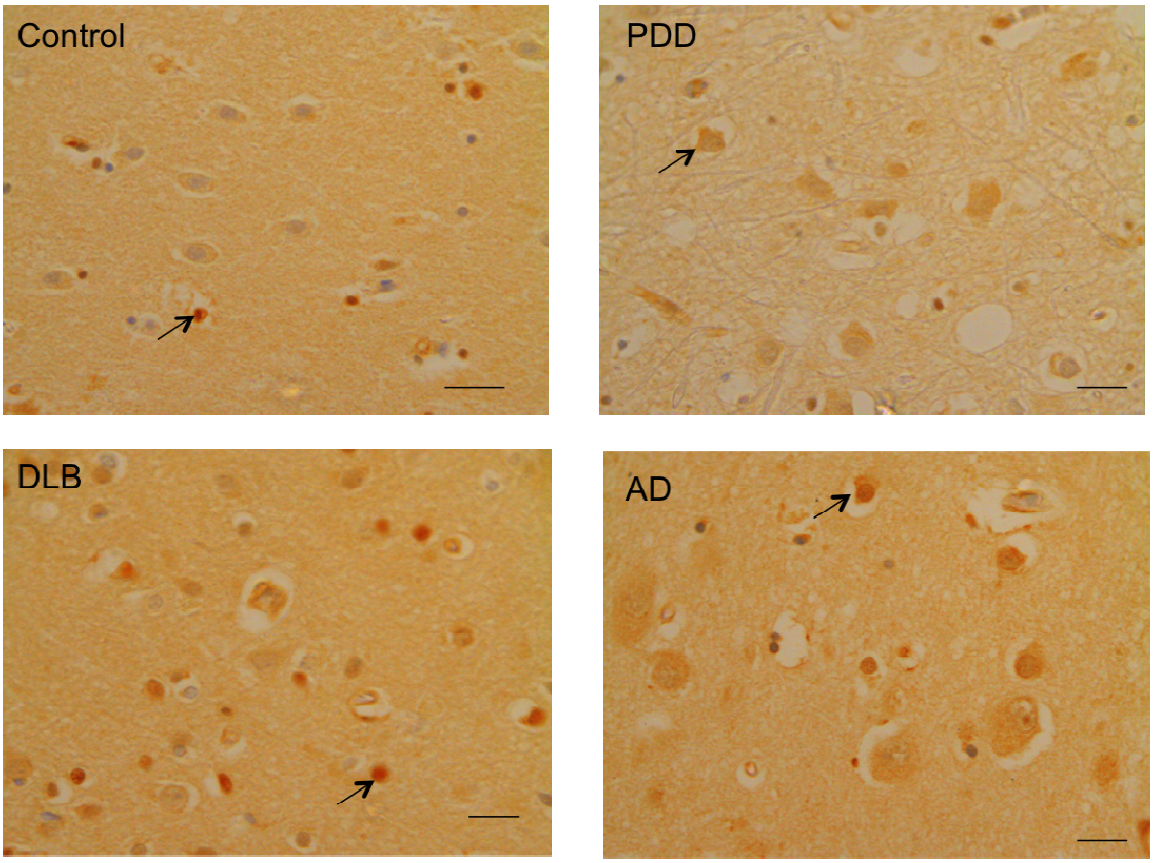


Figure 5-10: Photomicrographs of α-6 staining in the parietal cortex (BA40) region of the post-mortem human brain from control and dementia cases.

The photomicrograph illustrates immunohistochemistry for α-6 in the parietal cortex (BA40) of post-mortem brain from control, PDD, DLB and AD. The photomicrographs demonstrate the level of cytoplasm and nucleus staining was the same for all for groups. Scale bar represents 15 microns.

5.2.1.2.3 Proteasome α-3 sub-unit

Statistical analysis indicated that the α-3 expression level was significantly up-regulated in the parietal cortex in PDD (relative intensity 1.27 ± 0.02 n = 33) compared to the control subjects (1.12 ± 0.02, 11%, p = 0.012 n = 25), DLB (1.1 ± 0.01, 12%, p = 0.001, n = 53), and AD (1.16 ± 0.04, p = 0.016, n = 16) (one-way ANOVA, F = 10.49, d.f. = 3 and 123, p = 0.001; Bonferroni post hoc test). The post hoc test revealed that there was no significant difference between the control and DLB/AD groups ($p > 0.05$) (Figure 5-11).

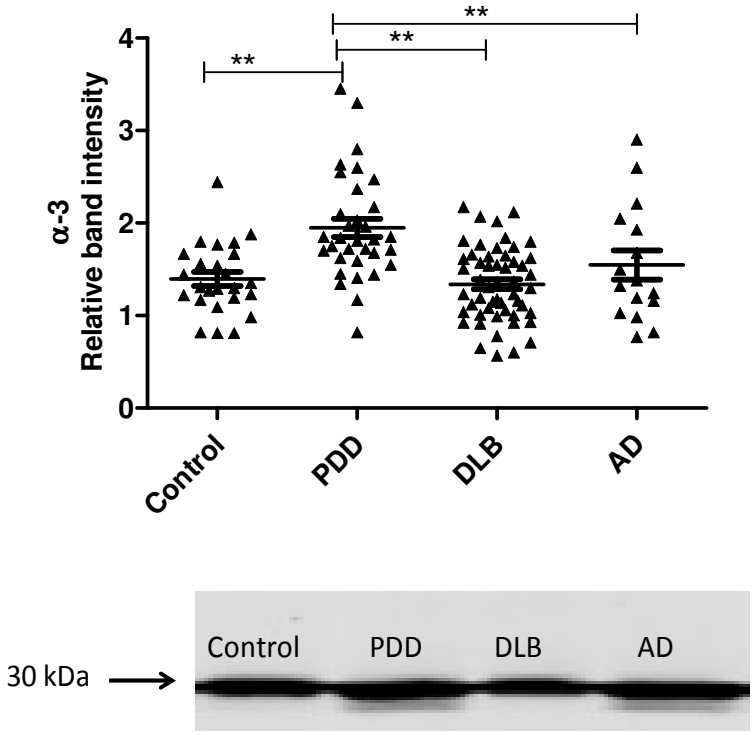


Figure 5-11: 20S α-3 proteasome sub-unit values, from semi-quantitative Western blotting in PDD, DLB, AD, and control in parietal cortex (BA40).

A) Scatter plot of 20S α-3 proteasome sub-unit values relative intensity determined by semi-quantitative Western blot analysis in DLB, PDD, AD, and age-matched control samples. Statistical analysis was performed using One-way ANOVA (F = 10.49, d.f. = 3 and 123, p = 0.001; Bonferroni post hoc test): mean α-3 values for the PDD (n = 33) group were significantly higher than the control (p = 0.001, n= 25), DLB (p = 0.001, n = 53) and AD (p = 0.004, n = 16) group. The horizontal bars within the data points in the graphs represent the mean values. **B)** Representative Western blot showing levels of the 20S α-3 proteasome sub-unit in DLB, PDD, AD, and control.

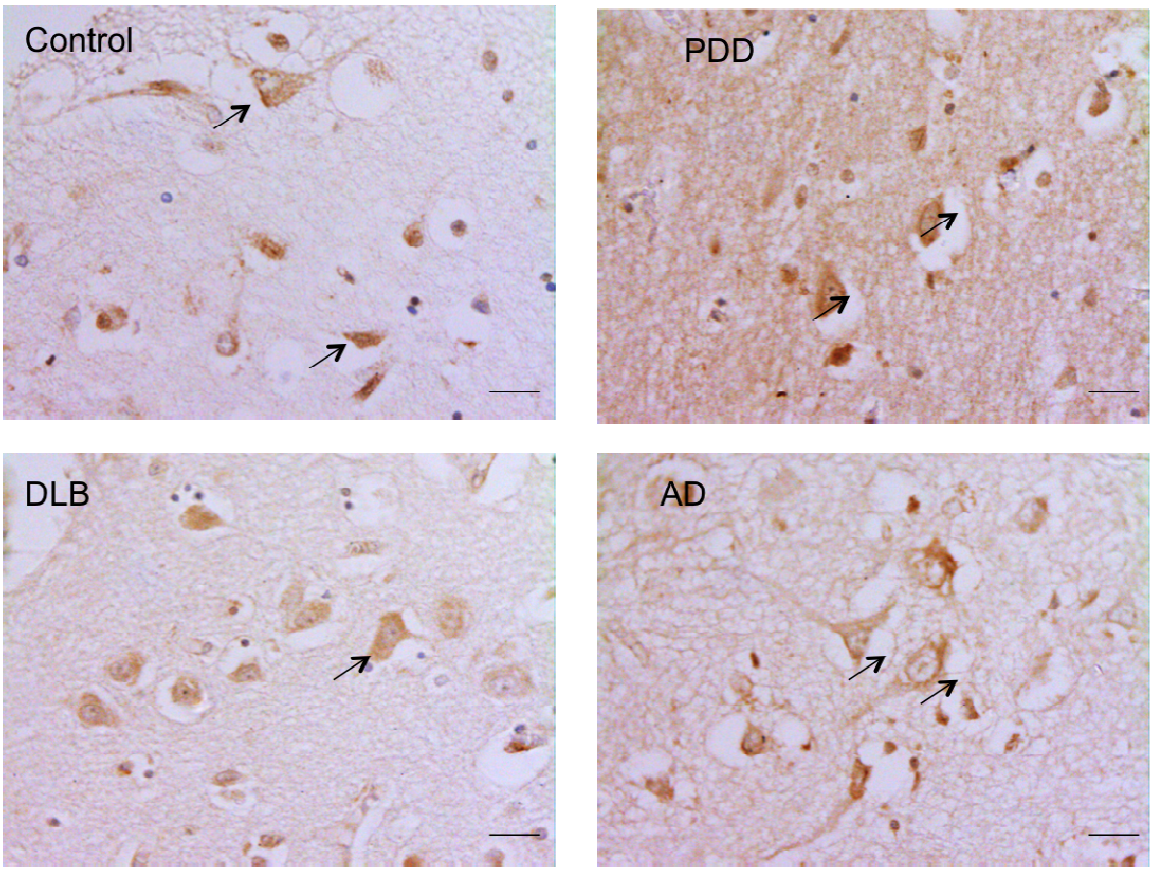


Figure 5-12: Photomicrographs of α-3 staining in the parietal cortex (BA40) region of the post-mortem human brain from control and dementia cases.

The photomicrograph illustrates immunohistochemistry for α-3 in the parietal cortex (BA40) of post-mortem brain from control, PDD, DLB and AD. The photomicrographs demonstrate no conspicuous difference in the level of cytoplasmic and nuclear staining when comparing the experimental groups. Scale bar represents 15 microns.

5.2.1.3 Anterior cingulate Brodmann area 24

5.2.1.3.1 Regulatory particles composed of ATPase (RPT6) proteasome sub-unit in BA24

Significant reductions in regulatory particles (RPT6) proteasome sub-unit mean levels were seen in DLB (13%, p = 0.001, n = 46) and AD (13%, p = 0.001, n = 16) compared to the controls (n = 23) (one-way ANOVA F = 13.5, d.f. = 3 and 113, p = 0.001; Bonferroni post hoc test). Mean RPT6 levels were significantly elevated in patients with PDD by 15%, p = 0.001 compared to AD and DLB groups. The post hoc test revealed that there was no significant difference between the control and PDD groups (p>0.05) (Figure 5-12).

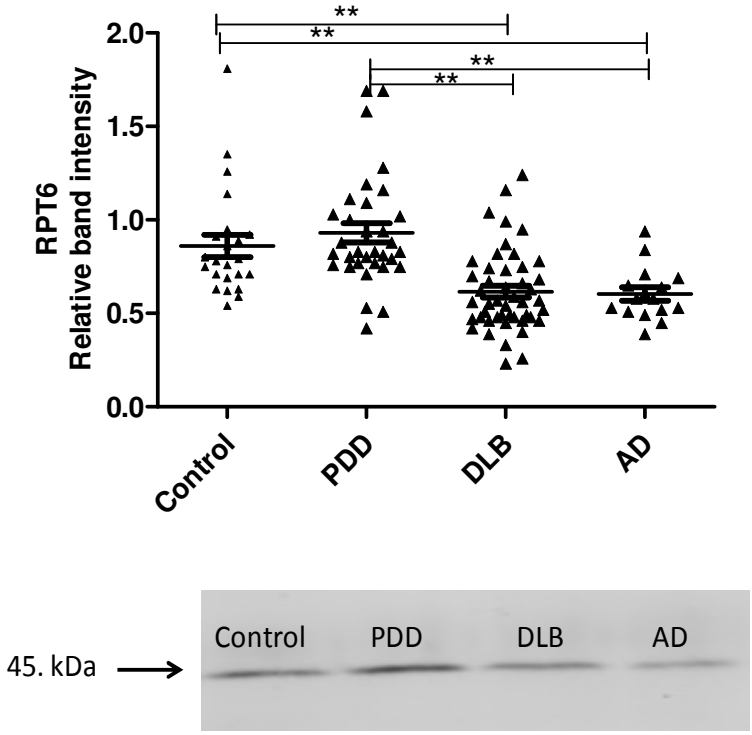


Figure 5-13: 19S ATPase RPT6 proteasome sub-unit values, from semi-quantitative Western blotting in PDD, DLB, AD, and control in cingulate cortex (BA24).

A) Scatter plot of 19S ATPase RPT6 proteasome sub-unit relative intensity determined by semi-quantitative Western blot analysis in DLB, PDD, AD, and age-matched control samples. Statistical analysis was performed using One-way ANOVA ($F = 13.56$, d.f. = 3 and 113, $p = 0.001$; Bonferroni post hoc test): mean RPT6 values for the control ($n = 24$) and PDD ($n = 33$) groups were significantly higher than DLB ($p < 0.05$, $n = 52$) and AD ($p < 0.05$, $n = 16$) groups. There was no difference between the control and PDD groups. The horizontal bars within the data points in the graphs represent the mean values. **B)** Representative Western blot showing levels of the 19S ATPase RPT6 proteasome sub-unit in DLB, PDD, AD, and control.

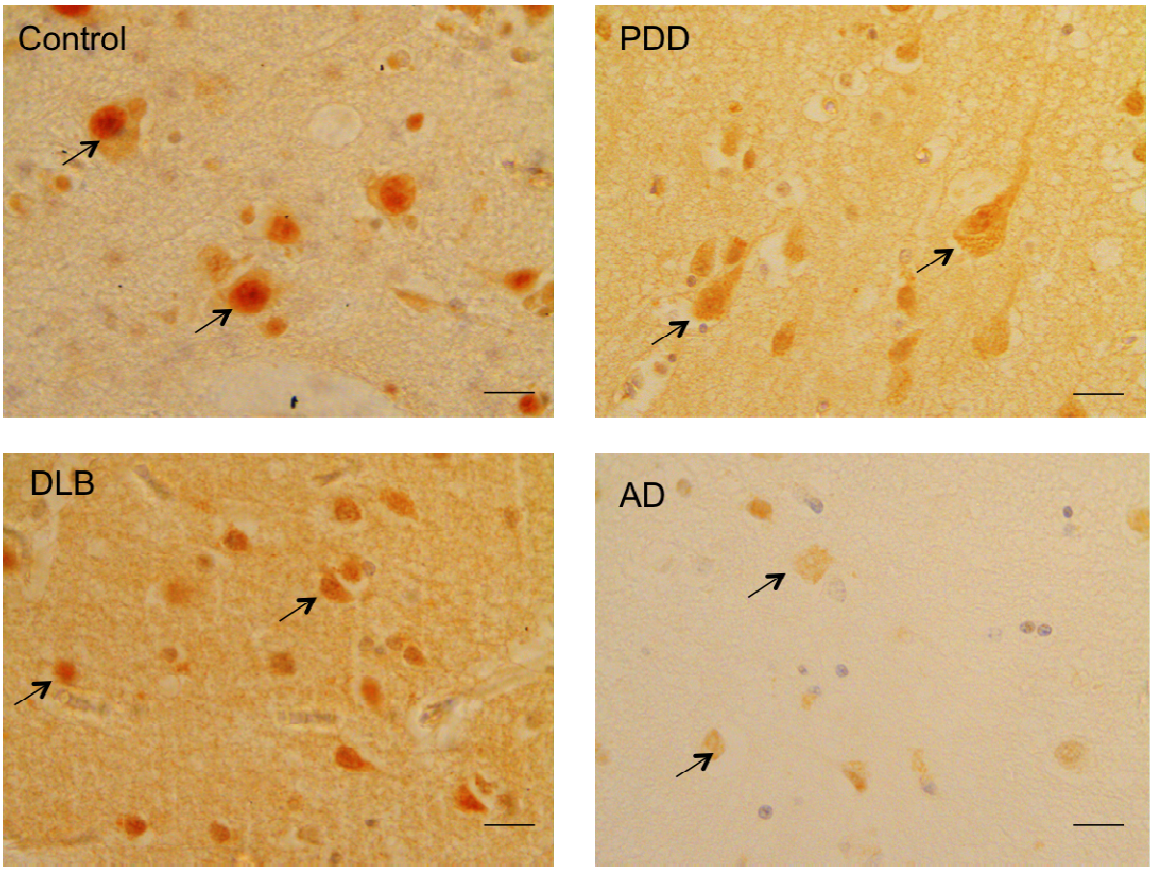


Figure 5-14: Photomicrographs of RPT6 staining in the cingulate cortex (BA24) region of the post-mortem human brain from control and dementia cases.

The photomicrograph illustrates immunohistochemistry for RPT6 in the cingulate cortex (BA24) of post-mortem brain from control, PDD, DLB and AD. The photomicrographs demonstrate a strong nuclear staining in the control as compared to DLB, and AD cases. Scale bar represents 15 microns.

5.2.1.3.2 Proteasome α-6 sub-unit

Analysis of data indicated a significant up-regulation in the expression level of α-6 sub-unit in both PDD (7%, $p = 0.002$, $n = 33$) and DLB (6%, $p = 0.007$, $n = 48$) compared to control ($n = 23$) (one-way ANOVA $F = 5.221$, d.f. = 3 and 116, $p = 0.002$; Bonferroni post hoc test). The post hoc test revealed that there was no difference between the control and AD groups ($p > 0.05$) (Figure 5-14).

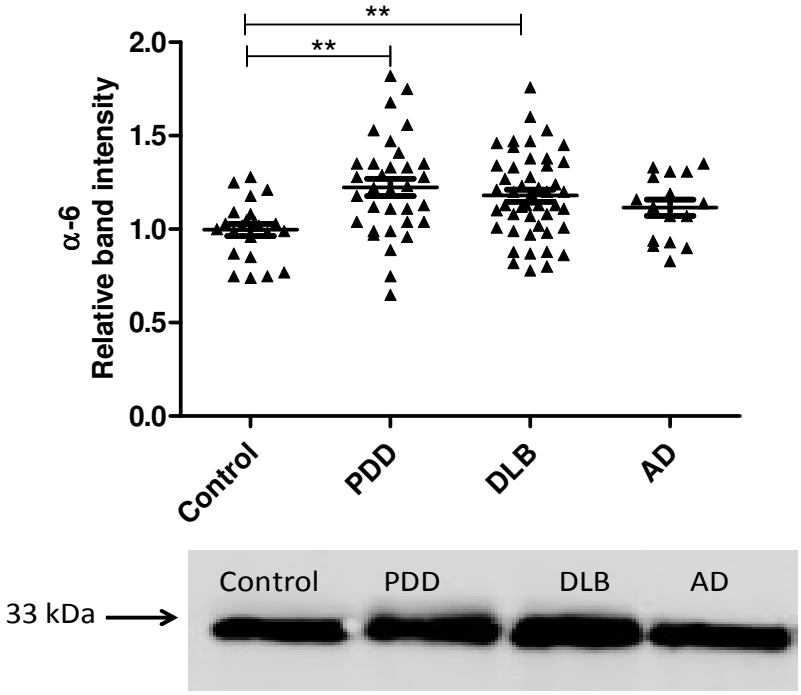


Figure 5-15: 20S α-6 proteasome sub-unit values, from semi-quantitative Western blotting in PDD, DLB, AD, and control in cingulate cortex (BA24).

Scatter plot of 20S α-6 proteasome sub-unit relative intensity determined by semi-quantitative Western blot analysis in DLB, PDD, AD, and age-matched control samples. Statistical analysis was performed using One-way ANOVA ($F = 5.221$, d.f. = 3 and 119, $p = 0.002$; Bonferroni post hoc test): mean α-6 proteasome sub-unit for α-6 values for the DLB ($n = 48$) and PDD ($n = 33$) group were significantly higher than the AD group ($p < 0.05$, $n = 16$) and the control ($p < 0.05$, $n = 23$) groups. The horizontal bars within the data points in the graphs represent the mean values. **B)** Representative Western blot showing levels of the 20S α-6 proteasome sub-unit in DLB, PDD, AD, and control.

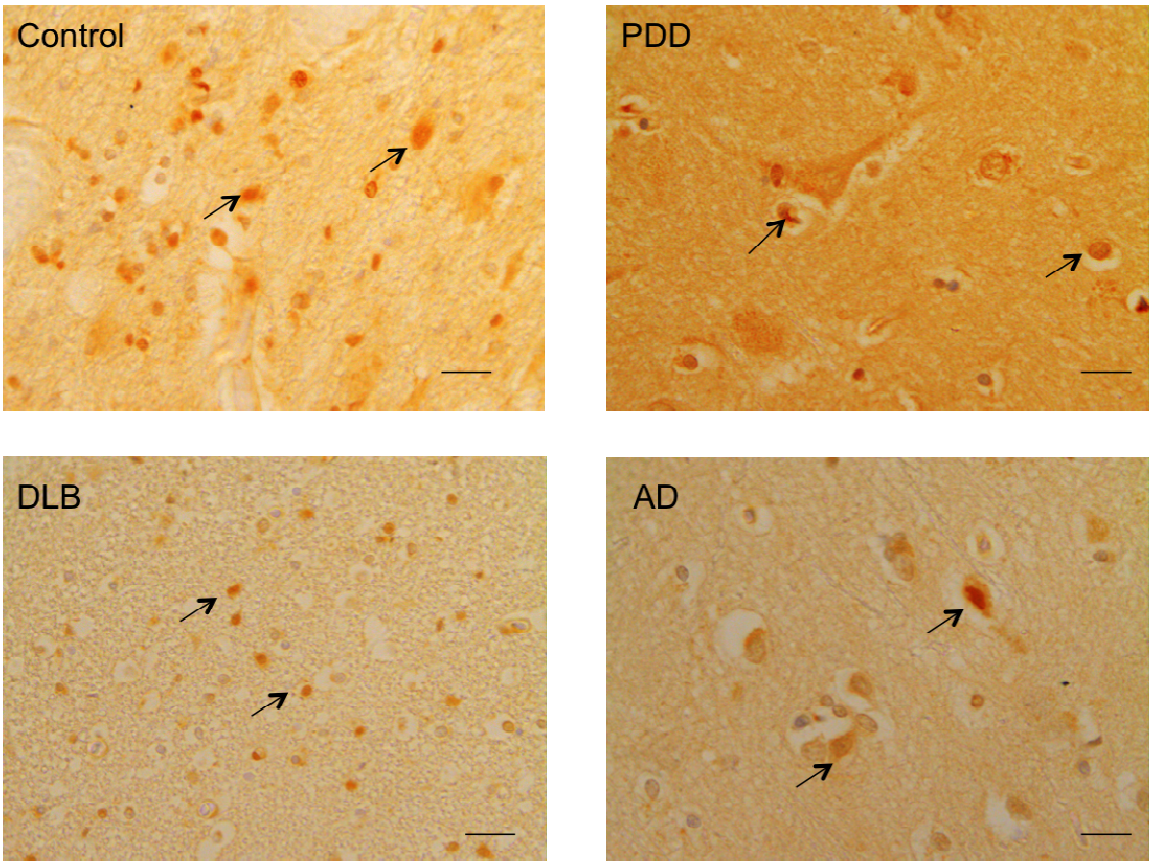


Figure 5-16: Photomicrographs of α-6 staining in the cingulate cortex (BA24) region of the post-mortem human brain from control and dementia cases.

The photomicrograph illustrates immunohistochemistry for α-6 in the cingulate cortex (BA24) of post-mortem brain from control, PDD, DLB and AD. The photomicrographs demonstrate the level of cytoplasmic and nuclear staining was the same for all for groups. Scale bar represents 15 microns.

5.2.1.3.3 Proteasome α-3 sub-unit

Significant reductions in 20S α-3 sub-unit mean levels were seen in PDD (relative intensity 0.88 ± 0.02, n = 33) compared to the control (1.00 ± 0.01, 12%, p = 0.019, n = 23), DLB (1.03 ± 0.02, 14%, p = 0.001, n = 48), and AD (1.11 ± 0.04, 9%, p = 0.001, n = 16) groups (one-way ANOVA F = 10.43, d.f. = 3 and 116, p = 0.001; Bonferroni post hoc test). The post hoc test revealed that there was no difference between the control and DLB and AD groups ($p > 0.05$) (Figure 5-16).

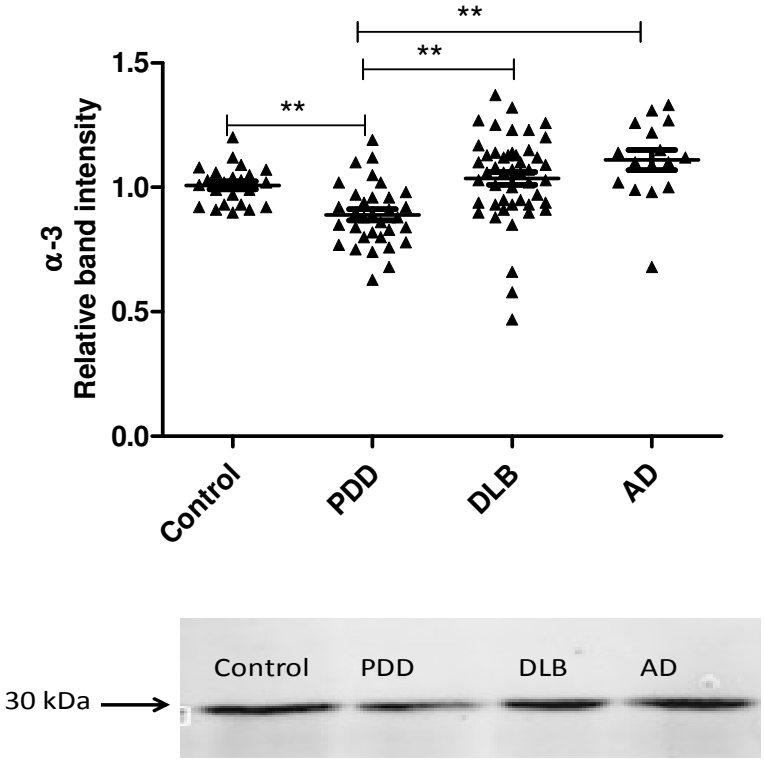


Figure 5-17: 20S α-3 proteasome sub-unit values, from semi-quantitative Western blotting in PDD, DLB, AD, and control in cingulate cortex (BA24).

Scatter plot of 20S α-3 proteasome sub-unit relative intensity determined by semi-quantitative Western blot analysis in DLB, PDD, AD, and age-matched control samples. Statistical analysis was performed using One-way ANOVA (F = 10.43, d.f. = 3 and 116, p = 0.001; Bonferroni post hoc test): mean α-3 value for the PDD (n = 33) group were significantly lower than the control (p = 0.019, n = 23), DLB (p = 0.001, n = 48) and AD (p = 0.004, n = 16) group. The horizontal bars within the data points in the graphs represent the mean values. **B)** Representative Western blot showing levels of the 20S α-3 proteasome sub-unit in DLB, PDD, AD, and control.

5.2.2 Proteolytic activity of 20S proteasomes are selectively impaired in all three brain regions examined in PDD, DLB, and AD

5.2.2.1 Patient demographic

As detailed in Chapter 2 Section 2.5, around 10 cases from each group for each brain region were chosen depending on the sufficient available of the frozen tissue. In BA9, samples from 11 DLB, 12 PDD, 6 AD patients, and 9 controls were analysed (Table 5-1). One-way ANOVA and Bonferroni post-hoc test revealed that pH was significantly higher in PDD patients compared to AD patients ($p = 0.023$) one-way ANOVA, $F = 3.52$, d.f. = 3 and 34, $p = 0.025$; Bonferroni post hoc test). The effect of pH was removed by (unstandardised) residuals calculated using linear regression as explained in detail in chapter 4 section 4.4. There were no significant differences between diagnostic groups for PMD and age at death $p > 0.05$.

In BA40, Samples from 9 DLB, 10 PDD, 12 AD patients, and 13 controls were analysed (Table 5-1). One-way ANOVA and Bonferroni post-hoc test revealed that were no significant differences between diagnostic groups and any of the variables shown (age at death, PMD and pH).

In BA24, Samples from 12 DLB, 9 PDD, 9 AD patients, and 13 controls were analysed (Table 5-1). One-way ANOVA and Bonferroni post-hoc test revealed that were no significant differences between diagnostic groups and any of the variables shown (age at death, PMD and pH).

Table 5-1: Patients' demographics.

Diagnosis		Gender (M/F) %	Age at death mean	PMD (mean hours)	pH (mean)
BA9	Control (9)	56/44	80.8 ± 2.76	47.83 ± 5.15	6.52 ± 0.09
	PDD (12)	50/50	80.08 ± 1.44	32.75 ± 4.91	6.68 ± 0.07
	DLB (11)	64/36	83.81 ± 1.79	45.9 ± 8.78	6.42 ± 0.1
	AD (6)	17/83	90.16 ± 4.64	25.95 ± 8.34	6.22 ± 0.1
BA40	Control (13)	54/46	80.15 ± 1.93	35.96 ± 5.9	6.53 ± 0.07
	PDD (10)	30/70	82.00 ± 1.5	30.7 ± 3.48	6.42 ± 0.06
	DLB (9)	67/33	82.22 ± 2.65	59.7 ± 12.76	6.54 ± 0.07
	AD (12)	25/75	87.92 ± 2.41	36.89 ± 6.54	6.31 ± 0.10
BA24	Control (13)	54/46	80.46 ± 2.09	35.07 ± 5.3	6.48 ± 0.07
	PDD (9)	33/67	81.56 ± 1.6	31.11 ± 3.89	6.49 ± 0.07
	DLB (12)	67/33	80.83 ± 2.14	48.19 ± 10.58	6.52 ± 0.08
	AD (9)	33/67	87.89 ± 1.98	29.21 ± 7.02	6.31 ± 0.09

Data are means ± SD age in years; PMD = post-mortem delay; DLB = dementia with Lewy bodies; PDD = Parkinson's disease dementia; AD = Alzheimer's disease. BA9) PMD were not significantly different between the groups in the one-way analysis of variance (ANOVA) ($P < 0.05$). Age at death of AD patients was significantly higher than subjects with PDD ($p=0.056$) and pH was significantly higher in PDD patients compared to AD patients ($p=0.023$). BA40 and 24) Age at death, PMD and pH were not significantly different between the groups in the one-way analysis of variance (ANOVA) ($P < 0.05$).

5.2.2.2 Assessment of PGPH-like proteasome activity

PGPH-like activity was measured in BA9, BA40, and BA24 homogenates from PDD, DLB, AD, and normal control using Z-Leu-Leu-Glu-AMC synthetic peptide substrate, as described in Chapter 2 Section 2.5 and previously by (Zeng et al., 2005). All assays were performed in the absence and presence of the proteasome inhibitor, MG132.

In BA9, a significant reduction in PGPH-like activity was seen in AD patients (7933 ± 273, n = 6) compared to the control (10342 ± 339, 23%, n = 9, p = 0.012) (one-way ANOVA, F = 3.816, d.f. = 3 and 34, p =0.019; Bonferroni post hoc test). DLB (9118 ± 371, 13%, n = 11) and PDD (9228 ± 533, 14%, n = 12) groups were lower compared to the control subjects, but the post hoc test revealed that there were no significant differences between the control and DLB or PDD groups ($p > 0.05$).

In BA40, analysis of data indicated a significant reduction in PGPH-like activity in the AD (p = 0.001), DLB (p = 0.001), and PDD (p = 0.02) groups compared to controls. The reduction in AD, DLB and PDD was 45 %, 39 % and 29 % with a mean ± SEM value of 3741.8 ± 587.5, n = 11, 4133.7 ± 640, n = 10 and 4809 ± 240, n = 9 compared to 6862 ± 253, n = 13 for the controls (Figure 5-17). The differences between the patients groups and the control were statistically different (one-way ANOVA, F = 10.263, d.f. = 3 and 42, p = 0.001; Bonferroni post hoc test)

In BA24, Analysis of data indicated a significant reduction in PGPH-like activity in DLB (28%, p = 0.013, n = 12), PDD (64%, p = 0.001, n = 9) and AD (62%, p = 0.001, n = 9) groups compared to control subjects (one-way ANOVA, F = 23.087, d.f. = 3 and 39, p =

0.001; Bonferroni post hoc test). The reduction in PGPH-like activity in PDD (51%, p = 0.002) and AD (47%, p = 0.004) was also significant different compared to DLB.

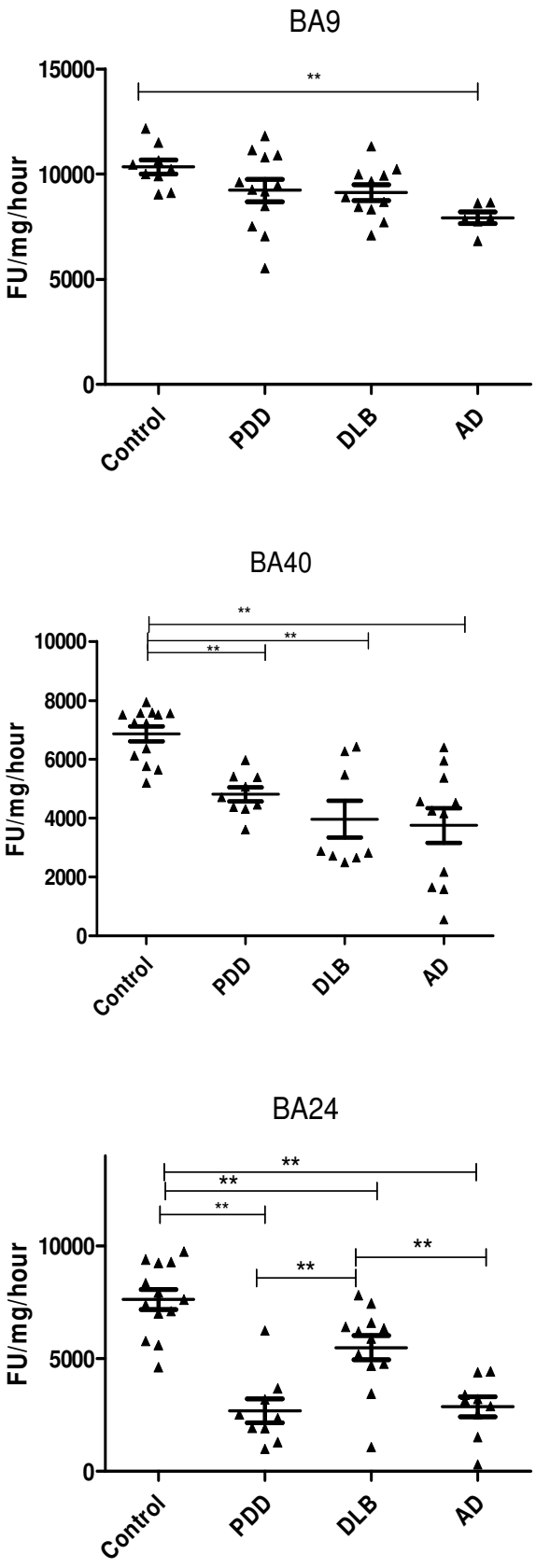


Figure 5-18: Analysis of PGPH-like activities in brain homogenates from BA9, BA40, and BA24 of DLB, PDD, AD, and controls.

Scatter plots of PGPH-like activity measurement in BA9, BA40, and BA24 homogenates from DLB, PDD, AD, and normal control samples using the fluorogenic substrate assay. Activities are expressed as fluorescence units (FU)/mg protein/hour. A) PGPH-like activity measurement in BA9; PGPH-like activity was decreased only in AD patients ($p = 0.012$, $n = 6$) compared to the control ($n = 9$); DLB and PDD groups were lower compared to the control subjects, but there was no statistically significant difference between them. The ANOVA for PGPH-like activity measurement in BA9 (one-way ANOVA, $F = 7.897$, d.f. = 3 and 34, $p = 0.001$; Bonferroni

post hoc test). B) PGPH-like activity measurement in BA40; the differences between the patients groups (PDD, DLB and AD) and the control were statistically different (one-way ANOVA, F = 10.263, d.f. = 3 and 42, p = 0.001; Bonferroni post hoc test). The reduction in PGPH-like activity was higher in the AD group with a mean ± SEM value of 3741.8 ± 587.5, 45 %, n = 11, compared to 1.28 ± 0.028, n = 24 for the controls. The reduction in both DLB and PDD were about 39% and 29 % with a mean ± SEM value of 4133.7 ± 640, n = 10 and 4809 ± 240, n = 9 compared to control. C) PGPH-like activity measurement in BA24; there was a significant difference between DLB (p = 0.013, n = 12), PDD (P = 0.001, n = 9) and AD (P = 0.001, n = 9) compared to the control (n = 13) (one-way ANOVA, F = 23.087, d.f. = 3 and 39, p = 0.001; Bonferroni post hoc test). PGPH-like activity measurements were significant lower in both AD (p = 0.004, n = 9) and PDD (p = 0.002, n = 9) compared to DLB subjects.

5.2.2.3 Assessment of chymotrypsin-like proteasome activity

In BA9, chymotrypsin-like activity was significantly reduced in PDD (27%, p = 0.004), DLB (24% p = 0.013), and AD (38%, p = 0.001), compared to control values (one-way ANOVA, F = 7.897, d.f. = 3 and 34, p = 0.001; Bonferroni post hoc test) (Figure 5-18). Chymotrypsin-like activity was lowest in the AD group compared to DLB and PDD; however, there were no significant differences among the three groups, PDD, DLB, or AD. In BA40 Analysis of data indicated a significant reduction in chymotrypsin-like activity in the AD, DLB, and PDD (1415.85 ± 9.9, n = 12, 1453.09 ± 11.77, n = 9 and 1436.88 ± 20.61, n = 10) groups compared to the control groups (1568.53 ± 10.2, n = 13) (one-way ANOVA, F = 30.033, d.f. = 3 and 40, p = 0.001; Bonferroni post hoc test).

In BA24, chymotrypsin-like activity was found to be significantly lower in PDD (878 ± 62, n = 9) and AD (906 ± 72, n = 9) samples compared to both control (1100 ± 39, n = 13) and DLB (1027± 23, n = 12) subjects (one-way ANOVA, F = 4.663, d.f. = 3 and 39, p = 0.007; Bonferroni post hoc test).

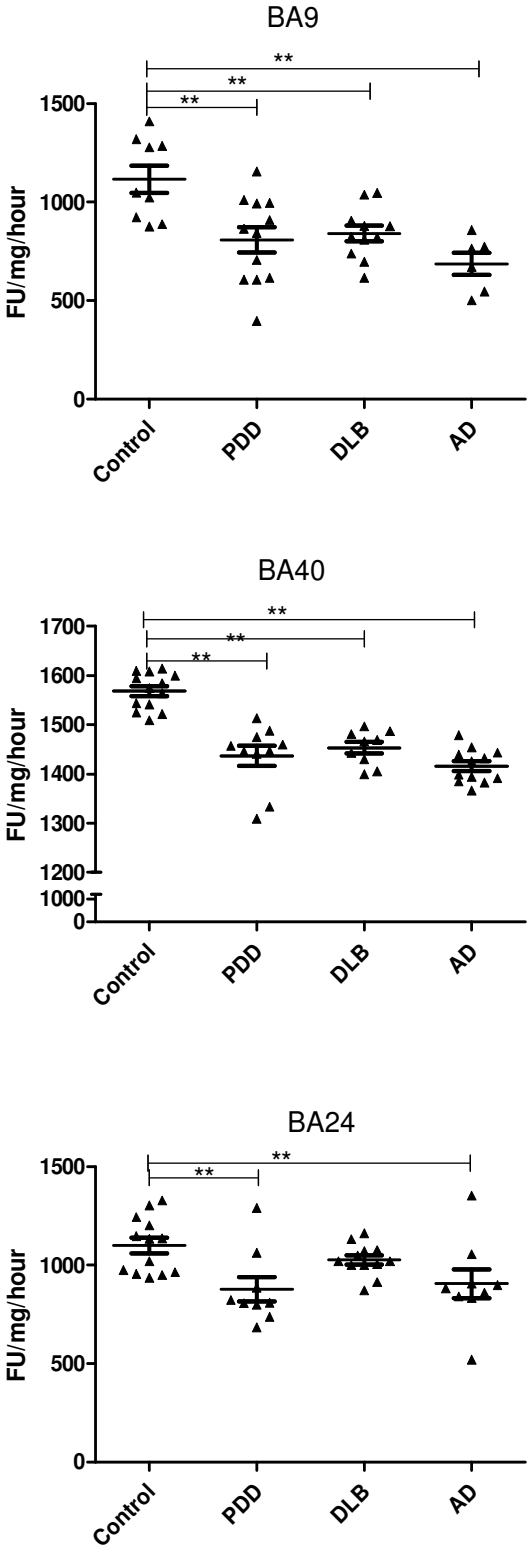


Figure 5-19: Analysis of chymotrypsin - like activities in brain homogenates from BA9, BA40, and BA24 of DLB, PDD, AD, and controls.

Scatter plots of chymotrypsin-like activity measurement in BA9, BA40, and BA24 homogenates from DLB, PDD, AD, and normal control samples using the fluorogenic substrate assay. Activities are expressed as fluorescence units (FU)/mg protein/hour. A) chymotrypsin-like activity measurement in BA9; the activities values for the control group were significantly higher than the PDD ($p = 0.004$, $n = 12$), DLB ($p = 0.013$, $n = 11$) and AD ($p = 0.001$, $n = 6$) groups. The ANOVA for chymotrypsin-like activity measurement in BA9 (one-way ANOVA, $F = 7.897$, d.f. = 3 and 34, $p = 0.001$; Bonferroni post hoc test). B) chymotrypsin-like activity in BA40; the activities values for the control group (n

=13) were significantly higher than the PDD (p = 0.001, n = 10), DLB (p = 0.001, n = 9) and AD (p = 0.001, n = 12) groups. The ANOVA for chymotrypsin-like activity measurement in BA40 (one-way ANOVA, F = 30.033, d.f. = 3 and 40, p = 0.001; Bonferroni post hoc test) C) chymotrypsin-like activity measurement in BA24; there was a significant difference between the PDD (p = 0.015, n = 9) and AD (p = 0.044, n = 9) groups compared to the control (n = 13)(one-way ANOVA, F = 4.664, d.f. = 3 and 39, p = 0.007; Bonferroni post hoc test).

5.2.3 Correlations between proteasome activity and expression level of RPT6 subunit

To test whether or not proteasome activity was associated with the protein levels of the proteasome subunits, Pearson and Spearman's rank correlation was determined between PGPH- and chymotrypsin-like activities, which were measured using synthetic peptide substrate, and the semi-quantitative protein values of RPT6, α6, and α3, which were measured using Western blot. The proteasome regulatory particle RPT6 was the only subunit associated with proteasome activity. In BA9, significant positive correlations were found between RPT6 and both chymotrypsin-like activity (Rs .418, p = .009, n = 38) and PGPH-like activity (Rs .363, p = .025, n = 38). While in BA40, there was a significant positive correlation with only chymotrypsin-like activity (Rs .409, p = .006, n = 44) (Figure 5-19).

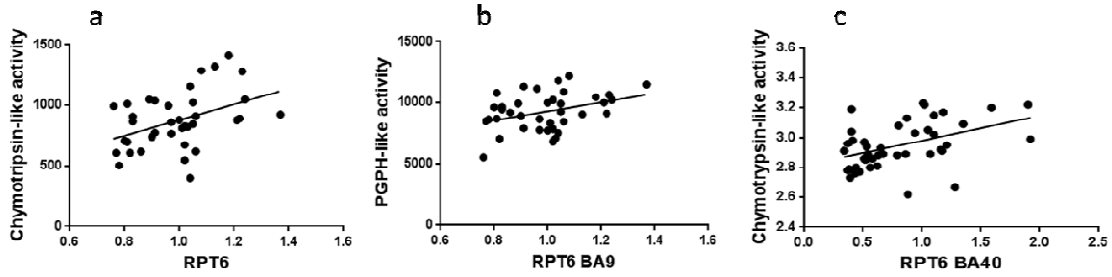


Figure 5-20: Correlations between proteasome activity and expressions level of RPT6 subunit.

Scatterplots of correlations between the expression level of RPT6 proteasome subunit relative intensity, determined by semi-quantitative Western blot analysis, and proteasome activity, measured using fluorogenic substrate assay in DLB, PDD, AD, and age-matched control samples: a) Significant positive correlations between RPT6 and chymotrypsin-like activity in BA9 (Rs .418, p = .009, n = 38); b) significant positive correlations between RPT6 and PGPH-like activity in BA9 (Rs .363, p = .025, n = 38); and c) significant positive correlations between RPT6 and chymotrypsin-like activity in BA40 (Rs .409, p = .006, n = 44).

Table 5-2: Summary of the results.

		RPT6	α-3	α-6	Chymotrypsin-like	PGPH-like
BA9	PDD	↓	→	→	→	↓
	DLB	↓	↑	→	→	↓
	AD	↓	→	↓	↓	↓
BA24	PDD	→	↓	↑	↓	↓
	DLB	↓	→	↑	↓	↓
	AD	↓	→	→	↓	↓
BA40	PDD	→	↑	→	↓	↓
	DLB	↓	→	→	↓	→
	AD	↓	→	→	↓	↓

Downward arrow (↓) indicated decreased, upward arrow (↑) indicated increased and right arrow (→) indicates unchanged.

5.3 Discussion:

These studies have shown a reduction in DLB, PDD and AD in a component protein (RPT6 ATPase 19S regulatory subunit) of proteasomes that is important in ridding the brain of damaged proteins and this reduction is related to the presence of more α-synuclein and AD pathology. Moreover, enzymatic activities related to proteasomes are down-regulated in all three groups and associated with the reduced RPT6 level.

5.3.1 Proteasome subunit expression

The main finding of the present study was the reductions of the RPT6 ATPase 19S regulatory subunit in DLB and AD in the frontal lobe neocortical area BA9, anterior cingulate BA24 and temporal cortex BA40. In PDD, reduced reduction in RPT6 level was found only in BA9. Interestingly, the reduction in RPT6 levels was associated with changes in proteasome proteolytic activities. There were positive correlations between RPT6 levels

and both chymotrypsin- and PGPH-like activity in BA9 but only with chymotrypsin-like activity in BA40. Neither α-3 nor α-6 was correlated with any of the proteolytic activity measured in this study. α-3 and α-6 were found to be altered differently depending on the brain region and the diagnostic group. In BA 9, significant reduction in α-6 was found only in the AD group, whereas upregulation was found in the level of α-3 in DLB in comparison with the controls. In BA40, significant upregulation was found in the level of α-3 in PDD; in contrast, the α-6 level did not differ significantly between the diagnostic groups and the controls. In BA24, increases in the protein expression level of α-6 were found in both PDD and DLB but not AD compared to the control group, with reduction in the level of α-3 in PDD only. Moreover, analysis of the enzymatic activities revealed reduction in both chymotrypsin- and PGPH-like activity in DLB, PDD and AD compared to the controls.

The significant reductions in RPT6 were more or less similar between DLB and AD in BA9 and 24, a greater reduction were observed in AD in BA40 compared to DLB ; in PDD, the reduction was significant only in BA9. RPT6 is one of the six ATPase subunits (RPT 1–6) of the 19S regulatory complex; it is a 45kDa subunit. Degradation of ubiquitinated substrate proteins by the 26S proteasome is dependent upon ATP (Rivett, 1989), which binds to the six ATPase subunits of the 19S regulatory complex. All six of the ATPase subunits contain the same substantial main functional domains: an N-terminus coiled-coil domain important for formation of the 19S base, and a C-terminus ATPase domain that is involved in ATP-dependent substrate unfolding and 20S CP opening (Marques et al., 2009). These ATPases provide the energy necessary for the degradation of multi-ubiquitin conjugated proteins by the 26S proteasome, and it is also believed that ATPase subunits participate in the substrate-unfolding step of the degradation pathway

(Strickland et al., 2000). It has been shown previously that the 19S RPT6 expression level decreased while α-synuclein was increased in mouse PD-like models (Liu et al., 2008) and a study of 9 PD cases, 7 PDD and 9 controls revealed a decrease in the 19S RPT3/S6 subunit in the inferior frontal gyri of PDD although the expression was similar in control and PD (Wills et al., 2010).

Calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase A (PKA) have been shown to phosphorylate the proteasome regulatory subunit RPT6 at Serine-120 and stimulate the proteasome function in vitro (Djakovic et al., 2012, Djakovic et al., 2009, Zhang et al., 2007). Consistent with this, Jarome et al. showed that phosphorylation of RPT6 by CaMKII increased proteasome activity in vivo and proteasome activity was necessary for long-term memory function (Jarome and Helmstetter, 2013, Jarome et al., 2013). Another significant finding is that RPT6 phosphorylation enhances proteolysis by promoting the assembly of the 26S proteasome, and RPT6 dephosphorylation promoted the dissociation of 26S into 19S and 20S components (Satoh et al., 2001). Previous studies in yeast have demonstrated that reductions in the expression of any single ATPase subunit are lethal, highlighting the importance and non-redundancy of these subunits in normal functioning of proteasomes (Rubin et al., 1998). Inactivation of the 19S regulatory particle (RPT2) subunit prevented the formation of the 26S proteasome, leaving the 20S proteasome subunit, which is ubiquitin-independent, unaffected (Bedford et al., 2008). Therefore, the reduction in RPT6 subunit expression identified in DLB, PDD and AD patients in three brain regions and the associated reduction in proteasome activity confirms and extends previous studies by demonstrating this phenomenon in the human brain and suggests that reduced subunit expression may directly lead to proteasome

impairment. The reason for the reduction in RPT6 ATPase subunit expression remains unexplained. However, it is possible that the reduction could be related to oxidative stress; indeed, proteasome subunits were demonstrated to be sensitive to oxidative stress (Yang et al., 2007, Zeng et al., 2006b). RPT6 was decreased in DJ-1-deficient mice treated with Paraquat (Yang et al., 2007). Furthermore, Sun et al. reported that proteasome subunits (Rpt5, Rpn10 and Rpn2) can be cleaved by caspase-3 following caspase activation during apoptosis; associated with the cleavage of these subunits, they found decreased proteasome activity (Sun et al., 2004).

The reduction of proteasome subunit α-3 in PDD subjects in the anterior cingulate (BA24) was demonstrated to be consistent with previous studies that have shown a selective loss of α-subunits within dopaminergic neurons of the substantia nigra of patients with PD (McNaught et al., 2003, McNaught et al., 2002c). However, 20S proteasome α-3 subunits protein levels were upregulated in DLB and PDD in BA9 and BA 40 respectively. It has been reported that the core particle (CP) of the yeast proteasome was autoinhibited by the N-terminal tails of the outer (alpha) ring subunits (Groll et al., 2000). Upregulation of the 20S proteasome α-3 subunits protein levels may promote autoinhibition of the core particle by the N-terminal tails of the α-3 subunits. Upregulation of α-3 has not been reported before in post-mortem brain tissue, as most of the studies investigated the level of the 20S core α-subunits, not the individual subunits (α1-α-7). α-3 did not show any significant difference between AD compared to the control in BA9, BA24 and BA40. This suggests that there was no relationship between AD pathology and the alteration in α-3 levels.

The level of the 20S proteasome α-6 subunits did not show any significant difference between PDD, DLB and the matched control within the frontal cortex (BA9) and the parietal cortex (BA40). The expression levels of the proteasome subunits α-6 were significantly increased in the anterior cingulate (BA24) in both DLB and PDD groups. This may suggest that the limbic BA24 region is affected earlier than the neocortical and increasing proteasome proteins levels may be one of the earliest processes that lead the compensatory mechanism to the formation of LBs. In contrast, another study has shown reduced α-subunit expression in the cortex of DLB patients (MacInnes et al., 2008) and also in PD SN (McNaught et al., 2002c). The variation in the expression levels between α-6 and α-3 in the same brain region for the same group suggests that each individual α-subunit is present in the cell at different rates. This suggests that the reduction found in the α-subunit in previous studies could result from changes in any of the α-subunits (α1−α7). On the basis of the current data, investigation of each individual subunit of proteasomes will provide more knowledge about the proteasome and will help in understanding the process that leads to the pathology of LB and AD by determining the subunit that most commonly associates with the impairment of proteasome function. To the best of our knowledge, this is the first study which aimed to investigate the expression level of the α3 and α6 proteasome subunits in LBD and AD. AD cases had significantly lower levels of the α-6 subunit in BA9. This suggests that there is a substantial relationship between the AD pathology and α-6 in this brain region. Considering the importance of α-subunits in proteasome stability, McNaught and colleagues have suggested that changes in the expression of α-subunits could cause proteasomes to become unstable, which may ultimately lead to the impairment of the

proteasome function (McNaught et al., 2002c). However, as there was no correlation between either α-3 or α-6 and any proteolytic activity, this explanation seems unlikely.

Immunohistochemistry staining was undertaken to examine proteasome subunit expression in an anatomical context. Overall the preliminary studies confirmed the findings from Western blotting and provided more information about the localisation of the proteasome subunits within the cell. The 19S regulatory RPT6 subunit presents mainly in the nucleus, where α-3 and α-6 resulted in cytoplasmic and nuclear staining. The appearance of RPT6 only in the nuclei is in agreement with other studies showing the presence of RPT6 and other ATPase subunits within the nuclei in non-diseased human and rat brains (Adori et al., 2006, Russell et al., 1999b). The nuclear localisation of RPT6 is unexplained; it could be due to unassembled subunits or associated with the 19S complex or in consistency with other findings that some regulatory ATPases of the 19S complex function independently of proteolysis in nucleotide excision repair (Muratani and Tansey, 2003, Russell et al., 1999a). The observation that α-3 and α-6 of the 20S immunoreactivity are both nuclear and cytoplasmic is in agreement with previous studies on 20S cellular localisation (Adori et al., 2006, Mengual et al., 1996). In contrast, other studies have reported the appearance of α-4 and α-6 only in the nuclei of tyrosine hydroxylase (TH)-positive cells in the SN (Bukhatwa et al., 2010b).

5.3.2 Proteolytic activity of 20S proteasomes

Compared to control groups, reduction of proteasome catalytic activities and chymotrypsin- and PGPH-like activities were found in all three regions of BA9, BA24 and BA40, in DLB, PDD and AD patients. Surprisingly, there was a significant difference between the PDD and DLB groups only in BA24 in both chymotrypsin- and PGPH-like activities, with PDD patients having smaller mean values compared to the DLB subjects. The same finding was indicated by comparing the AD and DLB groups; the mean values for the AD group were significantly lower than for the DLB group. The cause of the distinct difference between DLB and PDD or DLB and AD is unknown, and although upregulation occurred in the expression level of α-6 in PDD and DLB and in α-3 in DLB and AD in BA24, there was no association between the α-subunits and proteasome activity. It has been reported in AD that reduction in proteasome activity was not associated with alteration in the proteasome α-subunit (Keller et al., 2000). The proteasome catalytic activity in AD brains was reported to be lower compared to controls (Keller et al., 2000, Lopez Salon et al., 2000). Keller and colleagues found a reduction in proteasome activity in the hippocampus, parahippocampal gyrus, superior and middle temporal gyri and inferior parietal lobule of AD patients compared with controls (Keller et al., 2000). Impairment of proteasome function was previously reported to occur specifically in the substantia nigra pars compacta (SNc) in PD brains (McNaught et al., 2003, McNaught and Jenner, 2001).

It is possible that the reduction of proteasome activity is due to the decreased in the RPT6 level as there was a correlation between lower RPT6 protein levels and proteasome activity in BA9 and BA40 and due to the important role of RPT6 in promoting the activity of proteasomes. The reduction of the proteolytic activity could also arise from the

blockading of the entry pore to the 20S proteasome by protein aggregates, such as α-synuclein, which may in turn impede degradation of this and other proteins (Emmanouilidou et al., 2010, Liu et al., 2005, Zhang et al., 2008). Inhibition of the 26S proteasome with soluble oligomeric species of mutant and wild-type α-synuclein in PC12 cells has been demonstrated (Emmanouilidou et al., 2010). It is clear that these oligomers have to be degraded by the proteasomes, as they accumulate when proteasome function is inhibited. Proteasome inhibitors have been reported to induce α-synuclein aggregation and Lewy body-like inclusions, leading to neuronal loss among in vitro and in vivo models (Bedford et al., 2008). However, it is not clear whether the aggregation results from the impairment of the proteasomes or vice versa (Lansbury and Lashuel, 2006). Results from experimental studies have indicated that inhibition of the proteasomes causes the formation of aggregation (Bedford et al., 2008, McNaught et al., 2002b) and protein aggregation inhibits the proteasome activity (Emmanouilidou et al., 2010).

In view of the above, our data strongly suggest that the activation of the proteasomes may be a target to slow down the disease progression in DLB and PDD. Recently, Medina and his colleagues have found that methylene blue (MB) increases the clearance of Aβ in a mouse model of AD by increasing the proteasome activity (Medina et al., 2011). The effect of MB on α-synuclein aggregation remains to be determined, and it is strongly suggested that proteasome activation may reduce Lewy body formation

5.4 Conclusion

The present study has demonstrated that the activity of RPT6 ATPase 19S regulatory subunit protein levels of proteasomes in PDD/DLB and AD were decreased and correlated with proteasome activity. In contrast, the proteasome α-6 subunits were increased in the anterior cingulate and remain unchanged in the prefrontal and parietal cortex except for the AD group in BA9. Immunostaing did not indicate any abnormal accumulation of 26S proteasome subunits in BA9, BA40 and BA24. It will be very useful for a future study to use double staining of the proteasome subunits and the pathologic protein deposits (α-synuclein, Aβ, and tau) to detect any relationship and co-localiztion with both AD and LBs pathology. Further studies on the functions and interactions of the proteasome system subunits are needed to elaborate why proteasome α-3 and α-6 subunits show a parallel increase in some patient groups in single different areas of the brain (but not all), whereas RPT6 has an inverse pattern (i.e. decrease in the prefrontal, parietal cortex and in anterior cingulate). This may imply that selective (i.e. targeting specific subunits) inhibition and activation of subunits could have better therapeutic potential in LBDs rather than non-selective modification of the UPS activity.

Chapter 6 Expression levels of lysosomal proteins and cathepsin-D activity in human post-mortem brains of DLB, PDD, AD and age-matched control

6.1 Introduction

In the previous chapter, evidence was presented for a reduction in specific components of the proteasome system. Having examined the status of the proteasome in LBD it was now important to evaluate the lysosomal pathway.

Two lysosomal markers, cathepsin D and lysosomal-associated membrane protein 1 (LAMP1) were chosen for investigation. Cathepsin D is a member of a large hydrolytic enzyme family in the lysosome called cathepsins, derived from the Greek term meaning 'to digest' (Willstatter and Bamann, 1929). Cathepsin D is an aspartic protease, found in a high concentration within the lysosomes, and has a major role in apoptosis (Minarowska et al., 2007). Cathepsin D has been found to be involved in degradation of beta-amyloid (Hamazaki, 1996, McDermott and Gibson, 1996). Cathepsin D cleaves α-synuclein in vitro and its deficiency results in increased α-synuclein accumulation (Qiao et al., 2008). For more details about lysosomal pathway and its relevance to synucleinopathies, also cathepsin D and its role in α-synuclein degradation see (Chapter 1 section 1.10).

LAMP1 is one of the major components of the lysosome; it is a glycoprotein expressed extensively in the lysosomal membrane. LAMP1 is widely used as a lysosomal marker (Eskelinen, 2006, Kurzawa-Akanbi et al., 2012) and is found to be up-regulated in AD (Barrachina et al., 2006b) and down-regulated in PD (Chu et al., 2009, Dehay et al., 2010).

In this chapter we aimed to: 1) Investigate the expression level of cathepsin D and LAMP1, in regions of the human post-mortem brain from LBD and AD patients, and in controls using Western blot. 2) Determine if there is a correlative relationship between the two major proteolytic pathways—the ubiquitin-proteasome pathway and the lysosomal

pathway. 3) Investigate whether alteration of the expression level of the lysosomal markers in different brain areas correlated with semi-quantitative scores of AD and LBD pathology.

6.2 Result

6.2.1 Expression levels of lysosomal proteins in human post mortem brains of DLB, PDD, AD, and age-matched control

6.2.1.1 Frontal cortex – Brodmann area 9

6.2.1.1.1 LAMP1 expression

The immunoblot analysis were done using anti-LAMP1 antibody as a marker for the lysosome. Anti-LAMP1 was a rabbit polyclonal IgG (ab24170) that recognised a band of about 120 kDa and used before by (Kren et al., 2009)

No difference in expression level of LAMP1 was observed in the PDD, DLB, or AD groups compared with control subjects. Statistical analysis was performed using One-way ANOVA ($F = 0.443$, d.f. = 3 and 107, $p = 0.723$; Bonferroni post hoc test), results are shown in (Figure 6-1).

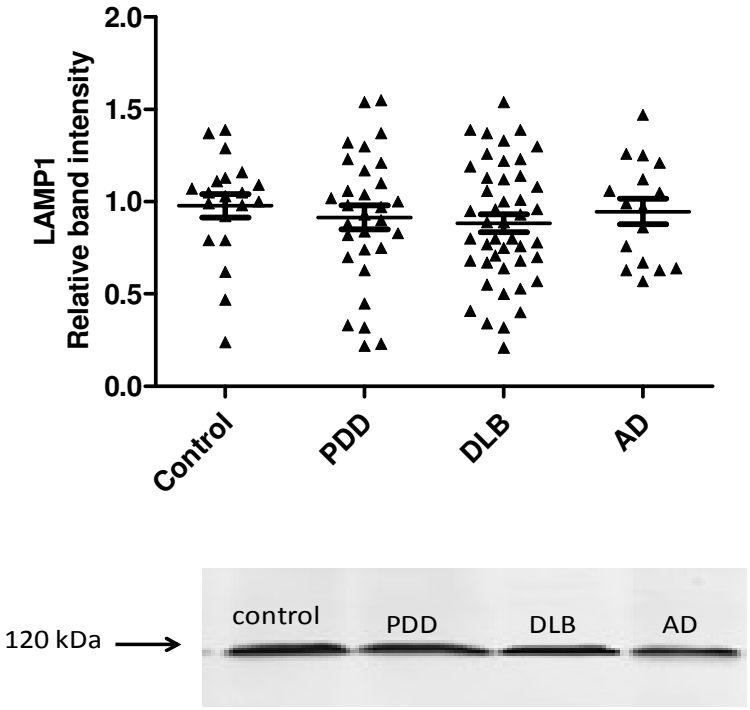


Figure 6-1: LAMP1 values, from semi-quantitative Western blotting in PDD, DLB, AD, and control in frontal cortex (BA9).

Scatter plot of LAMP1 relative intensity determined by semi-quantitative Western blot analysis in DLB, PDD, AD, and age-matched control samples. Statistical analysis was performed using One-way ANOVA ($F = 0.443$, d.f. = 3 and 107, $p = 0.723$; Bonferroni post hoc test): Statistical analysis confirmed there was no significant difference between diagnostic groups for LAMP1values. The horizontal bars within the data points in the graphs represent the mean values. **B)** Representative Western blot showing levels of the LAMP1 in DLB, PDD, AD, and control.

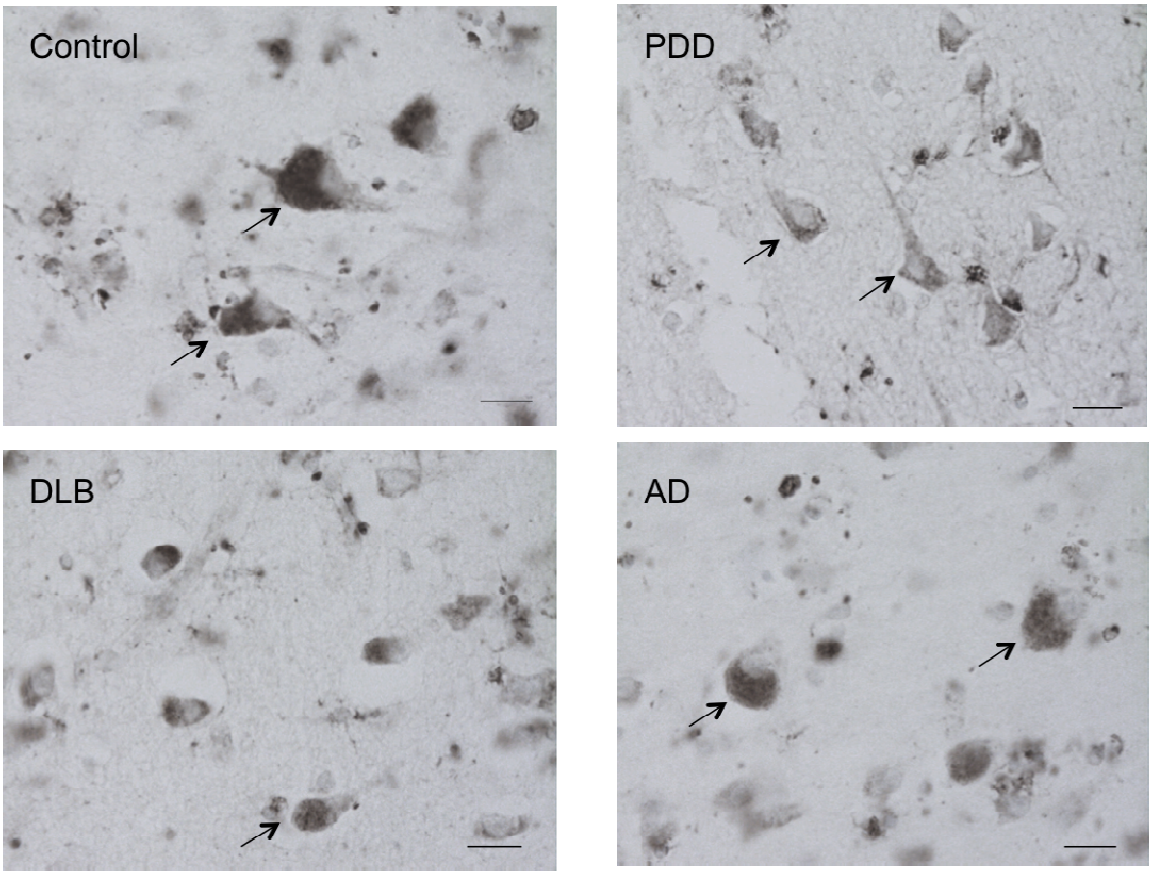


Figure 6-2: Photomicrographs of LAMP1 staining in the frontal cortex region of the post-mortem human brain from control and dementia cases.

The photomicrograph illustrates immunohistochemistry for LAMP1 in the frontal cortex of post-mortem brain from control, PDD, DLB and AD. The photomicrographs demonstrate the level of cytoplasm and nucleus staining was the same for all for groups. Scale bar represents 15 microns.

6.2.1.1.2 Cathepsin D expression

The immunoblot analysis were done using anti- cathepsin D as a marker for a major lysosomal enzyme involved in α-synuclein degradation by autophagy (Sevlever et al., 2008). Anti-cathepsin D was a goat polyclonal IgG (sc-6486) that recognised a band of about 52 kDa and used before by (Fan et al., 2010)

Cathepsin D expression was similar between individual cases and was not significantly altered across any of the diagnostic groups. Statistical analysis was performed using One-way ANOVA ($F = 1.652$, d.f. = 3 and 106, $p = 0.182$; Bonferroni post hoc test), result shown in (Figure 6-2).

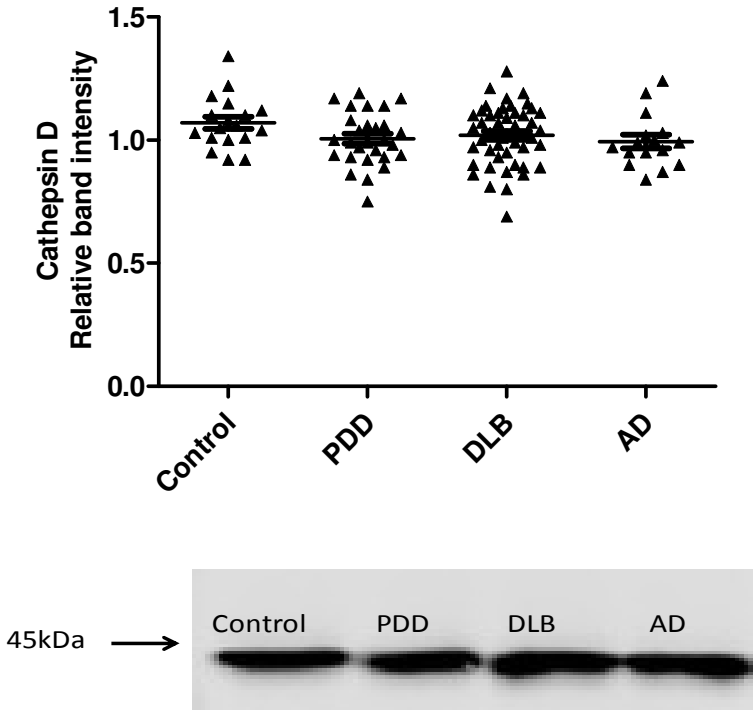


Figure 6-3: cathepsin D values, from semi-quantitative Western blotting in PDD, DLB, AD, and control in frontal cortex (BA9).

Scatter plot of cathepsin D relative intensity determined by semi-quantitative Western blot analysis in DLB, PDD, AD, and age-matched control samples. Statistical analysis was performed using One-way ANOVA ($F = 1.652$, d.f. = 3 and 106, $p = 0.182$; Bonferroni post hoc test): Statistical analysis confirmed there was no significant difference between diagnostic groups for cathepsin D values. The horizontal bars within the data points in the graphs represent the mean values. **B)** Representative Western blot showing levels of the cathepsin D in DLB, PDD, AD, and control.

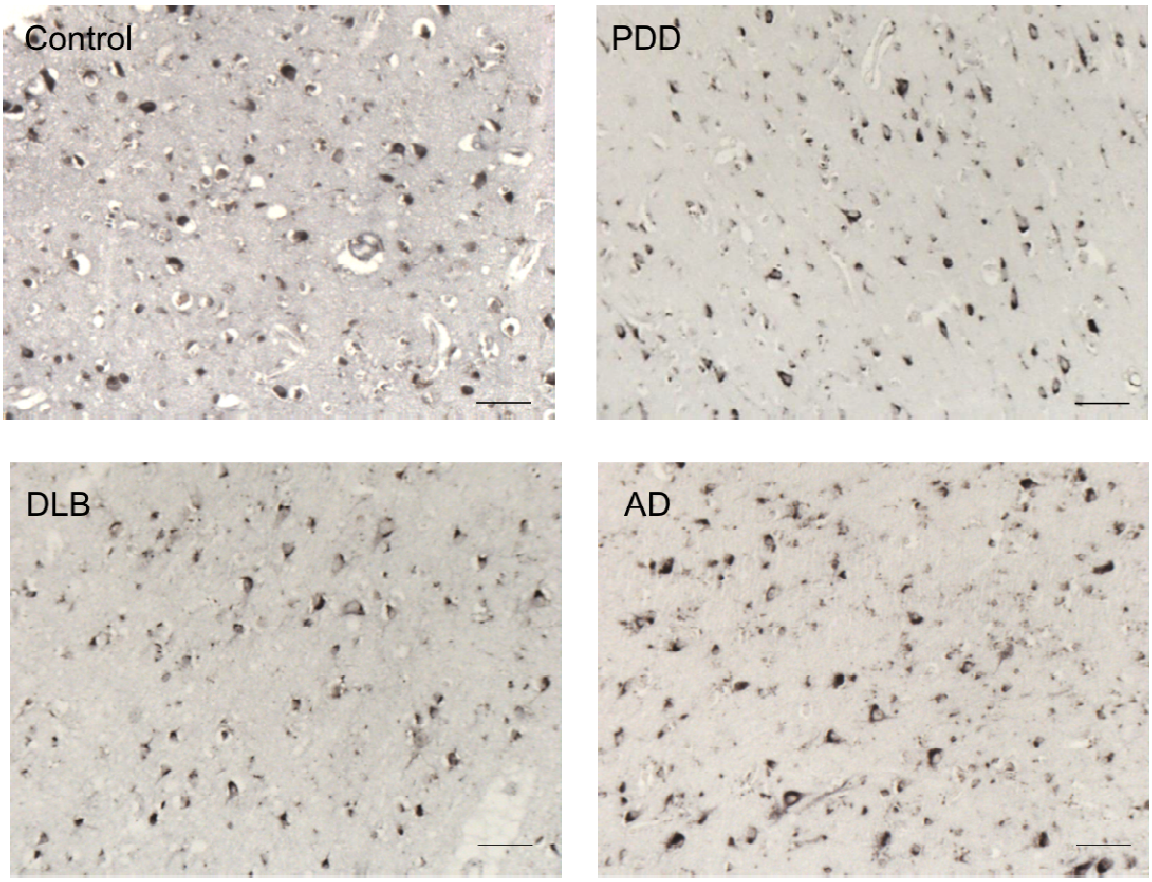


Figure 6-4: Photomicrographs of cathepsin D staining in the frontal cortex region of the post-mortem human brain from control and dementia cases.

The photomicrograph illustrates immunohistochemistry for cathepsin D in the frontal cortex of post-mortem brain from control, PDD, DLB and AD. The photomicrographs demonstrate the level of cytoplasm and nucleus staining was the same for all for groups. Scale bar represents 100 microns.

6.2.1.2 Parietal cortex—Brodmann area 40

6.2.1.2.1 LAMP1

A significant reduction in LAMP1was seen in DLB (29%, p = 0.001, 0.814 ± 0.04, n = 43) compared to the controls (1.148 ± 0.04, n = 20) (one-way ANOVA, F = 6.24, d.f. = 3, 104; p = 0.001; Bonferroni post hoc test) (Figure 6-3).

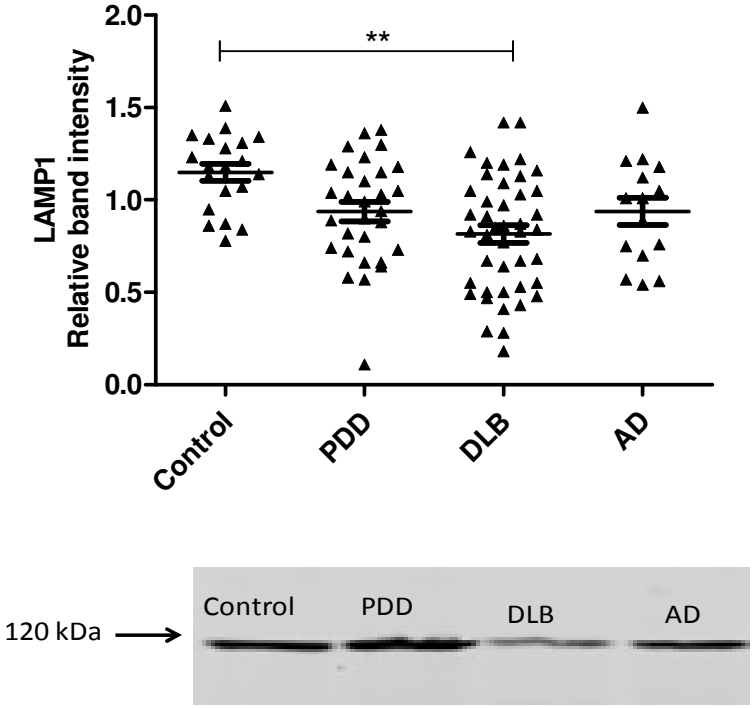


Figure 6-5: LAMP1 values, from semi-quantitative Western blotting in PDD, DLB, AD, and control in parietal cortex (BA40).

A) Scatter plot of LAMP1 relative intensity determined by semi-quantitative Western blot analysis in DLB, PDD, AD, and age-matched control samples. Statistical analysis was performed using One-way ANOVA (F = 6.242, d.f. = 3 and 104, p = 0.001): mean LAMP1 values for the control group (n = 20) were significantly higher than the DLB (p = 0.001, n =

43), and did not reach the significant compare to the PDD group (p = 0.069, n = 30) and AD (p = 0.193, n = 16) groups. The horizontal bars within the data points in the graphs represent the mean values. **B)** Representative Western blot showing levels of LAMP1 in DLB, PDD, AD, and control.

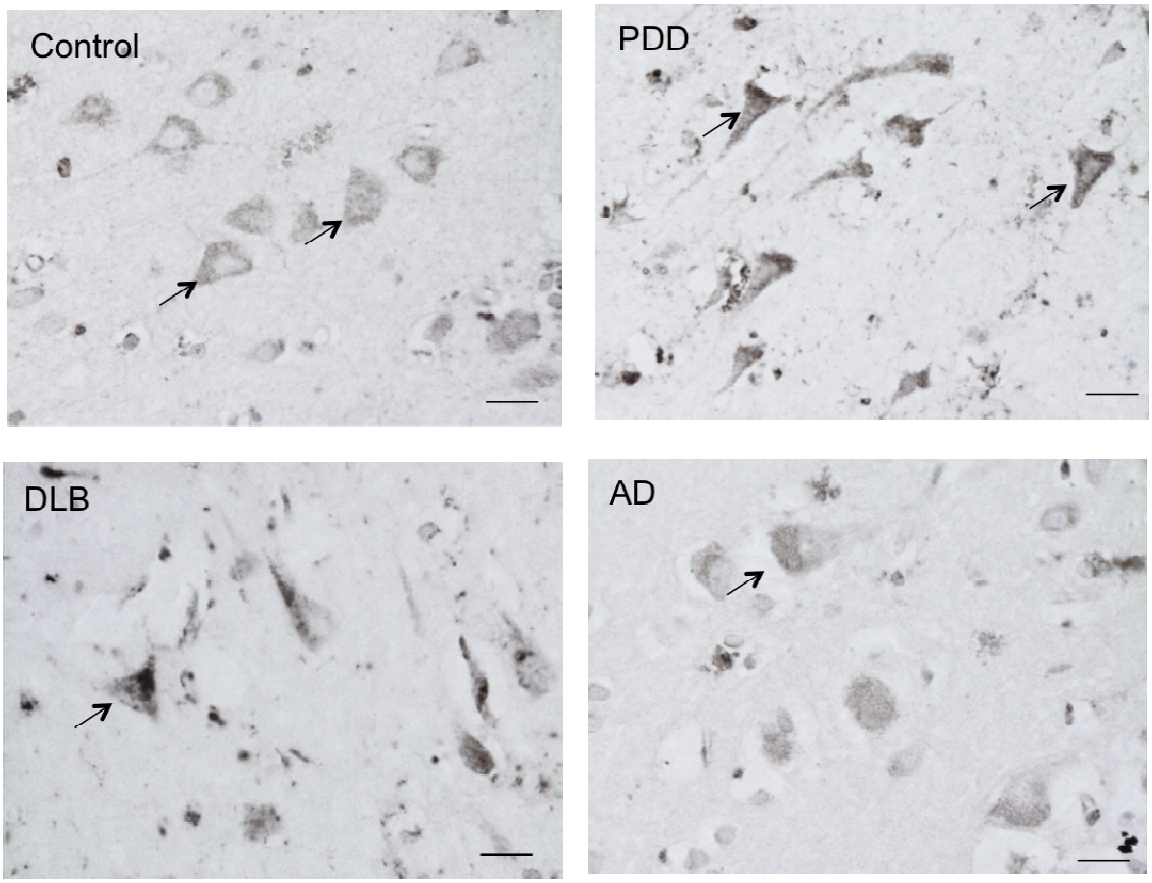


Figure 6-6: Photomicrographs of LAMP1 staining in parietal cortex (BA40) of the post-mortem human brain from control and dementia cases.

The photomicrograph illustrates immunohistochemistry for LAMP1 in parietal cortex (BA40) of post-mortem brain from control, PDD, DLB and AD. The photomicrographs demonstrate a strong staining in DLB and PDD as compared to control, and AD cases. Scale bar represents 15 microns.

6.2.1.2.2 Cathepsin D

There was no significant difference in the mean levels of cathepsin D between either DLB or AD patient groups compared to the controls according to the post hoc test (Figure 6-4, $p>0.05$). However, mean cathepsin D levels in patients with PDD were significantly elevated by 21% compared to AD ($p = 0.009$, $n = 16$) and by 20% compared to controls groups ($p = 0.005$ $n = 22$), ANOVA ($F = 5.336$, d.f. = 3 and 114, $p = 0.002$; Bonferroni post hoc test).

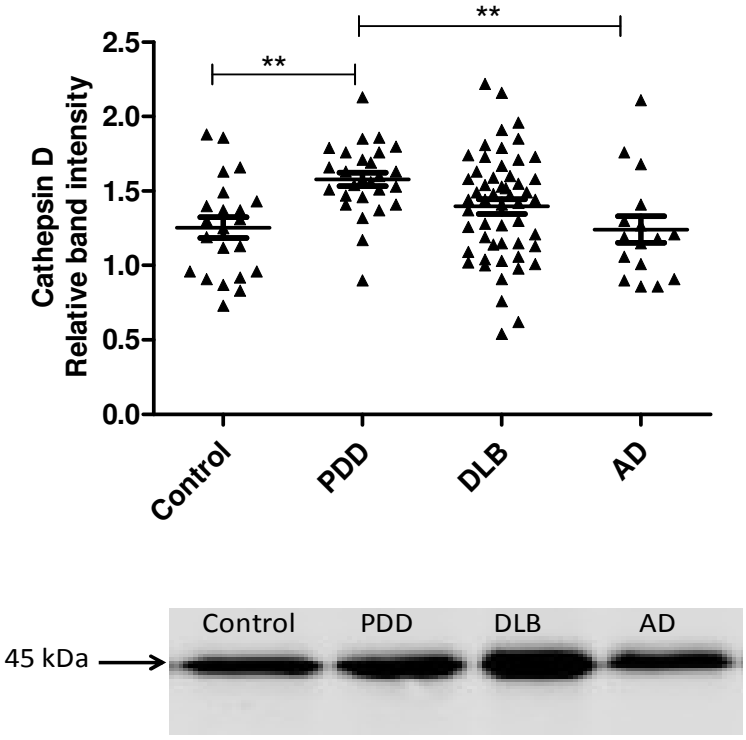


Figure 6-7: cathepsin D values, from semi-quantitative Western blotting in PDD, DLB, AD, and control in parietal cortex (BA40).

A) Scatter plot of cathepsin D relative intensity determined by semi-quantitative Western blot analysis in DLB, PDD, AD, and age-matched control samples. Statistical analysis was performed using One-way ANOVA (F = 5.336, d.f. = 3 and 114, p = 0.002; Bonferroni post hoc test): mean cathepsin D values for the for the PDD (n = 27) group were significantly higher than the control (p = 0.005, n = 22), AD (p = 0.009, n = 16) group. The horizontal bars within the data points in the graphs represent the mean values. **B)** Representative Western blot showing levels of the cathepsin D in DLB, PDD, AD, and control.

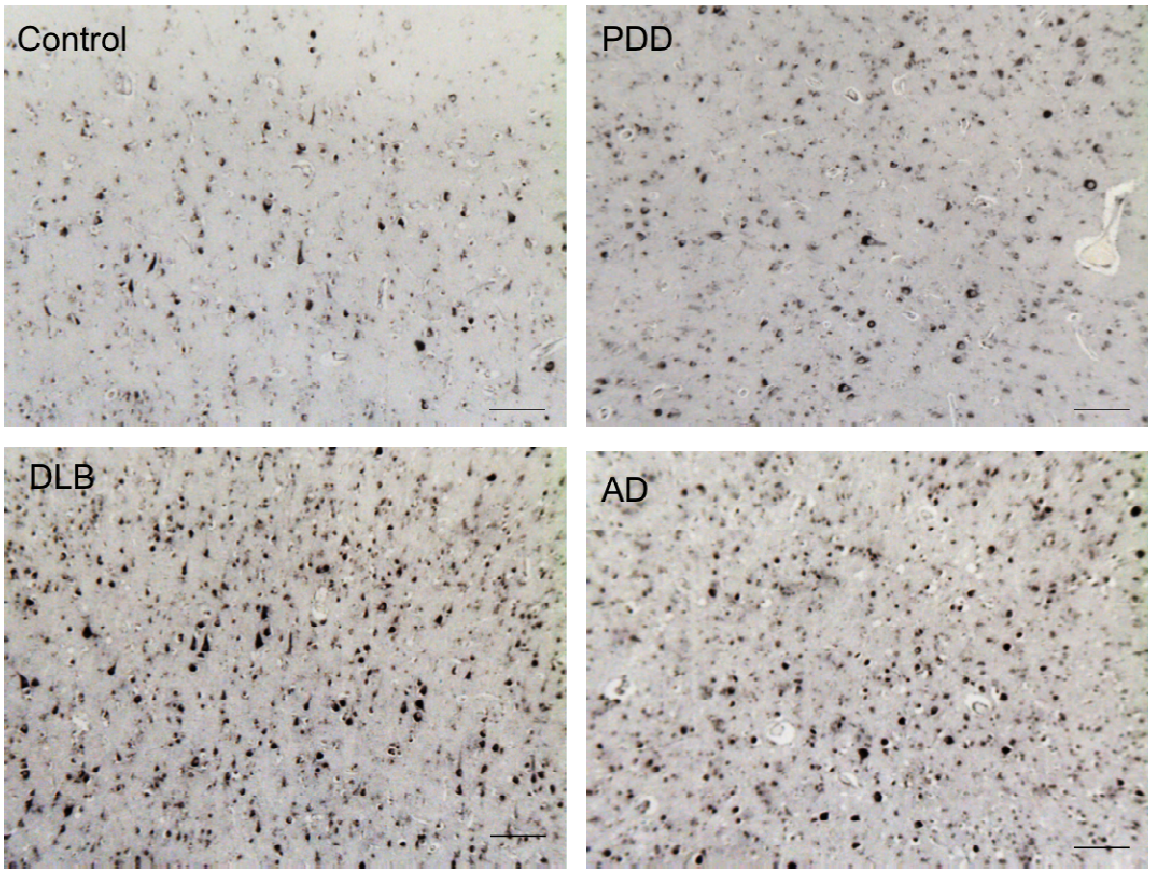


Figure 6-8: Photomicrographs of cathepsin D staining in the in parietal cortex (BA40). region of the post-mortem human brain from control and dementia cases.

The photomicrograph illustrates immunohistochemistry for cathepsin D in parietal cortex (BA40) of post-mortem brain from control, PDD, DLB and AD. The photomicrographs demonstrate the level of staining was the same for all for groups. Scale bar represents 100 microns.

6.2.1.3 Anterior cingulate gyrus Brodmann area 24

6.2.1.3.1 LAMP1

Significant reduction in the lysosomal-associated membrane protein 1 (LAMP1) was seen in DLB (51%, $p = 0.001$, 0.331 ± 0.04) and PDD, (46%, $p = 0.001$, 0.362 ± 0.05) compared to the controls (0.679 ± 0.07) (one-way ANOVA, $F = 8.149$, d.f. = 3, 114; $p = 0.001$; Bonferroni post hoc test) (Figure 6-5). The post hoc test revealed that there was no significant difference between the control and AD groups ($p > 0.05$).

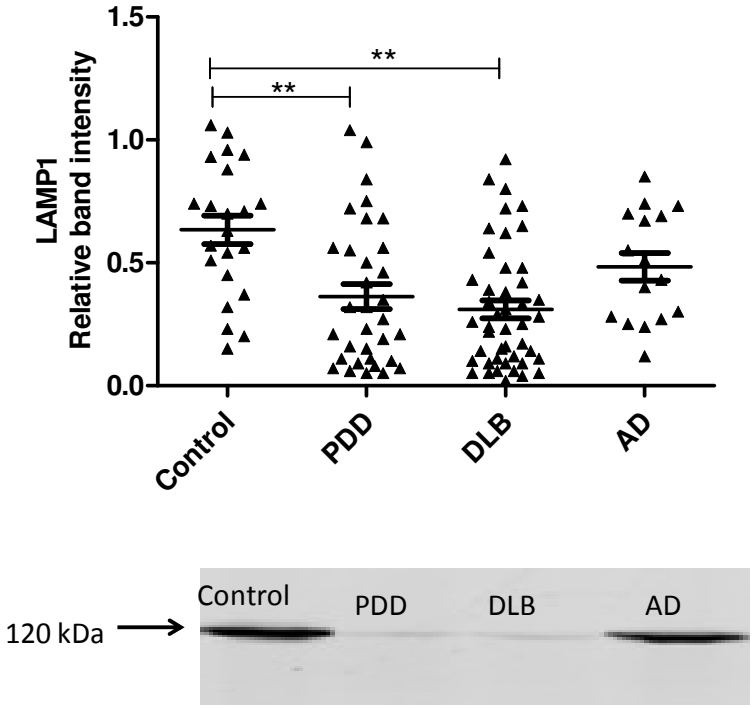


Figure 6-9: LAMP1 values, from semi-quantitative Western blotting in PDD, DLB, AD, and control in cingulate cortex (BA24).

A) Scatter plot of LAMP1 relative intensity determined by semi-quantitative Western blot analysis in DLB, PDD, AD, and age-matched control samples. Statistical analysis was performed using One-way ANOVA ($F = 8.149$, d.f. = 3 and 114, $p = 0.001$): mean LAMP1 values for the control group ($n = 23$) were significantly higher than the DLB ($p = 0.001$, $n = 33$) and PDD groups ($p = 0.001$, $n = 46$). The horizontal bars within the data points in the graphs represent the mean values. **B)** Representative Western blot showing levels of LAMP1 in DLB, PDD, AD, and Control.

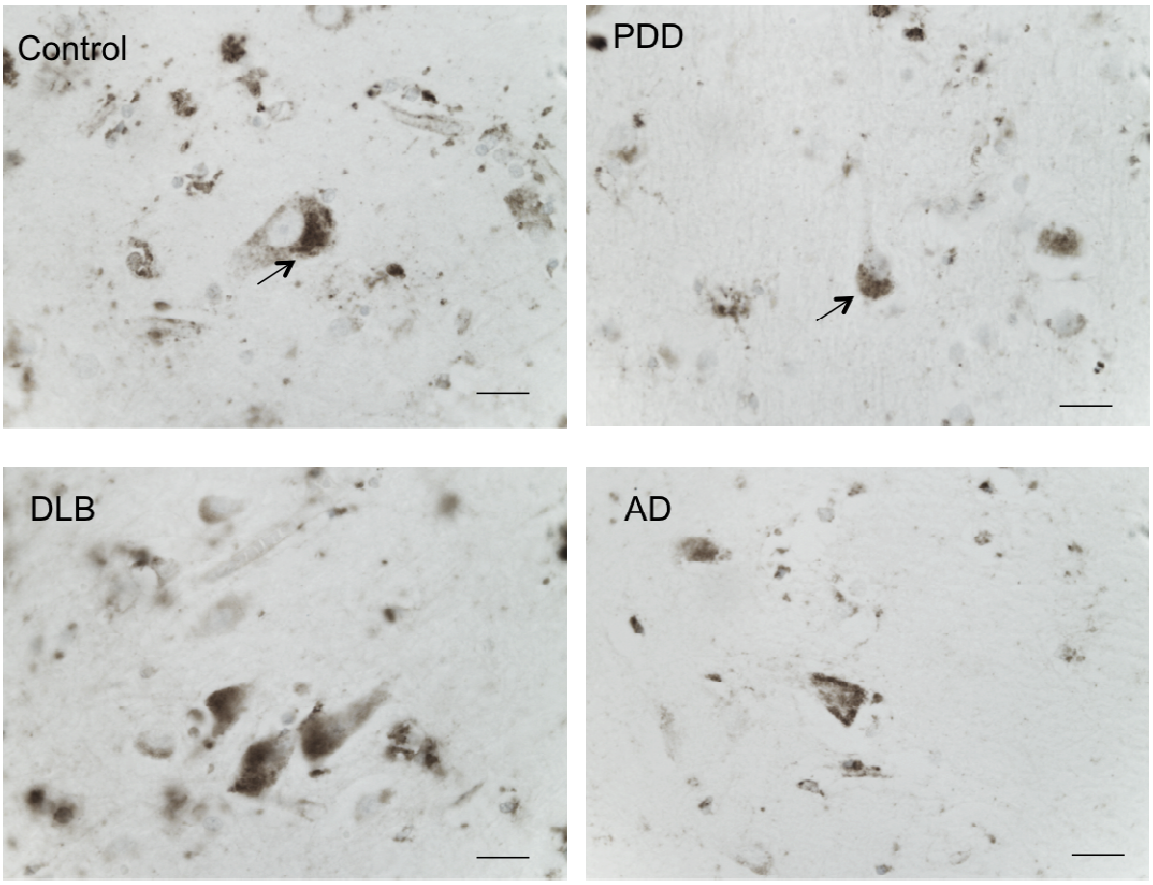


Figure 6-10: Photomicrographs of LAMP1 staining in in cingulate cortex (BA24) of the post-mortem human brain from control and dementia cases.

The photomicrograph illustrates immunohistochemistry for LAMP1 in cingulate cortex (BA24) of post-mortem brain from control, PDD, DLB and AD. The photomicrographs demonstrate the level of staining was the same for all for groups. Scale bar represents 15 microns.

6.2.2 Correlations between lysosomal markers and proteasome activities or proteasome subunits expression

To test whether or not lysosomal markers are associated with proteasome activity and proteasome subunits expression, Pearson and Spearman's rank correlation was determined between semi-quantitative protein values of LAMP1 and cathepsin D and between PGPH- and chymotrypsin-like activities. These activities were measured using synthetic peptide substrate and the semi-quantitative protein values of RPT6, α6 and α3, which were measured using a Western blot. There were no significant correlations between any of the protein levels or activities of the proteasome with any of the lysosomal markers measured in this study with $P > 0.05$.

6.2.3 Correlations between lysosomal markers and pathology scores

To investigate whether the expression levels of the lysosomal markers in different brain areas were associated with semi-quantitative scores of AD and LBD pathology, linear regression analyses were conducted with the two predictors LAMP1 and cathepsin D as independent factors and semi-quantitative scores for Aβ staining (plaque pathology), phospho-tau staining (tangle pathology) and α-synuclein pathology (in forms of Lewy bodies and dystrophic lewy neurites) as dependent variables. The pathological data was based on the semi-quantitative (on a scale of 0 (none), 1 (mild), 2 (moderate) and 3 (severe/frequent)) rating of IHC degree and distribution of plaque, tangle and α-synuclein immunostaining (for more details see Chapter 2 Section 2.2.2).

Reductions in LAMP1 in BA24 (Figure 6-6) and BA40 (Figure 6-7) were associated with the α-synuclein pathology. No one relationship was detected with the tangle and plaque pathology. Analysis of variance (ANOVA) followed by Bonferroni post-hoc tests also indicated a significant difference between LAMP1 levels and α-synuclein scores in BA24 (one-way ANOVA $F = 7.088$, d.f. = 3 and 109, $p = 0.001$; Bonferroni post hoc test) (Figure 6-6) and BA40 (one-way ANOVA $F = 4.5$, d.f. = 3 and 108, $p = 0.005$; Bonferroni post hoc test) (Figure 6-7).

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.353[a]	.125	.117	1.172

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	21.714	1	21.714	15.803	.000[b]
	Residual	152.516	111	1.374		
	Total	174.230	112			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	2.069	.183		11.282	.000
	LAMP1	**-1.373**	**.345**	**-.353**	**-3.975**	**.000**

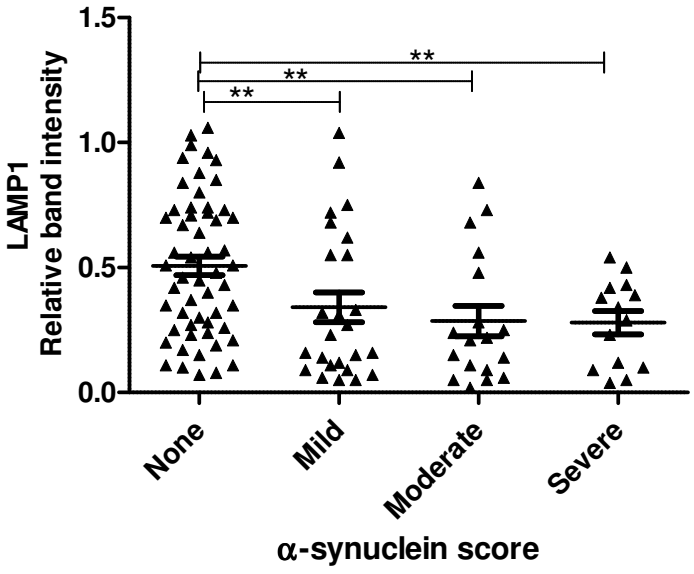


Figure 6-11: Relationship between expression levels of LAMP1 in BA24 and α-synuclein score.

Linear regression analyses were conducted with the LAMP1. In the analyses, the semi-quantitative α-synuclein were entered as dependent variables with LAMP1 values from semi-quantitative Western blotting in BA24 as independent factors. The α-synuclein scores in the dementia cases (DLB, PDD and AD) and control cases were significantly predicted by LAMP1 with (Beta = -0.353 and p = 0.001). One-way ANOVA was performed to compare semi-quantitative Western blotting values of LAMP1 and α-synuclein scoring groups. The analysis indicated a significant difference between the group with α-synuclein of none and the group with a α-synuclein score of sparse (p = 0.014), moderate (p = 0.014), or severe (p = 0.001) (one-way ANOVA F = 6.433, d.f. = 3 and 114, p = 0.001; Bonferroni post hoc test). The horizontal bars within the data points represent the mean.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.304[a]	.092	.074	.972

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	9.634	2	4.817	5.094	.008[b]
	Residual	94.560	100	.946		
	Total	104.194	102			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.295	0.447		2.898	.005
	LAMP1	**-.454**	**.143**	**-.304**	**-3.170**	**.002**
	Cathepsin D	-.009	.283	-.003	-.003	.974

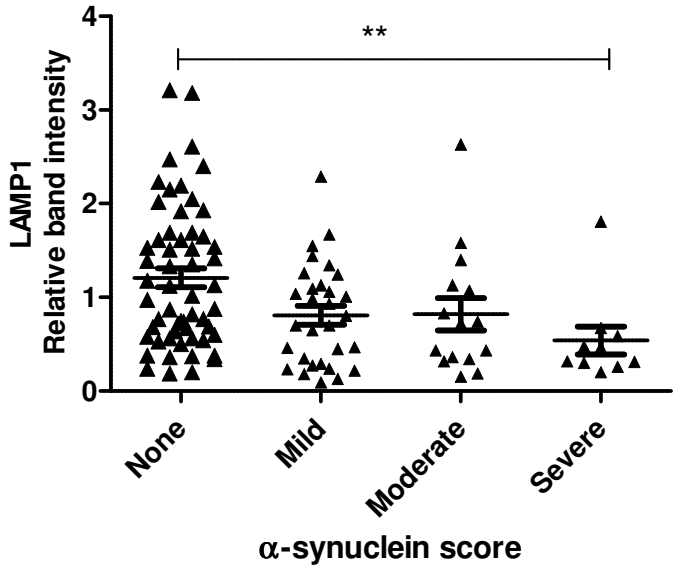


Figure 6-12: Relationship between expression levels of LAMP1 in BA40 and α-synuclein score.

Linear regression analyses were conducted with the two predictors to develop a model for the best predictor α-synuclein in BA40. In the analyses, the semi-quantitative α-synuclein were entered as dependent variables with LAMP1 and cathepsin D values from semi-quantitative Western blotting in BA40 as independent factors. The α-synuclein scores in the dementia cases (DLB, PDD and AD) and control cases were significantly predicted by LAMP1 with (Beta = -0.304 and p = 0.002). One-way ANOVA was performed to compare semi-quantitative Western blotting values of LAMP1 and α-synuclein scoring groups. The analysis indicated a significant difference between the group with α-synuclein of none and the group with a score of severe (p = 0.026) (one-way ANOVA F = 4.5, d.f. = 3 and 108, p = 0.005; Bonferroni post hoc test). The horizontal bars within the data points represent the mean.

6.3 Discussion

The main finding of the present study is that immunoreactivity for the lysosomal marker LAMP1 measured by Western blot was significantly decreased in BA24 and BA40 in DLB and in BA24 in PDD groups. The mean values of LAMP1 in PDD groups in BA40 was slightly decreased compared to the control, however, the results did not reach significance. Interestingly, these reductions were significantly associated with higher α-synuclein scores but did not correlate with any of the AD pathology. The non-significant reduction in PDD groups in BA40 can be explained by the lower distribution of α-synuclein inclusions in PDD groups compared to DLB. These results match those observed in earlier studies by Chu *et al*. who found that LAMP1 immunoreactivity was significantly decreased within PD nigral neurons and the decrease was significantly greater in nigral neurons that contained α-synuclein inclusions (Chu et al., 2009). These latter findings confirm the association between the α-synuclein inclusions and the lysosomal degradation pathway. Accumulation of α-synuclein can be a result of α-synuclein overexpression, impairment of the protein clearance pathway or both. There is a growing body of evidence suggesting a link between high levels of α-synuclein and dysfunction of the lysosomal degradation pathway. Increases in wild-type α-synuclein levels inhibit macroautophagy and increase the accumulation of other autophagy substrates such as p62 and huntington (Furlong et al., 2000, Winslow et al., 2010), in addition to α-synuclein abnormal aggregation and neuronal degeneration (Emmer et al., 2011, Outeiro et al., 2008, Zach et al., 2007). Aggregation of α-synuclein also has been shown to inhibit macroautophagy and the lysosomal degradation pathway and thereby impair the capacity of ALP to clear α-synuclein as well as other accumulated aggregation-prone proteins (Cuervo et al., 2004, Martinez-Vicente et al., 2008,

Snyder et al., 2003, Tanaka et al., 2001). Inhibition of the macroautophagy and the lysosomal degradation pathways enhances α-synuclein accumulation and aggregation (Rott et al., 2008). In contrast, autophagy activation facilitates the degradation of α-synuclein and neuroprotection (Spencer et al., 2009, Webb et al., 2003). Thus, the relationship between α-synuclein over-expression/aggregation and autophagy lysosomal degradation pathway dysfunction leads to a vicious cycle, where increased α-synuclein production could block its clearence mechanism, while impairement of the lysosomal pathway could result in α-synuclein accumulation and aggregation. In this study, no differences were found in LAMP1 protein levels in DLB and PDD in the frontal cortex (BA9). In PDD, BA9 was characterised by a relative scarcity of α-synuclein, while in DLB the distribution of α-synuclein was more in BA24 and BA40 than in BA9. It seems that in BA9 the autophagy-lysosomal pathway is still active after the impairment of the proteasome pathway in this region before it becomes inhibited by α-synuclein aggregation.

Cathepsin D protein values for the PDD group were significantly higher than the control in the parietal cortex (BA40), while in DLB they were slightly increased. LAMP1 protein values showed a significant reduction in DLB and a non-significant reduction in PDD. These results suggest that upregulation of cathepsin D serves as a compensatory mechanism for both proteasome dysfunction and other lysosomal defects and it is a response to protein accumulation and aggregation. Upregulation of cathepsin D has been reported in post-mortem brains in patients with AD (Cataldo et al., 1997). Few studies have investigated cathepsin D expression in LBD although a reduction in the expression levels of the protein in patients with PD has been reported, with this downregulation being more severe in neurons that contain α-synuclein inclusion (Chu et al., 2009). A possible

explanation for this might be that the overexpression of cathepsin D in DLB and PDD is a compensatory mechanism in cortical pathogenesis. The temporary increase is followed by deficiency after the exhaustion of this compensatory mechanism. This is perhaps due to proteasome dysfunction and an increase in α-synuclein aggregation. If this hypothesis is correct, the next question is: does overexpression of cathepsin D mean that it is in its active form and able to degrade α-synuclein inclusion or not? If cathepsin D is not in its active form then it is likely that activation of cathepsin D represents a possible treatment option for Lewy body dementia. Future studies on cathepsin D activity are therefore recommended.

There is a complementary relationship between the UPS and ALP. Inhibition of the proteasome activities has been previously reported to induce the autophagy-lysosomal pathway (Ding et al., 2007). In order to investigate the relationship between these two systems in this study, the expression level and the proteolytic activity of the proteasome were correlated with the protein levels LAMP1 and cathepsin D. As shown in the previous chapter (Chapter 5), there were reductions in the major proteasome proteolytic activities in the frontal, parietal and anterior cingulate cortices, which were related to reductions in the protein expression of the RPT6 ATPase 19S regulatory subunit of the proteasome. Changes in the lysosomal pathways did not correlate with either expression of the RPT6 ATPase 19S regulatory subunit of the proteasome or the proteolytic activities. The reason for this is not clear; because there are nearly 100 lysosomal proteins involved in the structure and activities of this pathway, it may be caused by other lysosomal proteins that have a direct link with the UPS.

Chapter 7 Relationships between pathology score, clinical data, synaptic biochemistry and expression levels of proteasome subunits/ proteasome activity

7.1 Introduction

Accumulation of ubiquitinated proteins and UPS-associated protein is a common feature in many neurodegenerative diseases. Currently it is unknown whether changes in proteasome function are driving synaptic changes, or if synaptic changes are driving proteasome dysfunction. However, it has become increasingly evident that the ubiquitin-proteasome system targets several key synaptic proteins and plays an important role in neuronal growth and development, synaptic function and plasticity, and neuronal survival (Yi and Ehlers, 2007). Any changes in the proteasome might, therefore, alter synaptic proteins and participate directly or indirectly in the pathogenesis of neuronal dysfunction. The early symptoms of many neurodegenerative disorders are characterized by synaptic impairment (Bagetta et al., 2010, Mallucci, 2009, Marcello et al., 2012, Picconi et al., 2012). Synaptic dysfunction play a central role in cognitive decline, as in AD among all the pathological changes in the brain, synapse loss is the strongest correlate of cognitive decline (DeKosky and Scheff, 1990, Terry et al., 1991). Given the role of the UPS in synaptic plasticity, the UPS may play a critical role in synaptic dysfunction that may underlie cognitive impairment in neurodegenerative diseases. Due to the plasticity of the synapses, they have the ability to regenerate after injury and hence mechanisms by which this occurs represent potential therapeutic strategies for the treatment of neurodegenerative diseases. This is a major factor as to why many researchers focus on the causes of synaptic dysfunction. Synaptic changes may also underlie the emergence of specific behaviours and mood disturbance. Ongoing work in our laboratory has highlighted relationships between mood and a zinc transporter (Whitfield et al., 2014a), zinc transporter and cognitive impairment (Whitfield et al., 2014b), and also between CAMKII and cognitive impairment

(Vallortigara et al., 2014). Therefore, we hypothesized that cognitive decline and non-cognitive symptoms would be associated with the proteasome impairment as a consequence of synaptic dysfunction and increased protein aggregation in LBD and AD. In Chapter-5, evidence was presented for a reduction in specific components, especially RPT6 and activities of the proteasome system. To address the link between proteasome impairment, AD and LBD pathology, cognition decline and non-cognitive symptom, and the synaptic dysfunction, the reduction of RPT6 and the alteration of the other proteasome components and activities were investigated in order to identify clinico-pathological correlations. These include:

1. Possible relationships between reduction of RPT6 and the alteration of the other proteasome components and semi-quantitative scores of AD and LBD pathology in different brain areas.
2. Possible relationships between proteasome dysfunction and cognition and non-cognitive symptom in LBD and AD.
3. Possible relationships between reduction of RPT6 and synaptic dysfunction.

The analysis of the relationships between non-cognitive symptoms, mood and proteasome markers were exploratory and unbiased as there were no compelling hypotheses linking them. It was therefore considered appropriate to set a level of statistical significance of 0.01 for rejection of the null hypothesis. It remains the case that all such associations are treated with caution because of the fact of multiple testing. Furthermore, due to the different regional patterns for the protein changes and the

linkage of particular behavioural symptoms to a specific brain area, each brain region was analysed separately.

Clinical and pathological data for more than 130 cases of PDD, DLB and AD were available for this study. Semi-quantitative pathology scoring (plaques/Aβ pathology, tangle/tau pathology and α-synuclein) was conducted using the following scale: 0 (none), 1 (mild), 2 (moderate) and 3 (severe/frequent) to score sections from BA9, BA24 and BA40. The cases were grouped by MMSE score as previously described (Whitfield et al., 2014b):- 'unimpaired cognition' for clinical control cases, 'mildly impaired cognition without dementia' (score of 25–30), 'mildly impaired cognition with dementia' (score of 17–24), 'moderately impaired cognition' (score of 10–16) and 'severely impaired cognition' (score of 9 or less). Individuals were categorized according to the duration and severity of the non-cognitive symptoms, such as depression on a scale of 0 to 3 where 0 was no depression, 1 was intermittent and mild depression, 2 was moderate (intermittent but significant) depression and 3 was persistent and/or severe depression (for more details on the clinical and pathological data see Chapter 2, Section 2.1).

The relative levels of synaptic proteins (PSD-95, ZnT3, synaptophysin, beta-III-tubulin and CAMKII) in BA9, BA24 and BA40 were measured by Dr David Whitfield and Dr Julie Vallortigara using quantitative Western blotting (Vallortigara et al., 2014, Whitfield et al., 2014a, Whitfield et al., 2014b). For full details of all synaptic proteins values from the Western blotting semi-quantifications, refer to the following tables in the Appendix: Table IX and X**.** For the differences in the relative levels of synaptic proteins between the diagnostic groups refer to Table XI in the Appendix.

7.2 Relationships between pathology scores and expression levels of proteasome sub-units and proteasome activity

To investigate whether the expression levels of the proteasome sub-units in different brain areas correlated with semi-quantitative scores of AD and LBD pathology, linear regression analyses were conducted with the three predictors RPT6, α-3 and α-6 as independent factors and semi-quantitative scores for Aβ staining (plaque pathology), phospho-tau staining (tangle pathology) and α-synuclein staining/pathology as dependent variables. The pathological data were based on the semi-quantitative (on a scale of 0 (none), 1 (mild), 2 (moderate) and 3 (severe/frequent)) rating of IHC degree and distribution of plaque, tangle and α-synuclein immunostaining (for more details see Chapter 2 Section 2.1).

7.2.1 Frontal cortex – Brodmann area 9

7.2.1.1 Proteasome components

Reductions in RPT6 were associated with the AD pathology. In three separate analyses (Figures 7-1, 7-2 and 7-3), tangle, plaque and α-synuclein pathology were entered as dependent variables with RPT6, α-3 and α-6 as independent factors. RPT6 was the best predictor for plaque scores and also predicted both tangle and α-synuclein pathology. The best predictor for the tangle score was α-6, and alpha 3 was the best for α-synuclein pathology.

Analysis of variance (ANOVA) followed by Bonferroni post-hoc tests also indicated a significant difference between RPT6 levels and plaque scores (one-way ANOVA $F = 2.9$, d.f. = 3 and 114, $p = 0.038$; Bonferroni post hoc test), a significant

difference between RPT6 level and tangle score (one-way ANOVA F = 2.7, d.f. = 3 and 115, p = 0.045; Bonferroni post hoc test); a significant difference between α-6 levels and tangle scores (one-way ANOVA F = 6,9, d.f. = 3 and 114, p = 0.001; Bonferroni post hoc test); and a significant difference between α-3 levels and α-synuclein scores (one-way ANOVA F = 6.433 d.f. = 3 and 114, p = 0.001; Bonferroni post hoc test).

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.302[a]	.091	.067	1.160

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	15.202	3	5.067	3.769	.013[b]
	Residual	151.943	113	1.345		
	Total	167.145	116			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	4.433	1.031		4.302	.000
	α-3	-.277	.783	-.033	-.354	.724
	RPT6	**-1.662**	**.723**	**-.213**	**-2.300**	**.023**
	α-6	-.849	.527	-.154	-1.612	.110

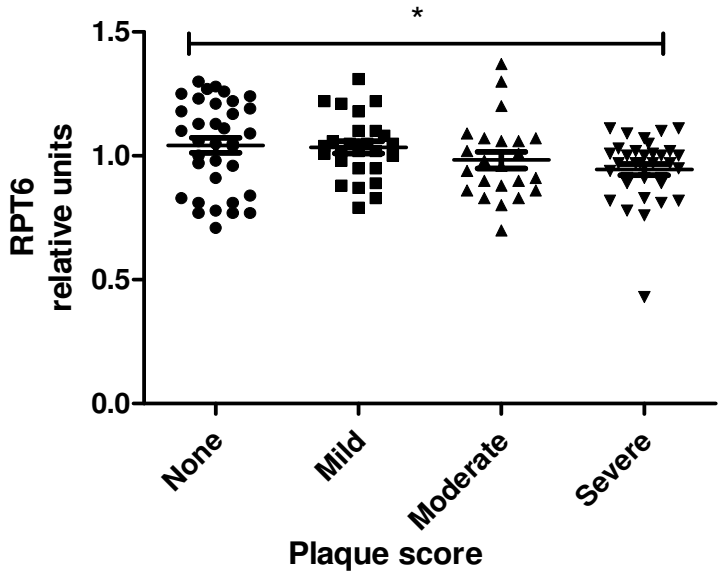


Figure 7-1: Relationship between RPT6 expression levels in BA9 and plaque score.

Linear regression analyses were conducted with the three predictors to develop a model for the best predictor plaque score in BA9. In the analyses, the semi-quantitative plaque scores were entered as dependent variables with RPT6, α-3 and α-6 values from semi-quantitative Western blotting in BA9 as independent factors. Plaque scores in the dementia cases (DLB, PDD and AD) and control cases were significantly predicted by RPT6 with (Beta = -0.213 and p = 0.023). One-way ANOVA was performed to compare semi-quantitative Western blotting values of RPT6 and plaque scoring groups. The analysis indicated a significant difference between the group with a plaque score of none and the group with a score of severe (one-way ANOVA F = 2.9, d.f. = 3 and 114, p = 0.038; Bonferroni post hoc test). The horizontal bars within the data points represent the mean.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.404[a]	.163	.141	.908

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	18.343	3	6.114	7.418	.000[b]
	Residual	93.971	114	.824		
	Total	112.314	117			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	2.773	.807		3.437	.001
	α-3	1.044	.607	.153	1.720	.088
	RPT6	-1.058	.567	-.165	-1.865	.065
	α-6	**-1.525**	**.412**	**-.339**	**-3.701**	**.000**

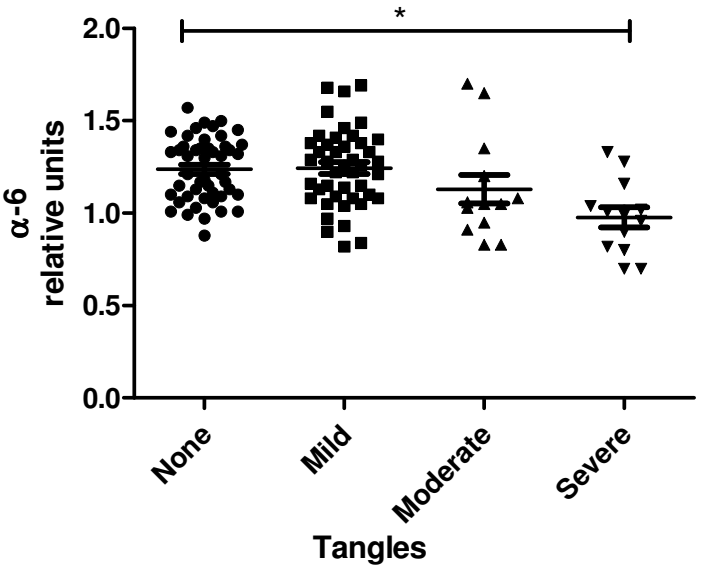


Figure 7-2: Relationship between α-6 expressions levels in BA9 and tangle score.

Linear regression analyses were conducted with the three predictors to develop a model for the best predictor tangle score in BA9. In the analyses, the semi-quantitative tangle scores were entered as dependent variables with RPT6, α-3 and α-6 values from semi-quantitative Western blotting in BA9 as independent factors. Tangle scores in the dementia cases (DLB, PDD and AD) and control cases were significantly predicted by α-6 (Beta = -0.336 and p = 0.001). One-way ANOVA was performed to compare semi-quantitative Western blotting values of α-6 and tangle scoring groups. The analysis indicated a significant different between the group with a tangle score of none and the group with a score of severe (one-way ANOVA F = 6,9, d.f. = 3 and 114, p = 0.001; Bonferroni post hoc test). The horizontal bars within the data points represent the mean.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.415[a]	.172	.150	.979

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	22.489	3	7.496	7.823	.000[b]
	Residual	108.280	113	.958		
	Total	130.769	116			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	-.899	.876		-1.027	.307
	α-3	**2.263**	**.657**	**.303**	**3.444**	**.001**
	RPT6	**-1.537**	**.598**	**-.226**	**-2.568**	**.012**
	α-6	.904	.445	.183	2.030	.045

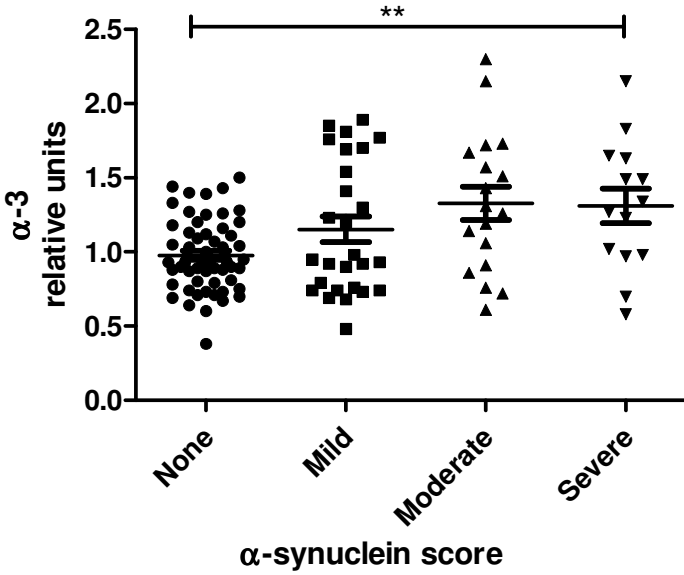


Figure 7-3: Relationship between expression levels of α-3 in BA9 and α-synuclein score.

Linear regression analyses were conducted with the three predictors to develop a model for the best predictor α-synuclein in BA9. In the analyses, the semi-quantitative α-synuclein were entered as dependent variables with RPT6, α-3 and α-6 values from semi-quantitative Western blotting in BA9 as independent factors. The α-synuclein scores in the dementia cases (DLB, PDD and AD) and control cases were significantly predicted by α-3 with (Beta = 0.303 and p = 0.001). One-way ANOVA was performed to compare semi-quantitative Western blotting values of α-3 and α-synuclein scoring groups. The analysis indicated a significant difference between the group with α-synuclein of none and the group with a score of severe (one-way ANOVA F = 6.433, d.f. = 3 and 114, p = 0.001; Bonferroni post hoc test). The horizontal bars within the data points represent the mean.

7.2.1.2 Proteasome activity

PGPH- and chymotrypsin-like activities were also associated with the AD pathologies in all three brain regions. Linear regression analyses were conducted with both predictors, PGPH- and chymotrypsin-like activities, to develop a model for the best predictor for each pathology score. In three separate analyses, tangle, plaque and α-synuclein scores were entered as dependent variables with PGPH- and chymotrypsin-like activities as independent factors. Tangle scores were significantly predicted by chymotrypsin-like activity with (Beta = -0.440 and p = 0.011) (Figure 7-4). Plaque scores were predicted by PGPH-like activity, but the analysis did not reach the significant level (Beta = -0.331 and p = 0.08).

Analysis of variance (ANOVA) followed by Bonferroni post-hoc tests also indicated a significant difference in chymotrypsin-like activity in different tangle scoring groups (one-way ANOVA F = 6.533, d.f. = 3 and 33, p = 0.001; Bonferroni post hoc test) (Figure 7-4) and a significant difference between PGPH-like activity in different plaque scoring groups (one-way ANOVA F = 4.279, d.f. = 3 and 33, p = 0.012; Bonferroni post hoc test) (Figure 7-5).

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.607[a]	0.368	0.331	0.767

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	11.653	2	5.826	9.893	.000[b]
	Residual	20.023	34	0.589		
	Total	31.676	36			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
	(Constant)	3.834	0.82		4.676	0
	Chymotrypsin-like	**-0.002**	**0.001**	**-0.44**	**-2.701**	**0.011**
	PGPH-like	0	0	-0.241	-1.476	0.149

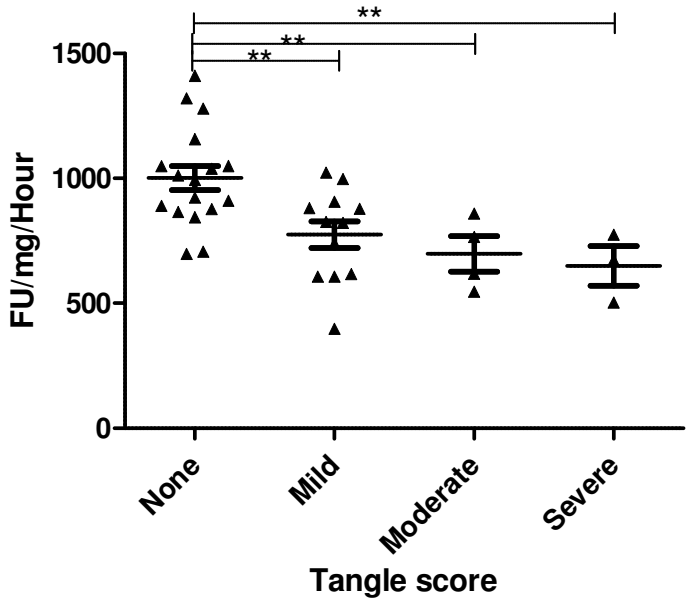


Figure 7-4: Relationship between chymotrypsin-like activity in BA9 and tangle score.

Linear regression analyses were conducted with both predictors, chymotrypsin- and PGPH-like activities, to develop a model for the best predictor for tangle scores in BA9. In the analyses, the semi-quantitative tangle scores were entered as dependent variables with chymotrypsin-like and PGPH-like activities values measured using fluorogenic substrates assay in BA9 as independent factors. Tangle scores in the dementia cases (DLB, PDD and AD) and control cases were significantly predicted by chymotrypsin-like activity with (Beta = -0.44 and p = 0.011). Analysis of variance (ANOVA) and Bonferroni post-hoc tests were performed to compare chymotrypsin-like activity and tangle scoring groups. ANOVA indicated a significant difference in chymotrypsin-like activity in different tangle scoring groups (one-way ANOVA F = 6.533, d.f. = 3 and 33, p = 0.001; Bonferroni post hoc test). The horizontal bars within the data points represent the mean.

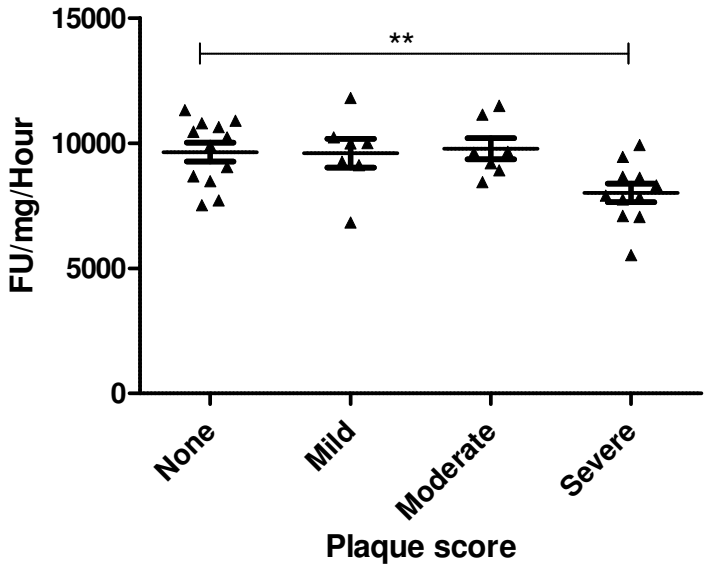


Figure 7-5: Relationship between PGPH-like activity in BA9 and plaque score.

Analysis of variance (ANOVA) and Bonferroni post-hoc tests were performed to compare PGPH-like activity and plaque scoring groups. ANOVA indicated a significant difference in PGPH-like activity between the none and severe groups (one-way ANOVA $F = 4.279$, d.f. = 3 and 33, $p = 0.012$; Bonferroni post hoc test). The horizontal bars within the data points represent the mean.

7.2.2 Parietal cortex—Brodmann area 40

7.2.2.1 Proteasome components

Analysis of the data in BA40 also indicated the association of proteasome dysfunction with the AD pathology. As with BA9, analyses were created to develop the best predictor using linear regression analysis. In three separate analyses, tangle, plaque and α-synuclein were entered as dependent variables with RPT6, α-3 and α-6 as independent factors. RPT6 was the best predictor for plaque (Figure 7-6) and tangle scores (Figure 7-7). Analysis of variance (ANOVA) and Bonferroni post-hoc tests also indicated a significant difference between RPT6 levels and plaque scores (one-way ANOVA $F = 5.610$, d.f. = 3 and 116, $p = 0.001$; Bonferroni post hoc test) and significant difference between RPT6 levels and tangle scores (one-way ANOVA $F = 7.491$, d.f. = 3 and 118, $p = 0.001$; Bonferroni post hoc test).

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.357[a]	.127	.105	1.108

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	20.611	3	6.870	5.599	.001[b]
	Residual	141.120	115	1.227		
	Total	161.731	118			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	4.667	1.125		4.149	.000
	α-3	-.376	.688	-.049	-.547	.586
	RPT6	**-2.434**	**.675**	**-.328**	**-3.604**	**.000**
	α-6	-.498	.807	-.055	-.617	.538

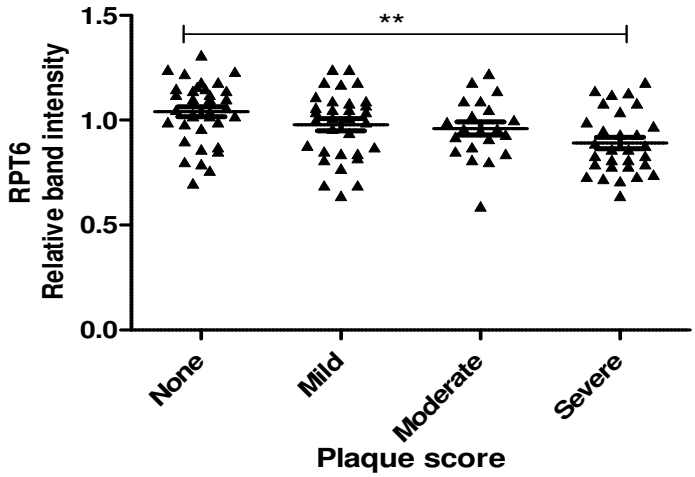


Figure 7-6: Relationship between RPT6 expression levels in BA40 and plaque score

Linear regression analyses were conducted with the three predictors to develop a model for the best predictor plaque score in BA40. In the analyses, the semi-quantitative plaque scores were entered as dependent variables with RPT6, α-3 and α-6 values from semi-quantitative Western blotting in BA40 as independent factors. Plaque scores in dementia cases (DLB, PDD and AD) and control were significantly predicted by RPT6 with (Beta = -0.328 and p = 0.001). One-way ANOVA was performed to compare semi-quantitative Western blotting values of RPT6 and plaque scoring groups. The analysis indicated a significant difference between the group with a plaque score of none and the group with a score of severe (one-way ANOVA F = 5.610, d.f. = 3 and 116, p = 0.001; Bonferroni post hoc test). The horizontal bars within the data points represent the mean.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.432[a]	.187	.166	.976

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	25.657	3	8.552	8.973	.000[b]
	Residual	111.517	117	.953		
	Total	137.174	120			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	5.102	.988		5.162	.000
	α-3	-.586	.606	-.083	-.967	.336
	RPT6	**-2.459**	**.593**	**-.361**	**-4.145**	**.000**
	α-6	-1.118	.706	-.134	-1.585	.116

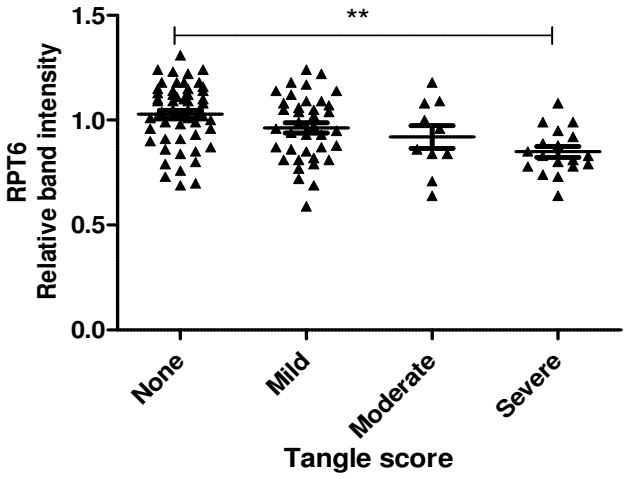


Figure 7-7: Relationship between RPT6 expression levels in BA40 and tangle score

Linear regression analyses were conducted with the three predictors to develop a model for the best predictor tangle score in BA40. In the analyses, the semi-quantitative tangle scores were entered as dependent variables with RPT6, α-3, and α-6 values from semi-quantitative Western blotting in BA40 as independent factors. Tangle scores in dementia cases (DLB, PDD and AD) and control were significantly predicted by RPT6 with (Beta = -0.361 and p = 0.001). One-way ANOVA was performed to compare semi-quantitative Western blotting values of RPT6 and tangle scoring groups. The analysis indicated a significant difference between the group with a tangle score of none and the group with a score of severe (one-way ANOVA F = 7.491, d.f. = 3 and 118, p = 0.001; Bonferroni post hoc test). The horizontal bars within the data points represent the mean.

7.2.2.2 Proteasome activity

In the parietal cortex, chymotrypsin-like activity was associated with both plaque and tangle scores. Linear regression analyses were conducted with both predictors, PGPH- and chymotrypsin-like activities, to develop a model for the best predictor for each pathology score. In two separate analyses, tangle and plaque scores were entered as dependent variables with PGPH- and chymotrypsin-like activities as independent factors. Both tangle (Beta = -0.479, and p = 0.006) (Figure 7-8) and plaque scores were significantly predicted by chymotrypsin-like activity with (Beta = -0.587 and p = 0.002) (Figure 7-9).

Analysis of variance (ANOVA) followed by Bonferroni post-hoc tests also indicated a significant difference in chymotrypsin-like activity in different plaque scoring groups (one-way ANOVA F = 8.921, d.f. = 3 and 38, p = 0.001; Bonferroni post hoc test) (Figure 7-8) and tangle scoring groups (one-way ANOVA F = 8.117, d.f. = 3 and 39, p = 0.001; Bonferroni post hoc test) (Figure 7-9).

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.552[a]	.305	.267	1.113

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	20.097	2	10.048	8.117	.001[b]
	Residual	45.803	37	1.238		
	Total	65.900	39			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	16.917	4.390		3.853	.000
	Chymotrypsin-like	**-.011**	**.003**	**-.587**	**-3.310**	**.002**
	PGPH-like	3.822E-05	.000	.058	.324	.747

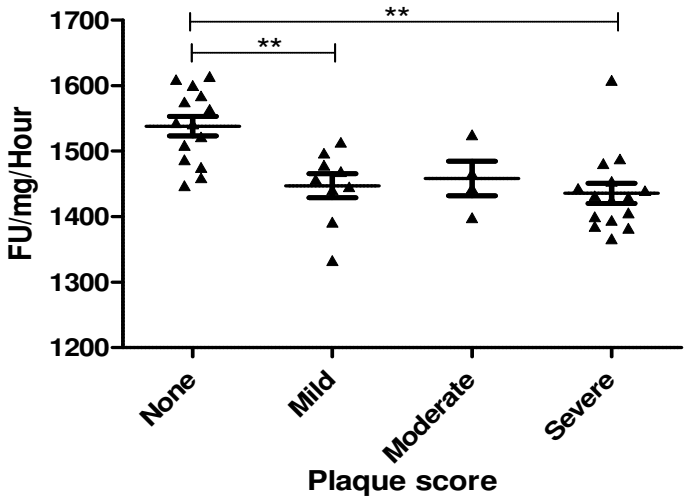


Figure 7-8: Relationship between chymotrypsin-like activity in BA40 and plaque score

Linear regression analyses were conducted with both predictors, chymotrypsin- and PGPH-like activities to develop a model for the best predictor for plaque scores in BA40. In the analyses the semi-quantitative plaque scores were entered as dependent variables with chymotrypsin-like and PGPH-like activities values measured using fluorogenic substrates assay in BA40 as independent factors. Plaque scores in the dementia cases (DLB, PDD and AD) and control cases were significantly predicted by chymotrypsin-like activity with (Beta = -0.587 and p = 0.002). Analysis of variance (ANOVA) and Bonferroni post-hoc tests were performed to compare chymotrypsin-like activity and plaque scoring groups. ANOVA indicated a significant difference in chymotrypsin-like activity in different plaque scoring groups (one-way ANOVA F = 8.921, d.f. = 3 and 38, p = 0.001; Bonferroni post hoc test). The horizontal bars within the data points represent the mean.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.551[a]	.303	.267	1.083

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	19.422	2	9.711	8.278	.001[b]
	Residual	44.578	38	1.173		
	Total	64.000	40			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	13.134	3.758		3.495	.001
	Chymotrypsin-like	**-.008**	**.003**	**-.479**	**-2.926**	**.006**
	PGPH-like	-7.418E-05	.000	-.114	-.695	.491

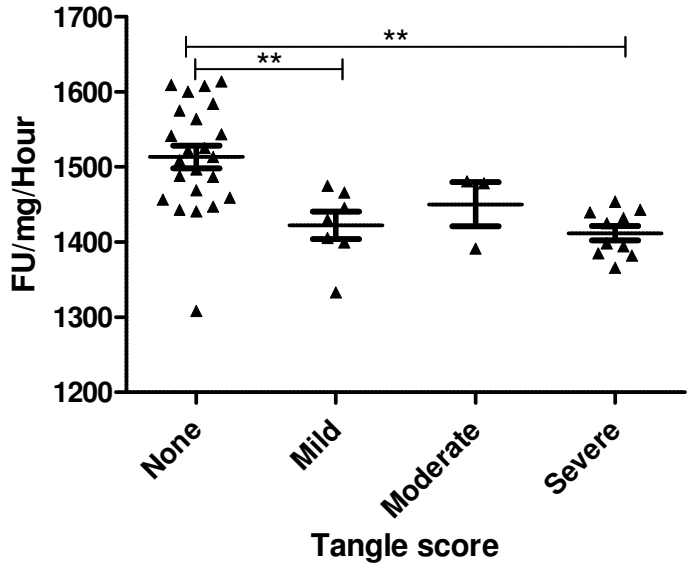


Figure 7-9: Relationship between chymotrypsin-like activity in BA40 and tangle score

Linear regression analyses were conducted with both predictors, chymotrypsin- and PGPH-like activities to develop a model for the best predictor for tangle scores in BA40. In the analyses the semi-quantitative tangle scores were entered as dependent variables with chymotrypsin-like and PGPH-like activities values measured using fluorogenic substrates assay in BA40 as independent factors. Tangle scores in dementia cases (DLB, PDD and AD) and control cases were significantly predicted by chymotrypsin-like activity with (Beta = -0.479 and p = 0.006). Analysis of variance (ANOVA) and Bonferroni post-hoc tests were performed to compare chymotrypsin-like activity and tangle scoring groups. ANOVA indicated a significant difference in chymotrypsin-like activity in different tangle scoring groups (one-way ANOVA F = 8.117, d.f. = 3 and 39, p = 0.001; Bonferroni post hoc test). The horizontal bars within the data points represent the mean.

7.2.3 Anterior cingulate Brodmann area 24

7.2.3.1 Proteasome component

In all three-brain regions, the levels of RPT6 were always associated with AD pathology. In the anterior cingulate, analyses were created to develop the best predictor using linear regression analysis. In three separate analyses, tangle, plaque and α-synuclein were entered as dependent variables with RPT6, α-3 and α-6 as independent factors. RPT6 was the best predictor for plaque and tangle scores (Figure 7-10 and 7-11), although analysis of variance (ANOVA) were not significant different between the RPT6 level in different scoring groups of both tangle and plaque.

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.365[a]	.133	.108	.952

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	14.708	3	4.903	5.414	.002[b]
	Residual	95.983	106	.905		
	Total	110.691	109			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.886	1.188		1.588	.115
	α-3	1.267	.589	.208	2.153	.034
	RPT6	**-1.785**	**.628**	**-.270**	**-2.843**	**.005**
	α-6	-.449	1.122	-.039	-.400	.690

Figure 7-10: Relationship between RPT6 expression levels in BA24 and plaque score.

Linear regression analyses were conducted with the three predictors to develop a model for the best predictor plaque score in BA24. In the analyses, the semi-quantitative plaque scores were entered as dependent variables with RPT6, α-3 and α-6 values from semi-quantitative Western blotting in BA24 as independent factors. Plaque scores in dementia cases (DLB, PDD and AD) and control cases were significantly predicted by RPT6 with (Beta = -0.270 and p = 0.005).

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.321[a]	.103	.077	1.004

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	11.931	3	3.977	3.942	.010[b]
	Residual	103.919	103	1.009		
	Total	115.850	106			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.647	1.257		.514	.608
	α-3	.996	.630	.159	1.581	.117
	RPT6	**-1.682**	**.667**	**-.249**	**-2.523**	**.013**
	α-6	1.037	1.205	.088	.860	.392

Figure 7-11: Relationship between RPT6 expression levels in BA24 and tangle score.

Linear regression analyses were conducted with the three predictors to develop a model for the best predictor tangle score in BA24. In the analyses, the semi-quantitative tangle scores were entered as dependent variables with RPT6, α-3 and α-6 values from semi-quantitative Western blotting in BA24 as independent factors. Tangle scores in dementia cases (DLB, PDD and AD) and control were significantly predicted by RPT6 with (Beta = -0.249 and p = 0.013).

7.2.4 Summary

Specifically, the reductions in RPT6 and proteasome activities were found to be associated mostly with the semi-quantitative scores (0-3) for plaques, and neurofibrillary tangles. In BA9, 40 and 24, semi-quantitative scores for plaques were significantly predicted by RPT6 while tangle scores were significantly predicted by RPT6 in BA40 and 24. α-synuclein semi-quantitative scores were significantly predicted by RPT6 in BA9 only. α-synuclein and tangle scores significantly predicted by α-3 and α-6 respectively in BA9 only. The major contributor to these relationships would appear to be RPT6 and chymotrypsin-like activity with the AD pathology (plaque and tangle) (Table 7-1).

Table 7-1: Relationship between pathology scores in PDD, DLB and AD patients and expression levels of proteasome sub-units and proteasome activity.

BA9	a-synuclein	Plaques	Tangles
RPT6	***-0.226***	***-0.213***	-0.165
Alpha6	0.183	-0.033	***-0.339***
ALPHA3	***0.303***	-0.154	0.153
chymotrypsin-like activity	-0.071	-0.153	***-0.44***
PGPH-LIKE activity	-0.028	-0.331	-0.241

BA24	a-synuclein	Plaques	Tangles
RPT6	-0.157	***-0.27***	***-0.249***
ALPHA6	0.154	0.208	0.159
ALPHA3	-0.118	-0.039	0.088
chymotrypsin-like activity	-0.083	0.229	0.171
PGPH-LIKE activity	0.051	***-0.514***	-0.288

BA40	a-synuclein	Plaques	Tangles
RPT6	0.068	***-0.328***	***-0.361***
ALPHA6	-0.089	-0.049	-0.083
ALPHA3	-0.146	-0.055	-0.134
chymotrypsin-like activity	-0.075	***-0.587***	***-0.479***
PGPH-LIKE activity	-0.216	0.058	-0.114

Pathology scores predicted by proteasome sub-units level and proteasome activities indicated by the standardised regression coefficients (β). Values that are presented in ***bold italics*** indicate statistically significance ($p<0.01$) except for RPT6 and plaque score in BA9 where $p<0.05$.

7.3 Relationships between clinical data and expression levels of proteasome subunits and proteasome activity

Regression analyses were undertaken to examine the relationship between the protein levels and behavioural symptom scores. Regression analysis using the SPSS method "Enter" was performed using the values from semi-quantitative Western blotting for RPT6, α-3 and α-6 as independent factors, and each of the behavioural symptom scores as dependent factors. One-way ANOVA was also performed to compare the value of the best predictor protein for each behavioural symptom and scoring groups for that symptom. For each behavioural symptom, individuals were categorized according to the duration and severity of the symptom on a scale of 0–3 (for more details see Chapter 2, Section 2-1). The Mental State Examination (MMSE) was used to measure the severity of cognitive impairment. The classification purposes for MMSE are described in Chapter 2, Section 2-1).

7.3.1 Frontal cortex —Brodmann area 9

7.3.1.1 Cognitive decline

Linear regression revealed a significant negative association between cognitive decline and RPT6 with a high β value (-0.56, P = 0.001). Analysis of variance (ANOVA) followed by Bonferroni post-hoc tests also indicated a significant difference between the groups (one-way ANOVA F = 17.82, d.f. = 4 and 99, p = 0.001; Bonferroni post hoc test) (Figure 7-12).

Linear regression analyses were conducted with both predictors, chymotrypsin-like and PGPH-like activities, to develop a model for the best predictor for cognitive decline. In

the analyses, MMSE scores (after grouping the cases) were entered as dependent variables with chymotrypsin-like and PGPH-like activities measured using fluorogenic substrates assay in BA9 as independent factors. MMSE scores were significantly predicted by chymotrypsin-like activity (Beta = -0.416 and p = 0.035).

One-way ANOVA was performed to compare chymotrypsin-like and PGPH-like activities and scoring groups for MMSE. The analysis indicated high chymotrypsin-like activity in the control cases compared to the cases with moderate (p = 0.014) and severe scores (p = 0.01) (one-way ANOVA F = 5.009, d.f. = 4 and 26, p = 0.004; Bonferroni post hoc test). Analysis of variance (ANOVA) followed by Bonferroni post-hoc tests also indicated a significant difference in PGPH-like activity between unimpaired cognition and moderate groups (one-way ANOVA F = 3.616, d.f. = 4 and 26, p = 0.004; Bonferroni post hoc test) (Figure 7-13).

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.576[a]	.332	.312	1.25222

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	77.151	3	25.717	16.400	.000[b]
	Residual	155.238	99	1.568		
	Total	232.388	102			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	9.356	1.176		7.956	.000
	α-3	-.719	.896	-.067	-.802	.424
	RPT6	**-5.270**	**.793**	**-.564**	**-6.647**	**.000**
	α-6	-.080	.594	-.012	-.134	.894

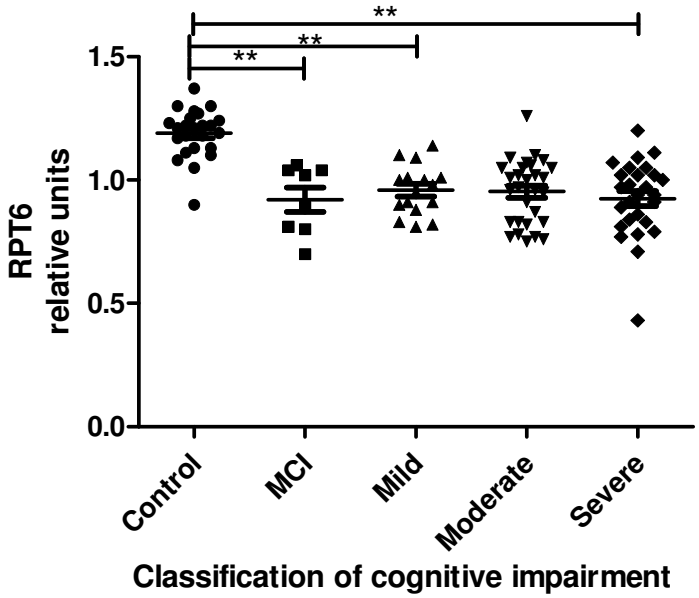


Figure 7-12: Relationship between RPT6 expression levels in BA9 and cognitive impairment based upon MMSE classification

Linear regression analyses were conducted with the three predictors to develop a model for the best predictor for cognitive impairment. In the analyses, MMSE scores (after grouping the cases) were entered as dependent variables with RPT6, α-3 and α-6 values from semi-quantitative Western blotting in BA9 as independent factors. Cognitive impairment in the dementia cases (DLB, PDD and AD) and control cases was significantly predicted by RPT6 with (Beta = -0.564 and p = 0.001). One-way ANOVA was performed to compare semi-quantitative Western blotting value of RPT6 and cognitive impairment groups. One-way ANOVA was performed to compare semi-quantitative Western blotting value of RPT6 and cognitive impairment groups. The cognitive impairment groups were: 'unimpaired cognition for the clinical controls cases'; 'mildly impaired cognition without dementia' for any case with the score 24 or above; 'mildly impaired cognition without dementia' = 25–30; 'mildly impaired cognition with dementia' = 17–24; 'moderately impaired cognition' = 10–16 and 'severely impaired cognition' = 9 or less. The analysis indicated high levels of RPT6 in the cases with unimpaired cognition compared to the other groups (one-way ANOVA F = 17.82, d.f. = 4 and 99, p = 0.001; Bonferroni post hoc test). The horizontal bars within the data points represent the mean.

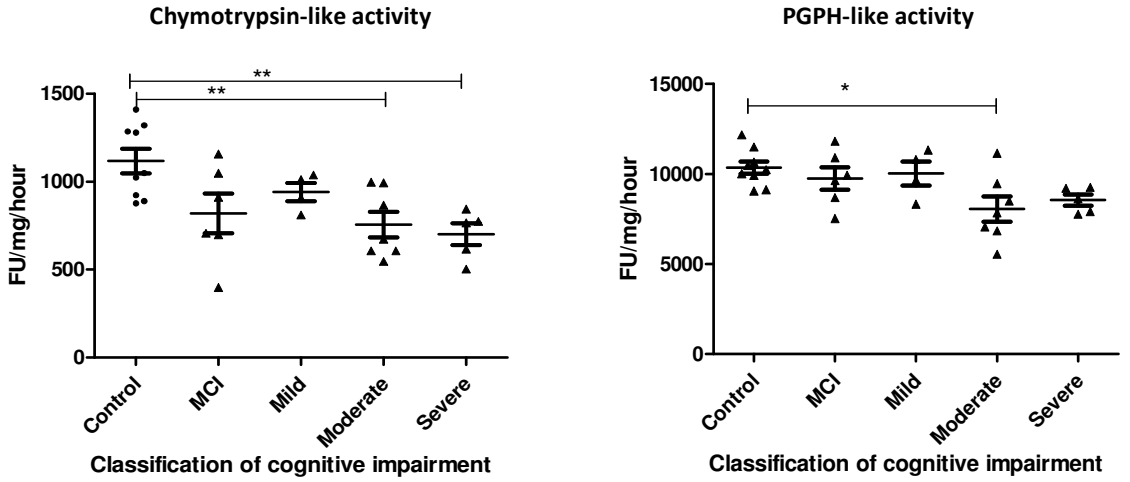


Figure 7-13: Relationship between chymotrypsin- and PGPH-like activities in BA9 and cognitive impairment based upon MMSE classification

One-way ANOVA was performed to compare the chymotrypsin- and PGPH-like activities and cognitive impairment groups. The cognitive impairment groups were: 'unimpaired cognition' for the clinical control cases; 'mildly impaired cognition without dementia' for any case with scores of 24 or above; 'mildly impaired cognition without dementia' = 25–30; 'mildly impaired cognition with dementia' = 17–24; 'moderately impaired cognition' = 10–16 and 'severely impaired cognition' = 9 or less. The analysis indicated high chymotrypsin-like activity in the control cases compared to the cases with moderate ($p = 0.014$) and severe scores ($p = 0.01$) (one-way ANOVA $F = 5.009$, d.f. = 4 and 26, $p = 0.004$; Bonferroni post hoc test). The difference in PGPH-like activity between cognitive impairment groups was significantly different between unimpaired cognition and moderate groups (one-way ANOVA $F = 3.616$, d.f. = 4 and 26, $p = 0.004$; Bonferroni post hoc test). The horizontal bars within the data points represent the mean.

7.3.1.2 Depression

Regression analyses were conducted using the values from semi-quantitative Western blotting for RPT6, α-3 and α-6 as independent factors and depression scores as dependent factors. A score of zero was assumed all the control cases in the analysis for any non-cognitive symptom, as there is no evidence of any neurological or psychiatric disease for these cases. RPT6 level was found to be significantly associated with severity of depression (the output of the regression analysis is shown in Figure 7-14). One-way ANOVA was also performed to compare the RPT6 values in BA9 from semi-quantitative Western blotting and scoring groups for depression. The analysis did not indicate a high significance level of $p<0.01$; however, higher level of RPT6 was noted in cases with no depression compared to the cases with severe depression ($p = 0.04$) (one-way ANOVA, $F = 3.451$, d.f. = 3 and 94, $p = 0.02$; Bonferroni post hoc test).

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.380[a]	.145	.117	1.000

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	15.726	3	5.242	5.245	.002[b]
	Residual	92.954	93	1.000		
	Total	108.680	96			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	4.336	.963		4.504	.000
	α-3	-.866	.768	-.110	-1.127	.263
	RPT6	**-1.809**	**.661**	**-.275**	**-2.739**	**.007**
	α-6	-.686	.486	-.142	-1.413	.161

Figure 7-14: Relationship between RPT6 expression levels in BA9 and depression score

Linear regression analyses were conducted with the three predictors to develop a model for the best predictor for depression. In the analyses, depression scores were entered as dependent variables with RPT6, α-3 and α-6 values from semi-quantitative Western blotting in BA9 as independent factors. Depression in the dementia cases (DLB, PDD and AD) and controls was significantly predicted by RPT6 (Beta = -0.275 and p = 0.007).

7.3.1.3 Persecution

Linear regression analyses were also conducted with the three predictors to develop a model for the best predictor for the persecution symptom. In the analyses, persecution was entered as dependent variables with RPT6, α-3 and α-6 as independent factors. Reduction of RPT6 was significantly associated with persecution (the output of the regression analysis is shown in (Figure 7-15). One-way ANOVA was also performed to compare RPT6 values in BA9 from semi-quantitative Western blotting and scoring groups for persecution. The analysis also did not reach a high significance level of $p < 0.01$ (one-way ANOVA, $F = 5.552$, d.f. = 3 and 89, $p = 0.02$; Bonferroni post hoc test).

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.365[a]	.133	.104	.973

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	12.835	3	4.278	4.516	.005[b]
	Residual	83.372	88	.947		
	Total	96.207	91			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	2.689	.960		2.801	.006
	α-3	.530	.760	.071	.698	.487
	RPT6	**-2.279**	**.653**	**-.363**	**-3.490**	**.001**
	α-6	-.108	.475	-.024	-.227	.821

Figure 7-15: Relationship between RPT6 expression levels in BA9 and persecution score

Linear regression analyses were conducted with the three predictors to develop a model for identification of the best predictor for persecution. In the analyses, persecution scores were entered as dependent variables with RPT6, α-3, and α-6 values from semi-quantitative Western blotting in BA9 as independent factors. Persecution in the dementia cases (DLB, PDD and AD) and the controls was significantly predicted by RPT6 with (Beta = -0.363 and p = 0.001).

7.3.2 Parietal cortex—Brodmann area 40

7.3.2.1 Cognitive decline

Linear regression also revealed a significant negative association between cognitive decline and RPT6 in BA40 with β value (-.441, P = 0.001). Analysis of variance (ANOVA) followed by Bonferroni post-hoc tests also indicated a significant difference between the groups (one-way ANOVA F = 6.472, d.f. = 4 and 102, p = 0.001; Bonferroni post hoc test) (Figure 7-16).

Linear regression analyses were conducted with both predictors, chymotrypsin-like and PGPH-like activities, to develop a model for finding the best predictor of cognitive decline. In the analyses, MMSE scores (after grouping the cases) were entered as dependent variables with chymotrypsin-like and PGPH-like activities measured using fluorogenic substrates assay in BA40 as independent factors. MMSE scores were significantly predicted by chymotrypsin-like activity with (Beta = -0.724 and p = 0.001).

One-way ANOVA was performed to compare chymotrypsin-like and PGPH-like activities and scoring groups for MMSE. The analysis indicated high chymotrypsin-like activity in the control cases compared to the cases with MCI (p = 0.001), mild (p = 0.001), moderate (p = 0.001) and severe scores (p = 0.001) (one-way ANOVA F = 21.845, d.f. = 4 and 37, p = 0.001; Bonferroni post hoc test). Analysis of variance (ANOVA) followed by Bonferroni post-hoc tests also indicated high PGPH-like activity in the control cases compared to the cases with MCI (p = 0.02), mild (p = 0.001), moderate (p = 0.001) and severe scores (p = 0.034) (one-way ANOVA F = 8.851, d.f. = 4 and 35, p = 0.001; Bonferroni post hoc test) (Figure 7-17).

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.446[a]	.199	.176	1.38713

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	48.805	3	16.268	8.455	.000[b]
	Residual	196.261	102	1.924		
	Total	245.066	105			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	7.410	1.536		4.823	.000
	α-3	1.025	.890	.105	1.152	.252
	RPT6	**-4.371**	**.910**	**-.441**	**-4.805**	**.000**
	α-6	-1.046	1.068	-.087	-.979	.330

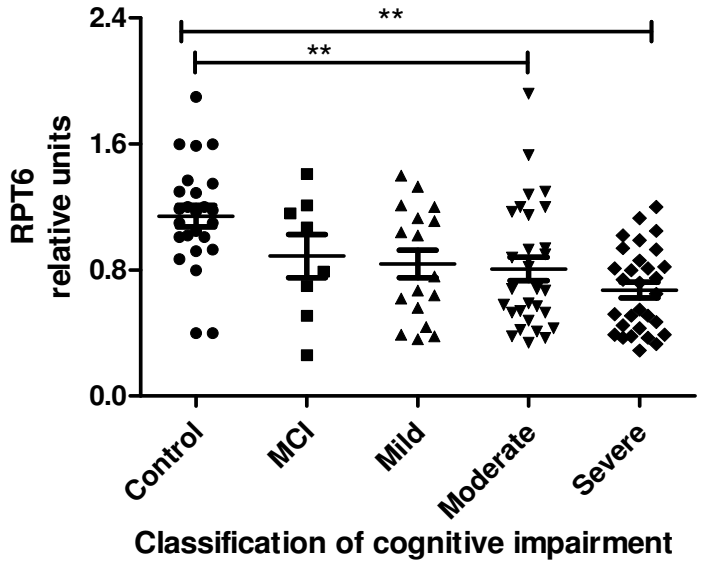


Figure 7-16: Relationship between RPT6 expression levels in BA40 and cognitive impairment based on MMSE classification

Linear regression analyses were conducted with the three predictors to develop a model for the best predictor for cognitive impairment. In the analyses, MMSE scores (after grouping the cases) were entered as dependent variables with RPT6, α-3 and α-6 values from semi-quantitative Western blotting in BA40 as independent factors. Cognitive impairment in the dementia cases (DLB, PDD and AD) and the control cases was significantly predicted by RPT6 with (Beta = -0.441 and p = 0.001). One-way ANOVA was performed to compare semi-quantitative Western blotting values of RPT6 and the cognitive impairment groups. The cognitive impairment groups were: 'unimpaired cognition' for the clinical control cases; 'mildly impaired cognition without dementia' cases with scores 24 or above; 'mildly impaired cognition without dementia' = 25–30; 'mildly impaired cognition with dementia' = 17–24; 'moderately impaired cognition' = 10–16 and 'severely impaired cognition' = 9 or less. The analysis indicated high levels of RPT6 in cases with unimpaired cognition compared to the other groups (one-way ANOVA F = 6.472, d.f. = 4 and 102, p = 0.001; Bonferroni post hoc test). The horizontal bars within the data points represent the mean.

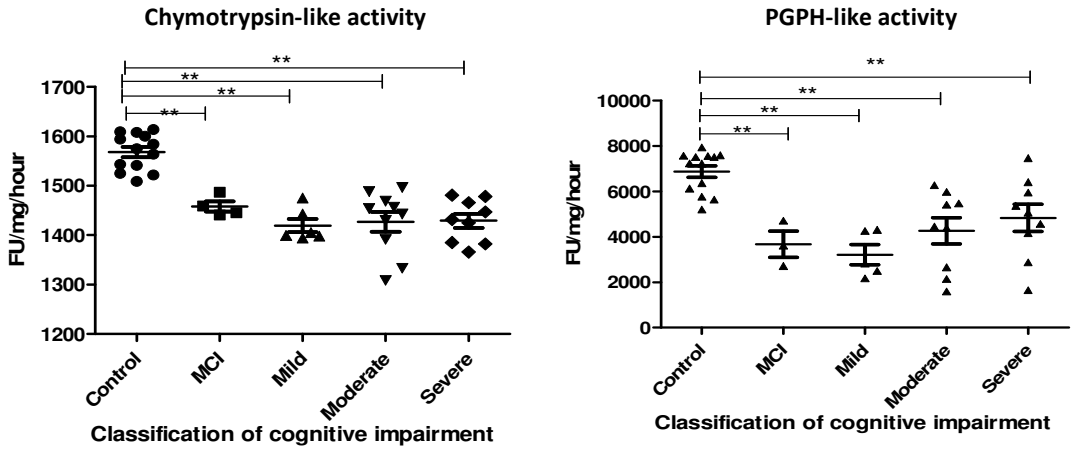


Figure 7-17: Relationship between chymotrypsin- and PGPH-like activities in BA40 and cognitive impairment based on MMSE classification

One-way ANOVA was performed to compare chymotrypsin- and PGPH-like activities and cognitive impairment groups. The cognitive impairment groups were: 'unimpaired cognition' for the clinical control cases; 'mildly impaired cognition without dementia' for cases with scores 24 or above; 'mildly impaired cognition without dementia' = 25–30; 'mildly impaired cognition with dementia' = 17–24; 'moderately impaired cognition' = 10–16 and 'severely impaired cognition' = 9 or less. The analysis indicated high chymotrypsin-like activity in the control cases compared to the cases with MCI ($p = 0.001$), mild ($p = 0.001$), moderate ($p = 0.001$) and severe scores ($p = 0.001$) (one-way ANOVA $F = 21.845$, d.f. = 4 and 37, $p = 0.001$; Bonferroni post hoc test). The difference in PGPH-like activity between the cognitive impairment groups was significantly different between unimpaired cognition cases compared to cases with MCI ($p = 0.02$), mild ($p = 0.001$), moderate ($p = 0.001$) and severe scores ($p = 0.034$) (one-way ANOVA $F = 8.851$, d.f. = 4 and 35, $p =$

0.001; Bonferroni post hoc test). The horizontal bars within the data points represent the mean.

7.3.2.2 Persecution

Regression analyses were also conducted with the following predictor variables: RPT6, α-3 and α-6 in BA40 with the semi-scoring for persecution. A significant model emerged ($F_{3,89} = 3.058$, $p = 0.032$, adjusted R square = 0.063). RPT6 was the only significant variable with a β value (-.289 and $p = .007$) (Figure 7-18), although analysis of variance (ANOVA) was not significant (one-way ANOVA $F = 2.185$, d.f. = 3 and 89, $p = 0.095$; Bonferroni post hoc test). Persecution scores were significantly predicted by RPT6 values in both brain regions BA9 and 40. Linear regression analyses were conducted with RPT6 expression level in BA9 and 40 and persecution scores to investigate in which brain region, reduction of RPT6 is best predicting persecution scores. Persecution scores were significantly predicted by RPT6 expression level in BA9 with (Beta = -0.27 and $p = 0.018$).

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.306[a]	.093	.063	.967

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	8.579	3	2.860	3.058	.032[b]
	Residual	83.228	89	.935		
	Total	91.806	92			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.003	1.061		.946	.347
	α-3	1.231	.686	.188	1.795	.076
	RPT6	**-1.809**	**.661**	**-.289**	**-2.736**	**.007**
	α-6	.061	.781	.008	.078	.938

Figure 7-18: Relationship between RPT6 expression levels in BA40 and persecution score.

Linear regression analyses were conducted with the three predictors to develop a model for the best predictor for persecution. In the analyses, persecution scores were entered as dependent variables with RPT6, α-3 and α-6 values from semi-quantitative Western blotting in BA40 as independent factors. Persecution in the dementia cases (DLB, PDD and AD) and the controls was significantly predicted by RPT6 with (Beta = -0.289 and p = 0.007).

7.3.3 Anterior cingulate Brodmann area 24

7.3.3.1 Cognitive decline

MMSE scores were also predicted by RPT level in BA24, as in the case of BA40 and BA9, but in BA24 MMSE scores were also predicted by α-6 the output of the regression analysis is shown in (Figure 7-19). Due to the significant relationship between RPT6 level and MMSE scores in BA9, 40 and 24, Linear regression analyses were conducted with RPT6 expression level in BA9, 40 and 24 and MMSE score to investigate in which brain region, reduction of RPT6 is best predicting MMSE scores. MMSE scores were significantly predicted by RPT6 expression level in BA9 and 40 with a higher beta value in BA9 (Beta = -0.448 and p = 0.001).

Linear regression analyses were conducted with both predictors, chymotrypsin-like and PGPH-like activities, to develop a model for the best predictor for cognitive decline. In the analyses, MMSE scores (after grouping the cases) were entered as dependent variables with chymotrypsin-like and PGPH-like activities measured using fluorogenic substrates assay in BA24 as independent factors. MMSE scores were significantly predicted by PGPH-like activity with (Beta = -0.596 and p = 0.001) (Figure 7-19).

One-way ANOVA was performed to compare chymotrypsin-like and PGPH-like activities and scoring groups for MMSE. There was no significant different in chymotrypsin-like activity in the control cases compared to all other groups. Analysis of variance (ANOVA) followed by Bonferroni post-hoc tests indicated high PGPH-like activity in the control cases compared to the cases with MCI (p = 0.03), mild (p = 0.024),

moderate ($p = 0.001$) and severe scores ($p = 0.001$) (one-way ANOVA $F = 9.839$, d.f. = 4 and 35, $p = 0.001$; Bonferroni post hoc test) (Figure 7-20).

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.373[a]	.139	.113	1.44060

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	32.554	3	10.851	5.229	.002[b]
	Residual	201.308	97	2.075		
	Total	233.861	100			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.642	1.961		.328	.744
	α-3	-.550	1.058	-.055	-.520	.604
	RPT6	**-2.741**	**1.013**	**-.269**	**-2.705**	**.008**
	α-6	**.5.695**	**1.773**	**.331**	**3.213**	**.002**

Figure 7-19: Relationship between RPT6 and α-6 expression levels in BA24 and cognitive impairment based on MMSE classification

Linear regression analyses were conducted with the three predictors to develop a model for the best predictor for cognitive impairment. In the analyses, MMSE scores (after grouping the cases) were entered as dependent variables with RPT6, α-3 and α-6 values from semi-quantitative Western blotting in BA24 as independent factors. Cognitive impairment in the dementia cases (DLB, PDD and AD) and the control cases was significantly predicted by RPT6 with (Beta = -0.269 and p = 0.008) and α-6 (Beta = 0.331 and p = 0.002).

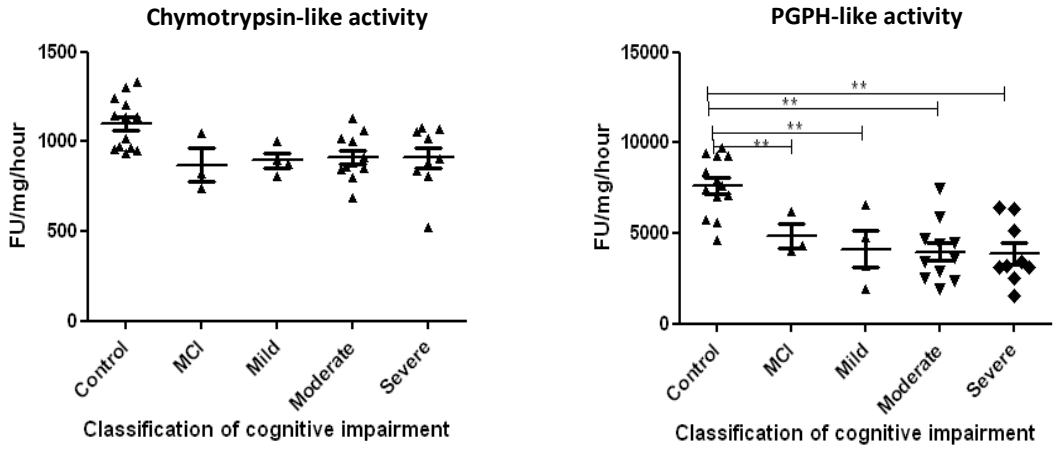


Figure 7-20: Relationship between chymotrypsin- and PGPH-like activities in BA24 and cognitive impairment based on MMSE classification.

One-way ANOVA was performed to compare chymotrypsin- and PGPH-like activities and cognitive impairment groups. The cognitive impairment groups were: 'unimpaired cognition' for the clinical control cases; 'mildly impaired cognition without dementia' for cases with scores 24 or above; 'mildly impaired cognition without dementia' = 25–30; 'mildly impaired cognition with dementia' = 17–24; 'moderately impaired cognition' = 10–16 and 'severely impaired cognition' = 9 or less. There was no significant difference in chymotrypsin-like activity in the control cases compared to all other groups. The difference in PGPH-like activity between cognitive impairment groups was significantly different between unimpaired cognition cases and the cases with MCI ($p = 0.03$), mild ($p = 0.024$), moderate ($p = 0.001$) and severe scores ($p = 0.001$) (one-way ANOVA $F = 9.839$, d.f. = 4 and 35, $p = 0.001$; Bonferroni post hoc test). The horizontal bars within the data points represent the mean.

7.3.3.2 Depression

The expression level of the proteasome sub-units in BA24 did not associate with any non-cognitive behaviours, but this was not the case with the proteasome activity. Regression analyses were conducted with the following predictor variables: PGPH-like activity and chymotrypsin like activity in BA24, with the semi-scoring for depression. A significant model emerged ($F_{2,34} = 4.892$, $p = 0.002$, adjusted R square = 0.258). Depression scores were significantly predicted by PGPH-like activity β value (-.526 and $p = .007$), analysis of variance (ANOVA) was significant (one-way ANOVA $F = 8.786$, d.f. = 3 and 33, $p = 0.001$), but with Bonferroni post hoc the difference in PGPH-like activity between were between depression absent and mild ($p = 0.001$) and did not reach the significant level $p < 0.01$ with intermittent ($p = 0.038$) and persistent ($p = 0.087$).

7.3.4 Summary

Having identified the relationship between the proteasome components and the underlying clinical pathology, we subsequently evaluated the relationship between proteasome dysfunction and cognition function and non-cognitive behaviours in LBD and AD. In BA9, 40 and 24, a significant relationship was found between RPT6 and MMSE scores. These data lead to further studies to investigate in which brain region the reduction in RPT6 values were more associated with MMSE scores. We discovered that RPT6 expression level in BA9 was the best predictor for MMSE scores (Beta = -0.448 and $p = 0.001$). In BA9 and BA40 MMSE scores were predicted by chymotrypsin-like activity, while in BA 24 by PGPH-like activity. As the analysis of the relationships between non-cognitive behaviours and mood and proteasome markers were exploratory and unbiased

since there were no compelling hypotheses linking them, a high significance level of $p<0.01$ was set. Persecution was significantly predicted by RPT6 expression in both brain regions BA9 and BA40 and PGPH-like activity in BA40 only. Depression was significantly predicted by RPT6 expression in BA9 and PGPH-like activity in BA24. However, both with persecution and depression scores, ANOVA did not reach the significance level at $p < 0.01$

Table 7-2: Relationships between clinical data and expression levels of proteasome sub-units and proteasome activity.

BA9	MMSE	Depression	Persecution
RPT6	***-0.564****	***-0.275****	***-0.363****
ALPHA6	-0.012	0.142	-0.024
ALPHA3	0.067	-0.110	0.071
chymotrypsin-like activity	-0.416*	-0.508*	-0.067
PGPH-LIKE activity	-0.269	0.116	-0.173

BA24	MMSE	Depression	Persecution
RPT6	***-0.269****	-0.048	-0.148
ALPHA6	***0.331****	0.19	0.054
ALPHA3	-0.054	-0.237	0.017
chymotrypsin-like activity	-0.022	-0.033	-0.052
PGPH-LIKE activity	***-0.596****	***-0.526****	-0.418*

BA40	MMSE	Depression	Persecution
RPT6	***-0.441****	-0.201	***-0.289****
ALPHA6	-0.087	-0.002	-0.008
ALPHA3	-0.105	0.218	0.188
chymotrypsin-like activity	***-0.724****	-0.227	-0.036
PGPH-LIKE activity	-0.044	-0.398*	***-0.550****

MMSE, depression and persecution predicted by proteasome sub-units levels and proteasome activities indicated by the standardised regression coefficients (β).

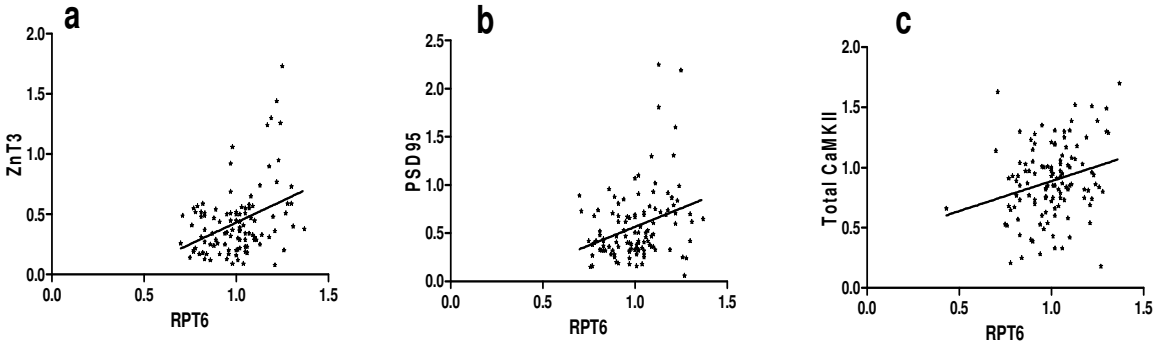
Values that are presented in ***bold italics with**** indicate statistically significant ($p<0.01$) and the values with with * indicate statistically significant ($p<0.05$) differences.

7.4 Relationships between synaptic biochemistry and expression levels of proteasome subunits and proteasome activity

Measurements of synaptic markers and proteins associated with various synaptic processes, such as PSD-95, ZnT3, synaptophysin and beta-III-tubulin measurements were available (measured by Dr David Whitfield) (Whitfield et al., 2014a, Whitfield et al., 2014b). Measurements of SNARE proteins such as munc18a and CAMKII were also available (measured by Dr Julie Vallortigara) (For full details of all values refer to Appendix table IX and X Synaptic proteins values from semi-quantification of Western blotting and to appendix tables XI for the differences in relative levels of proteins between diagnostic groups). The measurements were done using the same methods discussed in the Materials and Methods sections. To test whether synaptic dysfunction was associated with proteasomal impairment, Pearson and Spearman's rank correlations were determined between the synaptic protein measured by semi-quantitative Western blot in each brain region and the proteasomal sub-unit. The synaptic and SNARE proteins which correlated with any of the proteasome component at a significant level $p < 0.01$ were entered into a regression analysis (using the enter method in SPSS) to determine if they were significant predictors of the proteasome sub-unit to which the correlation occurred. The proteasome regulatory particle subunit RPT6 was the only sub-unit which correlated with synaptic and/or SNARE proteins across the three brain regions.

7.4.1 Frontal cortex – Brodmann area 9

In BA9 significant positive correlations were found between RPT6 and PSD95 (Rs .255, p = .008, n = 107), ZnT3 (Rs .356, p = .001, n = 110), and total CaMKII (Rs .255, p = .005, n = 122) (Figure 7-21 a, b, c,). Multiple linear regression analysis was conducted with the three predictors (PSD95, ZnT3, and total CaMKII) to develop a model for the best predicting RPT6 sub-unit. In the analysis PSD95, ZnT3, and total CaMKII were entered as independent factors and RPT6 as dependent variables. The ANOVA for the model was significant (p = 0.001, Rsq = 0.176) and RPT6 values from semi-quantitative Western blotting in BA9 was significantly predicted by ZnT3 (Beta = 0.240, p = 0.023) and total CaMKII (Beta = 0.248, p = 0.019) (Figure 7-21).



Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.419[a]	.176	.148	.14004

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.372	3	.124	6.328	**.001[b]**
	Residual	1.745	89	.020		
	Total	2.118	92			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.949	.067		14.267	.000
	ZnT3 BA9	**.155**	**.067**	**.240**	**2.311**	**.023**
	PSD95 BA9	-.097	.065	-.143	-1.483	.142
	total CaMKII	**.125**	**.052**	**.248**	**2.393**	**.019**

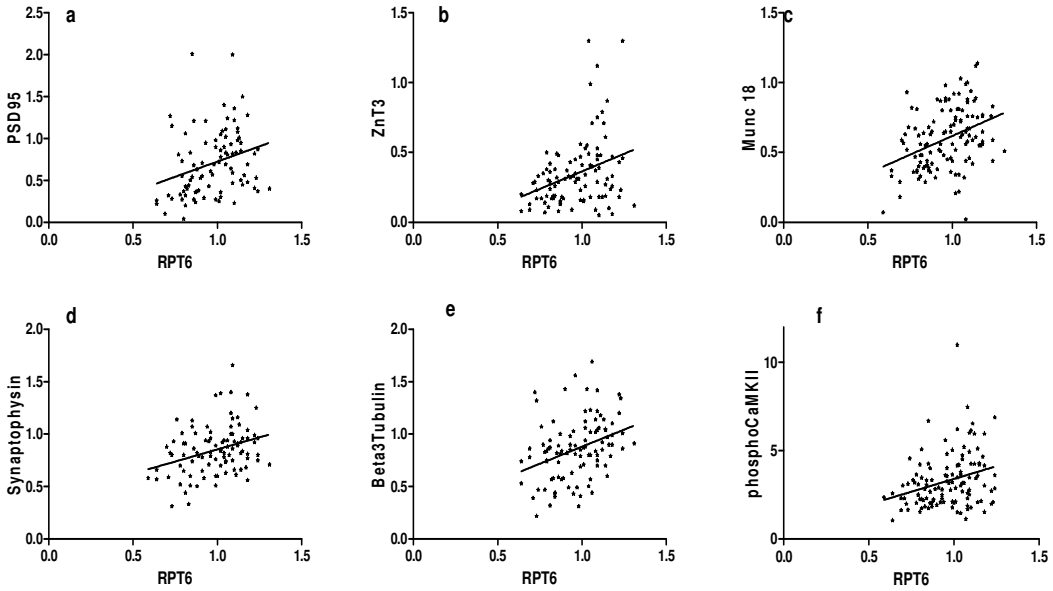
Figure 7-21: Relationships between expression of protesome sub-unit and synaptic proteins.

Pearson correlation was determined between the synaptic proteins (measured by semi-quantitative Western blot) and the proteasomal sub-units in BA9. Significant positive correlations were found between RPT6 and **a)** PSD95 (Rs .255, p = .008, n = 107), **b)** ZnT3 (Rs .356, p = .001, n = 110), and **c)** total CaMKII (Rs .255, p = .005, n = 122). Multiple linear regression analysis was conducted with the three predictors (PSD95, ZnT3, and total CaMKII) to develop a model for the best predicting RPT6 sub-unit. In the analysis PSD95, ZnT3, and total CaMKII were entered as independent factors and RPT6 as dependent variables. The ANOVA for the model was significant (p = 0.001, Rsq = 0.176) and RPT6 values from semi-quantitative Western blotting in BA9 was significantly predicted by ZnT3 (Beta = 0.240, p = 0.023) and total CaMKII (Beta = 0.248, p = 0.019)

7.4.2 Parietal cortex—Brodmann area 40

In BA40 significant positive correlations were found between RPT6 and PSD95 (Rs .286, p = .006, n = 90), ZnT3 (Rs .299, p = .006, n = 121), SPP (Rs .319, p = .002 n = 95), Munc18 (Rs .377, p = .001 n = 121), phospho-CaMKII (Rs .294, p = .001, n = 117) and Beta-3 Tubulin (Rs .347, p = .001, n = 98) (Figure 7-22 a, b, c, d, f, e). Multiple linear regression analysis was conducted with the 6 predictors (PSD95, ZnT3, Munc18, SPP, Beta-3 Tubulin and phospho CaMKII) to develop a model for the best predicting RPT6 sub-unit. In the analysis, PSD95, ZnT3, Munc18, SPP, Beta-3 Tubulin and phospho-CaMKII were entered as independent factors and RPT6 as dependent variables. The

ANOVA for the model was significant ($p = 0.001$, $Rsq = 0.176$) and RPT6 values from semi-quantitative Western blotting in BA40 was significantly predicted by ZnT3 (Beta = 0.367, $p = 0.003$), SPP (Beta = 0.303, $p = 0.033$) and phospho-CaMKII (Beta = 0.265, $p = 0.027$) (Figure 7-22).


a
PSD95
RPT6
b
ZnT3
RPT6
c
Munc 18
RPT6
d
Synaptophysin
RPT6
e
Beta3Tubulin
RPT6
f
phosphoCaMKII
RPT6

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.715[a]	.511	.442	.11917

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.637	6	.106	7.478	**.000[b]**
	Residual	.611	43	.014		
	Total	1.248	49			

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	.614	.092		6.689	.000
	PSD95_40	-.075	.049	-.206	-1.528	.134
	ZnT3_40	**.208**	**.067**	**.367**	**3.113**	**.003**
	Munc18 BA40	.128	.097	.162	1.319	.194
	Synaptophysin BA40	**.202**	**.092**	**.303**	**2.199**	**.033**
	Beta3Tubulin BA40	.088	.066	.179	1.328	.191
	phosphoCaMKII BA40	**.023**	**.010**	**.265**	**2.290**	**.027**

Figure 7-22: Relationships between expression of protesome sub-unit and synaptic proteins.

Pearson correlation was determined between the synaptic protein and the proteasomal sub-unit in BA40, as measured by semi-quantitative Western blot. Significant positive correlations were found between RPT6 and PSD95 (Rs .286, p = .006, n = 90), ZnT3 (Rs .299, p = .006, n = 121), SPP (Rs .319, p = .002 n = 95), Munc18 (Rs .377, p = .001 n =

121), phospho CaMKII (Rs .294, p = .001, n = 117) and Beta-3 Tubulin (Rs .347, p = .001, n = 98). Multiple linear regression analysis was conducted with the 6 predictors (PSD95, ZnT3, Munc18, SPP, Beta-3 Tubulin and phospho CaMKII) to develop a model for best predicting the RPT6 sub-unit. In the analysis, PSD95, ZnT3, Munc18, SPP, Beta-3 Tubulin and phospho CaMKII were entered as independent factors and RPT6 as dependent variables. The ANOVA for the model was significant (p = 0.001, Rsq = 0.176) and RPT6 values from semi-quantitative Western blotting in BA40 was significantly predicted by ZnT3 (Beta = 0.367, p = 0.003), SPP (Beta = 0.303, p = 0.033) and phospho CaMKII (Beta = 0.265, p = 0.027).

7.4.3 Anterior cingulate Brodmann area 24

In BA24 there was no significant correlation between RPT6 and any synaptic or SNARE proteins.

7.4.4 Summary

Since synaptic biochemistry data were available for these cases, a secondary exploratory analysis was perform to study the relationship between changes in proteasome function and components of the synaptic biochemistry. This helped to understand the relationship between the proteasome impairment, synaptic dysfunction, pathology score and cognitive decline. The results indicated a relationship between the reduction of RPT6 and the synaptic dysfunction. In BA9 significant positive correlations were found between RPT6 and PSD95, ZnT3, and total CaMKII. PSD95 and ZnT3 were also positively correlated with RPT6 in BA40. Significant positive correlations were found between RPT6 and SPP, Munc18, phospho-CaMKII, and Beta-3 Tubulin.

7.5 Discussion

7.5.1 Association between proteasome dysfunction and LBs/AD semi-quantitative pathology scores

The main findings of this study was that in BA24 and BA40, tangle and plaque scores inversely predicted RPT6 values, whereas in BA9, RPT6 values were predicted by α-synuclein and plaque scores. Subunit α-3 and α-6 expression were predicted by α-synuclein and tangle scores respectively, in BA9 only, whereas in BA24 and BA40, α-3 and α-6 had no associations with any of the semi-quantitative scores for AD and LBs pathology. In all three brain regions, RPT6 values had an inverse relationship with tangle and plaque scores (RPT6 values were decreased with higher tangle and plaque scores). RPT6 values also had a positive relationship with both activities measured in this project: chymotrypsin-like activity and PGPH-like activity (RPT6 values were decreased with low proteasome

activity) (Chapter 5 Section 5.2.3). Furthermore, chymotrypsin-like activity and PGPH-like activity were both inversely associated with AD pathology suggesting that there is a link between the regulatory particles subunit RPT6, proteasome activity and this aspect of the pathology of the disease.

It is possible that changes in RPT6 is the primary event in proteasome impairment. The observed reduction in proteasome activity may arise, therefore, as a result of lower RPT6 expression, which has been shown in previous studies to regulate proteasome function following phosphorylation at Serine-120 by Calcium/calmodulin-dependent protein kinase II (CaMKII) (Djakovic et al., 2012, Djakovic et al., 2009). Inhibition of CaMKII significantly decreases RPT6 phosphorylation and also proteasome activity (Jarome et al., 2013), indicating the important role of RPT6 in proteasome function and supporting the link between reduced RPT6 observed in this study and the reduction in proteasome activity. It is also possible that protein aggregates may block the entry pore to the 20S proteasome resulting in a reduction of proteasome proteolytic activity. This hypothesis is supported by evidence that degradation of proteins by the proteasome may be prevented by α-synuclein (Emmanouilidou et al., 2010, Lindersson et al., 2004, Liu et al., 2005, Zhang et al., 2008), tau (Keck et al., 2003) and Aβ (Almeida et al., 2006, Lopez Salon et al., 2003, Oh et al., 2005, Tseng et al., 2008). If this latter explanation is correct, it is unclear why aggregations would lead to a corresponding reduction in RPT6.

In this study, the association between semi-quantitive α-synuclein pathology score and the reduction of RPT6 values were observed only in BA9, and there were no similar associations observed in any other brain regions. Recent studies have demonstrated that

some soluble oligomeric species of mutant and wild-type α-synuclein inhibit the 26S proteasome in PC12 cells (Emmanouilidou et al., 2010). Emmanoulidou et al. revealed that decreased activation of the proteasome by oligomeric α-synuclein did not affect the expression level of the proteasome subunits (β1, β2 and β5 subunits) or the assembly of the 26S proteasome (Emmanouilidou et al., 2010). Furthermore, a small amount of 19S RPT6 ATPase bound to α-synuclein was detected, suggesting that localization of particular species of oligomeric α-synuclein impaired proteasome function by reducing the number of the substrates that normally interact with the 19S ATPase ring or by preventing the unfolding and degradation of other substrates (Emmanouilidou et al., 2010). Interestingly, pharmacological dissociation of oligomeric α-synuclein from the proteasome restored the proteasome function and reduced the level of polyubiquitinated protein (Emmanouilidou et al., 2010). These results agree with the findings of other studies, in which soluble α-synuclein protofibrils selectively inhibited the ubiquitin-independent proteasomal degradation of unfolded proteins and ubiquitin-dependent proteasomal degradation of folded proteins (Zhang et al., 2008). Zhang et al. also demonstrated that α-synuclein protofibrils bound both 26S proteasome and its substrates, preventing the degradation of the substrates by the proteasome, which may result in the accumulation of these proteins (Zhang et al., 2008). Further work is required to elucidate the exact sequence of events regarding RPT6/PS activity changes.

One unanticipated finding was the upregulation of α-3 values in BA9 and BA40 in DLB and PDD, respectively. The reason for this is not clear, but it may linked to auto-inhibition of the core particle of the proteasome that arises by N-terminal tails of the (α-3, α-4 and α-2) subunits, as mentioned previously in Chapter 1 Section 1.9.1.1 and as

discussed in Chapter 5 Section 5.3.1. Supporting this hypothesis, there was a positive relationship between upregulation of α-3 expression level in BA9 and α-synuclein score (α-3 values were increased with higher α-synuclein scores; the prediction was of a direct nature). The upregulation of α-3 was evident in DLB but not in PDD or AD, perhaps due to the absence of severe BA9 α-synuclein pathology in both PDD and AD cases. In contrast, the upregulation of α-3 in PDD in BA40 was not associated with either α-synuclein or AD pathology. Therefore, an increase in the α-3 subunit values in PDD and DLB may be a compensatory mechanism to alleviate the effects of pathological factors after the brain region has become affected by α-synucleinopathy.

The association of proteasome dysfunction with tau pathology was observed in BA9, BA24 and BA40. There were inverse relationships between the proteasome activity and tangle score as well as between RPT6 and tangle scores. Furthermore, α-6 values also had an inverse relationship with tangle scores. These findings are not surprising and are supported by previous research that links UPS with tau pathology in AD (review by (Lee et al., 2013) (explained in more detail in Chapter 1 Section 1.9.3.3.2). Tau is a substrate of the proteasome (David et al., 2002, Shimura et al., 2004, Wang and Mandelkow, 2012), and proteasome inhibition prevents the degradation of tau and results in its accumulation (Babu et al., 2005). Degradation of native unfolded tau was also shown to be catalysed by ubiquitin-independent 20S proteasome (David et al., 2002, Grune et al., 2010). Moreover, proteasome activity was found to be responsible for removing early tau pathology in the triple transgenic mouse model of AD (3xTg-AD) after treatment with Aβ immunotherapy (Oddo et al., 2004).

Dysfunction of the UPS has been implicated in Aβ pathology; as with α-synuclein and tau, Aβ was found to be a substrate of the proteasome, and inhibition of the proteasome prevented Aβ degradation (Lopez Salon et al., 2003, Tseng et al., 2008). There was an inverse relationship between plaque score and the reduction in RPT6 in BA9, 24 and 40. In addition, the same relationship is observed with PGPH-like activity in BA24 and with chymotrypsin-like activity in BA40. Thus, the present study confirms that Aβ pathology is associated with proteasome impairment although the detailed mechanisms underpinning this impairment remain unclear.

In summary, the main role of the UPS is the removal of damaged soluble proteins. LBD and AD both involve the accumulation of specific proteins, forming an aggregates with strong immunoreactivity to antibodies against ubiquitin (Lennox et al., 1988, Lowe et al., 1988, Mori et al., 1987), suggesting the important role of UPS in both LBD and AD. Previous studies have demonstrated that the main components of each of these aggregate—α-synuclein (Bennett et al., 1999), tau (David et al., 2002, Shimura et al., 2004, Wang and Mandelkow, 2012) and Aβ (Lopez Salon et al., 2003)—can be degraded by the proteasome. Furthermore, inhibition of the proteasome in tissue culture by proteasome inhibitors such as lactacystin or MG132 increased the aggregation and cytotoxicity of α-synuclein (Tanaka et al., 2001), tau (Babu et al., 2005) and Aβ (Lopez Salon et al., 2003). The results of the current study support a possible relationship between the reduction of RPT6 and the alteration of the other proteasome components on the one hand and semi-quantitative scores of AD and LBD pathology in different brain areas.

7.5.2 Association between proteasome dysfunction and clinical data

The purpose of this study was to assess the relationship between cognitive decline, non-cognitive symptoms and proteasome impairment. It was hypothesised that cognitive decline and non-cognitive symptoms would be associated with proteasome impairment as a consequence of synaptic dysfunction and increased protein aggregation in LBD and AD. In the previous sections, the relationship between the reduction of RPT6 and the alteration of the other proteasome components and semi-quantitative scores of AD and LBD pathology in different brain areas was reported. In this study, for the first time, the association between the reduction of RPT6 in addition to the proteolytic activity of the proteasome and cognitive decline and non-cognitive symptoms has been demonstrated.

RPT6 values in BA9, BA24 and BA40 predicted the cognitive impairment categories based upon MMSE scores. In BA9, control cases had high RPT6 values compared to all the other cognitive impairment categories, even in cases with high MMSE scores (the MCI group with scores ranging from 24 to 30). There was no significant difference between the nonsignificantly different cognitive impairment categories (MCI, mild, moderate and severe), but they all differed from the control group. This suggests that the reduction in the RPT6 levels appears to occur at a very early stage of dementia development, specifically in the BA9 region. This relationship is consistent with many evidences accumulated to date that cognitive and executive function are mediated by the prefrontal cortex (Cato et al., 2004, Funahashi, 2006, Smith and Jonides, 1999, Zhang et al., 2013). In BA40, the reduction in RPT6 was observed in all the cognitive impairment categories but it was significant only in the moderate and severe groups compared with the control group. This suggests that, after an initial decline in cognitive impairment, there were further significant reductions in the late stage of the disease. While there was no

significant difference seen in the ANOVA test between any of the cognitive impairment categories group compared to the control group, in BA24, regression analysis showed that RPT6 predicted cognitive impairment. This result suggests that the reduction in RPT6 in BA9 and BA40 has a greater impact on cognitive impairment than those seen in BA24. Regarding the proteolytic activity, in BA9 and BA40, the MMSE scores were predicted by chymotrypsin-like activity, while in BA24 the scores were predicted by PGPH-like activity. In reviewing the literature, no previous data was found on the association between proteasome dysfunction and cognitive decline/non-cognitive symptoms.

Dysfunction of the 20S/26S has been linked to different neurodegenerative diseases (Reviewed by (Dennissen et al., 2012, Paul, 2008) and Chapter 1 Section 1.9.4 for more details on the implication of the UPS in neurodegenerative disorders). Furthermore, there is a role for the UPS in various types of synaptic plasticity (Reviewed by (Hegde, 2010) and also Chapter 1 Section 1.9.3), it is not clear how proteasome impairment, specifically reduction in RPT6, could result in cognitive decline. Recently CaMKII, which plays an essential role in long-term synaptic plasticity and cognitive function (Giese et al., 1998, Miyamoto, 2006), has been shown to mediate proteasome activity and act as a scaffold to recruit proteasomes to dendritic spines and regulate its activity by phosphorylation of the RPT6 subunits (Bingol et al., 2010). Activation of NMDA receptors has been shown to induce this movement of the proteasome to the dendritic spine compartment (Bingol and Schuman, 2006). NMDA function can be positively regulated by CaMKII phosphorylation and also by interaction with Zn^{2+}. Consistent with this, the present study found a positive relationship between CaMKII and RPT6 expression levels in BA9 and in BA40; thus, with the decreased levels of RPT6 there was also a decrease in the protein levels of CaMKII.

Reductions in CaMKII affects signalling pathways, including phosphorylation of RPT6, and thus the proteasome activity, which could in turn impair synaptic plasticity and also contribute to cognitive dysfunction. Or it could the opposite direction; synaptic loss leads to decreased NMDA receptors, decreased CaMKII and hence decreased proteasome activity.

Furthermore, the BA9 RPT6 values predicted the semi-quantitative depression and persecution scores in an inverse manner, such that the cases with higher depression or persecution scores had lower RPT6 levels. Persecution was also predicted by the RPT6 values in BA40. It should be noted that the control cases were added to the analysis under the assumption that they did not have clinical depression or persecution (for more details on the clinical and pathological data for the control cases see Chapter 2, Section 2.1). Persecution and depression were also significantly predicted by PGPH-like activity in BA40 and BA24, respectively. The significant association between the RPT6 values and the depression semi-quantitative scores in individuals with DLB, PDD and AD was observed only in the prefrontal cortex (BA9). The same association has been reported between ZnT3 and depression in the same brain region and in the same cohort, and also between cognitive function and depression (Whitfield et al., 2014a). This relationship was not observed in either the BA24 or the BA40. Furthermore, this relationship is consistent with previous studies that have linked depressive symptoms to the prefrontal cortex (Khundakar et al, 2009a, Khundakar et al 2009b). ZnT3 plays an important role in sequestration of Zn^{2+} in vesicles and empty their contents into the synaptic cleft. Reduced ZnT3 corresponds to a loss of regulation of synaptic Zn^{2+}. Zn^{2+}dyshomeostasis has been linked to depression, and many studies (Grieger et al 2009, Van Kempen et al 1985, Yang et al 2005, Maes 1994, Siwek 2010), but not all studies, (Irmisch 2010, Narang 1991,

Crayton 2007), have suggested that depression might be associated with lower zinc concentrations. Zn^{2+} also plays an essential role in the deubiquitination step and in the whole proteolytic degradation cycle (Yao and Cohen, 2002). Furthermore, Zn^{2+}can induce an extensive structural rearrangement of the *Drosophila* 26 S proteasome, that, in the presence of Zn^{2+}, disassembles into regulatory particles and catalytic particles, a process that is fully reversible by removing Zn^{2+} (Kiss et al., 2005). Several questions remain unanswered. Does the downregulation of ZnT3 reported in this cohort (Whitfield et al., 2014a, Whitfield et al., 2014b) have an impact on proteasome dysfunction by reducing the Zn^{2+} concentration, which may be implicated in the role that UPS plays in synaptic function and, hence, the molecular pathophysiology of depression? Or, is the proteasome dysfunction responsible for regulating ZnT3 and, due to its dysfunction, this resulted in the reduction of the expression level of ZnT3, leading to a reduction of the Zn^{2+} concentration, which is implicated in the pathophysiology of depression (Swardfager et al 2013).

7.5.3 Association between proteasome dysfunction and synaptic proteins

The present study was designed to determine the relationship between dysfunction of synaptic proteins and proteasome impairment. As mentioned in Chapter 1, Section 1.9.3, there is evidence on the role of UPS in regulating synaptic function; thus, the correlation between different synaptic proteins and the proteasome component was not surprising. The most interesting finding was the relationship between RPT6 and ZnT3, PSD95 and CaMKII, which was observed in both BA9 and BA40. Correlation analysis, revealed that there was positive correlation between RPT6 protein levels and each of these synaptic

proteins (ZnT3, PSD95 and CaMKII), such that cases with higher synaptic proteins levels had higher RPT6 levels (For full details of all values refer to appendix table IX and X Synaptic proteins values from semi-quantification of Western blotting and to appendix tables XI for the differences in relative levels of proteins between diagnostic groups).

In the previous section 7.4.2, the relationship among RPT6, ZnT3, cognitive impairment and depression suggested a new hypothesis where the dysfunction of the UPS could be a result of an alteration in ionic Zn^{2+} concentration due to dysregulation of zinc transporters, such as ZnT3. Other studies have also implicated alterations in Zn^{2+} homeostasis and lysosomal activity (Hancock et al 2014). The association between ZnT3 and UPS is probably related to synaptic dysfunction, as proteasome dysfunction was also associated with the reduction of PSD95. A decrease in zinc could cause a reduction in PSD95, and this has been shown previously by (Grabrucker et al., 2011). Interestingly, the reduction of PSD95 also predicted the cognitive impairment in the prefrontal cortex in the present cohort (Whitfield et al., 2014b), suggesting that there is a strong link between a loss of ZnT3, a reduction in post-synaptic scaffolding PSD95 protein levels, proteasome dysfunction and cognitive deficits.

The role of CaMKII increased RPT6 phosphorylation and thus has been showed to regulate the proteasome function *in vitro* and *in vivo* (Djakovic et al., 2012, Djakovic et al., 2009, Jarome et al., 2013). In this study, we confirmed the relationship between CaMKII and RPT6 in subjects with PDD, DLB and AD. Reduction in CaMKII has been reported in our cohort, and this reduction was associated with a high plaque score (Vallortigara et al., 2014). As previously mentioned in section (7.4.2), reduction in CaMKII may affect

signalling pathways including phosphorylation of RPT6 and thus proteasome activity which could in turn impair synaptic plasticity and also contribute to cognitive dysfunction.

These results provide further support to our hypothesis that cognitive decline and non-cognitive symptoms would be associated with proteasome impairment as a consequence of synaptic dysfunction and increased protein aggregation in LBD and AD. Further research should be undertaken to investigate whether activation of the UPS could result in increases in ZnT3 transport activity, PSD95 protein levels, CaMKII activity and of NMDAR activity, and whether targeting ZnT3/CaMKII to increase proteasome activity may act as a pharmacological intervention in LBD.

In conclusion, our results indicated reductions in the key proteasome component RPT6 and proteasome activity in BA9, 24, and 40. These reductions were associated with cognitive decline, non-cognitive symptoms, protein aggregate and synaptic dysfunction. These data suggested that that enhancement of the UPS could be a therapeutic target for treating DLB, PDD and AD. Increasing the proteasome activity could be done by modulating Zn^{2+} or by activating CaMKII.

7.6 Summary

- Decreases in RPT6 and proteasome activities were found to be associated with the semi-quantitative scores for plaques and neurofibrillary tangles in BA9, 24, and 40.
- Semi-quantitative plaque scores were significantly predicted by RPT6 in BA9, 40 and 24 and by chymotrypsin-like activity in BA40.

- Semi-quantitative tangle scores were significantly predicted by RPT6 in BA40 and 24 and by chymotrypsin-like activity in BA9 and 40.
- Semi-quantitative α-synuclein scores were significantly predicted by RPT6 in BA9 only.
- Cognitive impairment was significantly predicted by RPT6 in BA9, 40 and 24 and by chymotrypsin-like activity in BA9 and 40.
- Persecution was significantly predicted by RPT6 expression in both BA9 and BA40 and PGPH-like activity in BA40 only.
- Depression was significantly predicted by RPT6 expression in BA9 and PGPH-like activity in BA24.
- Significant positive correlations were found among RPT6 and PSD95, ZnT3, and total CaMKII respectively, in both BA9 and BA40.

Chapter 8 General discussion

The aim of this thesis was to characterise the components of the UPS. Since disruption of the UPS appears to play a key role in DLB pathogenesis and could cause synaptic dysfunction, it was hypothesised, that cognitive decline and non cognitive symptoms in LBD were associated with synaptic dysfunction consequent upon alterations of proteasome subunit expression, proteasome activity and increased protein aggregation. Upon examination of the 26/20S components in selected brain regions, proteasome impairment was evident, including a reduction in the expression level of the RPT6 ATPase 19S regulatory subunit and a reduction in both chymotrypsin-like and PGPH-like proteasome activity in DLB, PDD and AD groups in all three selected brain regions: BA9, BA24 and BA40 (the results are summarised in Table 8-1).

A further aim of this thesis was to evaluate the second important degradation pathway, the lysosomal pathway, using the same cohort. Two lysosomal markers, cathepsin D and lysosomal-associated membrane protein 1 (LAMP1), were assessed in BA9, 24 and 40 (the results are summarised in Table 8-1).

Table 8-1: Summary of the results.

		RPT6	α-3	α-6	Chymotrypsin-like	PGPH-like	Cathepsin D	LAMP1
BA9	PDD	↓	→	→	→	↓	→	→
	DLB	↓	↑	→	→	↓	→	→
	AD	↓	→	↓	↓	↓	→	→
BA24	PDD	→	↓	↑	↓	↓	-	↓
	DLB	↓	→	↑	↓	↓	-	↓
	AD	↓	→	→	↓	↓	-	→
BA40	PDD	→	↑	→	↓	↓	↑	→
	DLB	↓	→	→	↓	→	→	↓
	AD	↓	→	→	↓	↓	→	→

Downward arrow (↓) indicated decreased, upward arrow (↑) indicated increased and right arrow (→) indicates unchanged.

The 26S proteasome is the major eukaryotic ATP-dependent protease that forms the proteolytic component of the UPS. The UPS is the key pathway for the degradation of α-synuclein (Alvarez-Castelao and Castano, 2011, Bennett et al., 1999, Ebrahimi-Fakhari et al., 2011, Ebrahimi-Fakhari et al., 2012, Tofaris et al., 2001), and other proteins that contribute to neurodegenerative diseases, such as Aβ, (Lopez Salon et al., 2003) and tau (David et al., 2002). Over the past decade, considerable evidence has addressed the role of UPS dysfunction in many neurodegenerative diseases such as AD (Riederer et al., 2011, Upadhya and Hegde, 2007), Huntington's disease (Schipper-Krom et al., 2012), PD and DLB (McNaught et al., 2003, McNaught et al., 2002a, McNaught et al., 2002b, McNaught and Olanow, 2009, McNaught et al., 2002c, McNaught and Jenner, 2001, McNaught and Olanow, 2006, McNaught et al., 2001). Furthermore, the important role that UPS plays in protein degradation and in processes specific to neurons, such as synaptic function and synaptic plasticity (Yi and Ehlers, 2007), has led to the hypothesis that alteration in the proteasome subunits or proteolytic activity in LBD may increase the severity of the cortical

pathology, such as cortical Lewy bodies and Lewy neurites, loss of cortical synapses and synaptic dysfunction, in addition to manifesting in the clinical phenotype of the disease.

The results of this study offer substantial support for the dysfunction of the 26S proteasome in LBD. The significant reduction in the expression level of RPT6 and both chymotrypsin-like and PGPH-like proteasome activity were associated with AD pathology in each brain region. A significant correlation was also found between RPT6 expression levels and the reduction in proteasome activity. Both RPT6 expression levels and overall proteasome activity were found to be associated with cognitive decline. This was supported by the observation that there was a strong link between RPT6 expression levels and the reduction in post- and pre-synaptic proteins PSD-95 and ZnT3. Both PSD-95 and ZnT3 were correlated with cognitive decline in this cohort (Whitfield et al., 2014a, Whitfield et al., 2014b). Thus, it could be suggested that proteasome impairment may result from synaptic dysfunction since the proteasome function in DLB, PDD and AD cases was impaired and correlated with a reduction in the synaptic proteins. Synaptic dysfunction has been linked to Aβ and tau pathology. One study found a positive correlation between both PSD95 and synaptophysin and soluble Aβ40 and Aβ42 (Shinohara et al., 2013)(Shinohara et al., 2013). Furthermore, decreases in pre-and post-synaptic terminal density have recently been reported in a mouse model of tau pathology (Garringer et al., 2013). Our group found a relationship between pathology and synaptic proteins, such as SPP, PSD95 and ZnT3 (Whitfield et al., 2014a, Whitfield et al., 2014b), as well as a relationship between proteasome impairment and the same synaptic proteins, PSD95 and ZnT3.

Table 8-2: Summary of the relationships between expression levels of the proteasome sub-units and proteasome activity with clinical symptoms, pathology and synaptic biochemistry.

BA9	pathology score			clinical data			synaptic biochemistry		
	a-synuclein	Plaques	Tangles	MMSE	Depression	Persecution	ZnT3	PSD95	CaMKII
RPT6	***-0.226***	***-0.213***	-0.165	***-0.564****	***-0.275****	***-0.363****	***.356****	***.255****	***.255****
Alpha6	0.183	-0.033	***-0.339***	-0.012	0.142	-0.024	.133	0.060	***.246****
ALPHA3	***0.303***	-0.154	0.153	0.067	-0.110	0.071	-.015	0.080	***.262****
chymotrypsin-like activity	-0.071	-0.153	***-0.44***	-0.416*	-0.508*	-0.067	***.429****	.217	.153
PGPH-LIKE activity	-0.028	-0.331	-0.241	-0.269	0.116	-0.173	***.385****	.287	.082

BA24	a-synuclein	Plaques	Tangles	MMSE	Depression	Persecution	ZnT3	PSD95	CaMKII
RPT6	-0.157	***-0.27***	***-0.249***	***-0.269****	-0.048	-0.148	.027	-.284	-.030
ALPHA6	0.154	0.208	0.159	***0.331****	0.19	0.054	-.023	0.088	.116
ALPHA3	-0.118	-0.039	0.088	-0.054	-0.237	0.017	-.053	.248	.170
chymotrypsin-like activity	-0.083	0.229	0.171	-0.022	-0.033	-0.052	.256	-.108	-.271
PGPH-LIKE activity	0.051	***-0.514***	-0.288	***-0.596****	***-0.526****	-0.418*	.295	-.057	-.245

BA40	a-synuclein	Plaques	Tangles	MMSE	Depression	Persecution	ZnT3	PSD95	CaMKII
RPT6	0.068	***-0.328***	***-0.361***	***-0.441****	-0.201	***-0.289****	***.224****	.172	***.217****
ALPHA6	-0.089	-0.049	-0.083	-0.087	-0.002	-0.008	-.056	-.080	-.009
ALPHA3	-0.146	-0.055	-0.134	-0.105	0.218	0.188	.153	.146	.189
chymotrypsin-like activity	-0.075	***-0.587***	***-0.479***	***-0.724****	-0.227	-0.036	-.131	.117	-.068
PGPH-LIKE activity	-0.216	0.058	-0.114	-0.044	-0.398*	***-0.550****	.040	-.094	-.053

Pathology scores, MMSE, depression and persecution predicted by proteasome sub-units level and proteasome activities indicated by the standardised regression coefficients (β). Values that are presented in ***bold italics with*** indicate statistically significant ($p<0.01$) and the values without ***bold italics*** and with * indicate statistically significant ($p<0.05$) differences.

Synaptic protein correlated with proteasome sub-units level and proteasome activities indicated by Pearson Correlation Coefficient (r). Values that are presented in ***bold italics with*** indicate statistically significant ($p<0.01$) and the values with ***bold italics with*** * indicate statistically significant ($p<0.05$) differences.

The observed correlation between proteasome impairment and AD pathology also supports the hypothesis that proteasome dysfunction may result from protein aggregation. It has recently been shown that Aβ accumulation directly inhibits proteasome activity (Tseng et al., 2008). Consequently, proteasome inhibition may further exacerbate other pathological proteins, such as tau and α-synuclein, by reducing protein degradation, resulting in more protein accumulation and further proteasome impairment (Tseng et al., 2008). The mechanism could also be reversed: proteasome impairment could cause both synaptic dysfunction and protein aggregation. Impairment of the UPS has been reported to cause cell death, resulting from impaired degradation and protein accumulation. Previous studies have demonstrated the importance of the UPS for normal cellular functioning (Bergink et al., 2006, Borissenko and Groll, 2007b, Bregere et al., 2006, Brodsky and McCracken, 1999, Zimmermann et al., 2001). Furthermore, numerous studies have reported that proteasome impairment is a pathological feature of several neurodegenerative diseases, and that *in vitro* and *in vivo* inhibition of proteasomes induces pathological features resembling those found in PD and LBD (McNaught et al., 2003, McNaught et al., 2002a, McNaught et al., 2002b, McNaught and Olanow, 2009, McNaught et al., 2002c, McNaught and Jenner, 2001, McNaught and Olanow, 2006, McNaught et al., 2001) .

On the basis of the present study, it is not possible to determine which of the three mechanisms, synaptic dysfunction, protein aggregation and proteasome impairment, comes first, merely the fact that they are related. Is it the synaptic dysfunction that causes proteasome dysfunction leading to protein aggregates? Is synaptic dysfunction the primary

cause of protein aggregation leading to proteasome dysfunction? Or, is proteasome dysfunction the primary cause of protein aggregate leading to synaptic dysfunction? This is currently a matter of considerable debate. Under physiological conditions, α-synuclein exists in an unfolded monomeric form or as an α-helical structure bound to lipid membranes (Davidson et al., 1998, Weinreb et al., 1996). Whereas under pathological conditions, α-synuclein monomers undergo misfolding and aggregate into small oligomers that further aggregate into higher-order structures forming protofibril β−sheets (Bandopadhyay and de Belleroche, 2010, Conway et al., 1998, El-Agnaf et al., 1998, Hashimoto et al., 1999). Misfolded α-synuclein is degraded by both the UPS and the ALP, specifically the CMA pathway, in addition to chaperone-mediated refolding (Ebrahimi-Fakhari et al., 2011, Ebrahimi-Fakhari et al., 2012). The formation of these oligomer species and protofibril β−sheet structures leads to primary failure of both the degradation pathways in the UPS and the CMA (Ebrahimi-Fakhari et al., 2011, Ebrahimi-Fakhari et al., 2012). Furthermore, overexpression of monomeric α-synuclein can also block the UPS (Ebrahimi-Fakhari et al., 2011, Ebrahimi-Fakhari et al., 2012). At this stage, the macroautophagy is upregulated to degrade α-synuclein oligomers and protofibril β−sheets (Ebrahimi-Fakhari et al., 2011, Ebrahimi-Fakhari et al., 2012). Uncontrolled accumulation of α-synuclein leads to further conformational changes of the β−sheet structure to form fibril structures (Caughey and Lansbury, 2003), which are the main component of Lewy bodies and Lewy neurites. The formation of fibril structures impairs the macroautophagy and the lysosomal pathway (Ebrahimi-Fakhari et al., 2011, Ebrahimi-Fakhari et al., 2012). Therefore misfolded synuclein leads to a sequential inhibition of both cellular pathways involved in synuclein degradation, starting with impairment of the UPS and progressing to

encompass inhibition of macroautophagy. In the opposite direction, impairment of the UPS or the ALP will increase the rate of the misfolding of α-synuclein, leading to the formation of oligomers, protofibril β−sheets and fibrils. In yet another direction, synaptic dysfunction could cause protein aggregation in either of the pathways and/or impair the protein degradation pathways (Figure 8-1).

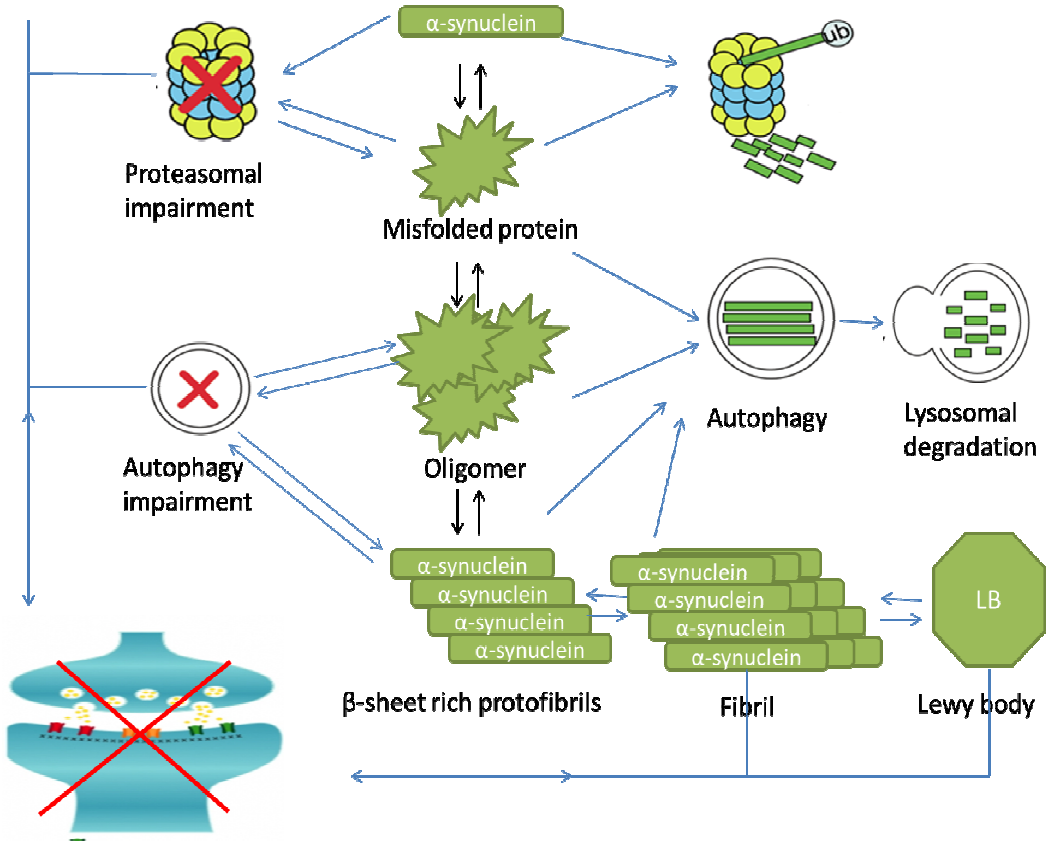


Figure 8-1: Accumulation of α-synuclein and the possibility of its toxicity.

Accumulation of misfolded proteins is likely to be a key event in neurodegeneration. Impairment of lysosomal and proteasomal protein-degradation pathways linked to synaptic dysfunction. Misfolded proteins linked to synaptic, proteasome and lysosome dysfunction (Figure adapted from (Bandopadhyay and de Belleroche, 2010, Engelender, 2012))

Since it would appear that each of these mechanisms could be the primary cause of neurodegenerative diseases and they are strongly linked together and to cognitive decline and other clinical features, then targeting any of these processes for therapeutic therapies could provide potential benefits for the treatment of neurodegenerative diseases and they may also have an effect on the other mechanisms.

In addition to proteasome dysfunction, this study also found a reduction in the expression level of LAMP1 in the anterior cingulate in PDD and DLB and in the parietal cortex in DLB. In both brain regions, the reduction in LAMP1 was associated with α-synuclein pathology. A defect in the lysosomal pathway could further exacerbate α-synuclein pathology and result in greater inhibition to both the lysosomal and proteasomal pathways due to a reduction in the proteolysis of α-synuclein (Ebrahimi-Fakhari et al., 2011, Ebrahimi-Fakhari et al., 2012). It is unknown whether the reduction of LAMP1 is one of the primary dysfunctions of the lysosomal pathway and whether or not LAMP1 reduction will lead to further reductions in the other components or if this reduction is a direct consequence of LB pathology. To better understand the role that the lysosomal pathway plays in DLB, further studies are needed by investigating other lysosomal markers as well as cathepsin D activity and the activity of other lysosomal proteases.

In view of the above, our data strongly suggest that proteasome activation may be a target for slowing down the disease progression in DLB and PDD. Recently Medina et al. (2011) have found that methylene blue (MB), a member of the phenothiazine family, increases the clearance of Aβ and rescues early cognitive deficit in a mouse model of AD

by increasing the proteasome activity (Medina et al., 2011). In addition, preventing α-synuclein aggregation by either activating the proteasomes and/or by using heat shock proteins could be an obvious target, such as the case with the use of Melittin for the treatment of Amyotrophic lateral sclerosis (ASL). Melittin, a component of bee venom that is comprised of 26 amino acids, reduced α-synuclein misfolding and increased proteasomal activity and the expression of heat shock protein 70 in the brain stem and spinal cord of ALS mice (Yang et al., 2011). Oral administration of 3H-1,2-dithiole-3-thione (D3T), a potential cancer chemopreventive agent, has been demonstrated to enhance proteolytic activity of the UPS by increasing the expression of the 26S proteasome subunits (β5, β6, β7) and the proteasome activities in various tissues of mice, including some brain regions such as the cerebral cortex and hippocampus (Kwak et al., 2007). Recently, sulforaphane (SFN), a naturally occurring isothiocyanate, was shown to enhance both proteasomal and autophagic activities in the brain and peripheral tissues of mice. In addition, treatment of cells expressing mutant huntingtin with SFN displayed reduced toxicity in both non-neuronal and neuronal cell cultures (Liu et al., 2014). Furthermore, Cabreiro and colleagues have demonstrated that zinc supplementation in healthy elderly people promotes an increase in proteasome activity and repair protein degradation systems in peripheral blood lymphocytes (Cabreiro et al., 2008). The effect of MB, Melittin, D3T and SEF on α-synuclein aggregation in Lewy body diseases remains to be determined. Based on promising results from a phase II clinical trial testing MB as a potential therapeutic agent for AD, where treated patients showed significantly improved cognitive functions after six months of MB administration compared to patients that received the placebo (Gura, 2008), MB may achieve the same beneficial result in LBD and it may play an important role in

preventing neurodegenerative diseases. Melittin and SEF are naturally occurring compounds that are safe after oral administration and they both have neuroprotective effects; moreover, they both appear to play an important role in preventing neurodegenerative diseases.

The strengths of this study are the large number of cases, our access to all the clinical and behavioural data and the number of projects undertaken on the same cohort, which provide chemical information on synaptic functions in addition to pathological and clinical data. Furthermore, this study examined three brain regions and compared the results from each of the regions separately to determine whether the biochemical changes are specific to a particular brain area or if all of the regions have the same alteration. However, despite these advantages, there are also a number of limitations regarding the use of post-mortem tissues which need to be taken into consideration, since these factors may have significant consequences for the outcome of the study. These include, ante-mortem factors such as medication history, the medication treatments for each individual patient were lacking, making it difficult to match the samples and to identify the association between the results and the medication treatments for these cases or to identify whether or not the medication had a considerable effect on the outcome. Post-mortem factors, including post-mortem delay, and the handling and storage of tissue are further problems that also should be addressed when performing studies with post-mortem tissue reviewed in (Hynd et al., 2003) . Furthermore, alteration of brain tissue pH, as a consequence of agonal state can affect sample quality for genetic and biochemical measurements. These factors were taken into consideration when planning the studies in this thesis. Post-/ante-mortem factors for controls, DLB, PDD and AD were matched as closely as possible for PMD and PH. In

addition, any relationships found between protein measurements and demographics/post-mortem factors were controlled for via the creation of unstandardized residuals. A further limitation of this study is that it applied a statistical approach using different statistical tests and, because of the high number of tests, there is a possibility of a false positive result.

In conclusion, the present study has demonstrated that, in PDD, DLB and AD, the activity of the RPT6 ATPase 19S regulatory subunit of the proteasome is decreased and inversely correlated with AD pathology in the prefrontal cortex, anterior cingulate and parietal cortex. The reduction is also associated with cognitive decline and other clinical features, such as depression and persecution. In contrast, the proteasome α-6 subunit is increased in the anterior cingulate in PDD and DLB and remains unchanged in the prefrontal cortex and parietal cortex. The changes in the proteasome α-3 subunits in BA9 were elevated in DLB; in PDD, they were elevated in BA40. Although this study observed a number of interesting proteasome alterations and provided evidence for impairment in the 26S proteasome in different brain areas in LBD, the molecular mechanisms of such changes are unclear. Considerably more work will need to be done to determine the functional consequences and potential causes of these alterations identified in these post mortem studies. It is also unclear why proteasome RPT6 was reduced and why it may be linked to different mechanisms such as synaptic dysfunction and protein aggregation, as this was not found to be the case with the α-3 and α-6 subunits. It is recommended that experimental research be undertaken to establish that whether the biochemical changes observed in this study will reflect both *in vivo* and *in vitro* studies. Furthermore, pharmacological studies targeting specific subunits and the activation of specific subunits could have better therapeutic potential in LBDs rather than non-selective modification of the UPS activity.

Chapter 9 References

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Appendix

Appendix I: Demographic and confounding variables.

Case ID	Diagnosis	Gender	Age	PMD	PH	Braak stage	Coded Braak	CERAD coded	CERAD	cLB score	Years of dementia	Years of PD
A011/06	Control	2	82	43	6.37	-	-	0	frequent	-	-	-
A047/02	Control	2	87	21	6	0	0	0	none	-	-	-
A048/09	Control	1	81	42	6.73	1	1	0	-	-	-	-
A049/03	Control	1	79	34	6.28	-	-	-	-	-	-	-
A063/10	Control	2	90	74	6.57	-	1	-	-	-	-	-
A133/95	Control	1	85	48	7.02	-	-	-	-	-	-	-
A134/00	Control	1	86	6	6.78	2	1	0	none	0	-	-
A136/10	Control	2	89	65	6.43	2	1	0	none	-	-	-
A153/01	Control	1	71	5	6.42	0	0	0	none	0	-	-
A170/00	Control	2	68	9	6.61	0	0	0	none	0	-	-
A185/04	Control	1	80	48	6.57	4	2	1	sparse	0	-	-
A219/97	Control	2	76	63	6.04	0	0	0	none	0	-	-
A223/96	Control	1	80	11	6.72	0	0	0	none	0	-	-
A239/95	Control	2	79	38	6.48	3	2	1	sparse	0	-	-
A283/96	Control	1	77	29	6.52	1	1	0	none	0	-	-
A308/09	Control	1	66	52	6.66	-	-	-	-	-	-	-
A31/96	Control	1	70	45	6.8	0	0	0	none	0	-	-
A316/95	Control	1	80	35	6.44	1	1	0	none	0	-	-
A320/94	Control	1	77	96	6.59	-	-	9	-	-	-	-
A33/96	Control	2	96	72	6.1	2	1	0	none	0	-	-
A346/95	Control	1	85	16	6.16	4	2	1	sparse	0	-	-
A359/08	Control	2	80	22	6.5	-	-	-	-	-	-	-
A401/97	Control	1	85	42	6.05	3	2	0	none	0	-	-
A61/96	Control	1	65	29	6.84	0	0	0	none	0	-	-
A94/95	Control	2	80	31	6.15	1	1	0	none	0	-	-
20020080	PDD	1	70	17	6.19	2	1	0	none	6	5	12
20030004	PDD	2	69	46	6.63	2	1	0	none	11	10	10
20030103	PDD	2	73	30	5.83	4	2	1	sparse	16	4	10
20030111	PDD	1	81	40	5.95	2	1	0	none	11	8	9
20030134	PDD	1	75	40	6.46	3	2	1	sparse	19	2	4
20040022	PDD	1	79	30	6.78	3	2	1	sparse	15	9	14

Case ID	Diagnosis	Gender	Age	PMD	PH	Braak stage	Coded Braak	CERAD coded	CERAD	cLB score	Years of dementia	Years of PD
20040076	PDD	1	76	17	6.45	2	1	0	none	12	7	11
20040105	PDD	1	68	11	6.15	5	3	3	frequent	18	6	8
20050096	PDD	1	73	31	5.83	0	0	0	none	8	5	23
20050099	PDD	1	89	64	5.99	3	2	1	sparse	9	3	16
A143/00	PDD	2	89	54	6.08	2	1	0	none	0		
ST01/01	PDD	2	83	24	6.63	-	1	2	moderate	11	4	19
ST02/01	PDD	1	83	37	6.45	-	1	0	none	6	1	15
ST03/01	PDD	1	75	36	6.31	-	1	1	sparse	14	1	13
ST04/01	PDD	2	85		6.81	-	2	3	frequent	18	4	8
ST09/02	PDD	1	79	72	6.8	-	3	3	frequent	3	1	9
ST10/02	PDD	1	82	24	6.44	-	1	1	sparse	12	1	4
ST11/02	PDD	2	73	60	6.68	2	1	1	sparse	-	-	-
ST12/02	PDD	2	80	28	6.34	-	1	0	none	3	1	6
ST13/02	PDD	2	81	28	6.85	2	1	1	sparse	-	-	-
ST14/02	PDD	1	78	24	6.48	3	2	2	moderate	-	-	-
ST15/02	PDD	2	88	72	5.9	2	1	0	none	-	-	-
ST16/02	PDD	1	80	26	6.58	2	1	0	none	-	-	-
ST17/02	PDD	1	72	9	6.9	1	1	0	none	-	-	-
ST18/02	PDD	1	79	30	6.8	-	1	1	sparse	2	5	16
ST19/02	PDD	2	84	27	6.18	-	1	0	none	6	-	6
ST20/02	PDD	2	85	36	6.36	-	1	2	moderate	20	2	16
ST21/03	PDD	2	83	24	6.45	-	1	3	frequent	15	2	26
ST22/02	PDD	2	75	24	7.19	-	1	3	frequent	6	2	7
ST23/03	PDD	2	82	33	6.74	-	1	2	moderate	14	2	14
ST24/03	PDD	1	88	24	6.49	-	2	1	sparse	-	6	15
ST25/04	PDD	2	86	24	6.3	2	1	1	sparse	-	7	21
ST29/04	PDD	2	88	32	6.15	-	2	1	sparse	-	-	-
ST30/04	PDD	1	86	32	6.73	-	0	0		-	-	-
27 7/82	DLB	2	87	13	6.36	5/6	3	3	frequent	-	6	0
36 7/81	DLB	1	85	19	6.19	5/6	3	3	frequent	-	10	4
51 7/96	DLB	1	82	80	6.44	5/6	3	3	frequent	-	3	0
52 1/13	DLB	1	82	29	6.21	3/4	2	2	moderate	-	7	1
55 2/25	DLB	1	81	38	6.7	3/4	2	1	sparse	-	9	24

Case ID	Diagnosis	Gender	Age	PMD	PH	Braak stage	Coded Braak	CERAD coded	CERAD	cLB score	Years of dementia	Years of PD
106 5/56	DLB	2	88	34	6.81	3/4	2	2	moderate	-	0	0
20030007	DLB	2	88	16	5.92	3	2	2	moderate	11	8	4
20030113	DLB	1	77	65	6	3	2	1	sparse	17	5	5
20040034	DLB	2	75	64	5.68	6	3	3	frequent	20	3	2
20040085	DLB	1	77	29	5.73	2	1	2	moderate	18	3	2
20050030	DLB	2	91	84	5.76	5	3	3	frequent	6	7	3
20050040	DLB	2	75	78	5.97	6	3	3	frequent	16	5	3
20060025	DLB	1	76	13	5.96	2	1	1	sparse	14	8	7
20070009	DLB	1	74	42	5.62	4	2	1	sparse	10	8	3
20070105	DLB	1	71	8	5.73	2	1	1	sparse	19	7	7
20080083	DLB	2	80	17	5.71	5	3	3	frequent	19	8	6
20100575	DLB	1	77	46	5.93	3	2	0	none	10	11	7
333 1/8	DLB	2	87	24	6.15	5/6	3	3	frequent	-	99	99
367 4/67	DLB	2	92	96	6.45	3/4	2	2	moderate	-	14	0
383 5/58	DLB	2	92	60	5.99	1/2	1	1	sparse	-	3	0
436 2/99	DLB	1	76	70	6.23	1/2	1	3	frequent	-	7	5
439	DLB	1	75	76	6.9	3/4	2	2	moderate	-	4	3
470	DLB	1	84	74	6.5	3/4	2	3	frequent	-	4	2
475	DLB	2	85	38	6.35	3/4	2	3	frequent	-	2	1
495	DLB	1	86	115	6.72	3/4	2	2	moderate	-	6	3
550 2/21	DLB	1	77	57	6.86	5/6	3	0	none	-	1	1
745 4/63	DLB	2	76	96	6.53	1/2	1	3	frequent	-	10	10
A014/07	DLB	1	74	20	6.85	-	3	0	frequent	18	-	0
A028/10	DLB	1	81	85	6.56	-	2	0	moderate	14	-	-
A035/08	DLB	2	83	14	6.2	-	2	0	sparse	18	-	-
A040/10	DLB	2	87	33	6.13	-	1	0	none	12	-	-
A046/07	DLB	1	76	53	6.52	-	2	0	moderate	9	-	-
A053/09	DLB	1	91	45	6.2	5	3	3	frequent	10	-	-
A055/09	DLB	2	87	30	6.31	5	3	3	frequent	13	-	-
A072/09	DLB	2	92	56	6.73	5	3	3	frequent	12	-	-
A084/09	DLB	2	85	31	5.91	5	3	3	frequent		-	-
A092/07	DLB	1	88	17	6.47	6	3	3	frequent	18	-	-
A109/01	DLB	1	65	5	6.63	3	2	1	sparse	19	9	7

Case ID	Diagnosis	Gender	Age	PMD	PH	Braak stage	Coded Braak	CERAD coded	CERAD	cLB score	Years of dementia	Years of PD
A148/08	DLB	2	84	13	6.09	4	2	2	moderate	11	-	-
A162/07	DLB	1	80	25	7.32	3	2	1	sparse	7	-	-
A190/03	DLB	1	83	38	6.24	3	2	1	sparse	16	-	-
A196/09	DLB	2	80	28	6.61	4	2	1	sparse	20	-	-
A204/07	DLB	1	74	18	6.71	2	1	0	none	9	-	-
A229/05	DLB	1	79	4	6.89	3	2	1	sparse	15	-	-
A231/06	DLB	2	70	22	6.89	3	2	2	moderate	9	-	-
A249/06	DLB	1	83	4	6.75	4	2	2	moderate	14	3	-
A273/05	DLB	1	86	8	5.87	2	1	1	sparse	11	4	-
A304/06	DLB	2	92	55	6.59	3	2	1	sparse	15	9	-
A335/08	DLB	1	79	12	6.78	1	1	0	none	0	-	-
A336/99	DLB	1	69	21	7.31	3	2	1	sparse	14	3	-
C1007	DLB	1	82	55	6.75	1/2	1	0	none	-	6	9
ST26/04	DLB	1	90	48	6.46	5	3	3	frequent	-	4	-
ST27/04	DLB	1	80	84	6.54	5	3	3	frequent	-	7	-
ST28/04	DLB	2	88		6.57	3	2	2	moderate	-	8	-
ST32/05	DLB	2	88	24	6.07	6	3	2	moderate	-	10	-
A071/09	AD	1	80	10	6.33	6	3	-	-	-	9	0
A108/09	AD	2	84	24	6.74	4	2	-	-	-	8	0
A120/09	AD	2	85	79	6.31	6	3	-	-	-	16	0
A147/10	AD	2	85	20	5.91	6	3	-	-	-	13	0
A216/09	AD	2	88	44	5.95	5	3	-	-	-	7	0
A267/09	AD	2	90	74	5.98	5	3	-	-	-	8	0
A349/08	AD	2	86	14	6.56	6	3	-	-	-	8	0
A350/09	AD	2	98	24	6.38	4	2	2	moderate	-	11	0
A37/09	AD	1	88	29	6.5	6	3	-	-	-	12	0
A371/08	AD	1	82	70	7.14	4	2	-	-	-	9	0
A38/11	AD	2	72	67	5.97	6	3	-	-	-	9	0
A61/09	AD	2	103	12	6.37	5	3	-	-	-	13	0
A7/10	AD	2	84	30	6.05	6	3	-	-	-	8	0
A76/09	AD	1	97	18	6.18	5	3	3	frequent	-	12	0
A8/10	AD	2	98	25	6.05	6	3	-	-	-	11	0
A92/09	AD	1	88	17	6.45	6	3	3	frequent	-	7	0

Appendix II: NPI and MMSE values and semi-quantitative scores.

Case ID	Diagnosis	MMSE at start	MMSE before death	MMSE decline year	MMSE coded	Hallucination CODED	Persecution CODED	Depression CODED	Agitation CODED
A011/06	Control	-	-	-	1	0	0	0	0
A047/02	Control	-	-	-	1	0	0	0	0
A048/09	Control	-	-	-	1	0	0	0	0
A049/03	Control	-	-	-	1	0	0	0	0
A063/10	Control	-	-	-	1	0	0	0	0
A133/95	Control	-	-	-	1	0	0	0	0
A134/00	Control	-	-	-	1	0	0	0	0
A136/10	Control	-	-	-	1	0	0	0	0
A153/01	Control	-	-	-	1	0	0	0	0
A170/00	Control	-	-	-	1	0	0	0	0
A185/04	Control	-	-	-	1	0	0	0	0
A219/97	Control	-	-	-	1	0	0	0	0
A223/96	Control	-	-	-	1	0	0	0	0
A239/95	Control	-	-	-	1	0	0	0	0
A283/96	Control	-	-	-	1	0	0	0	0
A308/09	Control	-	-	-	1	0	0	0	0
A31/96	Control	-	-	-	1	0	0	0	0
A316/95	Control	-	-	-	1	0	0	0	0
A320/94	Control	-	-	-	1	0	0	0	0
A33/96	Control	-	-	-	1	0	0	0	0
A346/95	Control	-	-	-	1	0	0	0	0
A359/08	Control	-	-	-	1	0	0	0	0
A401/97	Control	-	-	-	1	0	0	0	0
A61/96	Control	-	-	-	1	0	0	0	0
A94/95	Control	-	-	-	1	0	0	0	0
20020080	PDD	25	16	1.8	4	1	2	1	0
20030004	PDD	19	19	0	3	2	1	0	0
20030103	PDD	27	5	5.5	5	1	0	3	0
20030111	PDD	21	6	1.9	5	1	0	0	0
20030134	PDD	27	17	5	3	2	0	0	2

20040022	PDD	20	0	2.2	5	2	0	1	0
20040076	PDD	16	0	2.3	5	2	1	0	1
20040105	PDD	22	9	2.2	5	3	2	3	3
20050096	PDD	17	19	0	3	3	1	2	1
20050099	PDD	23	4	6.3	5	0	2	3	3
A143/00	PDD	-			9	0	0	0	0
ST01/01	PDD	-	12	2.1	4	1	0	1	0
ST02/01	PDD	-	16	0.5	4	1	2	2	0
ST03/01	PDD	-	16	1.5	4	2	2	0	2
ST04/01	PDD	-	2	3.4	5	0	0	0	3
ST09/02	PDD	-	9	2.5	5	2	0	2	1
ST10/02	PDD	-	20	1	3	0	1	2	3
ST11/02	PDD	-	25	0.3	2	3	2	1	2
ST12/02	PDD	-	29	0	2	0	0	0	0
ST13/02	PDD	-	26	0.2	2	1	1	1	1
ST14/02	PDD	-	5	2.4	5	0	1	1	0
ST15/02	PDD	-	13	3.8	4	2	0	3	2
ST16/02	PDD	-	20	1.1	3	0	2	0	0
ST17/02	PDD	-	27	-0.6	2	1	1	2	1
ST18/02	PDD	-	10	1.8	4	3	3	3	2
ST19/02	PDD	-		0	9	9	9	9	9
ST20/02	PDD	-	3	3.3	5	0	0	3	1
ST21/03	PDD	-	14	2.8	4	2	2	0	1
ST22/02	PDD	-	12	2	4	2	2	2	0
ST23/03	PDD	-	6	2.4	5	0	0	1	0
ST24/03	PDD	-	15	3.3	4	2	0	1	2
ST25/04	PDD	-	11	2.3	4	1	0	1	1
ST29/04	PDD	-	3	2.3	5	0	0	3	3
ST30/04	PDD	-	16	1.4	4	1	0	1	0
27 7/82	DLB	24	11	3.7	4	1	9	0	9
36 7/81	DLB	3	0	1	5	0	0	0	0
51 7/96	DLB	12	6	4	5	0	0	0	0
52 1/13	DLB	21	9	2.7	5	2	9	0	9
55 2/25	DLB	28	27	0	2	0	0	1	1
106 5/56	DLB	29	30	0	2	0	0	0	0
20030007	DLB	21	18	1.5	3	3	1	1	2
20030113	DLB	20	12	4	4	1	1	1	1
20040034	DLB	14	6	4	5	3	3	1	2

20040085	DLB	10	88	6	9	2	1	1	2
20050030	DLB	25	88	1	9	2	2	1	0
20050040	DLB	19	15	4	4	0	0	1	0
20060025	DLB	17	88	5	9	3	1	1	2
20070009	DLB	26	12	3.5	4	3	2	2	0
20070105	DLB	23	1	3.67	5	3	3	1	2
20080083	DLB	11	88	88	9	2	2	0	3
20100575	DLB	23	12	2.2	4	1	0	1	2
333 1/8	DLB	21	17	0.42	3	0	9	0	9
367 4/67	DLB	27	10	1.8	4	1	9	0	9
383 5/58	DLB	14	14	-1	4	2	9	9	9
436 2/99	DLB	22	16	2.2	4	0	1	0	0
439	DLB	23	15	5.3	4	2	0	0	0
470	DLB	23	18	3.3	3	1	0	0	0
475	DLB	22	7	15	5	0	2	0	0
495	DLB	5	7	0	5	0	1	1	1
550 2/21	DLB	23	16	7	4	0	0	0	0
745 4/63	DLB	14	14	14	4	2	9	0	9
A014/07	DLB	25	24	0.5	3	9	9	9	9
A028/10	DLB	30	10	6.67	4	9	9	9	9
A035/08	DLB	29			9	9	9	9	9
A040/10	DLB				9	9	9	9	9
A046/07	DLB	21	18	0.75	3	9	9	9	9
A053/09	DLB				9	9	9	9	9
A055/09	DLB	25	0	8.33	5	9	9	9	9
A072/09	DLB				9	9	9	9	9
A084/09	DLB				9	9	9	9	9
A092/07	DLB				9	9	9	9	9
A109/01	DLB	27	12	7.5	4	9	9	9	9
A148/08	DLB	24	18	3	3	9	9	9	9
A162/07	DLB	30	30	0	2	9	9	9	9
A190/03	DLB				9	9	9	9	9
A196/09	DLB				9	9	9	9	9
A204/07	DLB	29	0	2.64	5	9	9	9	9
A229/05	DLB	18	11	7	4	9	9	9	9
A231/06	DLB				9	9	9	9	9
A249/06	DLB		7	88	5	9	9	9	9
A273/05	DLB	29	29	0	2	9	9	9	9

A304/06	DLB	26			9	9	9	9	9
A335/08	DLB				9	9	9	9	9
A336/99	DLB	28	20	88	3	9	9	9	9
C1007 01/176	DLB	30	25	2.5	2	0	2	0	0
ST26/04	DLB	17		88	9	9	9	9	9
ST27/04	DLB	22	18	88	3	3	0	0	0
ST28/04	DLB	27	20	88	3	0	3	3	3
ST32/05	DLB	99		88	9	9	9	9	9
A071/09	AD	23	8	15	5	0	2	1	0
A108/09	AD	22	0	5.5	5	0	0	0	0
A120/09	AD	10	0	5	5	0	3	3	2
A147/10	AD	21	0	5.25	5	0	0	2	2
A216/09	AD	25	16	3	4	1	2	3	0
A267/09	AD	11	13	88	4	0	0	3	2
A349/08	AD	21	3	4.5	5	0	0	2	3
A350/09	AD	20	15	2.5	4	2	3	3	3
A37/09	AD	22	17	1	3	2	0	0	1
A371/08	AD	21	17	1.33	3	1	2	2	3
A38/11	AD	10	0	5	5	0	3	2	3
A61/09	AD			88	9	9	9	9	9
A7/10	AD	12	6	1	5	0	2	0	1
A76/09	AD	16	15	0.25	4	0	0	1	0
A8/10	AD	10	0	2.5	5	0	2	2	2
A92/09	AD	20	19	1	3	0	0	0	2

Appendix III: Semi-quantitative pathology scores.

Case ID	Diagnosis	Plaque BA9	Plaque BA24	Plaque BA21	Plaque BA40	Tangle BA9	Tangle BA24	Tangle BA21	Tangle BA40	Asyn BA9	Asyn BA24	Asyn BA21	Asyn BA40
A011/06	Control	0	0	0	0	0	0	0	0	0	0	0	0
A047/02	Control	0	0	0	0	0	0	0	0	0	0	0	0
A048/09	Control	0	0	1	0	0	0	0	0	0	0	0	0
A049/03	Control	0	0	0	0	0	0	0	0	0	0	0	0
A063/10	Control	0	2	2	0	1	1	1	0	0	0	-	0
A133/95	Control	-	-	-	-	-	-	-	-	-	-	-	-
A134/00	Control	0	0	0	0	0	0	3	0	0	0	-	0
A136/10	Control	2	0	3	3	0	0	1	0	0	0	-	-
A153/01	Control	0	0	0	0	0	0	0	0	0	0	0	0
A170/00	Control	0	0	0	0	0	0	0	0	0	0	0	0
A185/04	Control	1	-	1	2	0	-	0	0	0	-	0	0
A219/97	Control	0	0	0	0	0	0	0	0	0	0	-	-
A223/96	Control	0	0	0	0	0	0	0	0	0	0	0	0
A239/95	Control	1	3	1	1	1	1	1	1	0	0	0	0
A283/96	Control	0	0	0	0	0	0	0	0	0	0	0	0
A308/09	Control	1	0	1	1	0	0	0	0	0	0	-	0
A31/96	Control	0	-	0	0	0	-	0	0	0	-	0	0
A316/95	Control	2	0	2	1	1	0	0	0	0	0	0	0
A320/94	Control	-	-	-	-	-	-	-	-	-	-	-	-
A33/96	Control	0	0	0	0	0	0	0	0	0	0	-	-
A346/95	Control	1	1	1	1	0	0	0	0	0	0	0	0
A359/08	Control	0	0	0	0	0	0	0	0	0	0	0	0
A401/97	Control	0	0	1	0	1	0	1	0	0	0	-	0
A61/96	Control	0	0	0	0	0	0	0	0	0	0	0	0
A94/95	Control	0	0	0	0	0	0	0	0	0	0	0	0
20020080	PDD	0	0	1	0	-	0	0	-	1	0	1	1
20030004	PDD	1	0	0	0	0	1	1	0	1	3	1	1
20030103	PDD	3	2	2	-	1	1	0	0	2	3	1	1
20030111	PDD	2	1	1	1	0	1	0	0	1	3	2	0
20030134	PDD	2	2	1	1	1	2	1	1	2	3	1	1
20040022	PDD	3	1	-	-	1	1	2	1	1	3	1	1
20040076	PDD	2	1	1	2	0	1	1	1	1	1	2	2
20040105	PDD	2	3	2	2	2	2	2	2	1	2	2	3

Case ID	Diagnosis	Plaque BA9	Plaque BA24	Plaque BA21	Plaque BA40	Tangle BA9	Tangle BA24	Tangle BA21	Tangle BA40	Asyn BA9	Asyn BA24	Asyn BA21	Asyn BA40
20050096	PDD	1	3	1	1	1	2	0	0	1	2	2	1
20050099	PDD	0	1	1	2	1	1	1	1	2	2	1	1
A143/00	PDD	1	1	1	1	1	0	1	1	0	1	0	0
ST01/01	PDD	3	0	1	2	1	0	1	1	1	1	2	1
ST02/01	PDD	0	0	0	0	1	0	0	0	0	2	1	1
ST03/01	PDD	1	0	0	1	0	0	0	0	1	3	2	0
ST04/01	PDD	3	3	1	3	1	1	1	0	2	3	2	0
ST09/02	PDD	1	2	-	2	0	0	0	0	0	1	1	0
ST10/02	PDD	1	1	0	0	0	0	0	1	1	3	1	0
ST11/02	PDD	0	0	1	1	0	0	0	1	0	2	1	0
ST12/02	PDD	0	0	-	0	1	0	0	0	0	1	1	0
ST13/02	PDD	1	1	1	1	0	0	1	0	1	2	1	0
ST14/02	PDD	0	1	1	1	1	1	1	1	1	3	0	0
ST15/02	PDD	0	0	0	0	0	0	0	0	0	2	1	1
ST16/02	PDD	0	0	0	0	0	0	0	0	0	2	0	0
ST17/02	PDD	2	3	1	3	0	0	0	1	0	1	0	0
ST18/02	PDD	3	2	1	0	0	1	0	0	0	0	0	0
ST19/02	PDD	1	1	0	1	0	0	0	0	0	0	0	0
ST20/02	PDD	2	1	1	3	1	1	0	1	2	2	2	1
ST21/03	PDD	3	1	1	3	0	0	0	0	0	2	0	0
ST22/02	PDD	3	0	1	3	1	0	0	0	0	1	0	0
ST23/03	PDD	3	1	1	2	0	0	1	1	1	1	0	1
ST24/03	PDD	2	1	1	1	1	0	1	1	3	3	3	2
ST25/04	PDD	-	0	0	-	-	1	0	0	0	0	0	0
ST29/04	PDD	0	0	0	0	0	0	0	0	1	3	0	0
ST30/04	PDD	0	0	0	1	1	1	0	1	0	2	0	1
27 7/82	DLB	2	-	3	3	1	-	2	1	0	3	3	2
36 7/81	DLB	3	-	3	2	1	-	2	1	2	3	3	2
51 7/96	DLB	3	-	3	3	3	-	3	3	1	3	3	0
52 1/13	DLB	1	0	2	2	0	0	0	0	2	2	3	2
55 2/25	DLB	1	1	1	1	1	2	0	1	1	3	1	0
106 5/56	DLB	2	3	2	1	1	3	1	0	0	2	0	0
20030007	DLB	-	2	1	2	0	1	1	0	3	3	1	1
20030113	DLB	3	3	1	1	0	1	0	1	2	-	2	2

Case ID	Diagnosis	Plaque BA9	Plaque BA24	Plaque BA21	Plaque BA40	Tangle BA9	Tangle BA24	Tangle BA21	Tangle BA40	Asyn BA9	Asyn BA24	Asyn BA21	Asyn BA40
20040034	DLB	-	1	1	3	3	3	2	3	-	3	2	3
20040085	DLB	2	2	2	1	1	1	2	2	2	2	2	1
20050030	DLB	2	3	2	3	2	3	1	2	1	1	0	1
20050040	DLB	0	0	0	0	2	1	2	1	2	2	-	2
20060025	DLB	2	2	2	1	1	1	1	1	1	3	1	1
20070009	DLB	2	1	1	1	1	1	1	1	1	2	1	1
20070105	DLB	2	2	3	3	1	2	1	1	2	3	2	2
20080083	DLB	2	-	1	1	2	2	3	2	2	3	3	1
20100575	DLB	0	0	0	0	0	1	0	1	1	2	1	1
333 1/8	DLB	3	1	3	3	2	2	1	1	0	2	0	0
367 4/67	DLB	1	2	2	1	0	2	1	0	0	2	1	1
383 5/58	DLB	1	0	0	0	0	0	0	0	0	2	1	0
436 2/99	DLB	0	0	0	0	0	0	0	0	1	2	1	0
439	DLB	1	2	2	2	1	2	1	0	3	3	3	3
470	DLB	3	0	3	3	1	0	1	1	1	3	2	1
475	DLB	1	2	3	1	0	1	1	1	0	2	3	1
495	DLB	2	1	2	2	1	1	1	1	1	3	2	1
550 2/21	DLB	3	3	3	2	1	3	2	1	1	3	2	1
745 4/63	DLB	0	-	0	0	0	0	0	0	2	3	2	1
A014/07	DLB	3	3	3	3	3	3	3	3	3	3	3	3
A028/10	DLB	1	0	3	0	1	1	1	1	2	3	2	2
A035/08	DLB	3	3	1	3	1	1	1	2	3	3	3	3
A040/10	DLB	2	2	2	3	1	1	1	1	3	2	2	2
A046/07	DLB	2	0	2	0	0	0	1	0	2	2	2	2
A053/09	DLB	3	2	3	2	1	3	3	2	0	0	0	0
A055/09	DLB	3	3	3	3	2	3	3	2	3	3	2	3
A072/09	DLB	1	1	3	1	1	2	2	1	1	3	2	2
A084/09	DLB	2	3	3	2	1	3	2	3	2	3	2	1
A092/07	DLB	3	1	3	3	1	2	3	1	3	2	3	3
A109/01	DLB	2	0	1	1	1	0	1	0	3	3	3	3
A148/08	DLB	3	1	2	1	1	1	1	1	1	3	1	1
A162/07	DLB	3	0	1	2	0	1	3	0	1	1	0	0
A190/03	DLB	0	2	1	1	1	1	1	1	3	1	2	2
A196/09	DLB	1	1	3	1	1	2	3	1	3	3	3	3

Case ID	Diagnosis	Plaque BA9	Plaque BA24	Plaque BA21	Plaque BA40	Tangle BA9	Tangle BA24	Tangle BA21	Tangle BA40	Asyn BA9	Asyn BA24	Asyn BA21	Asyn BA40
A204/07	DLB	0	0	0	0	0	0	0	0	2	2	1	2
A229/05	DLB	0	1	2	0	1	0	1	1	3	2	3	3
A231/06	DLB	2	2	2	2	1	1	1	1	-	1	1	1
A249/06	DLB	1	1	1	1	0	1	1	0	2	3	3	1
A273/05	DLB	3	2	3	3	0	0	0	0	2	3	0	1
A304/06	DLB	1	1	1	1	1	0	1	1	3	3	3	1
A335/08	DLB	1	0	1	0	0	0	1	0	0	0	0	0
A336/99	DLB	1	0	1	1	1	0	1	1	3	3	3	2
C1007 01/176	DLB	0	0	0	0	0	1	0	0	1	3	1	1
ST26/04	DLB	1	1	1	2	1	1	2	0	-	0	-	-
ST27/04	DLB	2	2	1	1	1	1	2	1	2	2	3	1
ST28/04	DLB	0	0	1	0	0	0	0	1	0	0	0	0
ST32/05	DLB	3	1	1	2	3	2	3	3	1	1	0	1
A071/09	AD	3	1	3	3	3	0	3	3	0	0	0	0
A108/09	AD	3	1	3	3	2	1	3	3	0	0	0	0
A120/09	AD	3	3	3	3	3	3	3	3	0	0	1	0
A147/10	AD	3	2	3	3	3	1	3	3	1	0	-	0
A216/09	AD	3	2	3	3	3	3	3	3	0	0	0	0
A267/09	AD	3	-	3	3	3	1	3	3	0	0	0	0
A349/08	AD	3	1	3	3	2	1	1	2	0	0	0	0
A350/09	AD	1	0	1	1	2	0	2	2	0	0	0	0
A37/09	AD	3	0	3	3	2	0	3	3	0	0	0	0
A371/08	AD	3	1	3	2	2	0	3	3	0	0	0	0
A38/11	AD	3	3	3	3	3	3	3	3	0	2	0	0
A61/09	AD	3	3	3	3	2	2	3	3	0	0	0	0
A7/10	AD	3	1	2	3	3	3	3	3	0	1	1	1
A76/09	AD	3	2	3	2	3	2	3	3	1	2	1	1
A8/10	AD	3	2	3	1	2	3	3	2	0	0	0	0
A92/09	AD	2	0	3	3	3	0	3	3	0	0	0	0

Appendix IV: Medication details according to clinical diagnosis.

Control

		Medication classification according to BNF coding (first medication)	Medication classification according to BNF coding (second medication if applicable)	Medication classification according to BNF coding (3rd medication etc)	Medication classification according to BNF coding (4th etc)	Medication classification according to BNF coding (5th etc)
N	Valid	0	0	0	0	0
	Missing	25	25	25	25	25

PDD

		Medication classification according to BNF coding (first medication)	Medication classification according to BNF coding (second medication if applicable)	Medication classification according to BNF coding (3rd medication etc)	Medication classification according to BNF coding (4th etc)	Medication classification according to BNF coding (5th etc)
N	Valid	31	21	13	5	2
	Missing	3	13	21	29	32

Medication classification according to BNF coding (first medication)

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	donezepil	8	23.5	25.8	25.8
	anti-parkinsonism	23	67.6	74.2	100.0
	Total	31	91.2	100.0	
Missing	System	3	8.8		
Total		34	100.0		

Medication classification according to BNF coding (second medication if applicable)

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	donezepil	1	2.9	4.8	4.8
	anti-muscarinic for PD	1	2.9	4.8	9.5
	anti-parkinsonism	8	23.5	38.1	47.6
	anti-depressant	9	26.5	42.9	90.5
	anti-psychotic drugs	2	5.9	9.5	100.0
	Total	21	61.8	100.0	
Missing	System	13	38.2		
Total		34	100.0		

Medication classification according to BNF coding (3rd medication etc)

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	anti-depressant	6	17.6	46.2	46.2
	anti-psychotic drugs	3	8.8	23.1	69.2
	anxiolytic	2	5.9	15.4	84.6
	hypnotic	2	5.9	15.4	100.0
	Total	13	38.2	100.0	
Missing	System	21	61.8		
Total		34	100.0		

Medication classification according to BNF coding (4th etc)

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	anti-psychotic drugs	3	8.8	60.0	60.0
	hypnotic	2	5.9	40.0	100.0
	Total	5	14.7	100.0	
Missing	System	29	85.3		
Total		34	100.0		

Medication classification according to BNF coding (5th etc)

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	hypnotic	2	5.9	100.0	100.0
Missing	System	32	94.1		
Total		34	100.0		

DLB

		Medication classification according to BNF coding (first medication)	Medication classification according to BNF coding (second medication if applicable)	Medication classification according to BNF coding (3rd medication etc)	Medication classification according to BNF coding (4th etc)	Medication classification according to BNF coding (5th etc)
N	Valid	38	27	11	3	1
	Missing	17	28	44	52	54

Medication classification according to BNF coding (first medication)

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	9.00	1	1.8	2.6	2.6
	Tacrine	4	7.3	10.5	13.2
	donezepil	11	20.0	28.9	42.1
	anti-muscarinic for PD	2	3.6	5.3	47.4
	anti-parkinsonism	7	12.7	18.4	65.8
	anti-depressant	3	5.5	7.9	73.7
	anti-psychotic drugs	6	10.9	15.8	89.5
	anxiolytic	2	3.6	5.3	94.7
	hypnotic	2	3.6	5.3	100.0
	Total	38	69.1	100.0	
Missing	System	17	30.9		
Total		55	100.0		

Medication classification according to BNF coding (3rd medication etc)

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	59.00	1	1.8	9.1	9.1
	Tacrine	1	1.8	9.1	18.2
	Ginkgo	1	1.8	9.1	27.3
	anti-parkinsonism	1	1.8	9.1	36.4
	anti-depressant	2	3.6	18.2	54.5
	82.00	1	1.8	9.1	63.6
	anxiolytic	3	5.5	27.3	90.9
	hypnotic	1	1.8	9.1	100.0
	Total	11	20.0	100.0	
Missing	System	44	80.0		
Total		55	100.0		

Medication classification according to BNF coding (4th etc)

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	memantine	1	1.8	33.3	33.3
	anti-depressant	1	1.8	33.3	66.7
	hypnotic	1	1.8	33.3	100.0
	Total	3	5.5	100.0	
Missing	System	52	94.5		
Total		55	100.0		

Medication classification according to BNF coding (5th etc)

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	anti-muscarinic for PD	1	1.8	100.0	100.0
Missing	System	54	98.2		
Total		55	100.0		

Appendix V: Proteasome subunits (α3, α6 and RPT6) protein values from semi-quantification of Western blotting and data transformation in BA9.

		α3					α6	RPT6				
Case ID	**Diagnosis**	**α3**	**Res**	**Res+1**	**Log (Res+1)**	**Log(Res+1)+1**	**α6**	**RPT6**	**Res**	**Res+1**	**Log (Res+1)**	**Log(Res+1)+1**
A011/06	Control	0.71	-0.4	0.58	-0.23	0.77	1.3	0.9	0.27	1.27	0.1	1.1
A047/02	Control	0.73	-0.5	0.5	-0.3	0.7	1	1	0.4	1.4	0.14	1.14
A048/09	Control	0.97	-0.1	0.95	-0.02	0.98	1.1	1	0.29	1.29	0.11	1.11
A049/03	Control	1.03	-0.1	0.88	-0.06	0.94	1.4	1.1	0.4	1.4	0.15	1.15
A063/10	Control	0.98	-0.1	0.91	-0.04	0.96	1.2	0.6	0.03	1.03	0.01	1.01
A133/95	Control	1.05	0.11	1.11	0.05	1.05	1.3	0.7	0.08	1.08	0.03	1.03
A134/00	Control	1.5	0.49	1.49	0.17	1.17	1.3	1.1	0.42	1.42	0.15	1.15
A136/10	Control	1.11	0	1	0	1	1.5	1.4	0.75	1.75	0.24	1.24
A153/01	Control	0.93	-0.2	0.82	-0.09	0.91	1.3	0.9	0.16	1.16	0.06	1.06
A170/00	Control	1.04	-0	0.98	-0.01	0.99	1.3	0.9	0.19	1.19	0.08	1.08
A185/04	Control	1.03	-0	0.96	-0.02	0.98	1.3	1	0.35	1.35	0.13	1.13
A219/97	Control	0.9	-0.3	0.68	-0.17	0.83	1.2	1.2	0.51	1.51	0.18	1.18
A223/96	Control	1.33	0.3	1.3	0.12	1.12	1.5	1	0.35	1.35	0.13	1.13
A239/95	Control	1.07	-0	0.98	-0.01	0.99	1.2	1	0.28	1.28	0.11	1.11
A283/96	Control	1.25	0.17	1.17	0.07	1.07	1	1	0.28	1.28	0.11	1.11
A308/09	Control	1.13	0.09	1.09	0.04	1.04	1.4	1.2	0.43	1.43	0.16	1.16
A31/96	Control	0.95	-0.1	0.95	-0.02	0.98	1.5	0.9	0.19	1.19	0.08	1.08
A316/95	Control	1.28	0.17	1.17	0.07	1.07	1.3	1.3	0.59	1.59	0.2	1.2
A320/94	Control	0.91	-0.2	0.85	-0.07	0.93	1.5	0.5	-0.17	0.83	-0.08	0.92
A33/96	Control	0.87	-0.3	0.67	-0.18	0.82	1.4	1	0.46	1.46	0.16	1.16
A346/95	Control	0.81	-0.4	0.62	-0.21	0.79	1.2	1	0.35	1.35	0.13	1.13
A359/08	Control	0.91	-0.2	0.82	-0.09	0.91	1.2	1.2	0.57	1.57	0.2	1.2
A401/97	Control	1.43	0.21	1.21	0.08	1.08	1.4	1	0.35	1.35	0.13	1.13
A94/95	Control	1.2	0.01	1.01	0	1	1.2	0.8	0.12	1.12	0.05	1.05
20020080	PDD	0.74	-0.4	0.56	-0.25	0.75	1.3	0.5	-0.27	0.73	-0.13	0.87
20030004	PDD	0.98	-0.1	0.93	-0.03	0.97	1.1	0.5	-0.2	0.8	-0.1	0.9
20030103	PDD	1.14	-0.1	0.86	-0.07	0.93	0.9	0.5	-0.17	0.83	-0.08	0.92
20030134	PDD	0.76	-0.3	0.66	-0.18	0.82	0.8	0.6	-0.07	0.93	-0.03	0.97
20040022	PDD	0.74	-0.3	0.73	-0.14	0.86	1.1	0.7	-0.01	0.99	-0.01	0.99
20040076	PDD	1.81	0.71	1.71	0.23	1.23	1.2	0.6	-0.12	0.88	-0.06	0.94

		α3					α6	RPT6				
Case ID	Diagnosis	α3	Res	Res+1	Log (Res+1)	Log(Res+1)+1	α6	RPT6	Res	Res+1	Log (Res+1)	Log(Res+1)+1
20040105	PDD	1.76	0.57	1.57	0.2	1.2	1	0.7	-0.01	0.99	0	1
20050096	PDD	1.54	0.26	1.26	0.1	1.1	1	0.6	-0.11	0.89	-0.05	0.95
20050099	PDD	1.72	0.49	1.49	0.17	1.17	1.1	0.3	-0.27	0.73	-0.14	0.86
A143/00	PDD	1.12	-0.1	0.91	-0.04	0.96	1.2	0.7	0.11	1.11	0.04	1.04
ST01/01	PDD	0.95	-0.1	0.9	-0.05	0.95	1.7	0.8	0.12	1.12	0.05	1.05
ST02/01	PDD	0.93	-0.2	0.83	-0.08	0.92	1.7	0.8	0.1	1.1	0.04	1.04
ST03/01	PDD	0.74	-0.4	0.6	-0.22	0.78	1.4	0.7	0.04	1.04	0.02	1.02
ST04/01	PDD	0.61	-0.4	0.61	-0.22	0.78	1.4	0.7	0.09	1.09	0.04	1.04
ST09/02	PDD	0.71	-0.3	0.71	-0.15	0.85	1.4	0.7	0.04	1.04	0.02	1.02
ST10/02	PDD	0.9	-0.2	0.79	-0.1	0.9	1	0.8	0.13	1.13	0.05	1.05
ST11/02	PDD	0.96	-0.1	0.92	-0.03	0.97	1.4	0.8	0.05	1.05	0.02	1.02
ST12/02	PDD	1.4	0.27	1.26	0.1	1.1	1.1	0.7	0.02	1.02	0.01	1.01
ST13/02	PDD	0.76	-0.2	0.77	-0.11	0.89	1.1	0.7	0.02	1.02	0.01	1.01
ST14/02	PDD	1.89	0.8	1.8	0.25	1.25	1.4	0.6	-0.07	0.93	-0.03	0.97
ST15/02	PDD	0.67	-0.6	0.41	-0.39	0.61	0.9	0.4	-0.27	0.73	-0.14	0.86
ST16/02	PDD	0.97	-0.1	0.9	-0.04	0.96	1.3	0.4	-0.27	0.73	-0.13	0.87
ST17/02	PDD	0.69	-0.3	0.72	-0.15	0.85	1.1	0.4	-0.29	0.71	-0.15	0.85
ST18/02	PDD	0.38	-0.6	0.38	-0.42	0.58	1.4	0.4	-0.24	0.76	-0.12	0.88
ST19/02	PDD	0.64	-0.5	0.46	-0.34	0.66	1.2	0.5	-0.17	0.83	-0.08	0.92
ST20/02	PDD	0.86	-0.3	0.73	-0.14	0.86	1.3	0.4	-0.21	0.79	-0.1	0.9
ST21/03	PDD	0.6	-0.5	0.5	-0.3	0.7	1.1	0.4	-0.3	0.7	-0.15	0.85
ST22/02	PDD	0.7	-0.2	0.81	-0.09	0.91	1.3	0.4	-0.27	0.73	-0.13	0.87
ST23/03	PDD	0.73	-0.3	0.71	-0.15	0.85	1.5	0.5	-0.17	0.83	-0.08	0.92
ST24/03	PDD	0.7	-0.4	0.61	-0.22	0.78	1.5	0.5	-0.09	0.91	-0.04	0.96
ST25/04	PDD	1.39	0.24	1.24	0.09	1.09	1.4	0.3	-0.3	0.7	-0.16	0.84
ST29/04	PDD	0.48	-0.7	0.29	-0.54	0.46	1.1	0.4	-0.22	0.78	-0.11	0.89
ST30/04	PDD	1.2	0.18	1.18	0.07	1.07	1.1	0.4	-0.28	0.72	-0.14	0.86
27 7/82	DLB	0.79	-0.3	0.66	-0.18	0.82	1.2	0.6	-0.03	0.98	-0.01	0.99
51 7/96	DLB	0.68	-0.4	0.57	-0.24	0.76	1.2	0.6	-0.08	0.92	-0.04	0.96
52 1/13	DLB	0.72	-0.5	0.55	-0.26	0.74	1.1	0.7	0.04	1.04	0.02	1.02
55 2/25	DLB	1.69	0.66	1.66	0.22	1.22	1.1	0.7	-0.01	0.99	0	1
106 5/56	DLB	0.88	-0.1	0.88	-0.06	0.94		0.3	-0.33	0.67	-0.17	0.83
20030007	DLB	2.15	0.89	1.89	0.28	1.28	1.1	0.8	0.18	1.18	0.07	1.07

		α3					α6	RPT6				
Case ID	Diagnosis	α3	Res	Res+1	Log (Res+1)	Log(Res+1)+1	α6	RPT6	Res	Res+1	Log (Res+1)	Log(Res+1)+1
20030113	DLB	1.31	0.08	1.08	0.03	1.03	1.3	0.7	-0.02	0.98	-0.01	0.99
20040034	DLB	1.53	0.2	1.2	0.08	1.08	1.3	0.7	-0.02	0.98	-0.01	0.99
20040085	DLB	1.26	-0.1	0.95	-0.02	0.98	1.1	0.5	-0.21	0.79	-0.1	0.9
20050030	DLB	1.77	0.47	1.47	0.17	1.17	1.4	0.7	0.05	1.05	0.02	1.02
20050040	DLB	1.06	-0.2	0.82	-0.09	0.91	1.2	0.6	-0.09	0.91	-0.04	0.96
20070009	DLB	0.92	-0.4	0.58	-0.24	0.76	1.1	0.8	0.07	1.07	0.03	1.03
20070105	DLB	1.57	0.26	1.26	0.1	1.1	1.2	1.1	0.36	1.36	0.13	1.13
20080083	DLB	1.67	0.35	1.35	0.13	1.13	1.7	0.7	0.06	1.06	0.03	1.03
20100575	DLB	1.3	0.05	1.05	0.02	1.02	1.5	1.2	0.49	1.49	0.17	1.17
333 1/8	DLB	0.89	-0.3	0.7	-0.15	0.85	1.1	0.6	-0.04	0.97	-0.02	0.98
367 4/67	DLB	0.78	-0.3	0.68	-0.17	0.83	1	0.4	-0.18	0.82	-0.08	0.92
383 5/58	DLB	1.09	-0.1	0.85	-0.07	0.93	1.4	0.7	0.08	1.08	0.04	1.04
436 2/99	DLB	1.41	0.24	1.24	0.09	1.09	1.1	0.7	0.04	1.04	0.02	1.02
439	DLB	1.27	0.3	1.3	0.11	1.11	1.5	0.8	0.05	1.05	0.02	1.02
470	DLB	0.92	-0.2	0.83	-0.08	0.92	0.8	0.6	-0.02	0.98	-0.01	0.99
475	DLB	0.73	-0.4	0.6	-0.22	0.78	1.1	0.4	-0.27	0.73	-0.13	0.87
495	DLB	0.69	-0.3	0.67	-0.18	0.82	1.4	0.4	-0.23	0.77	-0.11	0.89
550 2/21	DLB	0.79	-0.2	0.8	-0.09	0.91	1.4	0.6	-0.04	0.96	-0.02	0.98
745 4/63	DLB	0.91	-0.2	0.83	-0.08	0.92	1.2	0.4	-0.31	0.69	-0.16	0.84
A014/07	DLB	0.98	-0	0.99	0	1	1.3	0.5	-0.16	0.84	-0.08	0.92
A028/10	DLB	1.19	0.12	1.12	0.05	1.05	1	0.4	-0.24	0.76	-0.12	0.88
A035/08	DLB	1.49	0.31	1.31	0.12	1.12	1.2	0.8	0.14	1.14	0.06	1.06
A040/10	DLB	1.02	-0.2	0.83	-0.08	0.92	1.1	0.7	0.05	1.05	0.02	1.02
A046/07	DLB	1.51	0.43	1.43	0.15	1.15	1.4	0.5	-0.17	0.83	-0.08	0.92
A053/09	DLB	1.18	0	1	0	1	1.3	0.5	-0.07	0.93	-0.03	0.97
A055/09	DLB	1.83	0.69	1.69	0.23	1.23	1.7	0.8	0.13	1.13	0.05	1.05
A072/09	DLB	1.7	0.68	1.68	0.22	1.22	1.3	0.5	-0.06	0.94	-0.02	0.98
A084/09	DLB	1.43	0.17	1.17	0.07	1.07	1.4	0.5	-0.19	0.81	-0.09	0.91
A092/07	DLB	1.65	0.55	1.55	0.19	1.19	1.4	0.6	0	1	0	1
A109/01	DLB	1.23	0.18	1.18	0.07	1.07	1.3	0.6	-0.18	0.82	-0.08	0.92
A190/03	DLB	0.97	-0.2	0.81	-0.09	0.91	1.4	0.6	-0.04	0.96	-0.02	0.98
A196/09	DLB	1.49	0.43	1.43	0.16	1.16	1.3	0.6	-0.05	0.95	-0.02	0.98
A204/07	DLB	1.73	0.7	1.7	0.23	1.23	1.3	0.3	-0.36	0.64	-0.2	0.8

		α3					α6	RPT6				
Case ID	**Diagnosis**	**α3**	**Res**	**Res+1**	**Log (Res+1)**	**Log(Res+1)+1**	**α6**	**RPT6**	**Res**	**Res+1**	**Log (Res+1)**	**Log(Res+1)+1**
A229/05	DLB	1.34	0.36	1.36	0.13	1.13	1.3	0.6	-0.1	0.9	-0.05	0.95
A231/06	DLB	1.15	0.17	1.17	0.07	1.07	1.1	0.5	-0.19	0.81	-0.09	0.91
A273/05	DLB	2.3	1.03	2.03	0.31	1.31	1.3	0.5	-0.17	0.83	-0.08	0.92
A304/06	DLB	1.63	0.57	1.57	0.2	1.2	1.2	0.5	-0.1	0.9	-0.04	0.96
A335/08	DLB	1	-0	0.99	0	1	1	1.3	0.63	1.63	0.21	1.21
A336/99	DLB	0.58	-0.3	0.72	-0.14	0.86	0.9	0.7	-0.02	0.98	-0.01	0.99
01/176	DLB	1.85	0.83	1.83	0.26	1.26	1	0.4	-0.25	0.75	-0.13	0.87
ST26/04	DLB	1.99	0.89	1.89	0.28	1.28	1.7	0.6	-0.01	0.99	0	1
ST27/04	DLB	2.15	1.07	2.07	0.32	1.32	1.6	0.4	-0.25	0.75	-0.12	0.88
ST28/04	DLB	0.89	-0.2	0.82	-0.09	0.91	1.6	0.5	-0.15	0.85	-0.07	0.93
ST32/05	DLB	1.2	-0	0.99	-0.01	0.99	0.9	0.5	-0.08	0.92	-0.04	0.96
A071/09	AD	0.88	-0.3	0.74	-0.13	0.87	0.7	0.4	-0.27	0.73	-0.13	0.87
A108/09	AD	0.89	-0.1	0.87	-0.06	0.94	1.1	0.5	-0.12	0.88	-0.05	0.95
A120/09	AD	1.27	0.13	1.13	0.05	1.05	1	0.6	-0.04	0.96	-0.02	0.98
A147/10	AD	0.93	-0.3	0.67	-0.17	0.83	1	0.4	-0.28	0.72	-0.14	0.86
A216/09	AD	0.75	-0.5	0.5	-0.3	0.7	0.7	0.5	-0.1	0.9	-0.05	0.95
A267/09	AD	1.05	-0.2	0.81	-0.09	0.91	1	0.6	0.03	1.03	0.01	1.01
A349/08	AD	0.8	-0.3	0.73	-0.14	0.86	0.9	0.6	-0.08	0.92	-0.04	0.96
A350/09	AD	0.87	-0.3	0.75	-0.13	0.87	1	0.6	-0.01	0.99	0	1
A37/09	AD	0.9	-0.2	0.81	-0.09	0.91	0.8	0.6	-0.04	0.96	-0.02	0.98
A371/08	AD	0.74	-0.2	0.84	-0.08	0.92	0.8	0.4	-0.24	0.76	-0.12	0.88
A38/11	AD	1.26	0.02	1.02	0.01	1.01	0.8	0.6	-0.16	0.84	-0.08	0.92
A61/09	AD	0.93	-0.2	0.8	-0.09	0.91	1.1	0.5	-0.06	0.94	-0.03	0.97
A7/10	AD	1.16	-0.1	0.94	-0.03	0.97	1	0.2	-0.48	0.52	-0.28	0.72
A76/09	AD	1.23	0.05	1.05	0.02	1.02	1	0.6	-0.01	0.99	0	1
A8/10	AD	1.44	0.22	1.22	0.09	1.09	1.1	0.6	0.07	1.07	0.03	1.03
A92/09	AD	0.93	-0.2	0.83	-0.08	0.92	0.8	0.7	0.1	1.1	0.04	1.04

Appendix VI: Proteasome subunits (α3, α6 and RPT6) protein values from semi-quantification of Western blotting and data transformation in BA24.

		α3				α6			RPT6				
Case ID	**Diagnosis**	**α3**	**Res**	**Res+1**	**SQRT**	**α6**	**LOG α 6**	**LOG +1**	**RPT**	**Res**	**Res+1**	**Log (Res+1)**	**Log(Res+1)+1**
A011/06	Control	0.83	-0.23	0.8	0.88	0.85	-0.1	0.93	0.8	0.07	1.07	0.03	1.03
A048/09	Control	1.16	0.22	1.2	1.11	1.21	0.1	1.08	0.79	0.05	1.05	0.02	1.02
A049/03	Control	1.06	-0.04	1	0.98	0.99	0	1	0.71	-0.05	0.95	-0.02	0.98
A063/10	Control	1.07	0.08	1.1	1.04	0.99	0	1	0.71	0.05	1.05	0.02	1.02
A133/95	Control	0.92	0.09	1.1	1.04	1.18	0.1	1.07	0.91	0.18	1.18	0.07	1.07
A134/00	Control	1.06	0.14	1.1	1.07	0.74	-0.1	0.87	0.63	-0.19	0.81	-0.09	0.91
A136/10	Control	1.04	-0	1	1	1.25	0.1	1.1	0.95	0.27	1.27	0.1	1.1
A153/01	Control	1.05	0	1	1	1.28	0.1	1.11	0.86	0.04	1.04	0.02	1.02
A170/00	Control	0.94	-0.04	1	0.98	0.96	-0	0.98	1.35	0.54	1.54	0.19	1.19
A185/04	Control	0.8	-0.19	0.8	0.9	1.03	0	1.01	0.76	0.04	1.04	0.02	1.02
A223/96	Control	1.05	0.11	1.1	1.05	1.04	0	1.02	0.75	-0.06	0.94	-0.03	0.97
A239/95	Control	0.78	-0.24	0.8	0.87	0.75	-0.1	0.88	0.71	-0.04	0.96	-0.02	0.98
A283/96	Control	1.49	0.48	1.5	1.22	0.98	-0	0.99	0.92	0.15	1.15	0.06	1.06
A308/09	Control	1.11	0.15	1.2	1.07	1.02	0	1.01	0.54	-0.17	0.83	-0.08	0.92
A31/96	Control	0.7	-0.21	0.8	0.89	0.87	-0.1	0.94	0.78	0.05	1.05	0.02	1.02
A316/95	Control	0.83	-0.21	0.8	0.89	1.05	0	1.02	0.69	-0.06	0.94	-0.03	0.97
A320/94	Control	1.02	0.03	1	1.02	1.08	0	1.03	0.62	0.01	1.01	0	1
A33/96	Control	1.04	-0.12	0.9	0.94	0.77	-0.1	0.89	0.59	-0.08	0.92	-0.04	0.96
A346/95	Control	1.02	-0.12	0.9	0.94	1.02	0	1.01	1.26	0.46	1.46	0.17	1.17
A359/08	Control	0.75	-0.27	0.7	0.86	0.75	-0.1	0.88	0.89	0.11	1.11	0.04	1.04
A401/97	Control	0.9	-0.28	0.7	0.85	1	0	1	1.81	1.07	2.07	0.32	1.32
A61/96	Control	0.89	-0.01	1	1	1.09	0	1.04	1.14	0.37	1.37	0.14	1.14
A94/95	Control	0.98	-0.16	0.8	0.91	1.03	0	1.01	0.63	-0.13	0.87	-0.06	0.94
20020080	PDD	0.95	-0.18	0.8	0.91	1.35	0.1	1.13	0.71	-0.08	0.92	-0.04	0.96
20030004	PDD	0.96	-0.01	1	0.99	0.96	-0	0.98	0.88	0.15	1.15	0.06	1.06
20030103	PDD	1.1	-0.16	0.8	0.92	1.35	0.1	1.13	0.8	0.04	1.04	0.02	1.02
20030111	PDD	1.25	0.03	1	1.02	0.99	0	1	0.83	0.09	1.09	0.04	1.04
20030134	PDD	0.74	-0.29	0.7	0.84	0.89	-0.1	0.95	0.82	0.08	1.08	0.03	1.03

		α3				α6			RPT6				
Case ID	Diagnosis	α3	Res	Res+1	SQRT	α6	LOG α 6	LOG +1	RPT	Res	Res+1	Log (Res+1)	Log(Res+1)+1
20040022	PDD	0.62	-0.3	0.7	0.84	0.75	-0.1	0.88	0.42	-0.34	0.66	-0.18	0.82
20040076	PDD	0.76	-0.28	0.7	0.85	1.28	0.1	1.11	0.51	-0.28	0.72	-0.14	0.86
20040105	PDD	1.65	0.51	1.5	1.23	1.56	0.2	1.19	0.8	-0.01	0.99	0	1
20050096	PDD	1.07	-0.19	0.8	0.9	1.35	0.1	1.13	0.83	0.07	1.07	0.03	1.03
20050099	PDD	0.84	-0.36	0.6	0.8	1.04	0	1.02	0.53	-0.16	0.84	-0.07	0.93
ST01/01	PDD	0.51	-0.46	0.5	0.73	0.97	-0	0.99	0.76	-0.02	0.98	-0.01	0.99
ST02/01	PDD	0.55	-0.48	0.5	0.72	1.04	0	1.02	0.77	0.02	1.02	0.01	1.01
ST03/01	PDD	0.69	-0.4	0.6	0.78	1.18	0.1	1.07	0.77	0.02	1.02	0.01	1.01
ST04/01	PDD	0.51	-0.4	0.6	0.78	1.11	0.1	1.05					
ST09/02	PDD	0.57	-0.34	0.7	0.81	1.12	0.1	1.05	0.75	0.08	1.08	0.03	1.03
ST10/02	PDD	0.67	-0.37	0.6	0.8	1.28	0.1	1.11	0.81	0.03	1.03	0.01	1.01
ST11/02	PDD	0.49	-0.46	0.5	0.73	1.13	0.1	1.05	0.75	0.05	1.05	0.02	1.02
ST12/02	PDD	0.48	-0.6	0.4	0.64	1.21	0.1	1.08	1.16	0.39	1.39	0.14	1.14
ST13/02	PDD	0.69	-0.2	0.8	0.89	1.29	0.1	1.11	0.83	0.06	1.06	0.03	1.03
ST14/02	PDD	1.01	-0.01	1	0.99	1.68	0.2	1.23	1.03	0.25	1.25	0.1	1.1
ST15/02	PDD	0.48	-0.75	0.3	0.5	0.65	-0.2	0.81	0.88	0.21	1.21	0.08	1.08
ST16/02	PDD	0.58	-0.41	0.6	0.77	1.11	0.1	1.05	0.94	0.17	1.17	0.07	1.07
ST17/02	PDD	1.12	0.24	1.2	1.12	1.47	0.2	1.17	0.79	-0.02	0.98	-0.01	0.99
ST18/02	PDD	0.72	-0.19	0.8	0.9	1.22	0.1	1.09	1.28	0.52	1.52	0.18	1.18
ST19/02	PDD	0.91	-0.22	0.8	0.88	1.53	0.2	1.18	1	0.23	1.23	0.09	1.09
ST20/02	PDD	0.63	-0.44	0.6	0.75	1.23	0.1	1.09	1.11	0.36	1.36	0.13	1.13
ST21/03	PDD	1.21	0.17	1.2	1.08	1.82	0.3	1.26	1.69	0.91	1.91	0.28	1.28
ST22/02	PDD	0.59	-0.18	0.8	0.91	0.99	0	1	1.02	0.24	1.24	0.09	1.09
ST23/03	PDD	0.82	-0.11	0.9	0.94	1.33	0.1	1.12	1.58	0.82	1.82	0.26	1.26
ST24/03	PDD	0.58	-0.44	0.6	0.75	1.04	0	1.02	1.69	0.91	1.91	0.28	1.28
ST25/04	PDD	0.73	-0.36	0.6	0.8	1.41	0.2	1.15	0.94	0.16	1.16	0.07	1.07
ST29/04	PDD	0.87	-0.27	0.7	0.85	1.75	0.2	1.24	1.09	0.33	1.33	0.12	1.12
ST30/04	PDD	0.6	-0.34	0.7	0.81	1.33	0.1	1.12	1.19	0.43	1.43	0.16	1.16
27 7/82	DLB	1.06	-0.01	1	1	1.24	0.1	1.09	0.42	-0.38	0.62	-0.21	0.79
36 7/81	DLB	1.79	0.66	1.7	1.29	1.47	0.2	1.17	0.39	-0.4	0.6	-0.22	0.78
51 7/96	DLB	1.12	0.08	1.1	1.04	1.36	0.1	1.13	0.56	-0.09	0.91	-0.04	0.96
52 1/13	DLB	1.41	0.29	1.3	1.14	1.28	0.1	1.11	0.64	-0.13	0.87	-0.06	0.94

		α3				α6			RPT6				
Case ID	Diagnosis	α3	Res	Res+1	SQRT	α6	LOG α 6	LOG +1	RPT	Res	Res+1	Log (Res+1)	Log(Res+1)+1
55 2/25	DLB	1.62	0.67	1.7	1.29	1.46	0.2	1.16	0.87	0.12	1.12	0.05	1.05
106 5/56	DLB	1.1	0.19	1.2	1.09	1.45	0.2	1.16	0.46	-0.3	0.7	-0.15	0.85
20030007	DLB	1.9	0.67	1.7	1.29	1.11	0.1	1.05	0.57	-0.23	0.77	-0.11	0.89
20030113	DLB	2.29	1.09	2.1	1.45	1.47	0.2	1.17	0.47	-0.21	0.79	-0.1	0.9
20040034	DLB	1.07	-0.24	0.8	0.87	0.82	-0.1	0.91	0.63	-0.06	0.94	-0.03	0.97
20040085	DLB	0.98	-0.31	0.7	0.83	0.88	-0.1	0.94	0.56	-0.21	0.79	-0.1	0.9
20050030	DLB	1.4	0.12	1.1	1.06	1.13	0.1	1.05	0.49	-0.15	0.85	-0.07	0.93
20050040	DLB	1.97	0.76	1.8	1.33	1.76	0.3	1.25	0.99	0.34	1.34	0.13	1.13
20060025	DLB	1.32	0.11	1.1	1.05	0.78	-0.1	0.89	0.52	-0.28	0.72	-0.14	0.86
20070009	DLB	1.31	-0.02	1	0.99	1.33	0.1	1.12	0.73	-0.01	0.99	0	1
20070105	DLB	1.44	0.15	1.2	1.07	1.07	0	1.03	0.67	-0.14	0.86	-0.07	0.93
20080083	DLB	1.65	0.35	1.4	1.16	0.86	-0.1	0.93	0.75	-0.04	0.96	-0.02	0.98
20100575	DLB	2.1	0.88	1.9	1.37	1.2	0.1	1.08	1.04	0.31	1.31	0.12	1.12
333 1/8	DLB	1.42	0.28	1.3	1.13	1.38	0.1	1.14	0.48	-0.3	0.7	-0.15	0.85
367 4/67	DLB	1.2	0.16	1.2	1.08	0.97	-0	0.99	0.48	-0.13	0.87	-0.06	0.94
383 5/58	DLB	0.94	-0.26	0.7	0.86	1.2	0.1	1.08	0.26	-0.44	0.56	-0.25	0.75
436 2/99	DLB	1.17	0.06	1.1	1.03	1.23	0.1	1.09	0.33	-0.34	0.66	-0.18	0.82
439	DLB	1.97	1.1	2.1	1.45	1.18	0.1	1.07	0.57	-0.09	0.91	-0.04	0.96
470	DLB	0.82	-0.2	0.8	0.9	1.08	0	1.03	0.54	-0.12	0.88	-0.06	0.94
475	DLB	1.34	0.27	1.3	1.13	1.13	0.1	1.05	0.46	-0.29	0.71	-0.15	0.85
495	DLB	1.25	0.31	1.3	1.15	0.8	-0.1	0.9	0.45	-0.12	0.88	-0.06	0.94
550 2/21	DLB	1.34	0.45	1.5	1.2	1.34	0.1	1.13	0.7	-0	1	0	1
745 4/63	DLB	1.29	0.28	1.3	1.13	1.12	0.1	1.05	0.51	-0.1	0.9	-0.05	0.95
A028/10	DLB	0.74	-0.26	0.7	0.86	0.98	-0	0.99	0.46	-0.18	0.82	-0.09	0.91
A040/10	DLB	0.31	-0.84	0.2	0.4	1.02	0	1.01	0.82	0.06	1.06	0.03	1.03
A053/09	DLB	1.05	-0.08	0.9	0.96	1.13	0.1	1.05	0.48	-0.25	0.75	-0.13	0.87
A055/09	DLB	1.32	0.23	1.2	1.11	1.01	0	1	0.4	-0.36	0.64	-0.2	0.8
A072/09	DLB	0.63	-0.31	0.7	0.83	1.1	0	1.04	1.16	0.45	1.45	0.16	1.16
A084/09	DLB	0.43	-0.8	0.2	0.45	0.87	-0.1	0.94	0.23	-0.53	0.47	-0.33	0.67
A109/01	DLB	0.79	-0.18	0.8	0.91	1.44	0.2	1.16	0.82	-0	1	0	1
A148/08	DLB	0.81	-0.36	0.6	0.8	1.01	0	1	0.49	-0.31	0.69	-0.16	0.84
A162/07	DLB	0.59	-0.13	0.9	0.93	0.88	-0.1	0.94	0.49	-0.29	0.71	-0.15	0.85

		α3				α6			RPT6				
Case ID	**Diagnosis**	**α3**	**Res**	**Res+1**	**SQRT**	**α6**	**LOG α 6**	**LOG +1**	**RPT**	**Res**	**Res+1**	**Log (Res+1)**	**Log(Res+1)+1**
A196/09	DLB	1.35	0.37	1.4	1.17	0.99	0	1	0.48	-0.29	0.71	-0.15	0.85
A204/07	DLB	1.02	0.08	1.1	1.04	1.38	0.1	1.14	0.78	-0.01	0.99	-0.01	0.99
A231/06	DLB	1.06	0.18	1.2	1.09	1.34	0.1	1.13	0.74	-0.04	0.96	-0.02	0.98
A249/06	DLB	1.16	0.23	1.2	1.11	1.13	0.1	1.05	0.66	-0.16	0.84	-0.08	0.92
A273/05	DLB	0.98	-0.26	0.7	0.86	1.16	0.1	1.06	0.68	-0.13	0.87	-0.06	0.94
A304/06	DLB	0.86	-0.13	0.9	0.94	1.21	0.1	1.08	0.55	-0.16	0.84	-0.07	0.93
A335/08	DLB	0.92	0	1	1	1.27	0.1	1.1	0.59	-0.21	0.79	-0.1	0.9
01/176	DLB	0.9	-0.03	1	0.99	1.2	0.1	1.08					
ST26/04	DLB	0.79	-0.24	0.8	0.87	1.22	0.1	1.09	0.95	0.23	1.23	0.09	1.09
ST27/04	DLB	0.79	-0.21	0.8	0.89	1.53	0.2	1.18	0.78	0.14	1.14	0.06	1.06
ST28/04	DLB	0.83	-0.16	0.8	0.92	1.6	0.2	1.2					
ST32/05	DLB	0.49	-0.68	0.3	0.56	1.08	0	1.03	0.62	-0.16	0.84	-0.07	0.93
A071/09	AD	1.05	-0.03	1	0.99	1.07	0	1.03	0.53	-0.28	0.72	-0.14	0.86
A108/09	AD	1.25	0.32	1.3	1.15	1.12	0.1	1.05	0.53	-0.25	0.75	-0.12	0.88
A120/09	AD	1.88	0.79	1.8	1.34	1.14	0.1	1.06	0.49	-0.16	0.84	-0.08	0.92
A147/10	AD	1.43	0.2	1.2	1.1	1.35	0.1	1.13	0.51	-0.28	0.72	-0.14	0.86
A216/09	AD	0.54	-0.68	0.3	0.57	0.83	-0.1	0.92	0.39	-0.34	0.66	-0.18	0.82
A267/09	AD	1.36	0.16	1.2	1.08	1.28	0.1	1.11	0.52	-0.14	0.86	-0.07	0.93
A349/08	AD	0.91	-0.09	0.9	0.96	0.91	-0	0.96	0.45	-0.35	0.65	-0.19	0.81
A350/09	AD	1.25	0.19	1.2	1.09	1.07	0	1.03	0.58	-0.2	0.8	-0.1	0.9
A37/09	AD	1.58	0.56	1.6	1.25	1.31	0.1	1.12	0.84	0.07	1.07	0.03	1.03
A371/08	AD	1.03	0.24	1.2	1.11	1.16	0.1	1.06	0.71	0.04	1.04	0.02	1.02
A38/11	AD	1.54	0.33	1.3	1.15	0.9	-0.1	0.95	0.65	-0.03	0.97	-0.01	0.99
A61/09	AD	2.02	0.96	2	1.4	1.31	0.1	1.12	0.64	-0.16	0.84	-0.08	0.92
A7/10	AD	1.99	0.81	1.8	1.35	1.33	0.1	1.12	0.58	-0.18	0.82	-0.09	0.91
A76/09	AD	0.99	-0.14	0.9	0.93	0.93	-0	0.97	0.69	-0.1	0.9	-0.05	0.95
A8/10	AD	2.34	1.16	2.2	1.47	1.19	0.1	1.08	0.61	-0.17	0.83	-0.08	0.92
A92/09	AD	0.97	-0.07	0.9	0.97	0.94	-0	0.97	0.94	0.15	1.15	0.06	1.06

Appendix VII: Proteasome subunits (α3, α6 and RPT6) protein values from semi-quantification of Western blotting and data transformation in BA40.

		α3			α6			RPT6				
Case ID	**Diagnosis**	**α3**	**LOGα3**	**LOGα3 +1**	**α 6**	**Res**	**Res+1**	**RPT6**	**Res**	**Res+1**	**Log (Res+1)**	**Log(Res+1)+1**
A011/06	Control	1.09	0.04	1.04	1.28	0.177	1.18	1.01	0.19	1.19	0.08	1.08
A047/02	Control	1.3	0.11	1.11	1.34	0.254	1.25	0.4	-0.43	0.57	-0.24	0.76
A048/09	Control	1.23	0.09	1.09	1.34	0.234	1.23	1.9	1.06	2.06	0.31	1.31
A049/03	Control	0.98	-0.01	0.99	1.06	-0.05	0.95	1.59	0.7	1.7	0.23	1.23
A063/10	Control	0.81	-0.09	0.91	1.01	-0.07	0.93	0.87	0.26	1.26	0.1	1.1
A133/95	Control	0.81	-0.09	0.91	1.1	0.007	1.01	0.4	-0.36	0.64	-0.2	0.8
A134/00	Control	1.17	0.07	1.07	1.29	0.201	1.2	1.2	0.31	1.31	0.12	1.12
A136/10	Control	1.35	0.13	1.13	1.29	0.211	1.21	1.02	0.36	1.36	0.13	1.13
A153/01	Control	1.8	0.26	1.26	1.16	0.019	1.02	0.8	-0.29	0.71	-0.15	0.85
A170/00	Control	1.56	0.19	1.19	0.98	-0.17	0.83	1.1	-0.01	0.99	-0.01	0.99
A185/04	Control	1.88	0.27	1.27	1.05	-0.06	0.94	1.05	0.22	1.22	0.09	1.09
A219/97	Control	1.77	0.25	1.25	1.05	-0.07	0.93	1.2	0.37	1.37	0.14	1.14
A223/96	Control	2.44	0.39	1.39	1.07	-0.04	0.96	1.18	0.23	1.23	0.09	1.09
A239/95	Control	1.79	0.25	1.25	1.06	-0.05	0.95	1.6	0.73	1.73	0.24	1.24
A283/96	Control	1.29	0.11	1.11	1.05	-0.07	0.93	1.6	0.67	1.67	0.22	1.22
A308/09	Control	1.22	0.09	1.09	1.48	0.322	1.32	1.01	0.01	1.01	0.01	1.01
A31/96	Control	1.19	0.08	1.08	1.03	-0.11	0.89	1.1	0.13	1.13	0.05	1.05
A316/95	Control	1.67	0.22	1.22	1.12	0.01	1.01	1.37	0.5	1.5	0.18	1.18
A320/94	Control	1.67	0.22	1.22	1.16	0.04	1.04	0.92	0.21	1.21	0.08	1.08
A33/96	Control	1.54	0.19	1.19	1.22	0.165	1.16	1.3	0.76	1.76	0.24	1.24
A346/95	Control	1.31	0.12	1.12	1.09	-0	1	1.17	0.3	1.3	0.11	1.11
A359/08	Control	0.82	-0.09	0.91	0.97	-0.14	0.86	1.35	0.44	1.44	0.16	1.16
A401/97	Control	1.45	0.16	1.16	1.23	0.137	1.14	1.29	0.51	1.51	0.18	1.18
A61/96	Control	1.44	0.16	1.16	1.03	-0.13	0.87	1.19	0.1	1.1	0.04	1.04
A94/95	Control	1.27	0.1	1.1	1.31	0.2	1.2	0.93	0.04	1.04	0.02	1.02
20020080	PDD	1.82	0.26	1.26	0.96	-0.18	0.82	1.2	0.14	1.14	0.06	1.06
20030004	PDD	1.71	0.23	1.23	1.01	-0.14	0.86	1.02	0.04	1.04	0.02	1.02
20030103	PDD	1.7	0.23	1.23	1.12	-0.01	0.99	0.8	-0.18	0.82	-0.09	0.91
20030111	PDD	0.82	-0.09	0.91	1.15	0.044	1.04	0.81	-0.03	0.97	-0.01	0.99

		α3			α6			RPT6				
Case ID	Diagnosis	α3	LOGα3	LOGα3 +1	α 6	Res	Res+1	RPT6	Res	Res+1	Log (Res+1)	Log(Res+1)+1
20030134	PDD	1.72	0.24	1.24	0.86	-0.27	0.73	1.04	0.12	1.12	0.05	1.05
20040022	PDD	1.55	0.19	1.19	1.01	-0.1	0.9	1.13	0.23	1.23	0.09	1.09
20040076	PDD	1.68	0.23	1.23	1.11	-0.01	0.99	1.2	0.22	1.22	0.09	1.09
20040105	PDD	1.44	0.16	1.16	1.1	-0.05	0.95	1.02	-0.09	0.91	-0.04	0.96
20050096	PDD	1.81	0.26	1.26	1.37	0.236	1.24	1.11	0.13	1.13	0.05	1.05
20050099	PDD	1.72	0.24	1.24	1.14	0.061	1.06	1.05	0.39	1.39	0.14	1.14
ST01/01	PDD	1.17	0.07	1.07	1.12	0.02	1.02	1.53	0.66	1.66	0.22	1.22
ST02/01	PDD	1.84	0.26	1.26	1.26	0.16	1.16	1.2	0.37	1.37	0.14	1.14
ST03/01	PDD	1.85	0.27	1.27	1.23	0.103	1.1	1.17	0.24	1.24	0.09	1.09
ST04/01	PDD	1.97	0.29	1.29	1.16	0.067	1.07	0.74				
ST09/02	PDD	1.45	0.16	1.16	1.21	0.097	1.1	0.82	0.06	1.06	0.02	1.02
ST10/02	PDD	3.3	0.52	1.52	1.3	0.197	1.2	1.21	0.33	1.33	0.12	1.12
ST11/02	PDD	2.8	0.45	1.45	1.33	0.196	1.2	1.07	0.19	1.19	0.08	1.08
ST12/02	PDD	2.6	0.41	1.41	1.23	0.12	1.12	1.16	0.27	1.27	0.1	1.1
ST13/02	PDD	2.47	0.39	1.39	1.07	-0.04	0.96	0.79	-0.09	0.91	-0.04	0.96
ST14/02	PDD	2.17	0.34	1.34	1.09	-0.03	0.97	0.81	-0.12	0.88	-0.06	0.94
ST15/02	PDD	1.96	0.29	1.29	1.05	-0.03	0.97	1.15	0.5	1.5	0.18	1.18
ST16/02	PDD	2.55	0.41	1.41	1.3	0.19	1.19	1.33	0.43	1.43	0.15	1.15
ST17/02	PDD	1.75	0.24	1.24	1.18	0.043	1.04	0.7	-0.36	0.64	-0.19	0.81
ST18/02	PDD	2.1	0.32	1.32	1.32	0.207	1.21	1.3	0.4	1.4	0.15	1.15
ST19/02	PDD	1.85	0.27	1.27	0.83	-0.27	0.73	0.94	0.09	1.09	0.04	1.04
ST20/02	PDD	2.63	0.42	1.42	1.08	-0.01	0.99	0.33	-0.47	0.53	-0.28	0.72
ST21/03	PDD	3.45	0.54	1.54	1.01	-0.09	0.91	0.41	-0.46	0.54	-0.27	0.73
ST22/02	PDD	2.37	0.37	1.37	1.09	-0.04	0.96	0.82	-0.15	0.85	-0.07	0.93
ST23/03	PDD	2.03	0.31	1.31	1.01	-0.1	0.9	0.75	-0.1	0.9	-0.05	0.95
ST24/03	PDD	1.41	0.15	1.15	0.92	-0.16	0.84	1.28	0.48	1.48	0.17	1.17
ST25/04	PDD	1.59	0.2	1.2	0.86	-0.23	0.77	0.88	0.05	1.05	0.02	1.02
ST29/04	PDD	1.62	0.21	1.21	1.01	-0.07	0.93	0.86	0.08	1.08	0.03	1.03
ST30/04	PDD	1.34	0.13	1.13	1.14	0.051	1.05	0.94	0.14	1.14	0.06	1.06
27 7/82	DLB	0.6	-0.22	0.78	1.19	0.104	1.1	0.58	-0.27	0.73	-0.14	0.86
36 7/81	DLB	1.23	0.09	1.09	1.19	0.097	1.1	0.72	-0.14	0.86	-0.07	0.93
51 7/96	DLB	0.65	-0.19	0.81	0.91	-0.19	0.81	0.37	-0.33	0.67	-0.17	0.83
52 1/13	DLB	0.78	-0.11	0.89	1.22	0.117	1.12	0.55	-0.32	0.68	-0.16	0.84

		α3			α6			RPT6				
Case ID	Diagnosis	α3	LOGα3	LOGα3 +1	α 6	Res	Res+1	RPT6	Res	Res+1	Log (Res+1)	Log(Res+1)+1
106 5/56	DLB	0.57	-0.24	0.76	0.91	-0.17	0.83	0.26	-0.51	0.49	-0.31	0.69
20030007	DLB	1.62	0.21	1.21	1.2	0.117	1.12	0.64	-0.19	0.81	-0.09	0.91
20030113	DLB	1.52	0.18	1.18	1.01	-0.11	0.89	0.57	-0.24	0.76	-0.12	0.88
20040034	DLB	1.23	0.09	1.09	1.12	-0.01	0.99	0.45	-0.39	0.61	-0.22	0.78
20040085	DLB	0.99	0	1	0.92	-0.2	0.8	0.37	-0.56	0.44	-0.36	0.64
20050030	DLB	0.92	-0.04	0.96	0.95	-0.12	0.88	0.3	-0.27	0.73	-0.14	0.86
20050040	DLB	1.19	0.08	1.08	1.26	0.133	1.13	0.53	-0.26	0.74	-0.13	0.87
20060025	DLB	1.12	0.05	1.05	1.05	-0.07	0.93	0.59	-0.41	0.59	-0.23	0.77
20070009	DLB	1.14	0.06	1.06	1.24	0.11	1.11	0.59	-0.34	0.66	-0.18	0.82
20070105	DLB	1.64	0.21	1.21	1.04	-0.1	0.9	0.93	-0.15	0.85	-0.07	0.93
20080083	DLB	1.51	0.18	1.18	1.27	0.16	1.16	1.17	0.24	1.24	0.09	1.09
20100575	DLB	1.39	0.14	1.14	1.26	0.14	1.14	0.93	0.06	1.06	0.02	1.02
333 1/8	DLB	0.71	-0.15	0.85	1.27	0.184	1.18	0.44	-0.38	0.62	-0.21	0.79
367 4/67	DLB	1.03	0.01	1.01	1.14	0.071	1.07	0.53	0.01	1.01	0.01	1.01
383 5/58	DLB	2.17	0.34	1.34	0.93	-0.14	0.86	0.43	-0.2	0.8	-0.1	0.9
436 2/99	DLB	1.77	0.25	1.25	1.21	0.087	1.09	0.42	-0.39	0.61	-0.21	0.79
439	DLB	1.01	0	1	1.25	0.123	1.12	0.54	-0.26	0.74	-0.13	0.87
470	DLB	0.91	-0.04	0.96	0.94	-0.16	0.84	0.62	-0.07	0.93	-0.03	0.97
475	DLB	1.08	0.03	1.03	0.84	-0.25	0.75	0.29	-0.51	0.49	-0.31	0.69
495	DLB	1.58	0.2	1.2	1.04	-0.05	0.95	0.65	0.12	1.12	0.05	1.05
550 2/21	DLB	1.3	0.11	1.11	1.05	-0.07	0.93	0.48	-0.36	0.64	-0.19	0.81
745 4/63	DLB	1.74	0.24	1.24	1.13	0.007	1.01	0.68	-0.04	0.96	-0.02	0.98
A014/07	DLB	1.06	0.03	1.03	1.15	0.02	1.02	1.2	0.2	1.2	0.08	1.08
A028/10	DLB	1.61	0.21	1.21	1.19	0.084	1.08	0.67	-0.02	0.98	-0.01	0.99
A035/08	DLB	1.66	0.22	1.22	1.09	-0.01	0.99	1.41	0.51	1.51	0.18	1.18
A040/10	DLB	1.15	0.06	1.06	1.12	0.034	1.03	0.88	0.09	1.09	0.04	1.04
A046/07	DLB	2.07	0.32	1.32	1.08	-0.04	0.96	1.13	0.27	1.27	0.1	1.1
A053/09	DLB	1	0	1	1.04	-0.03	0.97	0.69	-0.01	0.99	0	1
A055/09	DLB	1.11	0.05	1.05	1.08	-0.01	0.99	0.99	0.19	1.19	0.08	1.08
A072/09	DLB	1.54	0.19	1.19	1.06	-0.01	0.99	0.81	0.16	1.16	0.07	1.07
A084/09	DLB	0.93	-0.03	0.97	0.92	-0.17	0.83	0.53	-0.29	0.71	-0.15	0.85
A092/07	DLB	1.73	0.24	1.24	1.07	-0.01	0.99	1.2	0.37	1.37	0.14	1.14
A109/01	DLB	1.81	0.26	1.26	1.12	-0.04	0.96	1.92	0.76	1.76	0.24	1.24

		α3			α6			RPT6				
Case ID	Diagnosis	α3	LOGα3	LOGα3 +1	α 6	Res	Res+1	RPT6	Res	Res+1	Log (Res+1)	Log(Res+1)+1
A148/08	DLB	2.12	0.33	1.33				1.4	0.51	1.51	0.18	1.18
A162/07	DLB	1.55	0.19	1.19	1.07	-0.04	0.96	1.41	0.51	1.51	0.18	1.18
A196/09	DLB	1.44	0.16	1.16	0.99	-0.12	0.88	0.63	-0.26	0.74	-0.13	0.87
A204/07	DLB	1.16	0.06	1.06	1.13	-0	1	0.51	-0.5	0.5	-0.3	0.7
A229/05	DLB	0.92	-0.04	0.96	1.04	-0.07	0.93	0.9	-0.09	0.91	-0.04	0.96
A231/06	DLB	1.31	0.12	1.12	1.09	-0.05	0.95	0.43	-0.61	0.39	-0.41	0.59
A249/06	DLB	1.84	0.26	1.26	1.2	0.1	1.1	0.94	0	1	0	1
A273/05	DLB	2.02	0.31	1.31	0.93	-0.16	0.84	1.21	0.33	1.33	0.12	1.12
A304/06	DLB	1.04	0.02	1.02	1.03	-0.04	0.96	0.76	0.11	1.11	0.04	1.04
A335/08	DLB	1.65	0.22	1.22	1.24	0.127	1.13	0.59	-0.37	0.63	-0.2	0.8
A336/99	DLB	1.37	0.14	1.14	0.99	-0.16	0.84	0.76	-0.3	0.7	-0.15	0.85
01/176	DLB	1.8	0.26	1.26	1.08	-0.02	0.98	0.51	-0.27	0.73	-0.14	0.86
ST26/04	DLB	1.54	0.19	1.19	1.31	0.234	1.23	0.62	-0.08	0.92	-0.04	0.96
ST27/04	DLB	1.36	0.13	1.13	1.03	-0.08	0.92	0.36	-0.35	0.65	-0.19	0.81
ST28/04	DLB	1.57	0.2	1.2	1.16	0.077	1.08	0.39				
ST32/05	DLB	1.18	0.07	1.07	1.01	-0.07	0.93	0.44	-0.36	0.64	-0.2	0.8
A071/09	AD	0.82	-0.09	0.91	1.12	0.01	1.01	0.39	-0.56	0.44	-0.36	0.64
A108/09	AD	2.9	0.46	1.46	1.25	0.154	1.15	0.47	-0.38	0.62	-0.21	0.79
A120/09	AD	1.68	0.23	1.23	1.05	-0.04	0.96	0.43	-0.23	0.77	-0.11	0.89
A147/10	AD	0.98	-0.01	0.99	1.07	-0.02	0.98	0.39	-0.47	0.53	-0.27	0.73
A216/09	AD	0.77	-0.11	0.89	1.01	-0.07	0.93	0.34	-0.4	0.6	-0.22	0.78
A267/09	AD	1.38	0.14	1.14	1.11	0.034	1.03	0.38	-0.23	0.77	-0.12	0.88
A349/08	AD	1.16	0.06	1.06	1.2	0.111	1.11	0.38	-0.48	0.52	-0.29	0.71
A350/09	AD	1.32	0.12	1.12	0.76	-0.29	0.71	0.37	-0.31	0.69	-0.16	0.84
A37/09	AD	2.05	0.31	1.31	0.86	-0.22	0.78	0.67	-0.12	0.88	-0.05	0.95
A371/08	AD	1.19	0.08	1.08	1.19	0.087	1.09	0.56	-0.17	0.83	-0.08	0.92
A38/11	AD	1.03	0.01	1.01	1.16	0.023	1.02	0.52	-0.35	0.65	-0.19	0.81
A61/09	AD	2.6	0.41	1.41	0.95	-0.08	0.92	0.63	-0.02	0.98	-0.01	0.99
A7/10	AD	1.24	0.09	1.09	1.05	-0.05	0.95	0.51	-0.33	0.67	-0.17	0.83
A76/09	AD	2.21	0.34	1.34	0.96	-0.09	0.91	0.68	-0.03	0.97	-0.01	0.99
A8/10	AD	1.5	0.18	1.18	1.13	0.082	1.08	0.37	-0.3	0.7	-0.16	0.84
A92/09	AD	1.93	0.29	1.29	1.15	0.067	1.07	0.38	-0.45	0.55	-0.26	0.74

Appendix VIII: Proteasome activity (Chymotrypsin- and PGPH-like) values and data transformation in BA9, 24, and 40.

		BA9		BA24		BA40					
Case ID	Diagnosis	Chymotrypsin-like activity	PHPG- like activity	Chymotrypsin-like activity	PGPH-like activity	Chymotrypsin-like activity	Log(Chymo-like)	log(chymo-like)*500	PGPH-like activity	Log(PGPH-like)	log(PGPH-like)+5000
A011/06	Control	1411.16	10451.58	956.45	11996.35	1690.46	3.23	1614	13187.42	2589.714	7589.71
A047/02	Control	1049.2	10235.04			1105.23	3.04	1521.73	13758.39	2515.194	7515.19
A048/09	Control					1655.33	3.22	1609.44	11745.59	1117.861	6117.86
A049/03	Control	1279.92	10649.68	1244.74	11495.17	1586.43	3.2	1600.21	13418.17	2550.266	7550.27
A063/10	Control	1023.67	9907.71			1342.19	3.13	1563.91	12601.68	2934.688	7934.69
A133/95	Control	1285.56	12174.51	936.6	10531.32	1545.05	3.19	1594.47	13022.49	2574.9	7574.9
A134/00	Control			965.69	12590.52						
A136/10	Control	922.66	11498.87			1644.64	3.22	1608.04	12453.22	2516.021	7516.02
A153/01	Control					1209.98	3.08	1541.39	13104.83	1366.266	6366.27
A170/00	Control			1020.59	12103.82	1414.49	3.15	1575.3	13841.36	2222.886	7222.89
A185/04	Control	877.26	10009.69	1133.8	10804.81	1123.49	3.05	1525.28	12677.57	2229.986	7229.99
A223/96	Control			975.81	8423.81	1475.67	3.17	1584.5	11753.45	195.0181	5195.02
A239/95	Control			1203.51	9486.33						
A308/09	Control	889.05	9115.89	1329.87	12795.43						

		BA9		BA24		BA40					
Case ID	Diagnosis	Chymotrypsin-like activity	PHPG- like activity	Chymotrypsin-like activity	PGPH-like activity	Chymotrypsin-like activity	Log(Chymo-like)	log(chymo-like)*500	PGPH-like activity	Log(PGPH-like)	log(PGPH-like)+5000
A31/96	Control	1320.11	9041.1			1042.6	3.02	1509.06	11300.52	762.865	5762.87
A33/96	Control			950.17	11980.33						
A359/08	Control			1304.6	9578.57	1222.28	3.09	1543.58	11868.39	640.2093	5640.21
A401/97	Control			1147.7	13171.55						
A94/95	Control			1136.97	13076.41						
ST03/01	PDD			1063.9	7857.18	819.94	2.91	1456.89	10265.87	-541.992	4458.01
ST09/02	PDD	844.13	9254.07								
ST10/02	PDD			810.35	5561.55	891.78	2.95	1475.13	10475.13	-693.01	4306.99
ST11/02	PDD	910.4	10889.12	738.73	5324.24	779.13	2.89	1445.81	9789.65	-297.67	4702.33
ST12/02	PDD	398.21	7513.81			829.62	2.92	1459.44	9649.38	-1398.67	3601.33
ST13/02	PDD	1156.8	11801.32	823.31	5018.37	761.7	2.88	1440.89			
ST16/02	PDD	1011.42	10793.14								
ST17/02	PDD	706.72	9615.57								
ST18/02	PDD	866.22	9461.33								
ST19/02	PDD			1290.88	9746.8	1063.83	3.03	1513.44	11469.21	391.1383	5391.14
ST20/02	PDD	617.06	9192.51								
ST21/03	PDD	993.72	5528.42	799.55	5501.17	946.97	2.98	1488.17	11581.67	413.5384	5413.54

		BA9		BA24		BA40					
Case ID	Diagnosis	Chymotrypsin-like activity	PHPG- like activity	Chymotrypsin-like activity	PGPH-like activity	Chymotrypsin-like activity	Log(Chymo-like)	log(chymo-like)*500	PGPH-like activity	Log(PGPH-like)	log(PGPH-like)+5000
ST22/02	PDD	606.61	7059.4								
ST23/03	PDD			807.36	6832.13						
ST24/03	PDD	997.37	11149.51	885.54	5718.82	463.83	2.67	1333.18	10543.21	-624.921	4375.08
ST25/04	PDD			684.8	5708.18	414.39	2.62	1308.7	12141.52	973.3819	5973.38
ST29/04	PDD					784.07	2.89	1447.18	10991.71	63.76024	5063.76
ST30/04	PDD	607.29	8478.47								
27 7/82	DLB			1021.03	10714.08	723.67	2.86	1429.77	8640.11	-2858.28	2141.72
51 7/96	DLB			1073.31	8828.99	915.96	2.96	1480.94	11952.05	2465.188	7465.19
20050030	DLB	618.11	8439.51								
333 1/8	DLB			873.5	9869.44	629.91	2.8	1399.64	8992.88	-2175.26	2824.74
367 4/67	DLB			1133.52	8776.83	866.75	2.94	1468.95	10280.27	1273.778	6273.78
439	DLB			1000.21	7621.03	769.69	2.89	1443.16	10080.93	473.9831	5473.98
470	DLB	810.69	8324.2			647.75	2.81	1405.7	7158.9	-2508.1	2491.9
495	DLB			1076.4	9748.85	855.67	2.93	1466.15	6306.68	-2129.38	2870.62
A046/07	DLB			1000.1	8877.25						
A084/09	DLB			1162.52	11226.73				12387.1	1429.126	6429.13
A092/07	DLB	821.34	7087.77								

		BA9		BA24		BA40					
Case ID	Diagnosis	Chymotrypsin-like activity	PHPG- like activity	Chymotrypsin-like activity	PGPH-like activity	Chymotrypsin-like activity	Log(Chymo-like)	log(chymo-like)*500	PGPH-like activity	Log(PGPH-like)	log(PGPH-like)+5000
A109/01	DLB			914.37	9640.51	985.08	2.99	1496.74	9390.1	-2348.47	2651.53
A204/07	DLB			1020.65	10594.28						
A231/06	DLB	738.99	8923.14								
A249/06	DLB										
A273/05	DLB	1047.66	9928.5								
A335/08	DLB			1005.68	4956.74						
01/176	DLB	697.75	8677.71	1049.36	9847.48	941.05	2.97	1486.81	7952.81	-2284.62	2715.38
ST26/04	DLB	825.2	10233.99								
ST27/04	DLB	906.4	9652.27								
ST28/04	DLB	1037.72	11320.05								
A071/09	AD			906.46	5323.28						
A108/09	AD			883.4	6637.67	589	2.77	1385.06	12098.82	945.7008	5945.7
A120/09	AD			520.31	3730.27	581.61	2.76	1382.32	6161.52	-3355.36	1644.64
A147/10	AD	503.35	8613.79	1057.33	6823.37	539.98	2.73	1366.19	10432.02	-842.695	4157.31
A216/09	AD			1353.27	7586.81	809.05	2.91	1453.99	6117.23	-4450.45	549.55
A349/08	AD	765.99	7756.23	834.1	5878.03						
A350/09	AD	547.14	6830.32	841.9	6867.46	606.58	2.78	1391.44	7756.34	-3411.8	1588.2

		BA9		BA24		BA40					
Case ID	Diagnosis	Chymotrypsin-like activity	PHPG- like activity	Chymotrypsin-like activity	PGPH-like activity	Chymotrypsin-like activity	Log(Chymo-like)	log(chymo-like)*500	PGPH-like activity	Log(PGPH-like)	log(PGPH-like)+5000
A37/09	AD			898.82	6410.09	770.23	2.89	1443.31	10270.4	-747.624	4252.38
A371/08	AD					626.33	2.8	1398.4	6961.39	-2825.69	2174.31
A38/11	AD	773.78	7909.09			709.08	2.85	1425.35	11283.16	1406.009	6406.01
A61/09	AD	859.1	8659.96			757.32	2.88	1439.64	11037.95	-481.454	4518.55
A7/10	AD					730.35	2.86	1431.77	11348	360.0032	5360
A76/09	AD	672.63	7829.48	861.1	5397.72						
A8/10	AD					905.84	2.96	1478.53	10702.06	-436.049	4563.95
A92/09	AD					614.51	2.79	1394.26			

Appendix IX: Synaptic Protein values from semi-quantification of Western blotting.

		BA9				BA24				BA40			
Case ID	Diagnosis	ZnT3	PSD95	TotalCaMKII	PCaMKII	PSD95	ZnT3	TotalCaMKII	PCaMKII	PSD95	ZnT3	TotalCaMKII	PCaMKII
A011/06	Control	0.63	0.9	0.75	0.8	0	0.8	1.8	0.4		0.09	0.4	2.3
A047/02	Control	0.9	1	0.86	0.7						0.17	0.6	3.34
A048/09	Control	1.26	0.3	1.25	3.5		1.4	1.5	0.1	0.4	0.12		
A049/03	Control	1.3	0.8	0.94	3.4	1	1.1	2.1	0.2	0.53	0.23	0.5	2.08
A063/10	Control	0.95	0.3	0.79	2.8	1	0.4	3.5	0.6	0.23	0.05	0.3	1.77
A133/95	Control	0.55	0.4	0.84	2.1	0	0.4	2.5	0.6	0.04	0.3	0.4	1.54
A134/00	Control	0.65	2.2	1.39	5.2	0	0.5	2.3	0.2	0.98		0.8	5.97
A136/10	Control	1.73	0.7	1.7	5	0	1.3	3.7	1.4	1.01	0.71	1.3	3.43
A153/01	Control	0.38	1	1.39	4.5	1	0.5	2.5	0.7	2.01	0.49	1.1	6.69
A170/00	Control	0.29	2.3	1.07	3.9	0	0.3	2.3	0.3	0.98	0.23	0.7	5.04
A185/04	Control	0.25	1.3	0.54	3.4		0.4	2.3	0.5	0.82	1.12	0.6	6.01
A219/97	Control	0.08	0.1	0.18	0.6					0.85	0.61	0.9	3.59
A223/96	Control	0.51	0.8	0.69	2.7		0.3	1.3	1.1	2	0.53	1.2	3.47
A239/95	Control	0.36	0.7	0.82	2.4	0	0.3	1.4	0.4	0.87	1.3	0.8	2.86
A283/96	Control	0.31	0.7	1.03	1.8	0	0.5	1.5	0.6	0.82	0.43	0.8	2.72
A308/09	Control	1.24	0.7	1.51	4.4		0.9	1.5	0.2	0.25	0.16	0.5	2.88
A31/96	Control	0.77	1.8	1.52	3.9	1	0.4	1.5	0.3	0.83	0.41	1	4.51
A316/95	Control	0.74	0.4	1.3	3.1	1	0.2	1.5	0.5	1.28	3.68	1.4	4.16
A320/94	Control	0.59	0.4	1.05	5		0.4	1.7	0.3	0.64		0.5	7.49
A33/96	Control	0.26	0.2	0.8	3.9	0	0.3	0.9	0.6	0.37	0.46	0.8	6.88
A346/95	Control	0.59	0.6	0.96	3.8	0	0.3	1	0.1	0.83	0.48	0.9	6.54
A359/08	Control	1.44	0.7	1.49	2.6			1.3	0.6	0.56	0.1	0.2	2.14
A401/97	Control	0.73	1.6	0.74	3.4	0	0.1	0.8	0.2	0.69	0.47	3.2	5.97
A61/96	Control					0		1.2	0.1	0.9	1.3	0.6	3.81
A94/95	Control	0.8	0.9	1	3.1	1	0.4	1.6	0.5	1.05		0.3	1.51
20020080	PDD	0.45	0.4	0.85	2.2	1	0.4	2.8	1.3	1.24	0.71	1.1	5.21
20030004	PDD	0.51	0.3	0.77	3.9	1	0.5	2.1	0.5	0.78	0.53	1	11
20030103	PDD	0.24	0.2	0.7	2.3	1	0.5	3.5	0.9	0.71			

		BA9				BA24				BA40			
Case ID	Diagnosis	ZnT3	PSD95	TotalCaMKII	PCaMKII	PSD95	ZnT3	TotalCaMKII	PCaMKII	PSD95	ZnT3	TotalCaMKII	PCaMKII
20030111	PDD					0	0.3	1.2	0.4	0.27	0.16	0.5	1.79
20030134	PDD	0.22	0.3	1.01	1.3	0	0.4	1.1	0.5	1.11	0.4	1	4.73
20040022	PDD	0.37	0.3	1.2	0.9	0	0.3	1.3	0.3		0.75	0.5	2.51
20040076	PDD	0.44	0.9	0.28	1.9	2	0.6	1.1	0.5		0.48	1	2.78
20040105	PDD	0.25	0.5	0.33	2.2	2	0.6	3.5	1.3		0.29	0.9	3.26
20050096	PDD	0.22	0.5	0.6	1.9	1	0.4	1.8	1.2	1.02	0.99	1	3.16
20050099	PDD	0.5	0.3	0.91	2.2	1	0.3	0.9	0.5	0.5	0.19	0.6	2.08
A143/00	PDD	0.2	0.4	0.85	3.1								
ST01/01	PDD	0.29	0.6	1.31	1.3	0	0.5	1.5	0.7	0.41	0.18	0.4	1.98
ST02/01	PDD	0.29	0.4	0.58	2.1	1	0.5	2.4	0.8	0.95	0.27		
ST03/01	PDD	0.57	0.3	0.75	2.2	0	0.2	1.5	0.4	0.59	0.45	0.5	4.94
ST04/01	PDD	0.34	0.5	0.82	2.2	0	0.4	3	0.6		0.22	0.5	4.47
ST09/02	PDD	0.33	0.4	0.92	2.5	0	0.2	1.3	0.3	0.3	0.34	0.5	6.21
ST10/02	PDD	0.29	0.5	0.42	1.6	0	0.2	1.9	0.6	1.08	0.79		
ST11/02	PDD	0.31	0.5	0.33	1.6	0	0.2	1.4	0.5	0.93	0.37	0.9	2.61
ST12/02	PDD		0.4	0.68	2.4	1	0.5	2.8	0.8	1.22	0.18	1	6.14
ST13/02	PDD	0.41	0.2	0.82	2.5	0	0.4	1.3	0.5	0.61	0.45	0.8	3.56
ST14/02	PDD	0.09	0.4	0.54	2.1	1	0.1	2.2	0.8		0.09	0.4	2.1
ST15/02	PDD	0.09	0.4	0.21	1	0	0.1	0.9	0.3	0.48	0.06	0.4	3.02
ST16/02	PDD	0.52	0.5	0.89	2.9	1	0.4	2.4	0.8	0.45	0.31	0.8	4.21
ST17/02	PDD	0.31	0.7	0.67	2.5	1	0.3	2.6	0.5	1.06	0.36	0.8	5.08
ST18/02	PDD	0.17	0.4	0.44	1.9	0	0.7	2.6	0.7	1.5	0.87	1	4.29
ST19/02	PDD	0.13	0.3			0	0.3	0.6	0.3	1.4	0.49	1	3.02
ST20/02	PDD	0.29	1	1.03	2.1	0	0.4	1.1	0.5	1.27	0.29	0.9	1.98
ST21/03	PDD	0.12	0.2	0.52	1.4	1	0.2	2.3	0.6	1.15	0.17	0.7	3.12
ST22/02	PDD	0.41		0.93	2.8	0	0.5	0.3	0.3	1.21	0.43	0.9	2.48
ST23/03	PDD	0.59	0.3	0.4	2.6	0	0.4	1.3	0.5	0.43	0.33	0.8	5.59
ST24/03	PDD		0.4	0.49	2.8	1	0.3	2	0.5		0.24	1	4.65
ST25/04	PDD	0.2	0.4	0.53	1.9	0	0.4	1.4	0.7	0.86		1	4.15
ST29/04	PDD	0.14	0.3	0.25	0.9	1	0.7	2.3	1.1	1.09	0.55	1.2	4.19
ST30/04	PDD	0.17	0.2	0.68	1.6	0	0.2	1.9	0.8	0.81	0.4	0.6	3.17

		BA9				BA24				BA40			
Case ID	Diagnosis	ZnT3	PSD95	TotalCaMKII	PCaMKII	PSD95	ZnT3	TotalCaMKII	PCaMKII	PSD95	ZnT3	TotalCaMKII	PCaMKII
27 7/82	DLB	0.19	0.4	0.95	3.5	0	0.2	1.6	0.5	0.68	0.48	0.8	3.99
36 7/81	DLB					1	0.2	1.1				0.7	2.28
51 7/96	DLB	0.17	0.2	0.83	2.6	1	0.5	2.3	1.5	0.44	0.19	0.6	2.84
52 1/13	DLB	0.32	0.7	0.97	3.1	1	0.3	2.5	0.8	0.83	0.26	0.7	3.08
55 2/25	DLB	0.19	0.3	0.66	2.5	1	0.5	2.3	1.9				
106 5/56	DLB	0.36	0.9	1.14	2.9	2	0.5	2.6	0.9	0.1	0.13	0.3	2.3
20030007	DLB	0.26	0.7	1.18	1.7	0	0.4	2.3	0.8	0.55	0.37	0.9	2.9
20030113	DLB	0.33	0.2	0.85	2.1	1	0.3	2.3	1.9	0.63	0.18	0.7	2.23
20040034	DLB	0.21	0.4	0.85	2.5	0	0.1	1.9	1	0.33	0.14	1	3.64
20040085	DLB	0.37	0.4	0.97	2.6	0	0.2	1.7	1.1	0.26	0.2	0.5	2.58
20050030	DLB	0.25	0.5	0.95	2.3	0	0.1	0.8	1.5	0.27	0.08	0.8	2.67
20050040	DLB	0.23	0.3	0.75	2.2	1	0.3	1.1	1.4	0.24	0.13	0.8	3.34
20060025	DLB					1		1.5	0.8	0.81	0.35	0.9	3.19
20070009	DLB	0.37	0.4	1.25	2.4	1	0.3	1.4	1	0.4	0.17	0.8	2.19
20070105	DLB	0.34	0.9	0.81	2.8	1	0.2	2.1	0.7	0.95	0.32	0.8	2.8
20080083	DLB	0.48	0.5	1.3	3.7	0	0.3	2.2	1	0.47	0.29		
20100575	DLB	0.55	0.3	0.84	3.3	0	0.4	2.7	0.8	0.36	0.26	0.5	3.47
333 1/8	DLB	0.2	0.7	1.21	2.5	0	0.2	1.3	0.6	0.27	0.19	0.6	2.52
367 4/67	DLB	0.2	0.3	0.87	3	0	0.1	1.9	0.7	0.72	0.15	0.7	2.11
383 5/58	DLB	0.34	0.8	1.17	3	1	0.3	2	0.6	0.53	0.31	0.6	1.93
436 2/99	DLB	0.47	0.7	1.03	3.3	1	0.4	1.8	1	0.73	0.5	0.8	3.09
439	DLB	0.51		1.1	4.1	0	0.3	3.3	0.8	0.36	0.15	0.4	1.82
470	DLB	0.18	0.3	0.75	2.9		0.3	2.4	0.7	0.57	0.31	0.6	3.17
475	DLB	0.3	0.4	0.93	2.8		0.1	1.3	0.6		0.09	0.4	1.63
495	DLB	0.57	0.3	1.04	3.2	0	0.2	1.7	0.8	0.69	0.18	0.6	2.68
550 2/21	DLB	0.37	0.3	0.93	1.9	1	0.2	1.3	1.2	0.43	0.24	0.6	2.25
745 4/63	DLB	0.5	0.4	0.91	2.7	0	0.3	2.7	1.2	0.69	0.31	0.8	2.78
A014/07	DLB	0.55	0.6	1.2	5.2							0.5	1.88
A028/10	DLB			0.73	3.5			0.7	0.2			0.6	2.11
A035/08	DLB	0.49	0.8	0.86	3.3	1						0.6	1.53
A040/10	DLB			0.87	2.9			1.1	0.3			0.5	1.45

		BA9				BA24				BA40			
Case ID	Diagnosis	ZnT3	PSD95	TotalCaMKII	PCaMKII	PSD95	ZnT3	TotalCaMKII	PCaMKII	PSD95	ZnT3	TotalCaMKII	PCaMKII
A046/07	DLB		0.5	1.15	4.9					1.36	0.39	2	4.25
A053/09	DLB		0.2	0.76	3.2			2.6	0.7			0.2	2.06
A055/09	DLB	0.92	0.3	1.08	3.5			1.7	0.4			0.2	1.69
A072/09	DLB			0.85	2.9			0.7	0.2			0.3	1.12
A084/09	DLB	1.06	0.7	0.97	4.1			1.2	0.3			1.1	4.26
A092/07	DLB	0.47	0.6	0.91	2.9	1						1	5.12
A109/01	DLB	0.56	0.8	1.28	3.3	1		2.5	0.7			1.1	3.61
A148/08	DLB						0.3	0.5	0.4		0.26		
A162/07	DLB							1	0.2		0.33		
A190/03	DLB	0.48	0.5	1	5	1							
A196/09	DLB		0.7	0.99	4.8			1.1	0.5			0.8	2.05
A204/07	DLB	0.45	0.7	1.63	4.6	1	0.9	2.7	1.2			1.1	3.02
A229/05	DLB	0.49	0.7	1	3.3	1						1.8	4.8
A231/06	DLB	0.17	0.2	0.39	1	0		1.9	1			0.4	2.35
A249/06	DLB						1	2.7	1.3		0.41		
A273/05	DLB	0.61	0.9	1.23	2.9	1		3.9	0.8	1.12	0.18	1	4.25
A304/06	DLB	0.54	0.6	1.35	3.2	1		2.1	1.2			1.3	3.64
A335/08	DLB	0.35	0.6	1.29	2.8	1	0.4	1.2	0.7		0.3	1.2	4.22
A336/99	DLB	0.4	0.4	0.43	0.9					0.54	0.32	0.8	1.74
01/176	DLB	0.12	0.3	0.77	2.9	1	0.4	2.7	1.4	1.21	0.29	0.8	2.02
ST26/04	DLB	0.52	0.9	1.31	3.7	0	0.2	3.1	0.8		0.35	1.1	4.02
ST27/04	DLB	0.39	0.4	1.3	3.5	1	0.9	3.1	1.3	0.2	0.42	1.1	3.46
ST28/04	DLB	0.27	0.4	0.73	2.9	0	0.6	3	1.4	0.26		0.4	2.22
ST32/05	DLB	0.17	0.4	0.76	1.2	0	0.2	1	0.4	0.37	0.11	0.5	2.24
A071/09	AD	0.69	0.5	0.77	3	1	0.7	1.5	0.7	0.22	0.08	0.1	1.05
A108/09	AD	0.48	0.5	0.53	0.5	0	0.5	1.9	0.4	0.55	0.38	0.6	2.48
A120/09	AD	0.34		0.72	0.7	0	0.1	1.3	0.3	0.38	0.17	0.6	2.82
A147/10	AD	0.16	0.7	0.65	0.6	1	0.1	1.2	0.6	0.28	0.23	0.6	1.65
A216/09	AD	0.22		1.01	2.8	0	0.3	1	0.6		0.07	0.5	2.05
A267/09	AD	0.13	0.3	1.06	2.2	1	0.1	1.1	0.7	0.37		0.5	2.19
A349/08	AD	0.23	0.3	0.82	2.9	1	0.2	0.9	0.9	0.32	0.28	0.2	2.3

		BA9				BA24				BA40			
Case ID	Diagnosis	ZnT3	PSD95	TotalCaMKII	PCaMKII	PSD95	ZnT3	TotalCaMKII	PCaMKII	PSD95	ZnT3	TotalCaMKII	PCaMKII
A350/09	AD	0.45	0.4	1.19	1.8		0.5	1.9	1.1		0.38	0.8	3.34
A37/09	AD	0.5	1.1	0.97	3	2	0.5	1.4	1.1			1	3.61
A371/08	AD	0.52	0.3	0.57	0.6	0	0.5	1.2	0.9	0.29	0.33	0.4	2.07
A38/11	AD	0.18	0.7	0.95	1.9	1	0.2	0.7	0.4	0.32	0.29	0.4	1.73
A61/09	AD	0.44	0.7	0.63	0.7	1	0.1	0.8	0.9		0.47	0.8	3.2
A7/10	AD	0.51	0.9	0.66	0.6	1	0.3	1.4	0.6		0.17	0.6	1.82
A76/09	AD	0.77	1.1	0.6	0.6	1	0.3	1.9	0.8	0.9	0.56	1	3.64
A8/10	AD	0.29		0.73	0.6		0.3	1.5	1	0.5	0.19	0.5	2.31
A92/09	AD	0.58	1.3	1.02	3.7	1	0.7	1.2	0.7		0.33	1.2	4.56

Appendix X: Residual and normalised synaptic protein values.

		BA9				Residual BA24				BA40	
Case ID	Diagnosis	Residual ZnT3	Residual PSD95	Normalised TotalCaMKII	Residual PCaMKII	Residual PSD95	Residual ZnT3	Residual TotalCaMKII	Residual PCaMKII	Residual PSD95	Residual ZnT3
A011/06	Control	0.292	0.3	0.75	-1.49	0.65	0.395	0.62	0.11		-0.5
A047/02	Control	0.447	0.2	0.86	-1.61						-0.28
A048/09	Control	0.484	-0.2	1.25	0.62		0.55	0.3	-0.13	-0.175	-0.37
A049/03	Control	0.498	0.2	0.94	0.53	0.99	0.536	0.42	0.066	-0.106	-0.11
A063/10	Control	0.471	-0.1	0.79	0.46	0.73	0.087	0.76	0.625	-0.223	-0.67
A133/95	Control	0.124	-0.1	0.84	-0.79	0.66	-0.07	0.76	0.224	-0.588	0.04
A134/00	Control	0.197	0.5	1.39	2.28	0.51	0.126	0.39	0.153	0.0635	
A136/10	Control	0.731	0.2	1.7	2.62	0.63	0.664	1.17	0.688	0.2727	0.46
A153/01	Control	-0.04	0.2	1.39	1.61	0.84	0.07	0.85	0.204	0.488	0.14
A170/00	Control	-0.04	0.5	1.07	1.52	0.49	-0.23	0.56	0.268	0.0734	-0.18
A185/04	Control	-0.22	0.5	0.54	0.47		-0	0.7	0.145	0.1173	0.61
A219/97	Control	-0.6		0.18	-1.77					0.1831	0.39
A223/96	Control	0.091	0.1	0.69	-0.23		-0.12	1.03	-0.21	0.5043	0.19
A239/95	Control	0.049	0.2	0.82	0.06	0.48	-0.04	0.66	-0.08	0.1116	0.65
A283/96	Control	-0.12	0.1	1.03	-1.08	0.66	0.1	0.79	-0.12	0.0548	0.14
A308/09	Control	0.477	0.2	1.51	1.5		0.23	0.46	-0.14	-0.275	-0.22
A31/96	Control	0.27	0.6	1.52	1.05	0.73	-0.12	0.51	-0.14	0.113	0.17
A316/95	Control	0.253	-0.1	1.3	0.22	0.85	-0.16	0.72	-0.14	0.3004	1.09
A320/94	Control	0.155	0.1	1.05	2.16		0.023	0.51	-0.07	0.1698	
A33/96	Control	-0.09	-0.2	0.8	1.55	0.66	0.111	0.78	-0.31	-0.101	0.29
A346/95	Control	0.155	0	0.96	0.88	0.41	0.069	0.28	-0.36	0.0175	0.16
A359/08	Control	0.651	0.1	1.49	0.3			0.8	-0.1	-0.125	-0.51
A401/97	Control	0.247	0.5	0.74	0.47	0.47	-0.31	0.39	-0.47	0.0227	0.22
A61/96	Control					0.49		0.37	-0.26	0.098	0.62

		BA9				Residual BA24				BA40	
Case ID	**Diagnosis**	**Residual ZnT3**	**Residual PSD95**	**Normalised TotalCaMKII**	**Residual PCaMKII**	**Residual PSD95**	**Residual ZnT3**	**Residual TotalCaMKII**	**Residual PCaMKII**	**Residual PSD95**	**Residual ZnT3**
A94/95	Control	0.396	0.2	1	0.75	0.92	0.154	0.7	0.016	0.1806	
20020080	PDD	0.037	-0.1	0.85	-0.67	0.81	-0	1.12	0.321	0.2233	0.33
20030004	PDD	0.201	-0.2	0.77	1.55	0.71	-0	0.7	0.207	0.0884	0.28
20030103	PDD	-0.13	-0.4	0.7	-0.05	0.88	0.271	0.95	0.626	-0.005	
20030111	PDD					0.52	0.122	0.65	-0.29	-0.295	-0.26
20030134	PDD	-0.27	-0.2	1.01	-1.61	0.62	-0.04	0.68	-0.31		0.14
20040022	PDD	-0.05	-0.2	1.2	-2.01	0.69	-0.1	0.53	-0.23		0.39
20040076	PDD	0.027	0.2	0.28	-0.97	1.26	0.186	0.74	-0.31		0.16
20040105	PDD	-0.22	-0.1	0.33	-0.69	1.22	0.218	1.14	0.511		-0.08
20050096	PDD	-0.27	-0	0.6	-1	0.93	0.14	1.08	-0.03	0.1658	0.51
20050099	PDD	0.083	-0.2	0.91	-0.73	0.81	0.068	0.71	-0.43	-0.028	-0.12
A143/00	PDD	-0.21	-0.1	0.85	0.8						
ST01/01	PDD	-0.04	0	1.31	-1.02	0.42	0.169	0.85	-0.01	-0.227	-0.25
ST02/01	PDD	-0.15	-0.1	0.58	-0.8	0.77	0.165	0.89	0.185	0.1503	-0.04
ST03/01	PDD	0.14	-0.3	0.75	-0.72	0.4	-0.31	0.62	-0.15	-0.06	0.18
ST04/01	PDD	0.025		0.82	-0.12	0.67	-0.05	0.77	0.477		
ST09/02	PDD	-0.1	0	0.92	-0.37	0.2	-0.32	0.56	-0.22	-0.162	0.16
ST10/02	PDD	-0.15	-0.1	0.42	-1.32	0.37	-0.2	0.78	0.026	0.1721	0.4
ST11/02	PDD	-0.02	0.1	0.33	-0.76	0.6	-0.36	0.71	-0.08	0.2157	0.16
ST12/02	PDD		-0.2	0.68	0.04	0.79	0.206	0.89	0.411	0.2505	-0.24
ST13/02	PDD	0.106	-0.5	0.82	0.13	0.58	-0.02	0.71	-0.11	-0.073	0.16
ST14/02	PDD	-0.66	-0.2	0.54	-0.76	0.85	-0.42	0.88	0.118		-0.55
ST15/02	PDD	-0.55	0	0.21	-1.34	0.32	-0.33	0.53	-0.3	-0.016	-0.6
ST16/02	PDD	0.1	-0	0.89	-0.02	0.73	0.028	0.89	0.194	-0.19	-0.01
ST17/02	PDD	-0.12	0.1	0.67	-0.34	0.75	-0.29	0.71	0.243	0.113	0.01
ST18/02	PDD	-0.39	-0.1	0.44	-0.95	0.65	0.218	0.83	0.253	0.3773	0.45
ST19/02	PDD	-0.39	-0.3			0.56	0.026	0.52	-0.46	0.3259	0.2

		BA9				Residual BA24				BA40	
Case ID	Diagnosis	Residual ZnT3	Residual PSD95	Normalised TotalCaMKII	Residual PCaMKII	Residual PSD95	Residual ZnT3	Residual TotalCaMKII	Residual PCaMKII	Residual PSD95	Residual ZnT3
ST20/02	PDD	-0.04	0.3	1.03	-0.21	0.66	0.079	0.67	-0.18	0.2993	-0.01
ST21/03	PDD	-0.43	-0.6	0.52	-0.98	0.73	-0.17	0.76	0.262	0.2052	-0.27
ST22/02	PDD	0.106		0.93	0.49	0.46	-0.09	0.53	-0.73	0.2328	0.13
ST23/03	PDD	0.264	-0.3	0.4	0.3	0.5	0.041	0.69	-0.12	-0.182	0.04
ST24/03	PDD		-0.1	0.49	-0.12	1.11	-0.01	0.7	0.049		-0.12
ST25/04	PDD	-0.21	-0.1	0.53	-0.45	0.7	0.092	0.84	-0.06	0.0602	
ST29/04	PDD	-0.36	-0.2	0.25	-1.48	0.72	0.416	1.05	0.275	0.2032	0.26
ST30/04	PDD	-0.39	-0.5	0.68	-1.27	0.26	-0.24	0.9	0.013	0.0592	0.12
27 7/82	DLB	-0.23	-0.2	0.95	1.17	0.62	-0.29	0.74	0.026	-0.079	0.15
36 7/81	DLB					1.02	-0.15		-0.34		
51 7/96	DLB	-0.39	-0.3	0.83	-0.31	0.77	0.212	1.23	0.137	-0.02	-0.07
52 1/13	DLB	-0.11	0.1	0.97	0.23	0.71	0.059	0.87	0.229	0.0603	-0.07
55 2/25	DLB	-0.34	-0.2	0.66	-0.39	1.03	0.134	1.36	0.157		
106 5/56	DLB	0.049	0.2	1.14	0.55	1.48	0.135	0.92	0.375	-0.518	-0.36
20030007	DLB	-0.09	0.1	1.18	-0.68	0.67	0.209	0.89	0.28	-0.152	0.04
20030113	DLB	-0.1	-0.4	0.85	-0.74	0.74	-0.04	1.38	0.154	0.0615	-0.14
20040034	DLB	-0.18	0	0.85	0.13	0.56	-0.37	1.02	0.126	-0.161	-0.25
20040085	DLB	-0.05	-0.2	0.97	-0.24	0.55	-0.07	1.03	-0.05	-0.341	-0.19
20050030	DLB	-0.11	0.1	0.95	0.01	0.66	-0.15	1.23	-0.38	-0.15	-0.44
20050040	DLB	-0.15	-0.1	0.75	-0.12	0.75	-0.03	1.16	-0.19	-0.2	-0.24
20060025	DLB					0.84		0.87	-0.12	-0.003	0.01
20070009	DLB	-0.05	-0.1	1.25	-0.5	1	0.098	1.01	-0.2	-0.175	-0.22
20070105	DLB	-0.08	0.1	0.81	-0.06	0.72	-0.06	0.86	0.085	0.0549	-0.04
20080083	DLB	0.174	-0	1.3	1.33	0.62	0.106	1.01	0.233	-0.205	-0.06
20100575	DLB	0.124	-0.3	0.84	0.4	0.54	0.21	0.88	0.284	-0.195	-0.03
333 1/8	DLB	-0.21	0.1	1.21	0.21	0.7	-0.23	0.77	-0.1	-0.348	-0.22
367 4/67	DLB	-0.21	-0	0.87	0.68	0.57	-0.37	0.86	0.117	0.2183	-0.13

		BA9				Residual BA24				BA40	
Case ID	Diagnosis	Residual ZnT3	Residual PSD95	Normalised TotalCaMKII	Residual PCaMKII	Residual PSD95	Residual ZnT3	Residual TotalCaMKII	Residual PCaMKII	Residual PSD95	Residual ZnT3
383 5/58	DLB	0.025	0.3	1.17	0.64	0.88	0.075	0.8	0.167	-0.021	0.09
436 2/99	DLB	0.056	0.3	1.03	0.44	0.9	0.079	1.01	-0.03	0.1386	0.32
439	DLB	0.091		1.1	1.18	0.51	-0.22	0.88	0.443	-0.096	-0.19
470	DLB	-0.36	-0.1	0.75	0.05		-0	0.81	0.194	0.0524	0.12
475	DLB	-0.03	-0.1	0.93	0.44		-0.39	0.77	-0.09		-0.51
495	DLB	0.14	0.1	1.04	0.32	0.62	-0.31	0.9	-0.07	0.263	-0
550 2/21	DLB	-0.05	-0.1	0.93	-1.01	0.71	-0.31	1.09	-0.22	-0.103	-0.03
745 4/63	DLB	0.192	0.1	0.91	0.34	0.55	-0.09	1.11	0.401	0.2004	0.18
A014/07	DLB	0.124	0	1.2	2.28						
A028/10	DLB			0.73	0.58			0.47	-0.5		
A035/08	DLB	0.183	0.1	0.86	0.98	0.79					
A040/10	DLB			0.87	0.58			0.5	-0.2		
A046/07	DLB		0	1.15	2					0.3945	0.17
A053/09	DLB		-0.3	0.76	0.32			0.82	0.235		
A055/09	DLB	0.457	-0.2	1.08	1.16			0.6	0.052		
A072/09	DLB			0.85	0.57			0.38	-0.39		
A084/09	DLB	0.518	0.1	0.97	1.75			0.54	-0.13		
A092/07	DLB	0.056	-0	0.91	-0.03	0.72					
A109/01	DLB	0.132	0.1	1.28	0.45	1.03		0.85	0.215		
A148/08	DLB						0.021	0.62	-0.55		-0.12
A162/07	DLB							0.43	-0.39		0.02
A190/03	DLB	0.065	0	1	2.11	0.93					
A196/09	DLB		0.1	0.99	2.5			0.72	-0.2		
A204/07	DLB	0.037	0.1	1.63	1.71	1.1	0.279	1.09	0.274		
A229/05	DLB	0.074	0	1	0.4	0.79					
A231/06	DLB	-0.28	-0.5	0.39	-1.35	0.59		0.99	0.126		
A249/06	DLB						0.413	1.15	0.279		0.06

		BA9				Residual BA24				BA40	
Case ID	**Diagnosis**	**Residual ZnT3**	**Residual PSD95**	**Normalised TotalCaMKII**	**Residual PCaMKII**	**Residual PSD95**	**Residual ZnT3**	**Residual TotalCaMKII**	**Residual PCaMKII**	**Residual PSD95**	**Residual ZnT3**
A273/05	DLB	0.169	0.1	1.23	0.02	0.76		0.89	0.618	0.1385	-0.29
A304/06	DLB	0.226	0.2	1.35	0.89	0.79		1.1	0.197		
A335/08	DLB	-0.07	0	1.29	-0.12	0.72	-0.09	0.85	-0.26		-0.06
A336/99	DLB	-0.01	-0.2	0.43	-1.96					-0.142	-0.01
01/176	DLB	-0.54	-0.1	0.77	-0.01	0.73	-0.01	1.19	0.263	0.3348	0.04
ST26/04	DLB	0.1	0.3	1.31	0.79	0.65	-0.21	0.91	0.392		0.11
ST27/04	DLB	-0.03	0	1.3	0.6	0.99	0.365	1.14	0.398	-0.223	0.28
ST28/04	DLB	-0.08		0.73	0.6	0.62	0.269	1.16	0.498		
ST32/05	DLB	-0.28	-0.1	0.76	-1.18	0.47	-0.05	0.6	-0.25	-0.259	-0.46
A071/09	AD	0.223	-0.1	0.77	0.13	0.76	0.319	0.85	-0.13	-0.444	-0.64
A108/09	AD	0.174	-0	0.53	-1.84	0.58	0.13	0.67	0.138	-0.124	0.08
A120/09	AD	0.025		0.72	-1.63	0.66	-0.51	0.59	-0.11	-0.07	-0.12
A147/10	AD	-0.3	0.1	0.65	-1.7	0.95	-0.58	0.8	-0.15	-0.35	-0.15
A216/09	AD	-0.16		1.01	0.44	0.67	0.162	0.76	-0.23		-0.6
A267/09	AD	-0.39	-0.1	1.06	-0.11	0.84	-0.43	0.81	-0.18	-0.094	
A349/08	AD	-0.15	-0.3	0.82	0.6	0.9	-0.2	0.92	-0.32	-0.334	-0.08
A350/09	AD	0.146	-0.1	1.19	-0.54		0.336	1.03	0.128		0.08
A37/09	AD	0.083	0.3	0.97	0.08	1.39	0.163	1.04	-0.2		
A371/08	AD	0.1	-0.1	0.57	-2.3	0.66	-0.03	0.95	-0.28	-0.177	0.14
A38/11	AD	-0.25	0.2	0.95	-0.42	1.04	-0.24	0.63	-0.38	-0.16	0.08
A61/09	AD	0.137	0	0.63	-1.65	0.91	-0.49	0.95	-0.38		0.14
A7/10	AD	0.201	0.2	0.66	-1.73	1.08	-0	0.77	-0.06		-0.26
A76/09	AD	0.27	0.3	0.6	-2.24	0.82	0.128	0.88	0.022	0.0618	0.23
A8/10	AD	-0.04		0.73	-1.72		0.106	0.98	-0.02	-0.157	-0.22
A92/09	AD	0.147	0.3	1.02	0.77	0.96	0.375	0.83	-0.27		-0

Appendix XI: Differences in the relative levels of synaptic proteins between the diagnostic groups.

		BA9		BA24		BA40	
		N	Mean	N	Mean	N	Mean
PSD95	Control	24	.8692	17	.6571	23	.8235
	PDD	32	.4100	33	.6742	27	.8678
	DLB	46	.5061	40	.7600	31	.5603
	AD	13	.6762	14	.8729	10	.4130
	Total	115	.5743	104	.7312	91	.7019
ZnT3	Control	24	.7213	21	.0982	22	.6109
	PDD	31	.3052	33	-.0155	31	.3981
	DLB	44	.3925	35	-.0224	36	.2594
	AD	16	.4056	16	-.0478	14	.2807
	Total	115	.4394	105	.0000	103	.3791
Total CaMKII	Control	24	1.0254	23	.4501	24	.8250
	PDD	32	.6784	33	.6282	30	.7883
	DLB	50	.9816	47	.8715	49	.7561
	AD	16	.8050	16	.7239	16	.6098
	Total	122	.8875	119	.7027	119	.7584
Phospho CaMKII	Control	24	2.9993	23	.7479	24	4.0302
	PDD	32	1.9507	33	.7549	30	3.9167
	DLB	50	2.8902	48	.7979	49	2.7881
	AD	16	1.6301	16	.5992	16	2.5499
	Total	122	2.5000	120	.7500	119	3.2911

		BA9			BA24			BA40		
		df	F	Sig.	df	F	Sig.	df	F	Sig.
PSD95	Between Groups	3	9.922	.000	3	3.789	.013	3	6.440	.001
	Within Groups	111			100			87		
	Total	114			103			90		
ZnT3	Between Groups	3	14.007	.000	3	1.537	.209	3	4.078	.009
	Within Groups	111			101			99		
	Total	114			104			102		
Total CaMKII	Between Groups	3	10.856	.000	3	8.462	.000	3	1.094	.355
	Within Groups	118			115			115		
	Total	121			118			118		
Phospho CaMKII	Between Groups	3	12.790	.000	3	2.072	.108	3	7.559	.000
	Within Groups	118			116			115		
	Total	121			119			118		

Bonferroni

Dependent Variable			BA9 Sig.	BA24 Sig.	BA40 Sig.
PSD95	Control	PDD	.000	1.000	1.000
		DLB	.000	.587	.059
		AD	.571	.036	.022
ZnT3	Control	PDD	.000	.552	.312
		DLB	.000	.426	.007
		AD	.001	.415	.087
Total CaMKII	Control	PDD	.000	.353	1.000
		DLB	1.000	.000	1.000
		AD	.080	.095	.517
PhosphoCaMKII	Control	PDD	.001	1.000	1.000
		DLB	1.000	1.000	.004
		AD	.000	.607	.010