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The Role of -synuclein in the Pathological Mechanisms of Dementia with Lewy Bodies

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The role of β-synuclein in the pathological mechanisms of Dementia with Lewy bodies

by

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Abstract

The role of β-synuclein in the pathological mechanisms of Dementia with Lewy bodies

Dementia with Lewy bodies (DLB) is the second most prevalent neurodegenerative dementia following Alzheimer's disease (AD). DLB neuropathology is characterised by Lewy bodies (LBs) and Lewy neurites (LNs) resulting from the modification and aggregation of α-synuclein. The presence of small pre-synaptic accumulations of α-synuclein lead to structural synaptic alterations that may precede neuronal demise. Family member β-synuclein does not readily aggregate and has been shown to prevent α-synuclein aggregation in vitro and in vivo and regional changes in β-synuclein levels has been observed in the brains of DLB patients.

α-synuclein pathology is found in the limbic and cortical regions of the DLB brain producing fluctuating cognitive impairment, visual hallucinations and extrapyramidal motor disturbances. In order to examine whether regional changes in β-synuclein influence the course of DLB, examination of protein levels of α-synuclein and β-synuclein in the frontal cortex, occipital cortex and hippocampus of patients with DLB and age-matched controls was performed.

Evidence is provided here for a neuronal increase of β-synuclein within the frontal cortex and a decrease in occipital cortex of DLB patients, both regions see similar levels of oligomeric α-synuclein. Further examination of key pre-synaptic SNARE proteins reveal an increase of VAMP2 in the frontal cortex and a decrease of VAMP2 and SNAP25 in the occipital cortex. Autophagy markers LC3-II and p62 were also increased in the frontal cortex where an increase in β-synuclein was identified in DLB brains and in vitro overexpression of β-synuclein attenuates the autophagy flux.

Collectively, this data suggests that β-synuclein changes in the DLB cortex are regionally distinct and the possibility that β-synuclein may exacerbate neuronal dysfunction by influencing protein degradation pathways, cannot be excluded. This data highlights the possibility of a dual role for β-synuclein that may be both protective and antagonistic, advocating caution when considering the use of β-synuclein as a potential therapeutic.

Abbreviations

AD	Alzheimer's Disease
CMA	Chaperone Mediated Autophagy
CTD	Carboxyl Terminal Domain
DLB	Dementia with Lewy Body
FTD	Frontotemporal Dementia
GC	Glucocerebrosidase
LAMP	Lysosome Associated Membrane Protein
LB	Lewy Body
LC3	Microtubule-associated protein 1A/1B Light Chain 3B
LIMP2	Lysosome Membrane Protein 2
LN	Lewy Neurite
NAC	Non Amyloid Component
NACP	Non Amyloid Component Precursor
NFT	Neurofibrillary Tangles
NTD	amino-Terminal Domain
PD	Parkinson's Disease
PDD	Parkinson's with Dementia
pDLB	Pure Dementia with Lewy Bodies
PNS	Peripheral Nervous System
PTM	Post Translational Modifications
SNAP25	Synaptosome Associated Protein
SNARE	Soluble N-ethylmaleimide sensitive factor Attachment protein Receptor
TMR	Trans Membrane Region
UB	Ubiquitin
UBL	Ubiquitin Like
UPS	Ubiquitin Proteasome System
VAMP2	Vesicle Associated Membrane Protein2

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CHAPTER 1: INTRODUCTION

1.1. Dementia with Lewy bodies

Neurodegenerative conditions are predicted by the World Health Organisation (WHO) to become the world's leading cause of death by 2040 [1]. This class of disease constitutes a group of progressively debilitating conditions with unique disease-specific profiles, united by the selective loss of distinctive neuronal groups.

1.1.1. Neurodegenerative Dementias Epidemiology

Neurodegenerative Dementias represent a class of pathologies characterised by varying degrees of, but inexorably, the progressive decline in cognitive functions such that there is interference in an individual's ability to perform everyday duties, impacting on their social function and/or their capacity to perform usual occupational tasks [2].

Dementia has emerged as an epidemic with aging being the predominant risk factor. By 2050, the number of people aged ≥60 years will have increased by 1.25 billion, accounting for approximately 22% of the total global population, with 79% living in less developed regions [3]. Whilst the observed and projected increase in the number of people affected by dementia has largely been explained by the increase in population longevity, specifically in the developing world, dementia per se is not a natural part of the aging process.

Those affected by neurodegenerative dementias are principally aged 65 years and over with early-onset dementia accounting for only 2-5% of all cases, furthermore the prevalence nearly doubles with every additional 5 years of age following the age of

65 [4] underscoring an increase in an age-related risk of developing neurodegeneration, in parallel with an increase in longevity.

The most common forms of dementia include Alzheimer's disease (AD), Lewy body dementia (DLB), frontotemporal dementia (FTD) and vascular dementia [5]. Both AD and DLB continue to be the leading cause of degenerative dementia in the elderly population (Figure 1).

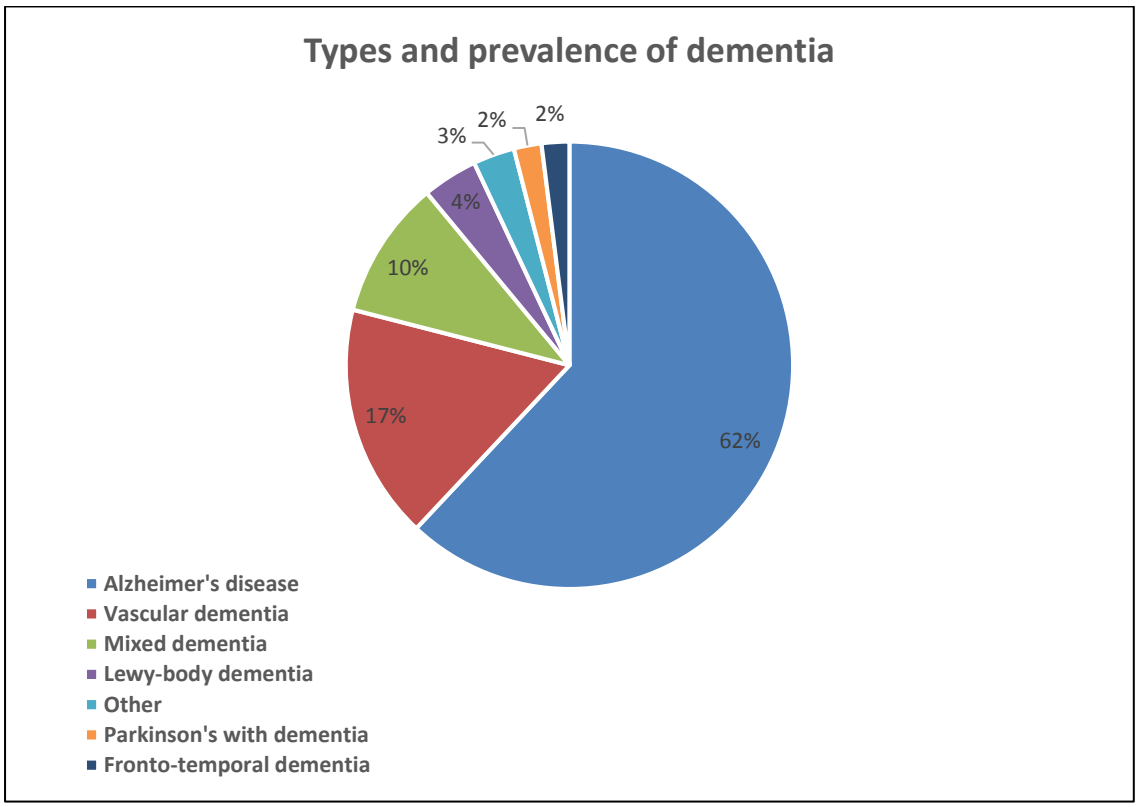

Figure 1 - Dementia subtypes and prevalence

Representative data from the Alzheimer's society's (UK) envisaged proportions of the subtypes of dementia and their prevalence in the UK alone. Adapted from (https://www.alzheimers.org.uk/info/20091/position_statements/93/demography), published September 2014.

In 2015 WHO reported 47.5 million people were afflicted worldwide by dementia, increasing from 35.6 million people in 2012 (WHO April 2012) and cases are predicted to rise by 7.7 million each year. It has been forecast that by 2050 the worldwide prevalence of dementia will reach 137.5 million [6]. In the UK alone, 850,000 people are affected, this bestows a substantial burden on the economy with the cost of health care in the region of £26 billion, annually. (https://www.mrc.ac.uk/research/spotlights/neurodegenerative-diseases-dementia/).

The broad-spectrum of dementia produces a gender bias with a predisposition towards females, 61% of dementia cases are seen in the female population when compared to 39% of males. This is a consistent observation that could be explained by the protracted longevity in females when compared to males. (http://www.dementiaconsortium.org/dementia-facts/).

A systematic review by Prince et al. [5] contemplates the global prevalence of dementia and identifies a higher incidence in Latin-America and lowest in Sub-Saharan Africa with the greater proportion of dementia cases being affiliated with low-middle range incomes. To corroborate this, more recent population based studies on “high-income” countries have contradicted previous projections regarding dementia prevalence, indicating a decline in the age-associated risk of dementia. This has been attributed to various factors, largely surrounding the fact that higher levels of education and advances in treatment and diagnostics may lead to the early intervention of dementia indicators; these include cardiovascular risk factors such as obesity and diabetes [7]. A recent UK based study on dementia has reported a 20% decrease in dementia incidence over the last two decades in males [8]. If the previous projections are proving variable, then it is possible that the predicted figures

may be regionally modulated in accordance with the scope of social health care support available and attainable by the general population.

1.1.2. Dementia with Lewy bodies

Population based studies show that Dementia with Lewy bodies (DLB) accounts for approximately 4% of all dementias [9]. DLB is the second most common form of neurodegenerative dementia after AD [10]. In contrast to general dementia demographics, DLB predominantly affects the male population [11] and symptom onset is usually after the 5th decade of life [12]. Diagnostically, DLB is associated with three predominant clinical presentations: cognitive fluctuation, extra pyramidal motor symptoms that present as parkinsonism [13] and visual hallucinations [14]. A single identified core symptom provides a possible diagnosis of DLB (Table 1) [15] whilst two of these presentations provide a probable diagnosis of DLB - it is possible to have two core features or one core characteristic in addition to suggestive features.

Central Characteristic of DLB	❖ The progressive cognitive decline such that an individual is no longer able to undertake conventional social or occupational functions
Core characteristic	❖ Fluctuating cognitive impairments o Likely, prominent alterations in alertness and attention & visual-spatial awareness ❖ Visual hallucinations ❖ Spontaneous Parkinsonism features ❖ REM sleep behaviour disorder
Suggestive or supportive characteristics	❖ Unexplained transient loss of consciousness ❖ Falls ❖ Autonomic dysfunction ❖ Depression ❖ Delusions ❖ Hallucinations in other modes

Table 1 - Characteristics to support a DLB diagnosis:

Table of central, core and suggestive characteristics that support a diagnosis of DLB (adapted from [15] [16] & The Lewy Body Dementia Association (LBA) (http://www.lbda.org/content/dlb-and-pdd-diagnostic-criteria).

1.1.3. DLB symptoms

1.1.3.1. DLB Core Symptoms – Cognitive

The underlying progressive cognitive decline in DLB may initiate as early as 55 years and be undetected for many years. The decline in cognitive functions is not dissimilar to those observed in AD although the deterioration of these events can proceed more rapidly in DLB (reviewed by [17]). An abating memory may be one of the earlier features of DLB with an impairment in the ability to encode new memories and a decline in the capacity to retrieve long term memories [18]; however, dementia is not a requisite for DLB diagnosis in the early stages. Attention and alertness are primary features that often represent changes in cognition and fluctuations [19] and may be identified initially by family members or colleagues. Visual-spatial awareness can also be disturbed in DLB [20] the manifestation of which include the inability to navigate otherwise familiar locations. Further, executive functions, including problem-solving and reasoning are affected, as is language and judgment [21]. These cognitive manifestations can vary dramatically, fluctuating between little evidence of cognitive dysfunction to incomprehension and confusion in a short duration; it is this fluctuating pattern of changes that is the defining feature of a DLB diagnosis.

1.1.3.2. DLB Core Symptoms – Hallucinations

Symptoms of DLB vary between patients although often early dementia will present in association with visual hallucinations [22]. Tiraboschi et al. [22] indicated that visual hallucinations were the most efficient predictor of a clinical diagnosis of DLB in contrast to AD. The visual hallucinations are typically well formed, recurring and complex in detail. They will involve the incorrect visual awareness of moving objects

and complex scenarios of people and objects that are non-existent [23]. It is noteworthy that hallucinations in DLB may also be audible although, this is relatively rare.

1.1.3.3. DLB Core Symptoms - Parkinsonism motor syndrome

The extrapyramidal symptoms seen in PD are also common in DLB with 25% of patients displaying Parkinsonism at disease onset, consistent with the loss of dopaminergic neurons in the substantia nigra [24]. Conversely, 25% of DLB patients will not develop Parkinsonism features during the pathological course, reflected by significantly less dopaminergic neuronal loss in these patients [25]. Bradykinesia, postural instability, rigidity and a resting tremor are representative of the predominant features of PD. These characteristics may appear in DLB though the resting tremor exists to a lesser extent than is observed in PD. Typically, in DLB, dementia will present prior to the advancement of motor symptoms, but motor symptoms could follow the disease progression within 1 year [2]. In view of the symptom similarity between DLB and PD/PDD, correct diagnosis requires the employment of The Unified PD Rating Scale (UPDRS), an assessment of the motor features in DLB. It is universally accepted that a DLB diagnosis acquiesces when cognitive dysfunction precedes parkinsonism or appears within a year [26]. A PDD diagnosis required dementia symptom to present >1year following motor dysfunction, [27]. In this regard, PDD differs only from DLB in the temporal course of the pathology. In DLB dementia precedes Parkinsonism features and in PDD Parkinsonism precedes dementia.

1.1.3.3. DLB Core Symptoms – Rapid-eye-movement (REM) sleep behaviour disorder (RBD)

REM-RBD is associated with vivid dreams with often simple or complex motor behaviour during the REM sleep [28] such that injuries may occur from the enactment of dreams, leading to kicking or punching.

1.1.3.4. DLB Supportive features

Supportive features include unexplained transient loss of consciousness and falls, delirium and depression, in addition to a decreased sense of smell, increased saliva production and intestinal changes (reviewed in [26]). Non-specific non-cognitive symptoms may precede the appearance of memory and other cognitive DLB related characteristics, by some years. Rapid eye movement sleep behaviour disorder (RBD) may be clinically representative of PD Braak stage 2 as RBD cases are known to progress and develop DLB or other α-synucleinopathies [29]. (Figure 3: Diagram of Braak staging). Whilst these symptoms alone would not necessarily be an immediate indicator of Lewy body disease; they could be suggestive of prodromal Lewy body disease and therefore, an early method of detection.

The variable symptoms between patients frequently leads to the mis-diagnosis of DLB. There presently is not an identified biomarker to enable dementia-specific diagnosis, so it is not unexpected that final clinical diagnosis will be made post-mortem.

1.1.4. The neuropathology of DLB

The neuropathology associated with DLB is characterised by α-synuclein protein accumulations that present in the form of Lewy bodies (LBs) and Lewy neurites (LNs) [30]. Whilst LBs were first observed over a century ago, the main component

of these structures was not identified as α-synuclein until 1997 [31]. These insoluble α-synuclein aggregates are found extensively distributed throughout the brain in the form of intracytoplasmic neuronal inclusions, in the neurite processes as LNs or as smaller aggregates located at pre-synaptic terminals; a defining pathological feature that is also exhibited in PD and PDD [32]. Furthermore, multiple system atrophy (MSA) is characterised by α-synuclein deposits located in glial cells [33]. The temporal expression and distribution patterns of these diseases are unique to each disease. Regardless of the differential expression pattern of the α-synuclein pathological spread, due to the common representation of LBs and LNs, these neurodegenerative pathologies are considered α-synucleinopathies, by virtue of the predominance of the α-synuclein aggregating protein in the form of LBs and LNs.

Dr. Fritz Jakob Heinrich Lewy (1885 – 1950, Friedrich Lewy) first described proteinaceous aggregates in 1912 when he published his initial findings from the postmortem brain examination of PD patients [34]. Lewy described these insoluble structures in the dorsal motor nucleus of the vagus nerve, the basal nucleus of Meynert and the lateral and periventricular nucleus of the thalamus. In 1919 similar inclusions were identified in the substantia nigra of PD patients by Tretiakoff and subsequently named as Lewy bodies [35]. It is well established that the substania nigra pars compacta of the basal ganglia is significantly affected by LB pathology in PD [36], with the specific and selective targeting of the dopamine producing neurons in this region [24]. Studies of PD and the substantia nigra pars compacta has enabled the advancement of the understanding of pathology in α-synucleinopathies. In combination with the findings of Lewy and the subsequent evidence, presence of inclusions that develop into “thread-like” LNs in the neuronal processes and neuronal

intracytoplasmic LBs underpin a clinic-pathological post mortem diagnosis of DLB or PD.

Those patients with PD follow a more specific pattern of LB distribution, as described above [37], in DLB, LBs are diffusely distributed throughout the neocortical regions [38] including the frontal, parietal, occipital and temporal cortex and in subcortical regions such as the substania nigra, the locus coeruleus [39] and the paralimbic regions [40].

Additional neuropathological characteristics of DLB include spongiform changes that are representative of fine neuropil microvacuolations [41]. It is observed that this spongiosis-effect is more severe in AD with concurrent LB pathology than in pure AD [42] and that the spongiosis distribution pattern corresponds to the LBs that are present, in particular in relation to the superior and inferior temporal cortex, amygdala and entorhinal cortex [43].

1.1.4.1. Lewy body structure

Lewy bodies are intracytoplasmic, eosinophilic structures, characteristically between 8μM and 30μM in diameter [44]. The morphology of the LBs is location dependent, presenting as classical or cortical LB depending on whether they are identified in the brainstem region or cortex, respectively [45]. Classical LBs typically are located in the brainstem and diencephalon regions, including frequently the substantia nigra, locus coeruleus, hypothalamus, dorsal motor nucleus of the vagus nerve and median raphe nucleus [46]. Spherical and uniform in shape, classical LBs have a dense core and an outer halo-like formation [47]; the dense core is encircled by radially positioned filaments [48]. Cortical LBs are more irregular and unstructured in appearance, the core of which is less distinctive with the absence of a peripheral

halo. Frequently it has been shown that cortical LBs will displace the nucleus of the cell [31, 49] however, from an ultrastructural perspective, cortical LBs have a similar filamentous morphology to those LBs seen in the brain stem. Insoluble α-synuclein filaments exhibit either a straight or a twisted conformation of approximately 5-10nm in diameter [49], they are 200-600nm in length with the core of the filament covering over 70 amino acids (reviewed in [50]; this conformation drives a typical beta-sheet formation [51], frequently associated with protein aggregate formations in neurodegenerative diseases.

1.1.4.2. Lewy body and Lewy neurite composition

LNs are dystrophic processes with an identical immunostaining profile as LBs [31]. The structural arrangement of both LBs and LNs is a complex formation, composed largely of the aggregated and fibrillary form of insoluble α-synuclein. These aggregates can be identified by α-synuclein immuno-staining, in addition there are over 70 other components of Lewy bodies [47] that can be further categorised to include: structural proteins, degradation pathway associated proteins and cell signalling molecules, providing evidence of multiple pathways potentially involved in the complexities of LB formation. The ubiquitin protein (UB), related to the ubiquitin-proteasome system (UPS), is readily used as a positive immuno-staining marker for the presence of LBs; its functional involvement with protein degradation pathways indicates defects in aggregate clearance in LB diseases.

1.1.4.3. Pale bodies

The presence of pale bodies is also associated with LB pathology; these cytoplasmic structures often co-occur in neurons with LBs. Ultra-structural examination shows that they contain vesicular and granular matter, dispersed amongst disorganised filamentous structures. The filamentous structures present in pale bodies are

identical to those observed in true Lewy bodies [47]. There is a strong correlation between the number of pale bodies and the UB-positive LBs in PD and DLB [52]. This correlation may indicate the possibility that pale bodies may represent pre-LB structures.

1.1.4.4. The process of Lewy body formation

Lewy bodies are critical to the neuropathology of DLB, whether this function is accountable for the pathological drive of neurodegeneration or whether the formation of Lewy bodies is a neuroprotective mechanism in the face of toxic insults, is still debatable. In contrast to its presence in LBs, under normal physiological conditions, α-synuclein is a soluble protein abundantly distributed throughout the brain with the predisposition for pre-synaptic terminals; where it is also associated with synaptic vesicles [53]. The cytoplasmic appearance of α-synuclein points to an early indicator of the erroneous behaviour of the protein. Increasing evidence suggests that the oligomeric form of α-synuclein is the toxic intermediate [54] and it is possible that these formations are occurring pre-synaptically. A common hypothesis for the formation of Lewy bodies is that monomeric α-synuclein merges to form oligomeric species of the protein, protofibrils [55] (To be discussed – Figure 8). In the monomeric or protofibril state, α-synuclein may still be soluble [56], although protofibrils can act as a template for nucleation, enabling the multimerisation of further protofibrils that proceed to larger fibril formations. The development of classical LBs has been described using α-synuclein immunoreactivity to map the aggregation process in the cytoplasm [47, 57] (Figure 2). Stage 1 presents as a diffuse, speckled pattern of α-synuclein staining in the neuronal cytoplasm (A). Stage 2 is more moderately intense but displays an irregular pattern (B). A pale body appears in stage 3, α-synuclein immunoreactivity detected a darker outer layer and a

less intensely stained core, the shape is still irregular (C). By stage 4 a ring like structure with the distinct halo and core associated with classical Lewy bodies is evident (D). Together, this demonstrated the temporal pattern of intraneuronal cytoplasmic α-synuclein aggregation leading to Lewy body formation and maturation.

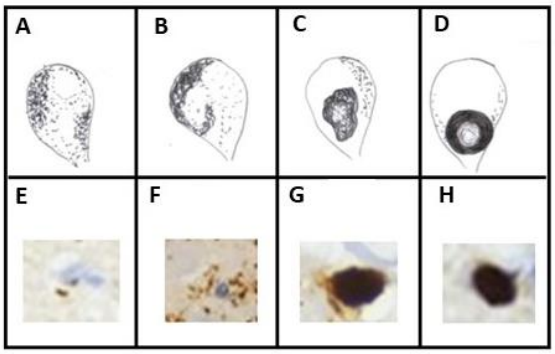

Figure 2 - LB formation and maturation

Hand drawn representations of LB formation and maturation stages (by Tracey Evans), as described by Wakabayashi, 1998. α-synuclein immunoreactivity of classical LB formation stage 1 (A): diffuse cytoplasmic staining; stage 2 (B): irregular pattern of staining; stage 3 (C): more discrete staining corresponding with a pale body and stage 4 (D). Cortical LB representation of the different stages (E – F, respectively). Immunoreactivity detected by 5G4 for oligomeric α-synuclein in the DLB brain.

The cytoplasmic and diffuse presence of α-synuclein that precedes the arrival of a pale body structure [57] and the subsequent LB formation is suggestive of an organised pathway in LB formation. Whether all Lewy bodies descend from pale bodies is not fully established, but they appear in some cases to provide the material on the basis of which the larger and more structured Lewy body may derive [58]. One considers the concept that all Lewy bodies may derive from pale bodies, pale bodies have a disorganised arrangement of α-synuclein, this is a stark contrast to the

concluding phase of LB formation; a structure that appears to be well structured [48]. This points to the possibility that the pale body is representative of early, potentially non-specific methods and attempts by multiple pathways to remove α-synuclein. This may provide the appearance of a more dis-organised structure, which all the same has sought to segregate and localise the aggregating proteins. In the event that these attempts are defeated, Lewy bodies may represent the structure that envelops the pathological species and bind the protein complexes into a more orderly structure to prevent further toxicity.

1.1.5. The temporal progression of Lewy body pathology - Braak staging

Lewy bodies in the midbrain are usually associated with idiopathic PD and are essential for a clinical-pathological diagnosis of PD. In DLB patients however the LB pattern of accumulation involves cortical regions [27] with pathology in the cerebral cortex affecting layers V-VI initially, advancement continues into layer III and ultimately layer II. This distribution pattern has been correlated with symptom severity [59]. In an attempt to provide a unifying pathological description of LB pathology in the brains of PD patients Braak & Braak, [37] proposed a stage scoring system. According to the proposed model for LB pathology in PD, specific neuronal phenotypes and regions of the brain are affected by deposits of α-synuclein in a predictable and temporal manner. The direction of the pathology is caudo-rostral over six stages from the lower brain stem to the neocortical structures (Figure 3), each stage refers to the anatomical location and progression of the pathology.

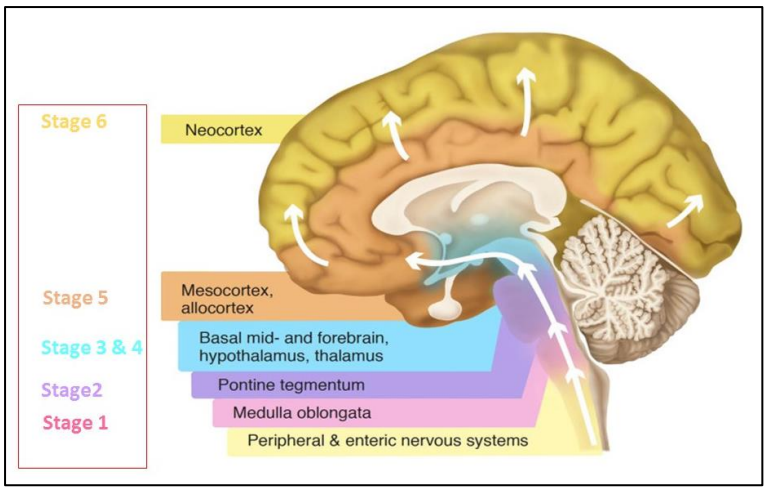

Figure 3 - Lewy body Braak staging

The image has been adapted from [60], the original is available under common creative license.

Stage 1 LB and LN appearance in the brain stem regions: the dorsal motor nucleus of the medulla oblongata and on occasions the intermediate reticular zone.

Stage 2: LBs and LNs in the caudal raphe nuclei, gigantocellular reticular nucleus and the melanin rich cells of the coeruleus-subcoeruleus complex.

Stage 3: LBs present in the midbrain especially, the melanin-rich neurons of the substantia nigra pars compacta. The magnocellular nuclei of the basal forebrain are also affected. Subcortical and cortical regions associated with the anterior olfactory bulb may see mild LB and LN pathology, but there are no LBs in the non-olfactory cortical areas in stage 3. Although there is a predilection for melano-neurons of the substantia nigra to be affected by pathology in stage 3, depigmentation is not yet detectable by the human eye.

Stage 4: There is a devastating loss of the melanin rich projecting neurons which may now be observed macroscopically. Projection neurons in the pedunculopontine

tegmental nucleus are severely affected and there is pathological spread into the basal forebrain, the paralimbic cortex and the allocortex. Stage 4 also sees the involvement of the temporal mesocortex, providing a point of access to the neocortex.

Stage 5 and 6 exhibits a further increase in the extent of LB and LN pathology in already affected regions.

Stage 5: The olfactory regions are severely afflicted and via the temporal mesocortex, the connecting sensory association region of the neocortex sees the involvement of the higher order sensory neurons. At this stage, the inclusion of some pre-frontal regions may be established

Stage 6: The progression at this stage will involve almost the entire neocortex.

Autopsy examination will confirm a definitive diagnosis between DLB, PD, PDD or AD based on the clinical picture of the patient, neuropathological observations correlated with the Braak staging and the presence of aggregating protein in target areas of the brain [61]. Whilst the Braak hypothesis is a powerful tool that enables the precise clinical diagnosis post-mortem, it is not universally accepted. α-synuclein deposits have been described in healthy elderly patients with no α-synucleinopathy-like symptoms or other neurodegenerative features [62]. Further, a peripheral rostrocaudal gradient of phosphorylation α-synuclein has been observed in PD patients [63] in contrast to the caudo-rostral pathway determined. Whilst a robust and reproducible diagnostic tool is required, inter-patient variability may see differing clinical and post-mortem outcomes, this is largely unavoidable until the precise mechanisms that initiate the pathological spread are understood.

1.1.6. DLB with concurrent AD-related pathology

The challenges facing a clinical diagnosis of DLB relate to the great crossover in pathology between DLB and AD. The neuronal presence of Lewy bodies supports a diagnosis of DLB and AD is characterised by neuritic amyloid beta plaques and neurofibrillary tangles (NFTs) although, DLB and AD may share neuropathological features such as Lewy bodies, amyloid plaques and NFTs collectively. DLB occurs in isolation as pure DLB (pDLB) and more commonly it presents with concurrent AD-type pathology [64]. There are conflicting reports on the prevalence of these conditions, although it is accepted that pDLB is less frequent in occurrence (reviewed by [65]).

Amyloid plaques are frequent in DLB and PDD; the incidence is lower in PDD and as many as 80% of DLB patients may exhibit amyloid pathology. Moreover, in up to 30% of AD cases Lewy bodies are present [66]. The mixed pathology associated with these neurodegenerative conditions leads to discrepancies in accurate clinical diagnoses which are not corroborated until an autopsy is undertaken; in this regard, it is reported that there is an astounding error rate of between 34% - 65% in correct diagnosis of DLB [67].

The knowledge that DLB and AD arise in the pure form and concurrently with amyloid-β plaques, α-synuclein and tau pathology (Figure 4) further complicates the multiplex field of dementia and fosters the questions of the driving force behind the neuropathology. Knowledge of the primary proteins in the initiation of the pathological process may expose the more toxic aggregates. The toxicity of these aggregating proteins may be revealed by life expectancy from diagnosis: the life expectancy in DLB is approximately 8 years from onset and approximately 8-12

years in AD [68]. It could potentially be argued that the presence of aggregating α-synuclein may lead to neuronal demise more rapidly.

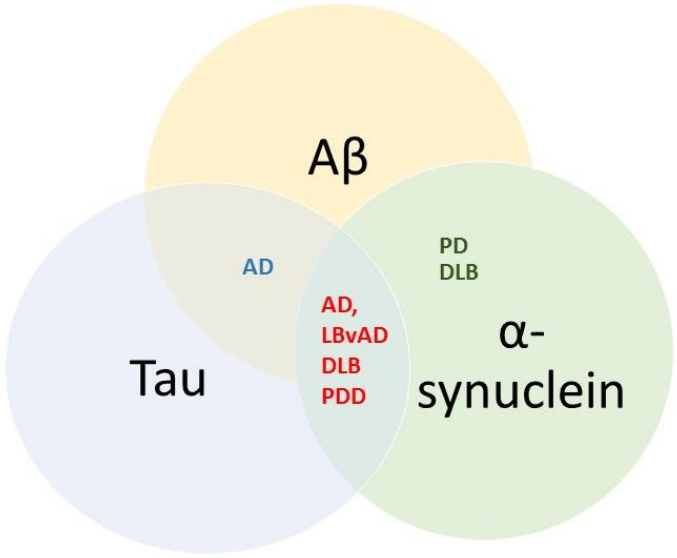

Figure 4 - α-synucleinopathies and AD share neuropathological characteristics

DLB, AD, LBvAD (Lewy body variant of AD) and PDD share Aβ, Tau and α-synuclein pathology. Adapted from [69].

The symptomatic similarities between DLB and AD relate to cognitive function and memory although, DLB have more pronounced attentional deficits, mild memory visual-spatial deficits [70] and executive function disparities [71] early in the disease course. Extrapyramidal signs and the psychiatric symptoms: hallucinations, depression, delusions & behavioural symptoms are also more pronounced in DLB when compared to AD, in the early stages, later in the disease course the disparity between the two diseases is less defined [71].

1.1.7. Treatment

There is no known cure for DLB although medications are prescribed to alleviate some of the symptoms. The management of DLB appears to be more complex than many other neurodegenerative diseases due to the convolution of symptoms that include: cognitive, neuropsychiatric, movement, autonomic, and sleep disparities (reviewed in [72]). Individual symptom categories require unique and specific treatments. DLB patients can have a high responsiveness to cholinesterase inhibitor (CHEI) treatment; these may be used to treat fluctuating cognitive functions, neuropsychiatric symptoms and visual hallucinations [2]. Neuroleptic drugs are prescribed to address some of the psychiatric symptoms present in DLB such as depression, however, due to DLB patients exhibiting an increase in sensitivity to neuroleptics, they are administered with caution [73]. Levodopa (L-Dopa) is used to treat the motor features associated with Parkinsonism in PD, this is the most successful treatment for extrapyramidal features although only one-third of DLB patients are as responsive to the treatment [74].

Collectively, DLB is a multifaceted, complex disease that reaches multiple brain regions. Symptoms may vary from patient-to-patient and a conclusive diagnosis is not able to be provided until post-mortem. The identification of disease specific bio-markers would enable a rapid diagnosis and the administration of therapeutics, early in the course of the pathology in order to manage disease symptoms.

1.2. The Synucleins

The synuclein family comprise of α-synuclein [75], β-synuclein [76] and γ-synuclein [77], they are small natively unfolded monomeric proteins associated with neurodegenerative conditions and cancer. α-synuclein and β-synuclein are primarily found in the pre-synaptic nerve terminals of the central nervous system (CNS) [78]. Conversely, γ-synuclein, although found in the CNS, is mainly associated with the peripheral nervous system (PNS) [79] and is linked to ovarian [80] and breast cancer [81]. For the purpose of this study, γ-synuclein will not be described in relation to DLB.

1.2.1. The history of α-synuclein and β-synuclein protein identification

The synuclein protein sequence was originally identified in the *Torpedo California* [82] with proteins of different molecular weights revealed. The regional distribution was limited to the nervous system only, found predominantly localised to the nuclear envelope and at pre-synaptic terminals.

Subsequently, the entire sequence of an amyloid preparation isolated from AD patients was analysed. In addition to the presence of amyloid-β, a 140-amino acid sequence was characterised and tentatively named the non-amyloid-β component precursor (NACP) and a fragment, of the full 140-amino acid sequence was named the non-amyloid-β component (NAC) [83]. The NAC region was predisposed to form β-sheet structures that associated with amyloid plaques in AD patients and the NACP was re-named α-synuclein. The protein previously known as phospho-neuroprotein 14 (PNP14) was renamed β-synuclein [76] [78].

1.2.2. The synuclein genes

1.2.2.1. SNCA

The α-synuclein gene is located on the long arm of chromosome 4: 4q21.3 – 4q22 [84]. There are five coding exons for the full-length α-synuclein protein [85], total transcript length is 3041base pairs (bp) [86] with at least three further splice variants of the gene: SNCA-98 [87], SNCA112 and SNCA-126 [88]. Transcript variants SNCA-112 and SNCA-126 have “in-frame deletions” of exon 3 and 5 respectively, in contrast, SNCA-98 lacks exon 3 and 5 [87]. The SNCA-126 transcript has an interruption in the N terminal domain (NTD) and it is thought this isoform reduces inherent aggregation propensity; conversely, SNCA-122 has a shorter C-terminal domain (CTD) than the full-length transcript and is more aggregation prone. The shortest transcript SNCA-98 has lower levels of expression when compared to the other isoforms [87]. The differential α-synuclein isoforms have been implicated in the pathogenesis of DLB with increased expression of SNCA-112 in the prefrontal cortices of DLB patients in parallel to a reduction in SNCA-140 [89] and SNCA-126 transcript [90]. The SNCA-112 transcript with a shortened, disordered CTD appears to favour aggregation propensity. Alternative splicing provides the platform for regulating transcriptional diversity, however, the physiological drive behind changes in the α-synuclein isoforms are not understood.

Transcription factors mediating the SNCA gene expression have not been fully elucidated. Inhibition of poly-(ADP-ribose) transferase/polymerase-1 (PARP1) via the REP1 region, a polymorphic microsatellite region situated approximately 10 kb upstream of the SNCA gene, leads to an increase in endogenous levels of α-synuclein [91]. Variations in the size of a dinucleotide repeat region in REP1 has

been linked to increased risk of PD [92], the increased risk was in parallel to an expansion of the repeat region. Furthermore, in vitro analysis has shown that a greater length of the repeat region can lead to a three-fold increase in expression of the SNCA gene [93]. This may in part be responsible for driving the over expression of α-synuclein leading in effect to an increase in gene-dosage and increase in the risk of protein aggregation. Promoter regions within the SNCA gene have been identified as potential transcriptional factors binding sites such as GATA [94] and zinc finger-and scan domain containing (ZSCAN) [95]; α-synuclein transcriptional regulatory factors are still under investigation. Not surprisingly, a GWAS study identified association at the SNCA locus in DLB, PD and PDD [96] [97].

1.2.2.2. SNCB

The β-synuclein gene (SNCB) is encoded by 7 exons and localised to the long arm of chromosome 5 (5q35.2) [98]. Two mRNA transcript variants of the SNCB gene are known; transcript 1 (SNCBtv1) includes exon 2 and the mRNA is 1594bp in length; the second transcript variant (SNCBtv2) lacks exon 2 and the mRNA size is 1437bp. Exon 2 is located within the untranslated 5' region producing an identical translation of the protein [98]. The SNCBtv2 transcript appears to be the predominant form and is expressed primarily in the frontal and temporal cortex and the caudate nucleus [89]. Transcriptional control of β-synuclein expression is not established, MTF-1, NRF2 and ZSCAN21 may regulate β-synuclein in vitro [99] although, supporting evidence for transcriptional regulation by these factors has not yet been established in the human brain. Other regulatory mechanisms may involve α-synuclein, this is based on of a potential inverse relationship present between β-synuclein and α-synuclein; supported by the findings that transcription factor, ZSCAN21, induces an increase in the levels of β-synuclein while repressing levels of α-synuclein [99]; thus,

indicative of a potential reciprocal synuclein relationship between the synuclein; in contrast, MTF-1 drives the expression of β-synuclein but does not affect the levels of α-synuclein [1].

Co-operative regulatory mechanisms are further corroborated by the finding that β-synuclein may act in a compensatory anti-apoptotic manner in relation to the loss of wild type α-synuclein. In vitro, both α-synuclein [100] and β-synuclein [101] have been shown to have anti-apoptotic properties. Wild type, α-synuclein is able to prevent apoptosis in response to toxic insults, and is associated with a reduction of caspase 3 [102] and p53 [100]; in contrast, this inhibitory effect of apoptosis is lost in the presence of PD associated mutant α-synuclein, A53T and in response to dopamine derivative 6-hydroxydopamine. Importantly, β-synuclein has been shown to reduce caspase 3 activity and p53 expression levels; furthermore, a reduction in p53 transcriptional activity in the presence of 6-hydroxydopamine is observed [101]. This provides evidence for a functional compensatory role such that the loss of function of α-synuclein in PD-type models can be compensated for by β-synuclein expression; thus, leading to the concept that there is inter-protein cross talk under toxic conditions.

1.2.3. Synuclein protein structure

1.2.3.1. α-synuclein

α-synuclein is a protein of 140 amino acids [82] (Figure 5) with a molecular weight of 14460 Da. The N-terminal domain (NTD) region of α-synuclein is crucial for membrane interactions with a partiality for alpha-helical conformations when interacting with lipids [103], mirroring the behaviour of apolipoproteins [104] and suggesting a role for α-synuclein as a lipid-binding protein. The NTD region contains

seven 11 amino acid imperfect repeats, each of which contains a highly conserved hexameric consensus sequence: KTKEGV [105]. The size of the repeats in the N terminal domain enables the protein to make three turns of a helix, conferring membrane binding capacity through multiple repeats (reviewed in [106]). These amphipathic regions confer binding properties to highly curved membranes [107].

The central NAC region sets α-synuclein apart from the other synuclein family members, it is highly hydrophobic [25] and this region is thought to enable the oligomerisation and fibrillisation of the α-synuclein protein producing, a “face-to-face” β-sheet formation [108]. Deletion of this region or inhibition decreases the aggregation propensity of α-synuclein [109].

The C-terminal domain (CTD) of α-synuclein is acidic, unfolded and disorderly when compared to the NTD and is subject to multiple post translational modifications that are found associated with α-synuclein in aggregates and Lewy bodies [110]. The CTD of α-synuclein has been implicated in the regulation of the nuclear location of the protein and has been shown to interact with histones [111] and double stranded DNA [112]. In addition, the CTD interacts with metal ions [113] and proteins including tau [114]. The CTD region may be able to directly or indirectly interact with the NAC region thereby inhibiting fibrillisation [115] with truncation of the CTD region leading to increased neurotoxicity and the number of α-synuclein inclusion bodies [116]. It is possible that the physiological conformation of the protein is such that the NAC is masked by the CTD region and that occupation of the CTD by other biomolecules alters the conformation exposing part of or all of the NAC region. Under stressful or dis-equilibrated conditions, this may effect a chain of events that leads to the fibrillisation of α-synuclein.

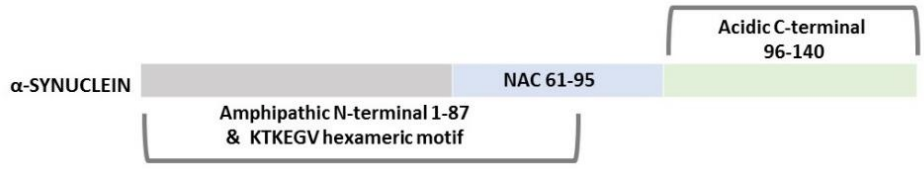

Figure 5 - α-synuclein protein structure

Schematic is based on reviews of the α-synuclein protein structure [117] & [118]

The natively unfolded conformation of α-synuclein protein [119] adopts an α-helical secondary structure associated with the membranes in vitro [120], once dissociated from the membrane the helical conformation is lost and the protein reverts to its native state [121]. It is possible that under normal physiological conditions, α-synuclein transitions between states of being monomeric and unfolded to an α-helical conformation when in association with lipid membranes.

1.2.3.2. β-synuclein

β-synuclein is 134 amino acids in length and under physiological conditions, is a monomeric natively unfolded protein (Figure 6). Like α-synuclein, β-synuclein has seven well conserved imperfect, eleven amino acid repeats, each containing the consensus sequence KTKEGV in the NTD domain, enabling the interaction between β-synuclein and synaptic membranes [122] and acidic phospholipids [123].

β-synuclein has a central hydrophobic region that does not confer fibrillisation propensity like α-synuclein [124] and as with the synuclein family, the CTD is rich in acidic residues [125]

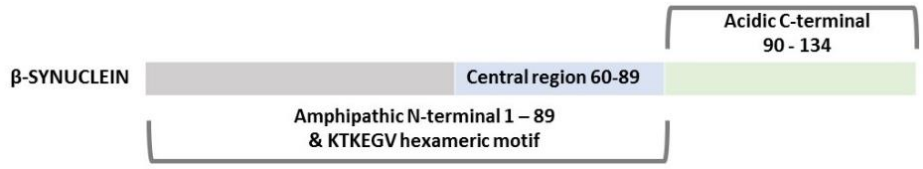

Figure 6 - β-synuclein protein structure

Schematic is based on reviews of the β-synuclein protein structure [118].

1.2.4. Structural similarities of β-synuclein to α-synuclein

α-synuclein and β-synuclein share 78% amino acid sequence homology [126] (Figure 7). 84% homology is seen in the NTD, a highly-conserved region in the synuclein family. It comprises almost half of the synuclein protein's sequence indicating a common biological mechanism among all of the synucleins such as lipid binding [127]. The absence of 11 amino acids in the central domain of β-synuclein that are present in α-synuclein [128] result in an interruption in the alpha-helical region of the β-synuclein protein that may contribute to the reduced ability of β-synuclein to self-aggregate and form fibrils [124]. It is the CTD of β-synuclein sees a greater divergence in homology from α-synuclein [129]. Despite, such similarities in the protein structure, it is thought that the differences that do occur in the amino acid configuration of β-synuclein, enables greater conformation outcomes in its native state when compared to α-synuclein [118].

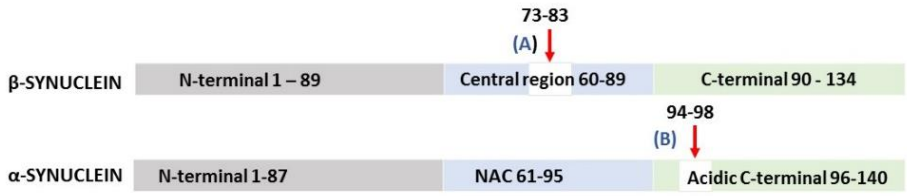

Figure 7 - Synuclein protein structure similarities

Structural similarities between the α-synuclein and β-synuclein protein. (A) region absent in β-synuclein that is present in α-synuclein. (B) Region absent in α-synuclein that is present in β-synuclein.

1.2.5. Regional distribution of the synucleins

1.2.5.1. α-synuclein

α-synuclein is an abundant protein accounting for between 0.5% and 1% of the cytosolic fraction [130] of the human brain and has only been identified in vertebrates [127]. α-synuclein is broadly expressed throughout the CNS but is present in the PNS, including intestinal tissue and the CSF (reviewed by, [131]). The highest regional levels of α-synuclein mRNA expression are observed in the neocortex, hippocampus, thalamus, striatum and cerebellum [130] and in pathological conditions such as MSA (multiple system atrophy), in glia cells [132].

Although α-synuclein was originally identified in association with the nuclear envelope, it has since been shown to be primarily located at pre-synaptic nerve terminals in close proximity to synaptic vesicles [82], with little evidence of dendritic or somata staining or along extra-synaptic sites.

1.2.5.2. β-synuclein

β-synuclein shows a strikingly similar pattern of regional distribution to α-synuclein throughout the human brain, high levels of expression are observed in the hippocampus, amygdala, substantia nigra, caudate nucleus and thalamus (Reviewed by [85]). β-synuclein mRNA is revealed in the neocortex, hippocampus, cerebellum, basal ganglia and substantia nigra; it is the most abundant synuclein in the neocortex and cerebellum; comprising approximately 75-80% of the total synuclein mRNA [133]. β-synuclein also presents with a limited peripheral expression including, in olfactory receptor neurons of olfactory epithelium [134].

In healthy controls, β-synuclein is present in astrocytes [135], α-synuclein is expressed in astrocytes and oligodendrocytes in relation to PD [136], DLB brains [137] and oligodendrocytes in MSA [33] but not the control brains. This relationship is redolent of β-synuclein supporting a possible physiological function in glial cells whilst α-synuclein may be more representative of a pathological presence only.

1.2.6. Physiological role for the synucleins

1.2.6.1. α-synuclein

Despite the abundant presence of α-synuclein in the central nervous system and its predominant feature in neurodegenerative α-synucleinopathies, the role of α-synuclein still remains elusive. Its physiological and pathological roles have been extensively scrutinised providing a plethora of possible functions and although the lipid-binding features go uncontested, there are distinct gaps in the scientific knowledge surrounding the role of α-synuclein at the pre-synaptic terminal.

In development, α-synuclein is one of the last pre-synaptic proteins to localise at developing synapses [138]. α-synuclein has been shown to be expressed after

synaptophysin and localised exclusively to the presynaptic terminals of mature neurons. [139] indicating it does not have a role in synaptogenesis. Moreover, early observations of α-synuclein in zebra finch pointed to a potential role for α-synuclein in regulating synaptic plasticity [104], however, no further evidence to support a role in synaptic plasticity has been identified.

The membrane binding properties of α-synuclein have been identified both in vitro and in vivo. The association of α-synuclein with synaptic vesicles was observed as early as 1991 when it was detected in the presence of cholinergic vesicles of the Torpedo electric organ [122]. Examination of its role at the synapse, in relation to vesicle trafficking, SNARE complex formation and neurotransmitter release has been undertaken to elucidate its physiological role. The knockdown of α-synuclein in hippocampal neurons leads to a reduction in the vesicular pools at the synapse [139] pointing to a role for α-synuclein in maintenance of the pool of vesicles. Human wild type α-synuclein expressed in mice has been shown to negatively affect neurogenesis, by reducing the survival rate of neuronal-committed progenitors in the olfactory bulb and dentate gyrus [140]. Furthermore, a role has been established supporting a chaperoning role for α-synuclein in the formation of the SNARE-complex [141], (this will be discussed at length in Chapter 3). The different conformations that α-synuclein is able to adapt may suggest multiple roles at the pre-synaptic terminal that have yet to be determined.

1.2.6.2. β-synuclein

The abundance and pattern of expression of β-synuclein throughout the human brain [98] and its synapse rich presence [82] does not reveal a physiological role for β-synuclein. β-synuclein expression is observed in the brain [142] and the testes [143] is significantly increased 2-3 weeks postnatally and is located within axonal growth

cones during axonal regeneration following damage [144] indicating a potential role in cellular proliferation. The knockout of the β-synuclein gene in murine models does not lead to any defining changes when compared to the wild type, life span is unchanged, neurotransmitter release is normal, including the size of the pool of vesicles and no abnormalities in long term potentiation [145]. In contrast, triple synuclein knock out mouse models do display changes, the absence of α-synuclein, β-synuclein and γ-synuclein leads to synaptic alterations, age-related neuronal dysfunction in addition to a decreased survival rate [146]. Whilst synaptic density is unchanged in the triple knockout model, the area of the pre-synaptic terminals is reduced in size, rescued by re-introducing α-synuclein alone. The age-related changes and survival may predispose some role for the synucleins that is more relevant in more advanced years as opposed to early in life.

The rescue effect by α-synuclein in the triple knock out model and the lack of obvious phenotype in the β-synuclein or α-synuclein knock out model is reinforcing the possibility of redundancy between the synucleins. It is possible that interplay between the synucleins is responsible for the lack of phenotype observed in a single knockout model, with this compensatory effect lost with deletion of all three synucleins. One must also consider the possibility of a hierarchy effect suggesting an existence of a delicate balance of the synuclein proteins whereby modifications in one may lead to the upregulation/downregulation of another producing a functional compensatory effect under physiological or pathological conditions.

1.2.7. Aggregation of synuclein proteins

1.2.7.1. α-synuclein

The aggregating process of α-synuclein that results in a neurodegenerative phenotype is complex; the triggering factor is usually indeterminable although gene [147] and pathological post translational modifications have been implicated in the initiation of protein fibrillisation such as phosphorylation [110], oxidation [148] and nitration [149]. Protein homeostasis and folding efficiency as forms of quality control in the normal physiological cellular environment are critical and these systems are progressively dysregulated in the pathogenesis of neurodegenerative disease (reviewed in [150]). Once cellular mechanisms that are in place to ensure correct folding and/or removal of the offending protein are breached, the protein creates a neurotoxic environment that can lead to dysfunction of routine cellular functions. The consequence of misfolding proteins that bring about protein aggregation is the potential for these proteins to fibrillise and to provide a “seed” [151] for the further propagation of pathology (Figure 8).

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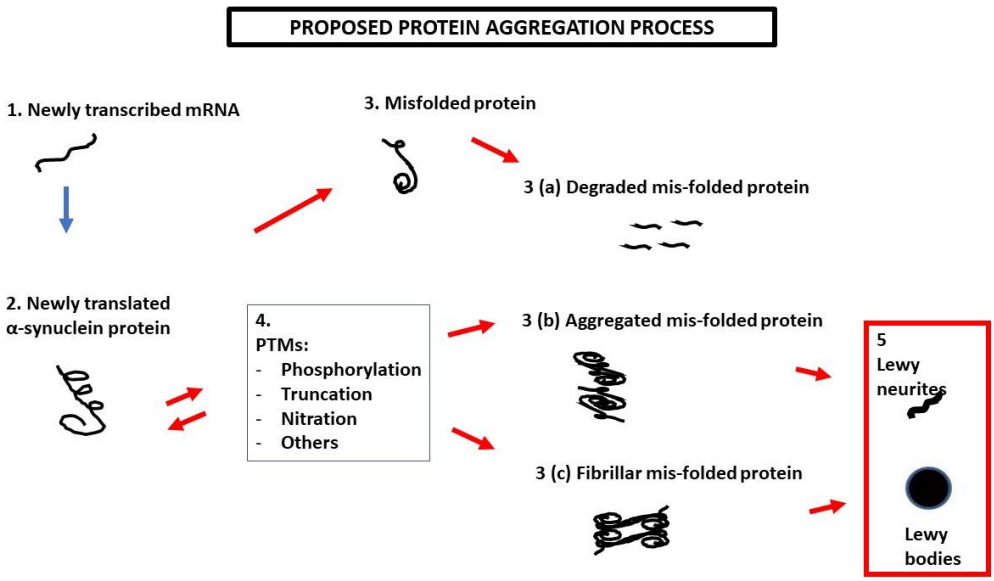


Figure 8 - Protein aggregation process:

α-synuclein mRNA (1); Newly translated monomeric α-synuclein (2); Misfolded α-synuclein protein (3), degraded by the UPS or autophagy pathway. 3(a). Misfolded α-synuclein can form aggregates 3(b) produce an antiparallel beta sheet formation (3c). Post-translational modifications (4) may lead to aggregated (3(b) or fibrillar form 3(c) of α-synuclein. 5. Aggregated or fibrillar α-synuclein can lead to Lewy neurites or Lewy bodies.

The process of aggregation may occur at various steps, during mRNA transcription (Figure 8-1) the polypeptide chain will predictably fold into its unique active 3D conformation determined by the sequence of hydrophobic and hydrophilic amino acids (Figure 8-2). The formation of a hydrophobic core induces a globular structure that limits the available conformational space [152] reducing the risk of a misfolded protein (Figure 8-3). Exposure of this region to the cytoplasm could induce a conformation change that is not conducive to the proteins final structure. In the event that α-synuclein is misfolded, the UPS or autophagy system will attempt to degrade the protein (Figure 8-3a). PTMs of α-synuclein (Figure 8-4) have been shown to

encourage protein aggregation (Figure 8-3b) or fibrils (Figure 8-3c), these species of α-synuclein may lead to LB or LN formation (Figure 8-5). Fibrils form interactions of variable strength and morphological associations but are often resistant to rigorous degradation processes [153]. The prevention of degradation leads to the formation of larger aggregates, LB formation, maturation and pathological spread.

1.2.7.2. β-synuclein

β-synuclein is not found in Lewy bodies [31] and wild type β-synuclein has significantly less intrinsic, fibrillogenic properties in comparison to α-synuclein [154]. β-synuclein is typically found in a soluble monomeric state, extensive periods of protein incubation see no changes to the protein conformation [155]. Isolation of the NAC fragment from α-synuclein leads to the rapid and efficient formation of proteolytic-digestion resistant, amyloid fibrils [154] although, when introduced into the central region of β-synuclein, it only marginally increases the protein fibrillisation propensity [129]. The fibrillogenic conferring properties of the NAC fragment, therefore, despite protein homology of the synuclein, does not predispose the same aggregating properties in the chimeric β-synuclein protein. It is possible that local or distal properties in the existing sequence of β-synuclein negate the aggregating effects of the amyloid fragment.

Enforced harsh conditions have been shown to induce the aggregation of β-synuclein, the presence of SDS led to a fibril-like morphology and protofilament structures [129], this multimerisation process is not conferred in the absence of the NTD. Metal copper, zinc and lead have been shown to induce structural changes and aggregation features in β-synuclein [156]. Following incubation with the metals the protein changed from a disorganised unfolded protein to a more structurally organised fibrillated β-synuclein, corresponding to a β-sheet formation.

Collectively, this evidence suggests that under certain enforced, artificial and harsh conditions, β-synuclein has the ability to form fibrils and to aggregate in much the same way that α-synuclein can. It can be argued that any polypeptide has capacity to aggregate under enforced conditions that do not represent the physiological environment.

1.2.8. Synuclein gene mutations

1.2.8.1. SNCA

The E46K mutation is the only mutation in the SNCA gene that has been associated with familial DLB [157] and there is presently no genetic marker for idiopathic DLB. In contrast, a genome wide association study (GWAS) identified that an increase in susceptibility to sporadic PD was linked to single nucleotide polymorphisms in the SNCA gene [158]. In PD, point mutations have been identified, including but not limited to: A53T [159], E46K [157], A30P [160], G51D [161], and H50Q [162]. Furthermore, duplications [163] and triplications [164] of the SNCA gene have been identified in families with autosomal-dominant PD. Point mutations in the SNCA gene and gene triplication lead to high penetrance, whilst duplication has incomplete penetrance [165]. Multiple gene copies see an increase in the wild-type expression of α-synuclein that is sufficient enough to cause early on-set PD [166]; the age of onset following duplication of SNCA induces clinical symptoms in the 50s and triplication as soon as the thirties (reviewed in [167]). The age of PD onset in those with gene duplication is similar to that observed with point mutations and thus, the argument is presented that increased gene dosage increases the propensity of α-synuclein to aggregate.

The point mutations A53T, A30P, E46K, H50Q and G51D in the SNCA gene, implicated in PD/DLB are all positioned in the NTD of the protein (Figure 9). This finding may suggest that alterations in the region of the protein associated with the formation of alpha-helices, leads to a change in a conformation such that favours oligomerisation/aggregation.

Figure 9 - SNCA mutations

Diagrammatic of mutations associated with α-synuclein. They are all located in the NTD.

1.2.8.2. SNCB

Missense mutations in SNCB gene have been associated with DLB [168]. The challenge is the lack of multi-generational-families with DLB, leading to the absence of the genetic power required for linkage analysis and positional cloning strategies. The ability to study families with a DLB history would enable the research into the molecular genetics of the disease, increasing the capacity to identify novel genes. A relatively large study screened sporadic DLB and familial DLB patients and found only two patients with two different two amino acid substitutions that occurred at conserved sites in the SNCB gene [169]. The sporadic DLB patient had a substitution in exon 4, at codon 70 of valine to methionine: the V70M mutation. The second patient they identified as an extended pedigree, with a substitution at codon 123 in exon 5 from proline to histidine: the P123H mutation. Immunohistochemical examination of the patient with the P123H mutation did not detect β-synuclein in

Lewy bodies, there were no β-synuclein positive aggregation or inclusions, although, a diffuse β-synuclein staining was observed in the hilus of the hippocampus and amygdala.

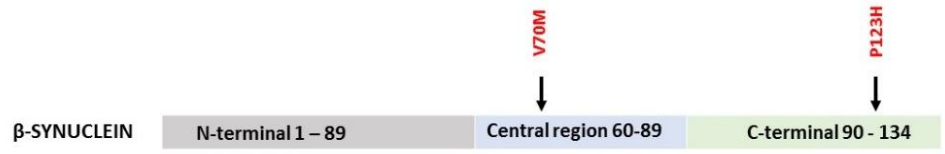


Figure 10 - SNCB mutations:

Diagrammatic of mutations associated with β-synuclein. Mutations are linked to the central domain and the CTD.

1.2.9. Pathological role of synucleins

1.2.9.1. α-synuclein

Lack of wild type α-synuclein does not produce a phenotype with severe neuropathological effects [170] but an increase in α-synuclein gene dosage leads to LB formation [164]. PTMs to α-synuclein can give rise to a toxic gain of function - α-synuclein is constituently phosphorylated occurring under both normal physiological conditions [171] and pathological [110]. It is not ascertained whether PTMs occur as a method of protein regulation or further propagates the disease. Extensive phosphorylation at serine 129 (PS129) of protein aggregates is associated with α-synucleinopathies and evidence suggests that phosphorylation at this site is promoting fibrillogenesis in vitro [110], contrasting reports suggest that PS129 may inhibit fibrillisation [172] Moreover, phosphorylation of α-synuclein at serine 87 (PS87) is increased in DLB brains, but is thought to inhibit fibrillisation and reduce

the ability of α-synuclein to bind to membranes [173]. Nitration of tyrosine residues in α-synuclein is a common feature in LBs in PD, DLB and LB variant of AD brains (LBVAD). It is proposed that nitration is a direct link to oxidation induced-cross linking of the protein that further encourages fibrillisation [148], nitration of α-synuclein is expressly neurotoxic for dopaminergic neurons [174].

Pre-fibrillar, soluble oligomeric α-synuclein is increasingly considered the predominant toxic species of the protein [54]. By inducing various mutations, associated with α-synucleinopathies, it was shown that the fibrillisation properties of the α-synuclein protein were reduced, but the propensity to form soluble oligomeric species was increased, correlating with increased neurotoxicity, neuritic defects, loss of dopaminergic neurons and behavioural deficits in multiple system models [175]. Moreover, examination of the DLB brain reveals at least ten times smaller α-synuclein aggregates when compared to the amount of Lewy bodies, 90% of these were outside of Lewy bodies and present at the pre-synapse in the DLB frontal cortex [176].

1.2.9.2. β-synuclein

In order to assess the functional effect of the P123H mutation, Fujita et al., [177] developed a mouse model overexpressing the human P123H transgene. Accumulations immuno-positive for β-synuclein were identified in hippocampal axons and apical dendrites in cortical regions. The aggregates were not Lewy-body-like and increased in insolubility with age. In the basal ganglia, small spheroidal axonal swellings composed of membranous components were observed that were non-functional as a pre-synapse and spatial learning and memory were affected. Although endogenous α-synuclein did not alter the pathology associated with P123H β-synuclein, the double transgenic mice over expressing α-synuclein in conjunction

with P123H saw augmented neurodegenerative pathology. Thus, a toxic gain of function for P123H- β-synuclein, requires overexpression of wild type α-synuclein to rescue the pathological phenotype. Furthermore, lysosomal dysfunction, representative of lysosomal storage disorders in the presence of both V70M and P123H SNCB mutations have been observed [168]. P123H overexpression produces various types and sizes of lysosomal structures including macro-lysosomes and giant autophagosomes.

1.2.10. Protective function of β-synuclein

Despite the lack of studies of β-synuclein, there are significant indications that the protein may bestow protective properties in the presence of increased levels of/or the modified structure of α-synuclein. Hashimoto et al., [178] developed a mouse model expressing human wild-type α-synuclein that develop intraneuronal inclusions, dopaminergic impairments and motor deficits. Co-expression of human β-synuclein and α-synuclein ameliorated the motor deficit in concurrence with significantly less intraneuronal inclusions. Co-incubation of α-synuclein with β-synuclein reduces the rate of α-synuclein fibrillation [124] and β-synuclein significantly reduces the generation of the mutant form of α-synuclein, A53T protofibrils and fibrils [179]. Finally, transient overexpression of β-synuclein is shown to enhance cell survival when exposed to potential toxins, such as α-synuclein-fibrillisation inducer, rotenone [180]. Indicative of direct or indirect neuroprotective responses by β-synuclein in toxic cellular conditions.

The evidence suggest that direct binding to α-synuclein by β-synuclein occurs, its regulation of α-synuclein aggregation and the fact that β-synuclein is not present in Lewy bodies [49] demonstrates that the protein is able to avoid being caught up in

the aggregating process. It could be argued that the neuropathological features of DLB in the patient become clinical when there is a loss of equilibrium between modification to α-synuclein and its aggregating properties and the presence of β-synuclein. It is important that levels of β-synuclein protein are examined in DLB neuropathological regions in relation to α-synuclein levels in order to establish the course of change in protein levels that may be occurring in the DLB brain.

1.3. Interaction between SNARE proteins and synucleins at the pre-synapse

Intercellular signaling, mediated at the synapse in the central nervous system, is highly organised. Neurotransmitters are packaged into synaptic vesicles, transported to the reserve pool of readily releasable pool of synaptic vesicles in anticipation of an action potential resulting in exocytosis. These constitutive and universal processes provide the foundation for many of the biological processes [181], the modulation of which can change the efficiency of the synapse such that it can impact on synaptic activity.

1.3.1. SNAREs at the pre-synaptic terminal

The soluble N-ethylmaleimide sensitive fusion attachment protein receptor (SNARE) proteins are a family of membrane bound proteins that facilitate the association of intracellular membranes with target membranes [182]. The exchange of materials between organelles or other cellular membranes such as the plasma membrane or pre-synapse requires the fusion of two phospholipid membranes [183]. The formation of the trans-SNARE complex and the association between SNAREs on independent membranes underpins the subsequent fusion of the membranes leading to the cis-SNARE complex formation, a conformation that sees the SNAREs associated with the same membrane [184]. Crucially, neurotransmitter release at the synapse relies on the efficiency of synaptic vesicles binding to the target membrane in order to release the neurotransmitter into the synaptic cleft. Each pre-synaptic portion of an axon includes hundreds of synaptic vesicles that contain neurotransmitter [185] and from the arrival of an action potential to the release of neurotransmitter can take as little as 100µs [186].The regulation of neurotransmitter

release underpins most functions of the nervous system, including those that mediate cognition and memory processes that are dysfunctional in DLB.

Depending on the pre-determined location for the SNAREs, they are either V-SNAREs, that are assembled onto the **v**esicle, such as VAMP2 (vesicle-associated membrane protein 2) or **t**-SNAREs that characterise the SNARE proteins that associate with the target membrane, such as syntaxin and SNAP25 [187].

1.3.2. The SNARE complex

VAMP2, syntaxin, and SNAP25 form the SNARE complex [188] at the pre-synapse (Figure 11). The SNAREs have a region of 70 amino acids [189] that is an extended coiled-coil motif. Most SNAREs including syntaxin and VAMP2, have a short linker that associated the SNARE motif to the carboxyl-terminal transmembrane region (TMR). SNAP25 deviates from this structure with the SNARE motifs being connected by a palmitoylated linker and the absence of the TMR [190].

The characteristics of these three SNARE proteins are unique in that they assemble into a ternary complex with 1:1:1 stoichiometry [187] forming a tight-SDS resistant complex [191]. The principal regions within each of the SNAREs that are important to ensure cooperative binding in the SNARE complex formation has been determined [191] - in VAMP2 this was identified in the central domain (residues 27-96); syntaxin relies on an H3 helix domain in the C-terminal region (194-261) and SNAP requires both the C-terminal and N-terminal regions (180-208 and 26-82, respectively). Each of these areas have a high predisposition towards alpha-helical coil-coiled formations [192] leading to a four-helix bundle as a result of the complex, two of the helices belonging to SNAP25 [193].

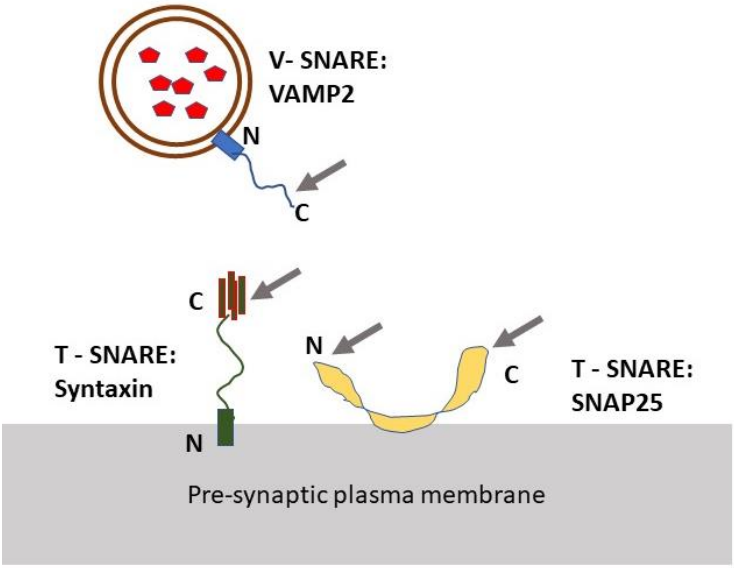


Figure 11 - SNARE proteins at the pre-synapse

Diagrammatic illustration of V-SNARE, VAMP2 in association with the vesicle. T-SNAREs syntaxin and SNAP25 are in association with the pre-synaptic plasma membrane. N denotes NTD and C denotes CTD. The arrow represents the region of the SNARE protein that is critical for producing the ternary structured SNARE complex.

A plethora of other proteins complement and support the role of the SNAREs ensuring efficient docking, priming and fusion of synaptic vesicle including, but not limited to, SM proteins [194] the N-ethylmaleimide-sensitive-factor (NSF), the soluble NSF attachment proteins (SNAPs), complexins, synaptotagmins [195] and Rabs [196]. This is an expansive area with much research and for the purpose of the thesis will remain limited.

1.3.2.1. SNARE complex assembly, priming, fusion and exocytosis

At the presynaptic terminal, vesicles cycle through a succession of trafficking phases including docking, priming, fusion, exocytosis, endocytosis and recycling (reviewed

in [197]). Some of these vesicles can be found already docked at specific regions of the plasma membrane, so-called “the active zone” [198]. At the active zone, this readily releasable pool (RRP) of vesicles is joined by multiple proteins that act in the manner of a scaffold to allow docking of the vesicle in preparation for an action potential, calcium flux and subsequent release of neurotransmitter.

Further co-operative components are required: SM protein, Munc 18-1, is a negative regulator of SNARE complex formation by its binding to syntaxin 1A, disassociation of syntaxin and Munc18 binding exposes syntaxin residues that are available for SNAP25 binding [194]. Tethering factors are important for bringing the vesicle into close proximity to the target site [199]. Subsequent assembly of the SNARE complex requires the contact of v-SNARE and t-SNAREs by associating in trans, starting from the NTD of the SNAREs and advancing towards the CTD, in a “zippering” effect anchoring the SNAREs [192].

Priming is the phase that sees docked vesicles transition into an exocytosis-proficient state, waiting for the calcium influx following an action potential that will induce fusion pore formation [200]. In the final stages of exocytosis, fusion of the vesicle membrane and the plasma membrane occurs. The development of a fusion pore ensues whilst maintaining the integrity of the hydrophobic components of the membranes. The precise mechanisms surrounding this process is still under investigation with one hypothesis focusing on the stalk hypothesis [201] once the fusion pore ruptures exocytosis of neurotransmitter at the synaptic cleft will occur.

1.3.2.3. Snare complex disassembly and endocytosis

Following fusion with the plasma membrane, the SNARE complex is released in a “spring-loaded” manner and unwinding of the four bundle helices arises [202]. Fast

neurotransmission in the human brain requires the efficient and rapid recycling of synaptic vesicles at the presynaptic terminals; this relies on competent endocytic processes that are relevant to the physiological requirements. There is only a little evidence suggesting a role for the SNAREs in endocytosis and reports are contradictory. Knock down of VAMP2 leads to a diminished response in the replenishment of readily releasable vesicles although, the overall number of pre-synaptic vesicles is unchanged [203]. A role for SNAP25 and VAMP2 in slow clathrin-mediated endocytosis is suggested [204] although, the role for SNAP25 is contradicted in other studies [205].

1.3.3. α-synuclein, SNAREs and neurodegeneration

1.3.3.1. SNAREs and α-synuclein in vesicle pool maintenance

α-synuclein is thought to play a role in synaptic transmission by the tempering of neurotransmission release through the supervision of SNARE complex assembly [141] and a role for α-synuclein is proposed in the maintenance of vesicle numbers [206, 207].

Partial α-synuclein knock down shows a reduction in the resting pool of vesicles but the number of docked vesicles is unchanged [139]. A mouse null α-synuclein model, had a significant decrease in the number of vesicles, in the resting pool of the hippocampus and vesicle replenishment was not readily available following repeated stimulation, leading to synaptic depression [206]. Thus, presenting a role for α-synuclein in the maintenance of the pool of resting synaptic vesicles.

1.3.3.2. α-synuclein in SNARE complex assembly and exocytosis

Age-dependent re-distribution of SNAP25, syntaxin and VAMP2 in a mouse model with C-terminal truncated α-synuclein is observed [208]. Triple-synuclein knock down sees a VAMP2 decrease in an age-dependent manner [141] but not when α-synuclein knockdown occurs alone [139]. Pre-synaptic accumulations of α-synuclein in addition to re-distributed SNARE proteins lead to synaptic failure associated with a decline in exocytosis and reduced dopamine release. Importantly, α-synuclein also chaperones the assembly of the SNARE complex by both binding to phospholipids by its NTD and via it's CTD binding to the NTD of VAMP2 [141] (Figure 12, A-D).

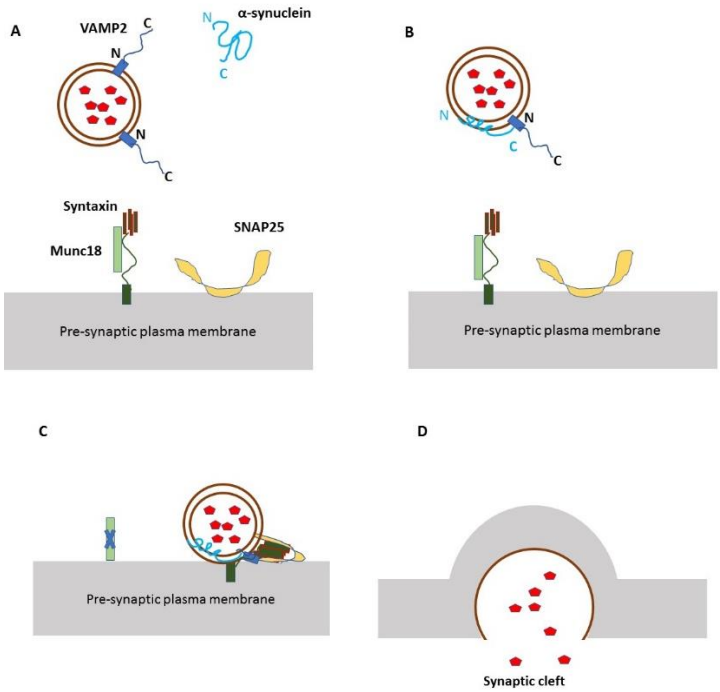


Figure 12 – An overview of SNARE complex formation

α-synuclein NTD anchors to the vesicle and its CTD binds to the VAMP2 NTD (A-B), this interaction stabilises the SNARE complex enabling VAMP2 to bind to syntaxin and SNAP25 (C). Fusion of the vesicle with the plasma membrane enables neurotransmitter exocytosis (D).

1.3.3.3. SNARES, α-synuclein and neurodegeneration:

The pre-synaptic terminal is increasingly linked to the initial location for the onset of neurodegeneration. Much focus has been given to examining the roles of pre-synaptic proteins to help elucidate the mechanisms that lead to synaptic de-regulation. VAMP2 has been shown to be decreased in DLB but increased in PDD [209]. The authors correlated the changes with the duration of dementia suggesting that an increase in VAMP2 may be an early compensatory mechanism at the synapse to overcome the toxic insults of α-synuclein. This is interesting as VAMP2 has been associated with replenishment of the pool of vesicles [203], a role also suggested for α-synuclein, indicating a compensatory effect.

α-synuclein pathology that occurs at the distal axon can proceed to cause retrograde degeneration [210]. In a rat model of PD, the levels of α-synuclein pathology reflect the dopaminergic terminal loss in the basal ganglia which parallels the reduced levels of dopamine available in the region, indicating a possible retrograde dying-back process [211]. α-synuclein may have multiple roles at the pre-synapse that include chaperoning and maintaining the integrity of the distal pool of vesicles. Dysregulation in these functions may underlie or contribute to neurodegenerative processes in an age-dependent manner. The knowledge that triple knock out models has a greater impact on the SNARE proteins than single knockout models requires further exploration, examining the potential of synuclein and family protein redundancy behaviours.

1.4. Synuclein regulation of Autophagy and neurodegeneration

Eukaryotic cells have two main pathways for degradation, lysosomal proteolysis and the ubiquitin-proteasome system (UPS). The UPS system is primarily responsible for the degradation of short-lived proteins that have been tagged with UB [212]. Lysosomal proteolysis incorporates several pathways including endocytic pathway, whereby extracellular components and plasma membrane are delivered directly to the lysosome and the autophagy pathway (reviewed by [213]).

Autophagy per se embodies three individual processes: macroautophagy**,** microautophagy and chaperone-mediated autophagy (CMA). Microautophagy refers to the invagination of the endosomal or lysosomal membrane, enabling direct engulfment of the target substrate from the cytoplasm, which is then degraded by lysosomal proteases [214]. CMA is mediated by chaperone proteins enabling the passage of proteins into the lysosomal lumen [215]. Macroautophagy sees the sequestering of components targeted for degradation by an autophagosome, this fuses with a lysosome, containing proteases that lead to the degradation of the autophagosome contents [216]. Macroautophagy is a bulk process that is able to degrade cytoplasmic proteins that have served a long-life in addition to aged and superfluous cellular organelles such as the mitochondria [217] and endoplasmic reticulum [218]. The mechanisms described in this chapter will predominantly focus on macroautophagy, from here on in, described only as autophagy.

1.4.1. The autophagy process

Saccharomyces cerevisiae is a model that has been extensively manipulated in order to elucidate the mechanisms surrounding autophagy. Over 30 ‘autophagy related genes’ (ATG) have been identified and are thought to be involved in the process, each regulating a different phase of the autophagosome formation [219].

The process of capturing cytoplasmic components for degradation occurs by the elongation of isolation membranes (phagophores), into doubled-membraned autophagosomes that encapsulate the material targeted for degradation [216]. Autophagosomes are shuttled to the microtubule-organising centre of the cell via the dynein machinery where it will be in close proximity to enzyme containing lysosomes [220] for fusion between the lysosome and autophagosome to ensue. Lysosomal proteases act on the sequestered cargo and break it down into down into smaller components that may be reused by the cell or in the case of protein aggregates, degraded in an attempt to prevent cellular dysfunction (Figure 13).

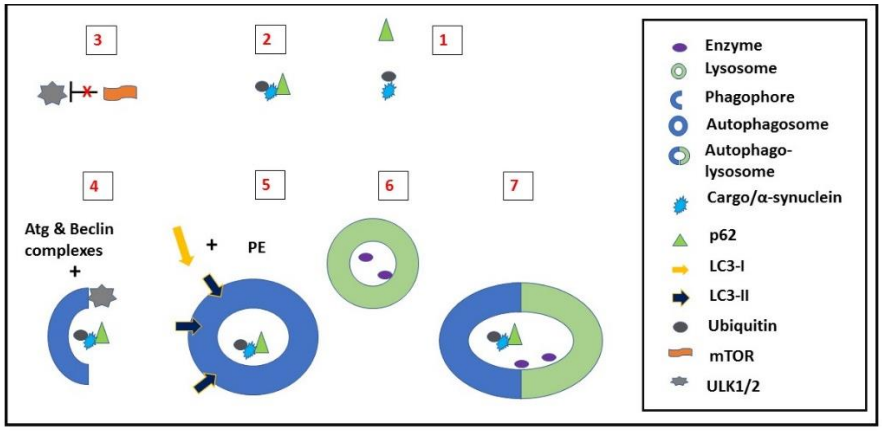


Figure 13 - An overview of the autophagy process

Substrate is tagged by UB (1) and recognised by p62 (2). mTOR is inactivated, dis-inhibiting ULK 1/2 (3) which is free to form the ULK complex on the phagophore (4). The ULK, Atg and Beclin complexes enable initiation and nucleation of the phagophore that will encapsulate the cargo destined for degradation. PE is conjugated to LC3-I (5), LC3-II binds the autophagosome and participates in the elongation process. The autophagosome is shuttled close to the lysosome (6) and autophagosome-lysosome formation occurs via fusion (7); thus, enabling enzymes access to the cargo for degradation.

1.4.1.1. Cargo delivery

Autophagosomes can non-selectively (in the case of starvation) and selectively sequester cytosolic components. The selection process is not fully understood although it is thought that cargo destined for degradation is tagged with UB. Protein aggregates are recognised by UB-binding receptors (UBR) such as p62, a multimodal adapter able to non-covalently bind to UB [221]. Concurrent binding of p62 to UB and ubiquitin-like (UBL) proteins associated with the autophagosome, will effectively deliver the cargo to the nascent autophagosome [222].

1.4.1.2. Autophagy regulation and initiation

The upstream modulator of autophagy, the (mammalian) target of rapamycin (mTOR) is a negative regulator of autophagosome formation by inhibiting UNC51-like kinase (ULK) 1 and 2 [223]. mTOR inactivation and resulting suppression are induced by various stress signals (reviewed by [224]). Once mTOR is inactivated, activation of ULK 1 and ULK2 occurs proceeding in the direction of autophagy; ULK1/2 is free to form the ULK complex initiating the extension of the phagophore that sequesters portions of the cytoplasm associated with the target cargo, forming an autophagosome. The process of autophagosome biogenesis involves - initiation, nucleation and elongation of the membrane.

The phagophore, a double membraned, small vesicular sac is destined to form the autophagosome [225]. The Atg proteins are crucial for initiating the formation of the autophagosome [226] this elaborate formation occurs at the phagophore assembly site, here the ULK complex gathers in order to begin the formation of the autophagosome. This complex comprises of ULK1/2, Atg13, FAK family kinase interacting protein of 200kDa (FIP200) and Atg10) and transmembrane At99 (reviewed by [227]). The activated ULK1 complex translocates to a region within the ER where it proceeds to activate a class III PI3K complex that consists of Beclin 1, vacuolar protein sorting 15 (VPS15), VPS34, AMBRA1 and ATG14 [227].

1.4.1.3. Nucleation

Activation of the ULK1 complex and the class III PI3K complex stimulates phosphatidyl-inositol 3-phosphate (PI(3)P) production, essential for phagophore elongation and the recruitment of other Atg proteins. Beclin 1 is necessary for the localisation of proteins involved in autophagy to couple with the phagophore and is

dependent on PI3KC (Vsp34) to form the complex that is composed of Beclin 1-Vsp34-Vsp15 [228].

1.4.1.4. Elongation

Membrane elongation requires the recruitment of complex Atg12-Atg5-Atg16 [229]. This enables the lipidation of microtubule-associated protein 1 light chain 3 (LC3B/Atg8) with phosphatidylethanolamine. Atg7 is an important facilitator in autophagosome formation; it acts as an E1-like activating enzyme [230] for UBL proteins, Atg12 and LC3; activation of the UBLs by Atg7 ensures their transfer to an E2 enzyme to support their final destination. Early in autophagosome formation, Atg7 will bind to Atg12 thus ensuring its transfer to Atg5, where a complex is formed between Atg12- Atg5. The complex forms a larger complex with Atg16 and is localised to the elongating phagophore where it acts as an E3 enzyme during the process of LC3-I lipidation [231].

LC3B is expressed as a full-length protein, however, upon stimulation of autophagy, the CTD of the LC3 precursor is proteolytically cleaved by Atg4, to form LC3-I [232]. LC3-I requires lipidation in order to become the active form of LC3 that is known as LC3-II. The Atg12- Atg5- Atg16 complex facilitates the transfer and conjugation of LC3-I to phosphatidylethanolamine (PE) [231]; during lipidation, Atg7 activates LC3. LC3-I-PE is then localised into the phagophore double membrane where it will be responsible for the recruitment of essential components of autophagy [233] including the proteins that associate with the cargo destined for degradation.

The Atg12-Atg5-Atg16 complex dissociates from the membrane once the autophagosome is formed [234]. A role for LC3 has been implicated in the closure of the autophagosome [177], creating a possible role for LC3-II in the later stages of

elongation and development of mature autophagosomes. The making of a fully enclosed autophagosome with encapsulated cargo is essential to ensure isolation from the cytosol and efficient passage to the lysosome.

1.4.1.5. Autophagosome-lysosome fusion

Once the autophagosome has formed and sealed it is ready to be fused to the lysosome, it will be shuttled along microtubules to the microtubule-organising centre using the kinesin and dynein motor proteins. A trans-SNARE complex, including members syntaxin 17 and SNAP29, enables the fusion of endosome or lysosomes with the autophagosome [235] leading to degradation of the cargo.

This is a finely tuned process that is still being explored; efficiency is needed at each step in the pathway to ensure that autophagy proceeds to completion.

1.4.2. Synucleins and autophagy-lysosomal degradation pathways

1.4.2.1. α-synuclein

The α-synuclein protein under normal physiological circumstances, is degraded by both the proteasomes and lysosomes [236] [237]. Pathological α-synuclein may impair all three of these degradation pathways leading to cellular accumulations. Monomeric α-synuclein can be degraded by the proteasome in a non-UB dependent manner [238] indicating that ubiquitination of the protein is potentially a pathological event [239]. In this regard, LBs are rich in UB [240] and UPS impairment is seen in PD brains [241]. Moreover, PTM species of α-synuclein obstruct the transition of PTM α-synuclein, into the lysosomal lumen [242] and mutant forms of α-synuclein, such as A30P and A53T, identified in familial PD, impair CMA and may also favour the autophagy pathway [237]. Ubiquitin-carboxy terminal hydrolase L1 (UCHL-1) [243] and Parkin [244] are both enzymes involved in the UPS, Parkin has been

associated with the early onset form of PD and although a UCH-L1 mutation was thought to have been implicated, this was in one family only and has not been further validated; interestingly however, UCH-L1 levels are reduced in the DLB cerebral cortex [245]. This provides evidence for the involvement of multiple pathways related to the removal or degradation of modified proteins and/or accumulations in DLB, the failure in these mechanisms builds the platform for LB formation.

Collectively, when considering the pathways involved in clearance of α-synuclein and other aggregation - prone proteins, it is likely that multiple pathways may exist in parallel, those operating at basal levels and those in response to changes in the microenvironment. Autophagy is the major degradation pathway for nonspecific and bulk degradation of intracytoplasmic components and it is entirely feasible that if the UPS and CMA attempt of clearance of the aberrant fail, the balance tips in favour of the autophagy pathway.

1.4.2.2. β-synuclein

Mutant β-synuclein P123H and V70M has been shown to induce lysosomal with the production of cytoplasmic inclusions similar to those observed in lysosomal storage disorders, such as Gaucher's disease, the phenotype is exacerbated in the presence of modified α-synuclein [168]. PD [246] and DLB [247] are linked to a lysosomal storage disorder, Gaucher's disease by mutations in the glucocerebrosidase (GC) enzyme. The high homology between β-synuclein and α-synuclein may provide the basis for mutant β-synuclein that enables it to act in a similar manner to its counterpart and that the presence of both proteins together may act in a cumulative manner in α-synucleinopathies.

1.4.3. Autophagy and neurodegeneration

In the event that autophagy is disrupted, the accumulation of proteins targeted for degradation will be seen with enlarged and increased numbers of autophagosomes and neurodegenerative phenotypes [248]. Defects in the autophagy pathway have been observed in neurodegeneration with the amassing of autophagic vacuoles in DLB [249] and PD [250].

Dysfunction at various stages of the autophagy pathway is associated with neurodegeneration, reduced levels of Beclin 1 has been identified in the brains of AD patients [251], mTOR and LC3 are increased in the DLB temporal cortex and Atg7 is decreased [249]; the increase in mTOR and LC3 was more profuse in neurons exhibiting α-synuclein pathology with the additional presence of an increase and enlargement of autophagosomes. Further neurodegenerative phenotypes are seen in knock down models of Atg7's alone [252]. The absence of the E1-like activating enzyme Atg7 would lead to mis-localisation of Atg12 to Atg5, required for lipidation of LC3-I therefore, conjugation of PE to LC3-I would be disabled, resulting in the lack of recognition of cargo carried by p62 and the potential disruption in autophagosome closure and maturation.

Finally, in the event that the fusion between the autophagosome and lysosome proceeds, it is crucial that the lysosome containing catalytic enzymes are in situ. The lysosome integral protein 2 (LIMP2) acts as a receptor and is crucial in targeting the GC enzyme to the lysosome [253] in addition to endosome/lysosome biogenesis [254]. The genetic linkage of LIMP2 has been associated with DLB [96] and mutations in the GC-encoding gene, GBA are associated with LB disorders, 12% of PD patients have the GBA mutation [247] implicating a role for both the enzyme and

its receptor in α-synucleinopathies. The mutations of the GBA gene and risk associated with α-synucleinopathies highlights the importance of this enzyme in the degradation of α-synuclein, the phenotype seen in these neurodegenerative conditions underscores the necessity for uninhibited and complete degradation of accumulation α-synuclein protein.

CHAPTER 2: Materials and Methods

2.1: Human post-mortem tissues

2.1.1: Tissue homogenisation

Fresh frozen tissues (Table 2) from the frontal cortex (BA9), occipital cortex (BA18-19) and hippocampus, were homogenized using a pestle and mortar in 10X volume of Tris-buffer saline (TBS) plus Triton-X (150mM NaCl, 50mM Tris-Hcl, 1% Triton & 2mM EDTA, pH7.6) and protease inhibitor (cOmplete, EDTA free, Roche). Dounce homogenisation was performed in 20 slow strokes. The process was undertaken on ice, in a fume hood. The homogenised tissue was centrifuged at 3000rpm, at +4ºC for 10 minutes and the pellet discarded. The supernatant was collected and total protein quantification was performed by BCA analysis (Pierce ^{TM}) and known standards of bovine serum albumin (BSA). Absorbance assessed at 450nm on the FLUOstar Omega plate reader (BMG labech). Total lysate then used for WB or stored at -80°C.

ID	Clinical diagnosis at death	Pathological diagnosis	Gender	Age	PMI	Disease duration	Braak stage tau	Braak stage LB
PDC016	Pneumonia	Ageing-related changes	F	91			2	0
PDC022	Lung cancer	Mild age-related changes	M	75			3	0
PDC23	N/A	Mild age-related changes	F	78	23		2	0
PDC24	N/A	Mild age-related changes	F	68			2	0
A002	N/A	Mild age-related changes	M	90	45		1	0
A114	Lung adenocarcinoma	Ageing-related process	M	82	24		2	0
A261	Colon cancer	Ageing-related process	M	63	23		1	0
A388	Prostate cancer	Ageing-related process	M	65	26		1	0
914	Pneumonia	normal aging changes	M	96	21		2	0
918	Ischemic enterocolitis	Control	F	85	13.5		3	0
927	Prostate cancer	Control mild SVD	M	78	51.5		2	0
941	Chronic kidney disease	Control, moderate CAA	M	92	56.5		1	0
948	Ovarian cancer	Mild AGD	F	82	36		2	0
957	COPD	Control	M	86	44		2	0
986	Acute leukemia	Control	M	86	42		3	0
988	Pancreatic cancer	Control, moderately severe CAA	M	78	56		2	0
265	AD	DLBD	F	80	68	4	3	3
449	N/A	DLB	M	84	12.5	2	3	3
452	Vascular dementia	Neocortical DLB, possible AD	M	82	4	3	3	3
478	N/A	DLB	M	78	21	3	3	3

743	N/A	Mild LBD with cortical involvement.	M	86	15	4	3	4
844	Pneumonia, DLB	DLB, AGD, severe CAA	M	93	16	8	3	4
901	N/A	LBD, vascular disease	M	94	32		3	3
A028	N/A	Diffuse neocortical LBD	M	81	57	6	3	4
A273	N/A	DLB	M	86	8	5	2	4
A375	N/A	Diffuse neocortical LBD	M	66	40	5	2	4
PD060	Dementia with hallucinations	Brain stem LBD	M	84		6	2	3
PD120	PD	Brain stem LBD	M	83		4	3	2
406	AD	LBD	M	66	23	8	3	6
1014	N/A	DLB/AD	M	84	56		3	6
A010	N/A	DLB, moderate CAA	M	67	35	4	2	6
N1	N/A	LBD	M	86	33	8	1	6
N2	N/A	LBD	M	73	47	11	3	6
N3	N/A	LBD	M	81	26	5	3	6
N4	N/A	LBD	M	80	34	10	3	6
N5	N/A	LBD	M	71	22	5	3	6
N6	N/A	LBD	M	84	72	10	2	6
PD294	PDD/DLB	Neocortical LBD	M	78		6	1	6

Table 2 - Post mortem tissue data

Details of post-mortem tissues used for the study. AD (Alzheimer's disease), AGD (argyrophilic grain disease), CAA (cerebral amyloid angiopathy), COPD (chronic obstructive pulmonary disease), DLB (dementia with Lewy Bodies), dLBD (diffuse Lewy body disease), PD (Parkinson's disease), PDD (Parkinson's disease with dementia), LBD (Lewy Body disease), MMSE (mini mental state examination).

2.1.2: Fluorescent Immunohistochemistry

Formalin fixed paraffin embedded tissues were de-paraffinised in Xylene for 2X 5 minutes and re-hydrated in a serial dilution of ethanol (100%, 95%, 70% & 50%) each for 2 minutes. Sections were washed briefly with H_2O before tissue equilibration with phosphate buffer saline (PBS) (1.3MNaCl, 70mM Na_2 HPO_4 & 30mM NaH_2 PO_4, pH 7.6) for 5 minutes. Auto fluorescence was quenched with Sudan black (Sigma, 4197-25-5) (0.1% Sudan black in 70% ethanol) for 10 minutes before washing (Figure 14- A-H). Antigen retrieval was performed by boiling in the microwave with 10mM citrate buffer (pH6.0) for 30 mins at 700watts. Tissue sections were blocked with 5% of corresponding serum for 1 hour at room temperature (RT) and incubated overnight with primary antibodies (table 3) or without primary antibodies. Incubation with Alexa Fluor conjugated secondary antibodies (Table 4) and nuclear stain hoerscht (New England Biolabs, UK) [1:1000] was performed for 1 hour at RT, tissues were cover slipped and mounted with Perma Fluor (Thermo Scientific, TA-006-FM). All washes were performed with PBS and antibodies diluted with PBS + 0.03% Triton X. The fluorescent signal from the tissue sections was visualised using the Leica Confocal Microscope. Secondary only antibodies in the cortical regions, hippocampus and substantia nigra were visualised (Figure 14-I-Q).

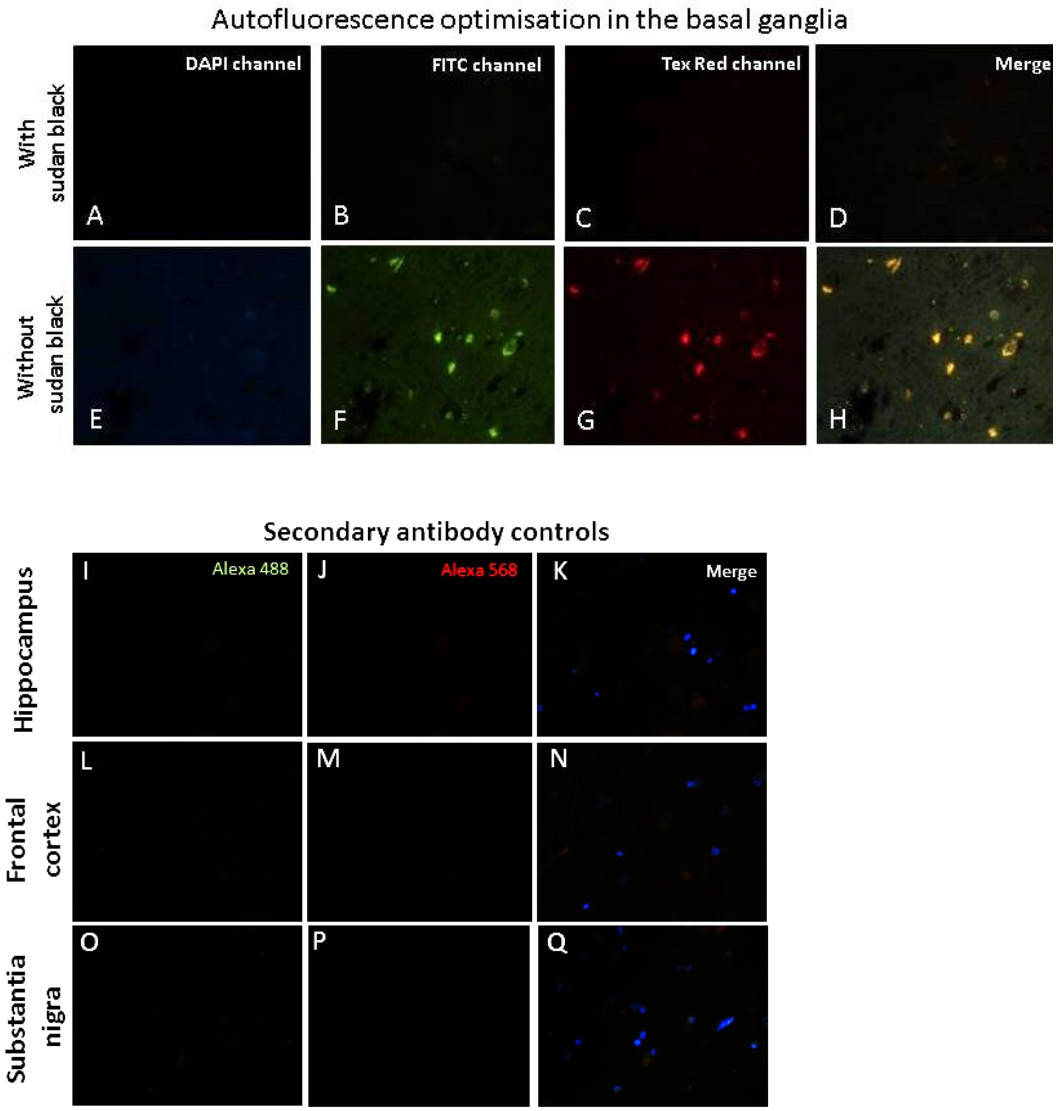


Figure 14 - Sudan black quenching of auto fluorescence and double, secondary antibody only controls

Quenching of auto fluorescence in the basal ganglia of the human brain using 0.1% Sudan black (A-D). Non-primary antibody controls in the double immunofluorescent protocol. A non-primary antibody control to examine non-specificity of secondary antibodies following incubation with two secondary antibodies simultaneously. Three brain regions were examined: the hippocampus (I-K), cortex (L-N) and substantia nigra (I-Q). Emission/excitation wave lengths of each channel – DAPI: 350nm/470nmm; Alexa: 488 (FITC) 490nm/525 nm; Alexa 568 (Tex Red): 578nm/603nm.

2.1.3: Manders overlap Coefficient analysis of co-localisation

Manders coefficient (M1 and M2) was performed for quantification of co-localisation of two proteins. The mean coefficient value was established from the analysis of 10 Z-stacks, per patient, using the Coloc2 plugin from Fiji freeware (FIJI - Image J software (NIH, USA)**.** Each image was split into two channels, green and red for our proteins and regions of interest. Background was subtracted and Costes randomisation was 10.

2.1.4: DAB Immunohistochemistry

Formalin fixed paraffin embedded tissues (6um) were de-paraffinised in Xylene for 2 X 5 minutes and then re-hydrated in a serial dilution of ethanol (100%, 95%, 70% & 50%) each for 2 minutes. Sections were washed briefly with H_2O before tissue equilibration with TBS for 5 minutes. Antigen retrieval was performed by boiling in the microwave with 10mM citrate buffer (pH6.0) for 30 mins at 700watts unless otherwise described. 5G4 pre-treatment involved Tris-EDTA (198mM Tris-base & 6.84mM EDTA) for 30 mins at RT minutes and 10% formic acid for 3 minutes. Endogenous peroxidase activity was quenched with a TBS buffer containing 3% H_2O_2, 20% methanol and 0.1% Triton X for 45 minutes. Tissue sections were blocked with 5% of corresponding serum for 1 hour at RT and incubated overnight with primary antibodies at +4ºC. On day 2, the Vectorstain Elite ABC system was used: secondary biotinylated antibodies were applied for 1 hour (Table 3) followed by avidin & biotin [1/200] for 1 hour, both at RT. Tissue sections were washed with TBS before applying the substrate, 3,3'-Diaminobenzidine (DAB). Tissue sections were washed with H_2O before applying Mayer's haemotoxylin (Thermo Scientific, TA125MH) for 1-2 minutes. Sections were again washed in H_2O before air drying overnight and cover-slipping with DPX mountant (Sigma, 44581).

2.1.5: Scoring β-synuclein positive cells

Following IHC, confocal analysis of cells positive for β-synuclein immunoreactivity was performed by randomly selecting ≥six areas throughout the cortical region, to achieve a minimum threshold of 100 neurons per patient. Cells were considered β-synuclein-positive with the appearance of strong β-synuclein immunoreactivity. The number of cells that are positive for β-synuclein are presented as a percentage of the total number of nuclei.

2.1.6: Pre-absorption

DAB immunohistochemistry was performed as described. Primary antibody, β-synuclein (0.096 mg/ml) and equivalent recombinant β-synuclein protein were co-incubated overnight with rotation. This antibody-antigen complex was added to the control slide at the primary antibody stage (Figure 15-A). Brightfield microscopy produced virtually no visual signal of β-synuclein in the tissues where pre-absorption was performed. The control slide was treated with β-synuclein antibody (Figure 15-B) without pre-absorption.

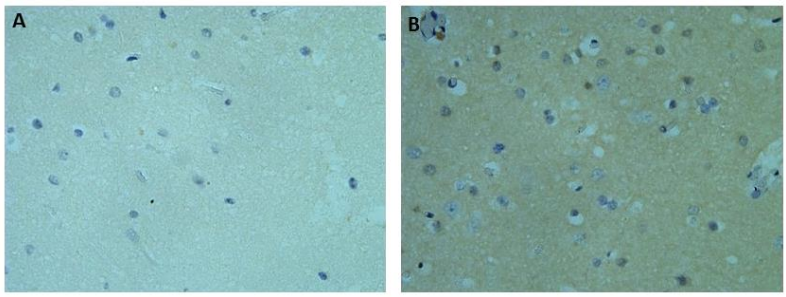


Figure 15: Pre absorption of the β-synuclein antibody

The β-synuclein antibody was incubated with (A) or without (B) β-synuclein recombinant protein to validate the specificity of the antibody.

2.1.7: Western blotting

Tissue homogenate proteins were separated by sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), using a 12-14% gel, dependent on protein size. Electrophoresis was performed at 120V for 1 hour and transferred to a 0.2uM polyvinylidene difluoride (PVDF) at 15V for 1.30 hours, both at RT. Membranes were blocked in 5% skimmed milk and TBS-T 0.05% tween for 1 hour and then incubated with primary antibodies (Table 3) overnight at +4ºC with rotation. The membranes were washed 3 X 30mins and HRP-conjugated secondary antibodies (Table 4) were applied at RT for 1 hour. The membranes were exposed to detection Amersham Prime ECL western blotting detection reagent (Fisher, 10308449) for 1 minute. Data was analysed by measuring the density of the specific band on the immunoblot using Image J software (NIH, USA) and presented as a ratio to the corresponding β-actin density. Washes were performed with TBS and antibodies diluted in TBS+ 0.05% Tween.

2.1.8: Synaptosomal isolation

Synaptosomal isolation was performed using the SynPer ™ (Thermo Scientific) synaptic protein extraction kit (Figure 16). Samples were homogenised using a hand-held pestle and mortar, on ice, in a fume hood with 10X the sample volume of the SynPer reagent and protease inhibitors. Dounce homogenisation was performed in 10 slow strokes. The homogenate was centrifuged 1200 × g for 10 minutes at 4°C. The pellet was discarded and total protein quantification was performed by BCA analysis (Pierce ™) and known standards of bovine serum albumin (BSA). A sample of the supernatant was taken for the total protein fraction analysis. The supernatant was further centrifuged at 15,000 × g for 20 minutes at 4°C and the cytosolic fraction was collected for analysis. 300-500µl of SynPer was used to resuspend the

synaptosome pellet. Western blot analysis was performed, or the samples were frozen at -80°C.

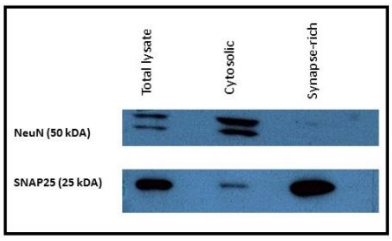


Figure 16 - Synaptosomal isolation optimisation

100mg of mouse cortical brain lysate homogenised and probed for neuronal marker NeuN and synaptic marker SNAP25. NeuN was barely visible in the synapse rich fraction and SNAP25 was abundant in this fraction.

2.1.9: ELISA

2.1.9.1: Oligomeric α-synuclein (5G4): The AnalytikJena human α-synuclein patho ELISA kit (0104000108) was used for this enzyme immunoassay. 50ug of total brain homogenate per patient was assayed by ELISA for 5G4 immunoreactivity. Antibody and homogenate were incubated overnight at RT and shaking at 300rpm. Following washing the samples were incubated in the dark at RT with 3,3',5,5'-Tetramethylbenzidine (TMB) and peroxide. Following termination of the reaction, the plate was briefly shaken and absorbance was read at the FLUOstar Omega plate reader (BMG labech) at 450nm and 650nm reference wavelength.

2.1.9.2: β-synuclein: The Abexxa (abx055444) human β-synuclein (SNCB) ELISA kit was used for quantitative detection of β-synuclein in the human brain lysates. 30ug of total brain homogenate per patient was assayed by ELISA for β-synuclein immunoreactivity. Samples were added to the plate and incubated for 30 mins at

37°C. Following washing HRP conjugated anti-β-synuclein secondary antibody was added to the samples and incubated for 30 mins at 37°C. Subsequent washing was followed by the addition of the TMB substrate and the plate was shaken for 30 seconds before being incubated at 37°C for 15 minutes. Once the reaction was terminated, absorbance was read (450nm) on the FLUOstar Omega plate reader (BMG labech).

2.2: In vitro work

2.2.1: Cell culture maintenance: BE(2)-M17 cells and Hela cells

Cells were grown in Dulbecco's Modified Medium (DMEM) (Gibco) with 10% fetal bovine serum (FBS)/ 5% penicillin/streptomycin and incubated at 37ºC with 5% CO_2. Media was changed every 48-72 hours.

Hela cells were gratefully received from Dr. Shouqing Luo's lab - Peninsula School of Medicine, University of Plymouth.

2.2.2: Cell culture maintenance: Inducible N27 (iN27) cells

N27 rat dopaminergic neuronal cells were grown in Roswell Park Memorial Institute (RPMI) media with 10% fetal bovine serum (FBS), 1% Geneticin® reagent, G418 and 0.4% hygromycin antibiotic. The media was changed every 48 hours.

N27 cells were gratefully received from Prof Kim Tieu's lab - Florida International University (FIU) Miami, Florida.

2.2.3: Induction of human α-synuclein in iN27

The expression of human α-synuclein was under the regulation of two vectors: vector pEGSH is PonA inducible and consists of SP1 and minimal heat shock promoter. PERV3 is under the CMV promoter, a positive control reporter.

Cells were treated with 1mM PonA to induce expression of human α-synuclein (h-α-synuclein), 24 hours post plating. Control cells were treated with the equivalent volume of 100% ethanol. Cells were collected at 24, 48 and 72 hours post induction (h.p.i) of human α-synuclein (Figure 17) for western blot or ICC.

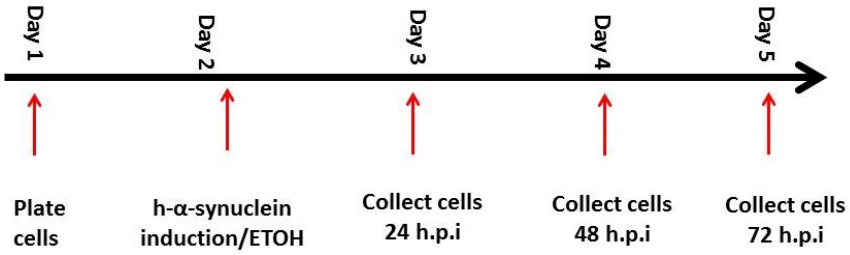


Figure 17 - Time course for human-α-synuclein expression in iN27 cells

Time course schematic of human-α-synuclein gene expression in iN27 cells. Gene is induced 24 hours post plating of cells and cells are harvested at 24 hpi, 48 hpi & 72 hpi.

2.2.4: Immunocytochemistry

Cells were plated on coverslips coated with 50ug/ml poly-L-lysine (PPL) (Sigma, P1399) on day 1 at a density of 7 X 10^4 in a 24-well plate. Upon collection of cells at various time points, cells were fixed with 4% paraformaldehyde for 15 minutes, washed with PBS and blocked with 5% of appropriate serum for 1 hour. The fixed cells were incubated overnight with primary antibodies at +4ºC (Table 3). Following appropriate washing, the fixed cells were incubated with both Alexa Fluor conjugated secondary antibodies (Table 4) and hoerscht [1/1000], for nuclei staining. After appropriate washing, cover slips were mounted onto slides with Perma Fluor. All washes were performed with PBS and antibodies diluted with PBS + 0.03% Triton X. 3 repeats were performed unless otherwise stated.

2.2.5: Scoring β-synuclein positive or negative cells for human-α-synuclein levels

The detection of h-α-synuclein (Syn204) immunofluorescent intensity was analysed using FIJI - Image J software (NIH, USA). Cells that were positive and negative for β-synuclein were examined for Syn204 immunoreactivity and the corrected total cell fluorescence (CTCF) was expressed in arbitrary units (AU). CTCF = integrated density – (area of selected cell × mean fluorescence of background readings). Subtraction of any background intensity was applied. In order for β-synuclein positive cells to be considered, a threshold of a mean-grey intensity density in excess of >200 AU (arbitrary units) for the region of interest (ROI) was set or the cells were excluded from the analysis. A minimum of 100 cells per group were analysed from 3 experimental repeats.

2.2.6: DNA transfection

Cells were grown in Roswell Park Memorial Institute (RPMI) media with 10% fetal bovine serum (FBS), 1% Geneticin® reagent, G418 and 0.4% hygromycin antibiotic. Cells were plated on day 1 and treated with β-synuclein (N27: 0.5µg & Hela: 0.25µg) (jetPRIME® polyplus DNA) on day 2 and media was changed after 4 hours.

2.2.6.1: Hela cells on day 4 cells were harvested and lysed with NP40 and a protease inhibitor in preparation for western blot analysis or fixed. Three repeats were performed.

2.3: Microscopy

Human brain fluorescent imaging was performed on the Leica TCS SP8 microscope; in vitro imaging was conducted on the Zeiss LSM 510meta or Leica TCS SP8 microscope and Brightfield images were taken on the Leica MC170HD microscope.

2.4: Statistics

Statistical analysis was performed using the analysis of variance (ANOVA) and/or the Student's t-test. Bonferroni posthoc test was applied where necessary. A 95% confidence interval was applied $P< 0.05$ (*), $P< 0.01$ (**) and $P<0.001$ (***). GraphPad Prism (version 5.01) software was used to perform statistical analysis and graphical representations of the data.

2.5: Primary and secondary antibody concentrations (see table 1 & 2)

2.6: Reagents

All reagents and chemicals were from ThermoFisher (UK) unless otherwise stated.

	Technique	Dilution	Supplier
Rabbit anti- β-synuclein	Western blot	1/1000	Abcam (ab76444)
	ICC	1/250	
	IHC	1/250	
Mouse anti-β-synuclein	ICC	1/250	Millipore (36-009)
Mouse anti-α-synuclein (Syn1)	Western blot	1/1000	BD Biosciences (610787)
	ICC	1/250	
Mouse anti-oligomeric alpha-synuclein (5G4)	IHC	1/2000	Millipore (MABN389)
Mouse anti-α-synuclein (Syn204)		1/500	Abcam (ab3309)
Mouse-anti-beta actin	Western blot	1/5000-10,000	Sigma, A5441
Rabbit anti-LC3B	Western blot	1/1000	Novus Biologicals (NB100-2220)
	ICC	1/500	
	IHC	1/500	
Rabbit anti-p62	Western blot	1/1000	MBL, PM045, 1/1000
	IHC	1/500	
Rabbit anti-LIMP2	Western blot	1/1000	ThermoFisher (PA5-20540)
	IHC	1/500	
Mouse-anti-synaptophysin	Western blot	1/5000	Cell signaling (12270)
Mouse anti-SNAP25	Western blot	1/2000	Abcam
	IHC	1/500	
Rabbit anti-VAMP2	Western blot	1/500	Cell signalling (14811)
	IHC	1/500	
Mouse anti-syntaxin	IHC	1/500	Gifted by Bristol labs
Mouse NeuN	WB	1/500	Gifted by Dr Bing Hu (UoP)

Table 3 - Primary antibodies and technique dependent concentrations

Secondary Antibody	Technique	Dilution	Supplier
Alexa Flour 488 - conjugated to anti-mouse IgG antibody	IHC/ICC	1/1000	Invitrogen Molecular Probes
Alexa Flour 488 - conjugated to anti-rabbit IgG antibody	IHC/ICC	1/1000	Invitrogen Molecular Probes
Alexa Flour 568 - conjugated to anti-mouse IgG antibody	IHC/ICC	1/1000	Invitrogen Molecular Probes
Alexa Flour 568 - conjugated to anti-rabbit IgG antibody	IHC/ICC	1/1000	Invitrogen Molecular Probes
HRP - conjugated to anti mouse IgG	WB	1/10,000	Amersham, ECL
HRP - conjugated to anti rabbit IgG	WB	1/10,000	Amersham, ECL
Biotinylated Goat Anti-Mouse IgG	IHC	1/200	Vector laboratories (BA-9200)
Biotinylated Goat Anti-Rabbit IgG Antibody	IHC	1/200	Vector laboratories (BA-1000)

Table 4 - Secondary antibodies and technique dependent concentrations

3.1: AIMS OF THE PROJECT

α-synuclein has been extensively examined in α-synucleinopathies, with modified and aggregated α-synuclein species being a primary feature of LBs and LNs. Moreover, aggregated α-synuclein appears to affect SNARE protein complex formation and function and the autophagy process of protein degradation. Family member, β-synuclein has been largely left unexplored. It is feasible that changes in β-synuclein protein levels may decrease its innate anti-fibrillisation effect on α-synuclein. These changes may be crucial for processes underlying the pathological function of α-synuclein, such as synaptic failure and protein degradation defects. If β-synuclein levels are diminished the pursuance of α-synuclein aggregation in DLB may proceed unabated.

3.1.1: Aim 1 of this project is to examine levels and the distribution of β-synuclein in the DLB brain. The pathological spread of LBs is seen throughout the brain and in order to fully elucidate the role of β-synuclein, DLB pathologically relevant brain regions were examined for β-synuclein changes, to be correlated with any changes in α-synuclein species.

3.1.2: Aim 2 is to examine key SNARE protein distribution in DLB brain tissues and to correlate this with changes in β-synuclein. It is possible that β-synuclein may take on a compensatory role in the event of loss of a monomeric α-synuclein function.

3.1.3: Aim 3 is to study a role of β-synuclein in the autophagy-lysosomal protein degradation pathway. For this, I will examine autophagy-lysosomal markers in human DLB tissues and in vitro models of β-synuclein overexpression and a stable cell line expressing the mRFP-GFP-LC3 tandem construct. This data will be

correlated with β-synuclein. Changes in autophagy are observed in neurodegenerative conditions, including DLB and mutant β-synuclein has been shown to induce lysosomal dysfunction. Clearance of aggregates or toxic proteins is critical to cell survival and therefore, the examination of β-synuclein in relation to key autophagy related proteins is necessary in the human brain.

Results chapter 4: β-synuclein and α-synuclein in the human brain

β-synuclein is an abundant pre-synaptic protein seen throughout most regions of the human brain. There is evidence supporting a neuroprotective role for β-synuclein with its ability to prevent fibrillisation of α-synuclein [124], reduce the rate of mutant α-synuclein fibril formation [179] and reduce the number of α-synuclein inclusions and associated motor deficits in a mouse-PD model [178]. Whilst β-synuclein and α-synuclein have a similar pattern of distribution throughout the brain, when compared to α-synuclein, mRNA expression levels of β-synuclein are more elevated in the cortex and cerebellum in the control brain [133]. In contrast, in DLB there is a decrease in some cortical regions of β-synuclein mRNA [98]. Due to the neuro protective qualities bestowed by β-synuclein, a decline in its protein levels will enable the pursuance of α-synuclein aggregation and the formation of LBs and LNs, promoting the neuropathology associated with DLB. This study aimed to identify any changes in the levels and distribution of the β-synuclein protein in the DLB human brain when compared to the control. The changes were correlated with α-synuclein protein levels thereby enabling the elucidation of synuclein proteins modifications in DLB.

4.1: β-synuclein and α-synuclein protein levels in the human brain

The frontal cortex (BA9), occipital cortex (BA18/19) and hippocampal regions were examined. These areas were selected due to the neocortical and subcortical degeneration observed in neurodegenerative conditions, including DLB [61, 255].

4.1.1: Changes in levels of total β-synuclein, α-synuclein and oligomeric α-synuclein protein in the human brain

The available literature relating to β-synuclein protein levels in the human brain is limited. It is reported that β-synuclein is reduced in the frontal cortex in pDLB and in the temporal cortex of DLB with concomitant AD pathology [98], no data for β-synuclein has been reported for the occipital cortex or hippocampus in DLB. Total protein levels in human brain tissue lysates from cortical and paralimbic regions were studied by western blot or ELISA in the frontal cortex, occipital cortex and hippocampus of controls and DLB patients.

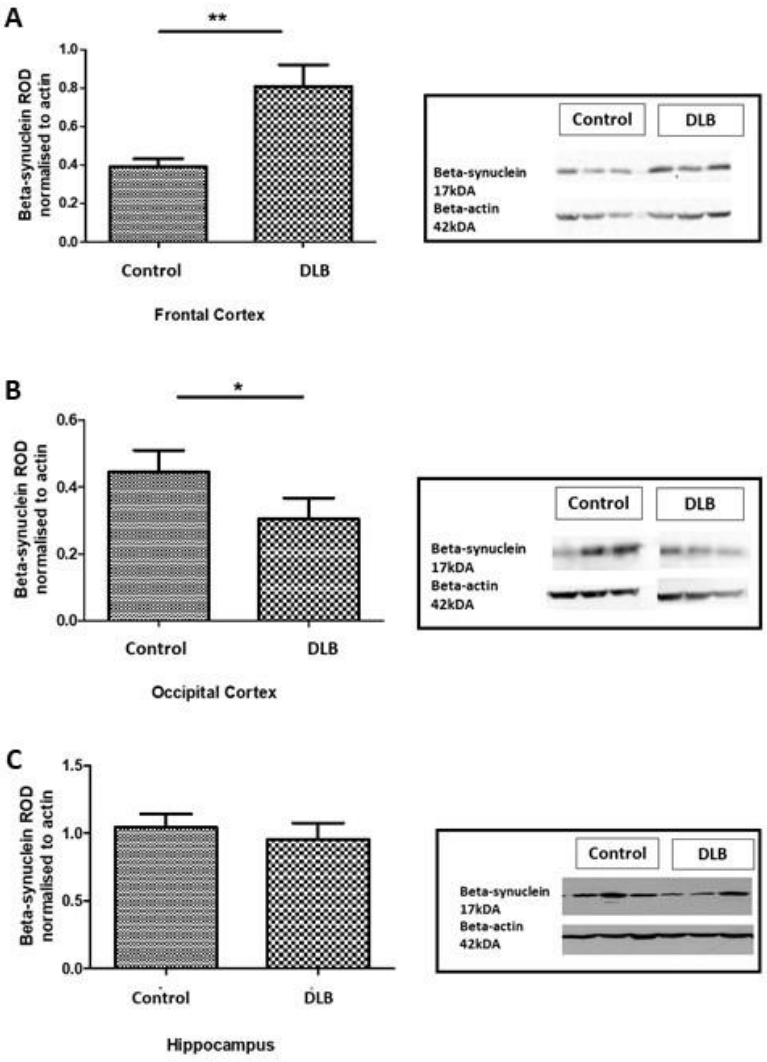


Figure 18 - β-synuclein protein is altered in the DLB brain

Western blot analysis of β-synuclein in the frontal cortex (A): control (n= 9), DLB (n= 17); occipital cortex (B): control (n= 10), DLB (n= 18) and the hippocampus (C): control (n= 4), DLB (n= 5). In DLB β-synuclein is increased in the frontal cortex (P=0.006) and decreased in the occipital cortex (P=0.03). Statistical analysis was performed by the Student's two-tailed t-test. *: P=<0.05; **: P=<0.01; ***: P=<0.001.

Examination of β-synuclein in the cortical regions and hippocampus shows a significant increase in β-synuclein levels in the frontal cortex of DLB patients (Figure 18-A) (P=0.006) and conversely, a significant decrease observed in the occipital

cortex (Figure 18-B) of DLB patients (P=0.03) when compared to controls. There are no changes in β-synuclein in the hippocampus (Figure 18-C) although, overall the hippocampus appeared to have higher levels of β-synuclein when compared to the cortical regions. Examination of monomeric α-synuclein protein levels was next performed in the same regions as β-synuclein in order to correlate any changes.

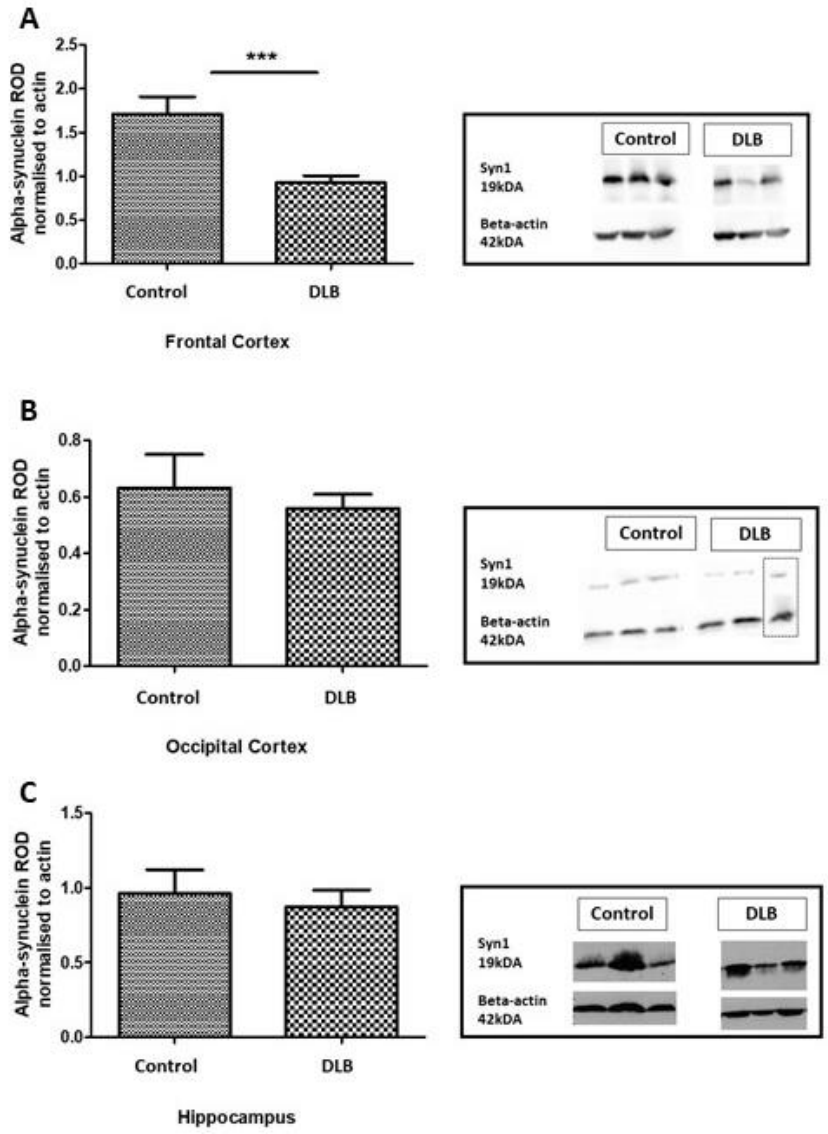


Figure 19 - Monomeric α-synuclein is reduced in the frontal cortex of the DLB brain

Western blot analysis of α-synuclein in the frontal cortex (A): control (n= 12), DLB (n= 23); occipital cortex (B): control (n= 10), DLB (n= 17) and the hippocampus (C): control (n= 4), DLB (n= 5). α-synuclein is decreased in the frontal cortex of DLB brains (P=<0.001). Statistical analysis was performed by the Student's two-tailed t-test. *: P=<0.05; **: P=<0.01; ***: P=<0.001.

The examination of α-synuclein in the frontal cortex (Figure 19-A), occipital cortex (Figure 19-B) and hippocampus (Figure 19-C) showed a significant decrease in the

frontal cortex of DLB patients (A) (P<0.001) however, no changes were observed in the occipital cortex (B) or the hippocampus (C).

In order to identify whether changes in β-synuclein are related to the presence of pathological α-synuclein species, the levels, of oligomeric α-synuclein, as detected by the 5G4 antibody, were quantified in these regions by ELISA.

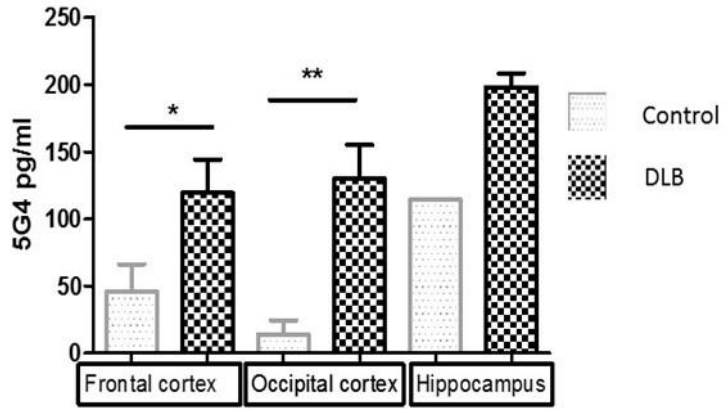


Figure 20 - Oligomeric α-synuclein is increased in the frontal and occipital cortex in the DLB brain

Enzyme-linked immunosorbent assay (ELISA) of human brain lysates from fresh frozen brain tissue. Immuno-detection of oligomeric α-synuclein (5G4) was detected in the frontal cortex: control (n= 5) and DLB patients (n= 16). 5G4 immunoreactivity is increased in DLB in the frontal cortex (P=0.03); the occipital cortex: control (n= 6) and DLB patients (n= 20) with an increase of 5G4 observed in DLB (P=0.004) in DLB. Examination of the hippocampus: control (n= 1) and DLB (n= 9) revealed no changes in 5G4. Absorbance was measured at 450nm reference 620nm. Absorbance data was converted to total protein concentrations (pg/ml) by correlating the absorbance with a standard curve of known concentrations. Statistical analysis was performed by one-way-ANOVA and Bonferroni post hoc test. *: P=<0.05; **: P=<0.01; ***: P=<0.001.

A significant increase in 5G4 immunoreactivity was observed in the frontal cortex of the DLB patients (P=0.03) and the occipital cortex of DLB patients (P=0.004) (Figure 20). No significant difference was observed between the frontal cortex and occipital cortex in DLB. Statistical analysis on the hippocampus was not performed as the control number was set at 1 patient.

4.1.2: *Correlations between β-synuclein and α-synuclein or oligomeric α-synuclein and β-synuclein and disease duration*

The data in this study shows an increase in β-synuclein in the frontal cortex (Figure 18-A) that correlates with a decrease in monomeric α-synuclein (Figure 19-A) and an increase in oligomeric α-synuclein (Figure 20). Conversely, in the occipital cortex a decrease in β-synuclein (Figure 18-B) is associated with an increase in oligomeric α-synuclein (Figure 20) but no change in monomeric α-synuclein (Figure 19-B). Transcriptional interplay between the synucleins is reported, decreased levels of one of the synucleins may lead to a transcriptional increase of the other [99]. I next wanted to examine the relationship between β-synuclein and α-synuclein at a protein level in the frontal and occipital cortex where modifications have been observed. Further, I aimed to explore whether the changes in β-synuclein levels may be associated with the duration of pathology. Regression analysis was performed using the western blot ROD values for Syn1 and β-synuclein and/or ROD β-synuclein in accordance with ELISA values for 5G4 and β-synuclein was correlated with disease duration as described in the patients' ID (Table 2).

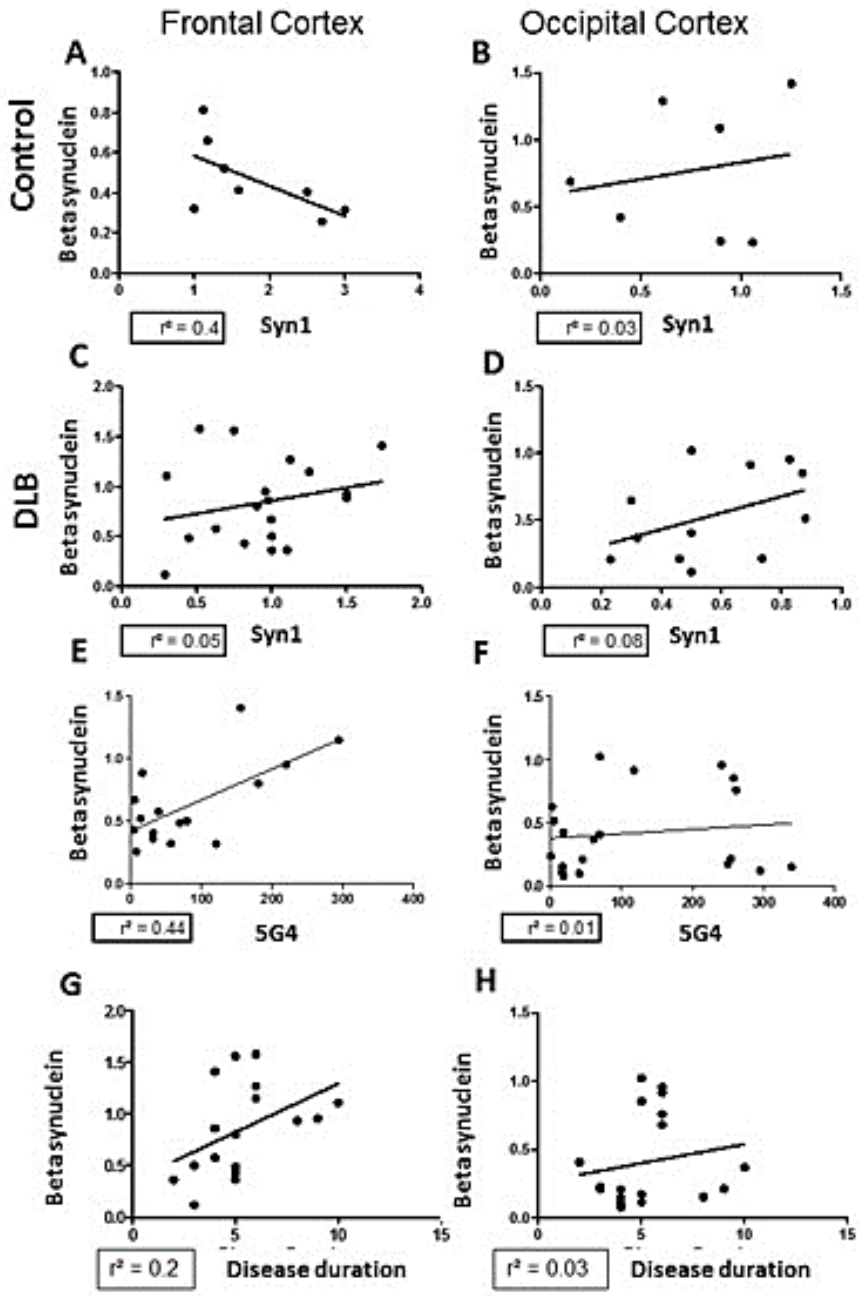


Figure 21 – In the frontal cortex a correlation exists between β-synuclein and α-synuclein and β-synuclein with disease duration.

Linear regression analysis: α-synuclein and β-synuclein ROD values in the Controls and DLB frontal cortex (A & C): (n= 8 & n= 19, respectively; occipital cortex (B & D) (n= 7 & n=12, respectively). β-synuclein (ROD) and 5G4 (pg/ml) in the frontal cortex (E) (n= 16) and the occipital cortex (F) (n= 20). β-synuclein (ROD) and disease duration (years) in the frontal cortex (G) (n= 17) and the occipital cortex (H) (n= 16).

Linear regression analysis on β-synuclein and α-synuclein, as detected by Syn1, identified a negative correlation between β-synuclein and syn1 in the frontal cortex (Figure 21-A) of the controls (R^2 = 0.4); this was indicative of high levels of β-synuclein correlating with low levels of α-synuclein. Correlation analysis between β-synuclein and 5G4 levels (Figure 21-E-F) reveal a significant positive relationship in the frontal cortex (R^2 = 0.44) (Figure 21-E). Higher levels of oligomeric α-synuclein positively correlated with higher levels of β-synuclein but no other significances were observed. In addition, analysis of β-synuclein in relation to disease duration in the frontal cortex showed a low-moderate positive correlation (Figure 21, G) (R^2 = 0.2), this effect was lost in the occipital cortex.

4.2: The distribution of β-synuclein in the frontal cortex, occipital cortex and hippocampus of controls and DLB patients

Following the identification of alterations in the synuclein protein levels, the β-synuclein pattern of immunostaining was explored in the frontal cortex, occipital cortex and hippocampus. Despite no changes in α-synuclein or β-synuclein having been observed in the hippocampus, intergroup variation was seen. In addition, this region physiologically has high levels of β-synuclein [76] and DLB patients often have concomitant AD pathology, a disease that sees extensive hippocampal degeneration. Examination of the control brains was undertaken in the first instance to provide a background for comparison to the DLB brain.

4.2.1: β-synuclein in the normal human brain

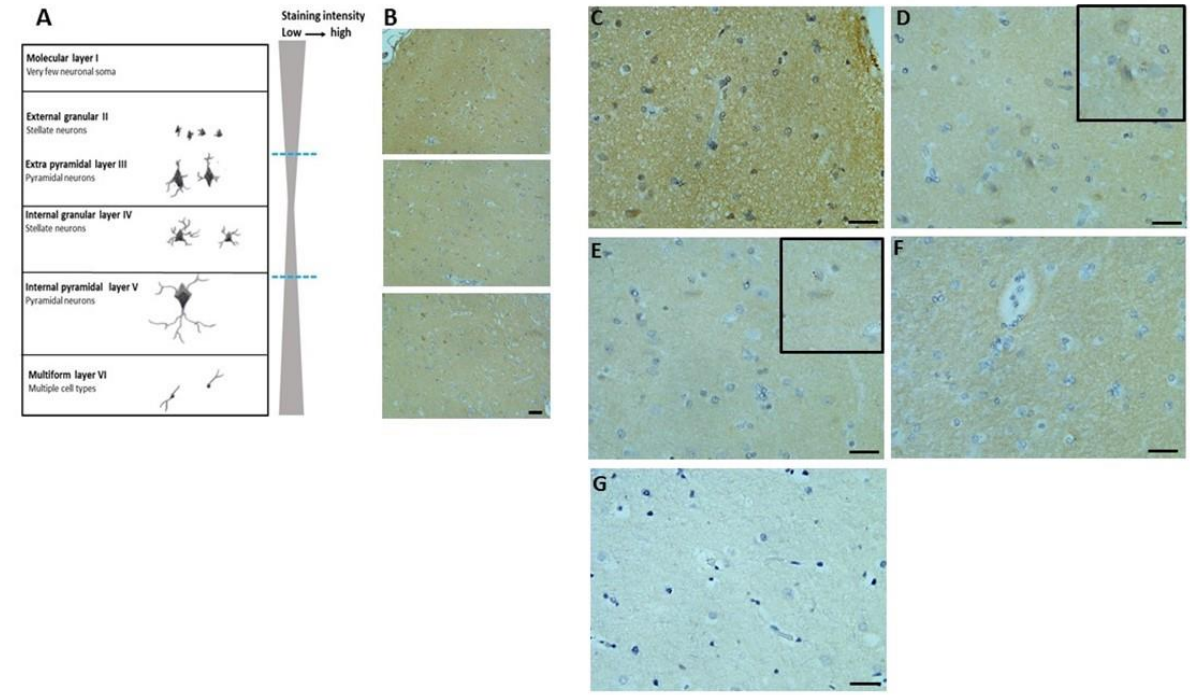


Figure 22 - β-synuclein in the frontal cortex of the control brain

Immunoreactivity for β-synuclein in the frontal cortex of the control brain (B – F) revealed by DAB substrate. Diagrammatic representation of cortical layers (A) and images captured by Brightfield microscopy moving from layer I to deeper frontal cortical layers V/VI (B). Differential staining of β-synuclein demonstrated by sequential images taken through the frontal cortical region (C-F) from Layer I/II (C) to deeper layers V/VI (F). Lowest staining intensity is depicted by the area between blue dotted lines (A). Non-Primary antibody control (G). N=6 control brains examined for the frontal cortex. Brightfield microscopy and magnification X40, scale bar 20μm (C-H) magnification X20, scale bar 50μm (B).

A β-synuclein immunopositive signal was detected throughout all layers (I to VI) in the frontal cortex of the control brains (Figure 22-B-F). Staining presented in a uniform manner with small granular speckles representing the neuropil. Although primarily homogenous, the staining was more prominent in the granular layer II

(Figure 22-B-C) and the deeper cortical layers V/VI (Figure 22-B & F). There appeared to be strong β-synuclein immunoreactivity around some neurons (Figure 22-D & E, enlarged).

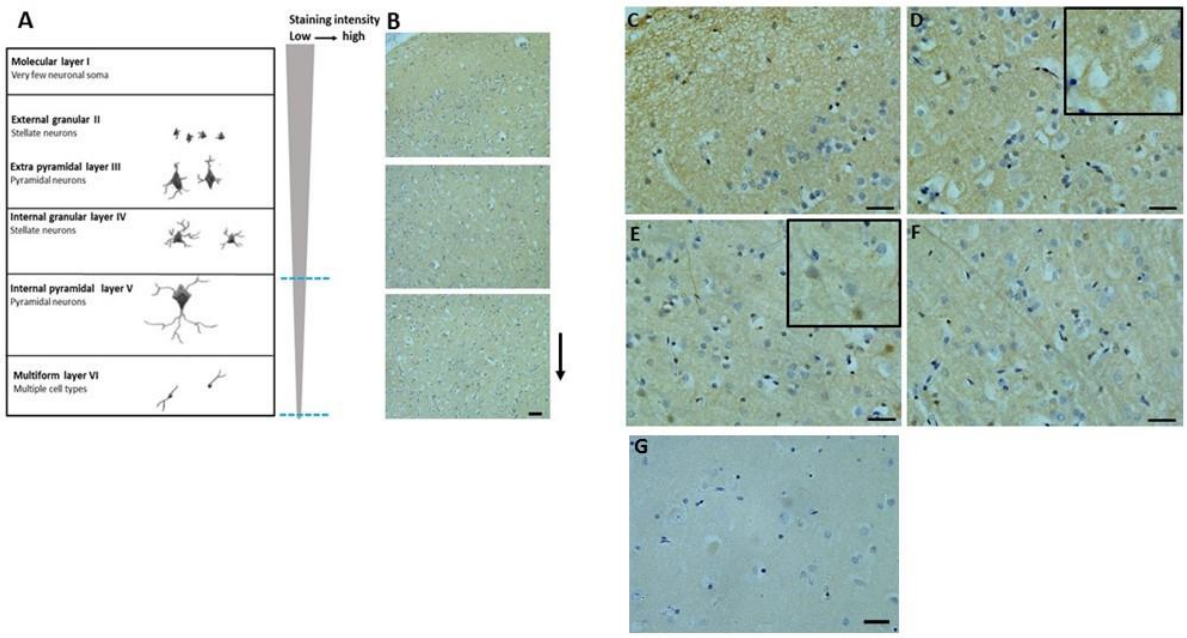


Figure 23 - β-synuclein in the occipital cortex of the control brain

Immunoreactivity for β-synuclein in the frontal cortex of the control brain (B – F) revealed by DAB substrate. Diagrammatic representation of cortical layers (A) and images captured by Brightfield microscopy moving from layer I to deeper occipital cortical layers V/VI (B). Differential staining of β-synuclein is demonstrated by sequential images taken through the occipital cortical region (C-F) from Layer I/II (C) to deeper layers V/VI (F). Lowest staining intensity is depicted by the area between blue dotted lines (A). N=6 control brains examined for the occipital cortex. Non-primary antibody control for DAB reactivity (G). Brightfield microscopy and magnification X40, scale bar 20μm (C-F) magnification X20, scale bar 50μm (B).

Examination of the pattern of β-synuclein immunoreactivity in the occipital cortex of the control brain showed that β-synuclein was present in all layers - layer I to VI

(Figure 23-B-F). The intensity of staining appeared more intense in layers II-IV (Figure 23-C-E) when compared to the deeper cortical layers V and VI (Figure 23-F). Darker regions of speckled staining around some neurons were observed throughout all layers (Figure 23-D & E, see enlargements).The comparison between the frontal cortex and occipital cortex showed that the level of intensity of β-synuclein immunoreactivity appears to be lower in the occipital cortex of the control brain.

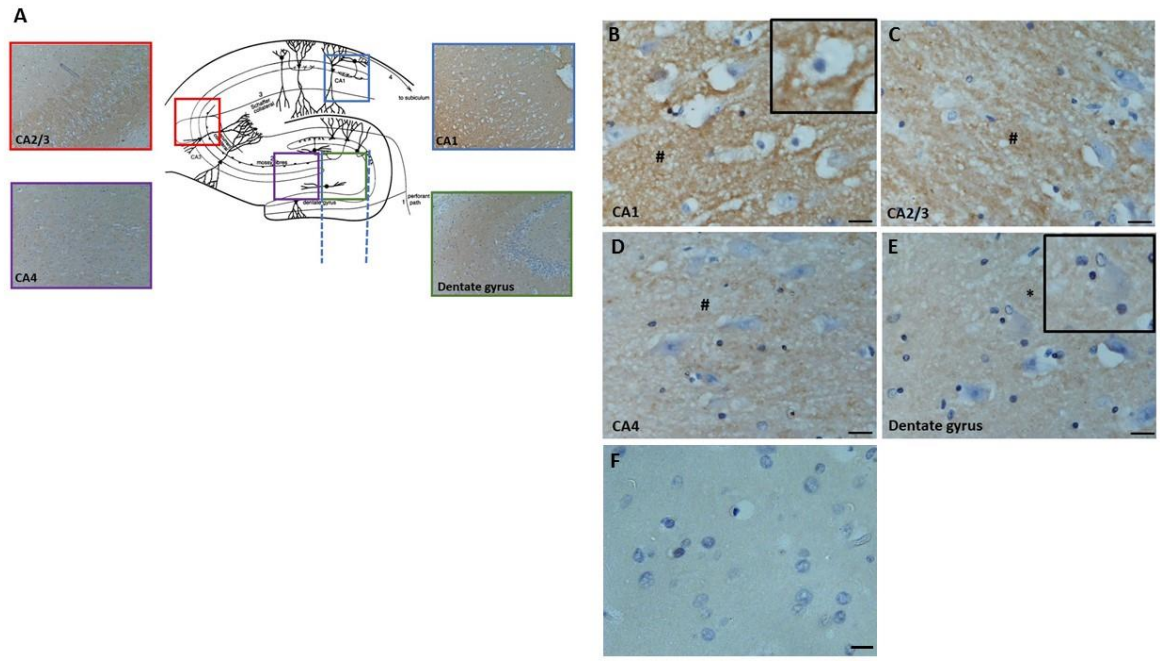


Figure 24 - β-synuclein in the hippocampus of the control brain

Immunoreactivity for β-synuclein in the hippocampus of the control brain (A – E) revealed by DAB substrate. Immunoreactivity was detected in the CA1 (B), CA2/3 (C), CA4 (D) and dentate gyrus (E). The regional depiction of β-synuclein in the hippocampus of the control brain (A) - CA1 (red), CA2/3 (purple), CA4 (blue) and dentate gyrus (green). β-synuclein is observed in the neuropil (#). Lowest staining intensity depicted by the area between blue dotted lines (A). Non-primary antibody control for DAB reactivity (F). Brightfield microscopy and magnification X10 (A). Magnification X40, scale bar 20μm (B-E). N=6 control brains examined for the hippocampus. Hippocampal region drawing – Adapted from [256].

Examination of β-synuclein was undertaken in the hippocampus of a control brain, in CA1 (Figure 24-B), CA2/3 (Figure 24-C), CA4 (Figure 24-D) and dentate gyrus (Figure 24-E). Differential regional intensities of immunoreactivity were observed in the hippocampus, with the CA1 having the most prominent staining (Figure 24-B) and the dentate gyrus, the lowest level of β-synuclein immunoreactivity (Figure 24-E). A strong distribution pattern of β-synuclein was observed around the perimeter of neurons in the CA1 region (Figure 24-B, enlarged) and varying sized granules speckled throughout the neuropil of differing staining intensity (B, #).

Collectively, the pattern of β-synuclein reactivity differed between the cortical regions and the hippocampus in the control brains; there was a unique, more granular and intense staining identified in the hippocampus when compared to the neocortex. Observations of some positively stained neuronal somata were seen in the cortex of the control brains. However, these were absent in the hippocampus, indicative of variance in β-synuclein protein distribution patterns between these regions.

4.2.2: β-synuclein in the DLB brain

Examination of the pattern of β-synuclein immunoreactivity was performed on the DLB patients in the frontal cortex, occipital cortex and hippocampus.

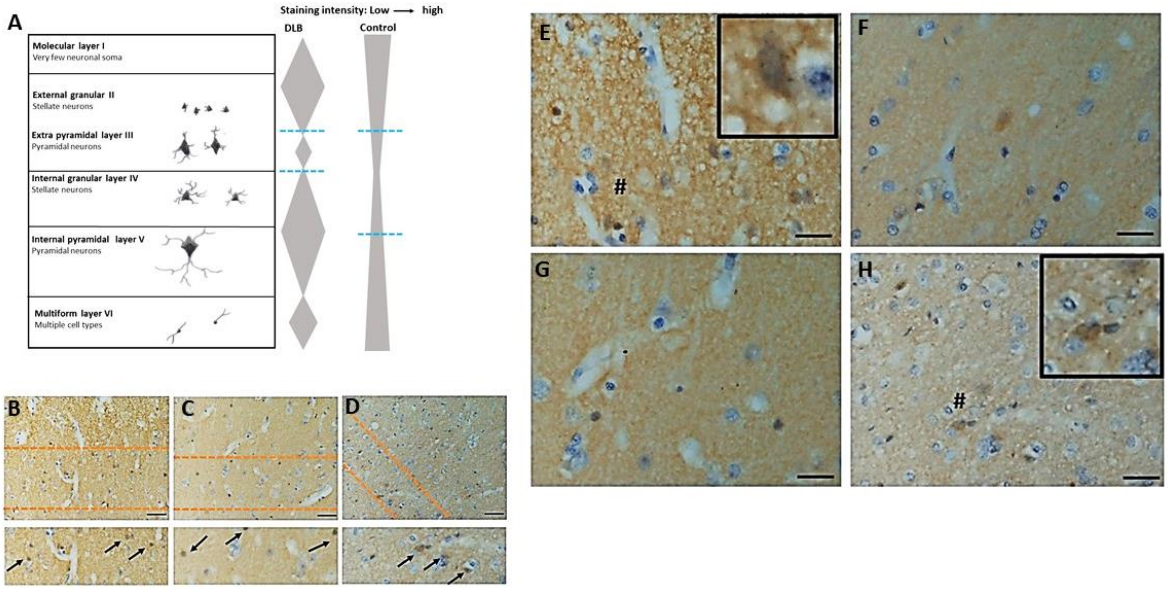


Figure 25 - β-synuclein is re-distributed in the frontal cortex of the DLB brain

Immunoreactivity for β-synuclein in the frontal cortex of the DLB brain (B – F) revealed by DAB substrate. Diagrammatic representation of cortical layers (A) and images captured by Brightfield microscopy moving from layer II (B) to deeper frontal cortical layers V/VI (D). The Orange dotted line represents the enlarged region. The arrows depict single stained neurons. Differential staining of β-synuclein is demonstrated by sequential images taken through the occipital cortical region (E-H) from Layer I/II (E) to deeper layers V/VI (H). Lowest staining intensity is depicted by the area between blue dotted lines (A). N=6 DLB brains examined for the frontal cortex. Pre-absorption of β-synuclein antibody was performed to validate the specificity of β-synuclein immunoreactivity (Figure 15). Non-primary antibody control for DAB reactivity (Figure 22-G). Brightfield microscopy and magnification X40, scale bar 20µm (E-H) magnification X20, scale bar 50µm (B-D)

A β-synuclein immunopositive signal was detected throughout all layers (I to VI) of the DLB brain (Figure 25-B-H). The staining was not homogenous throughout the layers; immunoreactivity was more intense in layers II (E) and deeper layers IV/V (G). The neuropil was variable in immunoreactivity and there was frequently

neuronal soma positive for β-synuclein immunoreactivity in DLB brains (Figure 25-B-D).

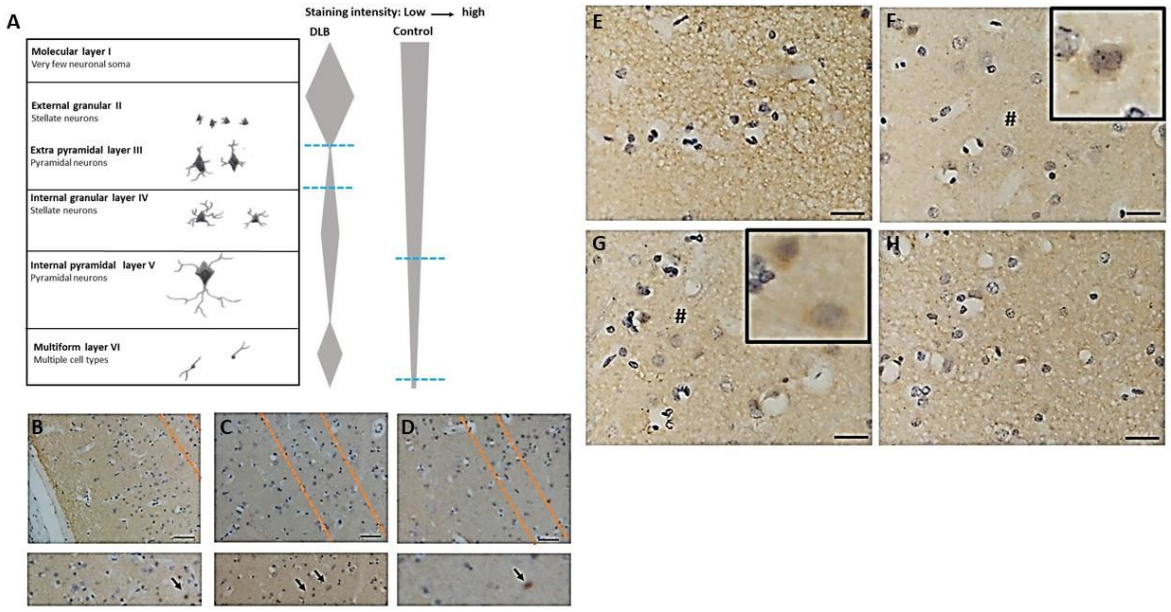


Figure 26 - β-synuclein is re-distributed in the occipital cortex of the DLB brain

Immunoreactivity for β-synuclein in the occipital cortex of the DLB brain (B – F) revealed by DAB substrate. Diagrammatic representation of cortical layers (A) and images captured by Brightfield microscopy moving from layer II (B) to deeper frontal cortical layers V/VI (D). The Orange dotted line represents the enlarged region. The arrows depict single stained neurons. Differential staining of β-synuclein is demonstrated by sequential images taken through the occipital cortical region (E-H) from Layer I/II (E) to deeper layers V/VI (H). Lowest staining intensity is depicted by the area between blue dotted lines (A). N=6 DLB brains examined for the occipital cortex. Pre-absorption of β-synuclein antibody was performed to validate the specificity of β-synuclein immunoreactivity (Figure 15) and non-primary antibody control for DAB reactivity (Figure 23-G). Brightfield microscopy and magnification X40, scale bar 20μm (E-H) magnification X20, scale bar 50μm (B-D)

Having examined β-synuclein in the occipital cortex of a DLB brain, it was revealed that β-synuclein is more intensely stained in layers II (Figure 26-E) and deeper layer VI (Figure 26-H). β-synuclein positive neurons are present throughout the layers (Figure 26-B-D, enlarged and arrows).

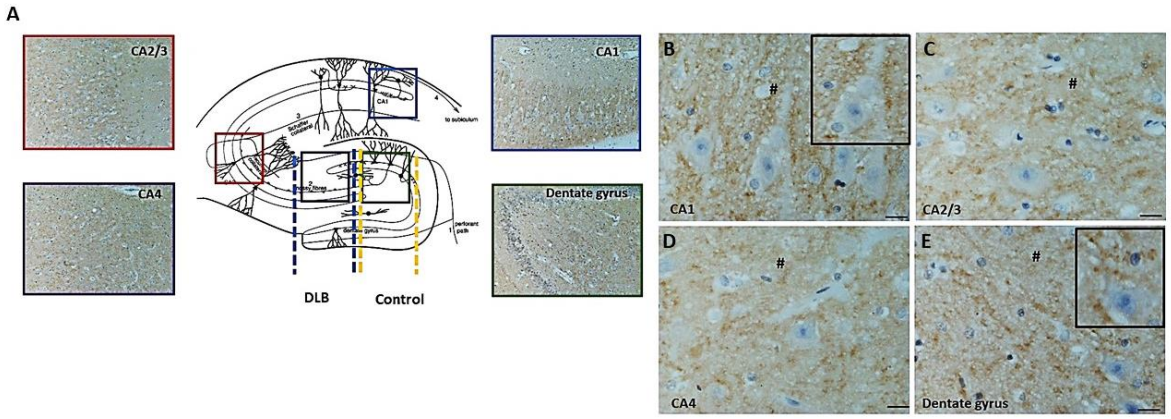


Figure 27 - Large β-synuclein positive granules are present in the hippocampus of the DLB brain

Immunoreactivity for β-synuclein in the hippocampus of the control brain (A – E) revealed by DAB substrate. Immunoreactivity was detected in the CA1 (B), CA2/3 (C), CA4 (D) and dentate gyrus (E). The regional depiction of β-synuclein in the hippocampus of the control brain (A) - CA1 (red), CA2/3 (purple), CA4 (blue) and dentate gyrus (green). β-synuclein is observed in the neuropil (#). Lowest staining intensity depicted by the area between blue dotted lines (A). N=6 DLB brains examined for the hippocampus. Pre-absorption of β-synuclein antibody was performed to validate the specificity of β-synuclein immunoreactivity (Figure 15) and non-primary antibody control for DAB reactivity (Figure 24-F). Brightfield microscopy and magnification X10 (A). Magnification X40, scale bar 20μm (B-E) Hippocampal region drawing – Adapted from [256].

The β-synuclein pattern of staining in the hippocampus of DLB patients (Figure 27-A-E) showed granular, multi-sized β-synuclein-positive speckles of varying degrees of intensity in the neuropil, throughout all regions shown for the hippocampus (Figure 27-B-E, #). There was frequently a clumping appearance throughout the neuropil. In the control brain (Figure 24), the highest level of reactivity was the CA1 (Figure 24-B) and the lowest observed in the dentate gyrus (Figure 24-E) however, in the DLB brain although, the highest immunoreactivity remained to be seen in the CA1 (Figure 27-B), the dentate gyrus (Figure 27-E) also produced noticeable levels of staining (Figure 27-E & enlarged). There was intense reactivity around large pyramidal neurons of the CA1 (B, enlarged), the other regions showed differential patterns of staining around neuronal soma, however, unlike the cortex there was no appearance of β-synuclein positive neuronal soma reactivity.

4.2.3: β-synuclein, α-synuclein or oligomeric alpha synuclein protein distribution in the human brain and β-synuclein positive cells.

I have seen changed patterns of β-synuclein immunoreactivity and protein levels in the cortical regions of the DLB patients. Moreover, a reduction in α-synuclein is observed in the frontal cortex in DLB, and a moderate correlation is found between β-synuclein and oligomeric α-synuclein in the same region. The neuropathological characteristic of DLB is the widespread distribution of Lewy bodies throughout the paralimbic and neocortical regions. Phosphorylation of serine 129 (ps129) of α-synuclein is extensively undertaken and well defined in α-synucleinopathies [110] with ps129 antibodies being commonly appropriated for Lewy body and Lewy neurite identification. More recently it has been established that pre-synaptic oligomers of α-synuclein may be more detrimental to the cell and Lewy bodies may be acting in a

protective manner by encapsulation of toxic oligomers. I next examined the regional morphological and distribution pattern of oligomeric-α-synuclein using the 5G4 antibody in the cortico-paralimbic areas of control and DLB brains. In addition, it is known that β-synuclein is able to prevent α-synuclein aggregation [178] but this protein is not observed in LBs or LNs. Therefore, examination of β-synuclein in the presence of monomeric or oligomeric α-synuclein was performed. I wanted to identify whether these proteins show mutually exclusive localisation “in situ” by performing a double fluorescent IHC. Moreover, in this study, IHC analysis reveals that β-synuclein immunopositive neuronal soma exists in the cortical regions. The presence of β-synuclein positive cells has not been previously reported, therefore, the examination of these immunopositive cells in the vicinity of oligomeric α-synuclein positive cells, was of particular importance, looking for any morphological changes or shifts in the distribution patterns of β-synuclein and oligomeric α-synuclein and observe the pattern of β-synuclein staining in and around cells that were positive for oligomeric α-synuclein (5G4). Having identified β-synuclein positive neuronal immunoreactivity in DLB and modifications in the levels of β-synuclein, I have then examined the number of β-synuclein positive cells in the cortical areas.

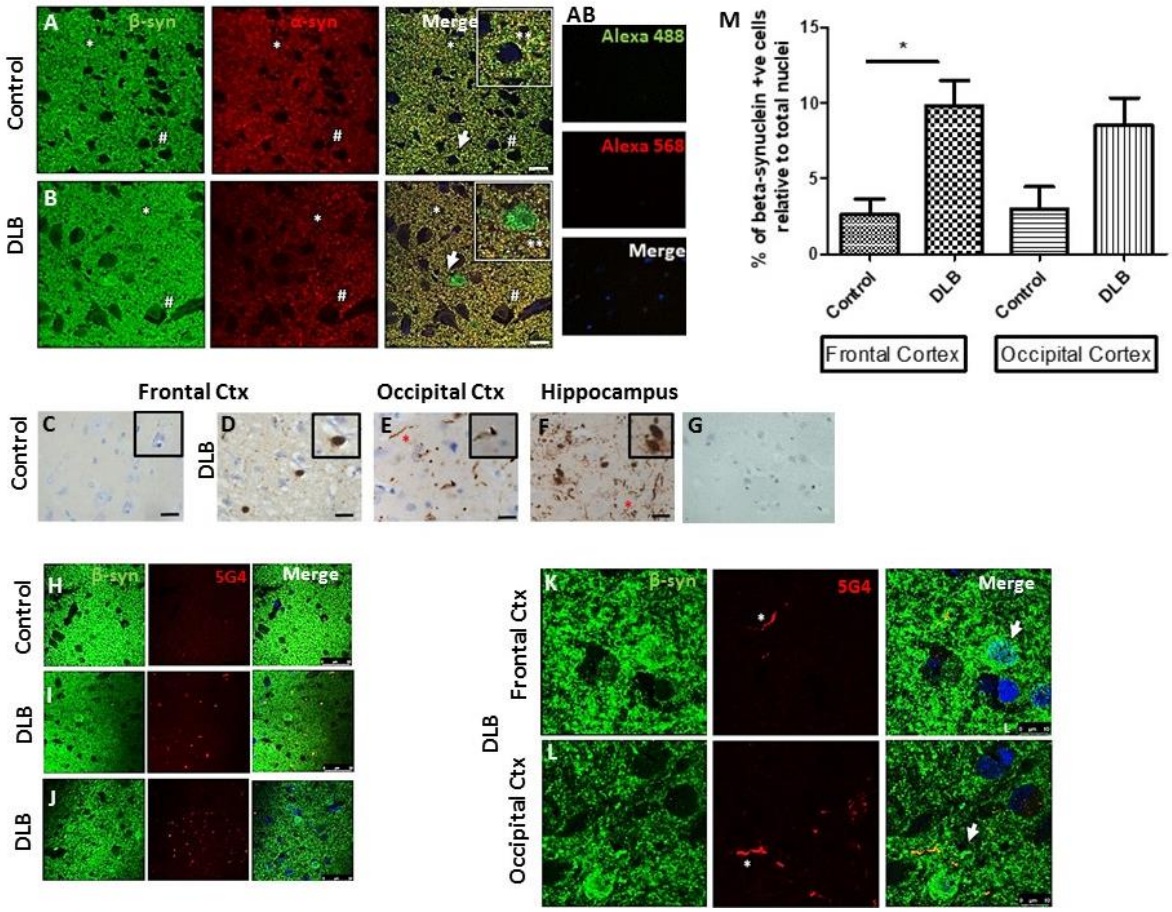


Figure 28 – β-synuclein positive cells are increased in the frontal cortex of the DLB brain and β-synuclein does not co-localise with oligomeric α-synuclein

Examination of β-synuclein (β-syn), α-synuclein (α-syn) and oligomeric α-synuclein (5G4) in control and DLB brains: double immunohistochemical examination of the frontal cortical regions of control (A) and DLB brains (B) detecting β-synuclein and α-synuclein (α-syn) reactivity. The neuropil-type pattern of the staining is marked with (*). Co-localisation is observed (A-B, **). Neuronal soma, positive for β-synuclein exclusive staining is observed in DLB (B, arrow & enlarged). Enlarged cell indicated by the arrow. N=3. Non-primary antibody controls performed (AB) and (Figure 14). Images A & B - Confocal microscopy, magnification X63. Scale bar 20µm.

Oligomeric α-synuclein (5G4) in the control frontal cortex (Frontal Ctx-C) (n=3), DLB (Frontal Ctx-D), DLB occipital cortex (Occipital Ctx-E) and DLB hippocampus (F) N=≥6 for DLB brains. Non-primary 5G4 antibody control (G). Brightfield microscopy, magnification X60. Scale bar

15µm. Double immunohistochemical examination of the frontal cortical regions of control (H) and DLB (I-K) and DLB occipital cortex (J-L), detecting β-synuclein and 5G4. Neuronal soma, positive for β-synuclein exclusive staining is observed (K & L, arrow). Multi-sized 5G4 deposits and filiform structures (K & L). Enlargements are shown by the arrow. N=≥3. Confocal microscopy, magnification X63. Scale bar 50µm (H-J) and (K-L) 10µm. β-synuclein positive cells were identified by confocal microscopy and expressed as a percentage of the total number of neurons visualised (M) ≥ 6 randomly selected regions from each control and DLB brain were examined to achieve a minimum total threshold of 100 neurons. There is a significant increase in β-synuclein positive neurons in the frontal cortex of DLB when compared to the controls (P=0.4) (M). Frontal cortex: control (n=8) and DLB (n=9); Occipital cortex: control (n=7) and DLB (n=8). Statistical analysis was performed by one-way-ANOVA and Bonferroni post hoc test. *: P=<0.05; **: P=<0.01; ***: P=<0.001. Data represents the mean and SEM.

As expected in the control (Figure 28-A) and DLB (Figure 28-B) tissue examination, β-synuclein and α-synuclein was primarily homogenous throughout the neuropil with small-medium sized puncta, observations include exclusive representation in the neuropil of both proteins as seen by green (β-synuclein) and red (α-synuclein) puncta. Co-localisation of both synucleins was observed throughout the neuropil (Figure 28-A & B, enlarged, **) and both β-synuclein and α-synuclein were located at the perimeter of the neurons, appearing as speckled borders (A & B, #) respectively. In the cohort of healthy control tissue sections and DLB, neuronal α-synuclein was not observed in the cytoplasmic form of neuronal inclusions; however, there were neuronal somata that stained immuno-positive for β-synuclein, these were devoid of α-synuclein (Figure 28-B, arrow & enlarged). In DLB the β -synuclein staining was evidently more intense in most of the DLB patients and frequently took on a more granular effect.

Examination of cortical regions and the hippocampus of control and DLB patients for 5G4 immunoreactivity (Figure 28-C-G) revealed notable distribution differences between regions and different morphological structures. In all regions examined the presence of intracytoplasmic inclusions was observed of differing shapes and sizes, reflecting differential distribution patterns of 5G4 stained aggregations. In the frontal cortex (Figure 28-D) was the appearance of typical cortical Lewy bodies, with a disorganised structure and dotted around were diffuse aggregates, small in size and with variable staining intensity. Additionally, faintly stained, Lewy neurite-like structures could be seen; these structures were short but may be representative of new Lewy neurite formations in progress.

In the occipital cortex (Figure 28-E) small-intermediate and mostly round structures that varied in size and staining intensity were observed. These smaller 5G4-positive aggregates were more abundant in the occipital cortex than the frontal cortex. Noted was the presence of longer filiform Lewy neurite-like 5G4 structures (E, *) that differed in staining intensity along the process and between processes. These structures were also more prominent in the occipital cortex than the frontal cortex.

The hippocampus (Figure 28-F) showed the greatest diversity of morphological immunoreactivity. There was a high burden of 5G4 immunoreactivity in this region with the presence of sizable Lewy bodies, irregular in shape and intensely stained. Surrounding and amongst the Lewy bodies were multiple structures of intermediate sized and smaller sized aggregates, irregular in shape (F, *) and were not diffuse throughout the hippocampus, rather the burden of the pathology showed few areas free from some form of oligomeric α-synuclein immunoreactivity. There were thick and longer Lewy neurites, compared to the cortex and multiple aggregates ranging in size. The staining intensity of the aggregates was variable, but overall the

hippocampus appeared to have the greatest burden of oligomeric α-synuclein pathology when examined by IHC.

Some immunoreactivity was also observed in the controls in the frontal cortex (Figure 28-C), this staining was minimal and appeared as small fuzzy edged structures (C, enlarged). It is possible that this may be indicative of the early signs of pathology in some patients.

When observing β-synuclein in the presence of 5G4 the DLB frontal cortex (Figure 28-I & K) and DLB occipital cortex (Figure 28-J & L) showed 5G4 reactivity for small aggregates and filiform α-synuclein structures in and throughout the neuropil of both the frontal cortex and occipital cortex (I, K & J, L, respectively). 5G4 immunoreactivity was absent in the control (Figure 28-H). There was no β-synuclein present in the cell bodies of any cells that were reactive for 5G4 although, in both regions, there was the presence of β-synuclein positive cells in the vicinity of 5G4 positive cells (Figure 28-K & L, arrow). Very rarely was co-localisation seen between β-synuclein and 5G4 in any of the sections examined. The appearance of 5G4 immunoreactivity was more prominent in the occipital cortex when compared to the frontal cortex with larger and thicker filiform structures that may be LN-type structures (K & L, *).

When examining the number of cells that were positive for β-synuclein in the frontal cortex and occipital cortex of DLB patients. An increase was detected in the frontal cortex of DLB when compared to the controls (P= 0.04)(Figure 28-M). Similar numbers of β-synuclein-positive cells were observed between the frontal and occipital cortex, with some patients showing greater variability.

4.3: Cellular location of the increase in β-synuclein

β-synuclein positive cells have been detected in addition to the increase in β-synuclein, revealed by the western blot. To determine whether this change is of a cytosolic or synaptic origin, I have isolated synaptosomes from the control and DLB tissue. Levels of β-synuclein were then examined in the synaptic rich fraction and cytosolic fractions of total lysate and compared to β-synuclein protein levels in the total lysate. This was performed in the frontal cortex/occipital cortex and β-synuclein levels were normalised to synaptophysin, a synaptic protein that is unchanged in DLB [209] or actin.

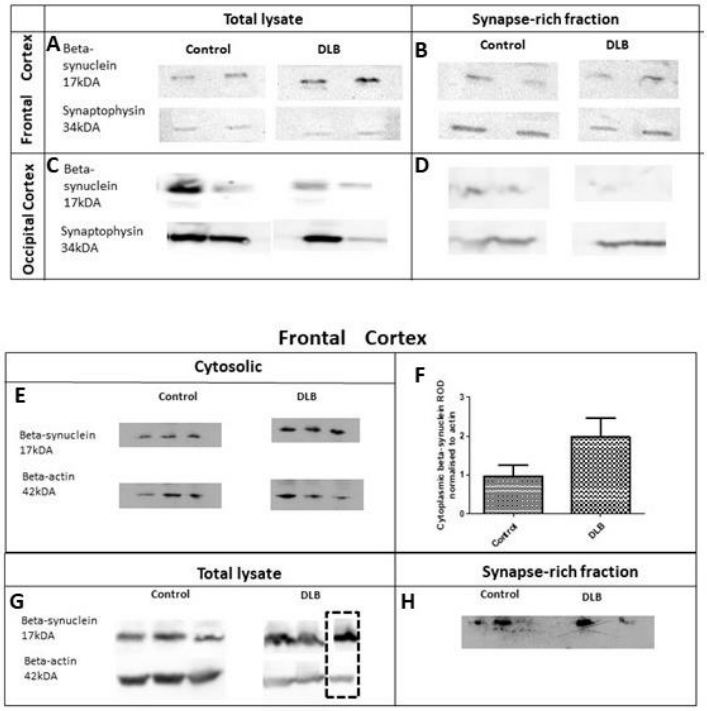


Figure 29 - β-synuclein protein changes may reflect changes in its cellular location

Western blot analysis of total lysates (A & C) compared to synapse-rich fractions (B & D) from human brain lysates with immuno-detection of β-synuclein and synaptophysin. Comparisons were made been control patients (≤3) and DLB patients (≤3) for the frontal cortex (A & B) and occipital cortex (C & D). Immunoblot images were collected by GSystems and data was analysed by measuring the relative optical density of the specific band on the immunoblot using FIJI-Image J software (NIH, USA) and presented as a ratio to the corresponding synaptophysin density. The frontal cortex cytosolic fraction (E) and synapse rich fraction of control (n=3) and DLB (n=3) were examined in patients with high β-synuclein levels in the total lysate (G). A trend for increased cytosolic β-synuclein is shown (F).

Analysis of the frontal cortex of DLB patients when compared to the controls, showed an increase in β-synuclein in the total lysate fraction (Figure 29-A & G), β-synuclein showed a differential synaptic presence among patients from increased, unchanged to absent at the synapse of DLB patients (Figure 29-B & H) and there was a trend towards an increase in the cytosolic fraction (Figure 29-E & F).

In the occipital cortex, total lysate levels of β-synuclein were decreased or unchanged (Figure 29-C) in the DLB patients when compared to the controls and there was an almost complete loss of β-synuclein at the synapse in this region for the DLB patients (Figure 29-D).

Chapter 4: Results discussion

The examination of total protein levels of α-synuclein and β-synuclein in the frontal cortex has shown an increase in β-synuclein (Figure 18) is associated with a parallel decrease in monomeric α-synuclein levels (Figure 19) but an increase in oligomeric α-synuclein (Figure 20). Although in the control frontal cortex, high β-synuclein negatively correlates with low α-synuclein (Figure 21), this effect was lost in DLB and replaced with a correlation between increasing levels of β-synuclein and oligomeric α-synuclein. The frontal cortex is variably affected in DLB with those patients with concomitant AD pathology, seeing greater synaptic loss [43]. In DLB fluctuating cognitive changes are considered a core symptom in the diagnosis of DLB. Therefore the cortical region is particularly important for cognitive function [257] such as executive decision-making planning and decision making [258].

In the occipital cortex, a decrease was observed in β-synuclein, although, while there was no change in α-synuclein levels there was an increase in oligomeric α-synuclein. Interestingly, for this region in controls and DLB, there were no significant correlations between β-synuclein and α-synuclein or oligomeric α-synuclein. Even though α-synuclein is unchanged in the occipital cortex; the oligomeric α-synuclein is increased; together, this produces increased levels of α-synuclein species overall for the occipital cortex. The levels of oligomeric α-synuclein in the occipital cortex of DLB brains are similar to that seen in the frontal cortex but in this regard, when comparing monomeric α-synuclein levels between regions, the levels of α-synuclein in the controls of the occipital cortex are approximately 50% less than the frontal cortex control, indicating regional differences under normal conditions. Changes related to the occipital cortex in DLB sets it apart from other dementias, including AD, due to the visual cortex being particularly affected [259] although Lewy bodies

are not abundant in the occipital cortex [27] there is hypometabolism [260] and occipital thinning [261]; thus, indicating pathological demise of the region is underway.

The cortical changes observed in α-synuclein and β-synuclein in DLB may reflect interaction on the transcriptional level, changes in protein stabilisation or protein turnover. If an increase in one of the synucleins occurs, there may be changes in relation to the stability of a family member. It is feasible that the synucleins employ mechanisms to re-address the synuclein protein balance. This may explain the increase in overall α-synuclein species levels in the occipital cortex leading to a decrease in β-synuclein protein levels. Moreover, although there is an increase in oligomeric α-synuclein levels in the frontal cortex there is a decrease in monomeric α-synuclein that may result in an increase in β-synuclein stability for this region that at some level is not affected by oligomeric α-synuclein levels. The pilot data from our laboratory show that mRNA levels of α-synuclein and β-synuclein are not changed in frontal cortex in DLB patients although, further work to progress and validate these findings is required.

IHC analysis of the β-synuclein pattern of distribution shows variations in the dissemination of the protein throughout the DLB brains in the cortex and the hippocampus (Figures 25-27). The differences in the pattern of re-distribution that is observed in the DLB brain in the cortical regions see the presence of large granules of β-synuclein in both the neuropil and around the neuronal perimeter, as opposed to small-medium homogeneous speckles primarily seen in the control brains (Figure 22-24). This provides evidence for changes of β-synuclein at the protein level that impact on its localisation status. These changes collectively may be representative of synaptic dysfunction or displacement in the presence of oligomeric α-synuclein at the

synapse. It is possible that either of these explanations see relocation as represented by protein “clumping.

The hippocampus is the anatomical region responsible for the consolidation of short term memories to long term memories, memory impairment is observed in DLB in addition to hippocampal atrophy, primarily in the CA1 region but to a much lesser extent than that seen in AD [262]. The hippocampus, shows a distinct morphological distribution when compared to the cortical regions, with a larger and intense staining around large pyramidal neurons observed in both the control and DLB, in particular, the CA1 region is most intensely stained in both groups however, DG accumulations are seen in DLB that are not present in the control brains. Interestingly, human wild type α-synuclein expressed in mice has been shown to impact on neurogenesis, in a negative manner, by reducing the survival rate of neuronal committed progenitors in the olfactory bulb and dentate gyrus [140]. It is possible that the presence of β-synuclein in this region is a neuroprotective effect; further, supported by the cell survival properties associated with β-synuclein under toxic conditions [180]. The dentate gyrus is an important location for neurogenesis; one could speculate that β-synuclein is preserving this process in the face of degenerating neurons in the presence of increasing pathology.

The hippocampus sees no modifications in β-synuclein or α-synuclein protein levels, a trend towards increased levels of oligomeric α-synuclein is observed in DLB for this region, also observed by oligomeric α-synuclein in the IHC analysis. The control also exhibited high levels of 5G4 and so statistical significance was not found in the ELISA assay. When considering this control patient, the clinical- pathological report indicates Braak stage 2 and ageing of this brain. Therefore, high levels of oligomeric

α-synuclein in the hippocampus of the control brain may be more representative of ageing or AD pathology.

I have shown that both monomeric α-synuclein and β-synuclein present a similar pattern of immunoreactivity in the cortex of the human brain (Figure 28-A-B) due to their similarities and pre-synaptic location, it is unsurprising that co-localisation is observed. Conversely, very rarely were there observations of β-synuclein and 5G4 co-localisation in this study. It could be speculated that β-synuclein transiently binds to α-synuclein in normal physiological circumstances, however, once pathological modifications occur that enable the oligomerisation and aggregation of α-synuclein, it may prevent direct contact between the two proteins due to contact sites being occupied or post-translational modifications preventing co-binding. This may see the re-localisation of either synuclein and once this neuroprotective effect is lost, pathological changes may proceed.

This study also provides evidence for the previously undescribed presence of cytoplasmic localisation of β-synuclein in the cortical regions by IHC, an otherwise pre-synaptic protein. In this context, α-synuclein is also pre-synaptic with its presence in the neuronal cell body more specifically associated with Lewy bodies; regardless of whether Lewy bodies are toxic or as a result of neuroprotective mechanisms, the presence of Lewy bodies is a pathological indicator of an α-synucleinopathy. The synaptosomal isolation work in this study (Figure 29) has shown that the total increase in β-synuclein levels in the frontal cortex is likely attributed to primarily cytosolic proteins although, an increase in the number of patients is required for validation. The examination of the cytosolic fraction saw all the DLB patients presenting with high levels of cytosolic β-synuclein. The findings were not proven signifant due to the presence of high levels of cytopsolic β-synuclein

in one of the controls. In contrast, the decrease in β-synuclein that has been observed in the cohort of DLB patients in the occipital cortex is likely related to loss of β-synuclein at the synapse. This is a possible indication of synaptic dysfunction occurring in this region which will be further explored in the examination of other pre-synaptic proteins (Chapter 6).

The occipital cortex sees an almost total loss of β-synuclein at the synapse in DLB (Figure 29), the absence of this protein may impair potential protective attributes, enabling α-synuclein to proceed in the direction of oligomerisation. In DLB, the α-synuclein aggregating process may start many years before clinical presentation which can 10 years prior. One could speculate that in the early course of the disease process, modifications to α-synuclein may lead to a loss of or impaired function that will see β-synuclein acting as a substitute at the synapse; potentially explaining the lack of DLB phenotype, early in the disease course. As α-synuclein pathology progresses and the burden becomes too high for the neurons it may be that β-synuclein is no longer able to act in a supporting role and the cell demise/synaptic dysfunction proceeds with the disease course favouring α-synuclein modification, oligomerisation, fibrillisation and Lewy body formation.

The data produced in figures 22-27 are descriptive and are intended to provide an indication of the general observations made in these cohort of patients, for each region. It must be pointed out that this is a qualitative and subjective analysis made on a minimum number of six patients. It is recognised that this analysis is not necessarily representative of changes directly attributed to DLB per se. It is also accepted that there are limitation regarding any observations due to subjectivity and therefore, caution in interpretation should be made. Indeed, when discussing the cortical layers and hippocampal regions, as I have done, it is not possible to be

absolutely certain of the correct layer in the field of vision, leading to possible morphological misinterpretation. The use of cell-specific markers to identify true cortical layers produces challenges due to the homogenous cell population in the human brain. The expertise of a neuropathologist would be beneficial, to validate the observations of changes that have been described in this thesis. The descriptions result from my own interpretation of the differences I see in control and DLB patients, of specific interest was the neuropil, β-synuclein positive cells and any changes in immunoreactivity intensity, as images were taken down through the cortical layers. The analysis was performed within a reasonable time constraint in order to maximise interpretation consistency and microscope settings optimised to ensure the comparisons were as reliable as possible.

Results Chapter 5: Modulation of β-synuclein in the presence of human-α-synuclein (h-α-synuclein) *in vitro*

Chapter 4 provides evidence for changes in β-synuclein levels in the human brain in parallel to alterations in oligomeric and monomeric α-synuclein. The available literature suggests that expression of α-synuclein and β-synuclein follow a reciprocal relationship, an increase of β-synuclein protein sees a decrease of α-synuclein protein expression, but not *SNCA* mRNA [263], *SNCA* mRNA is increased while *SNCB* mRNA is decreased in DLB patients [133] and transcription factor ZSCAN21, represses *SNCA* and increases *SNCB* expression in dopaminergic neurons [99]. Here I employed a rat dopaminergic neuronal line (N27) stably expressing human α-synuclein to identify whether changes in levels of α-synuclein have an impact on β-synuclein protein expression and distribution. The dopaminergic system is typically affected in PD and PD with dementia. Severe degeneration of nigrostriatal connections producing characteristic PD, motor symptoms of bradykinesia [264]. Although cholinergic neurons of the basal forebrain are primarily and most profoundly affected in DLB, the catecholaminergic (and dopaminergic) system also suffers – with a significant loss of dopaminergic cells in the ventral periaqueductal gray matter [265] and norepinephrinergic cells in the locus coeruleus (reviewed in [266]). In addition, advanced DLB patients display LB pathology in the substantia nigra and β-synuclein is highly expressed in the substantia nigra. Collectively this indicates a suitable cell type for examination of β-synuclein in the presence of α-synuclein. This cell line allows the precise temporal control of α-synuclein expression by ponasterone A (PonA) promoter activity induction, permitting examination of endogenous β-synuclein expression.

5.1: β-synuclein protein levels and distribution pattern of immunoreactivity in the presence of h-α-synuclein

5.1.1: β-synuclein protein is unchanged in the presence of h-α-synuclein

In order to reveal potential changes in β-synuclein levels, I examined h-α-synuclein over-expressing rat dopaminergic cells at 24, 48 and 72 hours post induction (hpi) of the transgene.

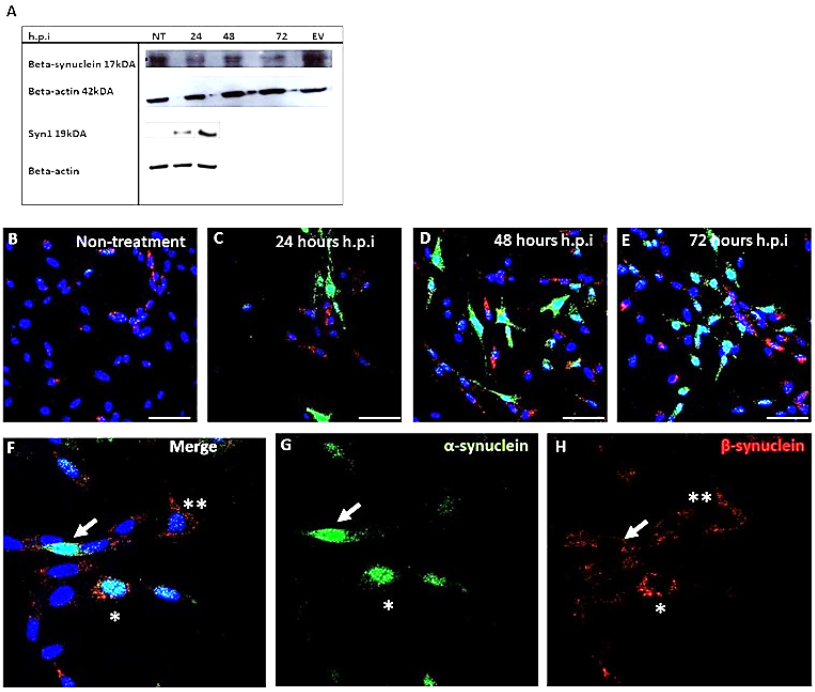


Figure 30 - β-synuclein protein levels are unchanged during the time course induction of h-α-synuclein

Western blot analysis of α-synuclein transgene expression (Antibody: syn1) (A). Time course of h-α-synuclein expression in N27 cell line (B – E). No treatment (B) and at 24, 48 & 72 hours h.p.i (C, D & E respectively). Pon A induction of human-alpha synuclein 24, 48 & 72 h.p.i. Syn204 & β-synuclein co-expression (F, *) 48 h.p.i β-synuclein (H) showed exclusive staining **.Higher levels of syn 204 immunoreactivity (G) was represented by low levels of β-synuclein (arrows, G & H). α-synuclein is detected by syn204 (green) and β-synuclein (red). Scale bar is 20μm.

The western blot analysis (Figure 30-A) confirmed that α-synuclein (revealed by syn1) is upregulated in response to PonA treatment. β-synuclein levels were unchanged between treatment groups when compared to the control.

The western blotting data was further supported by immunocytochemistry (ICC) (Figure 30-B-E). α-synuclein staining was consistently present following the transgene induction in the expressing cells, no significant overall change in β-synuclein (red) distribution was detected - similar levels of immunoreactivity were observed between controls (B) and 24-72 hpi groups (C - E). When examining co-localisation (F, *) between β-synuclein (H) and syn204-immunoreactivity (G), interestingly, a higher intensity of β-synuclein immunoreactivity was seen in cells that did not harbour high levels of α-synuclein (**) in contrast higher levels of syn204 saw lower levels of β-synuclein (arrows).

5.1.2: The β-synuclein pattern of immunoreactivity is redistributed in the presence of h-α-synuclein

There is no difference in the levels of β-synuclein in relation to α-synuclein expression in the N27 cells. However, it appeared that the cells that are harbouring higher levels of α-synuclein corresponded with lower levels of β-synuclein. Differential patterns of staining are also seen in the human brain with cells harbouring pathological α-synuclein, seemingly devoid of β-synuclein (Figure 28). Further, in the human brain, the β-synuclein IHC signal appears re-distributed in those cells that are positive for α-synuclein reactivity and enlarged granules immunoreactive for β-synuclein are observed in the cortex and the hippocampus of DLB patients (Figures 25-27). Next, the pattern of β-synuclein staining in cells

harbouring variable levels of h-α-synuclein was examined in rat dopaminergic cells from 24 – 72 h.p.i.

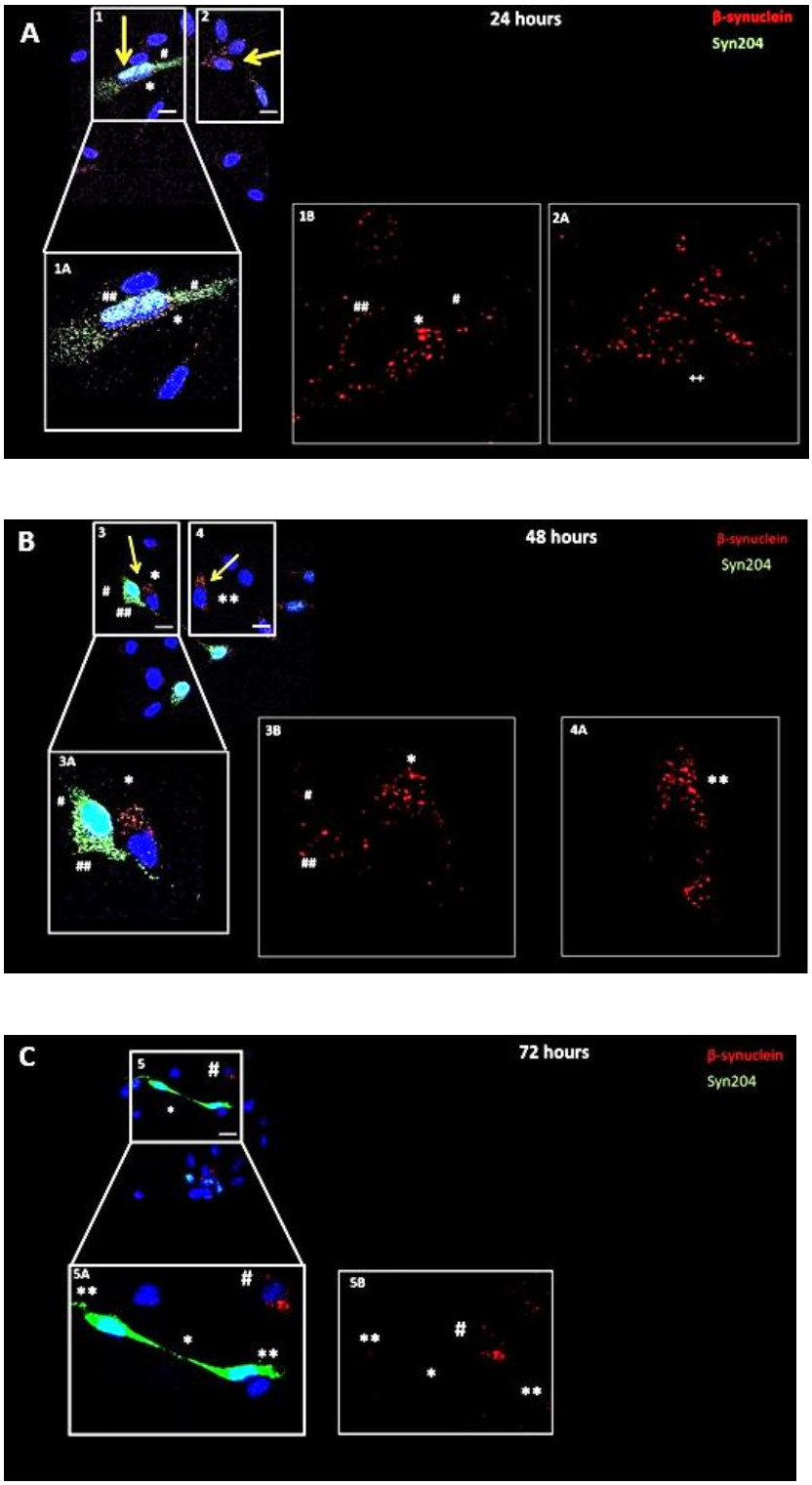


Figure 31 - β-synuclein immunoreactivity is low or absent in cells harbouring h-α-synuclein

β-synuclein (1B & 2A) and Syn204 immunoreactivity (1 & 1A) 24 hours post induction of h-α-synuclein. In cells, positive for syn204 (1 & 1A), β-synuclein is speckled with larger spots near the nucleus (*); β-synuclein is absent in areas of high syn204 staining (#) and β-synuclein shows a peri-nuclear localisation pattern (##). In cells, devoid of syn204 (2 & 2A), β-synuclein is speckled throughout the cytoplasm (**). B: β-synuclein (3B & 4A) and Syn204 immunoreactivity (3 & 3A) 48 hours post induction of h-α-synuclein. In cells, positive for syn204 (3 & 3A), β-synuclein is reduced (##) and absent in some regions (#); β-synuclein is speckled in cells in close proximity to syn204 expressing cells (*) and in cells negative for syn204 β-synuclein is speckled throughout the cytoplasm (**). C: β-synuclein (5B) exclusively stains syn204 -ve cells (#) and is devoid (*) or very sparse (**) in highly expressing α-synuclein cells (**). Scale bar 20μm. Images were taken with Zeiss confocal microscope.

24 hours following upregulation of h-α-synuclein, β-synuclein is present in syn204 positive neurons (Figure 31-A); the levels of α-synuclein are consistently low in these cells (Figure 31-A-1 & 1A). β-synuclein appears to be both speckled (1B) and with larger spots appearing close to the nucleus (lB, *). β-synuclein is absent (1B, #) in the region of the neuron with a higher intensity of syn204 staining (1A, #) indicating the cytoplasmic redistribution of β-synuclein protein within individual cells; further, some neuronal regions show a perinuclear pattern of β-synuclein (1B, ##). In contrast, the neurons that show no syn204 immunoreactivity (Figure 31-2) display a consistent diffuse cytoplasmic pattern of β-synuclein immunoreactivity (2A, ++).

48 hours post-treatment (Figure 31-B) syn204-positive cells, positive for β-synuclein immunoreactivity (B-3) was observed, in addition to β-synuclein positive cells, devoid

of syn204 staining (B-4). Cells expressing higher levels of α-synuclein had only low levels of β-synuclein immunoreactivity (3B ##) with the total absence of β-synuclein in some regions of the same neuron (3B, #). Cells in close proximity to an α-synuclein expressing cell show a diffuse pattern of staining of β-synuclein in a predominantly mono-polar manner (3B, *) with the appearance of low levels of α-synuclein at the opposite pole. Cells not closely associated with α-synuclein show a diffuse and uniformly speckled distribution throughout the cytoplasm (4A, **).

72 hours of α-synuclein expression (Figure 31-C-5) sees diminished β-synuclein (5B, *) or very low levels of speckled puncta (**) in those cells expressing α-synuclein.

5.1.3: β-synuclein positive cells do not express high levels of α-synuclein in iN27 cells

There is no change in β-synuclein protein levels but I identify a differential pattern of immunoreactivity when examining β-synuclein co-localisation with α-synuclein: cells with higher levels of α-synuclein immunoreactivity appear to present with lower levels of β-synuclein intensity. This may be indicative of β-synuclein single-cell modulations occurring, that are dependent on the local level of α-synuclein. To examine this hypothesis, the immunofluorescent signal of β-synuclein positive cells was examined for each time point and then the immunofluorescent signal of α-synuclein (revealed by Syn204 immunoreactivity) was analysed in cells that are both positive and negative for β-synuclein.

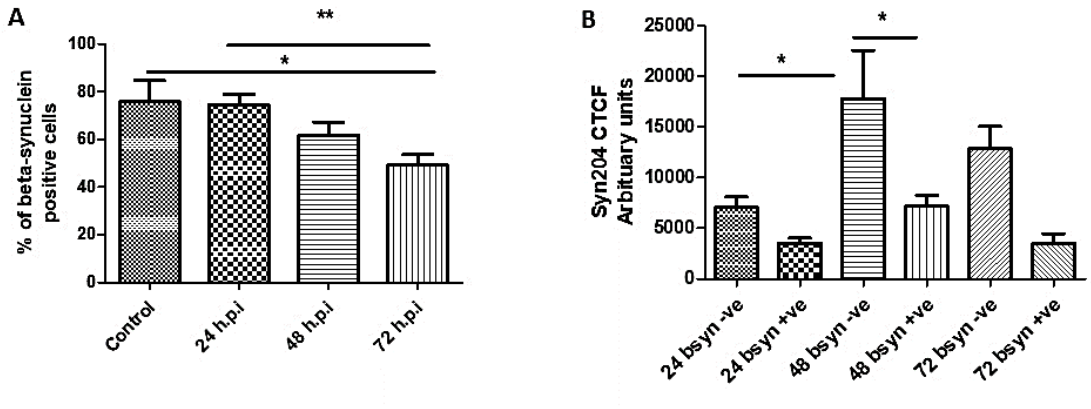


Figure 32 - β-synuclein positive cells are associated with low levels of h-α-synuclein

N27 cells treated with EtOH as a control or 24, 48 & 72 hpi (hours post induction) of human-α-synuclein gene expression. Data is expressed as the percentage of β-synuclein positive cells relative to the total number of cells examined in each group, nuclei ≥250 per group. A decrease in β-synuclein positive cells is seen at 72 hours (P = 0.02) (A). h-α-synuclein immunofluorescent intensity was measured in cells that were either positive (+ve) or negative (-ve) for β-synuclein (B). The detection of h-α-synuclein (Syn204) immunofluorescent intensity was analysed using FIJI - Image J software (NIH, USA). Cells that were positive and negative for β-synuclein were examined for Syn204 immunoreactivity and the CTCF (corrected total cell fluorescence) expressed in arbitrary units. β-synuclein positive cells required a mean-grey intensity density in excess of >200 AU (arbitrary units) for the region of interest (ROI) or were excluded from the analysis. A minimum of 100 cells per group were analysed from 3 experimental repeats. At 48 hours a significant decrease in Syn204 immunoreactivity is seen in β-synuclein positive cells (P = 0.03). Statistical analysis was performed using ANOVA and Bonferroni post-hoc test. ***: P=<0.05; **: P=<0.01; ***: P=<0.001.**

When examining the number of β-synuclein positive cells overall (Figure 32-A), there was a significant decrease in the 72 h.p.i group when compared to the control (P=0.02) and the 24 h.p.i and 72 h.p.i group (P=0.002). This data shows a decrease

in the number of β-synuclein positive cells 72 hours post expression of human-α-synuclein.

The immunofluorescent intensity of α-synuclein was examined in cells that were positive or negative for the presence of β-synuclein. To minimise subjectivity, a threshold level for grey mean intensity was set at >200AU for β-synuclein cells to be considered positive. A significant increase in levels of syn204 immunofluorescence was observed in β-synuclein negative cells at 48 hpi when compared to β-synuclein positive cells at 24 h.p.i. ($P=0.002$). Furthermore, at 48 h.p.i. there was significantly higher levels of syn204 immunofluorescence in cells that were β-synuclein negative when compared to β-synuclein positive ($P=0.03$).

Chapter 5: Results discussion

I provide here the evidence for a redistribution of the β-synuclein staining pattern in the neuronal culture system (Figure 30-32). The cytosolic representation of β-synuclein in the presence and absence of α-synuclein was distinctive and consistent. Single-celled differential patterns of immunoreactivity are observed: neurons expressing high levels of h-α-synuclein see changes in β-synuclein immunoreactivity, often the region positive for syn204-immunoreactivity shows low levels of, or is devoid of β-synuclein. In comparison, in the non-α-synuclein expressing cell, β-synuclein shows a diffuse cytosolic pattern of staining throughout the cell. This is representative of the findings in the human brain whereby the differential distribution of β-synuclein occurs in DLB brains and there is very little co-localisation of oligomeric-α-synuclein and β-synuclein (Figure 28).

I have shown that β-synuclein levels overall are unchanged in the presence of accumulating α-synuclein (Figure 30), in dopaminergic neurons in vitro. Whilst this does not mimic the findings in our human brains, where altered β-synuclein protein levels are seen, a significant decrease of β-synuclein positive neurons was seen at 72 hours (Figure 32). Therefore, it is a good indicator of a potential temporal change in the β-synuclein protein behaviour. When comparing the in vitro model with DLB brains, it must be considered that the majority of neurons in the cortex are glutamatergic large pyramidal neurons or GABAergic interneurons. It is, therefore, possible that the dopaminergic neuronal phenotype of these in vitro cells leads to differential changes in β-synuclein behaviours that may not support the change in protein levels in the cortical regions of the human brain, but more representative of the substantia nigra. It is known that β-synuclein is highly expressed in the substantia nigra, a region selectively targeted in PD, further indicating potential

mechanisms that seek to balance or minimise damage by α-synuclein. The findings that β-synuclein levels are not altered in response to α-synuclein, but rather, distribution of the protein is altered, in this dopaminergic cell model may be indicative of the type of mechanisms undertaken in regions rich in catecholamines. It may be considered, therefore, that β-synuclein behavioural may have neurochemical-specific responses.

The synucleins are known to colocalise and this study demonstrates that modified levels of either of the synucleins also appear to be represented by the lack of co-localisation. It is shown here (Figure 32-B) that at 48 hours h.p.i., α-synuclein levels are higher in cells that are not expressing detectable levels of β-synuclein when compared to cells positive for β-synuclein. It is possible that a) an increase in α-synuclein or β-synuclein leads to the loss of synuclein-synuclein interactions, b) β-synuclein is demonstrating a pattern of redistribution that may be associated with neuroprotective effects in an attempt to prevent or minimise α-synuclein toxicity rather than loss of interaction c) it is possible that the absence of β-synuclein in syn204 highly immuno-positive cells may result from α-synuclein-induced toxicity having deleterious effects although, diminished levels of β-synuclein in this cell model were not identified to support this, and finally d) transcriptional regulation of the synucleins may be occurring; therefore, increased levels of α-synuclein in a single neuron may see reduced levels of β-synuclein in the same cell.

Collectively, I have shown that in the presence of h-α-synuclein there is a temporal change in β-synuclein cellular localisation in rat dopaminergic neurons. But in the event that in vivo, β-synuclein is providing a protective function, this may present indications of how the protein behaves in the face of α-synuclein cellular burden. The observations that β-synuclein appears to be present only in cells primarily with lower

levels of α-synuclein may support a role that is indicative of the prevention of accumulations, that is at some point overwhelmed, seeing loss of β-synuclein in the same neurons. What is not clear is whether this redistribution is an effort to perform physiological roles that are typically attributed to α-synuclein, these roles include chaperoning SNARE complex assembly or whether this results from the toxic effects of α-synuclein that see trafficking of β-synuclein to different cellular locations.

Results Chapter 6: SNARE protein distribution changes in the DLB brain and in the presence of β-synuclein

Synaptic alterations are well described in neurodegeneration, with observations of synaptic loss [176] [43] [267], changes in protein composition [209], modifications of distribution patterns [208] and alterations in SNARE protein physiological activity, with impairments in SNARE complex formation observed [141]. T-SNAREs SNAP25 and syntaxin ensure the integrity of vesicle docking in association with the target membrane and therefore, any obstructions at the synapse will potentially lead to loss of functional integrity. Alterations in some SNARE protein levels have been reported in DLB, showing in general, a reduction of their levels [209]. Synaptic dysfunction, manifesting as redistribution of SNAREs, is observed in the striatum of patients with early-onset PD [208].

Although, Lewy body disease is characterised by Lewy bodies and neurites, small, oligomeric α-synuclein accumulations at the pre-synapse of DLB patients interfere with synaptic function [268] and may contribute to development of the clinical phenotype with suggestions synaptic malfunction and deregulation may be representative of the pre-clinical and early symptoms associated with these conditions (reviewed by [269]). To evaluate the damage entrapped oligomeric α-synuclein undertakes at the pre-synapse, it is necessary to examine other pre-synaptic proteins to appreciate synaptic changes that occur in the course of pathology.

6.1: Immunohistochemical distribution of the major SNARE proteins in cortico-paralimbic regions of the human brain

To examine whether α-synuclein aggregation leads to redistribution or accumulation of SNARE proteins in cortical and limbic areas of DLB brains, IHC was performed examining key synaptic proteins in the control brains, specifically observing patterns of distribution around the neurons and throughout the neuropil and comparisons are made with pattern of immunoreactivity occurring in the DLB brains.

6.1.1: VAMP2 and SNAP25 in cortical and hippocampal brain regions

α-synuclein is an essential chaperone in the process of SNARE complex formation [270] this role is performed by direct interaction with VAMP2, enabling an association of the pre-synaptic vesicle with the target membrane, facilitating the release of the neurotransmitter at the synapse. Moreover, SNARE-complex protein, SNAP25 is associated with the pre-synaptic plasma membrane having a dominant influence in the docking and fusion of vesicles at the synapse [185].

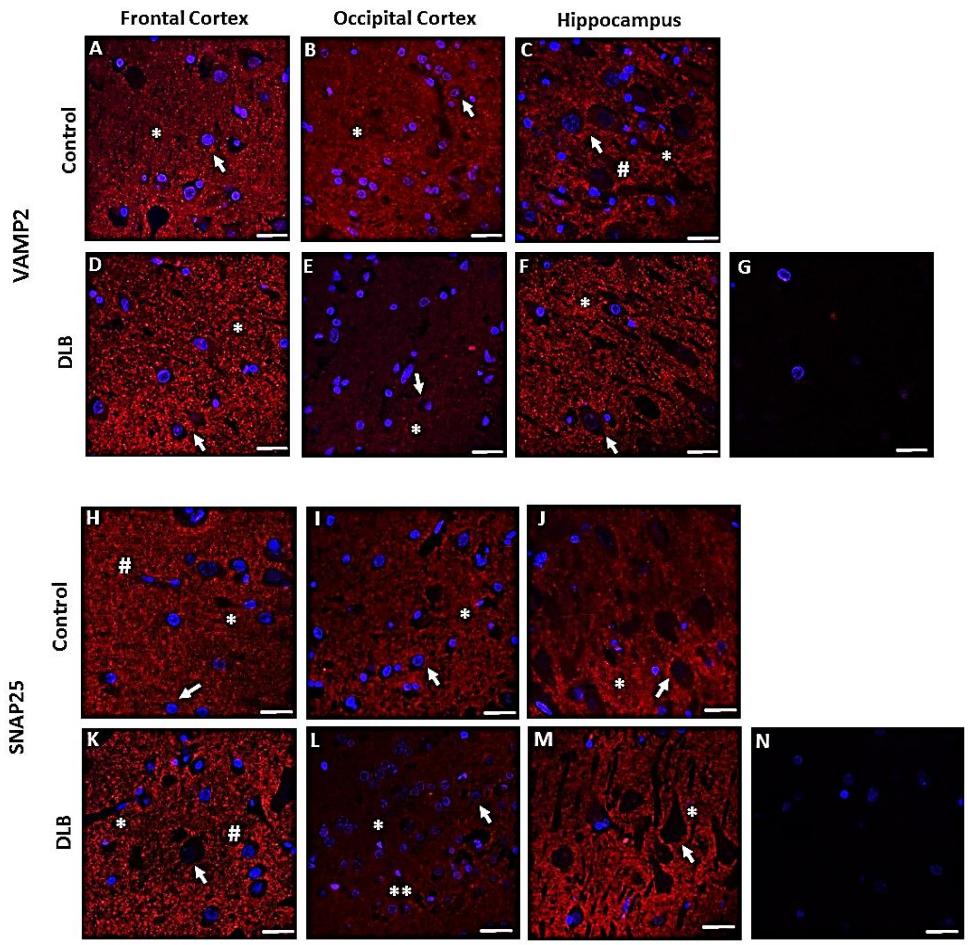


Figure 33 – In DLB occipital cortex VAMP2 and SNAP25 immunoreactivity is reduced and SNAP25-positive clusters are observed

Immunohistochemical detection of VAMP2 (A-F) and SNAP25 (H-M) proteins in the cortical and hippocampal regions of the human brain. VAMP and SNAP25 is observed in the control (A-C, H-J) and DLB (D-F, K-M) brains respectively. The neuropil-type pattern of the staining is marked with (*). Accumulation of immunopositive signal around the neuronal soma is marked with an arrow with the addition of immuno-positive clusters (#) and clumping (**). Non-primary control for VAMP2 and SNAP immunostaining (G & N, respectively). N = 3 per group and per region. Scale bar 19.5µm.

VAMP2 in the frontal cortex and occipital cortex of control patients gives the appearance of small puncta that both surround the neuronal periphery in addition to

a small and speckled distribution throughout the neuropil (Figure 33- A & B, respectively, *). The pattern of distribution in the hippocampus (Figure 33-C), when compared to the cortex, is more granular (C, *) and more immunoreactivity is seen surrounding some of the larger pyramidal neurons (C, arrow). Some puncta appear to have a more condensed pattern of staining in specific regions at the neuronal border (C, #)

The pattern of VAMP2 immunoreactive staining was changed in DLB tissues when compared to the controls. VAMP2 in the frontal cortex of DLB showed intermediate sized granular puncta that were dotted throughout the neuropil (Figure 33-D, *), often larger than that observed in the control tissues. The signal from VAMP2 in the occipital cortex was evidently diminished when compared to the frontal cortex with significantly reduced immunoreactivity appearing around the neurons and appearing non-homogenously throughout the neuropil (Figure 33-E). The hippocampus had a relatively intense pattern of staining (Figure 33-F) it was variably distributed with small to intermediately sized puncta throughout the neuropil (F, *). There was immunoreactivity surrounding the neuronal-border (F, arrow).

The detection of SNAP25 in the frontal and occipital cortical areas of the control brain presented similarly in dissemination with small puncta dotted throughout the neuropil (Figure 33-H-I). SNAP25 was present around the neuronal perimeter in the cortical regions. In the hippocampus (Figure 33-J) staining was evident around the border of the large neurons in the CA2/CA3 regions (J, arrow) with a speckled appearance throughout the neuropil (J, *).

The frontal cortex of the DLB patients (Figure 33-K) appeared to have less homogenous patterns of staining in the neuropil when compared to the control.

There was evidence of staining around the edge of the neurons although this appeared re-distributed in places (K, arrow). SNAP25 immunoreactivity in the DLB occipital cortex (Figure 33-L) was less pronounced overall when compared to the frontal cortex. There were also irregularities in the pattern of staining in this region: some neurons had positive staining at the perimeter of the somata but it appeared clumped and not uniformly distributed in places (L, **). The DLB hippocampus (Figure 33-M) had a distribution that was more coarse and granular in places, but not too dissimilar to the control, with SNAP25 immunoreactivity present around the neurons (arrow).

6.2: SNARE proteins in the cortical region of the DLB brain

The data related to SNAREs protein levels in the DLB brain is scarce. A significant correlation between levels of pre-synaptic proteins and advancement of dementia related neuropathological disorders has been reported in DLB, PDD and AD; indications are that VAMP2 levels negatively correlate with the duration of dementia [209]. A significant decrease in SNAP 25 is observed in the DLB primary and association visual cortex and the neocortex of LB variants of AD patients [271]. Overall studies point to a reduction in the SNAREs in cortical regions indicating the possibility of trans-synaptic degeneration. To confirm and expand these observations in DLB, I examined the levels of key v-SNARE and T-SNAREs, VAMP2 and SNAP25, respectively in human brain tissue lysates of control and DLB.

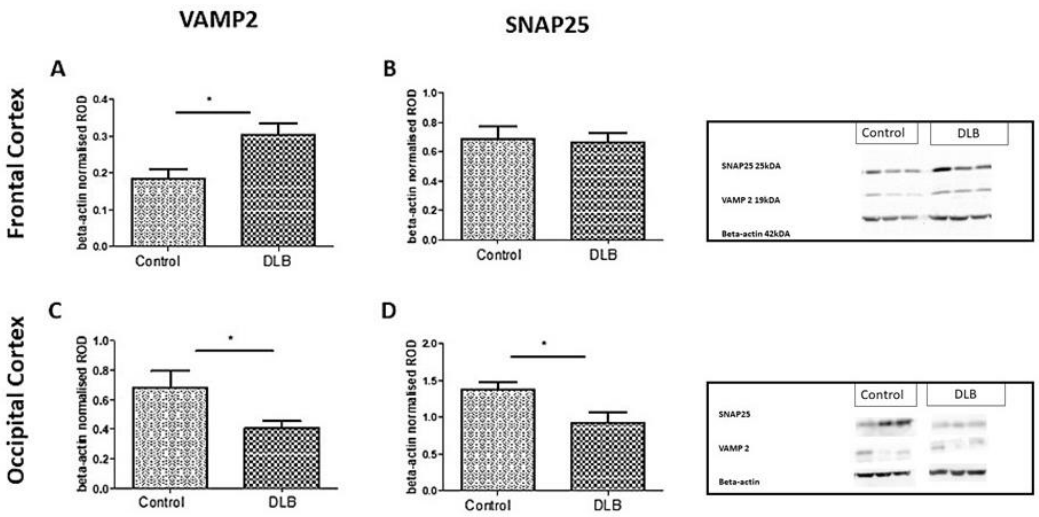


Figure 34 - VAMP2 protein levels are increased in the frontal cortex of the DLB brain and VAMP2 and SNAP25 are decreased in the occipital cortex in DLB

Western blot analysis of VAMP2: frontal cortex (A) (control n = 8) & (DLB n = 13) & occipital cortex (C) (control n = 5) & (DLB n = 14). VAMP2 is increased in DLB in the frontal cortex (P=0.012) and decreased in the occipital cortex (P=0.018). SNAP25: frontal cortex (B) (control n = 8) & (DLB n = 15) & occipital cortex (D) (control n = 7) & (DLB n = 13). SNAP25 is decreased in the occipital cortex of DLB brains (P=0.036). Statistics were performed using student's two-tailed t-test.

Examination of control and DLB human brain tissue lysates (Figure 34-A-D) led to the identification of a significant increase in VAMP2 in the frontal cortex (Figure 34-A) of the DLB brains (P= 0.012) and conversely, a significant decrease in the occipital cortex (Figure 34-C) (P= 0.018). Further, SNAP25 was reduced in the occipital cortex (Figure 34-D) of the DLB patients when compared to the controls (P = 0.036), no changes were observed in the frontal cortex for SNAP25 (Figure 34-B).

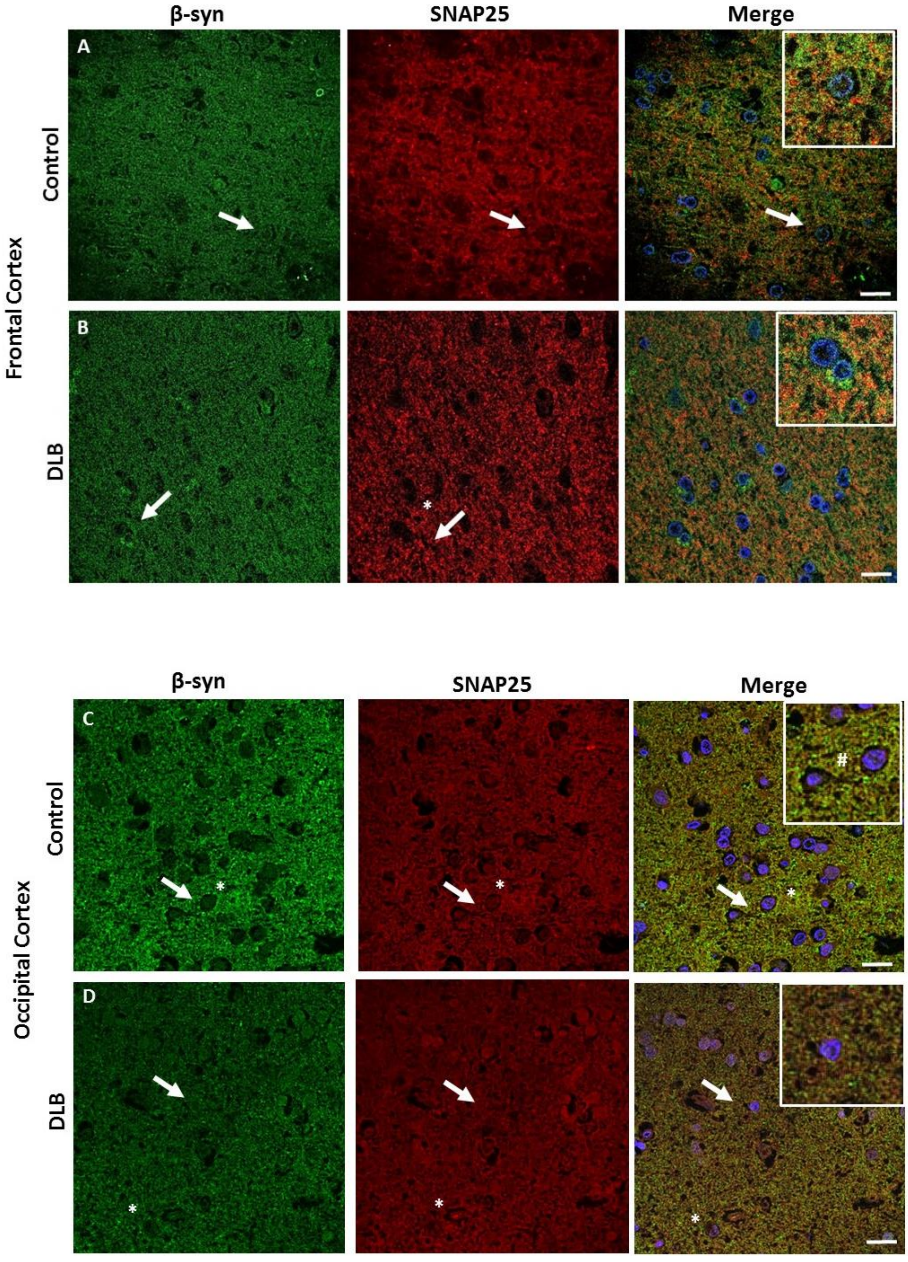
6.3: β-synuclein & key SNARE proteins changes in DLB

I have provided evidence for the re-distribution of β-synuclein and altered levels of β-synuclein protein in the cortex of the DLB brain in addition to SNARE protein redistribution and protein level modifications in the same regions. The increase of VAMP2 in the frontal cortex and decrease of VAMP2 and SNAP25 in the occipital cortex mimic the direction of change observed in β-synuclein. Despite the homology between the synucleins, and the close proximity of β-synuclein to the pre-synaptic vesicles [76] there is no evidence of a direct involvement of β-synuclein in SNARE complex formation or synaptic vesicle binding. In accordance with this, the precise role of β-synuclein is not entirely understood. It is, however, well established that α-synuclein and VAMP2 are binding partners with the coordinated effort of α-synuclein binding directly to VAMP2 assisting in the complex formation of the SNAREs. The phenotype for behavioural, biochemical and physiological changes associated with the triple knockout models is much more pronounced than those single or double knock out models, with detectable changes associated predominantly with ageing; thus, underscoring the potential for intra-protein redundancy. However, cooperative redundancy effects may be regionally or neuron specific since, α-synuclein is required to maintain the integrity of the nigrostriatal system, with β-synuclein failing to offer compensatory responses [272]. VAMP2 is key for fast endocytosis of synaptic vesicles [203] and deceleration of the kinetics of endocytosis in the absence of α/β/γ- synuclein in hippocampal neurons is observed. This effect is reduced with re-introduction with one of the synucleins [273]. The possibility that β-synuclein may act as a compensatory protein in the absence of α-synuclein or in the presence of α-synuclein modifications is intriguing and a role mediating SNARE protein complex cannot be excluded.

In order to examine β-synuclein in relation to SNAREs, a double fluorescence immunohistochemistry protocol was implemented to observe the localisation of β-synuclein in the cortical regions and hippocampus in relation to the SNARE proteins, paying particular attention to the SNARE protein distribution patterns in the vicinity of β-synuclein immunoreactivity.

6.3.1: Immunohistochemical distribution of β-synuclein and SNAP25 in the human brain

This study has presented evidence of redistribution of SNAP25 in DLB brains in the cortical regions and the hippocampus by IHC (Figure-34) and reduced levels of the SNAP25 protein in occipital cortex (Figure 34). Next, I examined the distribution of the proteins in the frontal cortex, occipital cortex and hippocampus.


β-syn
SNAP25
Merge
A
Control
Frontal Cortex
B
DLB
β-syn
SNAP25
Merge
C
Control
Occipital Cortex
D
DLB

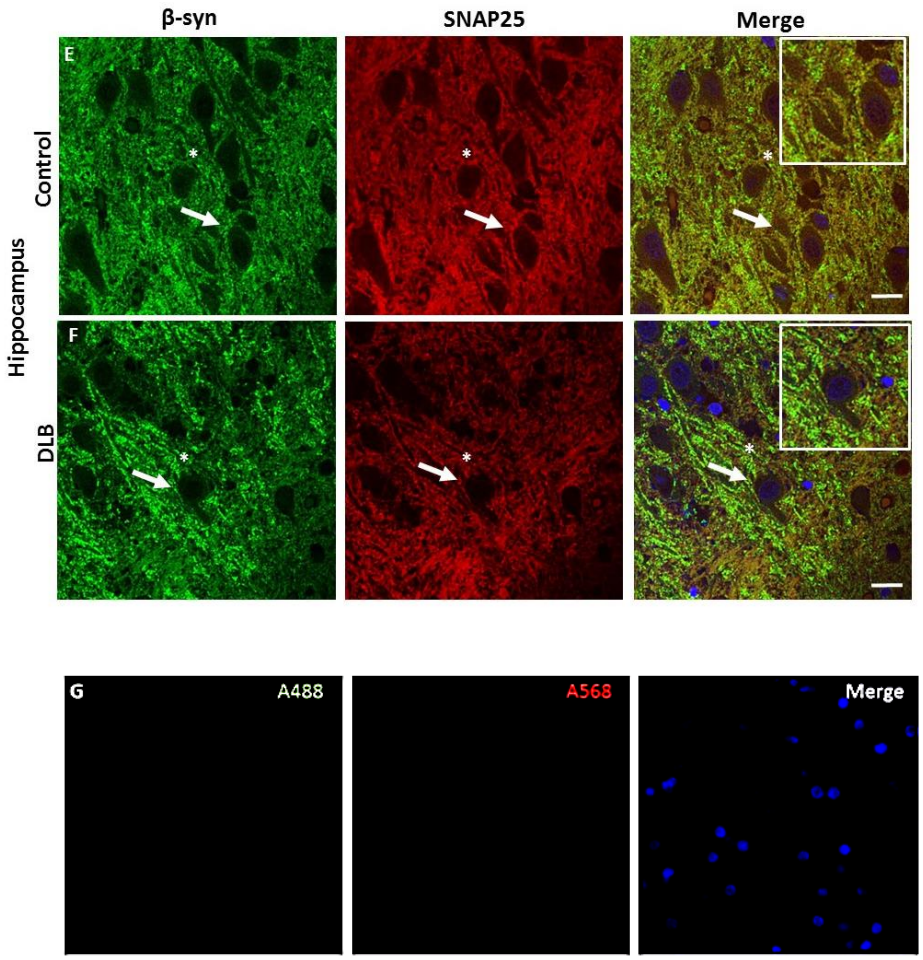


Figure 35 - SNAP25 is re-distributed in the frontal cortex and diminished in the occipital cortex

Immunohistochemical distribution of β-synuclein and SNAP 25 in the frontal cortex: control (A) and DLB (B) N=4; occipital cortex: control (C) and DLB (D), N = 3 and the hippocampus: control (E) and DLB (F) brains, N = 3. The neuropil-type pattern of the staining is marked with (*). Non-primary antibody, secondary antibody only controls (G). Accumulation of immunopositive signal around the neuronal soma and/or enlarged image insert is marked with an arrow. Scale bar 19.5μm.

In the frontal cortex and occipital of the control brain (Figure 35-A & C, respectively), β-synuclein and SNAP25 display a similar pattern of staining with a distinctly similar

pattern of β-synuclein and SNAP25 throughout the neuropil (A & C*) revealed by small speckles. Co-localisation is shown by yellow puncta in addition to the exclusive representation of both proteins (Arrow, enlarged).

In contrast to the control, the frontal cortex of the DLB patient (Figure 35-B) shows a differential pattern that often has a more granular appearance of β-synuclein and SNAP25 staining, with speckles of varied size and intensity of immunoreactivity. β-synuclein positive neurons were frequently surrounded by larger sized, coarsely patterned β-synuclein and SNAP25 granules (B, enlarged); both were independently stained along with grainy yellow puncta representing possible co-localisation. SNAP25 immunoreactivity was more prominent in the DLB patients when compared to the controls with the more obvious presence of red puncta, (B, enlarged).

In the occipital cortex of the DLB patients (Figure 35-D), overall both β-synuclein and SNAP25 presented with less immunoreactivity as I previously described for this region for both β-synuclein and SNAP25 single stains. Evidence of neuronal perimeter immunoreactivity of either protein is diminished when compared to control brains (Figure 35-D).

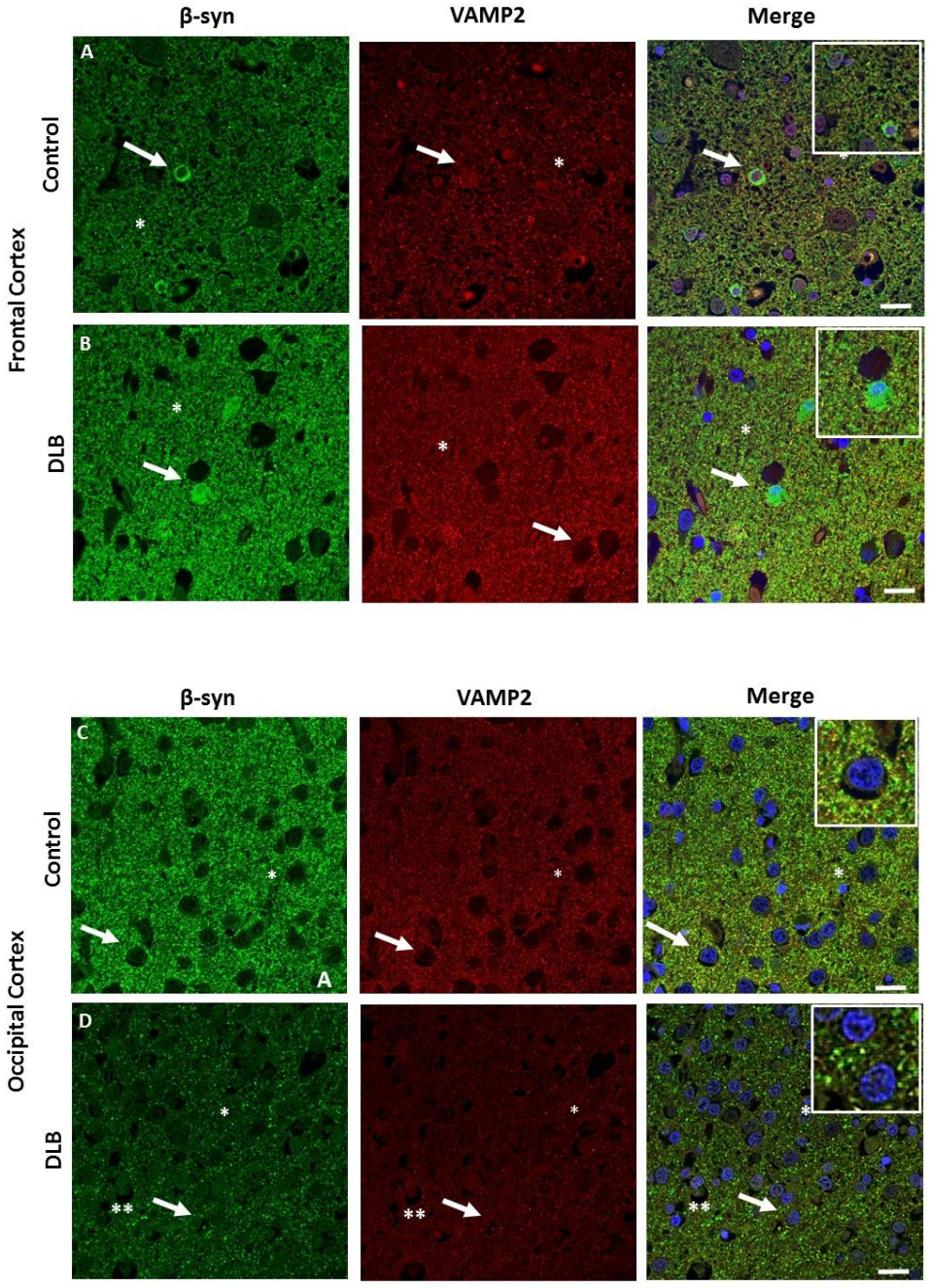
The control hippocampus (Figure 35-E) showed very similar patterns of staining between β-synuclein and SNAP25 around the perimeter of the neurons (see enlarged, arrow); presentation of immuno-positive punctate of β-synuclein or SNAP25 as singular stains was observed (enlarged).

As previously described for the hippocampus, the DLB brain (Figure 35-F) generally showed a very coarse pattern of medium sized granules that were immunopositive for β-synuclein. The SNAP25 pattern of distribution appeared, like the control brain,

as smaller speckles throughout the neuropil and around the neurons, (F, * & arrow respectively).

6.3.2: Immunohistochemical distribution of β-synuclein and VAMP2 in the human brain

Redistribution of the VAMP2 protein has been identified in the DLB patient cohort (Figure 33). Further, modulation of VAMP proteins has been identified (Figure 34) with an increase of VAMP2 seen in the frontal cortex and a decrease in the occipital cortex. The available literature provides no evidence of a binding association between VAMP2 and β-synuclein, however, by virtue of their location at the pre-synapse, they are in close proximity to each to each other. This study sought to examine β-synuclein and VAMP2 protein behaviours in the presence of double immunofluorescence; in particular, the frontal cortex and occipital cortex are of interest in view of altered β-synuclein levels.


β-syn
VAMP2
Merge
Frontal Cortex
Control
DLB
A
B
Occipital Cortex
C
D

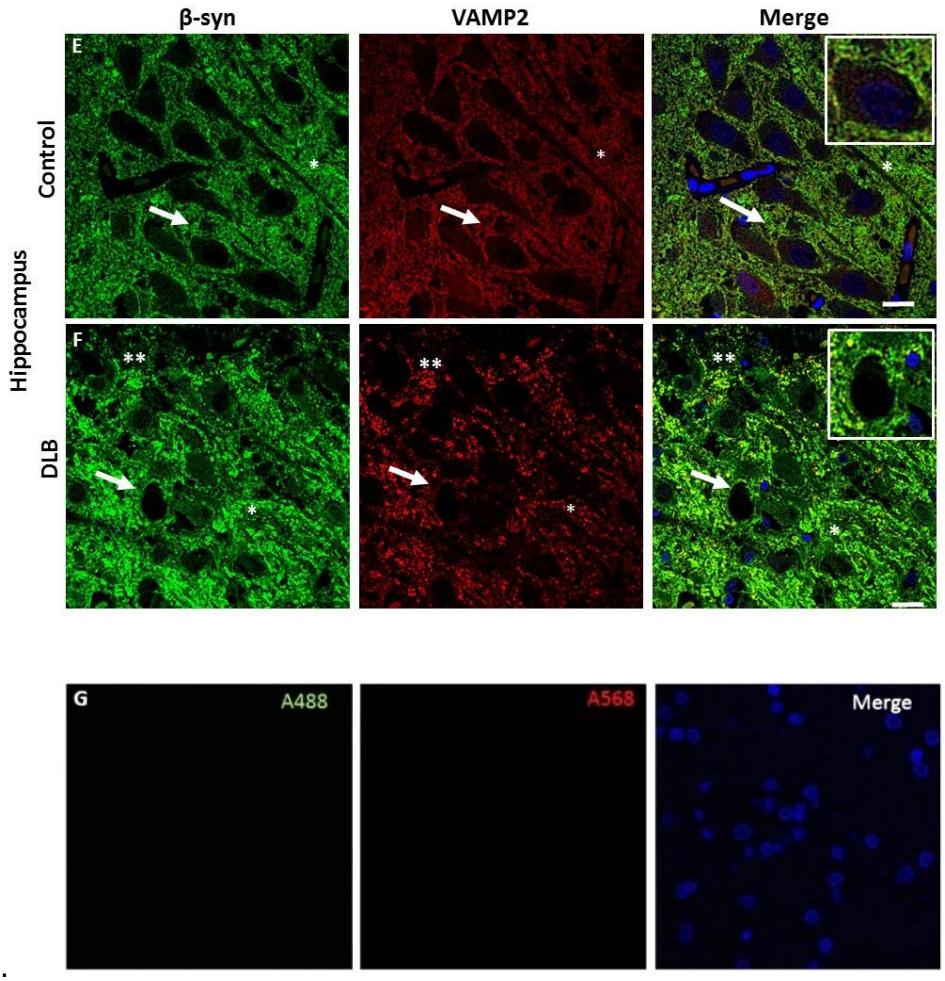


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Figure 36 – VAMP2 and β-synuclein immunoreactivity is diminished in the occipital cortex

VAMP2 and β-synuclein in the frontal cortex of the control (A) and DLB brain (B), N = 3; occipital cortex in the control (C) and DLB (D), N = 4 and hippocampus of the control (E) and DLB (F) brain, N = 3. Non primary antibody controls (G). ***The neuronal somata perimeter staining is shown (arrow) and the neuropil (*) represented for both proteins. High-intensity β-synuclein immunoreactivity is observed (**), in addition to β-synuclein positive cells. Scale bar 19.5μm.***

The frontal cortex control and occipital cortex (Figure 36-A & C, respectively) presented a staining pattern that was primarily uniformed for β-synuclein and VAMP2

throughout the neuropil (*). There was evidence of both proteins being distributed around neuronal cell bodies in the shape of small puncta (A & C, enlarged) and some yellow puncta indicating possible co-localisation.

Examination of the DLB brain (Figure 36-B) in the frontal cortex identified multiple β-synuclein positive cells (B & enlarged). There were regions with clustering that appeared to be immunopositive for both β-synuclein and VAMP2 (B, **) with both proteins presenting a coarser appearance in the DLB brain when compared to the control.

In the occipital cortex of the DLB patient (Figure 36-D) the level of immunoreactivity for β-synuclein and VAMP2 were both diminished when compared to the controls. The distributions of the proteins both in the neuropil (D, *) and around the neuronal periphery (arrow & enlarged), in relation to the levels of immunoreactivity was largely reduced. The appearance of some yellow puncta (enlarged) was indicative that there might be some co-localisation between β-synuclein and VAMP 2. There were regions throughout the neuropil that appeared to have clusters of β-synuclein (D, **) immunoreactivity, these areas were devoid of VAMP2 (E, **) staining.

The hippocampus control brain (Figure 36-E) pattern of staining was that of small-medium speckles for β-synuclein and small puncta for Vamp2. Both β-synuclein and VAMP appeared throughout the neuropil (E, *) and the pattern of staining was largely that of small-medium speckles for β-synuclein and small puncta for Vamp2. These puncta were clearly observed around the periphery of the large neurons in the hippocampus CA2/3 region (E, arrow).

As previously described for this region, the hippocampal DLB brain (Figure 36-F) showed a distinctively different pattern of staining for both β-synuclein and VAMP2

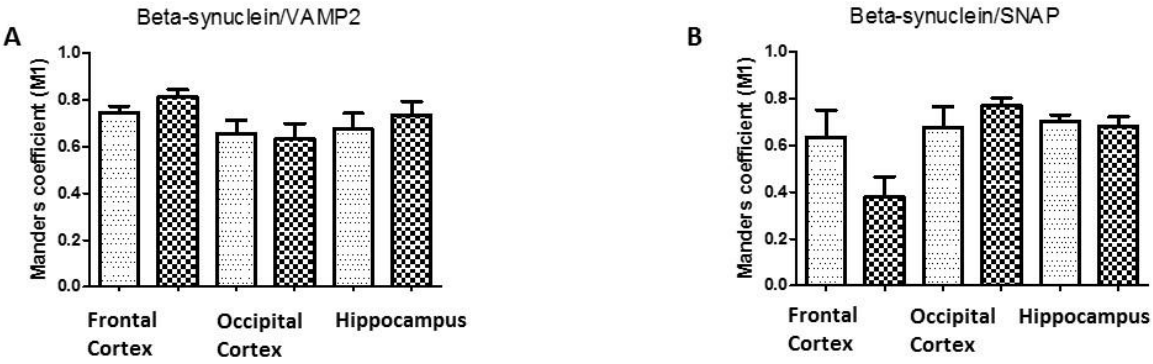
when compared to the control, both were displayed throughout the neuropil (F, *) and around the neuronal perimeter (arrow, enlarged) presenting with a grainy appearance of differential sized granules and the appearance of clumping (F, **).

6.4: β-synuclein colocalisation with SNAP 25 and VAMP2 in control and DLB brains

Super-imposing fluorescent images following IHC is a widely-used technique to evaluate co-localisation. Co-localisation is observed when the staining of two antigens in the same tissue section are labelled with differing excitation spectra and produce an overlap in colour; in this study, the overlap produces a yellow signal that occurs when the two different antigens are labelled independently with either a red or green fluorophore. This can provide evidence that the molecules are in close proximity to each other but it does not provide a rigorous proof of interaction. The quantification of two fluorescent probes is required in order to establish the validity of co-localisation meticulously; this is as opposed to the more subjective approach via visual interpretation [274]. This study provides evidence of yellow puncta following the IHC examinations of β-synuclein and the SNAREs, indicating the potential for co-localisation between these proteins. Evidence for these interactions has not been previously reported and could provide valuable evidence for inter-relationship between pre-synaptic proteins. To this end, I employed quantitative co-localisation analysis (QCA) [275] using the Manders coefficient [276] in order to estimate the level of antigenic co-localisation in multiple, confocal microscopy, immunofluorescent images. The examination focused on colocalisation between the β-synuclein

immunopositive signal with that of SNAP25 or VAMP2; thus, enabling a more accurate and objective predictor of co-localisation.

6.4.1 β-synuclein colocalises with VAMP2 and SNAP25 in control and DLB brains



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β-Synuclein and VAMP		Control			DLB	
	M1	M2	PCC (above threshold)	M1	M2	PCC (above threshold)
Frontal Cortex	0.75 ± 0.03	0.70 ± 0.01	0.35 ± 0.04	0.81 ± 0.03	0.80 ± 0.04	0.48 ± 0.03
Occipital Cortex	0.66 ± 0.06	0.66 ± 0.04	0.32 ± 0.06	0.63 ± 0.06	0.61 ± 0.04	0.3 ± 0.04
Hippocampus	0.67 ± 0.07	0.66 ± 0.05	0.31 ± 0.09	0.74 ± 0.06	0.7 ± 0.03	0.42 ± 0.03
β-Synuclein and SNAP		Control			DLB	
	M1	M2	PCC (above threshold)	M1	M2	PCC (above threshold)
Frontal Cortex	0.63 ± 0.16	0.67 ± 0.1	0.3 ± 0.11	0.38 ± 0.09	0.44 ± 0.07	0.27 ± 0.05
Occipital Cortex	0.68 ± 0.09	0.66 ± 0.08	0.33 ± 0.06	0.77 ± 0.03	0.75 ± 0.02	0.3 ± 0.07
Hippocampus	0.7 ± 0.03	0.67 ± 0.03	0.23 ± 0.06	0.68 ± 0.04	0.68 0.01	0.25 ± 0.05

Figure 37 – A moderate level of co-localisation between β-synuclein and Vamp2 or SNAP25 is observed in the cortex and hippocampus of control and DLB patients.

Manders coefficient for co-localisation between β-synuclein and VAMP2 (A) or SNAP25 (B) in control and DLB patients. N= 3 per group and per region; 10 z-stacks. Manders M1 indicates

the proportion of the green signal, β-synuclein, that coincides with a signal in the red channel, VAMP2 or SNAP25, in relation to the total intensity of the green channel. Tabulated results of M1, M2 and Pearsons correlation coefficient (PCC) above the Costes Threshold (C).

In order to validate an apparent co-localisation between β-synuclein and the SNAREs, Manders overlap coefficient of co-localisation has been applied. Manders calculates the proportion of overlap between the green (β-synuclein) and red channel (VAMP2 or SNAP25) in the stack of images for each patient [277]. The Manders coefficient value produced is between 0 and 1, '0' represents no co-localisation and '1' signifies100% co-localisation. In this study, a moderate index of co-localisation between β-synuclein and both VAMP2 (Figure 37-A) and SNAP25 (Figure 37-B) is observed in the cortical and hippocampal regions (M1 = 0.6 - 0.7). There was no discrimination in the co-localisation data when comparing control and DLB. The PCC index showed low-moderate level of co-localisation based on the relationship between the intensity of the pixels of the two fluorophores. PCC (R values) above the Costes threshold. $R \geq 0.5$ [278] describe strong co-localisation of two fluorophores [274].

Chapter 6: Results discussion

This study provided evidence for an increase in VAMP2 in the frontal cortex and in parallel, a reduction in the occipital cortex of the DLB patients (Figure 34). In conjunction with this, a reduction of SNAP25 in the occipital cortex was seen but not the frontal cortex (Figure 34). SNARE proteins underpin effective neurotransmitter exocytosis, with modifications of levels indicating potential dysfunction occurring at the pre-synapse. Changes in the synaptic proteins may be indicative of the level of α-synuclein pathology present in the region. It has been reported that reductions in SNAREs correlate with LB pathology and a decrease in ChAT levels [271]. It is possible to speculate that increasing α-synuclein pathology in DLB causes synaptic dysfunction, whether this results in synapse loss is unclear although, this is a feature in other dementias such as AD [279] and FTD [280]. The relationship between α-synuclein and VAMP2 is critical and the loss of α-synuclein in the frontal cortex of these DLB brains (Figure 19) is not reflected in VAMP2 levels with an increase observed (Figure 34). It is suggested that VAMP2 is important for the replenishment of the readily available pool of vesicles [203] a role described for α-synuclein, providing a possible protective role for VAMP2 in supporting the synapse in the face of declining levels of monomeric α-synuclein. In AD the synaptic protein loss is indicative of later stages of dementia with SNAP25 and syntaxin levels unchanged or increased in the frontal and temporal cortex early in dementia with a subsequent reduction with moderate to severe dementia [281]. If this holds true in DLB, the changes observed in the frontal cortex may indicate early cognitive decline and the occipital cortex may reflect greater synaptic dysfunction. Moreover, VAMP2 increases in the frontal cortex may be suggestive of possible attempts of synaptic

transmission reparation following α-synuclein toxicity; the loss of VAMP2 is able to induce neurodegeneration and autophagy defects in photoreceptor neurons [282].

In the cortex, the direction of change of either increasing or decreasing β-synuclein (Figure 19) is mimicked by the direction of change in important SNARE proteins for the same region (Figure 34). IHC analysis provides evidence for re-distribution in β-synuclein and the SNAREs in the frontal cortex, occipital cortex and the hippocampus (Figure 35 & 36).

When examining VAMP2 and SNAP25 in the presence or absence of β-synuclein, the data provided is descriptive and therefore, subjective. Whilst immunohistochemistry is a widely used tool, there are limitations when using this method to describe changes occurring in the human brain that may or may not be associated with DLB. In this regard, descriptive data is not suitable to correlate variables, perform statistical analysis or identify the cause or effect and should be interpreted cautiously. Misinterpretation of both positive and negative results are possible potentially leading to an incorrect morphological perspective.

Descriptions have focused on the intensity of immunoreactivity of the proteins of interest and the pattern of staining throughout the neuropil or around β-synuclein positive cells. Interpretation consistency was employed, I ensured that I was familiar with the specific observations I wished to make. From the beginning of the protocol to the image acquisition stage, all tissues were treated with the same level of consistency.

An increase in the number of patients per group examined would enable a greater appreciation of whether the changes observed in the study may be relevant to the

disease course. In addition, to ensure the exclusion of any bias in this method of analysis, it would be necessary for an additional examination to be undertaken independently, for each group and each region, by a neuropathologist; the observations would then be collated and compared.

In order to observe two proteins in tandem, the double immunofluorescent protocol was employed. In view of the implementation of fluorescent microscopy, it is more challenging to approximate the layer of the cortex/hippocampus and therefore, it is possible that different regions of the brain tissue have been compared between patients and groups. I attempted to be as consistent as possible to minimise such variation.

Potentially, damaged tissue, due to the disease process or human error during the tissue processing – from fixing to cover-slipping - may allow for accumulations of the antibody leading to a false-positive signal. In all cases of IHC processing, the brains were treated with the same protocol that was adhered to as strictly as possible and microscope settings optimised in order to enable comparable descriptions between groups and regions. In order to minimise the possibility of subjective bias, imaging was performed without the knowledge of whether the patient was a control or DLB, analysis was performed by myself only and within a reasonable period of time. Whilst one accepts that disease duration and other pathological considerations may interfere with the observations, where possible, patients and controls were age-matched to provide a relative and comparable discussion.

The IHC analysis indicated colocalisation that is supported by Manders colocalisation analysis (Figure 37). Surprisingly, the co-localisation analysis did not discriminate between controls and DLB indicating that these relationships are not

pathologically driven but rather represent previously unreported physiological roles. In this regard, one may assume that homology between the synucleins will dictate these potential interactions indicating a possible function for β-synuclein with regard to SNARE protein complex formation that cannot yet be excluded. Therefore, it is possible that β-synuclein acts in a supporting role at the pre-synapse attempting to modulate SNARE protein behaviour and ensure ongoing synaptic transmission and loss of β-synuclein at the pre-synapse will see the demise of any functional relationship.

One could speculate, that loss of SNARE complex formation would disrupt the neurochemical balance in relation to neurotransmitter release which could impact cognitive function. Furthermore, loss of the SNAREs may have a wider reaching impact, loss of VAMP2 is associated with impairment of protein degradation and slow neurodegeneration, that is not linked to its role in neurotransmitter release [282]. The yeast homolog of SNAP-25 is required for autophagosome and yeast vacuole fusion, the equivalent of the mammalian lysosome fusion [283]. It must, therefore, be considered that loss of /increases in SNARE proteins may disrupt processes such as autophagy, dysfunction of which is observed in DLB.

Results chapter 7: Autophagy in the cortical regions of the DLB brains and in vitro

Failure in the lysosomal-proteolytic degradation pathway has been implicated in neurodegeneration with the accumulation of enlarged and morphologically impaired autophagosomes/lysosomal-like organelles observed in DLB [249] and high levels of lysosomal-like vacuoles in PD [250]. Variations in some autophagy proteins are observed in Lewy body disease [284] and upregulation of autophagic factors leads to a recovery of neurodegenerative phenotypes [285]. β-synuclein has been shown to promote neuronal survival [178] and prevent neurotoxic cell death [180]. Conversely, increased levels of mutated β-synuclein lead to enhanced lysosomal dysfunction [168], is associated with autophagosome-like swellings [177] and in an AD mouse model, β-synuclein levels are increased and localised with enlarged lysosomes [286].This study has identified increased levels of β-synuclein protein in the frontal cortex of the DLB brain with increased cytosolic representation. The next step was to examine key autophagy factors in these regions to identify any autophagy-related changes that may be occurring.

7.1: Examination of p62, LC3, Beclin and LIMP2 protein levels in the cortical regions of the human brain

In order to identify whether potential alterations in the autophagy process occurs in regions with altered β-synuclein protein levels, I examined some key lysosomal and autophagosome-associated proteins in the cortical regions of human brain lysates by western blotting.

7.1.1: P62 protein levels in the human brain

Autophagy receptor p62 binds to ubiquitinated cargo destined for autophagy [287] and is a component of Lewy bodies [288], with loss of p62 seeing an increase α-synuclein fibrillisation properties [289]. P62 is degraded with the cargo by lysosomal enzymes, following the fusion of the lysosome and the autophagosome.

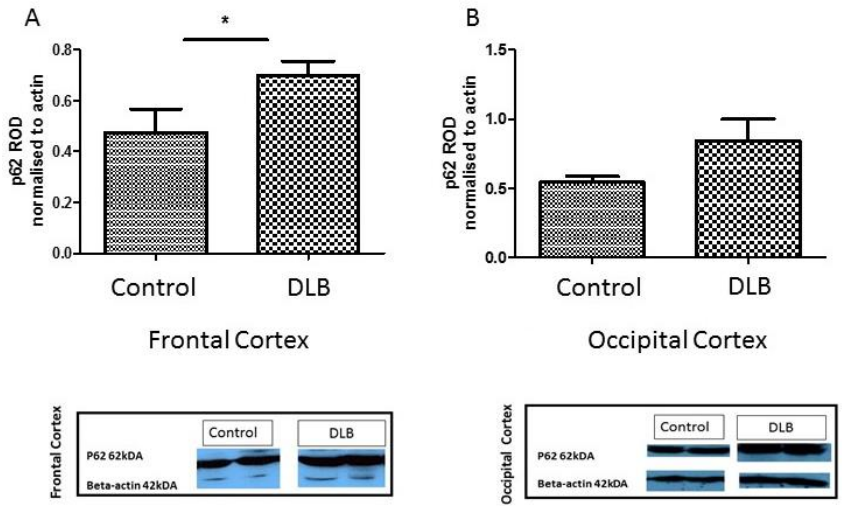

Figure 38 - p62 is increased in the DLB frontal cortex

Western blot analysis of levels of p62 in the frontal cortex (A) of controls (n= 8) and DLB (n= 15) and p62 in the occipital cortex (B) of controls (n= 7) and DLB patients (n= 13). P62 is increased in the frontal cortex in DLB (P=0.04). Statistics were revealed by Student's two-tailed t-test.

The examination of p62 in the frontal cortex (Figure 38-A) identified a significant increase in levels of the protein in the DLB patients when compared to the controls (P=0.04). In contrast, no changes were observed in the occipital cortex (Figure 38-B) between the groups.

7.1.2: LC3 protein levels and distribution in the human brain

The increase of p62 in the frontal cortex of the DLB brains led to the examination of the levels of LC3-II in the cortical regions. LC3-II serves as an anchor for P62 [284] and the reciprocal binding, between these two proteins, ensures the efficient delivery of the cargo to the autophagosome for degradation; further, LC3-II is required for elongation of the phagophore to an autophagosome [234]. An increase in LC3 in the temporal cortex [249] and the entorhinal cortex and amygdala [290] of DLB brains is reported. The frontal and occipital cortex was next examined.

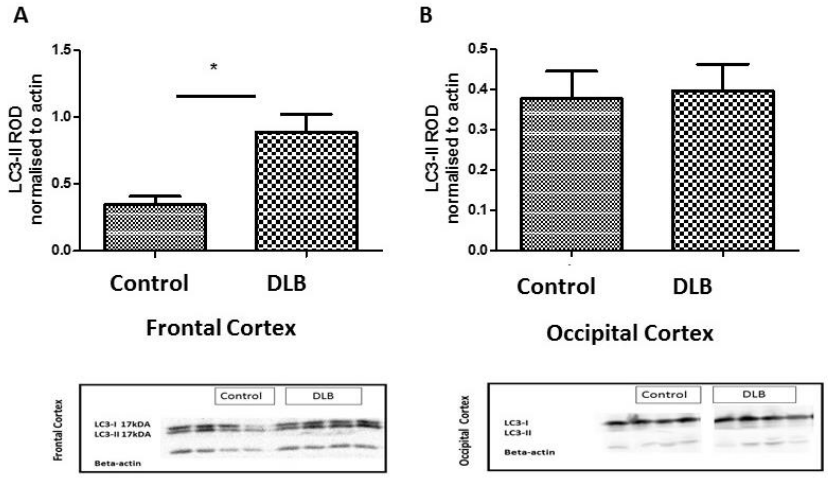

Figure 39 - LC3-II protein is increased in the DLB frontal cortex

Levels of LC3-II in the frontal cortex (A) of controls (n= 4) and DLB (n = 8) and LC3-II in the occipital cortex (B) of controls (n= 6) and DLB patients (n=7). LC3-II is increased in the frontal cortex of the DLB brain (P=0.02). As detected by western blot analysis. Statists revealed by Students two-tailed t-test.

Analysis of LC3-II in the cortical regions shows that LC3-II is significantly increased in the frontal cortex of the DLB patients when compared to the control (Figure 39)

(P= 0.02); however, no changes are observed in the occipital cortex. These changes mimic the changes that are observed when examining p62 in these regions.

7.1.3: LC3-I protein in the human brain

Under physiological conditions, LC3B is expressed as a full-length protein and stimulation of autophagy leads the proteolytic cleavage of its CTD to form LC3-I [232] the precursor to LC3-II [248]. Having identified changes in the frontal cortex of LC3-II next I sought to examine LC3-I in this region.

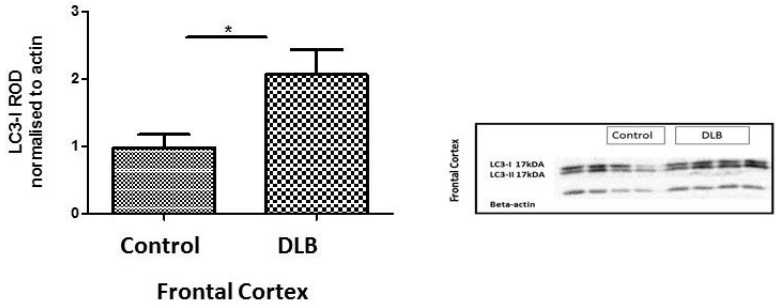

Figure 40 - LC3-I is increased in the frontal cortex of the DLB brain

Levels of LC3-I in the frontal cortex of controls (n= 4) and DLB (n= 7) as detected by western blot analysis. LC3-I is increased in the frontal cortex of the DLB brain (P=0.04). Statistics revealed by student's two-tailed t-test.

Examination of LC3-I in the frontal cortex identified a significant increase in LC3-I protein levels in the DLB brains when compared to the controls (Figure 40) (P= 0.04).

7.1.3: Beclin 1 protein in the human brain

The finding that the un-lipidated form of LC3, LC3-I, is increased in DLB patients in the frontal cortex indicates that there may be early events in the autophagy process that are involved in dysfunctional clearance functions. Beclin 1 is a major factor in the initiation of autophagosome formation and early in PD a significant reduction of beclin 1 is observed [291], indicating potential time-course events in protein levels under pathological conditions.

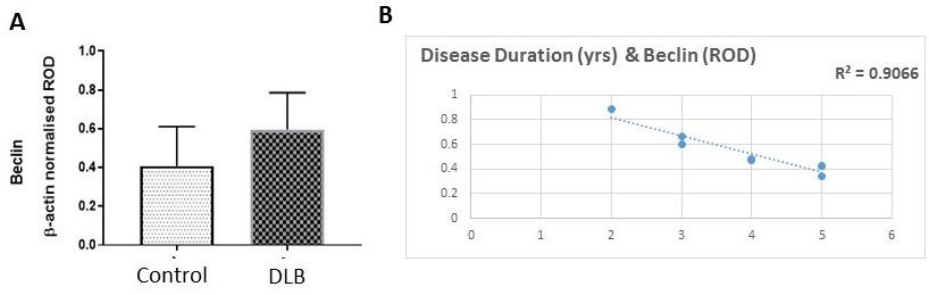

Figure 41 - Beclin levels are unchanged in the frontal cortex of the DLB brain when compared to the control and there is a negative correlation between protein levels of Beclin 1 and the disease duration

Levels of beclin 1 in the frontal cortex of controls (n= 7) and DLB (n = 12) as detected by western blot analysis. Statistics revealed by Students t-test. Regression analysis shows a negation correlation (R^2) between levels of beclin 1 and the duration of disease (2 – 5 years) in the frontal cortex (n=7). R^2 analysis performed by Excel.

There is no change in the levels of beclin 1 when comparing DLB patients to the controls in the frontal cortex (Figure 41); although large inter-group variations seen. I then examined the correlation between beclin levels and the disease duration by regression analysis. DLB patients with a disease duration spanning 2-5 years in the

frontal cortex, identified a strong relationship between a decline in beclin 1 levels as the pathology progressed (Figure 41), (R^2 0.9); indicating that levels of beclin 1 are higher earlier in the disease course in the frontal cortex of the DLB patient and significantly reduce over a period of 5 years of α-synuclein-induced pathology.

7.1.4: Lysosomal marker (LIMP2) protein levels in the human brain

DLB has been associated with lysosomal pathology, in particular, the SCARB2 gene (LIMP2) has been identified as a risk factor [96]. LIMP2 is a transmembrane lysosomal associated protein and a binding receptor for enzyme glucocerebrosidase (GC), ensuring the efficient delivery of the enzyme to the lysosome [292]. Importantly, GC may be involved in the degradation of α-synuclein, with mutations in the GBA gene increasing the risk of PD [293]. LIMP2 in the frontal cortex and occipital cortex of control and DLB patients were explored to identify whether protein levels of this protein are altered in our DLB brains, in the regions that we have identified as having modified β-synuclein levels.

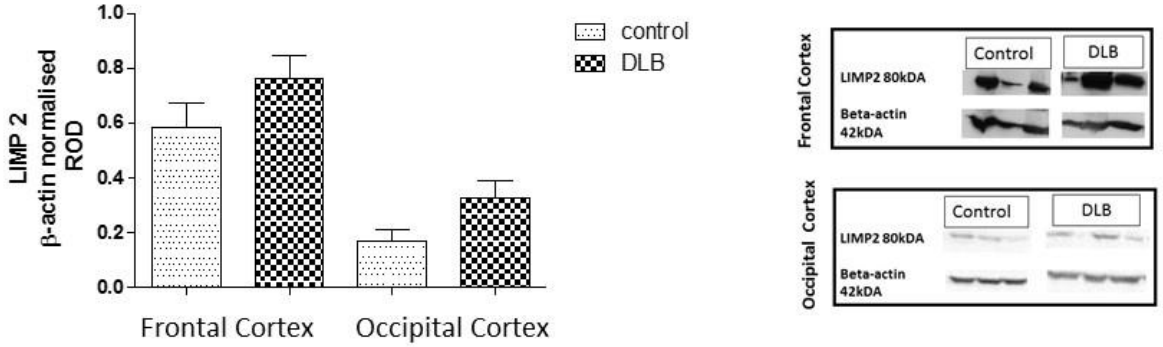

Figure 42 - LIMP2 is unchanged in the DLB brain when compared to the control

Levels of LIMP2 in the frontal cortex of controls (n= 7) and DLB (n = 15) and LIMP2 in the occipital cortex of controls (n= 7) and DLB patients (n=10). Statistical analysis was performed by one-way ANOVA and Bonferroni post hoc test.

There were no discernible differences of Limp2 in the DLB brain when compared to the controls in either the frontal cortex or occipital cortex (Figure 42). The frontal cortex showed large inter-group variation in both the controls and DLB patients that may be corrected by higher patient numbers.

7.2: β-synuclein and autophagy markers in vitro

This study has shown that p62 and LC3-II are increased in the frontal cortex of the DLB brain and strong evidence is provided for an increase in β-synuclein in the same region of the DLB brain. There is evidence to support a role for mutated β-synuclein in lysosomal dysfunction [168] and the observed changes in β-synuclein, may potentially have a direct or indirect relationship with the autophagy related changes that are occurring in the DLB patients. In the human brain, it is not possible to establish whether β-synuclein induces the autophagy-related changes that are observed in DLB patients. In order to examine this potential relationship, examination of autophagy related proteins was undertaken in Hela cells and BE(2)-M17, neuroblastoma cells in the presence or absence of β-synuclein.

7.2.1: β-synuclein and autophagy markers in Hela cells

In order to explore the relationship between β-synuclein and autophagy related components, the use of a mRFP-GFP-LC3-II quenching assay was employed. This assay enabled the autophagy flux to be examined in Hela cells expressing a human-β-synuclein construct. The mRFP-GFP-LC3 vesicle quenching assay is widely used to monitor autophagosome synthesis and autophagosome-lysosome fusion devised to label the autophagic compartments prior to and subsequent to lysosomal fusion and cargo degradation [294].

The labelling of the autophagosome with LC3-II in GFP (green) and mRFP re results in co-localisation of the channels producing a yellow signal. During the lysosomal acidic degradation process, the GFP-LC3 signal is quenched but mRFP is maintained. Thus, providing an assay that enables the monitoring of the autophagosome prior to and following fusion events with the mRFP signal being an indication that the autophagy flux has proceeded to completion. The presence of a red signal represents autophagosomes and autolysosomes, in contrast, a green signal is indicative of autophagosomes only. Therefore, the examination of the number of autophagosomes vs. autolysosomes, as quantified by puncta number, enables the autophagy flux to be more accurately observed [295]. Furthermore, investigation of the levels of autophagy related proteins in neuroblastoma cells was examined in the presence of human β-synuclein by western blot analysis. Enabling the characterisation of autophagy changes in relation to increased levels of β-synuclein.

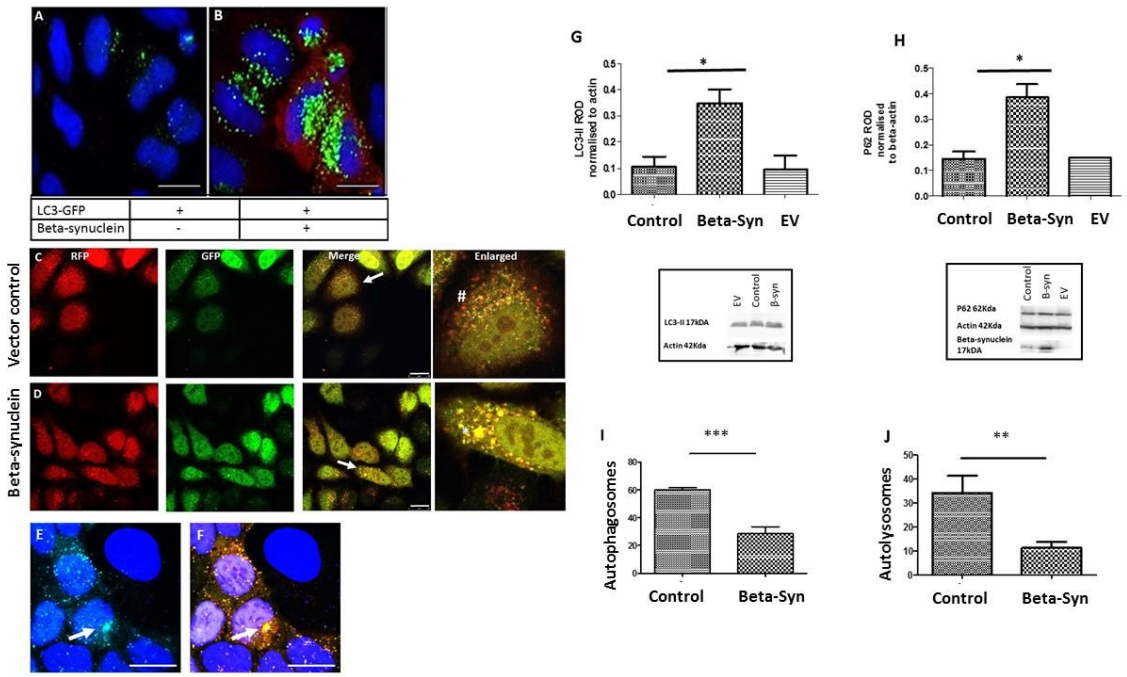

Figure 43 - LC3-II and p62 are increased in Hela cells in the presence of β-synuclein and autophagosomes and autolysosomes are depleted

Hela cells were transfected with or without human β-synuclein (A & B, respectively) in the presence of the LC3-GFP construct. There is an increase of LC3-GFP puncta (B) in β-synuclein containing cells (stained in red). mRFP-GFP-LC3 vesicle quenching assay (C & D) shows an increase in yellow puncta after overexpressing β-synuclein (D) versus control (C). LAMP2 in cyan blue (E) is associated with the large yellow structures (F). An increase of LC3-II (P=0.012) (G) and p62 (P=0.02) (H) levels are detected by western blotting in Hela cells. Autophagosomes are reduced in β-synuclein expressing cells (P=<0.0001) (I) and autophagy flux is decreased (P=0.003) (J). N = 3 experimental repeats. Statistical analysis performed by Student t-test. Scale bar 25μm.

In the presence of β-synuclein (Figure 43-B) there is a significant presence of LC3-II accumulations (B, arrow) in the cytoplasm of the Hela cells when compared to the cells not expressing the β-synuclein construct (Figure 43-A). The LC3-II labelling of the autophagosome (Figure 43-C & D) enabled the observation of the autophagy flux

in the presence (D) and absence (C) of human β-synuclein. In Hela cells over expressing human β-synuclein the presence of large yellow puncta was seen (D, enlarged, *) indicating a decrease in GFP-LC3-II quenching in cells overexpressing β-synuclein. Conversely, the controls (C) presented with small speckles that were labelled both green and red (C, enlarged, #). LAMP2 (cyan blue) (Figure 43-E) was associated with the large yellow structures (Figure 43-F). Furthermore, western blot analysis identified an increase in LC3-II (P = 0.012) (G) and p62 (P = 0.02) (H) in those cells expressing β-synuclein when compared to the control. The quantification of GFP and RFP structures demonstrated that in β-synuclein over expressing cell, autophagosome number (Figure 43-I) (P=<0.0001) and autolysosomes (Figure 43-J) (0.0033) are depleted.

7.2.2: β-synuclein and autophagy in neuroblastoma cells

The data I present (Figure 43) is indicative of a decrease in the autophagy flux in the presence of β-synuclein with the increase of LC3-II demonstrating that lysosomal degradation is not taking place. This is suggestive of β-synuclein interfering in upstream autophagy events. To validate these findings, the impact of β-synuclein on autophagy is addressed using neuronal cell lines. This ensures that the changes identified can be used to translate the changes presented in the human brain. Neuroblastoma cell line, BE(2)-M17 was used utilised with transient overexpression of β-synuclein or the control vector in the presence of a LC3-II construct in order to examine the behaviour of LC3-II in the presence and absence of β-synuclein. Furthermore, BE(2)-M17 cells were also treated with chloroquine, an inhibitor of autophagosome and lysosome fusion, in the presence and absence of β-synuclein in

an attempt to identify whether β-synuclein is preventing fusion and therefore, providing an explanation for the decrease in the autophagy flux.

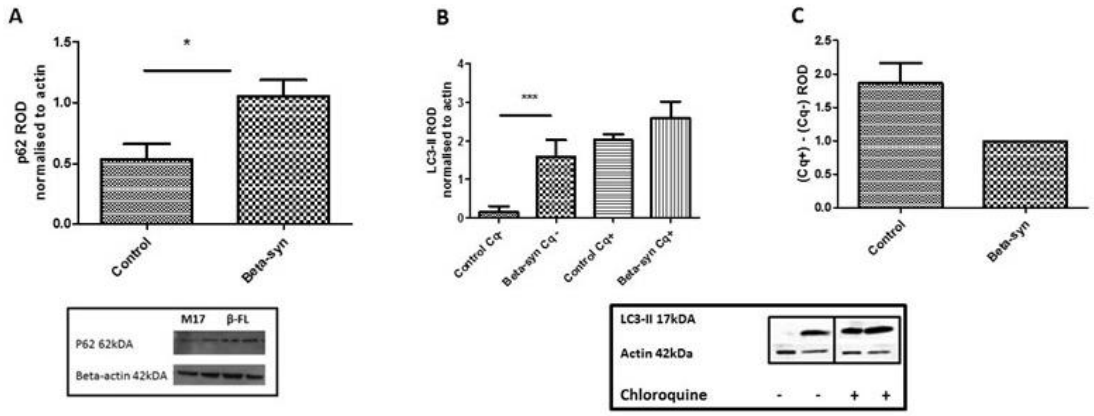

Figure 44 - β-synuclein impairment of the autophagy flux is comparable to chloroquine treatment indicating a reduction in autophagosome and lysosome fusion

An increase in p62 is revealed in β-synuclein stable expressers (P=0.03) (A). An increase in LC3-II levels is detected by western blotting in BE(2)-M17 (P=<0.0001) (B) after β-synuclein expression (FL Beta-syn) however, this dramatic increase was less pronounced in the presence of chloroquine (Cq). Autophagy flux (C) was calculated as the difference between the LC3-II levels in the cells treated with Cq and the vehicle. Statistics revealed by student's two-tailed t-test. N=3 experimental repeats.

In correlation with the data presented in the Hela cells (Figure 43), there is a significant increase in p62 in cells that stably express β-synuclein (Figure 44-A) (P=0.03). There is also an observed increase in LC3-II protein in the presence of β-synuclein when compared to the controls (P = < 0.0001), in the absence of chloroquine (Figure 44-B, Cq -). Whilst there is a trend for an increase, there is no

difference between the controls and β-synuclein expressing cells in the presence of chloroquine (Figure 44-B, Cq +). When performing quantification of the levels of LC3-II between chloroquine treated cells and non-treated, in the control group and β-synuclein expressing cells, the difference between the treatment and non-treatment is lower in the β-synuclein expressing cells when compared to the control (Figure 44-C).

Chapter 7: Results discussion

This study indicates that autophagy related proteins p62 (Figure 38), LC3-II (Figure 39) and LC3-I (Figure 40) are increased in the human brain lysates in the frontal cortex. These are essential autophagy markers that are critical for ensuring that the autophagy flux proceeds to completion. The increase in LC3-I may be a consequence of autophagy upregulation; alternatively, it may indicate that lipidation is not progressing at the rate required for protein removal. The presence of LC3-II shows that lipidation is occurring at some level, although, potentially if the insult is great, the cell may not be in a position to maintain the levels of autophagy required for clearance marked by the presence of LC3-II.

P62 and LC3-II are degraded with the cargo by lysosomal enzymes if the cargo does not reach the lysosome or a fusion event does not occur, these protein aggregates will accumulate in the cytoplasm leading to a backlog of multi-complex formations destined for removal. When considering the point at which the autophagy flux is impaired, it is possible that a) the autophagosome is not engulfing the p62 bound cargo and/or b) the autophagosome engulfed cargo is not being delivered to/fusing with the lysosome for degradation.

The increase in autophagy-related proteins is occurring only in the frontal cortex of the DLB patients, the region that sees an increase in β-synuclein protein levels in these patients. In correlation with the findings in the DLB human brain, the in vitro observations show an increase in β-synuclein is associated with an increase in LC3-II and p62. It is possible that this increase is related to the failure in the autophagy flux to move towards completion, as identified by large yellow structures. These may be autophagosome accumulations, indicating that there is a block in the early stages of the pathway and this is possibly being mediated by the presence of β-synuclein.

Furthermore, a reduction in the presence of autolysosomal fusion with autophagosomes is seen (Figure 43) however, surprisingly a decrease in autophagosomes is also seen despite an increase in LC3-II levels. In this regard, it is feasible that the large structures are indeed accumulations of autophagosomes or autophagosomes and lysosomes that are prevented from fusing. The traffic light cells are well reported in the literature. The quantification enables only the counting of single puncta; therefore, in reality, autophagosome number may not be reduced but rather backing up and accumulating in different regions of the cell leading to these large yellow structures that are observed (Figure 43). In neuroblastoma cells, LC3-II levels and P62 levels are also increased in the presence of β-synuclein and but there is no change when the cells were treated with chloroquine (Cq) (Figure 44). Cq is used to prevent autophagosome and lysosomal fusion indicating a similar role for β-synuclein that is not accentuated in the presence of chloroquine; in this regard, the difference between the Cq treatment and non-treatment ROD in the presence of β-synuclein sees lower levels of LC3-II indicating a potential threshold has been reached. Examination of LIMP2 levels by western blot analysis (Figure 42) show that in the frontal cortex and occipital cortex of the DLB patients when compared to controls there are no changes; however, LIMP2 levels are lower in the occipital cortex than the frontal cortex for both the control and DLB groups. This may suggest that the functional requirement for LIMP2 in the occipital cortex may be reduced and in this regard, it is also possible that in pathological conditions, lower expression of this protein leads to less trafficking of GC to the lysosome. In this situation, less lysosomal degradation of α-synuclein would occur in the occipital cortex when compared to the frontal cortex; therefore, an increase in deposits would be expected.

It is possible that regional differences in LIMP2 may lead to selective vulnerability in the clearance of protein aggregates.

Collectively, I show in the human brain, that autophagy dysfunction at different phases of the autophagy process may be implicated in the mechanisms that drive the neuropathology observed in DLB and potentially, other α-synucleinopathies. It is likely that various components of the autophagy pathway are implicated at different phases of the disease course. It is also possible that as the cellular system is over-powered by protein accumulations, its clearance capabilities become fatigued and less-efficient. Importantly, β-synuclein, like α-synuclein, has membrane binding properties, whilst under physiological conditions, this behaviour may not represent a threat, under the influence of pathology and increased levels of the β-synuclein protein, it is feasible that β-synuclein protein crowding can bind to the autophagosome or lysosome, leading to impairment of the autophagy process. Whether this is independent of α-synuclein or whether it accentuates α-synuclein-led pathology remains to be explored. These observations of an increase in β-synuclein in the frontal cortex, the abnormal granule deposits of the proteins in DLB patients, in addition to alterations in the autophagy pathway in this same region, give credence to the possibility that an increase in β-synuclein may present itself as playing a role in the pathological process.

Chapter 8: Final discussion and future work

8.1: Final discussion

Regional changes in the protein levels of the synucleins, key SNAREs and components of the autophagy pathway have been revealed in the cortical regions of the DLB human brain (Figure 45).

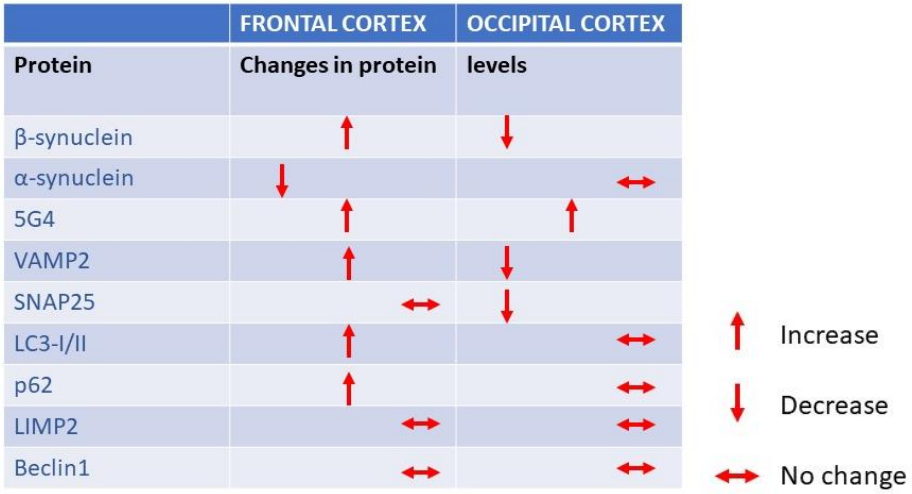

Figure 45 - An overview of protein changes identified in this study in the cortical regions of the DLB brain

The synucleins represent approximately 1% of the proteins in the cytosolic fraction of the human brain [25] indicating an important functional role for this family that for the most part remains elusive. β-synuclein and α-synuclein are binding partners with this relationship being described in relation to β-synuclein's attenuation capacity in α-synuclein pathology. This creates an important function for β-synuclein with the potential to inhibit the pursuance of α-synuclein aggregation and pathological behaviour in α-synucleinopathies. A role for β-synuclein has been explored in this study: (1) with examination of the levels of β-synuclein in the cortical-paralimbic

regions enabling the elucidation of whether β-synuclein levels are modified in relation to α-synuclein levels in the DLB brain; (2) in relation to key pre-synaptic SNARE proteins and (3) β-synuclein and the protein degradation pathway, autophagy.

I provide evidence for region-specific changes of β-synuclein protein levels in the DLB brain (Figure 18) in addition to its re-distribution and re-localisation into the neuronal soma. Modifications in the levels of β-synuclein are only observed in the cortical regions: an increase in the frontal cortex in parallel with a decrease in the occipital cortex. Despite the difference in the directional change in β-synuclein, both regions present with similar levels of pathological oligomeric α-synuclein (Figure 20) although, the frontal cortex sees a decrease in monomeric α-synuclein (Figure 19).

When comparing the synuclein proteins in the control brain, twice the level of α-synuclein in the frontal cortex is seen when compared to the occipital cortex (Figure 20), although α-synuclein is known to be highly expressed in the cortical regions, this indicates regional physiological preferences. In the DLB brains examined, the α-synuclein balance in the frontal cortex is reversed with an almost 50% depletion compared to the controls but a similar increase in β-synuclein is seen in the DLB patients (Figure 18). In the occipital cortex, α-synuclein remains unaltered in DLB with a decrease in β-synuclein levels; providing evidence for attempts in these regions to maintain levels of the synucleins overall.

The mechanisms behind these alterations in protein levels of β-synuclein is not clear. It was considered that protein level modifications might reflect changes in transcriptional activity by the synucleins, supported by previous studies that report a decrease in mRNA transcripts in the cortex of pDLB patients [98]. β-synuclein transcription in the frontal cortex has not been previously examined in isolation in the

DLB brain and pilot data indicate that β-synuclein mRNA is not significantly increased in the frontal cortex although future work will require an increase in patients to validate these findings and expansion into other regions. Therefore, based on the current evidence it is possible to surmise, that the increase in β-synuclein protein in the frontal cortex is attributed to protein stabilisation or impairment of protein degradation pathways, maintaining elevated levels of the β-synuclein protein in this region. In contrast, the decrease of β-synuclein in the occipital cortex may be explained by increased protein turnover or synaptic loss.

It is conceivable that an increase in β-synuclein protein levels and observed re-distribution of β-synuclein that I have identified in the frontal cortex of the DLB brain may be driven by a protein redundancy effect. Despite the lack of evidence for a role for β-synuclein at the pre-synapse, by virtue of its localisation properties it is likely to have a similar role that will see loss of α-synuclein being compensated for by β-synuclein. Evidence of synuclein family protein redundancy is provided by triple knockout models [146], a model that sees alterations in synaptic architecture and transmission deficits in addition to age-related neuronal dysfunction that does not appear with a single synuclein knockout. β-synuclein is specifically important in the cortex during the normal ageing process that sees cortical increases in β-synuclein mRNA and a decline in α-synuclein mRNA [296], prior to ageing similar levels of cortical synuclein expression is observed. This suggests that β-synuclein, under physiological conditions, is an important factor to consider in the ageing brain and changes in levels may lead to aberrant synaptic behaviours.

The significant loss of monomeric α-synuclein seen in the frontal cortex of the DLB brains may lead to an increase in the stability of the β-synuclein protein, resulting in a subsequent increase in β-synuclein protein levels. This may indicate an attempt by

β-synuclein in the frontal cortex to rescue any deficit in a physiological role resulting from declining monomeric α-synuclein levels or the increasing pathological behaviour of α-synuclein. This proposed role for β-synuclein is lost in the occipital cortex that sees the demise of β-synuclein. In the occipital cortex although monomeric α-synuclein is unchanged there is the additional burden of oligomeric α-synuclein. It is possible, therefore that these regional differences in β-synuclein may be more directly driven by the changes in total levels of the α-synuclein protein present in the region.

The appearance of β-synuclein, an otherwise pre-synaptic protein, in the cytosol of cortical neurons is novel and intriguing. The number of β-synuclein positive cells in DLB do not differ significantly between both cortical regions (Figure 28). This indicates that the reduced β-synuclein levels observed in the occipital cortex may be primarily associated with the cytosolic fractions of these brains with almost total loss of the protein in the synaptosomes. β-synuclein positive neurons are also present in the control brains, although at a reduced level. It is possible that the cytosolic presence of β-synuclein is a physiological effect. This effect may be accelerated in the wake of neurodegeneration, as identified by the increased number of β-synuclein positive cells in DLB patients when compared to the controls in the frontal cortex. Furthermore, β-synuclein has been shown to promote cell survival in the presence of cellular toxicity and inhibit apoptosis [101]; this may predispose a role for β-synuclein that involves re-localisation and upregulation of cell survival pathways to prevent cellular demise. Finally, it cannot be discounted that the cytosolic presence of β-synuclein may result from impaired trafficking events of the protein to or from the synapse. α-synuclein is associated with axonal trafficking defects [297] and it is thought that axonal dysfunction may also contribute to a “dying back” process that

leads to loss of neuronal function and loss of neurons from disease-specific neuronal populations [298]. In support of impaired trafficking, I observed 5G4-immunoreactive filiform structures in the cortical regions that appear more pronounced in the occipital cortex when compared to the frontal cortex (Figure 28). These structures may be regarded as LNs in differing phases of maturity, LNs are a predominant feature of DLB; although, it was not determined whether this immunoreactivity I observe is associated with axons or dendrites. In each case, the presence of oligomeric α-synuclein in neuronal extensions most likely will interfere with trafficking events and normal cellular functioning.

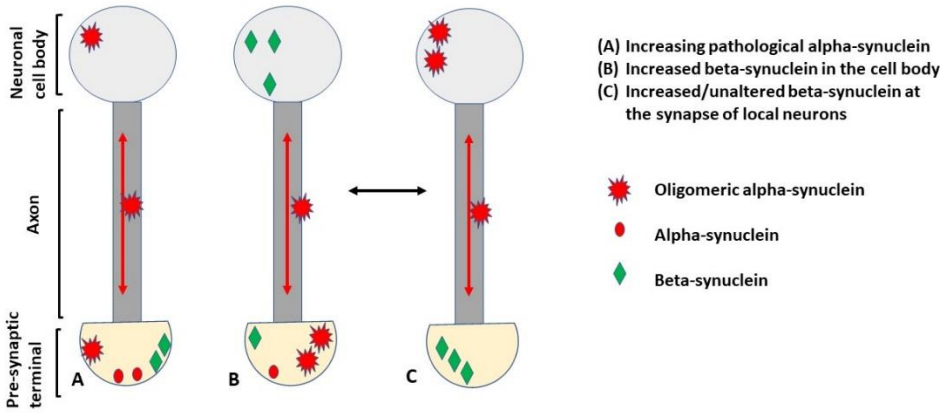

Figure 46 - An increase in the pathological presence of α-synuclein may lead to re-distribution of β-synuclein

(A) a cytosolic presence of β-synuclein in the absence of pathological α-synuclein in the cell body of local neurons or neurons with synaptic α-synuclein pathology (B) or β-synuclein may be increase in the pre-synapse and/or cytosol of neighbouring neurons (C) in an effort to modulate local neuronal activity or mediate the physiological function of α-synuclein with loss of α-synuclein.

In addition to modifications in the levels of β-synuclein, I identify alterations in SNARE proteins in the DLB brain with an increase in VAMP shown in the frontal cortex (Figure 34). The increase of VAMP2 that I observe in the DLB frontal cortex may also be driven by mechanisms attempting to rescue impending synaptic dysfunction in the face of increasing oligomeric α-synuclein and loss of monomeric α-synuclein in this region. α-synuclein assists in the cis-SNARE complex formation [141] but oligomers of α-synuclein have been shown to prevent SNARE complex formation and inhibit neurotransmitter release [299]. In ageing mice with triple synuclein knockout, an increase in VAMP2 is observed and VAMP2 levels have been shown to be increased in SNAP25 knockout mice [300], it is suggested that SNAREs such as VAMP2 may increase early in the pathology of α-synucleinopathies in an attempt to compensate for failing synapses [209], implying that changes in levels of key synaptic proteins may occur when others are lost in an effort to restore/maintain homeostasis at the synapse.

In contrast to the frontal cortex, VAMP2 and SNAP25 protein levels are reduced in the occipital cortex (Figure 34) supporting the idea that pre-synaptic loss or dysfunction is occurring in this region with a significant loss of pre-synaptic proteins. There is evidence that overexpression of α-synuclein leads to “vacant synaptic boutons” leading to the almost total loss of endogenous pre-synaptic proteins, including VAMP 2 [301]. This reinforces the idea that α-synuclein at the synapse may induce synaptic pathology that precedes neuronal cell death. The lack of SNARE proteins in the occipital cortex will see reduced neurotransmitter release resulting in a breakdown in the signalling circuitry. The concept that β-synuclein is acting in a compensatory manner to protect the synapse in the frontal cortex is supported by the evidence I provide for β-synuclein and SNARE protein co-localisation (Figure 37),

further, the changes I observed in β-synuclein protein levels are altered in the same direction as the SNARE protein level changes. This leads to the reasonable conclusion that β-synuclein may independently assist in the formation of the SNARE complex, like its family member α-synuclein. Although, co-localisation between β-synuclein and SNAREs is not changed between controls and DLB brains in the cortex it presents an important task for β-synuclein at the pre-synapse that is not primarily driven by pathological events but rather a physiological role. Under normal physiological conditions the primary driver may be α-synuclein and β-synuclein may act as a bystander until required. In this event, the loss of β-synuclein in the DLB brains will prevent any compensatory assistance that will potentially lead to enhancement of signalling dysfunction and synaptic demise (Figure 47); this may be representative of changes occurring in the occipital cortex of the DLB patients.

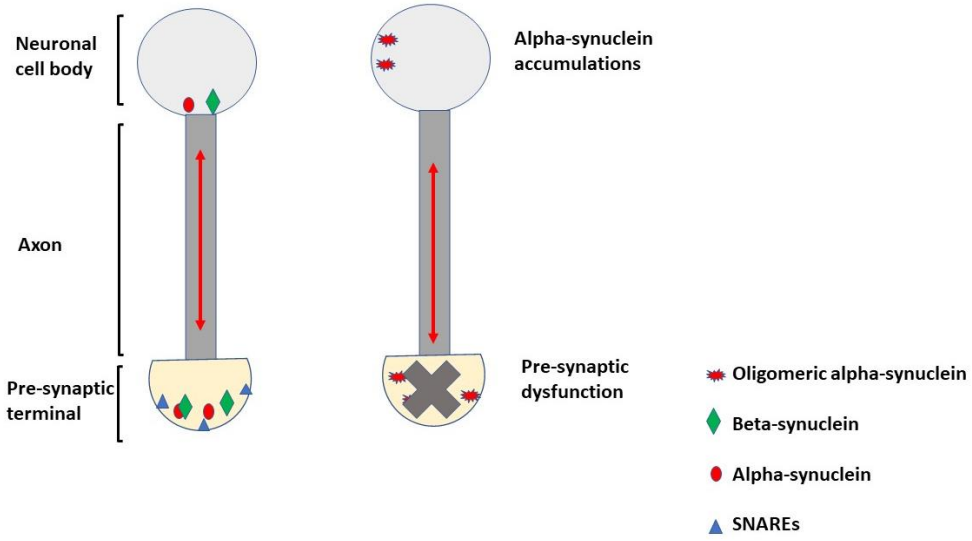

Figure 47 - Synaptic dysfunction in the absence of β-synuclein

Loss of β-synuclein and increasing α-synuclein levels see loss of pre-synaptic SNAREs

I have suggested that total α-synuclein regional levels may be the leading factor for the changes observed in β-synuclein protein levels and the consequential loss of other pre-synaptic proteins. In DLB the greatest burden of LBs is seen in the temporal lobe and limbic regions with lower levels seen in the frontal and the occipital cortex [27]. Indications are that the pathogenesis of α-synucleinopathies originates at pre-synapses with 90% of α-synuclein aggregates localised to the synapse [176].This study has provided evidence for high levels of oligomeric α-synuclein in the frontal and occipital cortex (Figure 20), that are likely causing pre-synaptic damage prior to sequestration of α-synuclein into LBs. It would be tempting to speculate that whilst LB pathology is considered to be low in the frontal cortex and occipital cortex of the DLB brain, the levels of oligomeric species of α-synuclein I observe in the DLB brain are undoubtedly producing a toxic effect. In both cases, this could explain the fluctuating cognitive impairment, an early sign of DLB that is associated with the frontal cortex in addition to the plethora of symptoms associated with the occipital cortex that include: visual hallucinations; poor responses to visuo-perceptive tasks, a severe deficit in neurotransmitters, involving the cholinergic system [259] and hypometabolism that is more severe than in other neurodegenerative conditions [302].

It is suggested that impaired synaptic activity, rather than neuronal loss, may fortify the symptoms associated with cortical neurodegeneration [303] & [304], it is likely that that the loss of SNAREs and increasing levels of α-synuclein in the occipital cortex would precede dysfunctional synaptic activity. Therefore, I propose that loss of the SNAREs and β-synuclein in the occipital cortex may contribute to the extensive cognitive dysfunction associated with the occipital cortex.

In the frontal cortex, increased β-synuclein correlates with disease duration (Figure 21), it is possible that loss of α-synuclein into oligomeric species, sees an increase in β-synuclein to balance the monomeric synucleins present in the frontal cortex. It needs to be established why this correlation does not occur in the occipital cortex. A potential explanation could be that an increase in β-synuclein is a disease-specific temporal event with the changes observed in the occipital cortex being more resonant of this region becoming overwhelmed by α-synuclein pathology prior to the frontal cortex. Therefore, it needs to be established whether these changes I observe are related to a temporal spread of pathology with the possible demise of one cortical region preceding another during the disease course. Alternatively, it cannot be ignored that the occipital cortex may be more susceptible to changes in the face of increasing pathology; clarification of β-synuclein levels in other cortical regions will help establish whether this is a stand-alone effect or whether there are region-specific changes that occur in tandem leading to the symptoms produced in the DLB patient.

When considering the direct interaction between the synucleins that may bestow protection against α-synuclein aggregation, co-localisation by IHC has shown that the pathological species of α-synuclein very infrequently co-localises with β-synuclein in the human brain (Figure 28), unlike monomeric α-synuclein (Figure 28). β-synuclein is not associated with LBs or LNs [49] reinforcing the theory that β-synuclein is not involved in the pathological process but may be an innocent bystander. It may be promoted that the interaction between β-synuclein and α-synuclein arises from non-modified forms of the protein and PTMs may prevent this association. It is likely that under physiological conditions when a balance in the synuclein proteins is present, transient interactions may occur but if the environment

sees an increase in α-synuclein or modified α-synuclein, then β-synuclein levels may not be sufficient to prevent oligomerisation and a pathological environment may ensue.

I speculate as to whether the changes observed in the frontal cortex and occipital cortex in respect of β-synuclein levels may be representative of temporal changes occurring that relates to the pathological spread. To address the temporal aspect of α-synuclein pathology, in relation to changes in β-synuclein, a dopaminergic in vitro model overexpressing human α-synuclein was employed. Surprisingly, β-synuclein levels were unchanged in this model at the different time points assessed during increasing α-synuclein levels. In contrast, there was clear evidence of β-synuclein cellular localisation changes - those cells that have high levels of β-synuclein display low α-synuclein and vice versa. This is fitting with the human IHC brain data with re-distribution of β-synuclein observed and its absence in oligomeric α-synuclein containing cells. The re-distribution and unchanged levels of β-synuclein may indicate that cellular modulations occur that see an overall balance in synuclein availability in the culture. This is demonstrative of either the potential cross-talk between synucleins that is performed at an inter-cellular level or the passage of the synucleins between cells. α-synuclein has been shown to have prion-like properties and in vitro has been shown to transfer between cells [305]. It is entirely feasible that β-synuclein may display similar properties and is an avenue to be explored further. An alternative explanation in the in vitro model I employed is more specifically related to the neuronal phenotype and the lack of changes in β-synuclein proteins levels may be explained by the use of dopaminergic neurons alone, the human brain is not homogeneous in cell types and models involving other neuronal phenotypes needs to explored, of particular interest would be cortical neurons.

Collectively, changes in key pre-synaptic proteins are observed in the frontal cortex and occipital cortex of the DLB brain in the presence of increasing levels of oligomeric α-synuclein. In order to prevent protein accumulations, it is essential for degradation pathways to remove any toxic threat to the cell. This requires efficient cellular homeostatic mechanisms that are able to address cellular dysfunction. Ageing is a risk factor for neurodegeneration as proofreading mechanisms and detection systems decline with age. It may be that any age-related decline in performance may further contribute to the failure of the cells to remove aggregating proteins. Furthermore, an increase in protein levels and aberrant behaviours produces additional stress in the cell, leading to interference in these pathways that augments the pathological process.

I have also provided evidence for changes in key autophagy-related proteins in the frontal cortex of the DLB brain, leading to the hypothesis that these changes in autophagy may be related to the increase in β-synuclein. These results were not mimicked in the occipital cortex where β-synuclein is diminished. Aggregating α-synuclein has been shown to interfere with the UPS and autophagy pathways, due to the similarities in synuclein properties it is intriguing that β-synuclein may also interfere with pathways associated with degradation processes in the DLB brain. In the frontal cortex LC3 protein (Figures 39 & 40) and p62 (Figure 38) are increased and the increased presence of these proteins demonstrates an early block in the autophagy pathway that is prevented from proceeding to completion.

In vitro methods were employed to validate the block in autophagy in the presence of β-synuclein. I present data that supports a role for β-synuclein-positive cells displaying dysfunctional autophagy-related behaviours with the presence of increased levels of β-synuclein appearing to prevent the autophagy flux, resulting in

increased levels of LC3-II and p62 (Figure 43). Further, transient expression of β-synuclein reveals large autophagosome-lysosome accumulations in Hela cells and BE-2-M17 cells, in parallel to increases in LC3 and p62 (Figure 44). The inherent membrane binding capabilities of the synucleins by their NTD cannot exclude the possibility that β-synuclein is able to bind to the autophagosome/lysosome directly and impair fusion events (Figure 48). A role for β-synuclein in the accumulation of large autophagosome-lysosomal structures has already been described. β-synuclein mutants P123H and V70M produce a phenotype that sees these large accumulations of abnormal structures that predisposes a toxic gain of function for β-synuclein in impairment of autophagy [168]. Moreover, lysosomal storage disorders are associated with α-synucleinopathies. Both cortical regions see similar levels of oligomeric α-synuclein therefore, it is proposed that the observed changes in the autophagy proteins is directly related to the levels of β-synuclein; providing strong evidence for β-synuclein autonomously inducing dysfunction. The use of a lysosomal fusion inhibitor, chloroquine, showed β-synuclein produced levels of p62 and LC3-II that were not dissimilar with or without treatment (Figure 44), indicating that a threshold had been reached.

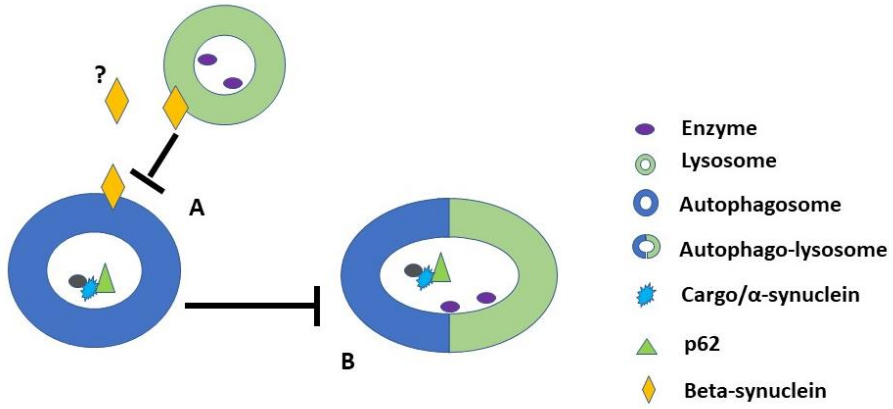

Figure 48 - β-synuclein prevents autophagosome and lysosomal fusion

β-synuclein prevents fusion of the lysosome and autophagosome (A) leading to inhibition of protein degradation.

An alternative explanation for the modifications occurring in the autophagy process may be as a direct result of the increase in β-synuclein, leading to upregulation of autophagy in order to enhance protein turnover of this protein. However, the increase in β-synuclein may contribute to protein crowding that then sees β-synuclein obstructing the process in the frontal cortex. α-synuclein is unable to be transitioned into the lysosomal lumen, in this regard the association of β-synuclein with the lysosome may be responsible for impairment of the fusion process. Whilst not intended to be pathological, the increase as a physiological response to pre-synaptic dysfunction may indirectly contribute to the failure of the autophagy system. Potentially, the finding that β-synuclein interferes with the autophagy flux in addition to the toxic effects of α-synuclein in the same region indicates two independent mechanisms that obstruct protein clearance strategies, resulting in the cellular accumulation of protein aggregates and autophagosome/lysosomes.

The role of α-synuclein in α-synucleinopathies is uncontested, but there are variable reports of the viability of α-synuclein as a biomarker in DLB. Increases in CSF-α-synuclein has been shown [306] in addition to decreases [307] and no changes [308]. Recently, β-synuclein in the CSF was shown to be consistently higher in the dementias when compared to α-synuclein, the β-synuclein to α-synuclein ratio was higher in PDD when compared to controls and PD further, β-synuclein highly correlates with tau levels in the CSF of AD patients [313]. This presents an important role for β-synuclein as a biomarker candidate in AD and PDD that requires further examination in relation to dementia. This study provides evidence for a positive correlation between increasing β-synuclein levels and disease duration in DLB in the frontal cortex only, an effect that is lost in the occipital cortex, a region that is relatively spared in the most common form of dementia, AD [309]. Therefore, further investigations into the regional levels of β-synuclein in other types of dementia, including AD, is necessary.

To surmise, the role of β-synuclein, previously considered a passive bystander, may be dependent on its cellular localisation status. Pre-synaptically, β-synuclein may act to maintain the integrity of the synapse in the absence of α-synuclein, in contrast, its cytosolic presence may represent a physiological or pathological response to α-synuclein toxicity that appears to directly impair essential protein clearance mechanisms in some brain regions.

8.2: Concluding remarks and future work

This study provides correlative evidence for a role for β-synuclein in the frontal cortex of the human brain, providing an interesting candidate for further exploration in the context of DLB and other dementias.

Evidence for a compensatory role of β-synuclein in the frontal cortex that may act to support the pre-synapse in the presence of increasing α-synuclein pathology and absence of monomeric α-synuclein requires further examination. Functional assays including the monitoring of neurotransmitter release in the presence of β-synuclein and absence of α-synuclein is required to clarify whether this protein is acting to preserve the integrity of the pre-synapse. In this regard, β-synuclein could be considered a valid therapeutic when considering its role at the pre-synaptic terminal.

Single cell transcriptomics from cells in the cortical regions that are positive for β-synuclein may identify whether α-synuclein mRNA is downregulated in these neurons and conversely, the level of β-synuclein mRNA in cells harbouring oligomeric alpha synuclein would also be useful to elucidate whether transcriptional changes are occurring in single cells. Investigations undertaken in this study have analysed the protein levels of whole tissue sections from a heterogenous cell population that needs to be narrowed down to single cell levels to reveal whether the changes in β-synuclein is restricted to specific cell populations.

In contrast to the protective role I propose for β-synuclein, a potentially detrimental role is observed with β-synuclein appearing to be directly or indirectly involved in the discontinuation of the autophagy flux. It is possible that the role of β-synuclein is to inhibit the process of one degradation pathway thereby, upregulating another. Future

work should also focus on β-synuclein in relation to the UPS and CMA pathways both in the presence and absence of α-synuclein.

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