THE ROLE OF THE ASCENDING RETICULAR ACTIVATING SYSTEM IN COGNITIVE FLUCTUATIONS IN DEMENTIA WITH LEWY BODIES

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Abstract

Background

Cognitive fluctuations occur in 80-90% of patients with dementia with Lewy bodies (DLB) and have a profound negative impact on the patient's quality of life. However, cognitive fluctuations are the least well characterised of the core clinical features. The ascending reticular activating system (ARAS) has an important role in arousal and awareness, therefore, has been implicated in the aetiology of cognitive fluctuations in DLB.

Methods

Post-mortem tissue from components of the ARAS, locus coeruleus, pedunculopontine nucleus, raphe and medial prefrontal cortex (mPFC), was obtained from DLB cases with a clinical history of cognitive fluctuations and compared to cognitively normal control, mixed Alzheimer's disease (AD)/DLB cases with cognitive fluctuations, and mixed AD/DLB and AD without cognitive fluctuations. Pathological burden of protein aggregates and neurotransmitter markers were quantified in individual cases. Immunoblots were performed on isolated synaptosomes from mPFC tissue to examine ARAS projection integrity. Influence of pathological and biochemical markers on the presence and severity of cognitive fluctuations were determined

Key findings

Neurodegenerative pathology was observed in all regions examined, with no specific pattern or level of pathological burden associated with the presence or absence of cognitive fluctuations. Alterations to the serotonergic system in DLB may relate to the severity of cognitive fluctuations. Synaptosomal isolation may preferentially select for intact, functional, synapses as neurotransmitter marker levels analysed did not differ from controls

Conclusions

This study identified that the severity of protein aggregates in the ARAS was not associated with the presence or absence of cognitive fluctuations. The results may indicate that DLB patients have a pathologically compromised arousal system, but that this likely acts in concert with other mechanisms to elicit cognitive fluctuations. As cognitive fluctuations are transient, one may speculate that static protein aggregates are unlikely to underlie a clinical feature defined by variable clinical presence.

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Abbreviations

Aβ- amyloid-beta
AChE- acetylcholinesterase
AChEI- acetylcholinesterase inhibitor
AD- Alzheimer's disease
APP- amyloid precursor protein
ARAS- ascending reticular activating system
BA- Brodmann area
Ca^{2+} - Calcium
CAF- Clinical assessment of fluctuation
ChAT- Choline acetyltransferase
DAB- 3' diaminobenzidine
DaTSCAN- dopamine transporter imaging
DBS-deep brain stimulation
DCFS- Dementia Cognitive Fluctuation Scale
DLB- dementia with Lewy bodies
DR- dorsal raphe
EDTA- Ethylenediamine tetra-acetic acid
EEG- electroencephalography
FTD- frontotemporal dementia
GAPDH- glyceraldehyde 3-phosphate dehydrogenase
GDS- Geriatric Depression Scale
H&E- Haematoxylin & Eosin
IHC- immunohistochemistry
LATE- limbic-predominant age-associated Transactive response DNA-binding protein 43 encephalopathy
LBD- Lewy body diseases
LC- Locus coeruleus
LFB- Luxol Fast Blue
MCFS- Mayo Composite Fluctuations Scale
MCI- mild cognitive impairment
MCI-LB- mild cognitive impairment with Lewy bodies
MIBG- metaiodobenzylguanidine
MMSE- mini mental state examination
MnR- median raphe
mPFC- medial prefrontal cortex
MRI- magnetic resonance imaging
NAC- non-amyloid-beta-component
NBTR- Newcastle Brain Tissue Resource
NET- Noradrenaline reuptake transporter
NFT- neurofibrillary tangles
NGS- normal goat serum
nLBD- neocortical/limbic pathologically Lewy body disease
ODFAS- One day fluctuation assessment scale
P1- pellet 1
P2- pellet 2
PAGE- polyacrylamide gel electrophoresis
PBS- phosphate buffered saline

PD- Parkinson’s disease
PDD- Parkinson’s disease dementia
PET- positron emission tomography
PFC- Prefrontal cortex
PIGD- postural instability gait difficulty
PPN- pedunculopontine nucleus
pS129- α-synuclein phosphorylated at Serine 129
RBD- Rapid eye movement sleep behaviour disorder
REM- rapid eye movement
S2- supernatant 2
SDS- sodium dodecyl sulphate
SERT- Serotonin reuptake transporter
SN- Substantia nigra
SPECT- single-photon emission computed tomography
TBS- tris-buffered saline
TBS-T- tris-buffered saline
TD- tremor dominant
TDP-43- Transactive response DNA-binding protein 43
TPH2- Tryptophan hydroxylase 2
vAChT- vesicular acetylcholine transporter
VaD- Vascular dementia
VMAT2- vesicular monoamine transporter 2
VTA- ventral tegmental area

Chapter 1: Introduction

1.1 Dementia

The global population is ageing, with people living longer than at any point previously. The United Nations World Population Ageing Highlights have stated that the global population of over 60s has increased from 205 million in 1950 to 962 million in 2017, with this projected to reach 2.1 billion by 2050, the number of people living until they are 80 and older is also set to triple by 2050 (United Nations, 2017). The result in this rapid increase in the aged population is a rise in age-related diseases and their associated societal, economic and medical problems.

Dementia is an 'umbrella' term used to define a group of conditions that are characterised by a noticeable, progressive and irreversible cognitive decline, through disease or dysfunction of the brain that impacts upon the independence of daily living (World Health Organization, 1992; American Psychiatric Association, 2013). Dementia can result from a number of conditions including Alzheimer's disease (AD), dementia with Lewy bodies (DLB) and frontotemporal dementia (FTD). With the incidence and prevalence of dementia increasing with age, the ageing global population has led to an increase in the number of people living with dementia (Prince *et al.*, 2014). In the United Kingdom presently there are approximately 850,000 individuals living with dementia, with this figure projected to rise to 1 million by 2025 (Prince *et al.*, 2014) . The current lack of available disease-modifying therapies is likely to exacerbate the impact that the rise in numbers presents.

1.1.1 Epidemiology

Globally the incidence and prevalence of dementia increases with age. The incidence of dementia increases from 0.1% at 60 to 8.6% at 90 years of age (Jorm and Jolley, 1998), with the overall prevalence doubling with every 5 years of age after 65 (Lobo *et al.*, 2000). In more economically developed countries this is 5-10% of those over 65 years of age, with a larger prevalence in women than men, although this is thought to be due to an increased life expectancy in women as there is no difference in incidence between the sexes (Hugo and Ganguli, 2014).

DLB is the second most common cause of neurodegenerative dementia after AD (Heidebrink, 2002), annually accounting for 3.2% of the new dementia cases (Zaccai *et al.*, 2005). Approximately 1-2% of the global population over 65 years are diagnosed with DLB,

with this rising to 5% in those of 75 years (Jellinger and Korczyn, 2018). The prevalence of DLB is estimated to be 4.2-4.6% of all dementia cases within a community setting, with this percentage rising to 7.5% in secondary care populations (Vann Jones and O'Brien, 2014; Kane *et al.*, 2018). However, post-mortem studies have suggested the prevalence of DLB was found to account for 20% of all dementia cases (McKeith, 2000). The disparity between clinical and post-mortem findings could be explained by an underdiagnosis clinically or an over representation at post-mortem.

1.2 Dementia with Lewy bodies

1.2.1 Clinical features

DLB is characterised by the presence of cognitive decline in conjunction with core and supportive signs and symptoms, along with indicative and supportive biomarkers. The four core clinical features of DLB are cognitive fluctuations, parkinsonian extra-pyramidal symptoms, rapid eye movement (REM) sleep behaviour disorder (RBD), and complex visual hallucinations (McKeith *et al.*, 2017). Supportive features of DLB include autonomic dysfunction and severe sensitivity to neuroleptics. Three indicative biomarkers are supportive to a clinical diagnosis of DLB: reduced dopamine transporter uptake in the basal ganglia demonstrated by single-photon emission computed tomography (SPECT) or positron emission tomography (PET); abnormal (low uptake) ^{123}iodine- metaiodobenzylguanidine (MIBG) myocardial scintigraphy; polysomnographic confirmation of RBD. Two core features or one core feature and a positive biomarker are needed for a probable diagnosis of DLB and one feature or one positive biomarker for a possible diagnosis (McKeith *et al.*, 2017). Recently there has been a move to try diagnosing DLB at an earlier stage, research has focussed on mild cognitive impairment (MCI) with Lewy bodies (MCI-LB) with new criteria having been developed (McKeith *et al.*, 2020), with MCI representing the stage between the expected cognitive decline of normal ageing and that seen in clinical dementia (Mayo Clinic, 2020). A number of the core and supportive features of DLB are present in the MCI-LB stage, including RBD, motor symptoms and autonomic symptoms (Donaghy *et al.*, 2015; Donaghy *et al.*, 2017; McKeith *et al.*, 2020), however, it is thought that the combination of these features occurs less frequently than in the those with clinical dementia. The mean age for presentation of DLB is 75, with an average life expectancy of 3.3-4.0 years post diagnosis; shorter than other dementia subtypes, which may reflect a delay in diagnosis (Vann Jones and O'Brien, 2014; Zweig and Galvin, 2014; Price *et al.*, 2017).

The core features of DLB are also observed in other neurodegenerative diseases including Parkinson's disease (PD) and PD with dementia (PDD). PDD and DLB possess overlapping clinical features, therefore for a diagnosis of DLB in a patient with parkinsonian features, the onset of dementia and the parkinsonism features must occur contemporaneously, defined for research purposes as the parkinsonism occurring within a year of the dementia (the 'one-year rule'). Thus, when parkinsonism precedes dementia by a year or more, a diagnosis of PDD is made (McKeith *et al.*, 2005).

1.2.1.1 Core clinical features

DLB is characterised by the presence of four core clinical features: cognitive fluctuations, parkinsonian extra-pyramidal symptoms, rapid eye movement (REM) sleep behaviour disorder (RBD), and complex visual hallucinations (McKeith *et al.*, 2017).

In DLB, fluctuating cognition, one of the four core features, has been shown to affect up to 90% of patients (Byrne *et al.*, 1989; McKeith *et al.*, 2005), with 56% of those with MCI-LB presenting with cognitive fluctuations (Donaghy *et al.*, 2017; McKeith *et al.*, 2020). The incidence of cognitive fluctuations in DLB is in contrast to those with a diagnosis of AD, where they are only encountered in 10-20% of patients (McKeith *et al.*, 1992b; McKeith *et al.*, 1994; Walker *et al.*, 2000b). Clinically fluctuating cognition in DLB manifests as spontaneous and transient alterations to arousal, attention and cognition (Zupancic *et al.*, 2011), although they have been shown to be independent to alterations in alertness (Bliwise *et al.*, 2014). The manifestation of fluctuating cognition in DLB is qualitatively distinct from those observed in AD, which typically are associated with transient episodes of confusion (McKeith *et al.*, 2005). Fluctuating cognition has a profound negative impact on the patient's quality of life and ability to function day to day (Gibb *et al.*, 1987; Yamamoto and Imai, 1988; Byrne *et al.*, 1989; McKeith *et al.*, 1992b; Zweig and Galvin, 2014). Although an important factor for the diagnosis of DLB, it has been found to be one of the most difficult to reliably identify and evaluate (Van Dyk *et al.*, 2016). Dysfunction to cholinergic systems is associated with fluctuations, particularly alterations to nicotinic receptors in the temporal cortex (Ballard *et al.*, 2002b; Gore *et al.*, 2015). Furthermore, the acetylcholinesterase inhibitor (AChEI) donepezil, has been shown to offer a modest improvement to cognitive fluctuations, as measured via the Cognitive Fluctuation Inventory score, compared to placebo in a clinical trial (Mori *et al.*, 2012).

Recurrent, complex visual hallucinations are also a core clinical feature of DLB, occurring in up to 80% of patients and 29% of those with MCI-LB, with their presence being a frequent clinical signpost to diagnosis (Donaghy *et al.*, 2017; McKeith *et al.*, 2017). Visual hallucinations have also been found to occur in other neurodegenerative disorders, for example AD and PD. However, compared to visual hallucinations in other neurodegenerative disorders those in DLB appear earlier in the disease progression (Fenelon et al., 2000), are more prevalent (Ballard *et al.*, 1997; Fenelon *et al.*, 2000), have increased severity (Chiu et al., 2016) and are more likely to be a persistent feature throughout the disease course

(Ballard et al., 1997). In DLB, visual hallucinations typically consist of objects including animals, people and faces, with frequent secondary delusional ideas, beliefs arising from and in an attempt to comprehend the primary hallucinations, that a stranger or intruders are in the home. Whereas, visual hallucinations reported by those with AD are briefer and simpler , where hallucinations lack a recognizable form such as shapes and patterns (Mosimann *et al.*, 2006; Urwyler *et al.*, 2016). As well as visual hallucinations, other visual abnormalities also occur in DLB, including impaired saccadic function and impairment to complex visual tasks for example drawing simple objects (McKeith *et al.*, 2005; Armstrong, 2012). However, although visual abnormalities occur in DLB, there is an absence of major pathological changes to the primary visual cortex and the lateral geniculate nucleus (Erskine *et al.*, 2016; Khundakar *et al.*, 2016), suggestive of an impairment to visual processing in the development of visual hallucinations. Preservation of the visual pathways may indicate a neurochemical origin to visual hallucinations and cholinergic deficits have also been associated with visual hallucinations (Perry *et al.*, 1990b). AChEIs have been shown to be effective in stopping visual hallucinations in DLB, supporting the neurochemical origin of this clinical phenomenon (McKeith *et al.*, 2000a).

RBD occurs in 76% of DLB cases, compared to the phenomenon being observed in only 4% of non-DLB cases, prompting its recent inclusion as a core feature in the latest consensus guidelines (Ferman *et al.*, 2011; McKeith *et al.*, 2017). RBD is defined as a parasomnia characterised by recurrent dream enactment behaviour that includes mimicking dream content and absence of normal REM sleep atonia. RBD can be particularly noticeable when the patient or partner sustains injuries due to the violent limb movements (Postuma *et al.*, 2009; Ferman *et al.*, 2011). In DLB, the onset of RBD can begin years or decades before the first signs of cognitive impairment (Boeve *et al.*, 2011).

The fourth core feature of DLB is spontaneous parkinsonian features, including parkinsonism; bradykinesia, rest tremor and rigidity which are common, but not invariant, in DLB. Approximately 60-92% of DLB patients develop parkinsonian features during their disease course (Ferman *et al.*, 2011). Unlike in PD where the presence of more than one parkinsonian feature is required for diagnosis, DLB patients commonly only display one of the cardinal features of parkinsonism; bradykinesia, rest tremor and rigidity (McKeith *et al.*, 2017).

1.2.1.2 Supportive clinical features

There are numerous common clinical features, often present early in the disease course but lacking in diagnostic specificity. These supportive clinical features include severe sensitivity to neuroleptics, autonomic dysfunction, hypersomnia, repeated falls and depression (McKeith *et al.*, 2017). A number of supportive clinical features are more common in DLB than in other dementias helping indicate a DLB diagnosis, especially if the features are present over an extended duration or in combination (McKeith *et al.*, 2005).

1.2.1.3 Indicative and supportive biomarkers

The Third Consensus report of the DLB Consortium introduced reduced dopamine transporter uptake in basal ganglia on SPECT or PET as a biomarker along with clinical features (McKeith *et al.*, 2005), with the Fourth Consensus report of the DLB Consortium introducing both indicative and supportive biomarkers to the diagnostic criteria for DLB (McKeith *et al.*, 2017). Reduced dopamine transporter uptake in basal ganglia on SPECT or PET, reduced uptake on ^{123}iodine-MIBG myocardial scintigraphy and polysomnography confirmation of RBD, were all included as indicative biomarkers of DLB (McKeith *et al.*, 2017). The presence of one indicative biomarker in conjunction with any of the core clinical features is necessary for a diagnosis of probable DLB (McKeith *et al.*, 2017). There are three supportive biomarkers: relative preservation of medial temporal lobe structures on a magnetic resonance imaging (MRI) scan; generalised low uptake on SPECT/PET perfusion/metabolism scan, reduced occipital activity and posterior cingulate island sparing on fluorodeoxyglucose-PET imaging; prominent posterior slow-wave electroencephalography (EEG) activity with periodic fluctuations in the pre-alpha/theta range. The three supportive biomarkers are consistent with DLB and aid diagnosis but lack diagnostic specificity (Harper *et al.*, 2016). It is an aspiration of the latest consensus report of the DLB consortium that genetic and fluid biomarkers should be developed to aid in the differential diagnosis of DLB (Guerreiro *et al.*, 2016; McKeith *et al.*, 2017).

1.2.1.4 Cognitive profile of DLB vs other Neurodegenerative diseases

Even at the MCI stage of DLB the characteristic profile of cognitive impairment observed at the later stages of the disease is present (Donaghy *et al.*, 2018; Ciafone *et al.*, 2020). The cognitive domains characteristic of DLB are deficits to attention, executive and visuospatial functions (Oda *et al.*, 2009; Kemp *et al.*, 2017), which are significantly more severe than those seen in AD (Yoshizawa *et al.*, 2013). Both MCI and late-stage AD are characterised by

impairments to memory, with those with MCI-AD tending to present with a more amnestic profile compared to those with MCI-LB (Oda *et al.*, 2009; McKeith *et al.*, 2016). The proportion of DLB patients with amnestic features is higher than that seen for Multiple System Atrophy or PD, likely due to the increased presence of AD pathology and hippocampal atrophy related to this (Goldman *et al.*, 2014). Evidence suggests that the overall cognitive profile of DLB patients is prominent deficits in attention and executive function, particularly tasks with a visuospatial component, such as the pentagon drawing component on the Mini-Mental State Examination (MMSE) (Oda *et al.*, 2009), and a relative preservation of memory in comparison to AD.

1.2.1.5 Clinical Management of DLB

Clinical management of DLB is often challenging due to the complex nature of the disease; key management strategies revolve around an early and accurate diagnosis, allowing for the correct medications and care provision to be put in place (McKeith *et al.*, 2017; Taylor *et al.*, 2020). As with other dementia types there are currently no treatments that slow the progression of the disease, with treatment options currently limited to symptomatic relief (Boot, 2015). The efficacy of AChEIs has been demonstrated in DLB, with evidence for donepezil and rivastigmine improving cognition (Stinton *et al.*, 2015; Wang *et al.*, 2015), as well as evidence for reducing apathy and improving visual hallucinations in DLB (McKeith *et al.*, 2000a). The use of neuroleptics for the management of behavioural disturbances or visual hallucinations is contraindicated in DLB due to the high risk of a serious sensitivity reaction (McKeith *et al.*, 1992a); there is no evidence to support the use of any antipsychotic in DLB (Taylor *et al.*, 2020). Levodopa/carbidopa can be used for treatment of motor symptoms in DLB, much like for PD, however, there is an increased risk of psychosis at higher doses in DLB (Goldman *et al.*, 2008).

1.2.2 Pathology

DLB is neuropathologically characterised by the presence of aggregated α-synuclein, the major protein component of abnormal neuronal aggregates known as Lewy bodies and Lewy neurites (McKeith *et al.*, 2005). Lewy bodies are spherical, dense cytoplasmic aggregates observed in the neuronal soma (Spillantini *et al.*, 1997), and they appear to affect certain neuronal subtypes and brain regions more preferentially than others (Erskine *et al.*, 2018), with Lewy neurites being found in the neuronal processes. Two types of Lewy body have been described: brainstem Lewy bodies comprise of a dense, acidophilic and argyrophilic

core surrounded by a halo of radiating fibrils, which are classically observed with Haematoxylin & Eosin staining; and cortical Lewy bodies which are eosinophilic, rounded, angular or reniform structures without a halo, cortical Lewy bodies are also not readily detectable by Haematoxylin & Eosin (Dening and Thomas, 2013; Outeiro *et al.*, 2019). Although Lewy bodies and neurites are the characteristic neuropathological feature of DLB, tau and amyloid-beta (Aβ) pathologies are commonly seen.

1.2.2.1 α-synuclein

α-synuclein is a small 14.5 kDa, 140 amino acid protein, which is divided into three functionally distinct sub-regions (figure. 1.1). The N-terminal, amphipathic region, amino

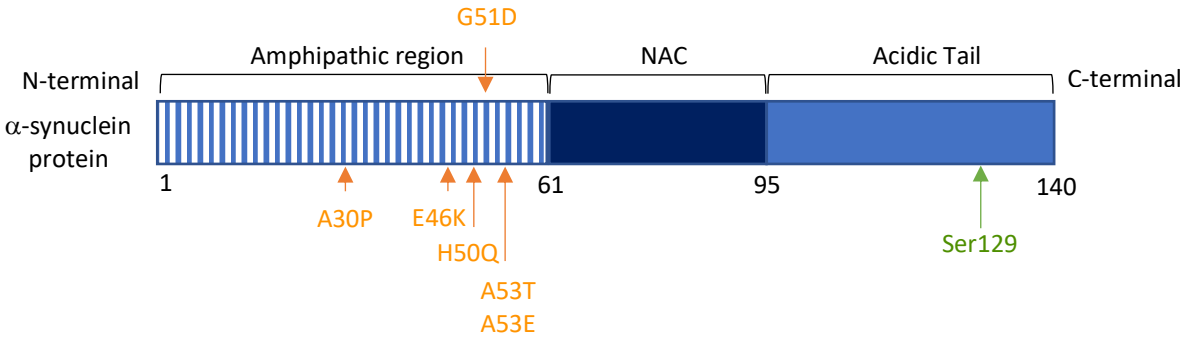


Figure 1.1. α-synuclein protein structure.
α-synuclein is a 140 amino acid protein, with three functionally distinct sub-regions. N-terminal region: highly conserved, contains 7, 11 residue repeats that form a helix upon membrane binding, the six known missense mutations that cause familial PD lie within this region (shown in orange). Central hydrophobic region (non-Aβ component or NAC domain): associated with an increased propensity of the protein to form fibrils. Acidic tail: contains mainly negatively charged residue and largely unfolded; all bar of the phosphorylation sites, serine (Ser) 87, are localised in the c-terminal domain, phosphorylation of α-synuclein at Ser129 (shown in green) has been found to be the predominant form of the α-synuclein in Lewy bodies (Wales et al., 2013).

acids 1-60, is highly conserved across species and contains a series of seven imperfect, 11 amino acid, repeats containing a KTKEGV consensus motif. The presence of the consensus motif, is thought to form an amphipathic helix upon lipid-binding, with other membrane binding proteins containing a similar motif (Segrest *et al.*, 1992; Bartels *et al.*, 2010). All mutations that have been associated with familial forms of PD (figure. 1.1) are located in the N-terminal domain (Bendor *et al.*, 2013). The central portion of the α-synuclein protein is termed the non-Aβ-component (NAC) domain, following its discovery when it was isolated from Aβ plaques as the non- Aβ element of plaques (Uéda *et al.*, 1993). The central, hydrophobic, NAC domain, amino acids 61-95, is responsible for the aggregational properties of the protein (Wales *et al.*, 2013). The C-terminal, acidic, region, amino acids 96-140 is composed of mainly negatively charged amino acid residues (George, 2002). α-synuclein has been shown to undergo an number of different post-translational

modification, with the most common being phosphorylation at serine 129 in the C-terminal domain (Wales *et al.*, 2013) . The function of the acidic tail region is unknown, with many hypothesised functions including mechanism of membrane binding regulation and conferring chaperone-like activity (Wales *et al.*, 2013) and it is the least conserved of the three sub-domains across species (Bendor *et al.*, 2013).

α-synuclein is an intrinsically disordered protein, although the protein is generally thought to be unstructured several structures for the native α-synuclein protein under certain conditions have been reported, including alpha-helix formation upon interaction with lipid membranes (Wales *et al.*, 2013; Meade *et al.*, 2019). Such dynamic structural characteristics have made determining the normal structure and related function of α-synuclein difficult. Recombinantly expressed α-synuclein, as well as α-synuclein isolated from the human brain has been suggested to have an unfolded monomeric structure in the native state (Weinreb *et al.*, 1996; Bertoncini *et al.*, 2005; Fauvet *et al.*, 2012; Bendor *et al.*, 2013). However, it has also been suggested that α-synuclein may remain helical in solution due to macromolecular crowding within the cell (McNulty *et al.*, 2006), and that a natively unfolded state had been identified due to the procedural methods utilised (Bartels *et al.*, 2011; Wang *et al.*, 2011). It has been reported that native α-synuclein behaves as a helical tetramer that can resist aggregation (Bartels *et al.*, 2011). Though, the debate over the native conformation of α-synuclein is still ongoing, with more recent studies focussing on α-synuclein isolated from human brain samples (Wales *et al.*, 2013; Meade *et al.*, 2019).

The physiological function of α-synuclein, like its native structure, is not entirely understood, with numerous functions proposed (Emamzadeh, 2016). The current main proposed function for α-synuclein is the involvement of the protein in neurotransmitter release, due to its location within presynaptic terminals and its propensity to bind to small spherical vesicular membranes (Bendor *et al.*, 2013; Wales *et al.*, 2013). It has been hypothesised that α-synuclein binds to the synaptic vesicles to modulate vesicular trafficking within the synapse (Emamzadeh, 2016), with the possible role of regulating the amount and speed of vesicular recycling within synapses and the presynaptic area. α-synuclein is also hypothesised to have chaperone functions, through both its N- and C-terminal domains, including for the SNARE proteins which are required for synaptic vesicle exocytosis (Burre *et al.*, 2010). Further reported functions for α-synuclein include chaperoning of heat shock proteins (Witt, 2013) and regulation of dopamine biosynthesis (Peng *et al.*, 2005). α-

synuclein has been shown to translocate to the nucleus of neurons, but there is no consensus on the putative role that the protein may play in the nucleus; however it has been reported that it may have a role in transcriptional regulation (Wales *et al.*, 2013; Pinho *et al.*, 2019).

Although the presence of intraneuronal α-synuclein aggregates (i.e. Lewy bodies) is the characteristic finding required for the neuropathological diagnosis of DLB, its role in the aetiology of the disease remains uncertain. However, α-synuclein is thought to be central to the pathogenesis of Lewy body diseases (LBD) (Kalia and Kalia, 2015), since mutations to the α-synuclein gene are causative for familial PD (Polymeropoulos *et al.*, 1997), as well as α-synuclein being the core component of Lewy bodies and neurites. The factors that underlie the conversion from native state α-synuclein into an aggregated state are not well understood. However, it is generally hypothesised that an imbalance between the normal proteostasis mechanisms that regulate α-synuclein synthesis, aggregation and clearance may favour the conversion of native to aggregated state (Kragh *et al.*, 2012). Polymorphisms in the α-synuclein promoter have been identified as a potential risk factor for sporadic PD (Maraganore *et al.*, 2006), which supports the hypothesis that increased levels of α-synuclein are sufficient to lead to the formation of Lewy bodies, in conjunction with multiplication of the α-synuclein gene leading to familial forms of PD (Lashuel *et al.*, 2013). Increased α-synuclein levels could more easily lead to oligomer formation which in turn form the characteristic neuropathological hallmark, Lewy bodies (Conway *et al.*, 2000a; Conway *et al.*, 2000c). This theory in regard to increased α-synuclein, has been further supported by Mittal *et al.* (2017) who identified that salbutamol, which interacts with the β2-adrenoreceptor which is a regulator of the α-synuclein gene, reduces the risk of developing PD. An increased propensity to form aggregated species is found with a number of familial PD mutations, including the A53T mutation (Bendor *et al.*, 2013). However, not all PD-associated α-synuclein mutations increase the aggregation propensity of the protein, with the G51D form being more resistant to aggregation than wildtype protein but is still associated with severe neurodegeneration implying α-synuclein aggregation independent mechanisms of neurodegeneration (Fares *et al.*, 2014). The N-terminal domains propensity to form a helical secondary structure can be disrupted or reduced in familial PD mutations, with some swinging to a more favoured beta sheet secondary structure (Wales *et al.*, 2013). Familial PD mutations can also interrupt the intramolecular interactions between the N and

C terminus of the α-synuclein protein which can destabilise the native form leading to a higher propensity to aggregate (Conway *et al.*, 2000b). The destabilisation and subsequent aggregation of α-synuclein as seen through the mutations in α-synuclein that lead to PD further support the role for α-synuclein in the pathogenesis of synucleinopathies.

1.2.2.2 Proposed neurodegenerative mechanisms of α-synuclein

There have been a number of mechanisms proposed through which α-synuclein can elicit neurodegeneration (Wales *et al.*, 2013; Bartels, 2019; Meade *et al.*, 2019). These mechanisms include different species of α-synuclein, interplay with cellular organelles and disruption to synaptic processes.

Different conformers of α-synuclein have been associated with protein aggregation and pathogenesis in DLB (Lashuel *et al.*, 2013). It is hypothesised that oligomeric species are more neurotoxic than fibrillar forms and may be the major causative agent for neurodegeneration in synucleinopathies (Winner *et al.*, 2011); although, recent evidence from Mahul-Mellier *et al.* (2020) implies that it is the transition from fibrillar to the formation of the Lewy body that is one of the major drivers of neurodegeneration. However, it is currently unclear whether it is a toxic gain or loss of function that underlies the neurodegenerative effects of α-synuclein oligomerisation and subsequent aggregation (Cookson, 2006; Ghiglieri *et al.*, 2018).

A large body of research has looked into how structurally altered or oligomeric α-synuclein can confer toxicity through interactions with membranes. Recent studies have found that the centre of Lewy bodies once thought to be composed of amyloid fibrils of α-synuclein are now thought to be mainly formed of membrane lipids from mis-trafficked vesicles (Shahmoradian *et al.*, 2019), with vesicle clustering thought to be neurotoxic (Bartels, 2019). The importance of membranes and trafficking in synucleinopathies is further highlighted by glucocerebrosidase mutations being a risk factor for DLB, with homozygous mutations leading to Gaucher disease, a disorder characterised by lysosomal dysfunction (Shiner *et al.*, 2016; Rongve *et al.*, 2019). Lysosomal and autophagy impairment has been observed in post-mortem studies of synucleinopathies (Arotcarena *et al.*, 2019). Alterations to the autophagy-lysosomal pathway has been associated with the accumulation of α-synuclein in both PD and DLB (Alvarez-Erviti *et al.*, 2010; Crews *et al.*, 2010) with the results suggestive of an accumulation through defective clearance of autophagosomes in these disorders (Arotcarena *et al.*, 2019). The dysfunction of the autophagy-lysosomal pathway observed in

PD and DLB is thought to increase the accumulation of α-synuclein, further exacerbating the neurodegenerative processes occurring within neurons (Arotcarena *et al.*, 2019).

α-synuclein oligomers have been shown to permeabilise membranes, altering ion permeability for the neuron (Volles *et al.*, 2001; Danzer *et al.*, 2007), interrupting the endoplasmic reticulum membrane leading to a disruption in calcium (Ca^{2+}) homeostasis that can promote changes to mitochondria (Wales *et al.*, 2013). As well as the reported affects to mitochondria downstream of α-synuclein impairment to the endoplasmic reticulum membrane, mitochondrial dysfunction has been shown to occur through direct contact with the α-synuclein protein (Bendor *et al.*, 2013; Wales *et al.*, 2013).

Mitochondrial involvement in synucleinopathies, especially PD, has been extensively studied in recent years, and it has been suggested that mitochondrial impairment is a major pathway for α-synuclein mediated neurodegeneration. A number of mutations that lead to familial forms of PD have been shown to induce mitochondrial fragmentation, including A53T (Park *et al.*, 2018). As well as mutations located in proteins essential for mitochondrial dynamics, including parkin and PINK1, leading to parkinsonian symptoms, although not always in the presence of Lewy body formation. Together the identification of mitochondrial dysfunction both directly and indirectly by mutations linked to Parkinson's disease gives further backing to a key mitochondrial role in the neurodegenerative processes associated with synucleinopathies especially PD (Ishihara-Paul *et al.*, 2008; Dawson and Dawson, 2010; Vives-Bauza and Przedborski, 2011). Although commonly thought a cytoplasmic protein, recent studies have shown that α-synuclein associates with mitochondria (Hu *et al.*, 2019; Wang *et al.*, 2019). α-synuclein has been shown to bind to complex I, part of the electron transport chain, inhibiting its function and leading to a decrease in ATP production within the cell and mitochondrial dysfunction (Schapira *et al.*, 1989; Wales *et al.*, 2013).

In the early stages of synucleinopathies it has been suggested that α-synuclein acts pre-synaptically on proteins related to mitochondria, for example heat shock protein 10, by sequestering the protein in the cytosol preventing it from translocating to the mitochondria (Leverenz *et al.*, 2007; Szego *et al.*, 2019). A recent study has shown in a cell model the sequestering of mitochondria during the formation of Lewy bodies, with different alterations to mitochondrial functions, including reduction in energy production, observed during the stages of α-synuclein fibrillisation and Lewy body formation (Mahul-Mellier *et al.*, 2020). The sequestering of proteins by α-synuclein preventing their native function is another proposed

mechanism for α-synuclein toxicity not just related to mitochondrial proteins (Yang and Hu, 2016; Choi *et al.*, 2018). Specific post-translationally modified species of α-synuclein have been shown to have an increased binding propensity to mitochondrial membranes compared to the native form which is thought to have a low affinity for mitochondrial membranes (Wang *et al.*, 2019), further post translational modifications for example C-terminal truncation and phosphorylation can increase membrane binding affinity furthering α-synuclein toxicity (Lashuel *et al.*, 2013). The alterations to mitochondria through direct and indirect α-synuclein mechanisms can lead to fragmentation of the mitochondria within the cell (Wales *et al.*, 2013). Mitochondrial fragmentation can lead to cellular damage though a number of mechanisms including increasing reactive oxygen species production, oxidative stress and inducing mitochondrially mediated apoptotic pathways (Wales *et al.*, 2013). Increased levels of reactive oxygen species and oxidative stress is thought to further lead to protein folding abnormalities perpetuating the cellular distress (Bendor *et al.*, 2013; Wales *et al.*, 2013). The data so far suggests that it is both a loss of normal function and a gain of toxic function as a result of abnormal α-synuclein that causes neuronal death through many hypothesised pathways.

1.2.2.3 Pathological Staging

Lewy body pathology in DLB is neuropathologically diagnosed according to its severity and topographical distribution in the brain and several stages are distinguished: neocortical, limbic, brainstem, amygdala-predominant and olfactory bulb only. The majority of DLB cases fall in either limbic or neocortical stage (McKeith *et al.*, 2017). α-synuclein within Lewy bodies and neurites is also associated with other intracellular proteins including intermediate filaments and chaperone proteins, similar to neuronal inclusions found in other neurodegenerative disorders (McNaught *et al.*, 2002; Tanaka *et al.*, 2004). α-synuclein is also the pathological hallmark of PD and PDD, and is central to the pathogenesis of the LBD spectrum (Jellinger, 2003; Kim *et al.*, 2014). Mild Lewy body pathology, is referred to as incidental Lewy body disease, and suggested to be more prevalent with increasing age (Saito *et al.*, 2004; Erskine *et al.*, 2020).

In LBDs, Lewy bodies are thought to follow a stereotypical temporal and topographic pattern, originating in the brainstem and spreading to the neocortex through the limbic system throughout the course of the disease (Braak *et al.*, 2003; McKeith *et al.*, 2005). The presence of Lewy body pathology within the neocortex is required for cognitive impairment

(Yamamoto *et al.*, 2005), with limbic or neocortical predominant pathology required for a diagnosis of DLB (McKeith *et al.*, 2005). There have been a number of staging criteria proposed for α-synuclein pathology in LBDs, including, Braak *et al*. (2003) for PD and PDD and McKeith *et al*. (2005) for DLB. Braak *et al*. (2003) suggests the topographical spread of α-synuclein pathology is formed of 6 stages, initiating in the medulla oblongata with pathological progression through the brainstem and into limbic and neocortical regions. The stereotypical spread of α-synuclein pathology within the brain has been proposed to be caused by propagation of α-synuclein in a prion-like manner, with transfer of disease related

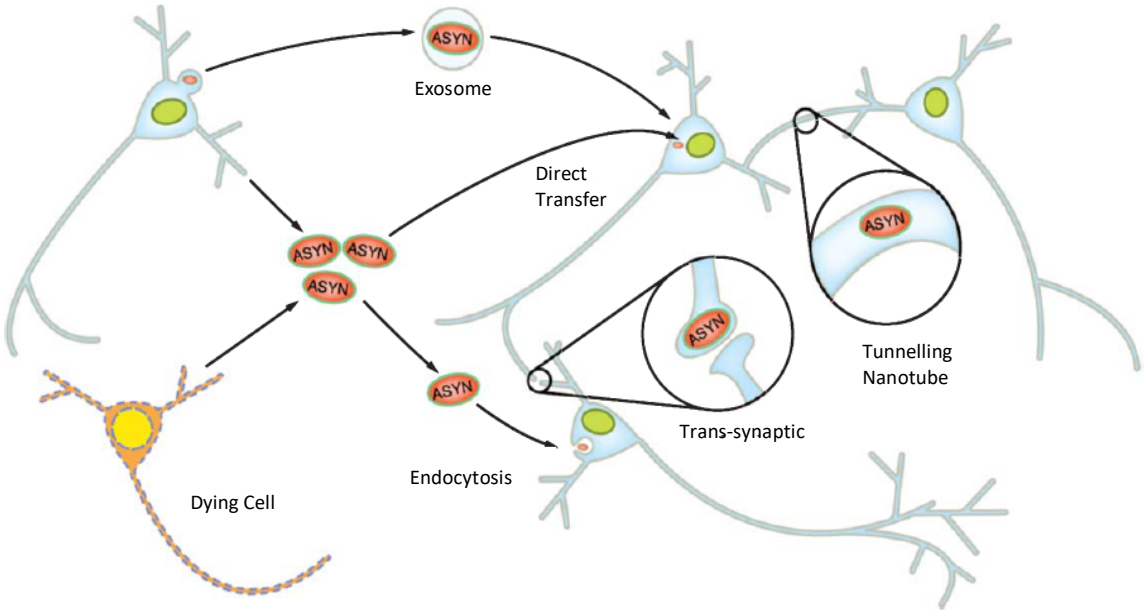


Figure 1.2. Mechanisms of a-synuclein propagation.
α-synuclein species may be transferred between neurons and induce spread of pathology to neighbouring brain regions. Once α-synuclein has been released from the originating cell into the extracellular environment, either via exosomes or non-classical exocytosis pathways, these α-synuclein proteins can enter other neurons in a variety of proposed ways, with either direct or indirect interactions between donor and recipient neurons. Indirect ways include through direct transfer, if there is membrane damage to the recipient neuron or via endocytosis. Direct mechanisms can occur through the formation of tunnelling nanotubes or through synaptic transmission. Figure adapted from (Wales *et al.*, 2013).

protein into functionally connected regions inducing native α-synuclein to undergo conversion into the disease like state (figure 1.2)

Although DLB and PDD are indistinguishable based upon the topographical location of terminal pathology (Ruffmann *et al.*, 2016), α-synuclein pathology in DLB is thought to progress in a different manner to the ascending pattern observed in the stages of PD pathology. The differences in topographical spread between DLB and PDD could underlie the different sequence of features seen clinically between the two disorders. Previous research into the pattern of α-synuclein deposition in DLB have proposed origins in the amygdala (Beach *et al.*, 2009) and the olfactory bulb (Cersosimo, 2017), this different topographical

pattern of spread has been supported by the fact not all DLB patients have an abnormal dopamine transporter scan (DaTSCAN), suggestive of differential spread or differences in severity burdens between DLB cases (van der Zande *et al.*, 2016). A recent study by Raunio *et al.* (2019) identified two common α-synuclein patterns of spread in a Finnish cohort. The group identified that cases with high levels of AD-type pathology were associated with an amygdala-based spread of pathology, rather than the caudo-rostral progression suggested by Braak *et al.* (2003) (Raunio *et al.*, 2019). Brainstem nuclei are affected in nearly all cases of LBD, although the severity of the pathology is highly variable (McKeith *et al.*, 2005).

1.2.2.4 Concomitant pathologies

Although DLB is characterised by the presence of α-synuclein aggregates, 50-80% of patients have concomitant AD type pathology in the form of extracellular Aβ plaques, intracellular neurofibrillary tangles (NFT), neuropil threads and neuritic plaques consisting of a focal core of Aβ surrounded by a distended halo of tau-positive neurites (Halliday *et al.*, 2011; McKhann *et al.*, 2011; Attems, 2017) (as discussed in 1.3.2). Although the presence of AD type pathology is not required for a neuropathological diagnosis of DLB, concomitant AD type pathology in DLB is thought to contribute to a faster cognitive decline (Howlett *et al.*, 2015; McKeith *et al.*, 2017), with tau co-pathology thought to underlie some of the heterogeneity observed in clinical impairment in Lewy body disorders (Coughlin *et al.*, 2019). Recent studies have identified that Lewy body disorder patients with concomitant AD pathology have higher levels of neocortical α-synuclein pathology than those without concomitant AD pathology (Coughlin *et al.*, 2019). The concomitant tau pathology within these Lewy body disorder cases was also found to diverge from the neocortical topographical pattern observed in AD and appeared to map more closely with the distribution of α-synuclein pathology, the pattern of Aβ pathology was found to be similar in both AD and Lewy body disorder cases (Coughlin *et al.*, 2019). Interactions between α-synuclein and tau proteins have been reported in *in vitro* studies (Giasson *et al.*, 2003), suggesting a possible reason why those with higher concomitant AD-type pathologies may have a faster cognitive decline and the divergence in tau's topographical pattern observed in AD (as discussed in more detail in 1.4 and 1.5).

Other pathologies have been described in DLB. Transactive response DNA-binding protein 43 (TDP-43) pathology is a hallmark of amyotrophic lateral sclerosis and a subtype of FTD, and has been located in the brains on cognitively intact cases as well as those with AD and DLB

(Ou *et al.*, 1995; Higashi *et al.*, 2007). TDP-43 has also been recently found to be the pathological hallmark for a newly discovered type of dementia, Limbic-predominant age-associated TDP-43 encephalopathy (LATE) (Nelson *et al.*, 2019). LATE has been described to mimic AD clinical features due to the accumulation of TDP-43 and damage to the hippocampus (Nelson *et al.*, 2019), which can occur in combination with AD and DLB-type pathology. However, the proposal of LATE as a distinct clinicopathological entity has been contested (Josephs *et al.*, 2019). TDP-43 can form intracytoplasmic inclusions under pathological conditions. Aggregation of TDP-43 occurs after the translocation of TDP-43 into the cytoplasm, where it is cleaved into C-terminal fragments that are abnormally hyperphosphorylated (Ou *et al.*, 1995). TDP-43 has been shown to affect structures that are vulnerable to Lewy bodies, such as the amygdala (Josephs *et al.*, 2016). Within TDP-43 positive DLB cases the severity of TDP-43 pathology is similar to that seen in cognitively intact controls and seems to be more associated with the concomitant AD-type pathology that the characteristic α-synuclein pathology (McAleese *et al.*, 2017). As well as TDP-43, further pathologies are present in DLB including microvacuolation and vascular pathologies although relatively few neuropathological studies have been conducted in this area (McKeith *et al.*, 1996). However, there is emerging evidence that supports the contribution of vascular changes that could exacerbate Lewy-body induced neuropathology (Raz *et al.*, 2016), however, risk factors that are associated with vascular changes are associated more with AD than DLB or PDD (Chan *et al.*, 2018). There are also a number of studies that suggest that vascular changes, such as white matter hyperintensities viewed on MRI, are associated with AD-type pathology in DLB and not the α-synuclein pathology (Joki *et al.*, 2018). Further research is required to fully understand the role that concomitant vascular changes play in the pathophysiology of DLB.

1.3 AD

1.3.2 Clinical features

AD is the leading cause of neurodegenerative dementia, accounting for 50-80% of all cases (Blennow *et al.*, 2006; Mayeux and Stern, 2012). Clinically AD is characterised by a progressive and irreversible decline in cognition, which overtime substantially impairs the patient's ability to perform activities of daily living (McKhann *et al.*, 1984). Although AD is primarily considered to be an amnestic disorder, impairments to visuospatial and language function are also commonly present (McKhann *et al.*, 2011). Impairments to memory are characterised by deficits in learning and recall of recent information, due to deficient episodic memory (McKhann *et al.*, 2011; Dubois *et al.*, 2014). AD patients have also been found to have cognitive fluctuations, although at a lower prevalence, 10-20%, than those seen in DLB (Lee *et al.*, 2012). Although cognitive fluctuations are clinically characterised by alterations to cognition, in AD and DLB the cognitive domains that are affected differ, with those observed in AD thought to reflect a reduced capacity to cope with the cognitive demands of the environment (Bradshaw *et al.*, 2004) (further discussed in 1.6).

1.3.3 Pathology

AD-type pathology consists of extracellular Aβ plaques, intracellular aggregates of hyperphosphorylated tau (NFTs and neuropil threads) and neuritic plaques consisting of a focal core of Aβ surrounded by dystrophic tau-positive neurites (Halliday *et al.*, 2011; McKhann *et al.*, 2011; Attems, 2017).

1.3.2.1 Aβ

Aβ plaques are accumulations of the 4kDa Aβ fragment, cleaved from the membrane bound amyloid precursor protein (APP), that form in the parenchyma (Murphy and LeVine, 2010). Formation of the Aβ fragments, in AD, is due to sequential proteolytic cleavage of the APP molecule by two secretases, β- and γ-secretase (Selkoe, 2001b). Dependent on the cleavage site of γ-secretase, two major isoforms of Aβ are present in the brain $A\beta_{40}$ and $A\beta_{42}$ with the variation in the C-terminus of the fragment (Murphy and LeVine, 2010) (figure 1.3).

Deposition of Aβ plaques within the brain are thought to be the result of a gradual and chronic imbalance in the production and clearance of Aβ from the brain (Selkoe, 2001a). The physiological role of Aβ is widely debated, with putative roles suggested in immunology, regulation of synaptic function and repair of blood-brain barrier leaks amongst others

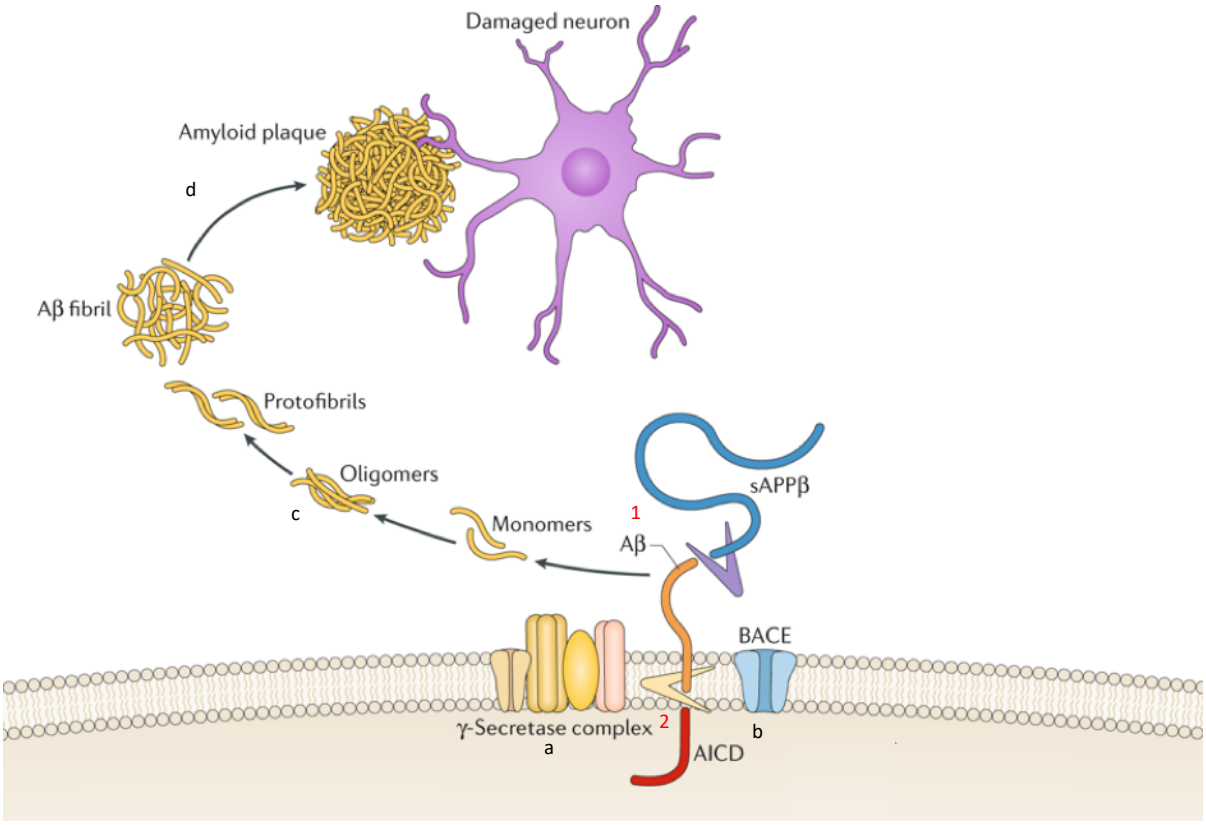


Figure 1.3. The amyloid cascade hypothesis.
The amyloid cascade hypothesis suggests that deposition of Aβ within the brain is an essential and crucial step that leads to AD. Autosomal dominant mutations that lead to familial forms of AD occur in three genes 1 presenilin 1 and 2 part of the γ-secretase complex and 2 APP. The cascade has also been the target for therapeutics which are currently in clinical trials. Targets include a) γ-secretase inhibitors and modulators, b) BACE inhibitors, c) monoclonal antibodies and compounds against monomers, oligomers and protofibrils and d) monoclonal antibodies and compounds preventing Aβ fibril aggregation into plaques. Figure adapted from (Panza *et al.*, 2019).

(Pearson and Peers, 2006; Brothers *et al.*, 2018). The lack of consensus on the putative role of Aβ, has been further supported by adverse effects observed in AD clinical trials that aim to reduce Aβ levels (Schneider *et al.*, 2014; Mehta *et al.*, 2017; Aisen *et al.*, 2020). Although the physiological role for Aβ is still elusive, Aβ has played a central role in AD research in the past 20 years, with the amyloid cascade hypothesis. The Aβ cascade hypothesis states that the production and deposition of Aβ within the brain parenchyma is the primary event which can initiate a sequence of events leading to formation of NFTs and eventually neuronal cell death (Hardy and Higgins, 1992).

Support for the amyloid cascade hypothesis has been shown through familial forms of AD whom have mutations, typically at or around the cleavage sites, or increased copy numbers of APP, as well as, in the secretases Presenilin 1 and 2, which form part of the γ-secretase complex (Rocchi *et al.*, 2003; Rosenberg *et al.*, 2016; Selkoe and Hardy, 2016). With additional support for the hypothesis from Down's syndrome, trisomy 21. The APP gene is located on chromosome 21, meaning those with Down's syndrome to possess an extra copy

of the gene, as well as an increased expression of β-secretase homologues, also present on the chromosome (Head and Lott, 2004). The increase in Aβ production due to the increased expression causes detectable AD-type pathology in Down's patients from the age of 40 (Annus *et al.*, 2016). Although there is an array of evidence to support the role of Aβ in familial AD and Down's syndrome, the role that the amyloid cascade plays in sporadic AD is a subject of continuing debate. This is evidenced by the knowledge that cognitively intact brains can have high levels of Aβ present (Maarouf *et al.*, 2011; Kovacs *et al.*, 2013), and that considerable amounts of NFTs can be observed in the absence of Aβ in the brain (Crary, 2016); along with the evidence that Aβ load does not correlate with cognitive impairment (Serrano-Pozo *et al.*, 2016).

1.3.2.2 Tau

NFTs and neuropil threads are aggregations of the microtubule-associated protein tau, a protein that under physiological conditions stabilises the microtubule network within axons (Mandelkow and Mandelkow, 2012). To stabilise the microtubule network whilst allowing for the dynamics of the network including axonal transport, tau is in a constant state of flux between phosphorylated/off and non-phosphorylated/on states (Brandt *et al.*, 2005). Under pathological conditions there is dysregulation of the phosphorylation/dephosphorylation cycle, leading to an accumulation of abnormally phosphorylated tau that disassociates from microtubules and aggregates within the neuron (Grundke-Iqbal *et al.*, 1986; Mandelkow and Mandelkow, 2012). In AD there is an over 300% increase in phosphorylation of tau in AD brains compared to normal physiological levels (Köpke *et al.*, 1993). Mutations, differential exon splicing, and increased expression levels (Myers *et al.*, 2007), have been associated with several dementias. These findings provide evidence for tau dysfunction playing a key role in tauopathies, including FTD, progressive supranuclear palsy and corticobasal degeneration (Poorkaj *et al.*, 1998; Spillantini *et al.*, 1998; Hardy and Singleton, 2008; Mandelkow and Mandelkow, 2012). As with Aβ pathology, tau pathology is commonly seen in aged individuals, both cognitively intact and impaired (Tomlinson *et al.*, 1970), although this is significantly less widespread than that seen in AD cases (Knopman *et al.*, 2003).

1.3.2.3 Pathological Staging

AD pathology has been shown to follow a classical topographical pattern of deposition within the brain, with a number of staging criteria developed in order to chart pathological progression. Aβ, in AD, is deposited in a descending pattern, following five phases during the

course of the disease, initially affecting the neocortex (Phase 1), before progressing to the allocortex (Phase 2), basal ganglia and basal forebrain (Phase 3) before finally being deposited in the brainstem (Phase 4) and cerebellum (Phase 5) (Thal *et al.*, 2002).

The stepwise topographical spread of tau pathology is divided into VI stages (Braak and Braak, 1991b; Braak *et al.*, 2006). Tau pathology originates in the trans-entorhinal cortex (Stage I), with pathology progressing to the hippocampus (Stage II), temporal neocortex (Stage III), insular cortex (Stage IV), superior temporal gyrus (Stage V) before spreading to the occipital cortex (Stage VI) in its final stages (Braak and Braak, 1991b; Braak *et al.*, 2006; Alafuzoff *et al.*, 2008). The severity of the pathology within affected brain regions increases with Braak stage, with severe tau pathology visible in the trans-entorhinal cortex in brains with Braak stage VI (Walker *et al.*, 2017). Braak stage has also been demonstrated, in several studies, to correlate with clinical severity of AD (Bancher *et al.*, 1996; Gertz *et al.*, 1996; Gold *et al.*, 2000). As well as staging criteria for both tau and Aβ individually, there is criteria to semi-quantitively assess neuritic plaque pathology in AD cases (Mirra *et al.*, 1991). Neuritic plaques, a convergence between the two AD pathologies, are a necessary feature for a neuropathological diagnosis of AD (Attems, 2003). The semi-quantitative scale assesses neuritic plaque pathology in the middle frontal gyrus, superior/middle temporal gyri and inferior parietal lobe, as either sparse moderate or frequent (Mirra *et al.*, 1991). All three staging criteria are combined to suggest an overall level of AD neuropathological change within the brain, either high, medium or low (Hyman *et al.*, 2012; Montine *et al.*, 2012).

1.3.2.4 Concomitant Pathologies

As with other common neurodegenerative disorders, AD brains also possess concomitant pathologies, alongside the hallmark deposition of Aβ and tau. Previous neuropathological studies have shown that cerebrovascular disease occurs commonly in ageing with up to 84% of those over 80 years old possessing vascular pathologies (Petrovitch *et al.*, 2005; Attems and Jellinger, 2014). Vascular pathology is common in AD cases, with 30% containing some cerebrovascular pathology and nearly all cases exhibiting some form of vascular lesion, including cerebral amyloid angiopathy and small vessel disease (Kalaria and Ballard, 1999; Yip *et al.*, 2005); with vascular pathology in AD more extensively investigated than that observed in DLB (Raz *et al.*, 2016), see 1.2.2.4. The interaction between vascular and AD pathologies are of great interest and it has been suggested that the combination of the two pathologies has either additive or synergistic effects on cognitive impairment (Attems and

Jellinger, 2014). AD patients with lower levels of Aβ and tau with concomitant vascular pathologies have been shown to be more severely cognitively impaired than those with comparable AD-type pathology without vascular pathology (Zekry *et al.*, 2002).

AD brains also contain neuropathological lesions commonly associated with other neurodegenerative disorders including TDP-43, found in 33-40% of cases and α-synuclein, discussed more in 1.4.2., found in 41-55% of AD cases (Robinson *et al.*, 2018). TDP-43, as discussed in 1.2.2.3, is the key hallmark for a subtype of FTD and the newly discovered LATE. TDP-43 pathology is found in 33-40% of AD cases, which increases to 75% in cases with the most severe AD-type pathology (Chang *et al.*, 2016).

1.4 Mixed AD/DLB

Neurodegenerative dementia had for a long time thought to have been caused by discrete diseases, which clinically manifest due to selective neuronal loss and dysfunction in distinctive anatomical regions by abnormal accumulations of endogenous proteins (Rahimi and Kovacs, 2014). Typically, a diagnosis of a neurodegenerative disease is based upon a clinico-pathological consensus, where neuropathological and clinical information is combined to form a definitive clinico-pathological diagnosis (Walker *et al.*, 2015). However, as previously described in sections 1.2 and 1.3, characteristic neuropathological deposits are not the only type of pathological lesion that can be found in the brain of dementia cases, concomitant pathologies usually characteristic of another neurodegenerative disorder can be found (Rahimi and Kovacs, 2014; Spires-Jones *et al.*, 2017). Mixed dementia was for a long time thought to apply, mainly, to a mixture of vascular and AD pathologies in the brain underlying the clinical symptoms. However, over the past few decades, the observation of concomitant pathologies across the neurodegenerative disease spectrum is increasingly recognised (Armstrong *et al.*, 2005; Jellinger, 2007; Jellinger and Attems, 2007; Kovacs *et al.*, 2008; Kovacs *et al.*, 2013), this has led to the updating of clinical and neuropathological criteria (Rahimi and Kovacs, 2014; Josephs *et al.*, 2016; McKeith *et al.*, 2017).

Numerous post-mortem and community-based studies have been undertaken with the aim to establish the rates of mixed pathologies within the ageing brain (Rahimi and Kovacs, 2014). A large, 1500 cases, post-mortem cohort from Vienna found that only 52% of the cases examined were ‘pure’ AD and 2% ‘pure’ vascular dementia (VaD) (Jellinger and Attems, 2007). 27.4-31.4% of the cases were either AD with additional vascular lesions or with additional α-synuclein pathology (Jellinger and Attems, 2007), however, there was no mention of whether the levels of the additional pathologies were likely to be symptomatic. In a larger multi-centre study led by BrainNet Europe, 3303 brains from 9 centres were analysed for the presence of co-occurring pathologies (Kovacs *et al.*, 2008). 55.3% of the cases examined in this multi-centre cohort were found to possess more than one pathology associated with a neurodegenerative disease including AD, vascular pathologies and synucleinopathies. The findings from the study also showed that multiple pathologies were found most frequently in PD (92%) with additional pathologies also found Lewy body dementias (61%) and AD (43%), highlighting the high frequencies of mixed pathologies within neurodegenerative diseases. This study has been further supported by research

suggesting that 89% of DLB cases had some level of AD pathology (Dugger *et al.*, 2014). Several community-based studies have also furthered the realisation that there is an under appreciation for mixed pathologies in neurodegenerative disorders (Schneider *et al.*, 2007; Kovacs *et al.*, 2013).

There are a number of hypotheses have been proposed to explain the co-pathologies found in neurodegenerative disorders. One hypothesis is that similarly to the incidence of neurodegenerative pathologies, age is associated with the accumulation of multiple co-occurring pathologies within the brain, with a brain from an older person more likely to possess more mixed pathologies (Jellinger and Attems, 2010; Kovacs *et al.*, 2013; Filfan *et al.*, 2017; Robinson *et al.*, 2018). Another hypothesis is that the primary pathology could seed the accumulation of a secondary pathology, this would suggest that cases with a more severe primary pathology would have an increased number or severity of co-pathologies (Clinton *et al.*, 2010). Along with the seeding of pathologies that are thought to associate with co-pathologies, genetic risk factors including apolipoprotein E ε4 that have been associated with specific neurodegenerative disorders are thought to also increase the levels of co-pathologies across neurodegenerative diseases, as shown with an increase in AD-type pathology in DLB cases with the apolipoprotein E ε4 allele (Robinson *et al.*, 2018). Although there has been an increased interest in how the co-pathologies occur and how they may affect the clinical phenotype (Irwin *et al.*, 2017; Kapasi *et al.*, 2017; Robinson *et al.*, 2018), the initial clinical diagnosis of mixed dementia, specifically mixed AD/DLB, is rare, with only a small percentage having a revised diagnosis of mixed AD/DLB dementia within their lifetime (Thomas *et al.*, 2018).

1.4.1 Clinical features

The clinical identification of mixed AD/DLB is of current interest due to the possible future development of protein targeted treatments, as well as the correct stratification in their clinical trials. Clinically, a diagnosis of mixed AD/DLB is uncommon, especially at baseline where it has hypothesised that the secondary pathology may not be advanced enough to elicit clinical symptoms (Thomas *et al.*, 2018). However, some initial diagnoses may be revised later on in the disease course, although this is mainly at specialist centres, with the vast majority not being identified until post-mortem (Nelson *et al.*, 2010; Lebouvier *et al.*, 2013; McKeith *et al.*, 2016; Thomas *et al.*, 2018).

Clinically mixed AD/DLB can present with clinical features of both AD or DLB, with this having been shown to be dependent upon which pathology occurs first within the brain (Walker *et al.*, 2015). It is currently unknown whether there is a different clinical course for those with initial AD-type and DLB-type dementia that then go onto develop mixed AD/DLB (Malek-Ahmadi *et al.*, 2019). Although clinically difficult to identify, clinicopathological studies have shown that DLB and mixed AD/DLB have a differing clinical presentation in regard more severity of impairments and a speed of decline, with mixed AD/DLB having both greater than pure DLB (Kraybill *et al.*, 2005; Nelson *et al.*, 2010). With Studies showing that the presence of mixed pathologies, including mixed AD/DLB have a great change in MMSE and Dementia Rating Scale scores per year that those with pure AD or pure DLB (Kraybill *et al.*, 2005).

In patients which present with a primarily AD-like dementia, studies have shown that an initial presentation which includes impairment to visuospatial and executive function is indicative of mixed AD/DLB, as this cognitive impairment is more commonly present in mixed AD/DLB than in pure AD cases (Hansen *et al.*, 1990; Weiner *et al.*, 2003; Malek-Ahmadi *et al.*, 2019). These mixed AD/DLB cases also tend to have a worse depression and Trail-Making A test score compared to pure AD cases, even though the neuropsychological scores do not differ (Malek-Ahmadi *et al.*, 2019). However, these differences between mixed AD/DLB and pure AD, done retrospectively, are not useful for clinical diagnosis. One third of mixed AD/DLB case present with a clinical DLB phenotype, which includes impairments to visuospatial function, this would thus lead to cases being diagnosed as DLB and not the correct mixed AD/DLB (Thomas *et al.*, 2018). Mixed AD/DLB, retrospectively, have also been shown to have an initial verbal memory and confrontational naming deficit that is more severe than those with pure DLB, however, again this does not help to discriminate against those with pure AD (Kraybill *et al.*, 2005), excluding a third of those with mixed AD/DLB that present with AD-like dementia (Thomas *et al.*, 2018). The majority of studies that have been undertaken so far into understanding the clinical aspects of mixed AD/DLB have focussed almost exclusively on retrospective analysis. In order for more thorough longitudinal research to be conducted, and the eventual ability to identify and diagnose mixed AD/DLB biomarkers for both AD and DLB-type pathology would need to be developed and utilised (Thomas *et al.*, 2018).

1.4.2 Pathology

Pathologically mixed AD/DLB is defined by the presence of both high neuropathologic change AD pathology, further described in 1.3.2, and neocortical DLB pathology, further described in 1.2.2, where the pathological criteria for both AD and DLB are met (Walker *et al.*, 2015). Although this criterion pathologically defines mixed AD/DLB dementia, a number of research studies have used varying definitions for mixed AD/DLB. These definitions include AD cases with α-synuclein pathology that does not meet the pathological criteria for DLB, as well as DLB cases with only mild AD pathology. This variation in what is defined as mixed AD/DLB has further reduced the small number of studies which look into the pathology and underlying mechanisms of mixed AD/DLB.

There have been a small number of clinicopathological studies which have tried to understand the pathological underpinning of certain clinical symptoms observed in mixed AD/DLB. EEG studies in DLB with and without AD pathology, not necessarily meeting the criteria for a diagnosis of mixed AD/DLB, compared to pure AD, found that severity and presence of AD pathology did not alter the EEG changes observed. This suggests that the EEG changes observed could be related to α-synuclein pathology present, rather than the AD-type pathology (van der Zande *et al.*, 2018). Studies similar to this have shown that both global and regional atrophy seen in mixed AD/DLB is more similar to that seen in AD than DLB brains, with the level of atrophy seen correlating with the severity of AD-type pathology (Nedelska *et al.*, 2015). Further studies examining the relationship of pathology on clinical symptoms have found that the amount of quantitative pathology does seem to reflect the clinical diagnosis, for example a clinically AD mixed AD/DLB will have a higher level of tau and Aβ compared to α-synuclein pathology (Walker *et al.*, 2015).

Currently it is unknown whether the initiation and spread of the secondary pathology is independent of the primary pathology. However, cases with higher levels of α-synuclein pathology tend to have higher amounts of AD-type pathology and a shorter clinical course suggesting that there is synergy between the two pathologies (Nelson *et al.*, 2009; Irwin *et al.*, 2017; Malek-Ahmadi *et al.*, 2019), further discussed in 1.5. This has been further evidenced by a study showing that there were no changes in MMSE score with varying α-synuclein pathology when AD-type pathology was controlled for in mixed AD/DLB, suggesting that the pathologies are additive (Nelson *et al.*, 2009). As with other dementia types, it has been proposed that it is the anatomical location of the secondary pathology

that could impact upon the cognitive impairment seen clinically (Kapasi *et al.*, 2017). Some studies have suggested that there could be changes to the topographical spread of the pathologies when they are present together in the brain, with higher tau and α-synuclein levels in neocortical areas compared to the hippocampus (Malek-Ahmadi *et al.*, 2019). As with the clinical aspect of mixed AD/DLB research, further studies need to be undertaken to be able to fully uncover the roles that the different pathologies have in mixed AD/DLB.

1.5 Pathological mechanisms of neurodegenerative disorders

Neurodegenerative diseases lead to a progressive loss of cognitive function, producing a spectrum of clinical syndromes. The majority are sporadic with the aetiology thought to be multifactorial with genetic, epigenetic, environmental and age-related factors (Jellinger, 2010). Although neurodegenerative diseases have a diverse array of clinical manifestations, and the initial event and genetic underpinning may vary among the diseases, it is thought common neurodegenerative pathways are shared between them (Santiago *et al.*, 2017). There have been a number of processes and pathways that have been proposed to be shared amongst neurodegenerative diseases, however, the basic molecular mechanisms and their pathogenic role in these common neurodegenerative pathways are not yet fully understood (Jellinger, 2010; Gan *et al.*, 2018).

Neurons are unique compared to other cell types in the central nervous system. Along with their terminally differentiated state (Frade and Ovejero-Benito, 2015), neurons face additional challenges that can make them susceptible to neurodegenerative processes. Due to the functional role that neurons play, trafficking of molecules and organelles over large distances, along axons and processes, as well as rapid endo-/exocytosis of molecules requires a continuous demand for high energy levels; impairment to the pathways involved in these functions are proposed to lead to neurodegeneration (Gan *et al.*, 2018). Currently the major basic processes that have been implicated in neurodegenerative pathways include (figure 1.4): abnormal protein dynamics, through impairment in the ubiquitin-proteasome system and autophagy-lysosomal pathway, and protein aggregation; oxidative stress, the formation of free radical and impaired bioenergetic through mitochondrial dysfunction; neuroinflammatory processes, with changes to extra-neuronal populations; disruption to neuronal transport and synaptic toxicity (Gitler *et al.*, 2017; Soto and Pritzkow, 2018).

Although the hallmark pathological proteins are different, in distinct neurodegenerative diseases, it is thought that the process of misfolding, aggregation and neuronal death, are similar (Soto and Pritzkow, 2018). The native, monomeric structures of these proteins are thought to misfold to form aggregates, that are rich in beta-sheet motifs, with intermediary oligomeric and protofibril structures (Soto, 2003; Ross and Poirier, 2004). Although in previous years it has been proposed that the large aggregates were toxic, recent data has proposed the ‘toxic oligomer hypothesis’, wherein the neurotoxic species is proposed to be

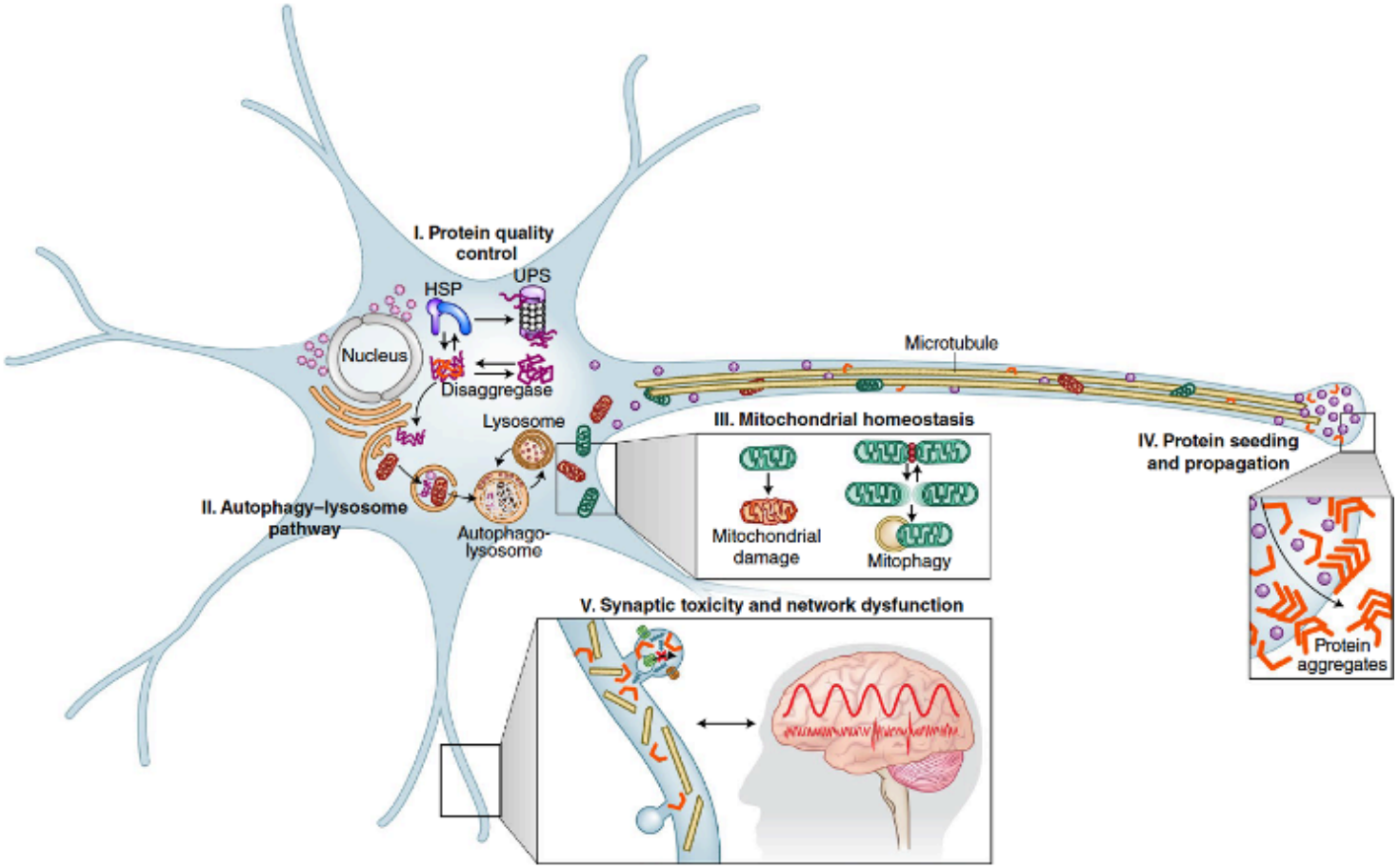


Figure 1.4. Common neuronal pathways altered in neurodegenerative disease.
Common neuronal pathways that can be altered in neurodegenerative disease include: I-protein quality control: molecular chaperones including heat shock proteins (HSP) regulate protein folding and ubiquitin-proteasome system (UPS) mediated degradation, while misfolded proteins could return to their native conformation state through interaction with disaggregase. II-autophagy-lysosome pathway (ALP): dysfunction to the ALP is thought to be a reason for the accumulation of protein aggregates and dysfunctional mitochondria within neurons. III-mitochondrial homeostasis: impairment of the clearance of dysfunctional mitochondria through mitophagy can lead to a reduction in energy production, signal for cell death pathways and impair proteostasis networks. IV-Protein seeding and propagation: prion like spread of pathological proteins could be the cause of disease progression and spread within the brain. V-Synaptic toxicity and network dysfunction: pathological proteins within pre- and post-synaptic terminals can lead to dysfunction in synaptic transmission, leading to network impairments. Figure adapted from (Gan *et al.*, 2018).

the oligomeric intermediates (Jellinger, 2010). In addition to researchers proposing that the formation of large aggregates, such as Lewy bodies, could be either protective for the neuron or a neutral component within the pathways to neuronal death (Gan *et al.*, 2018). The underlying mechanisms of the neurotoxic effects of the pathological proteins are still not completely understood. There have been three main mechanisms proposed; i) loss of function, ii) gain of function and iii) inflammatory mechanisms (Jellinger, 2010) and it is likely that a combination of these mechanisms occurs within the brain during neurodegenerative diseases, dependent on the protein sequence, cellular location and biophysical properties of the misfolded protein (Soto and Pritzkow, 2018).

The understanding of these pathways is confounded by the fact that the same neurodegenerative processes and pathways can be affected in different ways, in different neurodegenerative diseases. Neurodegenerative pathways can be associated with a variety of clinico-pathological phenotypes and vice versa identical phenotypes can be associated with a number of different neurodegenerative pathways (Golde, 2009; Sabir and Scholz, 2019). With the extensive pre-clinical phase seen in the neurodegenerative diseases, the elucidation of the role of these pathways is thought to be a critical step in order to locate drug targets for these diseases, as targeting the trigger may be too late at the point of clinical presentation, as well as the changes to neurodegenerative processes throughout the diseases progression (Golde, 2009; Kikuchi *et al.*, 2013).

1.5.1 Interactions between pathologies

Neurodegenerative diseases are characterised by the presence of distinct lesions, which are the prerequisite for its clinical development and later diagnosis (Spires-Jones *et al.*, 2017). However, the simultaneous accumulation of misfolded protein aggregates, both characteristic of and non-characteristic of the clinical disease is a common feature in neurodegenerative diseases (Badiola *et al.*, 2011). For example over 70% of DLB cases in a large multi-centre study where identified to have medium-high levels of AD pathology at post-mortem (Irwin *et al.*, 2017; Irwin and Hurtig, 2018). In the majority of instances the co-occurrence of pathologies is observed in different anatomical regions and cellular populations, although sometimes the proteins can be located within the same aggregates (Badiola *et al.*, 2011), for example tau and α-synuclein in Lewy bodies in DLB (Galloway *et al.*, 1988; Duda *et al.*, 2002; Ishizawa *et al.*, 2003; Galpern and Lang, 2006). The presence of

co-pathologies is thought to possibly be coincidental or, more likely, have an impact on disease progression or clinical presentation (Spires-Jones *et al.*, 2017). Research is currently focused upon whether there is a direct or indirect synergy between the co-pathologies when they are present within the same brain. Synergy and interaction between the proteins could be due to cross-seeding of the proteins or whether enhanced cellular vulnerability, impairments to proteostasis mechanisms result in an indirect synergy through an increased likelihood for protein misfolding (Soto and Pritzkow, 2018).

1.5.1.1 Tau and α-synuclein

Tau is frequently found as a co-pathology in neurodegenerative diseases, such as in DLB. From this observation research into the possible interactions between tau and α-synuclein have been undertaken and are ongoing. Giasson *et al.* (2003) were amongst the first to demonstrate that interactions between α-synuclein and tau can promote cross-fibrilization and could drive the formation of pathological inclusions. From their study it was found that when α-synuclein and tau were incubated together, α-synuclein may enhance a rate-limiting step in the initiation and early stages of tau fibrilization (Giasson *et al.*, 2003). Badiola *et al* (2011) provided further evidence for the interaction between tau and α-synuclein, reporting that tau enhanced α-synuclein aggregation and toxicity, in human neuroglioma cells and mouse cortical neurons. The study further identified that the presence of tau leads to smaller α-synuclein aggregates but with enhanced toxicity (Badiola *et al.*, 2011). The finding that smaller α-synuclein aggregates, have an enhanced toxicity, is consistent with human post-mortem findings that identified that presynaptic small α-synuclein aggregates associated with dendritic loss in the postsynaptic area, in DLB patients (Kramer and Schulz-Schaeffer, 2007). Human post-mortem brain tissue has supported the interaction between α-synuclein and tau with oligomeric forms located within the same aggregates using immunofluorescent techniques (Sengupta *et al.*, 2015). Not only has the interaction between tau and α-synuclein been shown to enhance aggregation, the interaction between the proteins has been shown to enhance phosphorylation of tau, utilising cellular and mouse models (Moussaud *et al.*, 2014; Li *et al.*, 2016). It has been proposed that α-synuclein can interact with glycogen synthase 3 beta to promote the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine induced phosphorylation of tau (Duka *et al.*, 2009) at a number of sites (Qureshi and Paudel, 2011) utilising cellular, mice and post-

mortem techniques. An increase in phosphorylated tau within neurons could therefore lead to increased microtubule instability, impairing intraneuronal trafficking and increasing the rate of neurodegenerative processes within the neuron.

1.5.1.2 Aβ and α-synuclein

α-synuclein was first identified as the NAC of Aβ plaques in AD (Uéda *et al.*, 1993), suggesting that α-synuclein and Aβ could interact (Chen, 2016). Studies have been undertaken to understand the interaction of α-synuclein and Aβ (Masliah *et al.*, 2001; Compta *et al.*, 2011; Marsh and Blurton-Jones, 2012). Cross seeding between α-synuclein and Aβ has been observed *in vitro* (Ono *et al.*, 2012), and α-synuclein has been shown to interact with Aβ oligomers to form hybrid oligomers in AD brains (Guerrero-Muñoz *et al.*, 2014). Within double transgenic mouse models, the presence of Aβ exacerbates α-synuclein accumulation and neuronal deficit (Masliah *et al.*, 2001). Aβ has also been shown to induce phosphorylation of α-synuclein at Serine 129 (pS129) *in vitro*, with the insoluble level of this post-translationally modified α-synuclein in brain homogenates related to the level of soluble and insoluble Aβ, with other studies suggesting that the concentration of ps129 is directly related to Aβ (Swirski *et al.*, 2014). An increase in pS129 levels, through interactions with Aβ, have been proposed to feed into a number of neurodegenerative mechanisms, including interactions with mitochondria (Oueslati, 2016). Wang *et al*. (2019) utilising primary rodent neuronal cultures and post-mortem brain tissue identified that pS129 preferentially accumulated in the mitochondria, compared to physiological α-synuclein which was found to not to be associated. The preferentially accumulation of pS129 in mitochondria was associated with defects in mitochondrial activity (Wang *et al.*, 2019), with further studies in rat brains identifying reduced protein import into the mitochondria due to pS129 interaction with Tom20 (Bernal-Conde *et al.*, 2019). Reduction to the import of proteins from the cytosol into the mitochondria could lead to alterations in energy production and excessive production of reactive oxygen species (Suhane *et al.*, 2013). The increased levels of reactive oxygen species could trigger apoptotic pathways leading to neuronal death.

1.5.1.3 Aβ and tau

The relationship between Aβ and tau has been extensively examined in the context of the amyloid cascade hypothesis in AD, with the relationship typically characterised as

unidirectional whereby the accumulation of Aβ induces hyperphosphorylation of tau (Chen, 2016). Although the link between tau and Aβ is clearly evidenced, for example their convergence in neuritic plaques in AD (Spires-Jones *et al.*, 2017), the exact species or conformations of Aβ that are responsible for this interaction is currently under debate (Chen, 2016). Many studies have suggested that species of Aβ interact indirectly via kinases with tau to enhance phosphorylation, leading to increased microtubule instability (Takashima *et al.*, 1993; Greenberg *et al.*, 1994; Ma *et al.*, 2009), with a few suggesting direct interactions (Jin *et al.*, 2011). The mechanistic link between Aβ and tau can be further supported by familial forms of AD, where genetic mutations which underlie alterations to Aβ processing not only leading to the formation of Aβ pathology but also leading to the production of tau pathology (Spires-Jones *et al.*, 2017). While there is an abundance of studies suggesting how Aβ exacerbates tau pathology, there have been fewer that have supported a role for tau in Aβ pathology (Chen, 2016; Spires-Jones *et al.*, 2017).

1.5.1.4 α-synuclein, Aβ and tau

Within mixed AD/DLB all three pathological proteins as discussed in 1.5.1 are present, although there has been a lack of studies that investigate all three pathological proteins together. As plaques, tangles and Lewy bodies occur in a way that cannot be merely explained by pure statistical overlap it is thought that the three proteins can promote the accumulation of one another, further necessitating the requirement to study the three together (Giasson *et al.*, 2003; Clinton *et al.*, 2010). The presence of α-synuclein pathology in AD is also associated with a more aggressive disease course and accelerated dysfunction (Kraybill *et al.*, 2005), highlighting a clinical need for the interaction for the three proteins to be investigated. Clinton *et al.* (2010) tried to address this by creating a DLB-AD mouse which exhibited all three pathological lesions. Data from the study supported the idea that α-synuclein, Aβ and tau interact *in vivo* leading to aggregation and accelerated cognitive decline. Within the study Clinton *et al.* (2010) also found an increase in the levels of insoluble Aβ at 12 months in the DLB-AD mice compared to AD-only mice, with the DLB-AD mice also possessing more plaques. Future work both *in vitro*, *in vivo* and using human post-mortem brains is required to fully understand the full interaction between α-synuclein, Aβ and tau.

1.5.2 Selective vulnerability

The clinical manifestation of neurodegenerative diseases is defined by the affected brain regions and neuronal subpopulations (Fu *et al.*, 2018; Jaunmuktane and Brandner, 2019). The selective loss of specific neuronal populations is thought to be due to these populations being more vulnerable to dysfunction or death in response to pathological states (Saxena and Caroni, 2011; Mattsson *et al.*, 2016), due to their intrinsic anatomy and biochemistry (Fu *et al.*, 2018). Although it has been known for a long time that specific neuronal populations are affected in different neurodegenerative diseases, the mechanisms underpinning this selective vulnerability have been difficult to uncover (Fu *et al.*, 2018).

Several hypotheses for selective vulnerability have been proposed, including the stressor-threshold model (Saxena and Caroni, 2011). The model hypothesises that the neurons that are selectively vulnerable in neurodegenerative diseases, on the background of environmental and genetic stress, have increased sensitivity to particular intrinsic stressors, organelle and energy homeostasis, which can combine to create a vicious cycle of increasing stressor load and lead to neuronal degeneration (Saxena and Caroni, 2011). A number of stressor pathways/mechanisms that were proposed to be involved in the model include enhanced physiological demands such as neuronal excitability, calcium fluxes and endoplasmic reticulum stress. Although the main neurodegenerative disease are characterised by the accumulation of misfolded proteins into aggregates, there has been research to say that this process alone is not sufficient to cause disease and that additional factors are required (Saxena and Caroni, 2011).

A number of mechanisms have been suggested that together or individually could underpin the selective vulnerability observed in neurodegenerative diseases (Fu *et al.*, 2018). Many of these mechanisms have been identified by studies that have mainly focused on a comparison of vulnerable neurons to their non-vulnerable neighbours, for example comparison of the dopaminergic neurons in the vulnerable substantia nigra (SN) and the non-vulnerable ventral tegmental area (VTA) in PD (Surmeier *et al.*, 2017b; Fu *et al.*, 2018).

Susceptibility to mitochondrial dysfunction has been implicated in selective vulnerability in PD (Surmeier *et al.*, 2010; Bolam and Pissadaki, 2012). Morphologically SN dopaminergic neurons have longer, sparsely myelinated axonal arbours and a higher number of synapses compared to their VTA neighbours (Brettschneider *et al.*, 2015). The morphological

differences are thought to result in a redistribution of mitochondria within the neuron towards the axonal terminals, leading to elevated energy demands and increasing the susceptibility to dysfunction in neuronal energy production (Surmeier *et al.*, 2010; Bolam and Pissadaki, 2012). The lower mitochondrial mass within SN dopaminergic neurons may mean that they are closer to mitochondrial failure than other neurons (Fu *et al.*, 2018), suggesting that they would be selectively vulnerable to neuronal insults that lead to mitochondrial dysfunction. It has been suggested that certain post-translationally modified species of α-synuclein, such as pS129, possess different properties than the physiological form. pS129 has been found to associate with mitochondria, leading to disruption of mitochondrial processes (Wang *et al.*, 2019), this could lead neurons that are more susceptible to changes in energy demands selectively vulnerable, including the SN in PD (Abbate, 2019; Lau *et al.*, 2019; Mrdjen *et al.*, 2019).

Furthermore, differential expression and levels of Ca^{2+} buffer proteins have been implicated in the selective vulnerability in PD (Fu *et al.*, 2018). The morphology of SN neurons, in combination with the pace-making oscillations, expression of different Ca^{2+} conductance channels and low Ca^{2+} buffering is what is currently thought to make these dopaminergic vulnerable compared to their VTA neighbours, with this leading to Ca^{2+} toxicity and mitochondrial stress and dysfunction (Sulzer, 2007; Brichta and Greengard, 2014; Surmeier *et al.*, 2017a; Fu *et al.*, 2018). The vulnerability conferred by the tonic pace-making activity and high branched axons are also observed in the cholinergic basal forebrain projection neurons that are commonly degenerated in DLB (Alegre-Abarrategui *et al.*, 2019).

1.6 Cognitive fluctuations

Cognitive fluctuations, are one of the least well characterised and understood of the four core clinical features of DLB, even though they have a prevalence of 80-90%, (McKeith *et al.*, 2000b; Ballard *et al.*, 2001; Matar *et al.*, 2019). Clinically defined as spontaneous variations in functional ability, cognition, attention and arousal, lasting from minutes to hours, as well as longer periods of weeks or months, cognitive fluctuations are thought to have no obvious environmental or situational cause (Walker *et al.*, 2000b; Ballard *et al.*, 2001; Escandon *et al.*, 2010; Lee *et al.*, 2012; Mainland, 2015). With cognitive fluctuations described as periods of inattention, behavioural confusion, hypersomnolence, appearing to be 'switched off' and incoherent speech alternating with periods of lucidity and 'normal' cognition, resembling a delirious state (Sin *et al.*, 2015), without acute motor or sensory loss; these fluctuations are thought to occur without an identifiable cause for the change in mental status and do not tend to follow a strict rhythm (Ballard *et al.*, 2001; Ferman *et al.*, 2004; McKeith *et al.*, 2017; Matar *et al.*, 2019). In DLB, fluctuations in cognition have been shown to be independent of alertness (Escandon *et al.*, 2010; Bliwise *et al.*, 2014).

Although cognitive fluctuations are a core feature of DLB, they have also been shown to affect those with VaD and AD, though with substantially lower prevalence than in DLB, 35-50% and 10-20% respectively compared to 80-90% (Lee *et al.*, 2012). While this is a feature found in a number of dementia subtypes, the cognitive domains affected can differ. Bradshaw et al (2004) found that in DLB, cognitive fluctuations tended to result in impairments to the flow of awareness and attention. Whereas, cognitive fluctuations observed in AD tended to reflect a reduced capacity to cope with the cognitive demands of the surrounding environment (table 1.1). It has also been suggested that fluctuations in DLB are more transient than those observed in AD, with caregivers stating that there were substantial changes in cognitive state on a daily basis (Bradshaw *et al.*, 2004). The differences in affected cognitive domains in fluctuations between AD and DLB could underlie the reportedly decreased quality of life in DLB compared to AD (Bostrom *et al.*, 2007).

The most obvious symptoms in those with neurodegenerative dementias is the loss of cognitive ability, however, it is the patient's ability to live an independent life that can have the most profound effect on their quality of life (Luttenberger *et al.*, 2012; Sikkes *et al.*,

Table 1.1. Care Giver Descriptions of Fluctuating Cognition.
Carer's answers to the questions 'Has the patient had a period or periods today when they seemed to be confused and muddled and then a period or periods when they seemed to be improved and functioning better? Give examples of the worst and best period of function.' Taken from the One Day Fluctuation Assessment Scale (Adapted from (Bradshaw *et al.*, 2004)).

Probable DLB	Probable AD
Worst: He was hallucinating, his character changed and he got loud, almost aggressive. Best: He was only slightly muddled.	Worst: She repeated the same question over and over 5–8 times in an hour. Best: She didn't repeat herself so much.
Worst: She required full direction with ADLs, was lethargic, dribbling and confused to time, place and routine. Best: She was alert, aware of her routine and familiar with the other residents.	Worst: He forgot the time and date and asked me 10 times in an hour. Best: He remembered the day.
Worst: He couldn't work our how to charge his electric razor or plug it in. Best: He attended to clerical work and paid the bills.	Worst: She repeated the same question numerous times over a few hours. Best: She recognised people by name.
Worst: She was nonsensical, confused, and mumbled incoherently. Best: She was almost as she was.	Worst: She was unsure of where she was going and why. Best: She was fleetingly objective and less repetitive.
Worst: She got up at 2:30 am and got dressed for an appointment. Best: Periods where she seems to think quite clearly, made sense and remembered things.	Worst: When he had to sort things out himself and remember what to do. Best: When there was someone to guide and remind him.
Worst: He woke in the morning and thought there was a drama somewhere and he had to be there, I couldn't convince him otherwise. Best: He woke up calm, and was more easily convinced not to worry.	Worst: He got snappy, agitated and couldn't think of what he wanted to say. Best: He was talkative and productive, making his own bread.
Worst: He kept looking for "the exit", couldn't find the bedroom or the bathroom and had trouble recognising me (wife) Best: He was alert, opened the door, and greeted me after work. He knew me and seemed pleased to see me.	Worst: After an argument she got agitated and couldn't think Best: Normal conversation and presented well to others who don't live with her.
Worst: She was seeing people, preparing extra meals, and asking how many people to cook for. Best: Normal conversation, made sense, nothing unusual.	Worst: After a small amount of alcohol she became confused and unsteady Best: When she relaxed and things were highly organised or centred around her.
Worst: Illogical discussion, all jumbled, and didn't make sense. Best: Made himself clearly understood.	

2012). The ability to be independent and perform fundamental activities of daily living, as well as their decision-making capacity, impacts not only the patient's quality of life, but the timing of possible institutionalisation and what degree of care they require (Jefferson *et al.*, 2006). Previous studies have demonstrated that cognitive fluctuations make a significant and independent contribution to the level of activities of daily living impairment (Ballard *et al.*, 2001; Sun *et al.*, 2018) and their decision-making capacity (Trachsel *et al.*, 2015). Cognitive fluctuations not only affect the ability of the patient to complete everyday tasks, but they can also contribute to increased caregiver burden (Ballard *et al.*, 2001; Zweig and Galvin, 2014). Several studies have investigated the impact of dementia subtype diagnosis and cognitive symptoms on the mental and physical demands of their caregiver (Kleinman *et al.*, 2004; Lee *et al.*, 2013; Zweig and Galvin, 2014). Studies have consistently found that a diagnosis of DLB or PDD resulted in a higher level of caregiver burden, even when compared with dementias such as AD or VaD, irrespective of age, gender and cognitive ability (Lee *et al.*, 2013). The increased caregiver burden resulting from cognitive fluctuations could largely be due to the unpredictability of the care requirements and level of support required over the course of the day (Mainland, 2015).

Cognitive fluctuations are highly disabling, above and beyond existing cognitive impairment, and can occur in varying frequencies both between subjects and within the same individual, from infrequent to several times a day (Ballard *et al.*, 2001; Mainland *et al.*, 2017). The wide variation in the clinical presentation of fluctuations presents the diagnostic challenge of how to accurately assess the feature whilst conveying all their characteristics, especially in the later stages of disease progression when cognitive fluctuations may be hidden by severe cognitive impairment (McKeith *et al.*, 1996). The presence of fluctuations can also impact upon other cognitive tests, such as MMSE, making it difficult to obtain a reliable and accurate assessment (Mainland, 2015; Izuhara *et al.*, 2019). The inability to accurately assess and characterise cognitive fluctuations seen clinically can provide difficulties when undertaking clinico-pathological correlative studies. Without an accurate assessment of cognitive fluctuations, it is not possible to correlate changes seen within brain regions to the severity of fluctuations.

Although the presence of cognitive fluctuations has been established in a variety of dementia subtypes and is of key diagnostic relevance in DLB, there is still little known about the changes that underlie this phenomenon (Matar *et al.*, 2019). Studies mainly utilising EEG and MRI in DLB patients have been undertaken in order to help understand what changes may underlie the aetiology of cognitive fluctuations. Bonanni and colleagues (2008) found that on EEG, DLB compared to AD patients tended to display an increase in slow wave activity posteriorly. Furthermore, in DLB patients the EEG frequency fluctuates in periodic patterns, particularly posteriorly, with such transient changes correlating with the frequency and severity of the fluctuating cognition (Walker *et al.*, 2000b). Research from Schumacher *et al.* (2019a; 2019b; 2019c) using a combination of MRI and EEG has investigated alterations to brain dynamics as well as the possible network underpinning of cognitive fluctuations. In Lewy body dementia patients it was found that there was an alteration to microstate dynamics compared to AD and controls, with a correlation found between the severity of the cognitive fluctuations and the microsite abnormalities, suggesting a less dynamic brain is related to cognitive fluctuations (Schumacher *et al.*, 2019c). However, the ability to change from low to high inter-network connectivity states as seen by functional MRI did not correlate with cognitive fluctuations, as measured by clinical assessment of fluctuation (CAF) score (Schumacher *et al.*, 2019b). However, the brain regions contributing

to fluctuating cognition are still unknown, with no obvious structural changes having been associated with the phenomenon (Taylor *et al.*, 2013). Further studies have found volumetric reductions to the hypothalamus in DLB, which have been suggested to relate to the alterations in attention and arousal seen clinically in DLB (Whitwell *et al.*, 2007). In DLB, alterations have been observed in cells responsible for cortical activation and the generation of wakefulness, including alterations to nicotinic receptor binding (Ballard *et al.*, 2002b; Pimlott *et al.*, 2006), that could influence cognitive fluctuations (Ferman *et al.*, 2004). Functional neuroimaging studies have associated fluctuations with changes in neural networks involving cholinergic and dopaminergic transmitter systems within the thalamus (Pimlott *et al.*, 2006; Peraza *et al.*, 2014; Delli Pizzi *et al.*, 2015). On the basis of these studies, a number of theories have been postulated, discussed further in 1.6.1, as well as the hypothesis that cognitive fluctuations result from alterations in networks, rather than obvious single structural changes (Taylor *et al.*, 2013).

In spite of the fact that there is a limited understanding of the underlying causes of cognitive fluctuations, there have been a number of studies that have investigated possible symptomatic treatments (O'Dowd *et al.*, 2019). Mori *et al* (2012) investigated the effect of donepezil, an AChEI, on cognitive fluctuations and found that it ameliorated fluctuating cognition on the Cognitive Fluctuation Inventory score and associated EEG abnormalities, compared to placebo. The efficacy of other currently licenced dementia drugs, including rivastigmine and memantine is less certain (O'Dowd *et al.*, 2019). Studies looking at deep brain stimulation of regions involved in attentional networks, including the nucleus basalis of Meynert have been contradictory in their ability to treat cognitive fluctuations (Freund *et al.*, 2009; Gratwicke *et al.*, 2018).

Although research has been undertaken to attempt to understand the aetiology of fluctuating cognition, it is understudied relative to other aspects of DLB and neurodegenerative dementias. The paucity of research is reflected in the limited reproducible biomarkers and lack of new major insights into the aetiology of cognitive fluctuations, as well as explaining the lack of treatments available that target this clinical feature (Matar *et al.*, 2019).

1.6.1 Theories behind cognitive fluctuations

Various brain regions have been implicated, through a variety of techniques including SPECT, diffusion and resting state MRI, and EEG, that could play a role in the generation of cognitive fluctuations. The identification and implication of these regions has in turn led to a number of mechanistic theories being posited (Matar *et al.*, 2019; O'Dowd *et al.*, 2019).

1.6.1.1 Theory of attentional dysfunction

One of the major theories for cognitive fluctuations in the disruption to attentional systems, with disruption in attentional processing thought to be an early feature of fluctuations (Matar *et al.*, 2019). Dysfunction to the attentional systems has been hypothesised to occur via a number of different mechanisms, with it likely that several components together are affected, rather than one single pathological change (Matar *et al.*, 2019; O'Dowd *et al.*, 2019). Overall the theory of attentional dysfunction suggests that in DLB the brain is less dynamic, with studies implicating network dysfunction and alterations to the cholinergic system in underlying the attentional changes. The alterations to brain dynamics could reduce the ability of the brain to adapt to changing loads, leading to variability within the attentional networks (Bradshaw *et al.*, 2004). Without the ability to quickly and easily adapt to changing attentional loads this could cause lapses of inattention, observed clinically as fluctuations in cognition.

Disturbances to large-scale neural networks has been implicated in the attentional hypothesis of cognitive fluctuations (O'Dowd *et al.*, 2019). The default mode network, where activity has been linked to attentional lapses (Weissman *et al.*, 2006), has been hypothesised to be involved in cognitive fluctuations, although the data in supporting this theory is conflicting (Franciotti *et al.*, 2013; Lowther *et al.*, 2014; Peraza *et al.*, 2014; Schumacher *et al.*, 2018; O'Dowd *et al.*, 2019). Within some of the studies examining default mode network, it has been found that a decrease in frontoparietal network connectivity negatively correlated with cognitive fluctuation severity in DLB (Franciotti *et al.*, 2013; Peraza *et al.*, 2014). Although, the default mode network alone has conflicting evidence supporting its involvement in cognitive fluctuations, the network in combination with other attentional networks has been implicated in dysfunctional attention in DLB and proposed to be involved in fluctuating cognition (Firbank *et al.*, 2016; Chabran *et al.*, 2018). As well as looking at discrete networks, studies have examined connectivity through dynamic analyses,

which have identified a loss of global variability in DLB (Ma *et al.*, 2019; Schumacher *et al.*, 2019b) and a disturbance to functional connectivity (Chabran *et al.*, 2020), suggesting that the brain in DLB is more rigid which could play a role in cognitive fluctuations, as well as analyses which using graph-theory have found that increased path lengths correlated with the severity of cognitive fluctuations (Peraza *et al.*, 2015). Alterations to the brain's dynamic properties have also been studied using EEG, with alterations in dynamics having been shown to be associated with (Bonanni *et al.*, 2008; Bonanni *et al.*, 2016; Stylianou *et al.*, 2018) or correlating with the severity of cognitive fluctuations (Bonanni *et al.*, 2015; Schumacher *et al.*, 2019c). Together the alterations in the brain's dynamics in DLB suggest that it is less able to adapt to changing loads; slower adaptations to changing loads could clinically be seen as durations of 'blankness' or an inability to focus properly (Bradshaw *et al.*, 2004).

Loss of cholinergic attentional drive has been speculated to play a role in the disruption of attentional networks in cognitive fluctuations (Ferman *et al.*, 2004). Cholinergic nuclei and their projections have been known to play an important role in attentional processing for over two decades, with lesion experiments of the cholinergic basal forebrain in primates impacting upon attention (Voytko *et al.*, 1994). Further studies using animal models have shown selective lesioning of the pedunculopontine nucleus (PPN) in rats lead to fluctuations in cognition (Cyr *et al.*, 2015). Nuclei within the basal forebrain including the nucleus basalis of Meynert, which is functionally linked to the PPN, as well as being important in attentional signalling are vulnerable to a-synuclein pathology in synucleinopathies (Liu *et al.*, 2015), with neuronal loss in the region having been shown to be correlated with a reduction in cortical choline acetyltransferase (ChAT) levels in DLB (Lippa *et al.*, 1999). Volumetric changes in cholinergic regions including, the substantia innominata a cholinergic nuclei within the basal forebrain (Colloby *et al.*, 2017), and midbrain, prefrontal cortex and thalamus overlapping with cholinergic systems (Chabran *et al.*, 2020) have been shown to correlate with fluctuating cognition in DLB utilising imaging techniques. Along with imaging and histopathological studies, support for a loss of cholinergic attentional drive underlying cognitive fluctuations comes from clinical (Mori *et al.*, 2012; Kazui *et al.*, 2017) and mechanistic (Onofrj *et al.*, 2003) studies that have examined the improvements to cognitive fluctuations observed with AChEIs.

The thalamus is one of the main structures involved in the dorsal pathway of the ascending reticular activating system (ARAS), further discussed in 1.7.1, where thalamocortical projections, controlled from brainstem inputs, mediate cortical activation and consciousness (Jang and Kwak, 2017). Due to its role in consciousness it has been speculated that thalamic dysfunction could be a possible mechanism involved in the attentional dysfunction hypothesis of cognitive fluctuations (O'Dowd *et al.*, 2019). α-synuclein pathology is readily located in the thalamic relay nuclei that from part of the ARAS (Braak *et al.*, 2003). Cholinergic denervation and higher choline levels in these thalamic sub-nuclei have been observed in DLB (Kotagal *et al.*, 2012b; Delli Pizzi *et al.*, 2015), with the ratio of total choline to total creatinine, a neuronal marker of energetic metabolism, in these regions correlating with severity of fluctuations, further supporting a mechanistic role of the thalamus in cognitive fluctuations. Imaging studies have also identified atrophy within thalamic nuclei of DLB patients (Watson *et al.*, 2017), which was more pronounced in those with cognitive fluctuations (Delli Pizzi *et al.*, 2015) and related to attentional deficits (Watson *et al.*, 2017). Within these studies a decrease in thalamic activity was also found, which was noted to be more marked in those with fluctuations (O'Brien *et al.*, 2005; Galvin *et al.*, 2011); as well as bilateral alteration of activity in DLB in thalamic regions known to project to prefrontal regions, although these alterations did not correlate with severity of cognitive fluctuations (Galvin *et al.*, 2011; Delli Pizzi *et al.*, 2015; O'Dowd *et al.*, 2019). Decreased activity between thalamic and cortical networks has also been identified within EEG studies (Schumacher *et al.*, 2019c). It is not only imaging studies that have identified thalamic alterations in cognitive fluctuations. A number of molecular studies have identified alterations to receptors, including cholinergic and dopaminergic, in specific attentional regions of the thalamus, known to be involved in the ARAS, and the cortical areas the thalamus projects to (Ballard *et al.*, 2002b; Pimlott *et al.*, 2006; Piggott *et al.*, 2007). However, cognitive fluctuations are generally thought to be non-dopaminergic due to Levodopa not having a beneficial effect on the symptom (Molloy *et al.*, 2006). These results further support and validate the idea of thalamic and cholinergic dysfunction possibly leading to attentional deficits that underlie cognitive fluctuations in DLB.

1.6.1.2 Theory of arousal/awareness dysfunction

Arousal/awareness is the general receptivity to external stimuli and the preparedness to respond to them, this is generally seen as a state without a specific target or stimulus, this is in contrast to attention which is the ability to focus and respond to a particular stimuli (Whyte, 1992). Arousal specifically describes the level of consciousness during wakefulness, with there being a lack of arousal when asleep. Sleep-wake systems in the brain have been identified to mediate arousal (de Lecea *et al.*, 2012). Alteration to arousal and awareness centres has already been shown in DLB (Pao *et al.*, 2013), with alterations to this system being theorised to underly the cognitive fluctuations clinically observed (Matar *et al.*, 2019; O'Dowd *et al.*, 2019). Dysfunction within the centres that maintain arousal and awareness could lead to durations where patients appear to be unreceptive to external stimuli, as Susan Schneider-Williams described 'blank, lost in confusion' (Williams, 2016) and Bradshaw *et al*. (2004) described as 'blank staring which the patient appeared to disengage from the on ongoing flow of activity or conversation'.

A key region involved in the maintenance of sleep-wake is the hypothalamus, a key centre of the ventral ARAS pathway (Jang and Kwak, 2017). The hypothalamus is known to be affected by pathology in DLB (Benarroch *et al.*, 2015). Wakefulness centres in the hypothalamus, including the histaminergic tuberomammillary nucleus are known to be affected through neuronal loss in DLB (Ferman *et al.*, 2004; Benarroch *et al.*, 2015), further supporting a dysfunctional arousal/awareness system in DLB possibly being associated with cognitive fluctuations. Whitwell *et al.* (2007) has found reductions in hypothalamus volume, which was suggested might relate to alterations in arousal/awareness, although they did not relate these findings to fluctuation severity (Taylor *et al.*, 2013). Alterations to these systems are proposed to underly not only cognitive fluctuations but also RBD, with some clinical studies identifying a correlation between the two core features (Escandon *et al.*, 2010; Cagnin *et al.*, 2017). This has suggested a possible universal alteration to the sleep-wake system may underlie both clinical features (Terzaghi *et al.*, 2013; Antelmi *et al.*, 2016); which could suggest that in DLB the systems for sleep or wake are not efficient enough to maintain adequate levels of global arousal (Cagnin *et al.*, 2017). Support for the arousal/awareness theory of cognitive fluctuation has also come from a limited number of studies looking into the efficacy of modafinil (Varanese *et al.*, 2013), although more

research is required to fully understand the possible role the arousal/awareness system plays in cognitive fluctuations (O'Dowd *et al.*, 2019).

Although the hypotheses of attentional and arousal/awareness dysfunction are distinct from each other, it is likely that a globally altered level of arousal will lead to impairment of attention. Furthermore, studies into cognitive fluctuations have identified that cognitive fluctuations in DLB may comprise of an attentional and an arousal/awareness component (Bliwise *et al.*, 2014; Ferman *et al.*, 2014). This could suggest that both theories in combination could underly cognitive fluctuations and the clinical phenomenon is due to dysfunction of a distributed neural system (Matar *et al.*, 2019; O'Dowd *et al.*, 2019) .

1.6.1.3 Theory of orthostatic hypotension

A third theoretical mechanism for cognitive fluctuations stems not from dysfunction within the brain but from variation in autonomic processes especially blood pressure (Riley and Espay, 2018; O'Dowd *et al.*, 2019). Orthostatic or paroxysmal hypotension is a supportive feature of DLB (McKeith *et al.*, 2017), this feature can lead to transient cerebral hypoperfusion. Transient cerebral hypoperfusion could lead to times where the brain is not able to obtain enough oxygen to sustain full cognitive function, leading to lapses or fluctuations in cognition associated with blood pressure. A case study by Riley and Epsay (2018) examined a PDD patient with cognitive fluctuations which reversed upon alteration of position and treatment with midodrine, providing evidence that in this case fluctuations were caused by underlying variations in blood pressure. Another small study demonstrated that attentional measure were detrimentally affected by orthostatic hypotension in PDD patients (Peralta *et al.*, 2007). Although, it is possible that orthostatic hypotension may underlie cognitive fluctuations in some Lewy body dementia cases, it is unlikely to be the cause in the majority of cases.

1.6.2 Clinical assessment of cognitive fluctuations

Although cognitive fluctuations are one of the four core features of DLB, there have been no operational criteria for defining cognitive fluctuations suggested in the consensus diagnostic criteria, making it the most difficult of the four core features of DLB to clinically identify and assess (Escandon *et al.*, 2010; Lee *et al.*, 2012; Kosaka, 2017; McKeith *et al.*, 2017). The lack of operational criteria has meant that examination of fluctuations within research studies has been inconsistent with it likely being even more so within clinical practice (Bliwise *et al.*,

2014). Lee and colleagues (2012) undertook an extensive literature search of all available literature to date on cognitive fluctuations in the major neurodegenerative dementias, as well as the psychometric tests to assess the fluctuations, reporting a lack of information to guide clinicians on an accurate assessment and identification of cognitive fluctuations.

1.6.2.1 Clinical assessment of fluctuation

The CAF (Walker *et al.*, 2000a) is a scale designed to be administered by experienced clinicians, consisting of a series of informant-directed questions regarding fluctuating confusion and impaired consciousness in the month prior to the assessment (Mainland, 2015). If fluctuating confusion is present, frequency and duration of the episodes are both rated from 0-4, with the scores multiplied together to produce an overall severity score. A score of zero would represent that no cognitive fluctuations were present, twelve would represent severe cognitive fluctuations and sixteen would represent a continuous clouded state of confusion, with no fluctuations from this state. Studies have identified that a score over 5 would indicated clinically significant fluctuating cognition, with interrater reliability increasing with increasing severity of fluctuations (Bradshaw *et al.*, 2004; Van Dyk *et al.*, 2016). However, due to the nature of the CAF scoring system, short duration but frequent fluctuations would receive the same score as longer duration, but more infrequent fluctuations, though it is thought that these two different fluctuation patterns may be due to differing underlying pathological changes (Matar *et al.*, 2019). Further caveats of the CAF, including the open-ended nature of questions and reliance on administration by an expert clinician, suggesting it is unlikely to be widely adopted in clinics outside a clinical research setting (Bradshaw *et al.*, 2004; Rongve *et al.*, 2010; Lee *et al.*, 2012).

1.6.2.2 One day fluctuation assessment

The One Day Fluctuation Assessment Scale (ODFAS) (Walker *et al.*, 2000a) was developed based on a validated delirium assessment scale (Inouye *et al.*, 1990), the Barthel index (Mahoney and Barthel, 1965) and the 'fluctuation' component of the DLB clinical diagnostic criteria (McKeith *et al.*, 1996). The ODFAS is a scale designed to be given by non-clinicians, consisting of seven items assessing behaviours indicative of confusion, including falls, fluctuation, drowsiness, attention, disorganised thinking, alterations to consciousness levels and communication, on the day prior to the assessment. For some of the seven items there are multiple related questions, scores are then summed to produce an overall severity score

for cognitive fluctuation from zero to twenty-one. Studies using the OFDAS found that scores could be altered by medication regimes, as well as the scale not detecting fluctuating cognition in a DLB cohort to the prevalence levels reported (Bradshaw *et al.*, 2004). A further caveat of the ODFAS is that the questionnaire relates specifically to the day prior to assessment which may not capture a transient clinical feature, in addition to scores being affected by falls which may not be related to cognitive fluctuations. The quantitative scoring systems utilised in both CAF and the ODFAS do not take into account any differences in the cognitive domains fluctuating, meaning that these scales may miss important difference between disease groups, such as AD and DLB (Bradshaw *et al.*, 2004; Lee *et al.*, 2012).

1.6.2.3 Mayo composite fluctuations scale

The Mayo Composite Fluctuations Scale (MCFS) was developed by Ferman and colleagues (2004), to ascertain whether the cognitive domains affected in the fluctuations could reliably distinguish DLB from AD or normal ageing (Mainland, 2015), the scale has been further translated into other languages (Thaipisuttikul *et al.*, 2018). The MCFS consists of a nineteen-item questionnaire that is administered to informants, which aims to elucidate the symptoms of the fluctuations and delirium experienced by the patient over the past month. The decision to administer the questionnaire to a patient's informant instead of the patient themselves, was supported by the fact that most family members or carers can describe the fluctuations in cognition or alertness that they have observed in patient (Ferman *et al.*, 2004). Although the MCFS would be able to score the severity of the fluctuations as seen by the informant, the nature of the test does not allow for examination of fluctuations to cognitive abilities, as such the test may be missing aspects of fluctuation that would only be 'visible' through a more specific cognitive task.

1.6.2.4 The dementia cognitive fluctuation scale

The Dementia Cognitive Fluctuation Scale (DCFS) was developed by Lee and colleagues (2013), to improve upon the CAF, ODFAS and MCFS tests already published. Items from the MCFS which have been previously shown to reliably distinguish between disease groups were incorporated. Relevant items from the CAF and ODFAS were also incorporated, including alertness and confusion. The final version of the DCFS is comprised of 17 items in four sections- confusion, sleep, alertness and communication, and was capable of distinguishing DLB from AD (Lee *et al.*, 2014).

1.7 The ascending reticular activating system

Consciousness is underpinned by two main components, arousal and awareness, of the environment and self (Weiss *et al.*, 2007; Jang and Kwon, 2015a), with the ARAS responsible for its regulation and providing 'context' to conscious experience (Paus, 2000; Zeman, 2001; Llinás, 2002). The ARAS, is a fibre bundle that originates from various nuclei within the pons and midbrain, that projects to the cortex, thalamus and basal forebrain to mediate wakefulness and arousal through cortical activation, suppression of slow rhythms in EEG and synchronisation of faster frequency rhythms (Sarter, 2006; Edlow *et al.*, 2012; Kovalzon, 2016a) (figure 1.5).

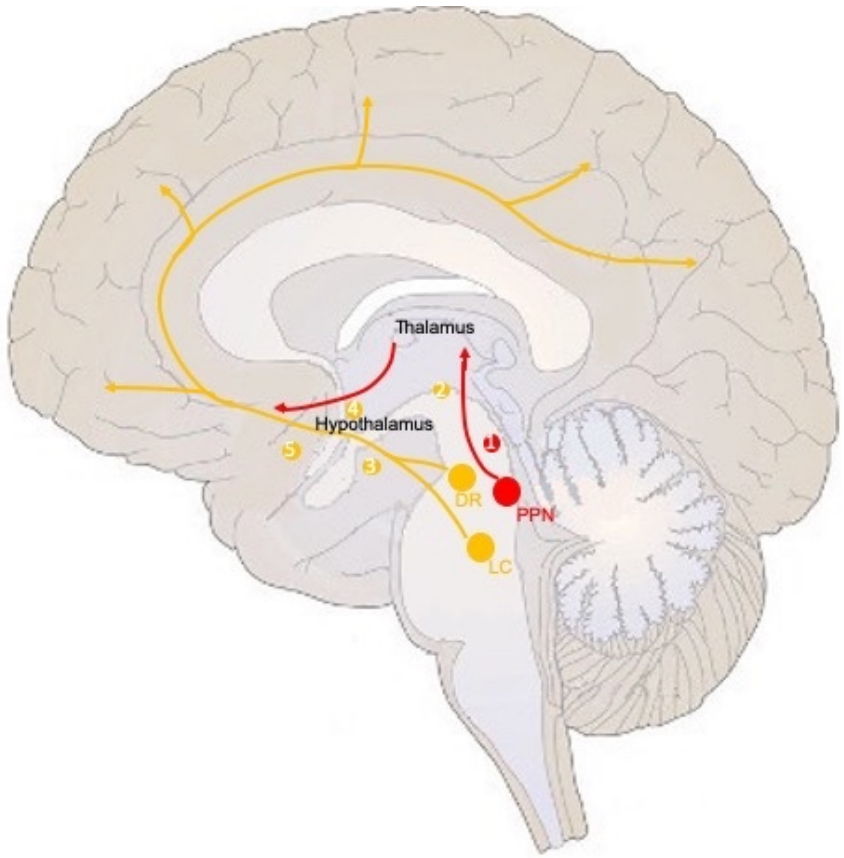


Figure 1.5. Wiring diagram for the ascending reticular activating system.
A wiring diagram summarising the nuclei and their neurotransmitter systems that form the ascending reticular activating system. Ventral pathway shown in orange and the dorsal pathway shown in red. Three main nuclei: cholinergic- pedunculopontine nucleus (PPN); serotonergic- dorsal raphe (DR); noradrenergic- locus coeruleus (LC). Other nuclei and neurotransmitter systems involved: 1) cholinergic-laterodorsal tegmental nucleus; 2) dopaminergic- ventral periaqueductal grey matter; 3) histaminergic- tuberomammillary nucleus; 4) orexinergic-hypothalamus; 5) cholinergic & GABAergic-basal forebrain. Figure adapted from (Saper et al., 2005).

The identification and investigation of the ARAS has occurred within the last century. Original experiments identified differing EEG states during different wakefulness states (Berger, 1929), with later animal studies undertaken to identify the origin of these EEG states (Bremer, 1937). With the first full description and identification of the brainstem

structures forming the ARAS by Moruzzi and Magoun (Moruzzi and Magoun, 1949). Although, initially considered to be a diffuse structure, currently it is thought to be comprised of a number of different neurotransmitter systems and brainstem nuclei ('waking centres'): cholinergic and glutamatergic PPN, noradrenergic locus coeruleus (LC), serotonergic dorsal (DR) and median raphe (MnR) and the dopaminergic VTA and SN (Zeman, 2001). The three major nuclei thought to be the core of the ARAS are the PPN, LC and DR/MnR (Mahaffey and Garcia-Rill, 2015), with further neurotransmitter systems playing a role in mediating the functioning of the ARAS, including orexin and histaminergic neurons (Kovalzon, 2016a). Further studies have suggested the ARAS consists of many ascending and descending connections to a wide range of brain regions that can mediate arousal and vigilance (Hyde and Garcia-Rill, 2015; Yates and Garcia-Rill, 2015).

The ARAS is thought to have two main pathways for its ascending projections, non-thalamic and thalamic, as well as direct projections to the cortex, with high connectivity to the prefrontal cortex (PFC) (Shute and Lewis, 1967; Daube, 1986; Paus, 2000; Jones, 2003; Jang and Kwon, 2015b; Krone *et al.*, 2017). The non-thalamic (ventral) pathway, contains noradrenergic and serotonergic projections to the basal forebrain, hypothalamus and the cortex, as well as the orexinergic and histaminergic neurons of the hypothalamus (Brown *et al.*, 2012). Whereas, the thalamic (dorsal) pathway originates in the cholinergic nuclei of the PPN and projects to intralaminar thalamic nuclei and medial thalamic nuclei, inhibiting the reticular thalamic nuclei, and activating thalamocortical pathways to mediate cortical activation (Jang and Kwak, 2017). The dorsal pathway has been shown to stimulate the cortex up to ten times faster than the ventral pathway, and is the pathway selectively activated in REM sleep (Jones, 2005b; Han *et al.*, 2014). Although the ARAS has been proposed to have two distinct pathways innervating the cortex, the dorsal and ventral pathways are not completely separate with the major nuclei of the ARAS have interconnecting projections (Shute and Lewis, 1967), specifically between the DR, LC and PPN, with this interconnectivity likely to play a vital role in the maintenance of arousal states (Edlow *et al.*, 2013). The multiple systems that comprise the ARAS suggest that there is some level of redundancy within the system, further supported by animal lesion models, show that no pathway is essential for the maintenance of arousal (Jones, 2005b).

Current research into the ARAS has mainly been undertaken in animal models, and a minority of human MRI studies, due to limited feasibility of post-mortem tracking and the only recent implementation of High Angular Resolution Diffusion Imaging (Edlow *et al.*, 2012). Through imaging and lesion research the ARAS is believed to control arousal and the maintenance of consciousness, through its control of thalamo-cortical activity, altering the patterns of neuronal firing to allow for global synchronisation (De Cicco *et al.*, 2017). With further experiments proposing a new ARAS model which implicates the thalamus as not only a relay centre but as an integral component allowing the integration of brainstem and cortical networks to modulate arousal (Edlow *et al.*, 2012).

1.7.1 Disorders of the ascending reticular activating system

The ARAS is directly involved in mediating arousal and awareness. Lesions within the brainstem, corresponding to ARAS nuclei are associated with disturbances of arousal and coma, leading to alterations in the level of cortical activation and cognition (Parvizi and Damasio, 2003; van Gaal *et al.*, 2012). Pathologies of wakefulness include coma and narcolepsy, the most studied area regarding the ARAS in humans, which evolve from a lack of drive from the ARAS to activate the cortex to produce the high frequency activity required for wakefulness and consciousness (Garcia-Rill and Simon, 2015). A number of pathologies of wakefulness can be due to changes to particular neurotransmitter pathways within the ARAS, for example narcolepsy can be caused through dysfunction of the hypocretin transmitter pathway in the lateral hypothalamus, in the ventral pathway (Siegel, 2004). Coma or disorders of consciousness, can be caused by damage in multiple locations within the ARAS affecting the ascending connections to the cortex, including the source nuclei, their projections or target regions including regions of the thalamus (Garcia-Rill, 2015; Jang and Lee, 2015). A case study of a patient who have suffered lesions to the ARAS, which have resulted in the presence of a minimally conscious state, was treated with deep brain stimulation (DBS) of the intralaminar thalamic nucleus, in the dorsal pathway (Schiff *et al.*, 2007). Stimulation of the intralaminar thalamic nucleus improved communication function, which dissipated once the stimulation was stopped (Schiff *et al.*, 2007), indicating that if the thalamocortical projections remain intact stimulation can be applied to increase cortical activation. It has also been hypothesised that subtle changes within ARAS activity can impact upon cognitive performance (Lee and Dan, 2012), suggesting that dysfunction

within the ARAS could underpin other disorders of consciousness. Within patients whom suffer from absent mindedness following a traumatic brain injury, imaging studies have identified injuries dysfunction within the lower aspects of both the dorsal and ventral ARAS pathways, with a decrease in connectivity to the thalamus, hypothalamus and PFC observed (Jang and Kwak, 2017).

The alterations and dysfunction observed in the ARAS in those with disorders of consciousness supports the idea that alterations due to neurodegenerative mechanisms in DLB could lead to the alterations in consciousness, cognitive fluctuations, observed clinically.

1.7.2 Raphe

The raphe nuclei is a heterogenous neuronal population, that borders the midline lie along the entire rostro-caudal aspect of the brainstem (Hornung, 2003). Serotonergic neurons are the main neuronal component of the raphe complex, allowing for easy identification with the serotonin synthesis enzyme, tryptophan hydroxylase 2 (TPH2), although other neurotransmitters including substance P in the DR are present (Baker *et al.*, 1990; Baker *et al.*, 1991). The raphe nuclei are comprised of a collection of sub-nuclei, defined upon their location within the complex as well as their main projections (Hornung, 2003). The two main sub-nuclei implicated in the ARAS are the DR located at the level of the oculomotor nucleus to the middle part of the pons, and the MnR located at the caudal end of the superior cerebellar peduncle to the level of the trigeminal motor nucleus, with neuronal overlap between the two nuclei evident in the lateral reticular formation (Hornung, 2003). The rostral group of subnuclei, including the DR and MnR, located in the rostral pons projects to cortical regions, whereas the caudal group located in the medulla oblongata have major connections to other brainstem regions and the spinal cord (Hornung, 2003).

Research has found that the DR and MnR play a role in a number of different brain systems (Hornung, 2003). The systems that the raphe is involved in include emotional processing and maintenance of wakefulness, through its connectivity with cortical, limbic and ARAS structures (Jones, 2005b); with the main research focus being the role of the serotonergic system depression and anxiety (Hale and Lowry, 2011), with the majority of antidepressant drugs targeting serotonergic neurotransmission. However, the role of the DR within the ARAS and maintenance of arousal has also been explored.

The earliest demonstration that the raphe and the serotonergic system played a role in the maintenance of arousal was when it was identified that injection of reserpine, which depletes the level of serotonin within the brain was observed to have a sedative effect (Brodie *et al.*, 1955). Further research, undertaken in cats, identified that lesioning of the total or partial lesioning of the raphe would lead to permanent wakefulness (Jouvet, 1968). However, a number of additional studies identified that after chronic depletion of serotonin that sleep would eventually return (Dement *et al.*, 1972); as well as the DR being thought to fire maximally during waking and cease during REM sleep (Trulson and Jacobs, 1979; Mahaffey and Garcia-Rill, 2015), which would indicate that the nuclei promotes wakefulness. The inconsistency between these studies suggest that the serotonergic system has a complex role in the maintenance of arousal and the differing effects on arousal could be region and receptor subtype dependent. Through the studies implicating the raphe in both the maintenance of sleep and wakefulness, it suggests that dysfunction to the nucleus in a disease state could lead to not only to depressive symptoms but alterations in the balance between arousal and sleep.

The DR is highly interconnected with other ARAS nuclei, receiving cholinergic inputs from the PPN through nicotinic receptors (Mihailescu *et al.*, 2002) and noradrenergic inputs from the LC through α_1-adrenergic receptors (Baraban and Aghajanian, 1981; Aghajanian, 1985); as well as sending reciprocal projections to these nuclei. It is thought that through this interconnectivity serotonin is able to attenuate cortical activation by inhibition of other neurons within the ARAS, including the cholinergic neurons of the PPN (Jones, 2005b). Dysfunction to the raphe nucleus through neurodegenerative mechanisms could alter the connectivity with other regions of the ARAS including the LC and PPN. Reductions or variability to serotonergic inhibition on cholinergic neurons in the PPN could lead to reductions in arousal due to activation of regions associated with sleep. Activation of sleep centres during wakefulness could create periods of inattention and low arousal, with reactivation of the serotonergic inhibitory effects leading to an increase in arousal.

The DR and MnR nuclei provide the majority of the ascending serotonergic projections to the forebrain and the hypothalamus (Leander *et al.*, 1998), in a parallel and overlapping manner (Beck *et al.*, 2004). Although the projections are overlapping there are differences within the projection fibres, as well as there being a number of distinct projection areas

specific to each sub-nuclei (Beck *et al.*, 2004). The DR projects to the prefrontal cortex, amygdala and striatum, with a high level of connectivity also observed with other nuclei of the ARAS (Vertes, 1991; Beck *et al.*, 2004; Michelsen *et al.*, 2007). In contrast, the MnR projects to the dorsal hippocampus, medial septum and cingulate (Molliver, 1987). Both the DR and MnR also receive afferent connections from similar regions, including forebrain and hypothalamus (Hornung, 2003), further cementing their role within the ARAS network. Both nuclei are involved in cognition, emotion, sleep-wake cycle and attention (Michelsen *et al.*, 2007; Andrade *et al.*, 2013; Pollak Dorocic *et al.*, 2014; Beliveau *et al.*, 2015).

In the DR, of rodents, the non-serotonergic neurons have similar electrophysiological properties to their neighbouring serotonergic neurons, in contrast to the MnR where the different neuronal populations have distinct characteristics (Beck *et al.*, 2004). Beck *et al*. (2004) identified that neurons in the MnR have a longer interspike interval, slower firing rate, than neurons in the DR; however, DR neurons were more susceptible to hyperpolarisation by inhibitory 5-HT_{1A} autoreceptors than MnR serotonergic neurons, with non-serotonergic neurons in the MnR not expressing 5-HT_{1A}. DR neurons express $5HT_{1A}$ auto-receptors on their cell bodies and dendrites (Bjorvatn *et al.*, 1997) which can inhibit further release of serotonin by DR neurons, through feedback inhibition of the raphe nuclei, possibly adding a further layer of control to the raphe's role in the modulation of cortical activity (McDevitt and Neumaier, 2011), with cortical regions known to feed back to the raphe (Beliveau *et al.*, 2015). Alterations in the expression of $5HT_{1A}$ auto-receptors could suggest dysfunction of the serotonergic projections from the DR, with a reduction to feedback inhibition. Although both raphe nuclei are considered to be predominantly serotonergic, there are other neurotransmitters, including dopamine and GABA used within the nuclei (Beck *et al.*, 2004; Michelsen *et al.*, 2007). Within the DR, serotonergic neurons have also been discovered to co-release neuropeptides including neuropeptide Y (Pau *et al.*, 1998) and substance P (Baker *et al.*, 1990; Baker *et al.*, 1991). The differences found in the electrophysiological and neurochemical properties of the raphe neurons could indicate that they play differing roles within the management of arousal and consciousness through the ARAS, with changes to a specific subset eliciting a different phenotype than dysfunction to another.

1.7.3 Locus coeruleus

The LC is the largest noradrenergic nucleus in the brain (Samuels and Szabadi, 2008a). Located in the upper pons, close to the fourth ventricle, the LC contains up to 19,000 neurons on each side of the brainstem, and these contain the lipoprotein pigment neuromelanin (Vijayashankar and Brody, 1979; Mann, 1983). Noradrenergic neurons from the LC are known to project to virtually all brain regions, including the basal forebrain, hippocampus, layer IV of the neocortex, thalamus and hypothalamic regions, as well as other nuclei in the ARAS (Levitt and Moore, 1978; Jones and Yang, 1985; Jones, 2005b; Samuels and Szabadi, 2008a; Samuels and Szabadi, 2008b), apart from the basal ganglia where projections are limited to the SN and the nucleus accumbens (De Cicco *et al.*, 2017), with projections thought to be denser in the right hemisphere than the left (Marrocco *et al.*, 1994). The regions that the LC sends noradrenergic projections to can be classified upon the subtype of noradrenergic receptor they express. α_1-adrenoceptors activation is thought to excite the post-synaptic cell, with the same believed to be true for β-adrenoreceptor expressing post-synaptic cells (Berridge and Waterhouse, 2003; Jones, 2004). However, neurons expressing α_2-adrenoceptors are thought to be inhibited following their activation (Jones, 2004).

The role of the LC has been extensively studied in regard to the promotion of wakefulness and arousal through both excitatory and inhibitory projections (Samuels and Szabadi, 2008a). Increases in LC activity are known to result in increases to cortical EEG activity indicative of an increase in arousal and attention, with stimulation to the noradrenergic neurons in the LC increasing awareness and lesions increasing slow-wave sleep and REM (Delagrange *et al.*, 1993). This suggests that dysfunction to the LC in a disease state could lead to alterations in wakefulness. Damage to the LC has been noted in the causation of comas in humans, consistent with the nucleus promoting wakefulness (Samuels and Szabadi, 2008b). The importance of the LC in the maintenance of wakefulness has been exemplified by research from Carter *et al*. (2012) whom found that orexinergic neurons acted through LC neurons in order to manifest waking. Through its projections and differential expression of receptor subtypes the LC selectively excites other systems that are also involved in waking whilst inhibiting those that are involved in sleep (Jones, 2005a; McKenna *et al.*, 2017), further supporting the role of the LC in the maintenance of

wakefulness. The LC further influences waking through regulation the neural activity of subsets of PPN cholinergic neurons, by exciting those involved in wakefulness or inhibiting those involved in REM through α_1 and α_2-adrenoceptor signalling respectively (Cespuglio *et al.*, 1982; Kaitin *et al.*, 1986; Delagrange *et al.*, 1993; Hou *et al.*, 2002; Samuels and Szabadi, 2008b).

The role of the LC in arousal have been examined in both humans and animals for a number of decades. The LC has been shown to be more active during wakefulness (Berridge and Foote, 1991), with GABA release into the nucleus at its lowest during waking, and increasing in slow-wave sleep and REM (Mahaffey and Garcia-Rill, 2015). Within the LC a local population of GABAergic neurons are able to modulate noradrenergic activity, this is thought to occur through electrical coupling, allowing neurons to fire in synchrony eliciting a coordinated output to regions receiving LC input (Christie *et al.*, 1989; Aston-Jones *et al.*, 2004). Afferent projections onto the local GABAergic interneurons from cortical areas including the PFC have been shown, in mice, to alter LC noradrenergic activity leading to alterations in global arousal levels (Breton-Provencher and Sur, 2019). Human studies have also shown similar results which pharmacological depletion of global noradrenaline reducing abilities in attentional tasks (Robbins and Everitt, 1995). Together this data from mice and humans support the role of the LC in maintain global arousal levels through its connectivity with various brain regions.

The LC receives input from all of the major ARAS nuclei to help maintain arousal and wakefulness (Mahaffey and Garcia-Rill, 2015); including excitatory cholinergic input from the PPN (Egan and North, 1985; Egan and North, 1986) and serotonergic input from the DR (Pickel *et al.*, 1977), as well as local noradrenergic feedback (Egan *et al.*, 1983). The noradrenergic neurons of the LC also receive inputs from the orexinergic neurons of the lateral hypothalamus, with this connection having been shown to be essential in order for the orexin system to be able to manifest waking (Garcia-Rill, 2015). The connectivity and experiments linking activity in the LC to increased arousal highlight the role the nucleus plays in mediating arousal/awareness; suggesting that changes to the LC, the inputs it receives from other ARAS nuclei or its projections to cortical and sub cortical regions could suggest a mechanism for clinically manifested changes to arousal.

1.7.4 Pedunculopontine nucleus

The PPN is a neurochemically heterogeneous nucleus, known to utilise cholinergic, glutamatergic and GABAergic neurotransmitters (Wang and Morales, 2009; Benarroch, 2013). Located in the caudal pontomesencephalic tegmentum of the midbrain and upper pons (Jenkinson *et al.*, 2009), with the most rostral end lying just below the red nucleus, dorsal to the SN, continuing until the LC at its most caudal part. The PPN is thought to contain 10,000 to 20,000 cholinergic neurons, with a proportion of these cholinergic neurons having been shown to co-express other neurotransmitters (Garcia-Rill *et al.*, 1996). Within the PPN the distribution of the neuronal subtypes varies along its axis. The PPN pars compacta, located in the caudal half of the nucleus, is comprised of 30% cholinergic, 50% glutamatergic and 20% GABAergic neurons; whilst the pars dissipata, is comprised of 20% cholinergic, 40% glutamatergic and, 40% GABAergic neurons (Mena-Segovia *et al.*, 2009; Wang and Morales, 2009). The subtype of neuronal output from the PPN also varies along its axis, with projections from the rostral PPN mainly GABAergic, where as those in the caudal PPN are mainly glutamatergic, although some projections are both cholinergic and glutamatergic (Martinez-Gonzalez *et al.*, 2011; Benarroch, 2013). This non-uniform neuronal subtype distribution coincides with the location of sites, which, when stimulated induce fast EEG rhythms indicative of arousal (Moruzzi and Magoun, 1949; Steriade, 1980). Dysfunction to all or a part of these neuronal subtypes could lead to a change in output from the PPN due to a lack of output or inhibition within the nucleus to coordinate neuronal firing.

Research has found that the PPN plays a role in number of different brain systems (Semba and Fibiger, 1992; Benarroch, 2013; Gut and Winn, 2016). The systems that the PPN plays a part in include, arousal and behavioural state control, through its many reciprocal connection to the thalamus and cortex (Benarroch, 2013; Garcia-Rill and Simon, 2015; Mahaffey and Garcia-Rill, 2015); as well as, locomotion and muscle tone suggesting it may contribute to previously reported, dopamine non-responsive, gait disturbances in PD (Hamani *et al.*, 2016). The PPN has been thought of as the main driver and critical cell group for waking and REM sleep in the ARAS (Garcia-Rill and Simon, 2015), this suggests that dysfunction to this nucleus in a disease state could severely affect not only locomotion but states of arousal. Dysfunction to the PPN has been reported in a number of clinical conditions. Pathologies of wakefulness, such as narcolepsy, have been shown to involve

dysfunction of the PPN either through direct alteration of the PPN neurons or indirectly via the inputs into the PPN (Yates and Garcia-Rill, 2015). Downregulation of PPN output either directly or indirectly would result in excessive daytime sleepiness through a reduction in stimulation to the cortex (Yates and Garcia-Rill, 2015).

The PPN is a highly interconnected structure with efferent projections, both cholinergic and noncholinergic, from the PPN projecting to the intralaminar and associative nuclei of the thalamus, SN, lateral hypothalamus and the globus pallidus internus (Shute and Lewis, 1967; Benarroch, 2013; Garcia-Rill and Simon, 2015). Similarly, to the raphe, the two regions of the PPN, the rostral and caudal ends, have been shown to not only possess different level of neuronal subtypes, but also project to different regions. In animal studies the rostral PPN has been shown to innervate the globus pallidus and SN; with the caudal aspect innervating the thalamus, LC and suprachiasmatic nucleus (Martinez-Gonzalez *et al.*, 2011) and thought to be the region predominantly involved in arousal. To aid the PPN in modulating arousal it also has reciprocal connections with all of the major neurotransmitter systems involved in the ARAS; receiving orexin, histaminergic, serotonergic, noradrenergic and cholinergic afferent inputs (Benarroch, 2013; Beck and Garcia-Rill, 2015; Yates and Garcia-Rill, 2015).

The PPN has been shown to be most active during waking and REM sleep, contributing to control of arousal through its ascending projections to the thalamus as well as its descending projections controlling behavioural responses to match the changes in cortical activation (Yates and Garcia-Rill, 2015). The PPN has been identified to control behavioural responses including changes to locomotion and postural control (Garcia-Rill and Skinner, 1988; Garcia-Rill, 1991; Beck and Garcia-Rill, 2015), and atonia during REM sleep (Chase and Morales, 2005). The role of subsets of PPN cholinergic neurons in the maintenance of wakefulness and REM is supported by studies in humans that have identified that when AChEIs were administered during waking it stimulated cortical activity, however if administered during sleep it promotes REM sleep (Jones, 2005b). Cholinergic efferent fibres innervate the PPN (Velazquez-Moctezuma *et al.*, 1989), projecting from the contralateral PPN and the laterodorsal tegmental nucleus (Semba and Fibiger, 1992). The modulation of the PPN through cholinergic input is thought to be mediated via both muscarinic and nicotinic receptors located on the dendrites or soma to modulate action potential discharge or on presynaptic axon terminals to regulate neurotransmitter release (Mahaffey and

Garcia-Rill, 2015). PPN cholinergic cells express muscarinic receptors whereas non-cholinergic cells typically express nicotinic cholinergic receptors (Lanca *et al.*, 2000). Alterations to receptor populations on PPN neuronal sub-populations could suggest dysfunction to the ARAS as a system, with inputs received from other major nuclei in the system to regulate the PPNs output to generate arousal. Changes to receptor populations could possibly influence PPN firing and control of arousal even in the absence of pathology or macroscopic changes to the nucleus.

In summary, the ARAS is a diffuse collection of neurotransmitter systems that has been implicated in controlling global consciousness and arousal levels. Studies examining pathologies of wakefulness, including coma and narcolepsy, have identified that the alterations to arousal and consciousness evolve from a lack of drive from the ARAS to activate the cortex to produce the high frequency activity required for wakefulness and consciousness. Furthermore, investigations into the main components of the neurotransmitter systems have identified how damage or dysfunction to brainstem nuclei or their transmitter systems could lead to alterations in global consciousness and arousal. The alterations and dysfunction observed in the ARAS in those with disorders of consciousness and from a number of animal models support the idea that alterations due to neurodegenerative mechanisms in DLB could lead to the alterations in consciousness and arousal, cognitive fluctuations, observed clinically.

1.8 The brainstem in neurodegenerative dementias

1.8.1 Brainstem pathology

The brainstem, divided into three parts, the medulla, pons and midbrain, consists of a number of nuclei and neurotransmitter systems and is thought to be the integration centre that links peripheral inputs from the spinal cord with cortical regions (Grinberg *et al.*, 2011). The brainstem's role as an integration centre allows it to play an important part in the regulation of several autonomic, behavioural and cognitive functions, a number of which have been shown to be dysfunctional in dementia disorders (Lee *et al.*, 2015).

Pathological lesions can affect the brainstem at an early stage of disease progression, such as α-synuclein and tau (Braak *et al.*, 2003; Grinberg *et al.*, 2009; Braak *et al.*, 2011). For Braak Lewy body stages, α-synuclein pathology is proposed to progress through the brainstem in a rostral manner. With α-synuclein pathology affecting the medulla in Braak Stage 1 progressing to the midbrain in Stage 3 and then into the cortex. Unlike for Lewy bodies, the original tau staging criteria did not account for brainstem pathology, instead focusing on that within the cortical regions. However, a number of studies had identified pathology within brainstem regions, including the LC and DR prior to pathology in the transenthorinal cortex, Braak Stage I (Grinberg *et al.*, 2009; Weinshenker, 2018). The identification of NFTs earlier than previously thought in AD, lead to an amendment to the original criteria and the addition of Stage 0 (a-b) with LC-specific tau pathology (Braak *et al.*, 2011; Weinshenker, 2018). Unlike tau and α-synuclein, the progression of Aβ within AD cases, as described in the Thal phases, is not observed in the brainstem until phase 4 (Thal *et al.*, 2002).

The LC, as previously discussed in 1.7.3, is highly involved in a number of cognitive and arousal functions which become altered in neurodegenerative dementias, leading to a number of studies investigating how pathological changes within the nucleus relate to these functions clinically (Samuels and Szabadi, 2008b; De Cicco *et al.*, 2017; Kelly *et al.*, 2017). Neuronal density has been observed to decrease within the LC with normal ageing (Vijayashankar and Brody, 1979; Tomlinson *et al.*, 1981; Mann, 1983; Chan-Palay and Asan, 1989b; Manaye *et al.*, 1995); with the neuronal loss occurring uniformly throughout the nucleus (Samuels and Szabadi, 2008b). The reduction in LC neuronal density is thought to be related to some of the memory impairments that can be seen in normal ageing (Marien *et*

al., 2004). This age-related decrease in neuronal density can be exacerbated by neurodegenerative disease such as AD, PD and DLB, this having been observed in post-mortem investigations (Samuels and Szabadi, 2008b; Brunnstrom *et al.*, 2011). Although tau pathology is detectable in the LC prior to cortical regions, studies have not been able to find a relationship between LC neuronal loss and NFT pathology (Weinshenker, 2018), with one study only finding significant neuronal loss in the LC at Braak stage III (Theofilas *et al.*, 2017). This lack of relationship between AD-type NFT pathology and LC neuronal loss has also been observed in DLB with LC neuronal loss shown to be independent of the levels of concomitant AD-type pathology (Brunnstrom *et al.*, 2011). Along with loss of neuronal density, alterations to the remaining LC neuronal morphology, synapses and dendrites have been reported (Baloyannis *et al.*, 2006).

Pathological lesions in a number of neurodegenerative diseases has also been observed in the DR and MnR (Lee *et al.*, 2015; Seidel *et al.*, 2015). As with the LC, tau pathology in AD is observed in the DR prior cortical involvement, with neuritic pathology negatively correlated with the loss of serotonergic cells within the region (Hendricksen *et al.*, 2004), with similar results having been found in the MnR (Halliday *et al.*, 1992). As the raphe serotonergic system is implicated in depression neurodegenerative changes to the nuclei in dementia disorders have been proposed to lead to depressive symptoms. Within AD, pathology and neuronal loss in the raphe was also found not to be different between those with and without depression (Hendricksen *et al.*, 2004). Within LBD, the raphe is affected by α-synuclein at Stage 2, the same timepoint as the LC (Braak *et al.*, 2003), with detectable alterations to the neurotransmitters thought to be present at this stage (Boeve, 2013). Degeneration of both DR and MnR neurons have been reported in DLB, although this loss was not related to any clinical features, including depression (Benarroch *et al.*, 2007), cell loss has also been observed in PD (Paulus and Jellinger, 1991).

The PPN, similar to the LC and DR, is vulnerable to Lewy body pathology at an early stage of disease progression (Braak *et al.*, 2003); however, in AD tau pathology in the PPN is thought to occur in the mid stages compared to the early observation of NFTs in the LC and DR (Dugger *et al.*, 2011). The PPN has been extensively investigated with regard to its role in PD and Parkinsonian symptoms, with degeneration in the nucleus observed in both disorders (Rinne *et al.*, 2008b; Jenkinson *et al.*, 2009). Neuronal loss within the PPN in PD has been

shown in post-mortem analysis to be correlated with the degree of dopaminergic loss within the patient (Di Giovanni *et al.*, 2019), with the neuronal loss found to affect cholinergic cells in the regions more than their non-cholinergic neighbours.

1.8.2 Neuroimaging studies

The brainstem and its projections to cortical and subcortical regions have been examined through various imaging modalities, with some, including the DaTSCAN looking at the dopaminergic SN projections to the striatum, having been implemented in clinical practice. Current imaging based studies undertaken so far have mainly been PET/SPECT ligand based studies, with the levels of transporters assumed to reflect the strength of connectivity and health of the neurotransmitter system (Stahl, 2000). A limited number of structural studies have been undertaken in the brainstem in neurodegenerative diseases. Structural imaging studies that have been published having conflicting results, likely due to the small size of the brainstem nuclei (Serra *et al.*, 2018), their ill-defined borders and no standard way to delineate them across studies (Ji *et al.*, 2020). With the development of stronger and higher resolution scanners, and standardised delineation software, it would be possible to more accurately assess smaller brain regions including brainstem nuclei and regions it projects to, including the hypothalamus, in both structural and ligand-based studies (Borgers *et al.*, 2013).

A limited number of structural imaging studies have looked at the brainstem in dementia; however, these have often reported inconsistent results. One study, which investigated the brainstem in early AD observed structural loss bilaterally within the pons and left part of the midbrain compared to controls (Ji *et al.*, 2020), whereas a similar study identified no asymmetry within the midbrain in AD (Lee *et al.*, 2015) and another study identified no structural changes in the pons in AD compared to controls (Mrzilkova *et al.*, 2012). Structural imaging studies have also been used to evaluate the cholinergic system in dementia. A structural MRI study found sub-region specific atrophy in the basal forebrain in DLB which was comparable to the atrophy observed in AD, with this atrophy in AD being associated with MMSE and in DLB associated with visuoperceptual function (Grothe *et al.*, 2014). A recent EEG study further identified an impairment to the alpha band reactivity, a potential marker for cholinergic system integrity, in PDD and DLB patients which was related

to reduced cholinergic drive from the nucleus basalis of Meynert in the PDD cases (Schumacher *et al.*, 2020).

The cholinergic system within neurodegenerative diseases, and its possible role in clinical symptoms, has been widely examined using imaging techniques, including PET/SPECT, with a particular focus on AD and DLB. The main targets for cholinergic imaging are acetylcholinesterase (AChE) and the vesicular acetylcholine transporter (vAChT) with a number of ligands available for each. There are two major cholinergic projections systems within the brain, one originating in the basal forebrain, which provides the main cortical cholinergic innervations and the other projecting from the PPN to the thalamus and other brainstem nuclei. Reductions in cortical AChE, reflecting basal forebrain projections, have been shown in AD and DLB compared to controls (Bohnen *et al.*, 2003), with cortical cholinergic losses increasing with disease progression in AD (Bohnen *et al.*, 2016). DLB have also been shown to have greater reductions in AChE activity than AD in a number of cortical regions, with the largest difference reported in the posterior cingulate gyrus (Shimada *et al.*, 2015). There have been studies examining the PPN-thalamic cholinergic pathway within neurodegenerative diseases, which have identified through AChE PET that a reduction was found in LBDs, but not AD at a mild disease stage (Kotagal *et al.*, 2012b). Alterations to the cholinergic system in neurodegenerative diseases have also been studied in regard to their influence upon clinical symptoms. A small number of studies have investigated the role of the cholinergic system in sleep disorders in AD (Pavese, 2014), with one AChE SPECT study found differences in perfusion between AD patients with and without sleep loss in Brodmann area (BA) 9 (Ismail *et al.*, 2009). Short latency afferent inhibition studies, a technique allowing for *in vivo* assessment of cholinergic systems, have found alterations were associated with hallucinations in DLB and disinhibition in AD patients (Marra *et al.*, 2012).

1.8.3 ARAS projections

The ARAS consists of brainstem nuclei, as well as its projections to regions within the thalamus and hypothalamus. Dysfunction to the ARAS and its projections has been reported in neurodegenerative diseases such as PD, AD and Huntington's disease (Luster *et al.*, 2015). Dysfunction to the ARAS has already been reported to underly some of the delirium symptoms observed post stoke (Boukrina and Barrett, 2017). With direct evidence of the

ARAS role in delirium through a study utilising functional MRI that identified acute reversible disruption between the thalamus and the nucleus basalis, two key integration centres of the ARAS (Middleton and Strick, 2000; Choi *et al.*, 2018).

The thalamus is the main relay centre for the dorsal pathway of the ARAS. The thalamus consists of up to 60 sub-nuclei and has been postulated to be involved in a vast array of networks and functions (Fama and Sullivan, 2015). With the its role in an array of networks, a number of studies have investigated its role within neurodegenerative disease, where these networks are dysfunctional (Pievani *et al.*, 2011). The PPN has been observed in a number of animal models to innervate a number of thalamic nuclei, including the mediodorsal nucleus, intralaminar nuclei and limbic regions (Martinez-Gonzalez *et al.*, 2011). Within the thalamus, studies have shown that certain sub-nuclei are more afflicted with pathology than others. In AD the mediodorsal nucleus is vulnerable to AD-type pathology and neuronal loss (Braak and Braak, 1991a; Paskavitz *et al.*, 1995). However, the lateral geniculate nucleus, which also receives inputs from the PPN has been shown to have widespread Aβ pathology but minimal tau deposition in AD (Dugger *et al.*, 2011). In PD, Lewy body pathology is most frequently observed in limbic regions of the thalamus, however, this does not seem to be associated with neuronal loss (Xuereb *et al.*, 1991; Rub *et al.*, 2002). In DLB, the intralaminar nuclei, which are a major site of inputs from the PPN, are severely affected by Lewy body pathology (Brooks and Halliday, 2009). However, neuronal loss is not observed in all these thalamic nuclei, suggesting that neuronal loss may not be solely mediated by Lewy body pathology.

The ventral pathway of the ARAS projects from the raphe and LC through the main relay, the hypothalamus, into the cortex. The hypothalamus, like the thalamus, consists of a collection of nuclei each with distinct functions (Vercruysse *et al.*, 2018). Dysfunction to the hypothalamus and the neurotransmitters within the regions have been implicated in sleep disturbances, including daytime somnolence and cognitive fluctuations are observed in dementia disorders. Within AD, atrophy of the hypothalamus has been observed using neuroimaging techniques, with changes thought to occur early in disease progression as well as being a site for AD pathology (Vercruysse *et al.*, 2018). Lewy body pathology has been observed within the hypothalamus in PD, with predominantly the posterolateral nuclei and tuberomammillary nucleus affected (Langston and Forno, 1978). Within PD it was noted

that the tuberoinfundibular nucleus of the hypothalamus, which contains a large concentrations of dopaminergic neurons was relatively spared from Lewy body pathology (Langston and Forno, 1978). Hypothalamic pathological lesions occur regularly in DLB, although with varying severity between patients (Londos *et al.*, 2019). However, the pathology seen in DLB does not seem to appear in orexinergic neurons, in the lateral hypothalamus, although a significant decrease in neuronal number has been observed (Kasanuki *et al.*, 2014). The orexin system has been implicated to be involved in AD and PD with it thought to have a role in excessive daytime sleepiness that is observed clinically (Lim and Szymusiak, 2015), with a reduction in orexin neurons seen in AD (Vercruysse *et al.*, 2018). Models of AD have also identified that sleep disturbances can also perpetuate the deposition of pathology (Kang *et al.*, 2009), suggesting dysfunction to hypothalamic systems could exacerbate neurodegenerative processes.

1.9 The ascending reticular activating system in Lewy body diseases

ARAS dysfunction is traditionally discussed in regard to coma and pathologies of wakefulness. However, within LBD, patients are frequently shown to manifest clinical phenotypes, including sleep (Jankovic, 2008) and mood (Boeve, 2013) disorders, that are thought to arise from dysfunction within ARAS and its components (Luster *et al.*, 2015), as well as pathology known to affect the core three nuclei, as well as their relay nuclei at early stages of the disease course (McKeith *et al.*, 2017). Alterations to specific neurotransmitter systems including the hypothalamic ARAS orexinergic and histaminergic systems have also been identified in a number of post-mortem studies (Kovalzon and Zavalko, 2013; Lim and Szymusiak, 2015). Dysfunction of the ARAS in LBDs, especially PD, is further supported by treatments such as Levodopa having been successfully utilised to treat those with alterations to consciousness related to ARAS dysfunction (Ciurleo *et al.*, 2013).

1.9.1 Locus coeruleus

Lewy bodies and Lewy neurites are known to accumulate in the LC early in LBD disease progression, even before the SN in PD (Vermeiren and De Deyn, 2017), with the presence of Lewy body pathology in the LC being definitive for the classification of Braak stage 2. Loss of LC neurons has been reported in LBD with that seen in DLB reported to be irrespective of the levels of concomitant AD pathology (Brunnstrom *et al.*, 2011), indicating, that this degeneration may be driven by α-synuclein pathology rather than concomitant AD-type pathology. The observed loss of noradrenergic neurons leads to noradrenergic deafferentation of cortical and subcortical regions, with eventual loss of projections from the LC, with the deafferentation hypothesised to play a role in cognitive decline observed in these disorders (Vermeiren and De Deyn, 2017). The hypothesised role of the LC and noradrenergic system in LBDs has been supported by several studies investigating the effects of noradrenergic drugs on cognition in PD. One study in PD identified a significant improvement in MMSE and excessive daytime sleepiness with atomoxetine, a selective noradrenaline reuptake inhibitor (Weintraub *et al.*, 2010). The finding of a reduction in excessive daytime sleepiness, suggestive of a restoration of arousal maintenance during wakefulness, through treatments that increase noradrenergic activity at the synapse, further implicates the LC and its role in the ARAS in underlying this clinical symptom, and other disorders of the sleep-wakefulness system including cognitive fluctuations and RBD in

LBDs (Schenck *et al.*, 1996; Boeve *et al.*, 2013). The finding that improvements to sleep disturbance in PD with noradrenergic treatments also suggests that although neuronal loss is known to occur there are still noradrenergic neurons present that can be targeted.

Although the LC has been extensively studied in various dementia subtypes and has been shown to be highly affected by pathology, it has not been evaluated in relation to the presence of cognitive fluctuations. Furthermore, there has not been a systematic study evaluating changes to the arousal system as a whole, the understanding of which could help understand the phenotypic consequences of such degeneration in DLB.

1.9.2 PPN

The PPN has been extensively examined in PD, in relation to a number of different clinical phenotypes, with less of a focus on the nucleus in DLB. Pathologically the PPN is affected by Lewy body pathology at Braak stage 3 (Braak *et al.*, 2003; Grinberg *et al.*, 2011), with the different cell types in the region more vulnerable to the accumulation of pathological lesions than others (Chambers *et al.*, 2019). Further neurodegenerative changes including accumulation of mitochondrial DNA deletions were observed to more preferentially affect PPN cholinergic neurons than there non-cholinergic neighbours (Bury *et al.*, 2017). Loss of cholinergic PPN neurons is observed in PD, with studies reporting a 50% reduction (Pahapill and Lozano, 2000), as well as a reduction in neuronal size compared with controls, (Rinne *et al.*, 2008b; Jenkinson *et al.*, 2009). Loss of cholinergic neurons in the PPN leads to a subsequent loss of cholinergic innervation to the thalamus (Francis and Perry, 2007).

PD patients experience a number of clinical symptoms, including sleep disorders, akinesia and gait impairments, that have been proposed to be due to PPN dysfunction (Jankovic, 2008). The collection of clinical features that patients can present with are suggestive of an overactive PPN in PD, as the PPN is known to have a key role in initiation of REM and for waking (Luster *et al.*, 2015). This has been supported through investigation into the P50 potential, a proxy measure of sensory gating, arousal level, ARAS functioning and PPN activity, in PD patients, where changes to this potential in both habituation and amplitude suggest overactivation of the PPN (Teo *et al.*, 1997; Hyde and Garcia-Rill, 2015). With similar clinical features known to be present in DLB, including sleep disturbances this could suggest a dysfunctional PPN within DLB.

PPN dysfunction has been reported to play a role in disorders of the sleep-wakefulness system, including RBD (Yates and Garcia-Rill, 2015). PD patients with RBD have been shown to have decreased cholinergic innervation to the cortex and thalamus compared to those without (Chambers *et al.*, 2019). Clonazepam, a benzodiazepine that enhances GABA receptor responses, has been shown to beneficial at reducing RBD symptoms (Schenck *et al.*, 1993; Olson *et al.*, 2000). Other non-benzodiazepine drugs that target GABA receptors, such as zopiclone and melatonin, have also be identified as effective in relieving the symptoms of RBD (Anderson and Shneerson, 2009; Taylor *et al.*, 2020). The efficacy of drugs that target GABA receptors in treating RBD, suggests that RBD is a disorder of REM sleep atonia disinhibition (Yates and Garcia-Rill, 2015). Although the specific mechanism for clonazepam is unknown, it could be that a lack or dysfunction of GABA-mediated inhibition to the PPN neurons that modulate REM sleep atonia could be restored or enhanced by administration of the drug (Anderson and Shneerson, 2009). RBD is a common feature in synucleinopathies, and can manifest decades prior to other clinical symptoms, perhaps suggesting changes to the PPN occur early in the disease course (Aurora *et al.*, 2010; Postuma *et al.*, 2019).

Direct dysfunction of the PPN has also been noted in PD, with cholinergic neuronal loss associated with gait-predominant parkinsonian phenotype (Benarroch, 2013), and severity of cholinergic loss within the PPN correlated with the severity of parkinsonian symptoms in PD (Zweig *et al.*, 1989; Rinne *et al.*, 2008b). Further support for the role of the PPN in gait disturbances come from a small number of clinical trials which identified a benefit of AChEI treatment on gait, balance or falls (Morris *et al.*, 2019). With the PPN having been discovered to play a role in the generation of parkinsonian symptoms in PD, a number of studies have investigated whether the nucleus would be a candidate for DBS (Hamani *et al.*, 2016). PPN DBS has been shown to not only improve gait disturbances, of which some are refractory to dopaminergic treatments (Ferraye *et al.*, 2010; Moro *et al.*, 2010), but also improve sleep disturbances (Stefani *et al.*, 2007) and executive and higher functions (Alessandro *et al.*, 2010; Tykocki *et al.*, 2011; Stefani *et al.*, 2013). Research suggesting that PPN DBS can improve cognitive function, could suggest that there are salvageable neuronal projections that could be targeted in a less invasive manner to help improve the cognitive dysfunction in PD and possibly other Lewy body type diseases.

Although the PPN is one of the more well-studied nuclei in the context of arousal and motor control, there is a lack of research into the role of the PPN in DLB, with research mainly having focused on cholinergic pathways, including the nucleus basalis of Meynert, and the role of the PPN in PD. With the DBS of the PPN shown to have an impact on cognitive functions in PD, there is a legitimate question as to whether dysfunction of the PPN could underlie the cognitive symptoms seen clinically in DLB.

1.9.3 Raphe

The DR and MnR have been shown to have high levels of Lewy body pathology in LBDs, in PD this burden is comparable to that observed in the SN (Seidel *et al.*, 2015). In PD, cell loss has been shown in the DR (Paulus and Jellinger, 1991) and MnR, with degeneration of MnR neurons thought to correlate with tremor severity (Halliday *et al.*, 1990; Loane *et al.*, 2013). Degeneration of both DR and MnR neurons have been reported in DLB, although this loss was not related to any clinical features, including depression (Benarroch *et al.*, 2007). A small number of studies have attempted to look at the role of the serotonergic system in DLB, one study used acute tryptophan depletion to examine the loss of serotonin function on global cognitive status (Mace *et al.*, 2016). However, the study failed to find a decrease in MMSE, or an effect upon depression, although this study utilised a small sample size.

Within DLB there have been a limited number of pathological studies that have comprehensively investigated both pathological and neuronal changes and the relation of these to clinical features within both the DR and MnR. A larger body of research has focused upon examining the integrity of serotonergic projections through imaging and examination of the regions to which they project in DLB, with the majority of this being related to depression or sleep disorders.

1.9.4 Ascending reticular activating system projections

Within LBD a number of neuroimaging studies, utilising different techniques, have examined the projections from nuclei of the ARAS and their possible role in clinical features. However, these studies have tended to focus on the cholinergic and serotonergic neurotransmitter systems separately and have not examined functioning of the ARAS as a whole.

Cholinergic projections have been widely evaluated in LBDs and have been proposed to be involved in a variety of clinical features, including alterations to cognition, sleep disorders (Kotagal *et al.*, 2012b) and visual hallucinations (Janzen *et al.*, 2012). Denervation of

cholinergic inputs from the PPN to the thalamus, as measured through PET, has been associated with an increased fall risk in PD (Bohnen *et al.*, 2009; Di Giovanni *et al.*, 2019). Reduction in brain AChE activity, measured through PET imaging, was also associated with the presence of RBD in PD patients, with the study further identifying that monoaminergic dysfunction was similar across patients with and without RBD (Kotagal *et al.*, 2012a). The role of cholinergic alterations in RBD has been further supported by imaging studies that have identified reduction in AChE binding in a number of cortical regions in those with idiopathic RBD, in this study no significant reduction in pontothalamic binding was observed, suggesting that this pathway may be affected later in disease progression (Gersel Stokholm *et al.*, 2019). A further structural imaging study identified an association between grey matter reductions in the PPN and thalamus with visual hallucinations in PD and PDD (Janzen *et al.*, 2012). In DLB, a multimodal MRI study identified alterations to thalamic cholinergic levels which associated with severity and frequency of cognitive fluctuations (Delli Pizzi *et al.*, 2015).

Alterations to white matter tracts, investigated via diffusion tensor imaging, have identified reductions to the fractional anisotropy, suggestive of diffusion along the axons becoming less directionally orientated in DLB (Mak *et al.*, 2014). Watson *et al*. (2012) identified reductions in fractional anisotropy in the pons and left thalamus in DLB when compared to AD, supporting the hypothesis for alterations in the pontothalamic cholinergic system in DLB. Alterations to other cholinergic pathways have also been identified in DLB. A recent study using a vAChT SPECT radiotracer evaluated the integrity of a number of cholinergic systems in DLB, including the pontothalamic and the innominatocortical pathways (Mazère *et al.*, 2017). The study identified reductions in the non-displaceable binding potential for the vAChT ligand in the terminal regions of the innominatocortical pathway, the anterior cingulate cortex, and the inferior and superior parietal cortices, and of the pontothalamic pathway, the thalamus; with the data suggestive of cholinergic neuronal loss in both pathways (Mazère *et al.*, 2017). However, Mazère *et al*. (2017) identified a relative preservation of another cholinergic pathway the septohippocampal, suggesting that not all cholinergic pathways are altered in DLB. Atrophy within the substantia innominata has also been observed in DLB, with the level of atrophy being negatively correlated with CAF scores

(Colloby *et al.*, 2017), providing further evidence for cholinergic involvement in cognitive fluctuations.

Along with cholinergic imaging studies, a limited number have aimed to examine the integrity of monoaminergic systems, including the serotonergic and noradrenergic systems. General monoaminergic and serotonergic PET studies have identified alterations to binding levels in the LC and raphe associated with sleep-disorders, including excessive daytime sleepiness and RBD (Pavese, 2014). Recently serotonergic integrity has been examined, utilising the DaTSCAN radioligand, which can also be utilised to image serotonin in vivo (Joling *et al.*, 2018). A limited number of studies have so far been conducted evaluating the serotonergic system in LBDs, with the conflicting results as to whether extrastriatal serotonin reuptake transporter (SERT) levels are altered in DLB compared to PD (Roselli *et al.*, 2010; Joling *et al.*, 2018). Further examination of the serotonergic system using imaging should be undertaken to try and understand the alterations that may occur in vivo in DLB and how they relate to clinical features. Unlike cholinergic imaging studies there have been less of a focus on the monoaminergic system in relation to cognitive alterations in LBD.

The possible dysfunction of the ARAS, and its constituent brain regions, in LBD has been investigated, yet a limited number of focus on cognitive fluctuations. Presently, there are no studies that have investigated the role of pathological lesions in regard to cognitive fluctuations in brainstem nuclei. However, although there are a limited number of pathological studies relating the ARAS or its constituent components to cognitive fluctuations, a number of imaging studies have identified alterations to thalamic cholinergic levels and connectivity changes that have been related to fluctuating cognition. Furthermore, a number of studies have identified associations with clinical features that could have similar pathological underpinnings to cognitive fluctuations, for example RBD. Alterations to the ARAS have been proposed to play a role in the underlying pathophysiology of RBD (Boeve *et al.*, 2007), with a universal alteration to the sleep-wake, arousal, systems hypothesised to underlie both RBD and fluctuating cognition (Terzaghi *et al.*, 2013; Antelmi *et al.*, 2016). Together these studies suggest that ARAS dysfunction, possibly leading to a system that is not efficient enough to maintain adequate levels of global arousal, is present in LBD and therefore warrants investigation into how this dysfunction could lead to cognitive fluctuations.

1.10 Summary and overview

Cognitive fluctuations are one of the core features of DLB (McKeith *et al.*, 2017) and cause significant distress to caregivers (Zweig and Galvin, 2014). Despite being a core feature, the underlying mechanisms that elicit cognitive fluctuations have not yet been identified. However, the mechanisms underlying fluctuating cognition have been hypothesised to be disturbances in attentional or arousal systems, either individually or in combination (Matar *et al.*, 2019; O'Dowd *et al.*, 2019). The proposed theory of arousal system dysfunction has centred around the ARAS, consisting of a diffuse network of neurotransmitter systems. There is evidence from studies investigating traumatic brain injuries that alterations to the ARAS, the LC, PPN, raphe and its projections, can elicit alterations in cognition and consciousness. Despite conceptual links between the clinical phenotype of DLB and ARAS dysfunction (Matar *et al.*, 2019), no study has yet evaluated the ARAS in entirety in this disorder. In DLB a number of studies have identified alterations to the components of the ARAS, with a subset relating these to the presence of cognitive fluctuations; including imaging studies have identified alterations to the cholinergic pathways of the ARAS that were associated with cognitive fluctuations (Delli Pizzi *et al.*, 2015; Colloby *et al.*, 2017). Pathological changes are known to affect the nuclei of the ARAS in the early stages of DLB. However, there is limited understanding about how these changes could alter the functioning of the ARAS in controlling arousal and awareness. The present study aimed to assess pathological changes within the three main ARAS nuclei, LC, PPN and raphe. The integrity of the projections to a cortical target of the system, the medial PFC (mPFC), was also additionally assessed.

It is important that the ARAS is investigated wholly due to the large number of overlapping pathways present allowing for a degree of redundancy within the system (Kovalzon, 2016a). However, a full comprehensive examination of the entire ARAS is not feasible due to a number of practical limitations, not limited to tissue availability and examination of the wide array of receptors expressed. The overall aim of the study was to evaluate whether pathological changes to the ARAS could contribute to cognitive fluctuations in DLB.

1.11 Research aims

The present study aims to investigate pathological changes within the different nuclei of the ARAS, and the integrity of their connections to a target region, within DLB. Alterations could provide an understanding into arousal deficits related to cognitive fluctuations in DLB.

The hypothesis of this study is that some, or all, of the assessed ARAS regions, possess a distinct pattern of neurodegenerative pathology and/or alterations to neuronal populations in DLB and mixed AD/DLB with cognitive fluctuations compared to AD, mixed AD/DLB without cognitive fluctuations and control cases.

This study aims to:

- To relate the burden of pathological lesions, α-synuclein, tau and Aβ, in the LC, using quantitative histological techniques, to the presence and severity of cognitive fluctuations (Chapter 1)
- To investigate the burden of α-synuclein, tau and Aβ within the PPN using quantitative histological techniques in relation to the presence of cognitive fluctuations and the parkinsonian subtype (Chapter 2)
- To determine the burden and neuronal location of pathological accumulations, in the raphe, using quantitative histological and immunofluorescent techniques. In addition to investigating the association to the presence of cognitive fluctuations (Chapter 3)
- To assess the neuronal location of pathological burden and innervation to the mPFC using quantitative biochemical techniques. (Chapter 4)

Chapter 2: Materials and Methods

2.1 Introduction

This chapter describes the histological, immunohistochemical and biochemical techniques that were performed on fixed and frozen human *post-mortem* brains. For fixed tissue it will outline the dissection methods and storage of the tissue, histological and immunohistochemical methods to stain tissues, and the assess pathological and neuronal changes. For frozen tissue, it will outline the acquisition of frozen tissue specimens, synaptosomal fractionation and the proteomic assay methods utilised to quantify protein expression in tissue.

2.2 Study Cohort

Cases were selected for inclusion in the present study on the basis that they fulfilled the clinical and neuropathological criteria for DLB (McKeith *et al.*, 2005), AD (Montine *et al.*, 2012), PDD (Emre *et al.*, 2007) or both AD and LBD, with the latter group being classified as mixed AD/DLB (figure 2.1). In most cases, DLB cases were included on the basis of a clinical history of cognitive fluctuations. Mixed AD/DLB cases without the clinical presentation of fluctuating cognition, fulfilling the pathological criteria for both AD and DLB, were included to provide a neocortical/limbic pathologically LBD (nLBD) control group without cognitive fluctuations. Within the nLBD group without cognitive fluctuations were a number of DLB cases which had no history of cognitive fluctuations. An AD group was included in the study, as a group with well-defined cognitive decline and neurodegenerative pathology, but without the presence of cognitive fluctuations to account for the role of AD-type pathology within the Mixed AD/DLB group. A second mixed AD/DLB, nLBD, group with clinical presence of cognitive fluctuations was included to assess whether any changes related to cognitive fluctuations in DLB were reflected in another neurodegenerative disease group. Control cases were included on the basis of an absence of neurological disorders during life and an absence of low neuropathological change on *post-mortem* analysis. Study characteristics are shown in table 2.1.

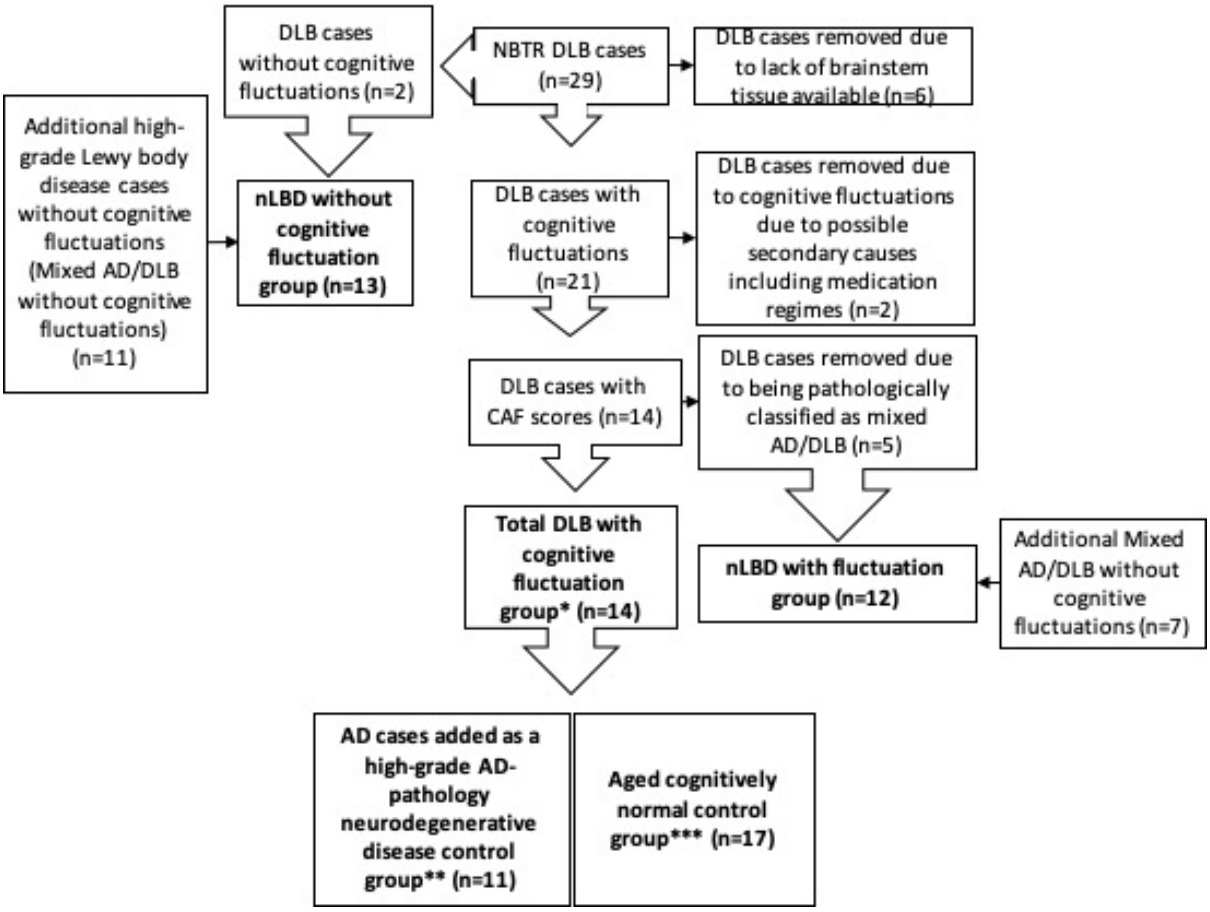


Figure 2.1. Study cohort identification and selection flow chart. * due to only 9 'pure' DLB cases possessing CAF scores an additional 6 cases which possessed well characterised cognitive fluctuations although were not assessed via CAF were included in order to increase the n for the DLB with cognitive fluctuations group. **/*** the main aim of the study was to examine the role of the ARAS in cognitive fluctuations; therefore, the main priority was selection of DLB cases which had the best characterised fluctuations. Both the AD and control cases were matched as best possible to the DLB cases for age and MMSE (in the case of AD cases), a further AD case was removed due to identification of a brainstem infarct upon examination of the pons.

Table 2.2. PhD study cohort characteristics.
Cohort characteristics for the PhD study. Abbreviations: AD-Alzheimer's disease; CERAD- Consortium to Establish a Registry for Alzheimer's disease; CFs- cognitive fluctuations; DLB- dementia with Lewy bodies; nLBD-neocortical/limbic pathologically Lewy body disease; NFT- neurofibrillary tangles; PM- post-mortem; SEM- standard error of the mean; w/o-without.

Case Number	Fluctuation group	Pathological diagnosis	Age	Sex	Disease Duration (years)	PM delay (hours)	Fixation length (weeks)	Cognitive Fluctuations	Braak NFT	CERAD	McKeith	Chapters used
1	DLB with CFs	DLB	75	Male	7	18	3	Present	II	None	Limbic	3,5,6
2	DLB with CFs	DLB	77	Male	2.5	29	4	Present	II	Moderate	Neocortical	3,4,5,6
3	DLB with CFs	DLB	76	Male	8	13	41	Present	II	Sparse	Neocortical	3,4,5,6
4	DLB with CFs	DLB	74	Male	8	42	8	Present	IV	Sparse	Neocortical	3,4,5,6
5	DLB with CFs	DLB	71	Male	7	8	10	Present	II	Sparse	Neocortical	3,4,5,6
6	DLB with CFs	DLB	72	Female	8	89	14	Present	III	None	Neocortical	3,4,5
7	DLB with CFs	DLB	77	Male	11	46	12	Present	III	None	Neocortical	3,4,5,6
8	DLB with CFs	DLB	73	Female	6	99	9	Present	III	None	Neocortical	3,5,6
9	DLB with CFs	DLB	81	Male	3	81	8	Present	III	Moderate	Neocortical	3,4,5
10	DLB with CFs	DLB	78	Male	6	8	12	Present	III	Moderate	Neocortical	3,5,6
11	DLB with CFs	DLB	81	Male	4	24	45	Present	IV	Moderate	Neocortical	3,4,5,6
12	DLB with CFs	DLB	73	Male	11	47	6	Present	III	None	Neocortical	3,4,5,6
13	DLB with CFs	DLB	74	Male	5	60	8	Present	II	None	Neocortical	3,4,5
14	DLB with CFs	DLB	84	Male	5	72	8	Present	II	None	Neocortical	3,4,5
Mean			**76.1**	**12M:2F**	**6.5**	**45.4**	**13.4**					
SEM			**1.0**		**0.7**	**7.9**	**3.3**					
15	nLBD w/o CFs	Mixed AD/DLB	79	Female	10	46	12	None	VI	Frequent	Limbic	3,4,5,6
16	nLBD w/o CFs	Mixed AD/DLB	82	Male	7	12	3	None	VI	Frequent	Neocortical	3,4,5,6
17	nLBD w/o CFs	Mixed AD/DLB	63	Female	14	38	16	None	VI	Frequent	Neocortical	3,4,5,6
18	nLBD w/o CFs	Mixed AD/DLB	78	Male	1	17	8	None	VI	Frequent	Neocortical	3,4,5,6
19	nLBD w/o CFs	Mixed AD/DLB	77	Male	10	78	8	None	VI	Frequent	Neocortical	3,5,6
20	nLBD w/o CFs	DLB	91	Female	4	10	7	None	II	Moderate	Neocortical	3,4,5
21	nLBD w/o CFs	Mixed AD/DLB	62	Male	-	28	14	None	VI	Frequent	Neocortical	3,4,5,6
22	nLBD w/o CFs	Mixed AD/DLB	73	Male	4	7	12	None	VI	Frequent	Neocortical	3,5,6
23	nLBD w/o CFs	Mixed AD/DLB	77	Female	5	51	12	None	VI	Frequent	Neocortical	3,4,5
24	nLBD w/o CFs	Mixed AD/DLB	80	Female	14	23	8	None	VI	Frequent	Limbic	3,4,5,6
25	nLBD w/o CFs	Mixed AD/DLB	101	Female	13	28	6	None	VI	Moderate	Limbic	3,4,5,6
26	nLBD w/o CFs	Mixed AD/DLB	84	Female	10	26	8	None	VI	Frequent	Neocortical	3,4,5,6

Case Number	Fluctuation group	Pathological diagnosis	Age	Sex	Disease Duration (years)	PM delay (hours)	Fixation length (weeks)	Cognitive Fluctuations	Braak NFT	CERAD	McKeith	Chapters used
27	nLBD w/o CFs	DLB	92	Male	9	91	5	None	III	Sparse	Neocortical	3,4,5
Mean			**79.9**	**6M:7F**	**8.4**	**35.0**	**9.2**					
SEM			**2.9**		**1.1**	**6.9**	**1.0**					
28	AD w/o CFs	AD	93	Female	13	53	10	None	VI	Frequent	Negative	3,4,5
29	AD w/o CFs	AD	86	Female	6	47	14	None	VI	Frequent	Amygdala Only	3,5,6
30	AD w/o CFs	AD	90	Female	7	90	10	None	VI	Frequent	Negative	3,4,5
31	AD w/o CFs	AD	89	Male	3	61	12	None	VI	Frequent	Negative	3,4,5,6
32	AD w/o CFs	AD	68	Male	-	24	15	None	VI	Frequent	Negative	3,4,5,6
33	AD w/o CFs	AD	76	Female	14	37	12	None	VI	Frequent	Negative	3,4,5,6
34	AD w/o CFs	AD	91	Male	6	72	14	None	VI	Frequent	Negative	3,4,5,6
35	AD w/o CFs	AD	80	Male	7	39	8	None	VI	Frequent	Negative	3,5,6
36	AD w/o CFs	AD	82	Male	10	12	8	None	VI	Frequent	Amygdala Only	3,4,5,6
37	AD w/o CFs	AD	95	Female	2	77	7	None	VI	Frequent	Negative	3,4,5,6
38	AD w/o CFs	AD	84	Female	12	79	8	None	VI	Frequent	Negative	3,4,5,6
Mean			**84.9**	**5M:6F**	**8.0**	**53.7**	**10.7**					
SEM			**2.3**		**1.2**	**7.1**	**0.8**					
39	nLBD with CFs	Mixed AD/DLB	78	Female	5	120	7	Present	V	Frequent	Neocortical	3,4,5
40	nLBD with CFs	Mixed AD/DLB	75	Female	3	64	5	Present	VI	Frequent	Neocortical	3,4,5,6
41	nLBD with CFs	Mixed AD/DLB	68	Male	8	11	2	Present	V	Frequent	Neocortical	3,4,5,6
42	nLBD with CFs	Mixed AD/DLB	91	Female	7	84	2	Present	V	Frequent	Limbic	3,4,5,6
43	nLBD with CFs	Mixed AD/DLB	75	Male	15	28	18	Present	V	Frequent	Neocortical	3,4,5,6
44	nLBD with CFs	Mixed AD/DLB	75	Female	5	78	7	Present	VI	Frequent	Neocortical	3,4,5,6
45	nLBD with CFs	Mixed AD/DLB	80	Female	8	17	6	Present	V	Frequent	Neocortical	3,5,6
46	nLBD with CFs	Mixed AD/DLB	83	Male	15	-	6	Present	VI	Frequent	Neocortical	3,4,5
47	nLBD with CFs	Mixed AD/DLB	78	Male	6	18	10	Present	VI	Frequent	Limbic	3,4,5,6
48	nLBD with CFs	Mixed AD/DLB	91	Female	15	84	12	Present	VI	Frequent	Neocortical	3,4,5,6
49	nLBD with CFs	Mixed AD/DLB	62	Male	-	46	12	Present	VI	Frequent	Neocortical	3,4,5,6
50	nLBD with CFs	Mixed AD/DLB	73	Male	5	39	10	Present	VI	Frequent	Neocortical	3,4,5,6
Mean			**78.1**	**6M:6F**	**8.4**	**53.5**	**8.1**					
SEM			**2.0**		**1.3**	**7.9**	**1.3**					

Case Number	Fluctuation group	Pathological diagnosis	Age	Sex	Disease Duration (years)	PM delay (hours)	Fixation length (weeks)	Cognitive Fluctuations	Braak NFT	CERAD	McKeith	Chapters used
51	Control	Control	69	Female	NA	16	6	None	I	None	Negative	3,5,6
52	Control	Control	68	Male	NA	54	8	None	0	None	Negative	3,5,6
53	Control	Control	72	Male	NA	17	4	None	I	Sparse	Negative	3,5,6
54	Control	Control	103	Female	NA	21	4	None	II	Sparse	Negative	3,4,5,6
55	Control	Control	58	Female	NA	39	5	None	0	None	Negative	3,4,5
56	Control	Control	78	Female	NA	23	6	None	II	Sparse	Negative	3,4,5
57	Control	Control	72	Female	NA	27	9	None	I	Sparse	Negative	3,4,5,6
58	Control	Control	74	Female	NA	45	4	None	II	Frequent	Negative	3,4,5,6
59	Control	Control	85	Female	NA	95	8	None	II	None	Negative	3,5
60	Control	Control	99	Male	NA	111	30	None	II	None	Negative	3,4,5,6
61	Control	Control	70	Male	NA	72	10	None	0	None	Brainstem	3,5
62	Control	Control	98	Female	NA	59	8	None	III	None	Negative	3,5
63	Control	Control	73	Male	NA	25	11	None	II	None	Negative	3,4,5,6
64	Control	Control	97	Female	NA	21	10	None	II	Moderate	Negative	3,4,5,6
65	Control	Control	85	Male	NA	57	7	None	II	Sparse	Negative	3,5,6
66	Control	Control	101	Female	NA	104	8	None	IV	Sparse	Negative	3,4,5,6
67	Control	Control	94	Male	NA	39	10	None	II	Sparse	Brainstem	3,4,5,6
Mean			**82.1**	**8M:10F**		**48.5**	**8.7**					
SEM			**3.3**			**7.3**	**1.4**					

2.3 Clinical Assessment

Cases underwent neuropsychological testing *intra vitam.* MMSE was gathered as a measure of cognitive function, if more than one MMSE measurement was available then rate of cognitive decline was determined as previously described (Olichney *et al.*, 1998). CAF scores were collected in order to quantify the degree of severity of the cognitive fluctuations experienced (Walker *et al.*, 2000b). If multiple CAF scores were collected, last, average and maximal values were calculated. Cognitive fluctuations are a transient, that appear to vary in severity throughout the disease course. Utilising the last, average and maximal CAF values should produce a clearer picture on the severity of a transient clinical phenomenon. Especially as CAF scores rate the severity of the fluctuations in the preceding month, meaning analysis with only one score could omit critical information. This is supported by 4 cases which were identified as having cognitive fluctuations during life had a last CAF score of 0, suggestive of no fluctuations.

2.4 Tissue Preparation

2.4.1 Tissue preparation at the Newcastle Brain Tissue Resource

Human *post-mortem* brain tissue was obtained from the Newcastle Brain Tissue Resource (NBTR), ethical approval was granted by the Newcastle and North Tyneside Research Ethics Committee (ref: 08/H0906/136).

At *post-mortem*, brains were removed and time interval from death noted. The left hemisphere, brainstem and cerebellum was sliced into approximately 1cm slices and snap frozen at -120^{o}C between copper plates. The right hemisphere, brainstem and cerebellum was fixed in 10% neutral-buffered formalin for 6 weeks, however in the cohort there are cases which vary from the routine protocol with fixation lengths ranging 3-45 weeks. After fixation the tissue is dissected into 7mm coronal blocks. From the coronal slices a pre-defined number of sections were sub-dissected into blocks used for diagnosis and research. All fixed brain tissue then underwent processing, to dehydrate and clear the tissue, before being embedded into paraffin wax.

Cases were neuropathologically assessed using standardised diagnostic guidelines (Braak and Braak, 1991b; Mirra *et al.*, 1991; Thal *et al.*, 2002; Braak *et al.*, 2003; McKeith *et al.*, 2017), by a neuropathologist, with neuropathology data combined with clinical data acquired during the patient's life in order to give a definitive clinico-pathological diagnosis.

2.4.2 Locus coeruleus

The LC is a small nucleus, located 1mm below the fourth ventricle and 3mm from the midline, extending from the level of the widest portion of the fourth ventricle, at its most caudal, to the inferior colliculus, at its most rostral (German *et al.*, 1988; Fernandes *et al.*, 2012). The LC is easily identifiable due to the presence of neuromelanin within the noradrenergic neurons (figure 2.2). Sections for analysis were taken from the upper mid pons, Block P on the NBTR dissection protocol (figure 2.5).

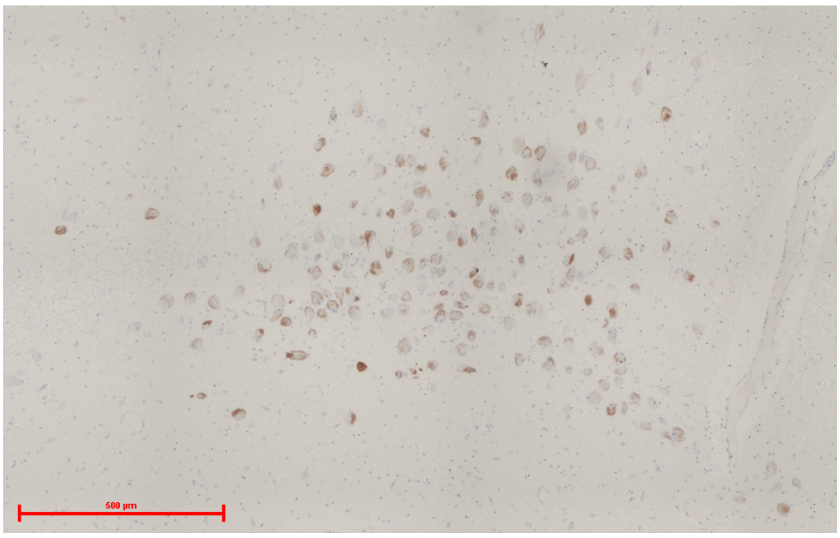


Figure 2.2. Location and identification of the locus coeruleus.
Pigmented neurons of the locus coeruleus, at the level of the upper-mid pons, stained with haematoxylin. Image shown is of a control case. Scale bas = 500µm.

2.4.3 Raphe nucleus

The raphe nucleus is a large heterogenous collection of serotonergic neurons, within this study both the DR and MnR were examined. The DR extends from the mid pons, caudally, to its rostral end at the level of the oculomotor nucleus, with the MnR extending from the level of the trigeminal motor nucleus to the decussation of the superior cerebellar peduncle at its rostral limit (Hornung, 2003). Sections for analysis were taken from the upper mid pons, Block P on the NBTR dissection protocol (figure 2.5). The nuclei were visualised utilising TPH2 immunohistochemistry (IHC) (figure 2.3).

Figure 2.4. Location and identification of the raphe.
Tryptophan hydroxylase 2 immunohistochemistry to identify the serotonergic neurons of the raphe within the upper-mid pons. Image shown is of a control case. Scale bar = 1000µm, inset scale bar 100µm. IV- IV ventricle

2.4.4 Pedunculopontine nucleus

The PPN is a large nucleus located in the upper pons and midbrain. It is commonly accepted that the PPN extends from its caudal limit at the level of the retrorubral field to its rostral end at the level of the SN pars reticulata (Hamani *et al.*, 2016). Sections for analysis were taken from the lower midbrain, Block O on the NBTR dissection protocol (figure 2.5). The nucleus was visualised utilising ChAT IHC (figure 2.4).

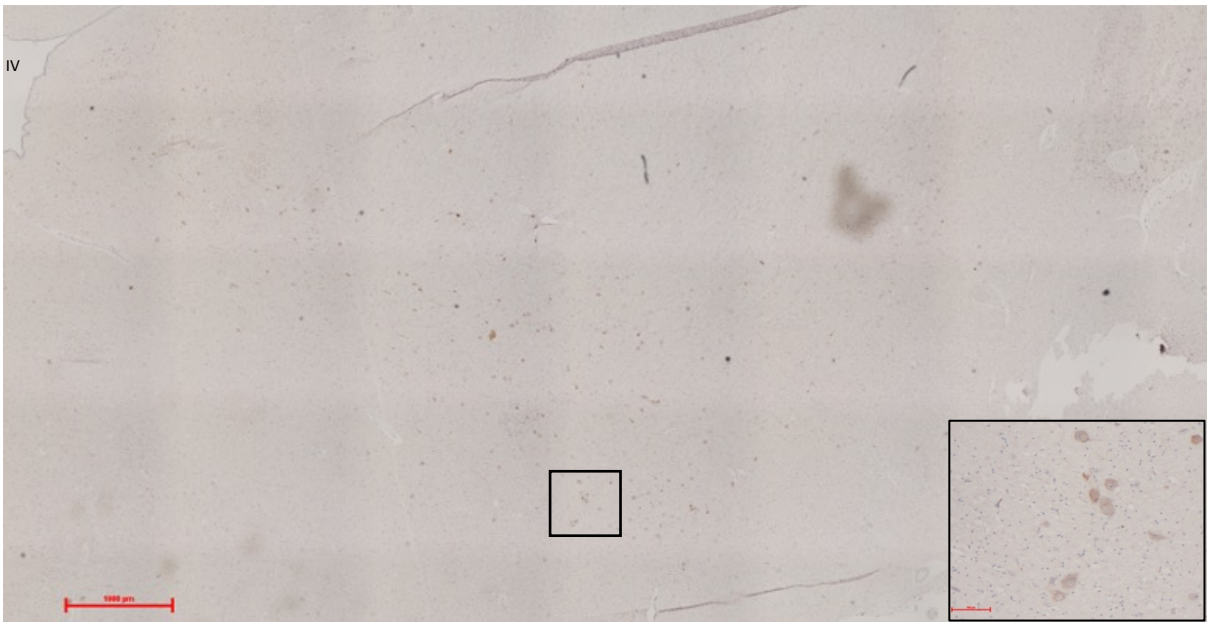


Figure 2.5. Location and identification of the pedunculopontine nucleus.
Choline acetyltransferase immunohistochemistry to identify the cholinergic neurons of the pedunculopontine nucleus within the lower midbrain. Image shown is of a control case. Scale bar = 1000µm, inset scale bar 100µm. IV-IV ventricle

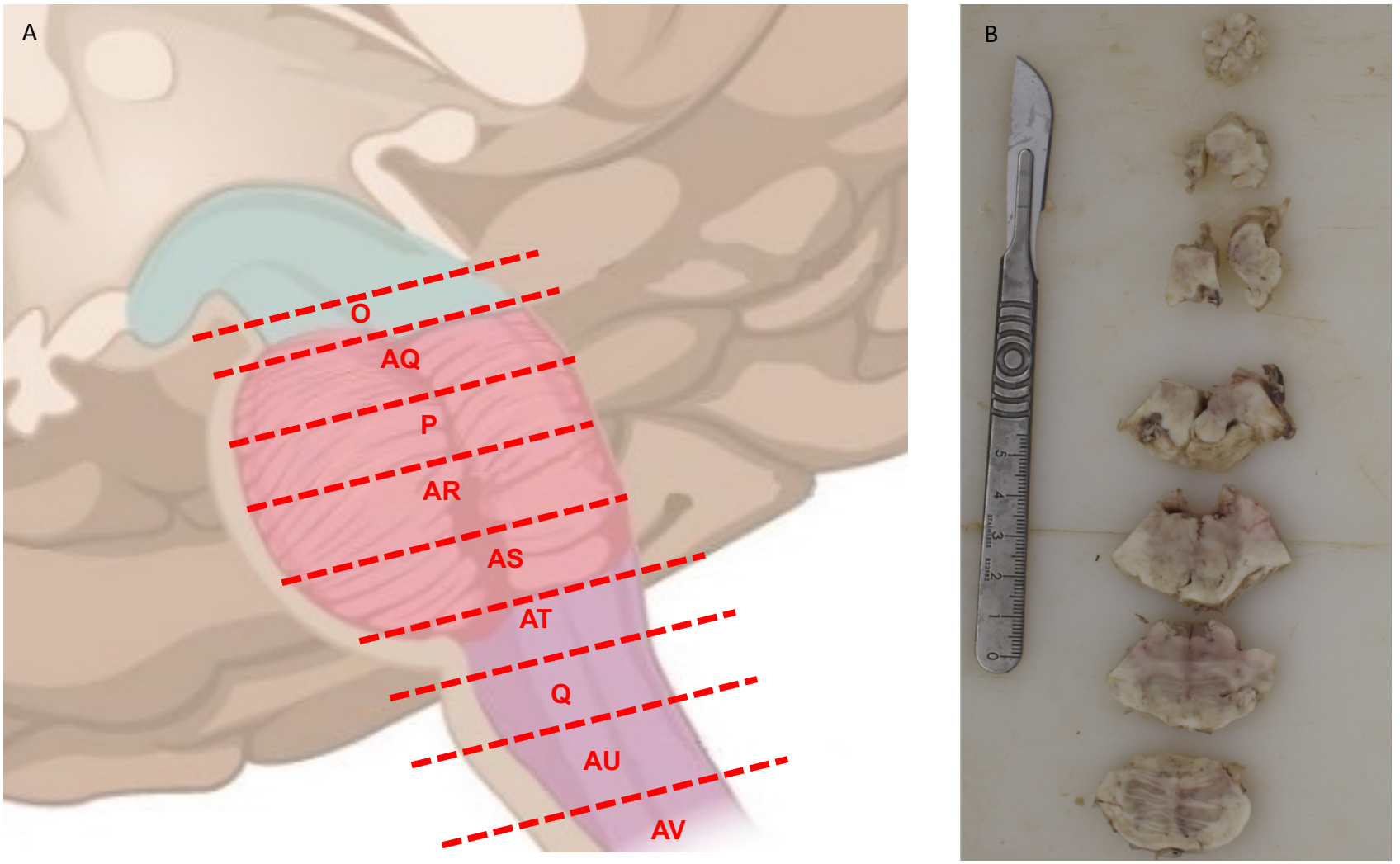


Figure 2.6. Newcastle Brain Tissue Resource brainstem dissection protocol.
(A) Medulla, lilac, is divided into 4 blocks AV to AT; Pons, pink, is divided into 4 blocks AS to AQ; midbrain, green, is divided into 2 blocks, only the lower midbrain block is shown. Blocks are cut with the ventral side as the cut face, apart from Block O which is cut from the dorsal side. (B) Formalin fixed brainstem tissue from the medulla and pons blocks AU-AQ from the top to the bottom. Blocks P, AQ and O were sampled for analysis within this study.

2.4.5 Tissue sectioning

Blocks containing the regions of interest were sectioned using a rotary microtome (ThermoFisher Scientific HM355S). 7µm sections for histological analysis were mounted onto charged glass slides (Superfrost Plus, Thermo Scientific, UK). Mounted sections were then dried at 37°C for 48 hours. Once dried sections were stored at room temperature prior to staining. Tissue sectioning was completed by NBTR technicians.

2.5 Immunohistochemistry

IHC was undertaken on 7µm formalin-fixed tissue section. Samples for each region underwent IHC in as few batches as possible to minimise any variability within the staining. IHC was conducted to visualise pathological proteins and to locate specific neuronal populations.

Paraffin-embedded sections containing the region of interest were placed in a 60°C oven for 45 minutes, to aid dewaxing and adhesion of the tissue to the slides, prior to immersion in two changes of xylene (10 minutes each). Sections were then rehydrated through a series of ethanol concentrations (99%, 99%, 95%, 70% for 5 minutes each) through to water. Following tissue rehydration, sections underwent antigen retrieval to reveal epitopes that had been cross-linked due to the fixation process. The technique for antigen retrieval varied dependent upon the antibody utilised. Citrate buffer antigen retrieval was performed by pre-heating 0.1M trisodium (Sigma-Aldrich, Dorset, UK), pH 6.0, in an 800-watt microwave (SLS, Hessle, UK) until 'bubbling', before immersing slides into the buffer and heating on full power for 10 minutes. Slides were then cooled in the buffer solution at room temperature for 20 minutes, before being rinsed in water. Formic acid antigen retrieval was performed by immersing the tissue slides in room temperature 99% formic acid (Sigma-Aldrich, Dorset, UK) for antibody specific duration, before slides being rinsed in water. Ethylenediamine tetra-acetic acid (EDTA) antigen retrieval was performed by heating 1mM EDTA buffer (Sigma-Aldrich, Dorset, UK), pH 8.0 in a pressure cooker until boiling, before immersing tissue sections in the EDTA buffer for two minutes at high pressure. Following depressurisation, sections were rinsed in water.

Following antibody specific antigen retrieval all slides were quenched for endogenous peroxidase activity incubation in 3% H_2O_2 (Sigma-Aldrich, Dorset, UK) solution in water for 15 minutes. Tissue sections were then thoroughly rinsed in water before rinsing in tris-

buffered saline (TBS) (Fisher Scientific, Waltham, MA, USA), pH7.6, twice for 5 minutes followed by a 1-minute rinse in TBS with Tween20 (VWR, Lutterworth, UK) (TBS-T), pH 7.6. Tissue sections were outlined using an ImmEdge hydrophobic barrier pen (Vector Laboratories, Burlingame, CA, USA). Sections were subsequently incubated with primary antibody, at an antibody specific optimal dilution, suspended in TBS, for one hour at room temperature. Slides were then rinsed twice in TBS (5 minutes each) and once in TBS-T (1 minute) before application of the universal probe, from the Menarini MenaPath kits (Menarini, diagnostics, Berkshire, UK), for 30 minutes at room temperature. Sections were then washed as previously described prior to application of the polymer solution, conjugated to horseradish peroxidase (Menarini MenaPath), for 30 minutes at room temperature. Tissue sections were then washed twice in TBS (5 minutes each). To visualise antibody binding, tissue sections were incubated in 3' diaminobenzidine (DAB) chromogen in DAB substrate buffer (Menarini MenaPath), 1 drop (32µl) per ml, for 3 minutes at room temperature. Sections were then washed thoroughly under running water for 15 minutes.

A haematoxylin counterstain was applied to the tissue sections to identify cellular nuclei. Tissue sections were counterstained by immersion in haematoxylin solution (700mls of distilled water: 0.5g sodium iodate, 50g aluminium potassium sulphate, 5g haematoxylin, 40mls acetic acid and 300mls glycerine) for 30 sections, before rinsing in water. Tissue sections were then differentiated quickly with 1% acid alcohol, before washing in water and then immersed in 1% ammonia water until the nuclei turned blue.

Following counterstaining sections were dehydrated through a series of ethanol concentrations (70%, 95%, x2 99%) before clearing in xylene and being mounted with DPX and coverslipped.

2.6 Histological staining: Luxol Fast Blue/Haematoxylin & Eosin

Luxol Fast Blue (LFB)/ Haematoxylin & Eosin (H&E) histological staining was undertaken on slides from the upper pons and midbrain to help locate the correct brainstem level for the PPN, prior to ChAT IHC. LFB stains myelin/myelinated axons, with haematoxylin staining cell nuclei and eosin staining the extracellular matrix and cytoplasm within formalin fixed *post-mortem* brain tissue. Following dewaxing and rehydration up to 95% alcohol, as outlined in 2.5, sections were placed in preheated, 60°C, LFB for 45 minutes.

Sections were then rinsed in 95% alcohol and then water. LFB was differentiated in a weak lithium carbonate solution, followed by further differentiation in 70% alcohol until the grey and white matter were clearly distinguishable. Sections were counterstained with H&E. Firstly, sections were incubated in haematoxylin for 5 minutes before rinsed in water and differentiated as outline in 2.5. Followed by incubation in eosin for 3 minutes, before being rinsed in water and dehydrated and cleared in xylene and mounted with a coverslip using DPX (CellPath, Newtown, UK).

LFB staining solution was prepared by combining 500ml 95% alcohol with 0.5g Luxol Fast Blue (Fisher Scientific, Waltham, MA, USA) and 2.5ml 10% acetic acid. Haematoxylin was prepared as outlined in 2.5. Eosin was prepared by combing 1000ml 20% alcohol with 10g Eosin Y, 0.25g Phloxine and 0.25g Erythrosin.

2.7 Immunofluorescence

Immunofluorescence was undertaken on 7µm formalin-fixed tissue section containing the DR and MnR. Samples for the DR and MnR were stained in batches to ensure that there was limited variability in time from completion of immunofluorescence to imaging. Immunofluorescence was conducted to visualise the expression levels of neurotransmitter-specific markers and to visualise colocalization of pathological lesions in neuronal subpopulations.

Tissue sections containing the DR and MnR were dewaxed and rehydrated and underwent citrate antigen retrieval, as previously described in 2.5. Sections were rinsed twice in TBS (5 minutes each) then in TBS-T (1 minute). Tissue sections were outlined with an ImmEdge hydrophobic barrier pen, before blocking in 3% normal goats' serum (NGS) in TBS for 1 hour at room temperature. Block was tipped off before incubation in primary antibody serum. Primary mouse and rabbit antibodies were diluted in 3% NGS in TBS, at an antibody specific dilution. Primary antibody serum was incubated for 21 hours at 4°C. Following two rinses in TBS-T (5 minutes each) and one in TBS (2 minutes), tissue sections were incubated in secondary antibody serum in the dark for 1 hour at room temperature. Secondary antibodies, Alexa Fluor® 488 Goat Anti-Mouse IgG (Invitrogen, Waltham, MA, USA), fluoresces green and Alexa Fluor® 594 Goat Anti-Rabbit IgG (Invitrogen, Waltham, MA, USA), fluoresces red, were diluted both 1:200, in 3% NGS in TBS. Sections were then rinsed, in the dark, twice in TBS-T (5 minutes each), once in TBS (5 minutes) before being immersed

in water. Tissue sections were quenched for autofluorescence by incubation with Sudan Black. Sections were dipped in 70% ethanol, to prevent precipitation, before being covered with Sudan Black (Sigma Aldrich, Dorset, UK) (1% Sudan Black in 70% ethanol) and incubated for 10 minutes at room temperature. Sections were then dipped in 70% ethanol and immersed in water prior to being mounted with UltraCruz hardset mounting medium containing DAPI (4',6 diamino-2-phenylindole) (Santa Cruz, Heidelberg, Germany) to visualise all neuronal nuclei, and cover slipped.

Sections were then covered, to block light, and stored at 4°C before being imaged the following day.

2.8 Image analysis

2.8.1 Analysis of immunoreactivity

Analysis assessed the percentage area stained by an antibody within the LC, PPN and DR/MnR. Multiple adjacent single images were captured at x100 magnification using a Nikon 90i microscope, with a fully motorised stage, and DsFi1 camera microscope coupled to a PC. The multiple adjacent images were stitched together, by NIS-Elements AR3.2 software (Nikon, Surrey), to create one large image which covered the whole region of interest. For the LC the large image was formed of 5x5 single images image representing an area of 10.1 mm^2, for the DR and MnR an 8x14 image was taken representing an area of 46.1mm^2 and for the PPN a 10x10 image was taken representing an area of 41.5mm^2.

The brainstem nuclei were delineated prior to calculation of immunoreactivity (figure 2.6). For DR and MR nuclei were delineated on the pathology slides by comparison to a consecutive TPH2 slide; for the PPN the comparison was made with a consecutive ChAT slide. The images were analysed using a standardised red-green-blue threshold on NIS elements AR3.2 software, determined separately for each antibody, to selectively detect only immunopositive signals without detection of non-specific background (figure 2.6). In addition to the red-green-blue thresholds for 4G8 analysis, a size threshold was also applied, based upon empirical observations of size, to ensure intracellular physiological APP which

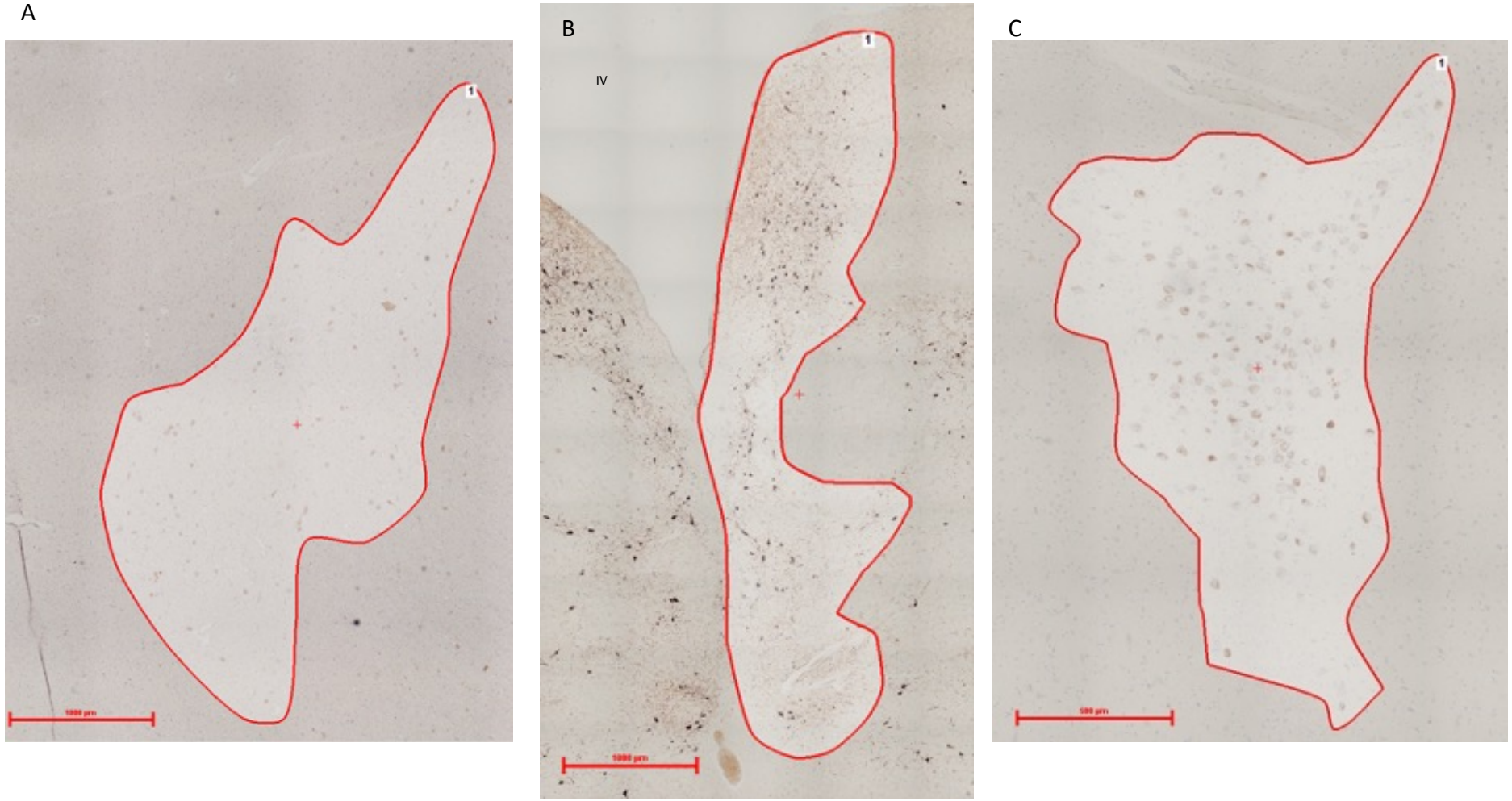


Figure 2.7. Example images of delineation of the brainstem nuclei for image analysis.
(A) pedunculopontine nucleus stained with choline acetyltransferase, scale bar = 1mm; (B) raphe nuclei stained with tryptophan hydroxylase, scale bar = 1mm; (C) locus coeruleus with a haematoxylin stain, scale bar = 500µm. IV- IV ventricle

the 4G8 antibody also detects was not included. Percentage area stained was calculated for all regions analysed, the DR and MnR were combined to give a total percentage area stained for the raphe.

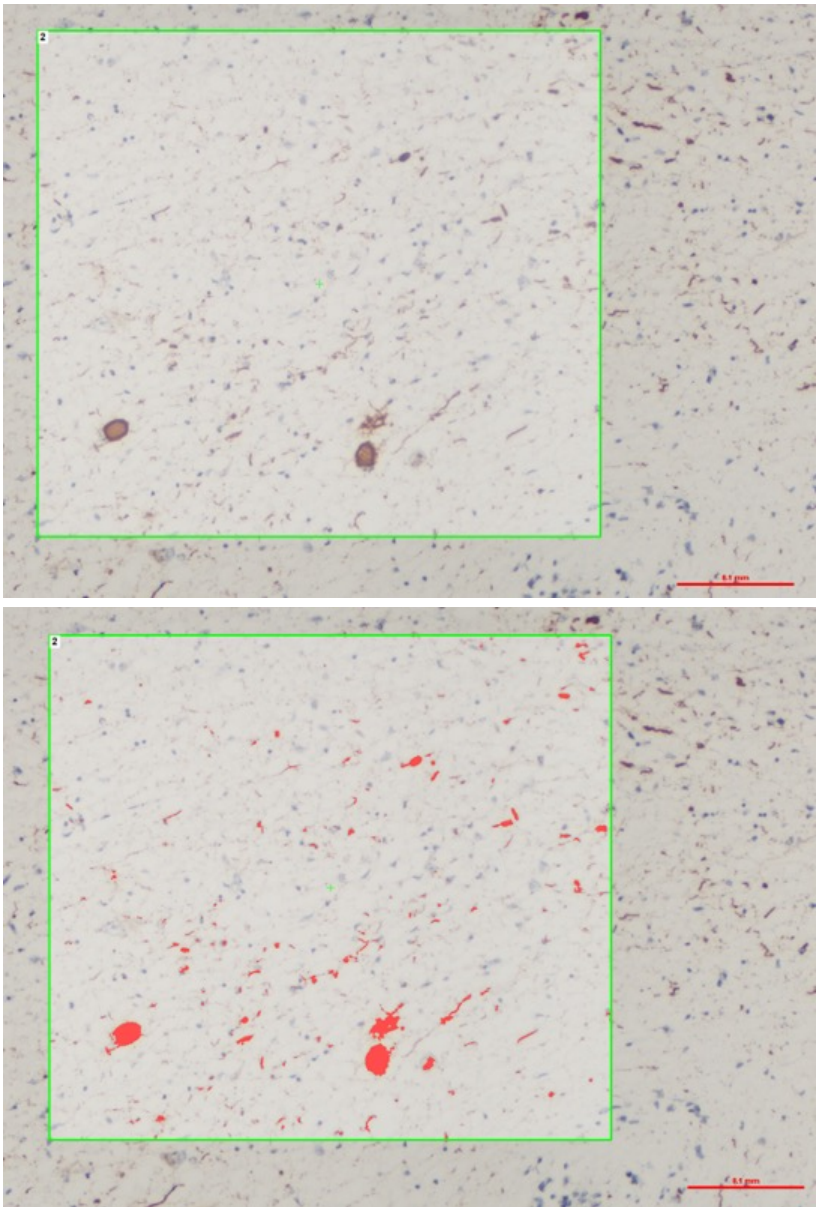


Figure 2.8. Analysis of immunoreactivity.
Images taken at x100 magnification had the nuclei of interest delineated, as shown in figure 2.6. A colour threshold was then applied which detected only the immunoreactivity labelled by the DAB chromogen within the delineated region of interest but does not detect non-specific background staining. Representative image shows a small section of the raphe from an Alzheimer's disease case with AT8 immunohistochemistry, both with and without the red-green-blue colour threshold. Scale bar = 100µm.

2.8.2 Analysis of immunofluorescence

Sections that underwent immunofluorescence staining had multiple single adjacent images at x100 magnification captured using a Nikon 90i microscope, with a fully motorised stage, and DsQi1Mc camera microscope coupled to a PC. Images were taken of DAPI (excitation

358 nm and emission 461nm), FITC (excitation 495 nm and emission 519nm) and TRITC filter (excitation 544 nm and emission 570nm). The multiple adjacent images were then manually stitched together based upon overlapping landmarks, to create a large image of the entire region of interest (figure 2.8).

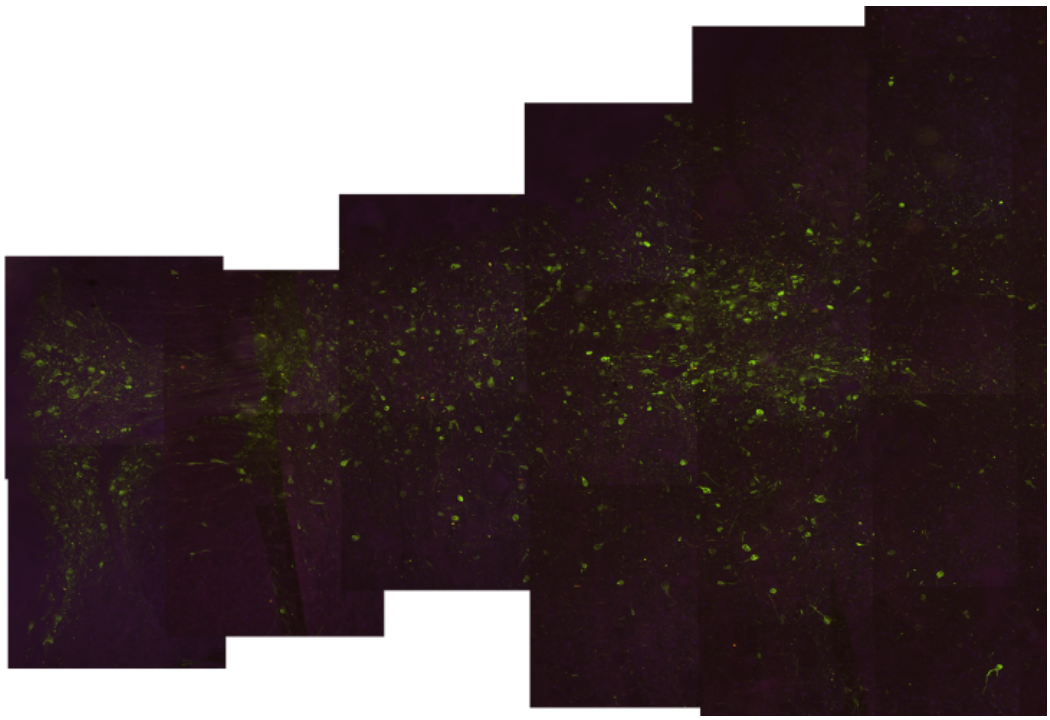


Figure 2.9. Example manually stitched image of immunofluorescent double labelling.
Manually stitched immunofluorescence image for tryptophan hydroxylase 2 and pS129 α-synuclein double labelling, with DAPI mountant. Image shown is of a DLB case**.**

2.8.2.1 Intensity analysis of immunofluorescence

Individual TPH2-positive neuron intensity was assessed within the raphe utilising FIJI software (Schindelin *et al.*, 2012). The intensity was measured on an individual neuron basis following the methods as outlined by Fitzpatrick (2014). Large stitched images of the FITC (green) channel only, were altered to greyscale, in order to make the background pixels have a value close to zero. Individual TPH2-positive neurons were delineated and the area integrated intensity and mean grey value were measured. Background measurements from 30-40 areas within the raphe were taken provide an average background measurement for the region of interest. The corrected TPH2 intensity for each individual TPH2-positive neuron was calculated using the following formula:

$$\frac{\text{integrated density} - (\text{area of selected cell x mean fluorescence of background readings})}{\text{area of selected neuron}}$$

The median corrected TPH2 intensity for all assessed TPH2-positive neurons was calculated for each individual section analysed.

2.8.2.2 Analysis of co-localisation

Manually stitched images, of all three fluorescence channels merged, were assessed for the presence α-synuclein aggregates, Lewy bodies, within TPH2-positive neurons (figure 2.9). Total number of TPH2-positive within the section were counted and the percentage bearing α-synuclein calculated. Through comparison of the merged image to the green only, CTCF adjusted for area was calculated for the TPH2-positive neurons containing Lewy bodies. Lewy body-containing neurons were also delineated, excluding the Lewy body, to calculate the CTCF adjusted for area of the TPH2-positive neuron that did not include the Lewy body.

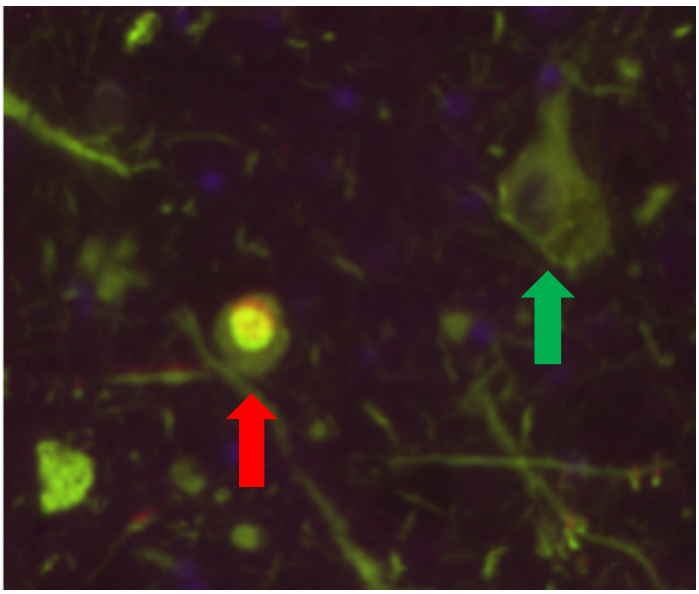


Figure 2.10. Colocalisation between Tryptophan hydroxylase 2 and α-synuclein.
Tryptophan hydroxylase 2 positive neurons containing α-synuclein (red arrow) and with no α-synuclein present (green arrow). Image shown is of a DLB case.

2.9 Biochemical techniques

2.9.1 Acquisition of frozen tissue

Frozen tissue was acquired from 10 DLB with cognitive fluctuations, 9 AD, 10 Mixed AD/DLB without cognitive fluctuations, 10 Mixed AD/DLB with cognitive fluctuations and 10 controls. Tissue was taken from the mPFC, levels 4 and 5 on the NBTR dissection protocol (figure 2.10) corresponding to BA12.

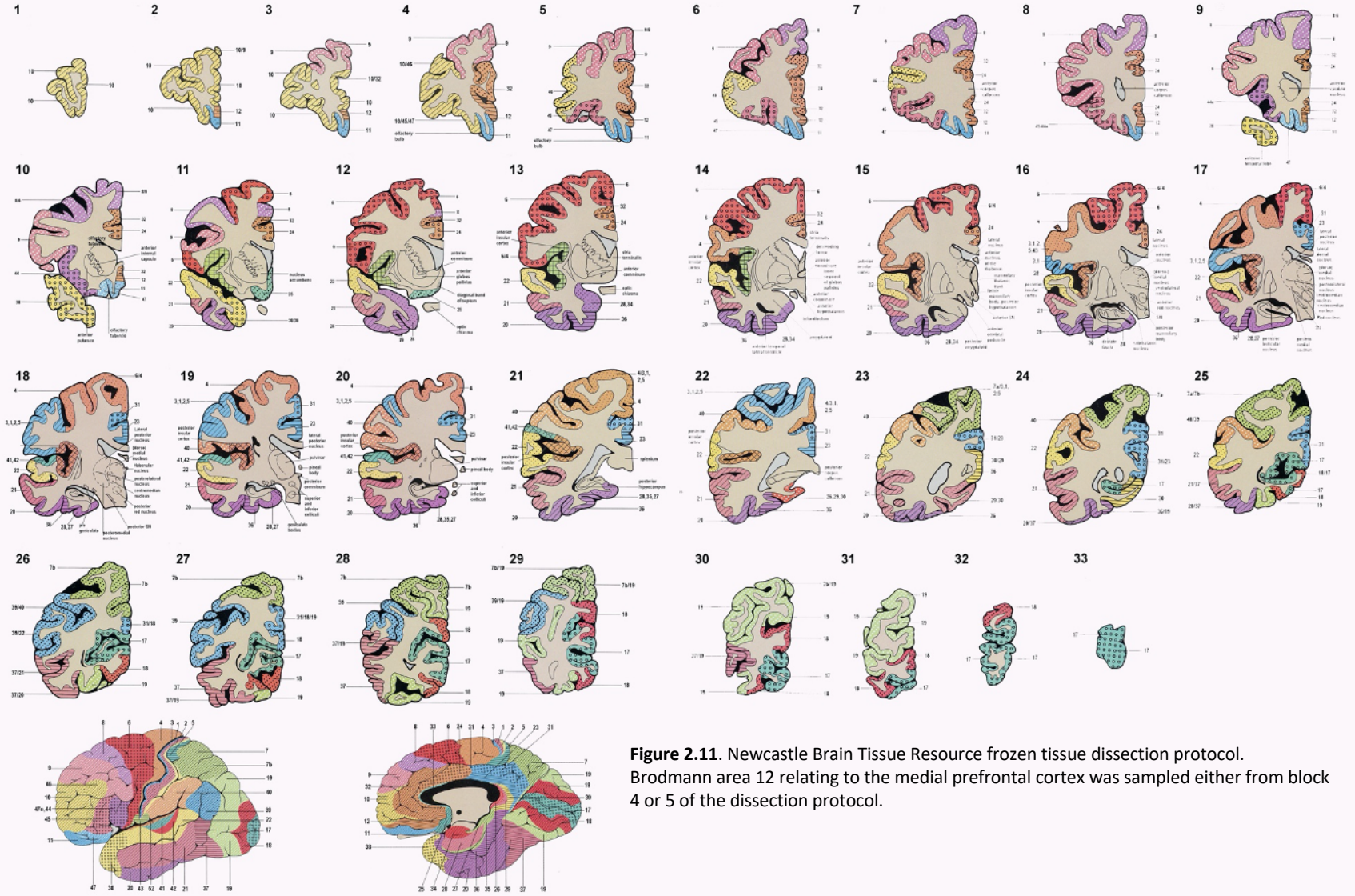


Figure 2.11. Newcastle Brain Tissue Resource frozen tissue dissection protocol. Brodmann area 12 relating to the medial prefrontal cortex was sampled either from block 4 or 5 of the dissection protocol.

Frozen tissue was brought to -20°C and tissue containing the mPFC was removed. Approximately 250mg tissue was dounce homogenised, 10 strokes, in ice cold lysis buffer at a tissue weight:volume ratio of 1:6. Lysis buffer, pH 7.5 was prepared containing 0.32M sucrose (Sigma Aldrich, Dorset, UK), 5mM HEPES (Sigma Aldrich, Dorset, UK), cOmplete Mini^{TM} protease inhibitor (Sigma Aldrich, Dorset, UK) (1 tablet per 10ml) and phosphatase inhibitor cocktail (Roche, Mannheim, Germany) (1 tablet per 10ml), made up in deionised water. Homogenised aliquots were then put on ice to undergo synaptosomal fragmentation.

2.9.2 Synaptosomal fragmentation

Homogenised aliquots underwent synaptosomal fragmentation by sequential centrifugation (figure 2.11) as previously described by Wirths (2017), immediately after tissue homogenisation. Tissue homogenate was centrifuged at 1,000 x g for 10 minutes at 4°C in a 1.5ml Eppendorf tube. Following the first centrifugation the supernatant was transferred into a new 1.5ml tube, and the pellet, pellet 1 (P1) containing nuclei, was resuspended in 500µl of 0.01M Phosphate buffered saline (PBS) (Sigma-Aldrich, Dorset, UK). The supernatant was then centrifuged at 12,000 xg for 20 minutes at 4°C. Following the second centrifugation the supernatant (S2), containing microsomes and soluble enzymes, was removed and transferred to a new 1.5ml tube. The pellet was then resuspended in the lysis buffer, pellet weight:volume ratio of 1:5, and recentrifuged at 12,000 xg for 20 minutes at 4°C, in order to wash the pellet, following the removal of the wash supernatant the pellet was again washed, in lysis buffer with a pellet weight:volume ratio of 1:5. After the second wash of pellet 2 (P2), the crude synaptosomal fraction, the pellet was resuspended in 250µl PBS. All fractions were then stored at -80°C until further use.

2.9.2.1 Fractionation validation

Fractionation was validated by assessing the levels of nuclear, mitochondrial and synaptic markers within the three fractions via western blotting.

Protein quantification of each frozen tissue fraction was performed using Bradford assay. Quantification of protein concentration was undertaken prior to every blot undertaken. Protein standards (1000, 800, 600, 400, 200, 100, 50 µg/ml BSA) were prepared in both 0.01M PBS for pellet fractions and lysis buffer for supernatant fractions. 10µl of relevant protein standards and 2µl of tissue sample were loaded into a 96-well plate, in duplicate.

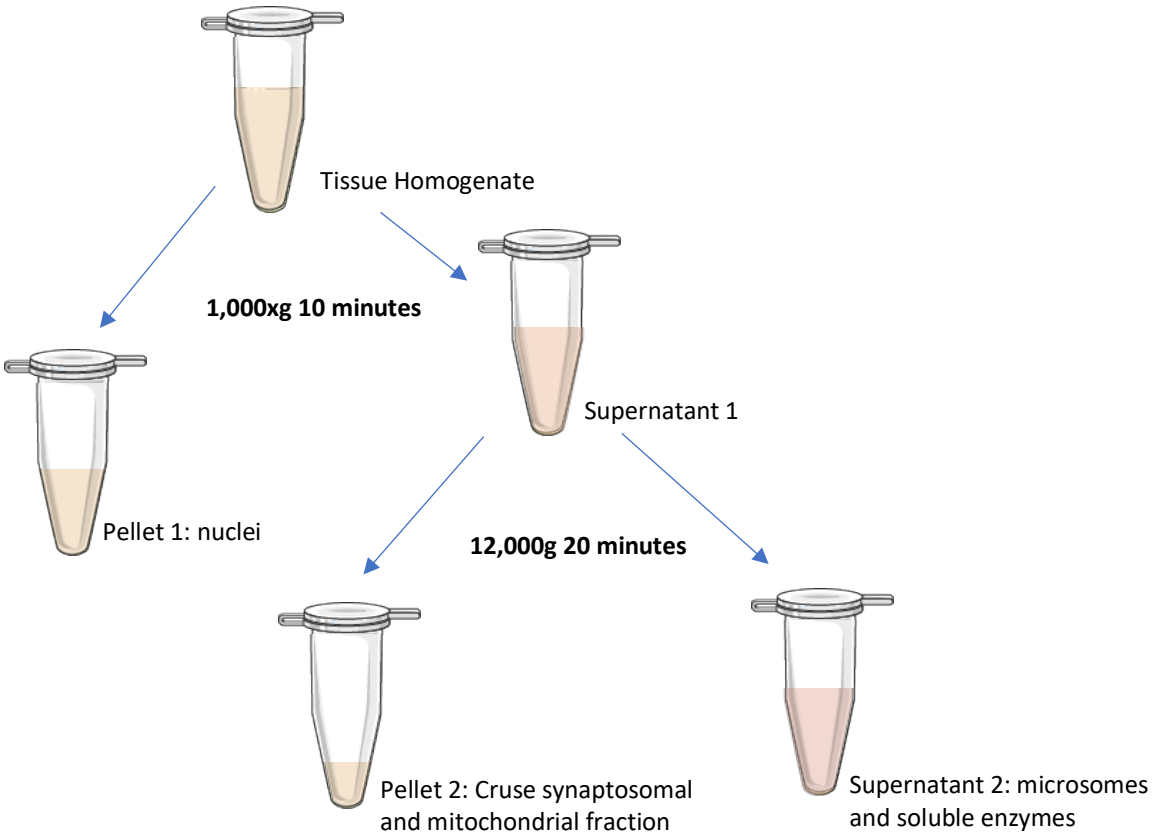


Figure 2.12. Synaptosomal fractionation protocol.
Flow chart describing the sequential centrifugation for preparation of a crude synaptosomal fraction.

200µl of Bradford reagent was then added to each well (Sigma-Aldrich, Dorset, UK). A visual inspection of the wells to ensure a colour change from brown (470nm) to blue (595nm) had occurred was done prior to absorbance readings. 96-well plates were loaded into FLUOstar Omega (BMG Labtech, Aylesbury, UK) and absorbance was read at 37°C at 595nm. Absorbances were measured within 10 minutes of Bradford reagent being added to the wells. Average absorbance values from the protein standards were plotted to create a protein standard curve. A linear line of best fit was fitted to the protein standard curve, with an R-value calculated to show the strength of the best fit line. Any R-value below 0.95, was deemed not to be sufficient and the Bradford assay was performed again. Using the equation of the protein standard curve the concentration (µg/µl) of the tissue samples was calculated. These concentration values were then used to prepare the 10µg samples loaded onto the blots. Samples were prepared for sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) by adding sample buffer (4x Novex NuPAGE LDS sample buffer, Invitrogen, Waltham, MA, USA), a reducing agent (10x NuPAGE sample reducing agent, Invitrogen, Waltham, MA, USA) and made up to 0.5 µg/µl with deionised water.

Samples were then heated for 10 minutes at 70°C, before being returned to ice and briefly centrifuged in a benchtop centrifuge, to ensure all sample was at the bottom of the tube following heating. Prepared samples were loaded onto a NuPAGE 4-12% Bis-Tris gel (Invitrogen, Waltham, MA, USA), 10µg of sample was loaded per well. Gels were run at 200V constant voltage for 35 minutes, with SDS NuPAGE MOPS Running Buffer (Invitrogen, Waltham, MA, USA). All blots were run with the SeeBlue™ protein ladder (ThermoFisher, Waltham, MA, USA) to ensure correct molecular weight of the protein bands. Proteins were transferred to a nitrocellulose membrane via IBlot2 (Invitrogen, Waltham, MA, USA), 20mV for 7 minutes.

Proteins within the nitrocellulose membranes where then detected following the protocol outlined in 2.9.3. Size and intensity of the protein bands on the blots was quantified using FIJI (Schindelin *et al.*, 2012) with the target protein expression normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression within the fractions. Target proteins were normalised to GAPDH to remove variations due to experimental errors, for example unequal loading of the three fractions into the blot. Furthermore, GAPDH was utilised over other proteins due to its ubiquitous expression throughout the neuron, therefore it should not be preferentially identified in any one of the fractions

2.9.3 Immunoblots

Dot blots were undertaken to assess the levels of a number of different proteins within the synaptosomal, P2, and soluble enzyme fragment, S2. Prior to all blots protein quantification was undertaken to obtain the concentration of total protein within each sample fraction via Bradford assays (as described in 2.9.2.1).

Samples from both fractions analysed were adjusted to 1µg/µl with distilled water and directly dotted in duplicate onto 0.2µm nitrocellulose membrane (10µl per sample, 10µg per dot). Sample dots were allowed to dry prior to incubation in TBS-T. Optimisation of the protocols identified one antibody, NDuFB8, that required sample pre-treatment where lysates were heated at 100°C for 5 minutes prior to being dotted. All blots had samples added in a randomised order to remove any experimental differences between the first and last blotted samples.

All immunoblots, both dot and western blots, were washed three times in TBS-T (5 minutes each) prior to being blocked for 1 hour at room temperature in 10% milk powder in TBS-T.

After blocking immunoblots were washed three times in TBS-T (5 minutes each). Immunoblots were then incubated with primary antibody at 4°C overnight under continuous agitation. Primary antibody solution was prepared in TBS-T, 5% BSA (Sigma-Aldrich, Dorset, UK) and 0.05% of sodium azide in a 50ml tube, with the primary antibody dilution having been empirically determined. Following incubation with primary antibody, immunoblots were washed three times with TBS-T (5 minutes each) prior to incubation with an appropriate, goat anti-mouse or goat anti-rabbit horseradish peroxidase conjugated secondary antibody (both 1:5000, Merk Millipore, Watford, UK) diluted in 5% milk powder in TBS-T. Immunoblots were incubated with secondary antibody for 1 hour at room temperature. All immunoblots were then washed in TBS-T (x3 5 minutes) prior to visualisation with enhanced chemiluminescence (1.25 mM Luminol, 30 µM coumaric acid, 0.015 % H_2O_2). Processed blots were subsequently stained for total protein measurements utilising ponceau (Sigma-Aldrich, Dorset, UK) total protein stain (0.1% ponceau in 5% acetic acid). Images were captured using a Fuji LAS 4000 with imaging software (Fuji LAS Image, Raytek, Sheffield, UK) at 8-bit for illustration and 16-bit for analysis.

To ensure specificity of the detected signal, appropriate secondary antibody control dot blots were undertaken, in which the primary antibody was excluded. For heat treated and soluble enzyme fraction immunoblots a small detectable immunoreactive signal was observed, following an exposure time comparable to the primary treated immunoblot. Non-specific immunoreactive signal was subtracted from the signal detected on the primary incubated immunoblot. No other immunoblots possessed a detectable non-specific immunoreactive signal at an exposure time comparable to the primary incubated blot.

2.9.4 Blot analysis

Immunoreactivity was quantified from 16-bit digitised images utilising FIJI (Schindelin *et al.*, 2012) based on area under curve measurements normalised to total protein load established via area under the curve for ponceau processed blots, as previously described by Koss *et al* (2016). Immunoblot intensity data were normalised within blot using total protein adjusted values and expressed relative to the control cases, prior to being pooled across blots.

2.10 Statistical analysis

Statistical analysis was conducted using SPSS v.26 (IBM). All data were assessed for normality by the Shapiro-Wilk test and inspection of histograms and Q-Q plots, prior to statistical analysis of significance between groups. Immunohistochemistry and immunoblot data did not fulfil normal distribution criteria; therefore, Kruskal-Wallis tests were employed to determine overall group differences. A paired t test (Mann-Whitney U) was used to determine differences between two clinical groups. Spearman's rank correlation co-efficient was used to determine associations between variables. Corrections such as Bonferroni were not applied due to the fact that there is not a true null hypothesis because of the classification of neurogenerative diseases, for example AD will have higher levels of AD-type pathology than DLB, therefore a p-value of <0.05 was considered significant.

Chapter 3: Pathological studies in the locus coeruleus

3.1 Introduction

Cognitive fluctuations are one of the four core clinical features of DLB (McKeith *et al.*, 2017). Despite this they are one of least understood and most difficult to assess clinically, with the underlying pathophysiological framework poorly understood (Matar *et al.*, 2019). Imaging studies have demonstrated connectivity alterations in the basal ganglia and thalamus which have been associated with cognitive fluctuations (Schumacher *et al.*, 2019c); as well as dysconnectivity within the ARAS, having been shown to lead to alterations in consciousness in other disorders (Siegel, 2004; Jang and Lee, 2015). Regions that form part of the ARAS, including the thalamus (Braak *et al.*, 2003), basal ganglia (Liu *et al.*, 2015) and hypothalamus (Benarroch *et al.*, 2015) are known to be vulnerable to the accumulation of pathological proteins in DLB and are hypothesised to play a role in the pathophysiology of cognitive fluctuations. However, there have been a limited number of studies that have examined how these accumulations could relate to the presence and severity cognitive fluctuations (Matar *et al.*, 2019; O'Dowd *et al.*, 2019).

The LC, the largest source of noradrenergic projections in the brain, has been extensively studied in in respect to its role in the maintenance of arousal and wakefulness (Samuels and Szabadi, 2008a). Activity within the LC is known to relate to cortical EEG levels (Berridge and Foote, 1991), indicative of arousal, with the nuclei thought to control this through selective excitement of systems that are also involved in waking, whilst inhibiting those that are involved in sleep (Jones, 2005a; McKenna *et al.*, 2017). A number of studies have investigated the LC in neurodegenerative diseases. Age-related neuronal loss in the LC has been shown to be exacerbated by neurodegenerative changes, which in DLB seem to be driven by α-synuclein pathology and not concomitant AD-type pathology (Samuels and Szabadi, 2008b; Brunnstrom *et al.*, 2011). Accumulation of α-synuclein pathology in the LC is thought to occur early in DLB (Vermeiren and De Deyn, 2017). Tau pathology has also been shown to be located in the LC prior to the transenthorinal cortex (Weinshenker, 2018). Although the LC has been extensively studied in many different dementia subtypes and has been shown to be highly affected by pathological protein deposits, there has not been a systematic study evaluating these changes and alterations to the ARAS as a whole, in relation to cognitive fluctuations. A recent opinion paper has suggested a role for the ARAS

and its constituent parts in the pathophysiological framework of cognitive fluctuations (Matar *et al.*, 2019). Therefore, the aim of this present study was to investigate whether pathological changes in the LC are specific to the cases with cognitive fluctuations and that may contribute to the severity of the cognitive fluctuations.

3.1.1 Aims

It was hypothesised that the LC will display a pattern of pathological protein deposition that relates to the presence of cognitive fluctuations. Using *post-mortem* tissue sections containing the locus coeruleus from DLB and nLBD cases with fluctuations, nLBD and AD cases without cognitive fluctuations, and aged cognitively normal controls, this study aims to

1. Quantify the burden of neuropathological protein lesions, utilising immunohistochemical techniques for Aβ, tau and α-synuclein
2. Assess whether the neurodegenerative changes seen are related to the presence or severity of cognitive fluctuations, utilising neuropsychiatric data obtained *intra vitam*

3.2 Methods

3.2.1 Study Cohort

Five groups were included in the LC study with a total of 67 cases (table 2.1). 14 DLB cases that had been recorded to have experienced cognitive fluctuations during life; 13 nLBD cases who did not experience cognitive fluctuations during life, containing a mixture of mixed AD/DLB and DLB cases; 11 AD cases with no recorded experience of cognitive fluctuations during life; 12 nLBD cases who did experience cognitive fluctuations during life, mixed AD/DLB; and 17 aged matched controls with no history of cognitive impairment. Cases who during life were uncooperative with neuropsychiatric testing, were postulated to have cognitive fluctuations due to medicine regimes and contained brainstem infarcts were excluded from the study. Within the study cohort a number of those with a history of cognitive fluctuations had the severity of these measured via CAF (9 DLB and 9 nLBD with cognitive fluctuations) allowing associations with severity of fluctuations to be assessed.

3.2.2 Tissue Acquisition

The LC is located 1mm below the fourth ventricle and 3mm from the midline (German *et al.*, 1988; Fernandes *et al.*, 2012). The LC is easily identifiable, even by the naked eye, due to the presence of neuromelanin within the noradrenergic neurons (figure 3.1). Sections for analysis were taken from the paraffin embedded formalin fixed tissue blocks containing the upper mid pons, as described in 2.4.5.

3.2.3 Pathology

Sections containing the LC underwent IHC staining, as described in 2.5, with antibodies against hyperphosphorylated tau (AT8), Aβ (4G8) and α-synuclein (KM51) (table 3.1). The three pathology antibodies were utilised as they have been widely validated and utilised for diagnostic within the NBTR**.** A large image, with an area of 10.1mm^2, was created by capturing and stitching 25 adjacent, 5x5, single images at x100 magnification using NIS-Elements AR3.2 software (Nikon, Surrey), a Nikon 90i microscope, with a fully motorised stage, and DsFi1 camera microscope coupled to a PC.

Stitched images were analysed using a standardised red-green-blue threshold on NIS elements AR3.2 software. Thresholds were determined for AT8, 4G8 and KM51 separately to detect only immunopositive signals, without detection of non-specific background and the neuromelanin pigment within the noradrenergic neurons. The threshold for 4G8 also

included a size threshold to ensure intracellular APP was excluded from the analysis.

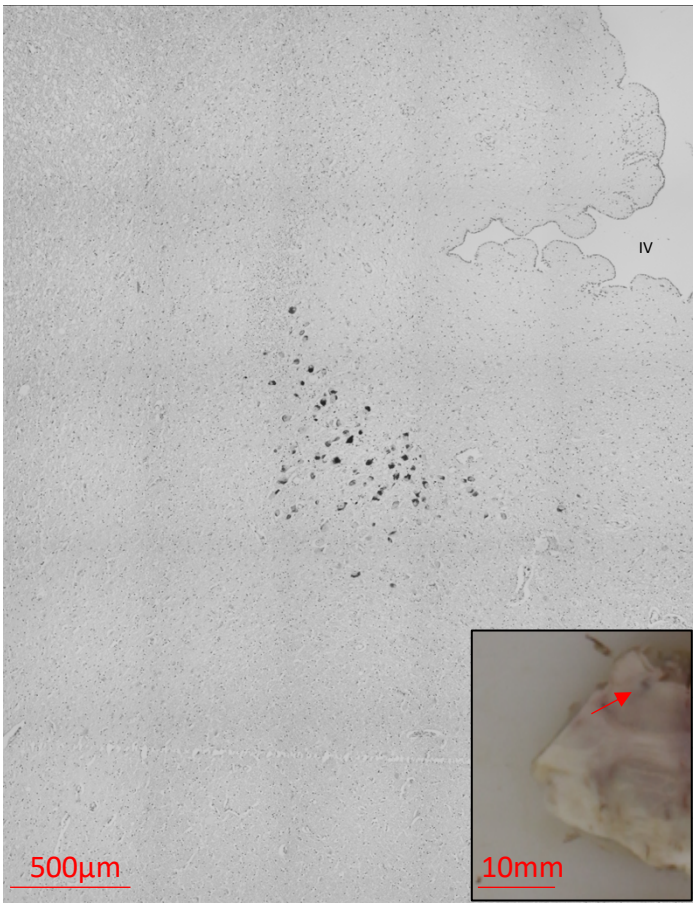


Figure 3.1. Location and identification of the locus coeruleus.
Neuromelanin containing noradrenergic neurons of the locus coeruleus located close to the fourth ventricle in the upper-mid pons in a fixed tissue control case section counterstained with haematoxylin. Scale bar = 500µm. Inset displays fixed tissue block with red arrow indicating the location of the locus coeruleus as can be seen with the naked eye. Inset scale bar = 10mm. IV-IV ventricle

Table 3.1. Antibodies utilised in the locus coeruleus study.
Optimised dilutions and antigen retrievals protocols for the antibodies utilised in the locus coeruleus study

Antibody	Manufacturer	Dilution	Antigen Retrieval
AT8, hyperphosphorylated tau	Autogen, MA, USA	1:4000	0.1M Citrate pH 6.0
4G8, Aβ	Covance, NJ, USA	1:15000	Formic acid (1 hour)
KM51, α-synuclein	Leica Biosystems, UK	1:200	Formic Acid (10 minutes), 1mM EDTA pH 8.0

3.2.4 Statistical analysis

Statistical analysis was conducted using SPSS v.26 (IBM). Variables were assessed for normality by the Shapiro-Wilk test and inspection of histograms and Q-Q plots. Pathology data was found to be non-normal leading to non-parametric tests being employed. To determine differences in pathological burden between the study groups Kruskal-Wallis and post-hoc Mann-Whitney were undertaken. To determine whether there was an association between pathological burden and severity of cognitive fluctuations as measured by CAF scores, Spearman's rank correlations were conducted, firstly in both DLB and nLBD (mixed AD/DLB) with cognitive fluctuations together and then both groups separately. Corrections such as Bonferroni were not applied due to the fact that there is not a true null hypothesis because of the classification of neurogenerative diseases, for example AD will have higher levels of AD-type pathology than DLB, therefore a p-value of <0.05 was considered significant.

3.3 Results

3.3.1 Demographics

No significant differences were observed in *post-mortem* delay, fixation duration, and age between the study groups. There was no significant difference observed in disease duration between the neurodegenerative disease groups. Between the groups, a significant difference was found in the proportion of males to females, with the DLB group having a significantly higher proportion of males compared to nLBD with fluctuations ($\chi(1)=3.869$, $p=0.049$), nLBD without fluctuations ($\chi(1)=4.747$, $p=0.029$), AD ($\chi(1)=4.588$, $p=0.032$) and controls ($\chi(1)=6.419$, $p=0.011$).

CAF scores were available for 25/67 cases. 18/26 of the cases with cognitive fluctuations possessed at least one CAF score. No significant difference was observed between the last ($p=0.927$), maximal ($p=1.000$) or average ($p=0.789$) CAF score between the DLB with fluctuations or the nLBD with fluctuation groups.

Final MMSE scores were available for 55/67 cases: 13 DLB cases with fluctuations, 12 nLBD with fluctuations, 11 nLBD without fluctuations, 10 AD and 9 Controls. No significant difference in the interval between the last MMSE and death was observed between the groups. Significantly lower last MMSE scores were observed between all disease groups and controls: DLB cases with fluctuations ($z=-21.363$, $p=0.002$), nLBD with fluctuations ($z=28.722$, $p<0.001$), nLBD without fluctuations ($z=-26.510$, $p<0.001$) and AD cases ($z=-27.156$, $p<0.001$). No significant differences in last MMSE score were observed between the 4 disease groups.

3.3.2 Pathology

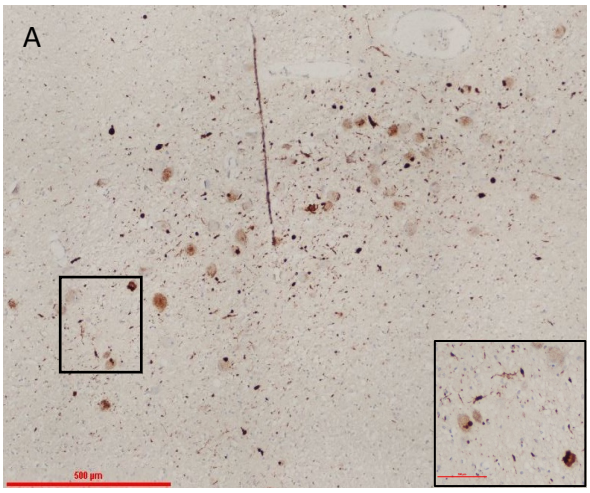
Alpha-synuclein pathology was observed in all cases with cognitive fluctuations, DLB and nLBD, with a mixture of Lewy bodies and Lewy neurites observed in the majority of cases (figure 3.2A). A significant main effect of disease group on α-synuclein was observed in the LC ($\chi^2=39.505$, $p<0.001$) (figure 3.2B). Alpha-synuclein pathological burden was higher in DLB with fluctuations ($z=32.315$, $p<0.001$), nLBD with fluctuations ($z=-30.363$, $p<0.001$) and nLBD without fluctuations ($z=30.376$, $p<0.001$) than controls. Pathological burden of α-synuclein was significantly higher in DLB with fluctuations ($z=29.604$, $p<0.001$), nLBD with fluctuations ($z=-27.652$, $p<0.001$) and nLBD without fluctuations ($z=27.664$, $p<0.001$) than AD cases. No significant differences between the level of α-synuclein burden were observed between the

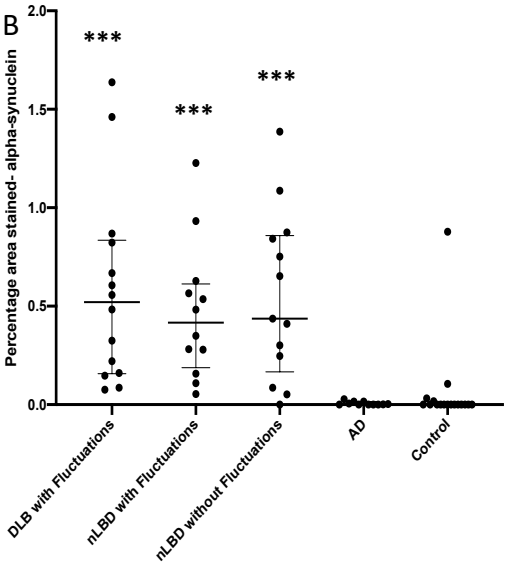
nLBD groups with and without fluctuations (p=0.999) or between the DLB with fluctuations group and the nLBD groups with (p=0.797) and without (p=0.794) fluctuations. No significant difference was also observed between AD and control cases.

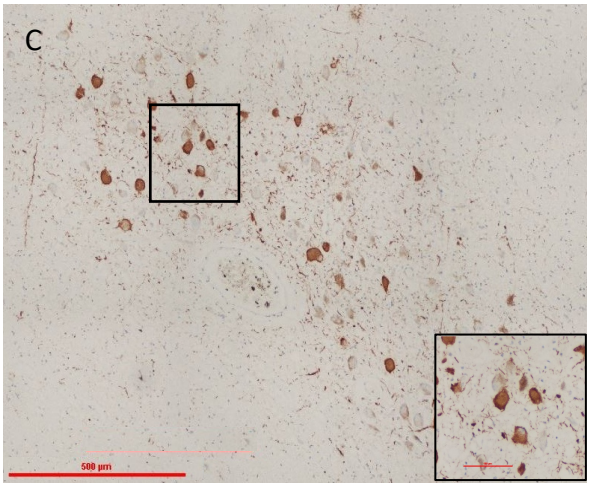
Tau pathological burden was observed in all cases, including control cases which typically possessed one or two small AT8 positive neuropil threads or neurites. The highest LC tau burdens were observed in the AD and nLBD groups, both with and without fluctuations, with cases containing neurofibrillary tangles, neuropil threads and neurites (figure 3.2C). A significant main effect of disease group on tau pathological burden was observed in the LC (χ^2=34.079, p<0.001) (figure 3.2D). Tau pathological burden was higher in nLBD with fluctuations (z=-21.069, p=0.004), nLBD without fluctuations (z=29.312, p<0.001) and AD cases (z=32.963, p<0.001) than controls. Pathological burden for tau was significantly higher in nLBD with fluctuations (z=-19.333, p=0.012), nLBD without fluctuations (z=-27.577, p<0.001) and AD cases (z=-31.277, p<0.001) than DLB with fluctuations. No significant differences between the level of tau burden were observed between the nLBD groups with and without fluctuations (p=0.291) or between the AD group and the nLBD groups with (p=0.144) and without (p=0.647) fluctuations. No significant difference was also observed between DLB with fluctuations and control cases

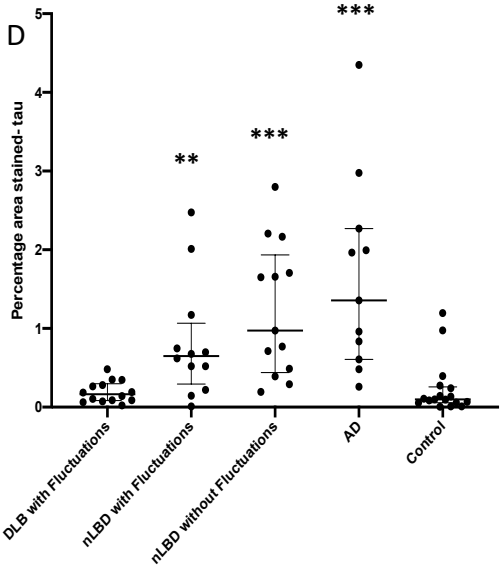
The percentage area stained for Aβ, as measure by 4G8 was the lowest of the three pathological protein observed, with variable pathological burden observed within the cases and disease groups (figure 3.2E). The majority of plaques were ill-defined aggregations of fine argentophilic processes, type 3 as previously described by (Iseki *et al.*, 1989). A significant main effect of disease group on Aβ pathological burden was observed in the LC (χ^2=30.216, p<0.001) (figure 3.2F). Aβ pathological burden was higher in nLBD with fluctuations (z=-27.995, p<0.001), nLBD without fluctuations (z=19.335, p=0.005) and AD cases (z=32.366, p<0.001) than controls. Pathological burden for Aβ was significantly higher in nLBD with fluctuations (z=-21.619, p=0.003) and AD cases (z=-25.990, p=0.001) than DLB with fluctuations. In the nLBD without fluctuations group a trend

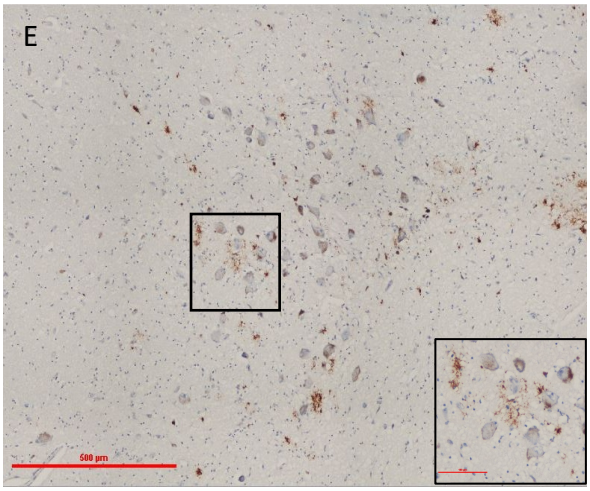
Figure 3.2. Pathology in the locus coeruleus.
Representative images of A-α-synuclein (KM51), C- tau (AT8), E-Aβ (4G8). Pathological burden data for B- α-synuclein, D- tau and F- Aβ. Data is shown as median with interquartile range. ** p<0.01 ***p<0.001 compared to controls. Scale bar 500μm, inset 100μm. Abbreviations: AD- Alzheimer's disease; DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease.

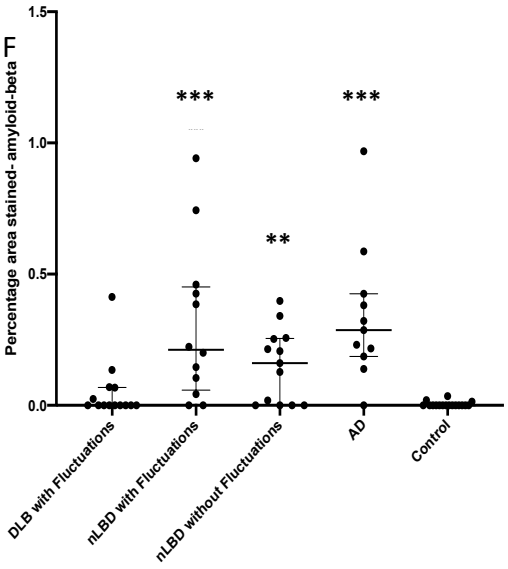

A
500 µm


B
Percentage area stained- alpha-synuclein
2.0
1.5
1.0
0.5
0.0
***
***
***
DLB with Fluctuations
nLBD with Fluctuations
nLBD without Fluctuations
AD
Control


C
500 µm


D
Percentage area stained- tau
5
4
3
2
1
0
**
***
***
DLB with Fluctuations
nLBD with Fluctuations
nLBD without Fluctuations
AD
Control


E
500 µm


F
Percentage area stained- amyloid-beta
1.5
1.0
0.5
0.0
***
**
***
DLB with Fluctuations
nLBD with Fluctuations
nLBD without Fluctuations
AD
Control

towards a significantly higher Aβ burden was observed compared to the DLB with fluctuations (p=0.070) group and a trend towards a significantly lower Aβ burden was observed compared the AD cases (p=0.087). No significant differences were observed between DLB with fluctuations and control cases, between the nLBD groups with and without fluctuations (p=0.245) and between nLBD groups with fluctuations and AD cases (p=0.573).

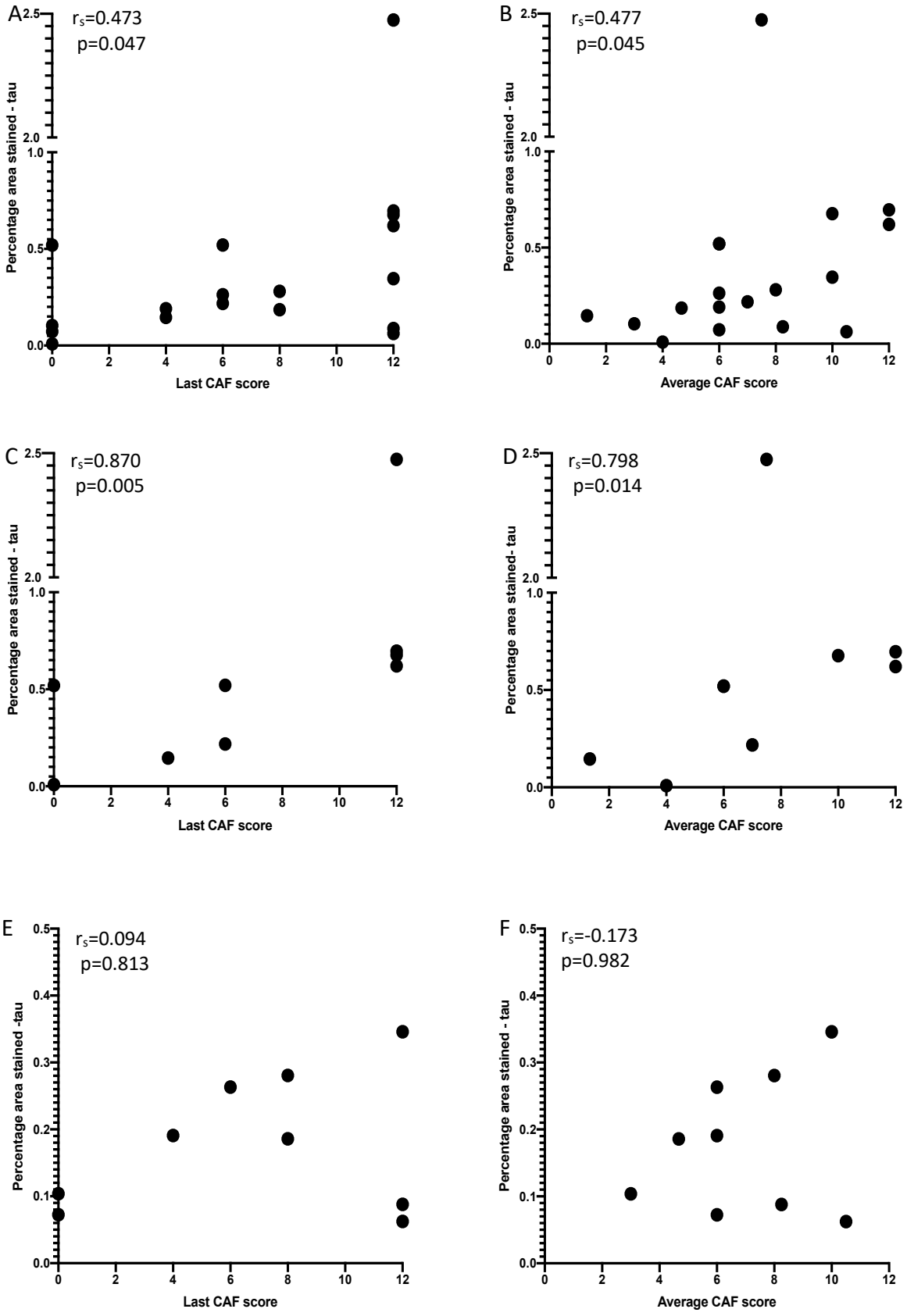
No associations were found between disease duration, across the disease groups, and α-synuclein (p=0.903), tau (0=0.976) or Aβ (p=0.360) pathological burden.

3.3.3 Relationship with cognitive fluctuations

Within the groups that contained cases which during life had experienced cognitive fluctuations, both DLB and nLBD, a significant positive Spearman's rank correlation was observed between tau pathology in the LC and last CAF score (r_s=0.473, p=0.047) (figure 3.3A) and average CAF score (r_s=0.477, p=0.045) (figure 3.3B). No other associations were observed between pathological burden and severity of cognitive fluctuations in the combined DLB and nLBD with fluctuations group.

When the combined cognitive fluctuation group was separated into DLB and nLBD, the previously observed association between cognitive fluctuation severity and LC tau burden was lost in the DLB only group, last CAF (r_s=0.094, p=0.813) (figure 3.3E) and average CAF (r_s=-0.017, p=0.982) (figure 3.3F). The positive association of cognitive fluctuation severity, as measured by last and average CAF score, and LC tau pathological burden was only observed within the nLBD with fluctuations group (r_s=0.870, p=0.005; r_s=0.798, p=0.014) respectively (figure 3.3C&D).

Figure 3.3. Correlations between locus coeruleus tau pathology and cognitive fluctuation severity.
A and B- LC tau pathology against last CAF scores and average CAF scores respectively shown all cases whom experienced cognitive fluctuations during life and had at least one CAF score in both the DLB and nLBD groups. C and D- nLBD cases only for LC tau pathology against last and average CAF score respectively. E and F- DLB cases only for LC tau pathology against last and average CAF score respectively. Abbreviations: LC- locus coeruleus; CAF-clinical assessment of fluctuation; DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease.


A
r_s=0.473
p=0.047
Percentage area stained - tau
Last CAF score
B
r_s=0.477
p=0.045
Percentage area stained - tau
Average CAF score
C
r_s=0.870
p=0.005
Percentage area stained - tau
Last CAF score
D
r_s=0.798
p=0.014
Percentage area stained- tau
Average CAF score
E
r_s=0.094
p=0.813
Percentage area stained -tau
Last CAF score
F
r_s=-0.173
p=0.982
Percentage area stained - tau
Average CAF score

3.4 Discussion

The present study identified significant differences in LC pathological burden between the disease groups utilised. As expected, α-synuclein pathology was found to be the significantly higher in the groups that were pathologically classified as LBDs; with tau and Aβ pathology significantly higher in the groups that were pathologically classified as having high AD neuropathological change. Increased CAF score, suggestive of an increased severity of cognitive fluctuations, was observed to be positively associated with an increased tau burden in nLBD with cognitive fluctuations but not in DLB with cognitive fluctuations. No relationship was observed between α-synuclein pathological burden or Aβ and presence or severity of cognitive fluctuations.

Cognitive fluctuations are one of the four core features of DLB; however, little is known about their pathophysiology. Clinical research into this phenomenon, has implicated both arousal and attention in cognitive fluctuations (Bliwise *et al.*, 2014; McKeith *et al.*, 2017; O'Dowd *et al.*, 2019). The LC and its noradrenergic projections have been heavily implicated in the maintenance and control of arousal and attention, both directly and indirectly through the ARAS (Jones, 2005a; Samuels and Szabadi, 2008a), with a recent opinion paper theorising that the ARAS and its constituent parts could be involved in the neural underpinning of cognitive fluctuations (Matar *et al.*, 2019).

3.4.1 Pathology alone does not distinguish fluctuating from non-fluctuating groups

This present study aimed to investigate the involvement of LC pathology, α-synuclein, tau and Aβ, on the presence and severity of cognitive fluctuations in DLB. Although, significant differences were identified between the groups within the study, these differences were related to the pathological diagnosis rather than the presence or absence of cognitive fluctuations.

Within the current investigation there was no observed difference between the nLBD groups with and without fluctuations. The majority of cases within each of the nLBD groups are classified as mixed AD/DLB, meeting the pathological criteria for both AD and DLB. The segregation based on the clinical presence of cognitive fluctuations between the nLBD groups also leads to a division based upon clinical phenotype. For nLBD cases with cognitive fluctuations, ten out of the twelve cases assessed were clinically classed as DLB; whereas nLBD without cognitive fluctuation, ten out of twelve cases assessed were clinically

classified as AD. A previous study investigating the effect of clinical phenotype on the pathological burdens of mixed AD/DLB identified a significant difference in tau, but not α-synuclein or Aβ, burden in the LC between the mixed AD/DLB cases that were clinically AD compared to clinically DLB (Walker *et al.*, 2015). Within the previous study, tau pathological burden was found to be significantly higher in those that were classified as clinically AD compared to those that were clinically DLB, a finding that was not replicated within the current study.

The LC has been shown to be affected at a very early stage of disease progression by tau and α-synuclein pathology, Braak stage 0 b and Braak 2 respectively (Braak *et al.*, 2003; Braak *et al.*, 2011). The difference observed between tau pathology burden in clinically AD and DLB by Walker *et al.* (2015) was hypothesised to be due to an earlier progression of tau in the clinically AD cases, in the current study classified as nLBD without fluctuations. The clinically AD cases were hypothesised to have originated as AD cases, which then developed Lewy body pathology further along the disease course. Conversely, the clinically DLB cases, in the current study nLBD cases with fluctuations, were hypothesised to start as DLB which later developed AD-type pathology. Pathology in earlier affected regions has been shown to increase with disease progression (Walker *et al.*, 2017). Therefore, it could be expected that the nLBD cases with cognitive fluctuations, clinically DLB, would possess higher levels of α-synuclein in the earliest affected regions, including the LC, than clinically AD nLBD cases. However, this was not found in the present study or the previous study by Walker et al (2015).

There has been a debate among researchers as to whether the progression of α-synuclein pathology occurs in the same manner in all LBDs, with different initial clinical presentations suggestive of differing initially affected regions. In DLB the spread of pathology has been proposed to originate in the amygdala (Beach *et al.*, 2009) and the olfactory bulb (Cersosimo, 2017). If α-synuclein pathology was to originate in non-brainstem structures in DLB, and progress in an anterograde manner (Henderson *et al.*, 2019), it would explain why α-synuclein pathological burdens in the LC do not differ between clinically AD and clinically DLB nLBD cases. Studies examining the SN in PD have identified an association between the number of Lewy bodies within the region and the number of dopaminergic neurons remaining (Parkkinen *et al.*, 2011); the more neurons remaining the more Lewy bodies

present. The LC, similarly to the SN, is subject to neuronal loss in neurodegenerative diseases. Therefore, a further hypothesis as to why α-synuclein pathological burdens in the LC did not differ between clinically AD and clinically DLB nLBD is due to loss of the neurons bearing Lewy bodies. The current study did not investigate neuronal loss in the LC; thus, it is not possible to control for the level of neuronal loss in the α-synuclein burden analysis.

Previous studies have shown that it is pre-synaptic accumulations of α-synuclein and not Lewy bodies that leads to neuronal death and possible cellular dysfunction in DLB (Kramer and Schulz-Schaeffer, 2007). The presence of disease-relevant α-synuclein pathology in the presynaptic compartments of noradrenergic neurons could elicit neuronal dysfunction without detectable pathology present in the cell bodies in the LC. These presynaptic alterations could explain why, although alterations to the LC have been implicated in the pathophysiology of cognitive fluctuations, no specific pathological alterations within the soma of LC neurons were attributed to the presence of cognitive fluctuations.

3.4.2 Tau burden was associated with CAF score

The present study identified a positive association, when both DLB and nLBD with fluctuations groups were combined, but only in the nLBD group when analysed individually, between LC tau burden and CAF score. The association between tau and last CAF score was lost in the DLB group, when analysed separately from the nLBD with fluctuation group.

Stimulation of the LC increases awareness and attention, with lesions leading to an increase in REM and SWS (Jones, 2005a; Jones, 2005b). The LC most densely innervates frontal regions, most specifically the PFC, where the noradrenergic projections have been implicated in behavioural flexibility and attentional systems (Borodovitsyna *et al.*, 2017). Although tau pathology has been identified within the nucleus at a very early stage of disease progression, the presence and severity of tau specifically has been found not to relate to the level of neuronal loss observed within the nucleus both in AD and in DLB (Brunnstrom *et al.*, 2011; Weinshenker, 2018). The lack of association between tau pathological burden and neuronal loss suggests that the presence of the pathology may not be the direct cause for the loss of neurons within the LC. However, tau could be a marker of dysfunction within the neuron and possibly the noradrenergic system as a whole, with alterations to the remaining LC neuronal morphology, synapses and dendrites having been reported (Baloyannis *et al.*, 2006). In an animal model where tau was injected into the LC it

was the frontal region that was affected first (Iba *et al.*, 2015), and further studies have identified a distinct noradrenergic pathway from the LC to frontal regions (Chandler *et al.*, 2014). Together, this suggests how tau in the LC could lead to dysfunction within the noradrenergic system and its terminal regions. However, it does not explain how it could be specifically related to cognitive fluctuations, as although a relationship between tau and severity was observed in the nLBD group, there was no difference in the LC tau burden between the nLBD groups with and without fluctuations.

Although an association was observed when both fluctuation groups were analysed together, when the two groups were analysed individually the association between tau and CAF score only remained in the nLBD group. The burden of tau in the nucleus could be representative of more severe degeneration within the LC or more severe tau deposition globally within the brain or general disease progression, that could lead to more severe cognitive fluctuations being present. However, as the association was only identified in the nLBD and not the DLB group when analysed individually, the relationship could be spurious with no meaningful biological effect. Due to the relatively small cohort sizes within the current study it is not possible to make firm conclusions. Any conclusions or hypotheses drawn from the present study, should be treated with caution until further experiments with additional cases can be undertaken, to assess if the results can be confirmed.

3.4.3 Limitations

A major caveat of the current study was that the LC was not sampled at a consistent level in all cases. Although all sections analysed came from within the same tissue block, containing the upper mid pons. Previous studies have identified that noradrenergic neurons in different LC regions preferentially innervate different cortical and sub-cortical regions (Ward and Gunn, 1976), and neuronal loss varies along the extent of the LC in AD (German *et al.*, 1992), although in PD this loss is more uniform in nature (Chan-Palay and Asan, 1989a). The variability within the level sampled could have affected made it harder to find relevant real changes if present. If regions that are known to highly innervate the frontal region had been consistently sampled it would have strengthened the ability to test the generated by the hypotheses.

Another limiting factor of the study was the sample size, although 67 cases were utilised within the study, the maximum number within one of disease groups was 14. As the study

was retrospective, the sample size was further limited, as not all cases utilised had been assessed for cognitive fluctuations. This limited the number of cases that could be utilised to test associations with severity of cognitive fluctuations using CAF score. Although the pathological analysis that was undertaken was quantitative, rather than qualitative, in nature a large cohort would be required to attain more meaningful results. Therefore, the conclusions from this study should be validated with a larger cohort before meaningful results can be inferred. Pathological correlations with cognitive fluctuation measure were not corrected for multiple comparisons within this study. Although corrections such as Bonferroni adjustment can reduce type I error it is considered too conservative to be utilised in certain situations where it can increase the likelihood of type II errors (Armstrong, 2014). Further investigations with a larger cohort, specifically a larger number of cases who have had their cognitive fluctuations assessed would be required to determine if the associations within the current study could withstand robust statistical testing.

The current study only investigated the pathological burden of protein aggregates. However, limited relationships were observed between pathological protein burdens and clinical data. It could be possible that investigations into markers of cellular stress, neuronal loss or specific species of protein aggregates may have been more informative. Therefore, future investigations should focus on more specific changes to noradrenergic neurons.

3.4.4 Conclusions

This study aimed to investigate the pathological differences in the LC between cases that clinically presented with cognitive fluctuations and those that did not, along with investigating whether there was an association between pathological burden and cognitive fluctuation severity.

The data showed pathological differences related to the pathological diagnosis of the cases, with no changes related specifically to the presence or absence of cognitive fluctuations. An association was observed between CAF score and tau pathological burden, only in the nLBD with fluctuation group. This association could reflect dysfunction in the noradrenergic system; however, it is difficult to assess whether the association is suggestive of a meaningful biological effect from the data obtained in the current study. It is likely that cognitive fluctuations are related to a global system failure rather than one specific change, with the finding in the current study possibly representing one change which in combination

with alterations to other neurotransmitter systems could lead to the presence of cognitive fluctuations. Investigations into cortical regions which receive noradrenergic projections and other ARAS systems are required to fully understand cognitive fluctuations.

Chapter 4: Pathological studies in the pedunculopontine nucleus

4.1 Introduction

The PPN has been thought of as the main driver and critical cell group for waking and REM sleep in the ARAS (Garcia-Rill and Simon, 2015); and is highly interconnected with all of the major neurotransmitter systems involved in the ARAS (Benarroch, 2013; Beck and Garcia-Rill, 2015; Yates and Garcia-Rill, 2015). Lesions to the PPN have been shown to alter or eliminate REM sleep (Shouse and Siegel, 1992; Deurveilher and Hennevin, 2001) and stimulation increasing waking like EEG activity (Steriade *et al.*, 1991; Datta and Siwek, 1997). Alterations to the PPN have been observed in pathologies of wakefulness, such as narcolepsy, either directly though dysfunction to PPN neurons or indirectly via the inputs into the PPN (Yates and Garcia-Rill, 2015). Dysfunctional PPN output, directly or indirectly, could result in alterations to arousal and awareness through a changes to cortical stimulation (Yates and Garcia-Rill, 2015).

Pathologically the PPN is affected by Lewy body pathology at an early stage of disease progression, Braak stage 3 (Braak *et al.*, 2003; Grinberg *et al.*, 2011). The PPN has been extensively examined in PD, in relation to a number of different clinical phenotypes, though there has been less focus on the nuclei in DLB. Loss of cholinergic PPN neurons is observed in PD, where studies have reported a 50% reduction (Pahapill and Lozano, 2000), as well as a reduction in neuronal size compared with controls, (Rinne *et al.*, 2008b; Jenkinson *et al.*, 2009). The neuronal loss observed in PD was shown to correlate with the degree of dopaminergic loss (Di Giovanni *et al.*, 2019). Loss of cholinergic neurons in the PPN leads to a subsequent loss of cholinergic innervation to the thalamus (Francis and Perry, 2007).

The PPN is also known to play a role in locomotion and muscle tone through its ascending connections with the SN and globus pallidus internus and descending connections with the spinal cord (Shute and Lewis, 1967; Benarroch, 2013; Garcia-Rill and Simon, 2015). The role of the PPN regarding locomotion and muscle tone has led to researchers investigating its contribution to the postural instability gait difficulty (PIGD) parkinsonian phenotype (Hamani *et al.*, 2016). The tremor dominant (TD) phenotype has mainly been ascribed to dopaminergic deficits, as the phenotype typically responds to dopaminergic treatments (Hughes *et al.*, 1992; Thenganatt and Jankovic, 2014). To support this hypothesis researchers have shown that the severity of PPN cholinergic loss correlated with the

severity of parkinsonian symptoms in PD (Zweig *et al.*, 1989; Rinne *et al.*, 2008a); and targeting the nucleus for DBS improved gait disturbances (Ferraye *et al.*, 2010; Moro *et al.*, 2010).

Although the PPN is one of the more well-studied nuclei in the context of arousal and motor control, there is a lack of research into the role of the PPN in DLB, and research has mainly having focused on cholinergic pathways rather than the nucleus. Stimulation of the PPN has been shown to have improved measures of executive function and working memory (Alessandro *et al.*, 2010; Tykocki *et al.*, 2011; Stefani *et al.*, 2013) and lead to beneficial changes in sleep architecture (Stefani *et al.*, 2007) in PD. Therefore, the improvement to sleep architecture and executive function through stimulation of the PPN, could suggest that there are salvageable neuronal projections that could be targeted in a less invasive manner to help improve the cognitive dysfunction in PD and possibly other Lewy body type diseases. Hence, the present study aimed to assess neuropathological changes to the PPN that could relate to, the presence and severity cognitive fluctuations and, the phenotype and severity parkinsonian features.

4.1.1 Aims

Using *post-mortem* tissue sections containing the PPN from DLB cases with fluctuations, compared to nLBD cases, both with and without cognitive fluctuations, AD cases without cognitive fluctuations, to control for the high levels of AD-type pathology in the nLBD cases, and aged cognitively normal controls, this study aims to:

3. Quantify the burden of neuropathological protein lesions, utilising immunohistochemical techniques for Aβ, tau and α-synuclein
4. Assess whether the neurodegenerative changes seen are related to the presence or severity of cognitive fluctuations, utilising neuropsychiatric data obtain *intra vitam*
5. Assess whether the neurodegenerative changes seen are related to the different parkinsonian subtypes and the severity of parkinsonian symptoms.
6. Evaluate whether levels of pathology in the PPN relate to levels of the pathology observed in the LC

4.2 Methods

4.2.1 Study Cohort

Five groups were included in the PPN study with a total of 52 cases (table 2.1): 11 DLB cases that had been recorded to experience cognitive fluctuations during life; 11 nLBD cases whom did not experience cognitive fluctuations during life, containing a mixture of mixed AD/DLB and DLB cases; 9 AD cases with no recorded experience of cognitive fluctuations during life; 11 nLBD cases whom did experience cognitive fluctuations during life, mixed AD/DLB; and 10 aged matched controls with no history of cognitive impairment. Cases that were uncooperative with neuropsychiatric testing or postulated to have cognitive fluctuations due to medicine regimes were excluded from the study. A subset of cases in the present study had cognitive fluctuation severity measured via CAF, 7 DLB with cognitive fluctuations and 8 nLBD with cognitive fluctuations. A further subset of 11 cases had their parkinsonian symptoms classified into either the PIGD or TD subtype.

4.2.2 Tissue Acquisition

The PPN is a large nucleus extending through the upper pons and midbrain (Hamani *et al.*, 2016). Sections for analysis were taken from the paraffin embedded formalin fixed tissue blocks containing the lower midbrain, as described in 2.4.4 and 2.4.5.

4.2.2.1 Pedunculopontine nucleus localisation

The PPN was identified within the sections by first assessing an LFB/H&E section from the lower midbrain to identify neuronal structures to ascertain the brainstem level in the brainstem atlas (Paxinos and Huang, 2013). If the section assessed by LFB/H&E contained structures known to be present at the same level as the PPN a sequential section underwent ChAT IHC. Three ChAT antibodies were utilised to locate the PPN for analysis (table 4.1). The rationale for the three ChAT antibodies being utilised was that the different epitopes of the antibodies enabled the identification of the PPN in more cases, as described below. The cases that were identified more preferentially by one antibody over another did not relate to the diagnosis of the case and it is currently unknown why some PPN regions were more immunopositive than others.

Sections first underwent staining the goat-polyclonal ChAT antibody. With the antibody being raised in goat the normal IHC protocol, following antigen retrieval could not be followed as outlined in 2.5. Briefly, sections following antigen retrieval were incubated in

Table 4.1. Antibodies utilised in the pedunculopontine nucleus study.
Optimised dilutions and antigen retrieval protocols for the antibodies utilised in the pedunculopontine nucleus study.

Antibody	Manufacturer	Dilution	Antigen Retrieval
AT8, hyperphosphorylated tau	Autogen, MA, USA	1:4000	0.1M Citrate pH 6.0
4G8, Aβ	Covance, NJ, USA	1:15000	Formic acid (1 hour)
KM51, α-synuclein	Leica Biosystems, UK	1:200	Formic Acid (10 minutes), 1mM EDTA pH 8.0
ChAT, mouse monoclonal	Atlas antibodies, Bromma, Sweden	1:1000	0.1M Citrate pH 6.0
ChAT, rabbit polyclonal	Atlas antibodies, Bromma, Sweden	1:500	0.1M Citrate pH 6.0
ChAT, goat polyclonal	Merck, Darmstadt, Germany	1:200	1mM EDTA pH 8.0

0.9% H_2O_2 in 0.01M PBS with 0.1% triton (Sigma-Aldrich, Dorset, UK) for 30 minutes. Sections were then washed in PBS, prior to blocking in normal rabbit serum (Vector Laboratories, Burlingame, CA, USA), diluted 1:60 in PBS for 30 minutes. Block was washed off before immediate incubation in ChAT primary antibody serum for 1 hour at room temperature. Sections were then washed in PBS, prior to incubation in secondary biotinylated anti-goat antibody (Vector Laboratories, Burlingame, CA, USA) diluted 1:200 in 1:60 normal rabbit serum in PBS for 30 minutes. Sections were then washed in PBS prior to incubation in ABC solution for 30 minutes at room temperature. Sections were washed in PBS and then acetate buffer, 100mM sodium acetate trihydrate (VWR, Lutterworth, UK) solution in H_2O adjusted to pH 6.0 with acetic acid. Sections were then incubated for 10 minutes at room temperature in DAB/Nickel solution: 233mM DAB, 7.5mM ammonium chloride, 11mM β-D+ glucose and 0.01% glucose oxidase, added to an 87mM diammonium nickel sulphate solution in acetate buffer. Sections were subsequently washed in acetate buffer prior to counterstaining and rehydration and mounting as described in 2.5. Upon examination of the slides stained using the goat-polyclonal ChAT antibody it was noted that although cholinergic structures within the sections were recognised (figure 4.1A), there was a high level of non-specific staining with non-cholinergic structures being immunopositive for ChAT staining. A number of cases at this stage, however, where identified as being too rostral to contain the PPN due to the presence of the oculomotor nucleus. Furthermore, the

levels stated by the brainstem atlas were observed to be misleading, as the fixed tissue utilised was dissected at a different angle. Assessment of cases, for the PPN, at this stage was aided by the help of Dr. Chris Morris, an expert in brain anatomy.

Sections thought to contain the PPN were then stained with mouse monoclonal ChAT antibody (figure 4.1B) following the protocol outlined in 2.5. Although, staining was intense when optimised in the striatum (figure 4.1D) when IHC was undertaken in the midbrain staining was much weaker. Utilising the mouse monoclonal ChAT, a number of cases were positively identified as containing the PPN, allowing for delineation of the region. Cases which were thought to contain the PPN but were immunonegative with the mouse monoclonal ChAT antibody underwent staining with a rabbit polyclonal ChAT antibody (figure 4.1C). Following assessment with the third ChAT antibody the PPN was identified in a total of 52/67 cases within the PhD cohort.

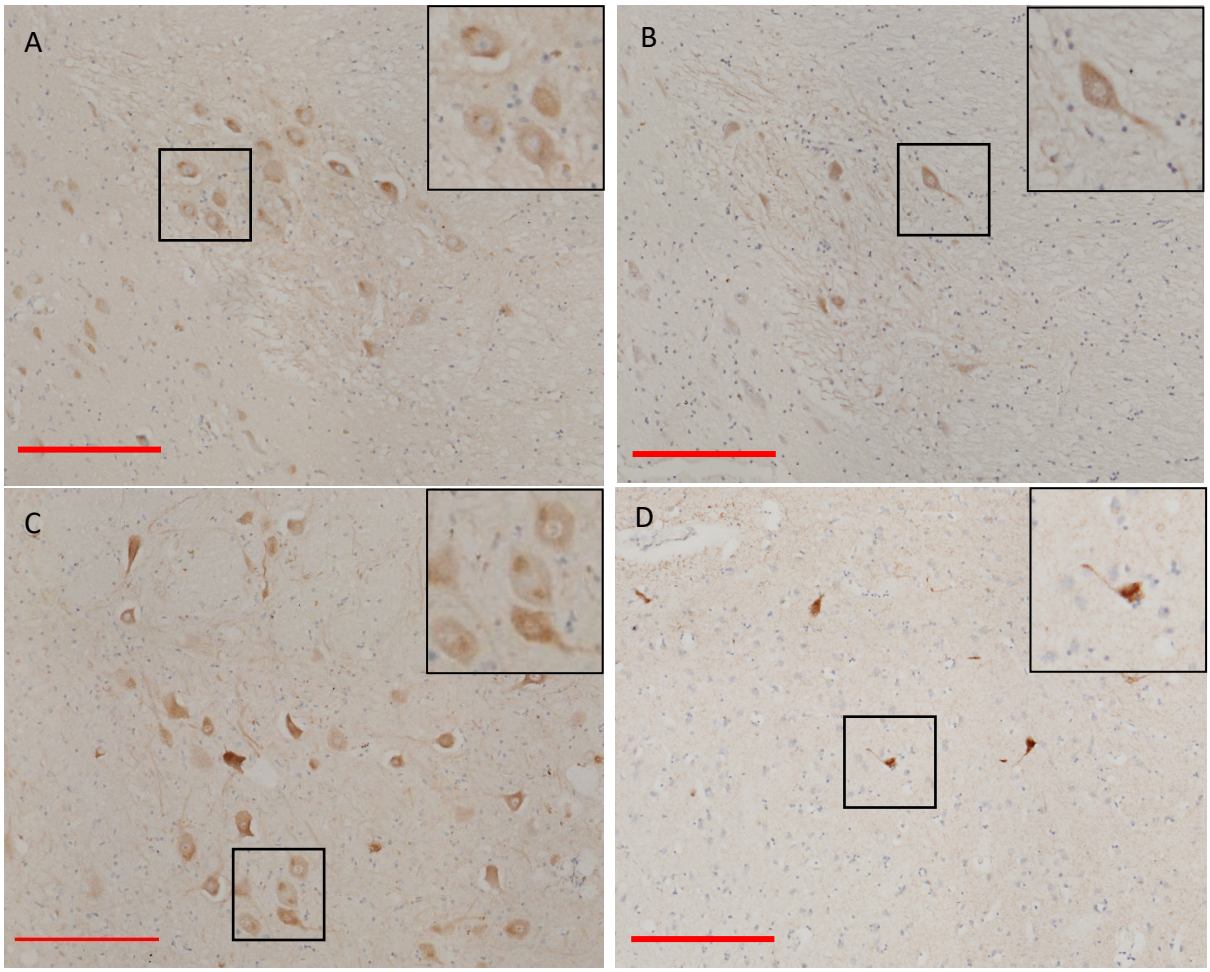


Figure 4.1. Representative choline acetyltransferase staining in the midbrain and striatum.
Choline acetyltransferase (ChAT) positive staining in the midbrain (A-C) and striatum (D). Representative images of A- goat polyclonal ChAT, B- mouse monoclonal ChAT, C- rabbit polyclonal ChAT and D- mouse monoclonal ChAT in the striatum. Scale bar 250µm. Inset images are x2 the original image.

4.2.3 Pathology

Following identification of the PPN, consecutive sections underwent IHC staining, as described in 2.5, with antibodies against hyperphosphorylated tau (AT8), Aβ (4G8) and α-synuclein (KM51) (table 4.1).

A large image, with an area of 41.5mm^2, was created by capturing and stitching 100 adjacent, 10x10, single images at x100 magnification using NIS-Elements AR3.2 software (Nikon, Surrey), a Nikon 90i microscope, with a fully motorised stage, and DsFi1 camera microscope coupled to a PC.

Stitched images were analysed using a standardised red-green-blue threshold on NIS elements AR3.2 software. Thresholds were determined for AT8, 4G8 and KM51 separately to detect only immunopositive signals, without detection of non-specific background. The PPN was delineated on the pathology sections by comparison to the ChAT section prior to application of the threshold (figure 2.6).

4.2.3.1 LC pathology

All cases within the current study also had the pathological burdens for hyperphosphorylated tau, Aβ and α-synuclein assessed and analysed in the LC, as described in 3.2.3.

4.2.4 Statistical analysis

Statistical analysis was conducted using SPSS v.26 (IBM). Variables were assessed for normality by the Shapiro-Wilk test and inspection of histograms and Q-Q plots. Pathology data was found to be non-normal, therefore, non-parametric tests were employed. To determine differences in pathological burden between the study groups Kruskal-Wallis and post-hoc Mann-Whitney were undertaken. To determine whether there was an association between pathological burden and severity of cognitive fluctuations as measured by CAF scores, Spearman's rank correlations were conducted, firstly in both DLB and nLBD (mixed AD/DLB) with cognitive fluctuations together and then both groups separately. Corrections such as Bonferroni were not applied due to the fact that there is not a true null hypothesis because of the classification of neurogenerative diseases, for example AD will have higher levels of AD-type pathology than DLB, therefore a p-value of <0.05 was considered significant.

4.3 Results

4.3.1 Demographics

No significant differences were observed in *post-mortem* delay, fixation duration, and age between the study groups. There was no significant difference observed in disease duration between the neurodegenerative disease groups. Between the groups a significant difference was found in the proportion of males to females, with the DLB group have a significantly higher proportion of males compared to nLBD with fluctuations ($\chi(1)=7.071$, $p=0.008$), AD ($\chi(1)= 5.089$, $p=0.024$) and controls ($\chi(1)=8.240$, $p=0.004$). A trend to a significantly higher proportion of males in DLB compared to nLBD without fluctuations ($\chi(1)=3.667$, $p=0.056$) was seen.

CAF scores were available for 20/52 cases, and 15 cases with cognitive fluctuations possessed at least one CAF score. No significant difference was observed between the last ($p=0.668$), maximal ($p=0.684$) or average ($p=0.536$) CAF score between the DLB with fluctuations or the nLBD with fluctuations groups.

Final MMSE scores were available for 44/52 cases: 10 DLB cases with fluctuations, 11 nLBD with fluctuations, 10 nLBD without fluctuations, 8 AD and 5 Controls. No significant difference in the interval between the last MMSE and death was observed between the groups. Significantly lower last MMSE scores were observed between: DLB cases with fluctuations ($z=-16.050$, $p=0.021$), nLBD with fluctuations ($z=2.027$, $p=0.001$), nLBD without fluctuations ($z=-19.600$, $p=0.005$) and AD cases ($z=-20.330$, $p=0.005$) and controls. No significant differences in last MMSE score were observed between the four disease groups.

A number of cases were classified as either having the tremor predominant (n=3) or postural instability gait difficulty (n=8) parkinsonian symptom subtype.

4.3.2 Pathology

α-synuclein pathology was observed in all DLB cases with cognitive fluctuations, and the majority of nLBD cases with cognitive fluctuations, most frequently as Lewy bodies (figure 4.2A). A significant main effect of disease group on α-synuclein was observed in the PPN ($\chi^2=27.470$, $p<0.001$) (figure 4.3A). α-synuclein pathological burden was higher in DLB with fluctuations ($z=23.495$, $p=0.001$), nLBD with fluctuations ($z=-16.041$, $p=0.015$) and nLBD without fluctuations ($z=15.495$, $p=0.019$) than controls. Pathological burden of α-synuclein was significantly higher in DLB with fluctuations ($z=30.379$, $p<0.001$), nLBD with fluctuations

(z=-22.924, p=0.001) and nLBD without fluctuations (z=22.379, p=0.001) than AD cases. No significant differences between the level of α-synuclein burden were observed between the nLBD groups with and without fluctuations (p=0.933) or between the DLB with fluctuations group and the nLBD groups with (p=0.248) and without (p=0.215) fluctuations. No significant difference was observed between AD and control cases.

Tau pathological burden was observed in the majority of cases. The highest PPN tau burdens were observed in the AD and nLBD groups both with and without fluctuations, with cases containing neurofibrillary tangles, neuropil threads and neurites (figure 4.2B). A significant main effect of disease group on tau pathological burden was observed in the PPN (χ^2=27.621, p<0.001) (figure 4.3B). Tau pathological burden was higher in nLBD with fluctuations (z=-26.400, p<0.001), nLBD without fluctuations (z=25.400, p<0.001) and AD cases (z=22.178, p=0.001) than controls. Pathological burden of tau was significantly higher in nLBD with fluctuations (z=-21.182, p=0.001), nLBD without fluctuations (z=-20.182, p=0.002) and AD cases (z=-16.960, p=0.013) than DLB with fluctuations. No significant differences between the level of tau burden were observed between the nLBD groups with and without fluctuations (p=0.877) or between the AD group and the nLBD groups with (p=0.535) and without (p=0.636) fluctuations. No significant difference was observed between DLB with fluctuations and control cases.

Aβ pathology was observed in 70% of cases analysed, with 6 DLB and 9 control cases possessing no positive 4G8 staining (figure 4.2C). In the majority of cases Aβ pathology was higher in neighbouring regions rather than the PPN itself. A significant main effect of disease group on Aβ pathological burden was observed in the PPN (χ^2=24.569, p<0.001) (figure 4.3C). Aβ pathological burden was higher in nLBD with fluctuations (z=-28.186, p<0.001), nLBD without fluctuations (z=19.050, p=0.003) and AD cases (z=23.994, p<0.001) than controls. Pathological burden of Aβ was significantly higher in nLBD with fluctuations (z=-19.182, p=0.002) and AD cases (z=-14.990, p=0.025) than DLB with fluctuations; DLB with fluctuations and nLBD without fluctuations did not have a significantly different Aβ burdens (p=0.112). No significant differences between the level of Aβ burden were observed between the nLBD groups with and without fluctuations (p=0.149) or between the AD group and the nLBD groups with (p=0.530) and without (p=0.458) fluctuations. No significant

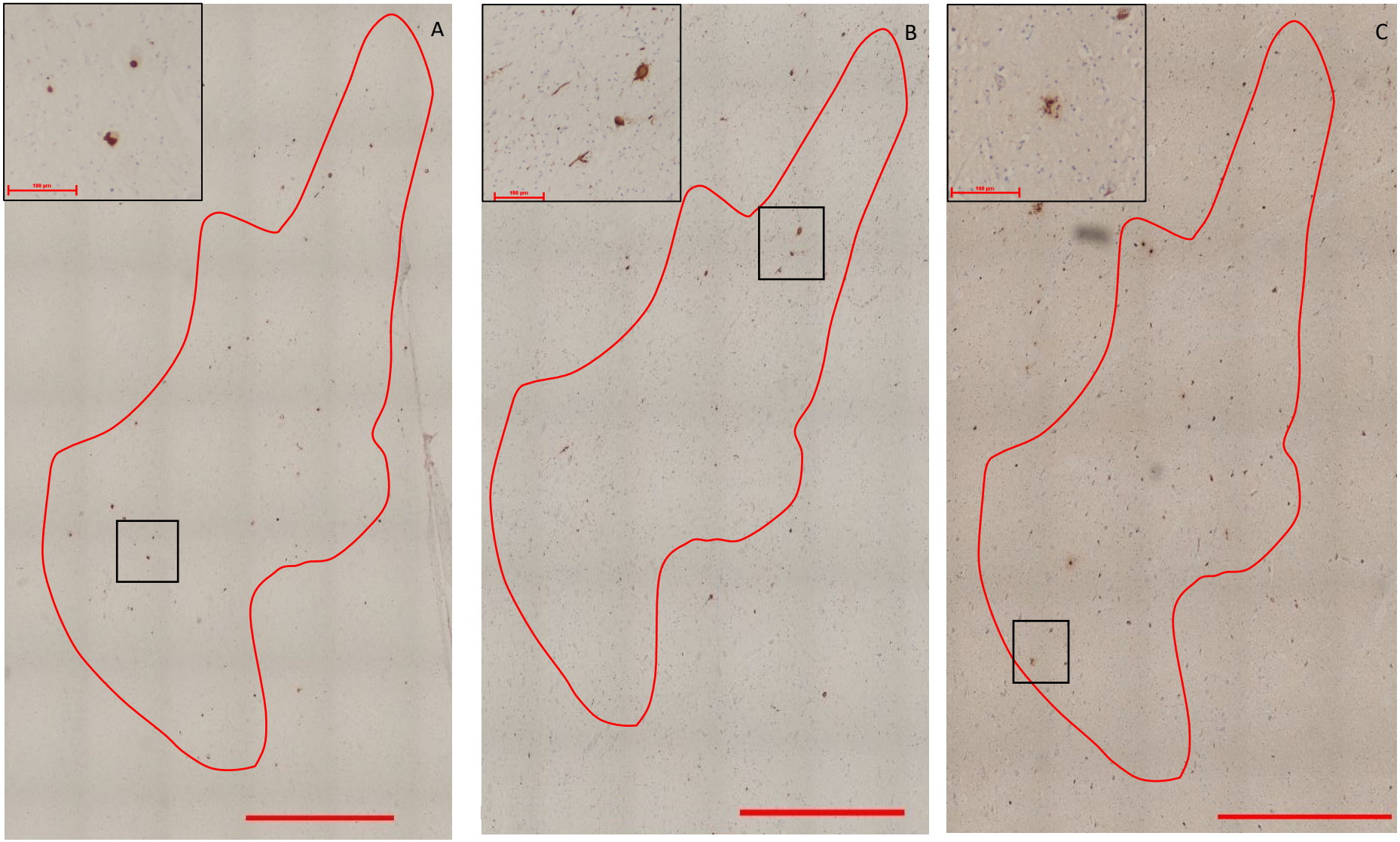


Figure 4.2. Representative pathology in the pedunculopontine nucleus.
Representative images of A-α-synuclein (KM51), B- tau (AT8), C-Aβ (4G8) pedunculopontine pathology. Scale bar 1000µm, inset 100µm.

difference was also observed between DLB with fluctuations or control cases. No other significant differences were observed between the disease groups.

No associations were found for disease duration, across the disease groups, with α-synuclein (p=0.110), tau (p=0.467) or Aβ (p=0.330) pathological burden.

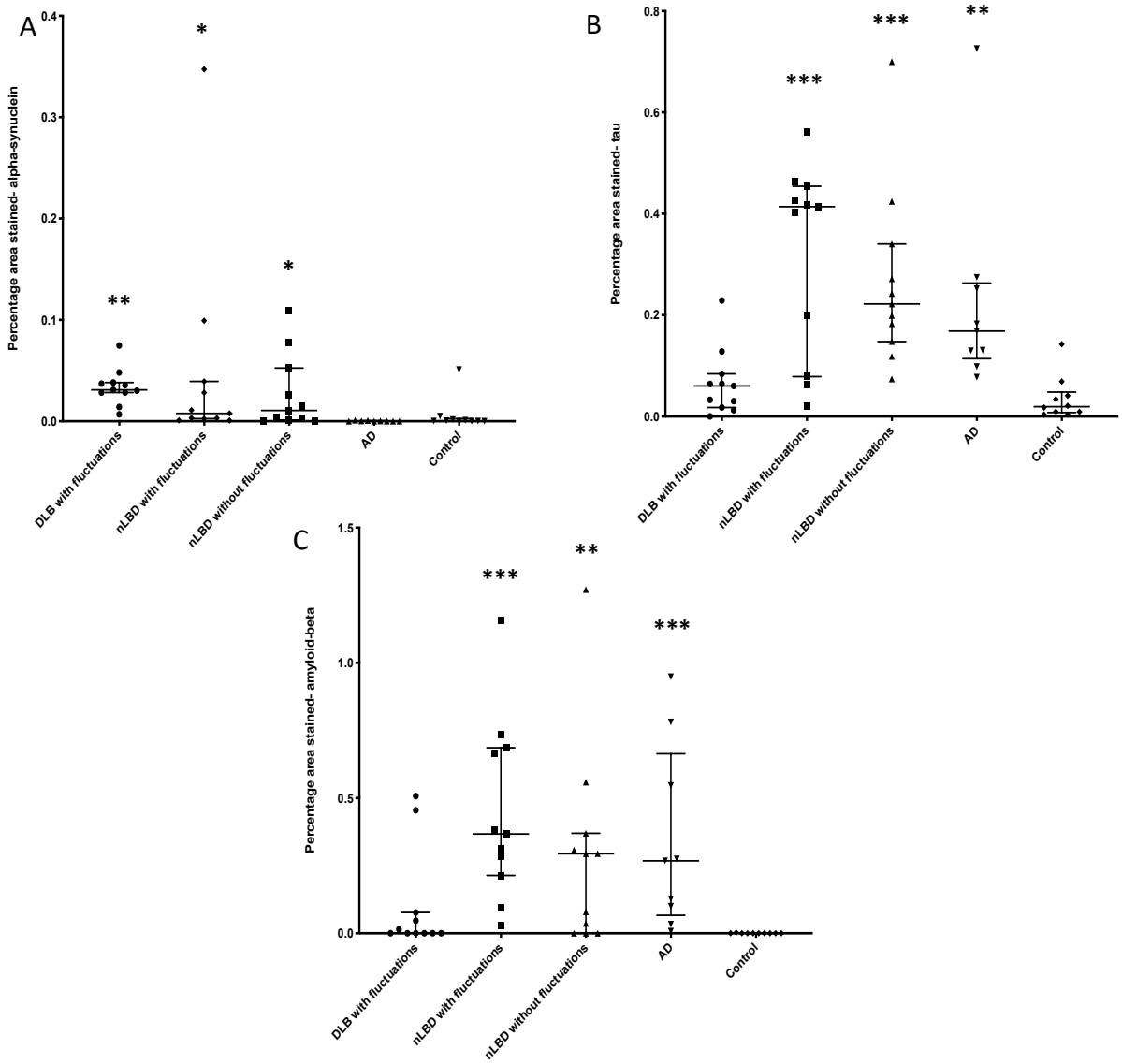


Figure 4.3. Pathology in the pedunculopontine nucleus.
Pathological burden data for A- α-synuclein, B- tau and C- Aβ in the pedunculopontine nucleus. Data is shown as median with interquartile range. ** p<0.01 ***p<0.001 compared to controls. Abbreviations: AD- Alzheimer's disease; DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease.

4.3.3 Relationship to locus coeruleus pathology

All cases that had pathological burden assessed for the PPN, also had the pathological burden assessed within the LC.

In the whole cohort, excluding the control group, Spearman's rank correlations revealed associations between pathological burden of α-synuclein, tau and Aβ in the LC and the pathological burdens in the PPN (table 4.2). PPN α-synuclein pathology was associated with LC pathological burden of α-synuclein positively (r_s=0.641, p<0.001), tau negatively (r_s=-0.530, p<0.001) and Aβ negatively (r_s=-0.432, p=0.004). PPN Aβ burden was associated with LC pathological burden of tau positively (r_s=0.321, p=0.038) and Aβ burden positively (r_s=0.386, p=0.012). PPN tau pathological burden was positively associated with the LC pathological burden of tau (r_s=0.445, p=0.003). No other associations between PPN pathological burden and LC pathological burden were associated (table 4.2).

When the fluctuation groups were assessed independently from each other no associations were observed between PPN pathological burden and LC pathological burden in the DLB, nLBD with cognitive fluctuations and AD cases (table 4.2). Within the nLBD without cognitive fluctuations group a negative association was observed between PPN α-synuclein pathology and LC Aβ pathology (r_s=-0.618, p=0.043), no other associations between PPN and LC pathological burdens were observed within the fluctuation group (table 4.2).

4.3.4 Relationship to cognitive fluctuations

No association was observed between α-synuclein, tau or Aβ pathological burden and severity of cognitive fluctuations as measured by CAF score, in all cases that during life possessed cognitive fluctuations (table 4.3). When fluctuation groups were separated into DLB and nLBD with fluctuations no associations were observed between PPN pathological burden and any of the measures of cognitive fluctuation severity, (table 4.3).

Table 4.4. Associations between pedunculopontine nucleus and locus coeruleus pathological burdens.
Data shown for the Spearman's rank correlation between PPN pathology and LC pathological burden for α-synuclein, tau and Aβ. Correlations shown for: All-whole cohort minus the control cases), DLB with cognitive fluctuations, nLBD with cognitive fluctuations, nLBD without cognitive fluctuations and AD cases. An Asterix indicates a significant result: * p<0.05 ** p<0.01 ***p<0.001. Abbreviations: AD- Alzheimer's disease; DLB- dementia with Lewy bodies; LC- locus coeruleus nLBD- neocortical/limbic Lewy body disease; PPN- pedunculopontine nucleus.

PPN Pathology	Cognitive fluctuation group	LC Pathology	Spearman's rank correlation
Alpha-synuclein	All	Alpha-synuclein	r_s=0.641, p<0.001 ***
		Tau	r_s=-0.530, p<0.001 ***
		Amyloid-beta	r_s=-0.432, p=0.004 **
	DLB with cognitive fluctuations	Alpha-synuclein	r_s=-0.510, p=0.109
		Tau	r_s=-0.116, p=0.734
		Amyloid-beta	r_s=-0.501, p=0.116
	nLBD without cognitive fluctuations	Alpha-synuclein	r_s=0.533, p=0.091
		Tau	r_s=-0.618, p=0.043 *
		Amyloid-beta	r_s=-0.333, p=0.318
	nLBD with cognitive fluctuations	Alpha-synuclein	r_s=0.418, p=0.201
		Tau	r_s=-0.500, p=0.117
		Amyloid-beta	r_s=-0.159, p=0.640
	AD without cognitive fluctuations	Alpha-synuclein	r_s=0.070, p=0.858
		Tau	r_s=0.522, p=0.149
		Amyloid-beta	r_s=0.226, p=0.558
Tau	All	Alpha-synuclein	r_s=0.079, p=0.620
		Tau	r_s=0.445, p=0.003 **
		Amyloid-beta	r_s=0.244, p=0.120
	DLB with cognitive fluctuations	Alpha-synuclein	r_s=0.373, p=0.259
		Tau	r_s=0.309, p=0.355
		Amyloid-beta	r_s=0.200, p=0.555
	nLBD without cognitive fluctuations	Alpha-synuclein	r_s=0.182, p=0.593
		Tau	r_s=-0.200, p=0.555
		Amyloid-beta	r_s=-0.093, p=0.786
	nLBD with cognitive fluctuations	Alpha-synuclein	r_s=0.064, p=0.853
		Tau	r_s=0.291, p=0.385
		Amyloid-beta	r_s=-0.378, p=0.252
	AD without cognitive fluctuations	Alpha-synuclein	r_s=0.268, p=0.486
		Tau	r_s=-0.067, p=0.865
		Amyloid-beta	r_s=-0.300, p=0.433
Amyloid-beta	All	Alpha-synuclein	r_s=-0.123, p=0.436
		Tau	r_s=0.321, p=0.038 *
		Amyloid-beta	r_s=0.386, p=0.012 *
	DLB with cognitive fluctuations	Alpha-synuclein	r_s=0.506, p=0.113
		Tau	r_s=-0.015, p=0.965
		Amyloid-beta	r_s=0.431, p=0.186
	nLBD without cognitive fluctuations	Alpha-synuclein	r_s=-0.532, p=0.092
		Tau	r_s=-0.147, p=0.667
		Amyloid-beta	r_s=0.080, p=0.816
	nLBD with cognitive fluctuations	Alpha-synuclein	r_s=-0.082, p=0.811
		Tau	r_s=0.091, p=0.790
		Amyloid-beta	r_s=0.005, p=0.989
	AD without cognitive fluctuations	Alpha-synuclein	r_s=0.000, p=1.000
		Tau	r_s=0.267, p=0.488
		Amyloid-beta	r_s=-0.183, p=0.637

Table 4.7. Associations between cognitive fluctuation severity and pedunculopontine pathology.
Spearman's rank correlations between pedunculopontine pathology and severity of cognitive fluctuations as measure by last, average and maximum CAF score. Associations were undertaken in dementia with Lewy bodies and neocortical/limbic Lewy body disease both combined and individually. Abbreviations: CAF- clinical assessment of fluctuation: DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease; r_s- spearman's rank coefficient.

Measurement of cognitive fluctuation severity	Cognitive fluctuation group	Pathology	Cognitive fluctuation severity
Last CAF	nLBD and DLB with cognitive fluctuations	α-synuclein	r_s=0.244, p=0.380
		Tau	r_s=0.233, p=0.403
		Aβ	r_s=-0.095, p=0.737
	DLB with cognitive fluctuations	α-synuclein	r_s=0.037, p=0.937
		Tau	r_s=-0.206, p=0.658
		Aβ	r_s=-0.208, p=0.655
	nLBD with cognitive fluctuations	α-synuclein	r_s=0.421, p=0.298
		Tau	r_s=0.511, p=0.196
		Aβ	r_s=-0.243, p=0.563
Average CAF	nLBD and DLB with cognitive fluctuations	α-synuclein	r_s=0.384, p=0.157
		Tau	r_s=0.289, p=0.296
		Aβ	r_s=0.016, p=0.954
	DLB with cognitive fluctuations	α-synuclein	r_s=0.180, p=0.699
		Tau	r_s=0.072, p=0.878
		Aβ	r_s=0.055, p=0.908
	nLBD with cognitive fluctuations	α-synuclein	r_s=0.551, p=0.157
		Tau	r_s=0.359, p=0.382
		Aβ	r_s=-0.192, p=0.649
Maximum CAF	nLBD and DLB with cognitive fluctuations	α-synuclein	r_s=0.225, p=0.420
		Tau	r_s=0.284, p=0.305
		Aβ	r_s=0.167, p=0.553
	DLB with cognitive fluctuations	α-synuclein	r_s=-0.279, p=0.545
		Tau	r_s=0.378, p=0.402
		Aβ	r_s=0.563, p=0.188
	nLBD with cognitive fluctuations	α-synuclein	r_s=0.332, p=0.422
		Tau	r_s=0.485, p=0.223
		Aβ	r_s=-0.013, p=0.976

4.3.5 Relationship to parkinsonian subtype

In the cases that had been classified as being either tremor predominant or postural instability gait difficulty parkinsonian subtype, there was no difference in the level of PPN α-synuclein (p=0.376), tau (p=0.194) or Aβ (p=0.376) pathological burden observed between the two subtypes (figure 4.4A-C).

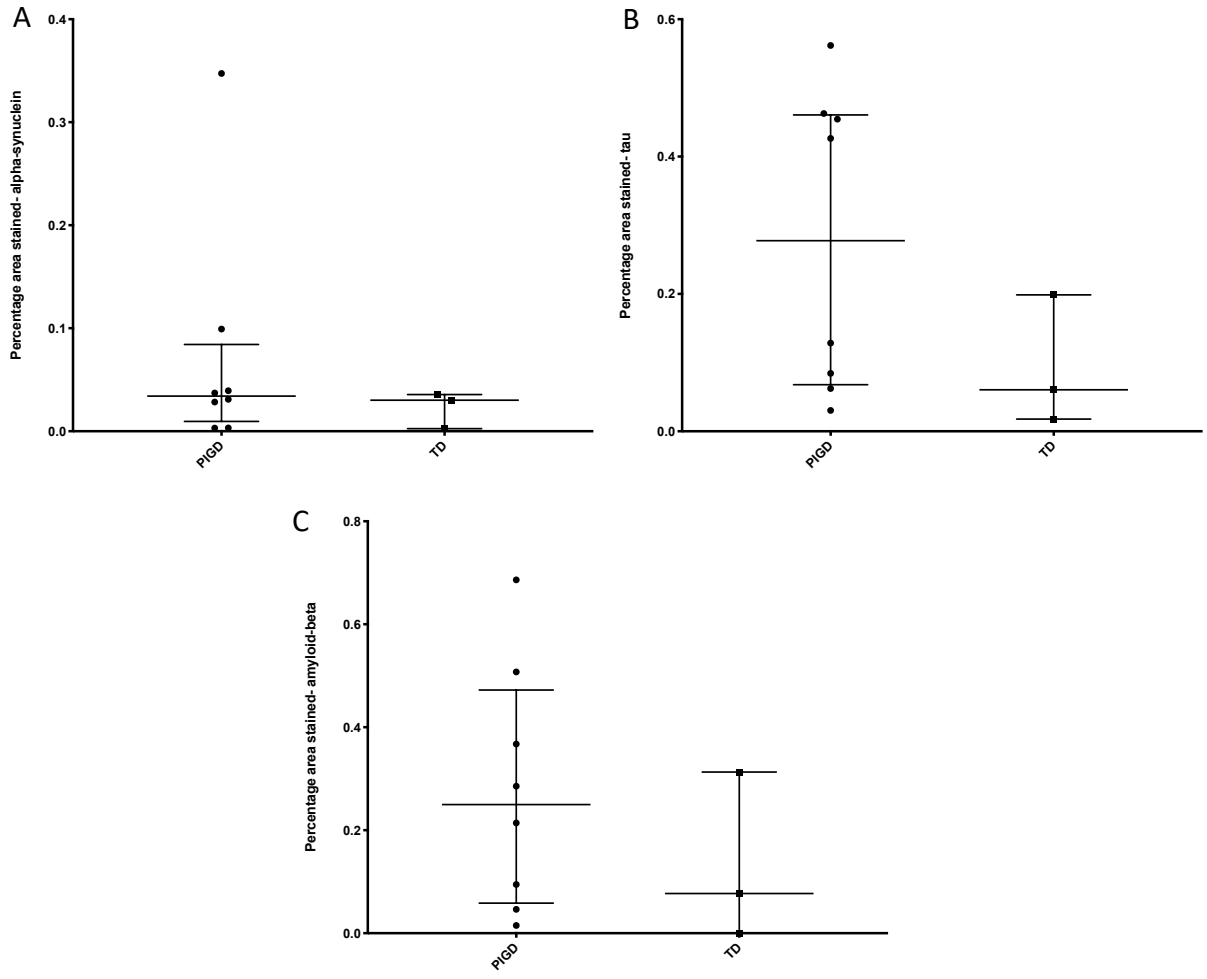


Figure 4.4. Pedunculopontine nucleus pathology in parkinsonian subtypes.
Pathological burden for A- α-synuclein, B- tau and C- Aβ between the PIGD and TD parkinsonian subtypes.
Abbreviations: PIGD- postural instability gait difficulty; TD- tremor predominant.

4.4 Discussion

The present study identified no relationship between pathological burden of α-synuclein, tau and Aβ, and the presence of cognitive fluctuations. Similarly, no association was found between pathological burden and the severity of cognitive fluctuations as measured by CAF scores in either the combined or separate DLB and nLBD with cognitive fluctuation groups. However, associations were found between that pathological burdens in PPN and those in the LC within the whole cohort, excluding controls. Although, the majority of these associations were lost when the cohort was split into individual fluctuation groups. Furthermore, the study identified no difference in the PPN pathological burden between those classified as having a PIGD or TD parkinsonian subtype.

4.4.1 Pathology in the PPN does not relate to the presence or severity of cognitive fluctuations

Previous studies have investigated the vulnerability of the PPN to accumulation of pathological proteins and neuronal loss in a number of neurodegenerative diseases, including AD and LBD (Rinne *et al.*, 2008b; Dugger *et al.*, 2012; Seidel *et al.*, 2015). The results from the current study are in agreement with previous findings that the PPN is vulnerable to α-synuclein and AD-type pathology. However, the present study extends the current findings to include the pathological vulnerability of the region in those diagnosed with mixed AD/DLB, with data showing that the region is vulnerable to all three pathological proteins investigated in these cases.

The PPN has been described as a key centre in the ARAS, and is most active during waking and REM sleep (Jenkinson *et al.*, 2009; Benarroch, 2013). Alterations to the PPN have been implicated in a number of clinical phenotypes including cognitive fluctuations, PIGD subtype and RBD; with an MRI study identifying an association with volumetric changes in the PPN and visual hallucinations in PD and PDD (Janzen *et al.*, 2012). A previous retrospective study undertaken by Dugger *et al.* (2012) examined the role of α-synuclein and tau pathology in the PPN and LC in regards to RBD. The study identified no differences in the pathological burden between LBD cases with and without RBD, similar to the results from our study where no difference was observed in pathological burden between cases with and without cognitive fluctuations. As suggested by Dugger *et al.* (2012), although the PPN is a key component within the systems maintaining atonia during sleep, as well as maintaining the

high frequency EEG required for arousal, it could be possible that the clinical phenotype of RBD, or in this case cognitive fluctuations could be a product of a network dysfunction. Dysfunction to the PPN could be a factor but not a key role within the dysfunctional network suggesting why no associations were observed between pathological burdens and the presence of cognitive fluctuations.

Rodent models have also identified that lesions of the PPN can elicit fluctuations in consciousness (Cyr *et al.*, 2015). However, these lesions were acute in nature and do not represent the chronic and progressive nature of changes seen in neurodegenerative diseases. Whereas, other studies have identified that lesions to the PPN do not have the effect on wakefulness as would be expected, due to there being compensation from other regions, with lesions affecting REM and SWS but not affecting the ability of the PPN to maintain high frequency EEG activity which is required for the maintenance of arousal and waking (Mahaffey and Garcia-Rill, 2015). The lesions are also not known to affect higher cognitive functions (Mahaffey and Garcia-Rill, 2015). These studies further suggest that single lesions may not be the sole underpinning factors for complex clinical phenotypes and that network disturbances may be the main factor.

The PPN is a highly interconnected nucleus (Mahaffey and Garcia-Rill, 2015). The midlatency auditory P50 potential is functionally related to arousal state and has been postulated to be a proxy measure of PPN activity (Hyde and Garcia-Rill, 2015). Dysfunction to PPN activity has been identified in PD patients, as studies have identified increases to the amplitude and decreased habituation of the P50 potential that suggest overactivation of the PPN (Teo et al., 1997). A further study has demonstrated the P50 potential returned to normal levels immediately after a bilateral pallidotomy, which could indicate that the increased PPN output was reinhibited and restored to normal levels (Teo *et al.*, 1998). With no pathological findings within the PPN having been found to relate to the presence of cognitive fluctuations it could be that dysfunction to the PPN is in part caused by dysfunction to its inputs including the LC, which synapses with both the cholinergic and non-cholinergic neurons in order to control activity levels in the PPN. The main output centre for the PPN arousal-mediating neurons is the thalamus. A number of different studies have identified molecular alterations, including increased thalamic dopamine D2 receptor levels in DLB with parkinsonism compared to those without, and structural connectivity differences, with

patients with more severe fluctuations related to greater connectivity to the left thalamus and decreases connectivity for the right thalamus (Chabran *et al.*, 2020), to the thalamus in DLB. Studies have observed alterations to the cholinergic system in the thalamus relating to cognitive fluctuations; including preservation of thalamic nicotinic receptors in those with fluctuations compared to those without (Pimlott *et al.*, 2006) and increased levels of acetylcholine precursors which were closely related to the presence and severity of fluctuations (Delli Pizzi *et al.*, 2015). Studies have also noted that loss of cholinergic neurons in the PPN leads to a decrease in cholinergic innervation to the thalamus (Francis and Perry, 2007). From these studies it could be suggested that neurodegenerative and molecular alterations occur to PPN cholinergic neurons both within the PPN and its synaptic regions that relate to the presence and severity of cognitive fluctuations. However, these changes may be independent from the pathological protein accumulation within the region. Quantification of cholinergic neuronal number was not undertaken within the current study and thus neuronal counts would be required to assess whether pathological burden was related to loss of neurons within the region.

4.4.2 PPN pathology relates to LC pathology in the whole cohort but not in individual groups

When the whole cohort, excluding the controls, was analysed a number of relationships between the level of pathological proteins in the PPN and LC were identified. However, upon examination of the fluctuation groups individually the majority of these relationships were lost, with only an association between PPN α-synuclein and LC tau in the nLBD without cognitive fluctuations group remaining. The likely cause of the associations observed in the whole cohort are group effects due construction of the cohort, with pathologically AD cases having higher AD-type pathology in both the LC and PPN compared to DLB cases. These group differences would drive an apparent association which was then lost upon examination of the groups separately. The association observed within the individual nLBD without cognitive fluctuations group could again be driven by the fact that the group is formed by both mixed AD/DLB and DLB cases who did not possess cognitive fluctuations clinically. As DLB and mixed AD/DLB, within the current study had been shown to have significantly different levels of PPN and also LC pathology (Chapter 3) this could have driven the association observed between α-synuclein and tau. The splitting of these groups into

pathological mixed AD/DLB and DLB would identify whether this association is driven by group factors.

The LC is highly interconnected with the PPN, with the PPN receiving both excitatory and inhibitory projections from the PPN in order to control PPN neuronal firing (Mahaffey and Garcia-Rill, 2015). With the PPN and LC thought to be vulnerable to α-synuclein at an early stage of disease progression it is surprising that pathology within the two nuclei does not progress at a similar rate. With neuronal loss known to occur in both the PPN and LC in a number of neurodegenerative dementias (Dugger *et al.*, 2012), neurons bearing α-synuclein pathology could have died leading to a loss of relationship between the pathological burden in the two nuclei in pathologically DLB cases.

4.4.3 PPN pathology does not distinguish parkinsonian subtypes

Loss of cholinergic neurons within the PPN has been previously described to associated with gait and postural parkinsonian symptoms (Karachi *et al.*, 2010); with gait and postural symptoms resistant to levodopa treatment unlike tremor symptoms which have been proposed to be due to nigrostriatal dopaminergic denervation (Grabli *et al.*, 2012). The results from the current study identified no difference in the pathological burden of α-synuclein, tau or Aβ in the PPN between those classified as either having the PIGD or TD subtype.

The underlying differences between the TD and PIGD subtypes has been a focus for PD-related research for a number of years. However, the classification of the symptoms into two subgroups has not been without controversy. A number of studies have investigated the progression and pathological differences between the two subgroups with varying results. Studies have reported that patients with a TD subtype have a better prognosis and mild progression rate compared to those with a PIGD subtype (Lee *et al.*, 2019), which could suggest a more 'pure' dopaminergic pathology in those classified as TD. However, studies investigating the pathologies have identified differing and conflicting results about dopaminergic dysfunction between the two subtypes, with more dopaminergic dysfunction observed via imaging in PIGD groups than TD at baseline (Lee *et al.*, 2019). Together the data from Lee *et al*. (2019) could suggest that differences between the two subtypes may not be as clear cut as dopaminergic vs cholinergic dysfunction, which could explain why no difference was observed in pathological burden between the two subtypes in the current

study. Alterations to DaTSCAN ligand binding have been shown to reflect changes to neuronal number and not pathological burden in the SN (Colloby *et al.*, 2012), with further studies observing no relationship between imaging findings and Braak stage (Burke *et al.*, 2008; Lee *et al.*, 2019) and no associations between α-synuclein pathology and motor dysfunction (Parkkinen *et al.*, 2005). From these studies it could be suggested that loss of PPN cholinergic neurons, which have previously been shown to relate to gait impairments (Rinne *et al.*, 2008b), could differ between the two subtypes, irrespective of the level of pathological burden. However, due to the group numbers utilised and lack of neuronal counting, a follow-up study with large cohort numbers and assessment of cholinergic neuronal numbers in the TD and PIGD groups are needed to validate this result. It could also be suggested that it is the balance between dopaminergic and cholinergic deficits that underlie the PIGD and TD subtypes and thus further studies investigating the ratio between SN and PPN neurodegenerative changes are required.

4.4.4 Limitations

The most influential caveat of this study was the relatively small cohort, 56 cases across five groups. Although quantitative neuropathological assessment provides an accurate representation of the pathological burden, large cohorts are required to obtain significant statistical results. Therefore, the results should be confirmed utilising a larger cohort. Correlations within the current study were not corrected for multiple comparisons. Although corrections such as Bonferroni adjustment can reduce type I error, it is considered too conservative to be utilised in certain cases where it can increase the likelihood of type II errors (Armstrong, 2014). In the current study corrections would have been too conservative, previous studies have inferred relationships between pathology in the PPN and LC due to their close proximity and inter connectivity, along with previously described patterns of spread for pathological proteins (Braak *et al.*, 2003; Braak *et al.*, 2006; Braak *et al.*, 2011).

A limitation to a retrospective anatomical study was that stereological analysis of the entire region was not possible. The PPN is known to be heterogeneous structure and different locations within the PPN project to different anatomical locations and have differing cognitive and motor effects. This has been exemplified by the fact PPN DBS does not have the same outcome when different regions of the nucleus are stimulated (Hamani *et al.*,

2016). Although the pathological findings are in agreement with previous studies (Dugger *et al.*, 2012), the lack of a consistent level analysed within the region could explain why no relationship was observed with cognitive fluctuations. Lack of neuronal count data also limited the study's ability to relate neuropathological findings to changes in neuronal number and clinical data which would have further strengthened the study. Future studies should aim to utilise a single ChAT antibody using a stereological method to assess neuronal levels within cases that have experienced cognitive fluctuations.

The cohort utilised within this study was limited as it required tissue samples that had information relating to cognitive fluctuations status obtained during life. With the structuring of the cohort related to cognitive fluctuations as well as it being a retrospective study not all cases had additional clinical data including parkinsonian subtype. This led to a smaller subset of cases being utilised for investigation into parkinsonian phenotypes. Secondly due to the remit of the study, the pathological burden within the SN was not investigated which would have strengthened the study in relation to the role of the cholinergic system in the PIGD subtype, with the possible calculation of the relative dopaminergic to cholinergic dysfunction. Although a limiting factor to further examine this by increasing the number of TD cases is that pathways involved in cognitive impairment are thought to overlap with those impaired in the PIGD subtype, meaning DLB or mixed AD/DLB cases are more likely to be classified as PIGD (Zuo *et al.*, 2017), with TD cases slower to progress to dementia (B. Tilley 2020, personal communication, 22nd May 2020).

4.4.5 Conclusions

The study aimed to investigate the pathological difference in the PPN between cases that clinically presented with cognitive fluctuations and those that did not. Along with investigating the difference in pathological burden in the PPN between TD and PIGD parkinsonian subtypes.

The data showed pathological differences in the PPN related to the pathological diagnosis of the cases, with no changes specifically related to the presence or absence of cognitive fluctuations. This could suggest that cognitive fluctuations could be the result of more widespread changes within the brain or changes that are 'upstream' of the PPN that are independent from PPN pathological burden, with changes having already been observed to the cholinergic system in the thalamus (Pimlott *et al.*, 2006; Delli Pizzi *et al.*, 2015). The data

also showed that there were no pathological differences in the PPN between those classified as having the TD or PIGD subtype.

Chapter 5: Pathological studies in the raphe nucleus

5.1 Introduction

The rostral raphe, including the DR and MnR subnuclei, has been proposed to play an important role in the maintenance of sleep and wake states (Monti, 2011), and forms part of the ventral ARAS pathway (Zeman, 2001). Serotonergic neurons in the DR are thought to fire maximally during waking and cease during REM sleep (Trulson and Jacobs, 1979; Mahaffey and Garcia-Rill, 2015), indicating that the nucleus promotes wakefulness. However, studies have identified lesions of the raphe can elicit states of permeant wakefulness (Jouvet, 1968), with depletion of cortical serotonin having a sedative effect (Brodie *et al.*, 1955), suggestive that the raphe plays a complex role in the maintenance of arousal states. Alterations to sleep-wake states, including insomnia, increase in proportion of REM sleep and reduction in slow-wave sleep duration, have been observed in serotonergic system disturbances, including depression (Murphy and Peterson, 2015); with antidepressant drugs, including selective serotonin reuptake inhibitors, altering the levels of arousal, sleeping efficiency (Dorsey *et al.*, 1996) and exacerbating (Gagnon *et al.*, 2006) or inducing RBD symptoms (Schenck *et al.*, 1992).

In DLB, clinical features, including the presence of depression and anxiety, would imply serotonergic dysfunction, including RBD, depression and alterations to arousal levels, are suggestive of serotonergic dysfunction (McKeith *et al.*, 2017). Pathologically the raphe is affected by Lewy body pathology at Braak stage 2, the same timepoint as the LC (Braak *et al.*, 2003), with detectable alterations to the neurotransmitters thought to be present at this stage (Boeve, 2013). Degeneration of both DR and MnR neurons have been reported in DLB, although this loss was not related to any clinical features, including depression (Benarroch *et al.*, 2007). Alterations to regions that the serotonergic system innervates, including atrophy of the basal forebrain (Grothe *et al.*, 2014) and volumetric reductions in the hypothalamus associated with changes to arousal (Whitwell *et al.*, 2007), have been identified in DLB. Despite the link between the raphe, serotonergic system and arousal, no neuropathological studies have investigated the role of the raphe in cognitive fluctuations in DLB. Therefore, the present study aimed to assess neuropathological changes to the raphe that could relate to the presence and severity cognitive fluctuations.

5.1.1 Aims

Using *post-mortem* tissue sections containing the raphe from DLB cases with fluctuations; nLBD cases, both with and without cognitive fluctuations; AD cases without cognitive fluctuations; PDD cases and aged cognitively normal controls, this study aims to:

1. Quantify the burden of neuropathological protein lesions, utilising immunohistochemical techniques for Aβ, tau and α-synuclein
2. Quantify the expression levels of TPH2, utilising immunofluorescence techniques
3. Assess whether the neurodegenerative changes seen are related to the presence or severity of cognitive fluctuations, utilising neuropsychiatric data obtain *intra vitam*

5.2 Methods

5.2.1 Study Cohort

5.2.1.1 Raphe pathology and immunofluorescence cohort

Five groups were included in the raphe pathology study and the immunofluorescent analysis with a total of 65/47 cases respectively (table 2.1): 13/10 DLB cases with cognitive fluctuations; 13/10 nLBD cases without cognitive fluctuations; 10/7 AD cases without cognitive fluctuations; 12/10 nLBD with cognitive fluctuations; and 17/10 aged matched controls with no history of cognitive impairment. One DLB case with a history of cognitive fluctuations underwent immunofluorescence analysis but did not undergo pathological analysis in the raphe.

Within the study cohort a number of those with a history of cognitive fluctuations had the severity of these measured via CAF. For the raphe pathology analysis, 8 DLB with cognitive fluctuations and 9 nLBD with cognitive fluctuations; within the raphe immunofluorescence subset CAF scores were available for 9 DLB with cognitive fluctuations and 9 nLBD with cognitive fluctuations, allowing associations with severity of the fluctuations to be assessed.

Within the cases that had a history of cognitive fluctuations, in both the main cohort and subset utilised for immunofluorescence, 16 cases had neuropsychiatric scores relating to depression severity. 9 DLB and 7 nLBD with fluctuations had at least one Geriatric depression scale (GDS) score available. The average GDS score was calculated for each case. Average GDS score was utilised due to the large variation in time from last GDS score till death, as well as taking into account any fluctuations in depression severity if multiple scores were recorded.

5.2.2 Tissue Acquisition

The raphe nuclei is a collection of sub-nuclei located along the entire rostro-caudal aspect of the brainstem (Hornung, 2003). Sections for analysis were taken from paraffin embedded formalin fixed blocks containing the upper mid pons, as described in 2.4.3 and 2.4.5.

5.2.2.1 Raphe localisation

The raphe nuclei, DR and MnR, were identified within sections by immunostaining against the serotonin synthesis enzyme tryptophan hydroxylase, as outline in 2.5. Sections were stained with antibodies against tryptophan hydroxylase 1 and TPH2 (figure 5.1), to assess which marker would best identify the serotonergic neurons of the raphe. TPH2 was identified as the best marker for the raphe, consistent with the fact TPH2 is the more abundant isoform expressed in the brain (Hasegawa and Nakamura, 2010). Following the validation of TPH2 all sections underwent TPH2 IHC to identify the raphe. The raphe nuclei, both the DR and MnR were identified in a total of 65/67 cases.

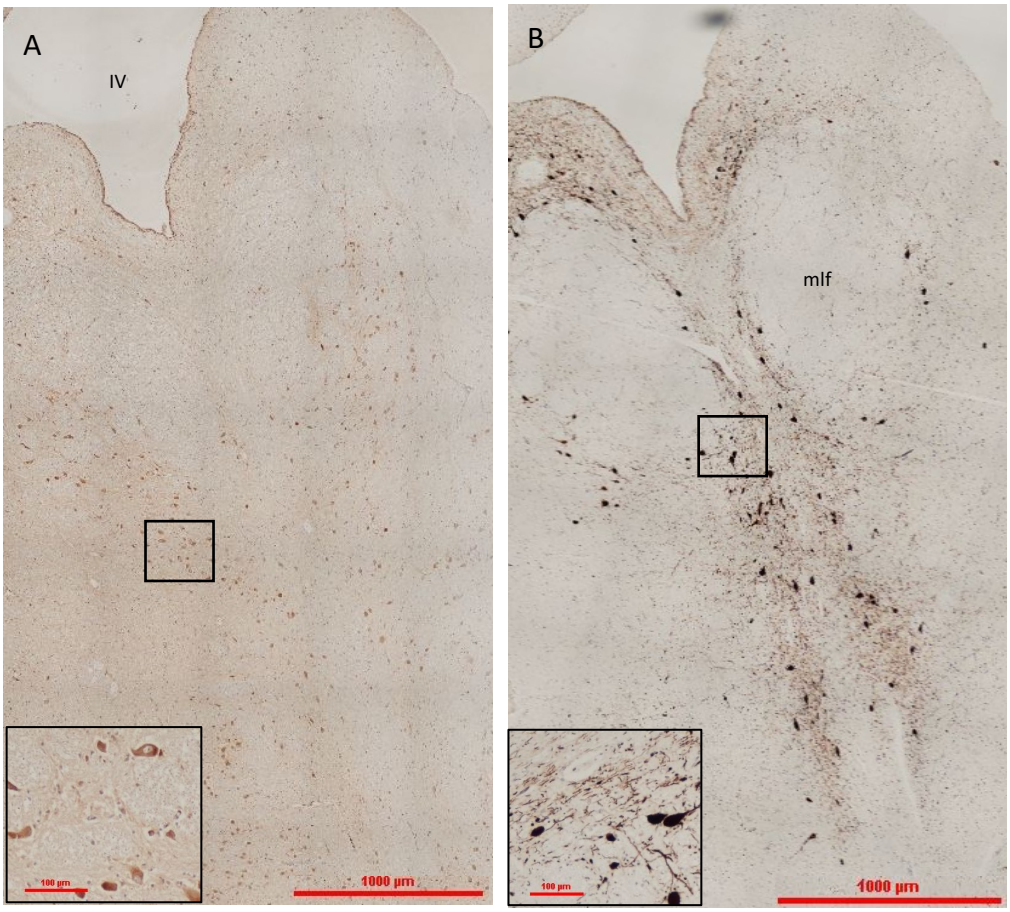


Figure 5.1. Representative tryptophan hydroxylase 1 and 2 positive staining.
Representative images of A- tryptophan hydroxylase 1 and B- tryptophan hydroxylase2 in the upper pons. Scale bar 1000µm, inset 100µm. IV- IV ventricle mlf- medial longitudinal fasciculus

5.2.3 Pathology

Consecutive sections, following the section assessed with TPH2 identifying the raphe, underwent IHC staining, as described in 2.5, with antibodies against hyperphosphorylated tau (AT8), Aβ (4G8) and α-synuclein (KM51) (table 5.1). A large image, with an area of 46.1mm^2, was created by capturing and stitching 112 adjacent, 8x14, single images at x100 magnification using NIS-Elements AR3.2 software (Nikon, Surrey), a Nikon 90i microscope, with a fully motorised stage, and DsFi1 camera microscope coupled to a PC.

Stitched images were analysed using a standardised red-green-blue threshold on NIS elements AR3.2 software. Thresholds were determined for AT8, 4G8 and KM51 separately to detect only immunopositive signals, without detection of non-specific background. The threshold for 4G8 also included a size threshold to ensure intracellular Aβ, APP, was excluded from the analysis. The raphe was delineated on the pathology sections by comparison to the TPH2 section prior to application of the threshold (figure 2.6). The DR and MnR were delineated and analysed as a single unit. A further threshold was determined for TPH2 to detect immunopositive signals, to assess the percentage area stained for the serotonin synthesis enzyme.

Table 5.1. Antibodies utilised in the raphe study.
Optimised dilutions and antigen retrieval protocols for the antibodies utilised in the raphe study. Abbreviations: IF- immunofluorescence; IHC- immunohistochemistry.

Antibody	Manufacturer	Dilution	Antigen Retrieval
AT8, hyperphosphorylated tau	Autogen, MA, USA	1:4000	0.1M Citrate pH 6.0
4G8, Aβ	Covance, NJ, USA	1:15000	Formic acid (1 hour)
KM51, α-synuclein	Leica Biosystems, UK	1:200	Formic Acid (10 minutes), 1mM EDTA pH 8.0
TPH1, tryptophan hydroxylase I	Merck, Darmstadt, Germany	1:200	0.1M Citrate pH 6.0
TPH2, tryptophan hydroxylase II	Atlas antibodies, Bromma, Sweden	1:1000 IHC 1:250 IF	0.1M Citrate pH 6.0
pS129, α-synuclein phosphorylated at Serine 129	Abcam, UK	1:200	0.1M Citrate pH 6.0

5.2.3.1 LC and PPN pathology

All cases within the raphe pathology study also had the pathological burdens for hyperphosphorylated tau, Aβ and α-synuclein assessed and analysed in the LC, as described in 3.2.3. 50/65 cases also had neuropathology evaluated in the PPN: 10 DLB with cognitive fluctuations, 11 nLBD without fluctuations, 11 nLBD with fluctuations, 8 AD and 10 controls, as described in 4.2.3.

5.2.4 Immunofluorescence

A consecutive section to those that were assessed for pathological staining underwent double immunofluorescent labelling, as described in 2.7, for TPH2 and α-synuclein (pS129) (table 5.1).

Sections were manually imaged at x100 magnification, captured using a Nikon 90i microscope with a fully motorised stage, and DsQi1Mc camera microscope coupled to a PC. Images obtained covered the whole raphe, including the DR and MnR. Sections were imaged twice, firstly to obtain a TPH2 (FITC/green channel) only image for analysis of TPH2 intensity. Secondly the section was captured to obtain a merged (FITC, TRITC and DAPI channels) image to identify TPH2-positive neurons that contained Lewy bodies. The multiple adjacent images, both single channel and merged, were then manually stitched together based upon overlapping landmarks to create a large single image of the region of interest (figure 2.8).

5.2.4.1 Intensity analysis of immunofluorescence

Individual TPH2-positive neuron intensity was assessed within the raphe as outlined in 2.8.2.1. Individual TPH2-positive neurons were delineated with the area integrated intensity and mean grey value measured. The number of TPH2-positive neurons delineated per case ranged from 16-688, with a mean of 192. Background measurements from 30-40 areas within the raphe were taken to provide an average background measurement for the region of interest. The corrected total cell fluorescence for each individual TPH2-positive neuron was calculated using the following formula:

$$\frac{\text{integrated density} - (\text{area of selected cell x mean fluorescence of background readings})}{\text{area of selected neuron}}$$

The corrected TPH2 intensity was adjusted for area to account for atrophic neurons. The median corrected TPH2 intensity for all assessed TPH2-positive neurons was calculated for each individual section analysed.

z-scores were calculated to assess variability within the corrected TPH2 intensity values across all the disease groups and controls. As the corrected TPH2 intensity data was not normally distributed the data was log transformed to a natural logarithm (log_{10}). The mean and standard deviation for the log transformed corrected TPH2 intensity data for all control cases that underwent immunofluorescent analysis was calculated. The individual z-scores were then calculated using the following formula, where the mean and standard deviation are those calculated from the control data:

$$\frac{log10(corrected\ TPH2\ intensity) - \ mean}{standard\ deviation}$$

A z-score was calculated for each TPH2-positive neuron analysed. The proportion of neurons in each case that had a z-score less than -1 and less than -2 was then calculated.

5.4.4.2 Analysis of colocalization

Utilising the merged image, the total number of TPH2-positive within the section were counted and the percentage bearing α-synuclein calculated (figure 2.9). Through comparison of the merged image to the green, TPH2, only, corrected TPH2 intensity adjusted for area was calculated for the TPH2-positive neurons containing Lewy bodies. The cytoplasm of the Lewy body containing neurons was also delineated, to calculate the corrected TPH2 intensity adjusted for area of the cytoplasm of TPH2-positive neurons bearing Lewy bodies, (figure 5.2).

A ratio was calculated to calculate the magnitude of difference in the median corrected TPH2 intensity value for the TPH2-postive neurons without Lewy bodies and the median corrected TPH2 intensity value for the TPH2-postive neurons with Lewy bodies. A further ratio was calculated between the median corrected TPH2 intensity value for the TPH2-postive neurons without Lewy bodies and the median corrected TPH2 intensity value for the part of the neuron that the Lewy body did not occupy in Lewy body bearing TPH2-postive neurons. For the two ratios calculated a value of 1 would suggest that the corrected TPH2 intensity values are the same for the TPH2-positive neurons without Lewy bodies and those with Lewy bodies, including or excluding the Lewy body respectively. A ratio value >1 would suggest the corrected TPH2 intensity value is higher in the TPH2-positive neurons with Lewy bodies. A ratio value <1 would suggest the corrected TPH2 intensity value is higher in TPH2-positive neurons without Lewy bodies.

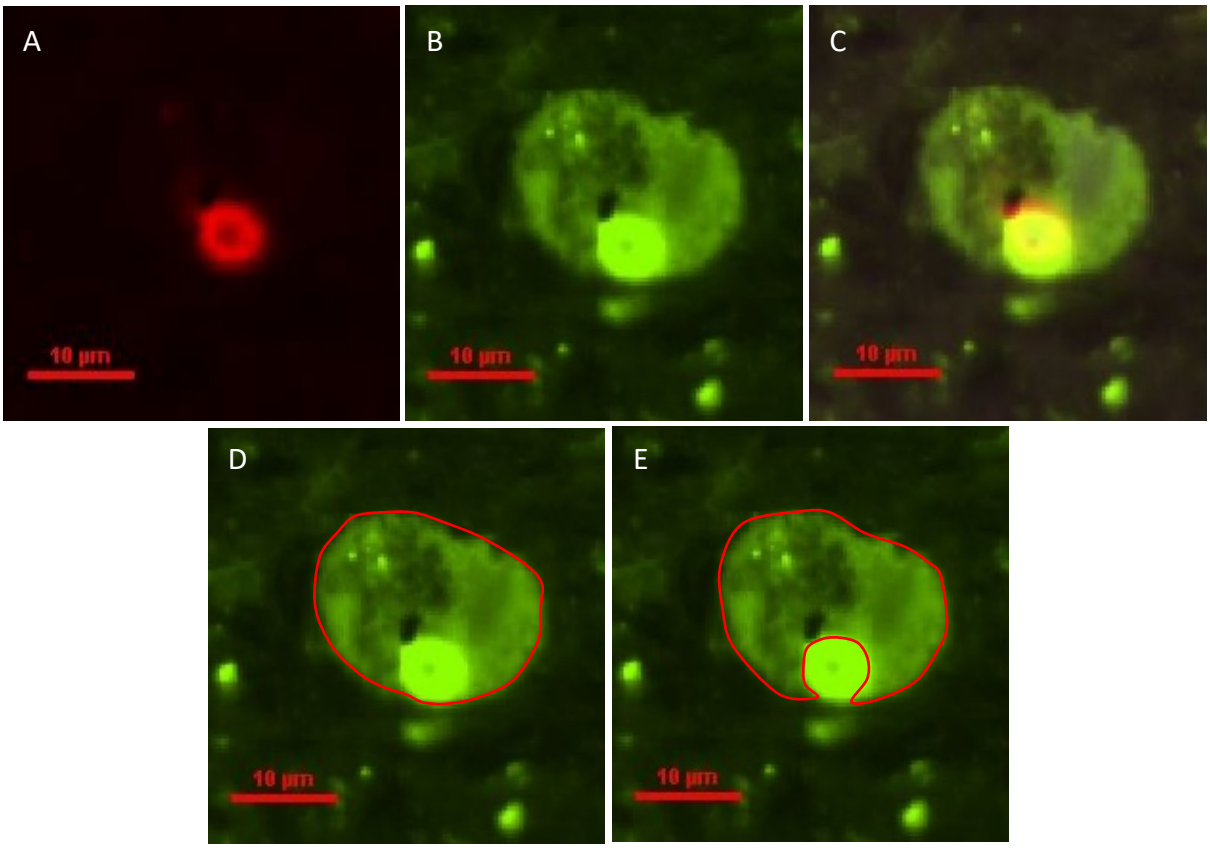


Figure 5.2. Analysis of α-synuclein and tryptophan hydroxylase 2 colocalization.
A- ps129 only, B- TPH2 only, C- merge all channels: DAPI, pS129 and TPH2, D- delineation of the Lewy body containing TPH2-positive neuron, E- delineation of the cytoplasm of TPH2- positive neuron bearing a Lewy body. Scale bar 10µm.
Abbreviations: pS129- α-synuclein phosphorylated at serine 129; TPH2- tryptophan hydroxylase 2.

5.2.6 Statistical analysis

Statistical analysis was conducted using SPSS v.26 (IBM). Variables were assessed for normality by the Shapiro-Wilk test and inspection of histograms and Q-Q plots. A normal distribution could be assumed for the two ratios and the proportion of TPH2-positive neurons that contain Lewy bodies. Therefore, on the normally distributed data ANOVA was conducted with Tukey's HSD as the post-hoc test. However, pathology data, corrected TPH2 intensity data, and the z-scores were found to be non-normal leading to non-parametric tests being employed. To determine differences in the non-normally distributed data Kruskal-Wallis and post-hoc Mann-Whitney were undertaken. A one-sample Wilcoxon signed rank test was utilised to determine whether the corrected TPH2 intensity ratios calculated deviated from a median of 1.

To determine whether there was an association between any pathology measures or immunofluorescence intensity and neuropsychological data obtained *intra vitam* Spearman's rank correlations were conducted. Corrections such as Bonferroni were not

applied due to the fact that there is not a true null hypothesis because of the classification of neurogenerative diseases, for example AD will have higher levels of AD-type pathology than DLB, therefore a p-value of <0.05 was considered significant.

5.3 Results

5.3.1 Raphe pathology

5.3.1.1 Demographics

No significant differences were observed in *post-mortem* delay, fixation duration, and age between the study groups. There was no significant difference in disease duration between the neurodegenerative disease groups. Between the groups a significant difference was found in the proportion of males to females, with the DLB group have a significantly higher proportion of males compared to nLBD without fluctuations ($\chi(1)=4.248$, $p=0.039$ and controls ($\chi(1)=5.792$, $p=0.016$). No further differences in the proportion of males to females were observed.

CAF scores were available for 24/65 cases. 17/25 of the cases with cognitive fluctuations possessing at least one CAF score. No significant difference was observed between the last ($p=0.728$), maximal ($p=0.592$) or average ($p=0.837$) CAF score between the DLB with fluctuations or the nLBD with fluctuations groups.

Final MMSE scores were available for 53/65 cases: 12 DLB cases with fluctuations, 12 nLBD with fluctuations, 12 nLBD without fluctuations, 9 AD and 9 Controls. No significant difference in the interval between the last MMSE and death was observed between the groups. Significantly lower last MMSE scores were observed between all disease groups and controls: DLB cases with fluctuations ($z=-20.889$, $p=0.002$), nLBD with fluctuations ($z=27.222$, $p<0.001$), nLBD without fluctuations ($z=-25.237$, $p<0.001$) and AD cases ($z=-26.056$, $p<0.001$). No significant differences in last MMSE score were observed between the four disease groups.

At least one GDS score was available for 29/65 cases: 9 DLB cases with fluctuations, 7 nLBD with fluctuations, 3 nLBD without fluctuations, 3 AD and 7 Controls. No significant difference in average GDS score was observed across the groups within the study.

5.3.1.2 Pathology

α-synuclein pathology was observed in all DLB cases with cognitive fluctuations, most frequently as Lewy bodies (figure 5.3B). A significant main effect of disease group was observed on α-synuclein burden in the raphe ($\chi^2=34.538$, $p<0.001$) (figure 5.4A). α-synuclein pathological burden was higher in DLB with fluctuations ($z=34.527$, $p<0.001$), nLBD

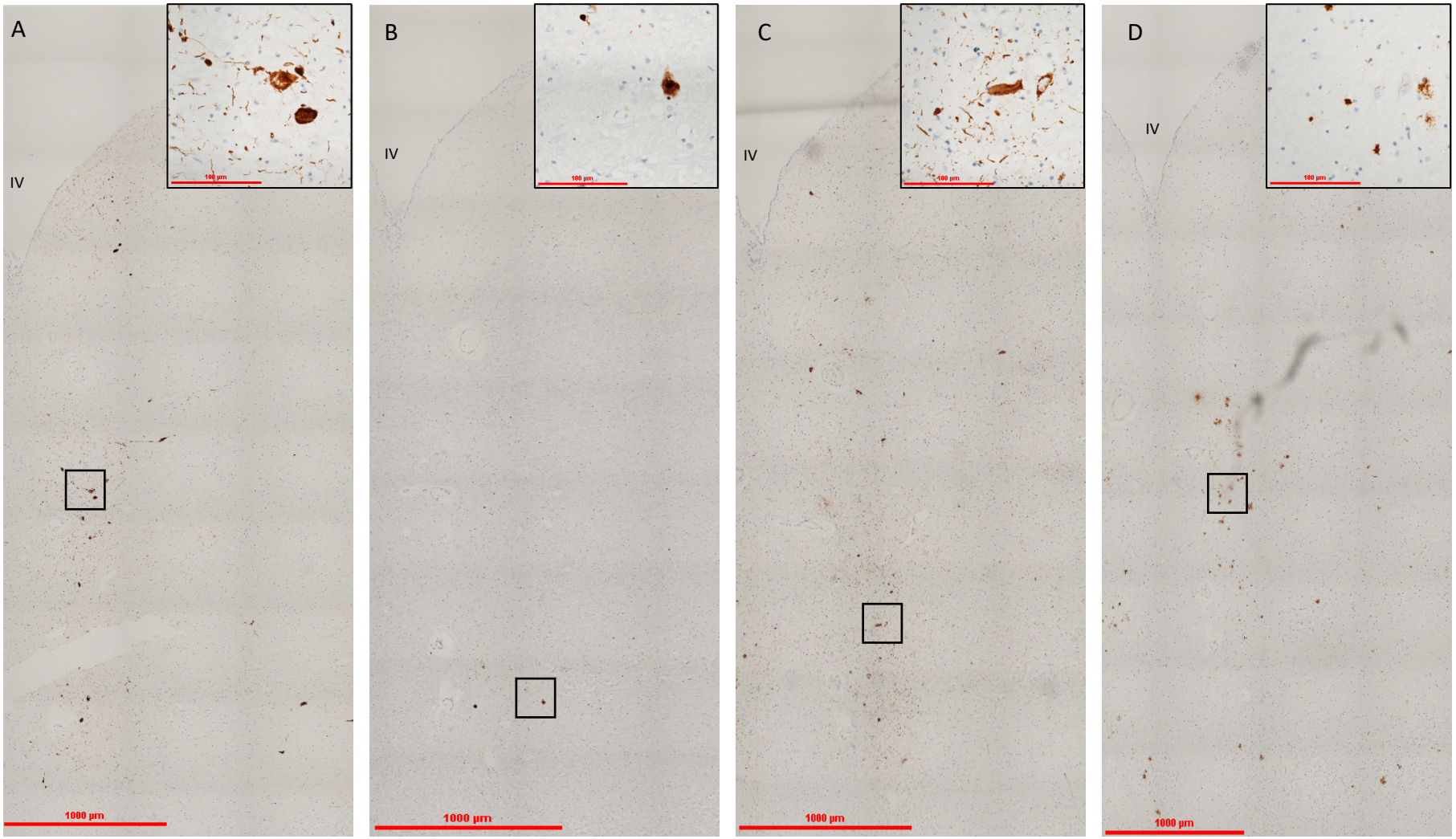


Figure 5.3. Representative pathology in the raphe.
Representative images of A- tryptophan hydroxylase 2 (TPH2) B- α-synuclein (KM51), C- tau (AT8), D-Aβ (4G8). Scale bar 1000µm, inset 100µm. IV- IV ventricle

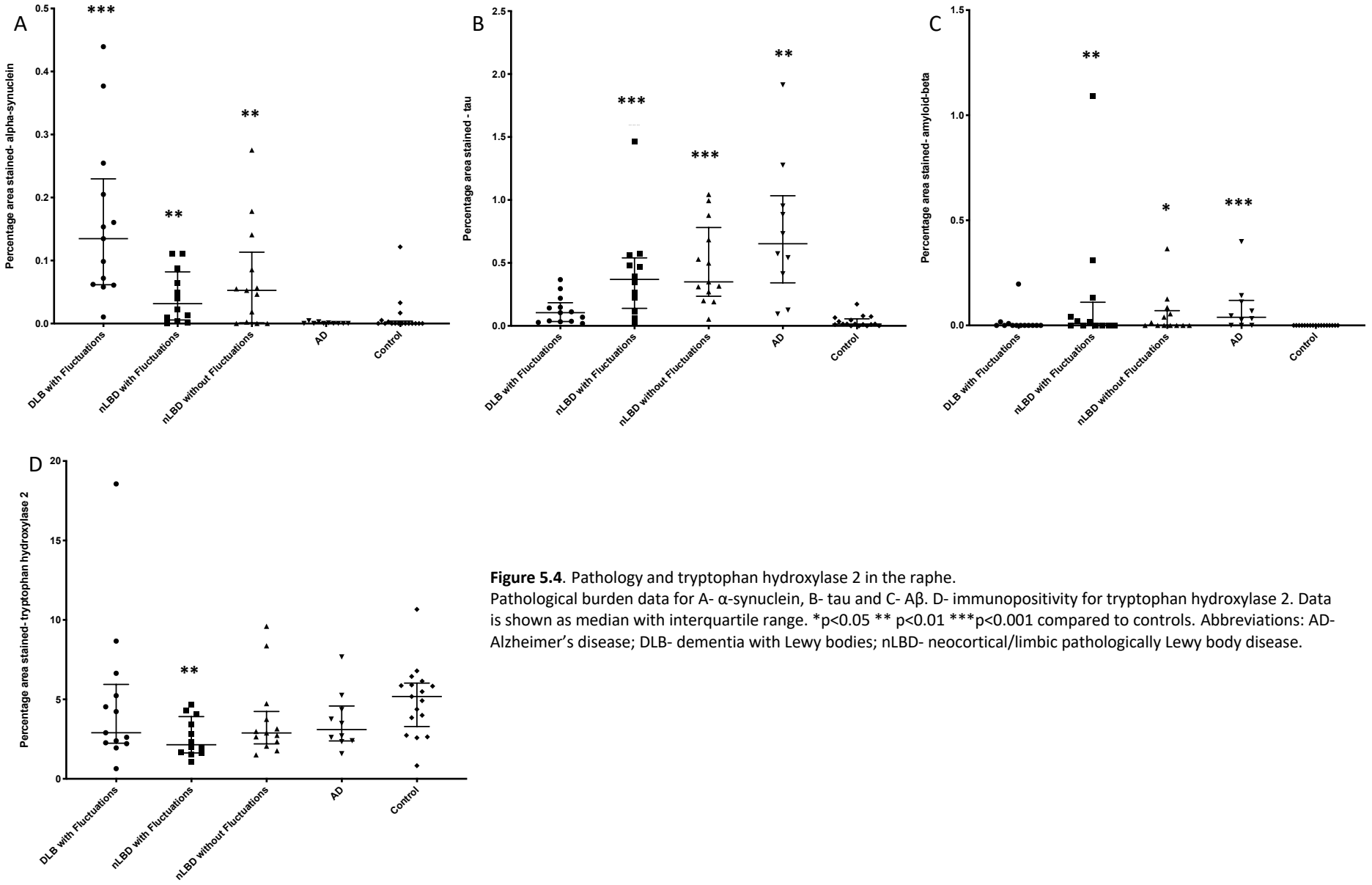


Figure 5.4. Pathology and tryptophan hydroxylase 2 in the raphe.
Pathological burden data for A- α-synuclein, B- tau and C- Aβ. D- immunopositivity for tryptophan hydroxylase 2. Data is shown as median with interquartile range. *p<0.05 ** p<0.01 ***p<0.001 compared to controls. Abbreviations: AD- Alzheimer's disease; DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease.

with fluctuations (z=-19.662, p=0.005) and nLBD without fluctuations (z=18.566, p=0.007) than controls. Pathological burden of α-synuclein was significantly higher in DLB with fluctuations (z=36.715, p<0.001), nLBD with fluctuations (z=-21.850, p=0.006) and nLBD without fluctuations (z=20.754 p=0.008) than AD cases. DLB with fluctuations had a significantly higher pathological burden in the raphe compared to nLBD with cognitive fluctuations (z=15.962, p=0.030) and nLBD without cognitive fluctuations (z=14.865, p=0.047). No differences in pathological burden were observed between controls and AD cases (p=0.769) or nLBD with and without cognitive fluctuations (p=0.884).

Tau pathological was observed in the all DLB and nLBD cases. The highest raphe tau burdens were observed in the AD and nLBD groups both with and without fluctuations, with cases containing neurofibrillary tangles, neuropil threads and neurites (figure 5.3C). A significant main effect of disease group on tau pathological burden was observed in the raphe (χ^2=39.952, p<0.001) (figure 5.4B). Tau pathological burden was higher in nLBD with fluctuations (z=-29.108, p<0.001), nLBD without fluctuations (z=33.557, p<0.001), AD cases (z=39.241, p=0.001) and DLB with fluctuations (z=14.095, p=0.043) than controls. Pathological burden for tau was significantly higher in AD cases (z=-16.862, p=0.012), nLBD with fluctuations (z=-15.013, p=0.047) and nLBD without fluctuations (z=-19.462, p=0.009) than DLB with fluctuations. No significant differences between the level of tau burden were observed between the nLBD groups with and without fluctuations (p=0.557) or between the AD group and the nLBD groups with (p=0.211) and without (p=0.475) fluctuations.

Aβ pathology was observed in all AD and nLBD with fluctuation cases, with a number of DLB and control cases possessing no positive 4G8 staining (figure 5.3D). A significant main effect of disease group on Aβ pathological burden was observed in the raphe (χ^2=17.549, p=0.002) (figure 5.4C). Aβ pathological burden was higher in nLBD with fluctuations (z=-16.750, p=0.005), nLBD without fluctuations (z=15.000, p=0.011) and AD cases (z=23.400, p<0.001) than controls. Pathological burden for Aβ was significantly higher in AD cases (z=-14.990, p=0.025) than DLB with fluctuations. No significant differences between the level of tau burden were observed between the nLBD groups with and without fluctuations (p=0.784) or between the AD group and the nLBD groups with (p=0.330) and without (p=0.210) fluctuations. No significant difference was also observed between DLB with fluctuations and

the nLBD with (p=0.110) or without fluctuations groups (p=0.176) or control cases (p=0.266).

No associations were found between disease duration, across the disease groups, and α-synuclein (p=0.230), tau (p=0.622) or Aβ (p=0.194) raphe pathological burden.

5.3.1.3 TPH2 Immunopositivity

TPH2 immunopositivity was measured utilising IHC, data measured represented TPH2 levels in both neuronal soma and in surrounding neuronal fibres (figure 5.3A). A significant main effect of disease group on TPH2 immunopositivity was observed in the raphe (χ^2=10.476, p=0.033) (figure 5.4D). A significantly higher TPH2 percentage area stained was observed in the control group compared to those in the nLBD with fluctuations (z=22.623, p=0.002). A trend towards a higher TPH2 immunopositivity in the control group was observed with the nLBD without fluctuations group (p=0.060). No significant difference was observed between the DLB with fluctuation groups and the nLBD with (p=0.104) and without (p=0.709) fluctuation groups. No further significant differences in TPH2 immunopositivity were observed between the disease groups.

TPH2 immunopositivity did not relate to the raphe pathological burden of α-synuclein (p=0.579), tau (p=0.405) and Aβ (p=0.521) or disease duration (p=0.275).

5.3.1.4 Relationship to PPN and LC pathology

Cases that had pathological burden assessed for the raphe, also had the pathological burden examined in other ARAS regions, the LC (65/65) and PPN (50/65).

In the whole cohort, excluding the control group, Spearman's rank correlations revealed associations between pathological burden of α-synuclein, tau and Aβ in the LC and the pathological burdens in the raphe (table 5.2). Raphe α-synuclein pathology was associated with LC pathological burden of α-synuclein, positively (r_s=0.707, p<0.001), tau, negatively (r_s=-0.422, p=0.003) and Aβ, negatively (r_s=-0.392, p=0.006). Raphe tau pathological burden was associated with the LC pathological burden of α-synuclein, negatively (r_s=-0.367, p=0.010), tau, positively (r_s=0.684, p<0.001) and Aβ, positively (r_s=0.510, p<0.001). Raphe Aβ pathological burden was associated with the LC pathological burden of α-synuclein, negatively (r_s=-0.286, p=0.049), tau, positively (r_s=0.409, p=0.004) and Aβ, positively (r_s=0.554, p<0.001).

Table 5.3. Associations between raphe and locus coeruleus pathological burdens.
Data shown for the Spearman's rank correlation between raphe pathology and LC pathological burden for α-synuclein, tau and Aβ. Correlations shown for: All-whole cohort minus the control cases, DLB with cognitive fluctuations, nLBD with cognitive fluctuations, nLBD without cognitive fluctuations and AD cases. An Asterix indicates a significant result: * p<0.05 ** p<0.01 ***p<0.001. Abbreviations: AD- Alzheimer's disease; DLB- dementia with Lewy bodies; LC- locus coeruleus; nLBD- neocortical/limbic Lewy body disease.

Raphe Pathology	Cognitive fluctuation group	LC Pathology	Spearman's rank correlation
Alpha-synuclein	All	Alpha-synuclein	r_s=0.707, p<0.001 ***
		Tau	r_s=-0.422, p=0.003 **
		Amyloid-beta	r_s=-0.392, p=0.006 **
	DLB with cognitive fluctuations	Alpha-synuclein	r_s=0.720, p=0.006 **
		Tau	r_s=-0.066, p=0.831
		Amyloid-beta	r_s=-0.110, p=0.721
	nLBD without cognitive fluctuations	Alpha-synuclein	r_s=0.541, p=0.056
		Tau	r_s=0.055, p=0.858
		Amyloid-beta	r_s=-0.546, p=0.053
	nLBD with cognitive fluctuations	Alpha-synuclein	r_s=0.552, p=0.063
		Tau	r_s=-0.405, p=0.746
		Amyloid-beta	r_s=0.340, p=0.280
	AD without cognitive fluctuations	Alpha-synuclein	r_s=-0.517, p=0.126
		Tau	r_s=-0.252, p=0.482
		Amyloid-beta	r_s=0.601, p=0.066
Tau	All	Alpha-synuclein	r_s=-0.367, p=0.010 *
		Tau	r_s=0.684, p<0.001 ***
		Amyloid-beta	r_s=0.510, p<0.001 ***
	DLB with cognitive fluctuations	Alpha-synuclein	r_s=0.385, p=0.194
		Tau	r_s=0.484, p=0.094
		Amyloid-beta	r_s=0.194, p=0.525
	nLBD without cognitive fluctuations	Alpha-synuclein	r_s=-0.456, p=0.117
		Tau	r_s=0.203, p=0.505
		Amyloid-beta	r_s=0.774, p=0.002**
	nLBD with cognitive fluctuations	Alpha-synuclein	r_s=-0.420, p=0.175
		Tau	r_s=0.622, p=0.031*
		Amyloid-beta	r_s=0.546, p=0.066
	AD without cognitive fluctuations	Alpha-synuclein	r_s=0.546, p=0.102
		Tau	r_s=0.394, p=0.260
		Amyloid-beta	r_s=-0.333, p=0.347
Amyloid-beta	All	Alpha-synuclein	r_s=-0.286, p=0.049 *
		Tau	r_s=0.409, p=0.004 **
		Amyloid-beta	r_s=0.554, p<0.001 ***
	DLB with cognitive fluctuations	Alpha-synuclein	r_s=-0.249, p=0.412
		Tau	r_s=0.026, p=0.933
		Amyloid-beta	r_s=0.532, p=0.061
	nLBD without cognitive fluctuations	Alpha-synuclein	r_s=-0.078, p=0.801
		Tau	r_s=0.340, p=0.255
		Amyloid-beta	r_s=0.612, p=0.026*
	nLBD with cognitive fluctuations	Alpha-synuclein	r_s=-0.295, p=0.352
		Tau	r_s=0.396, p=0.203
		Amyloid-beta	r_s=0.350, p=0.265
	AD without cognitive fluctuations	Alpha-synuclein	r_s=0.031, p=0.932
		Tau	r_s=0.117, p=0.748
		Amyloid-beta	r_s=0.129, p=0.723

When the disease groups were assessed independently, a number of associations observed between raphe pathological burden and LC pathological burden in the whole cohort were lost (table 5.2). Within the DLB with cognitive fluctuations groups a positive association was observed between raphe and LC α-synuclein pathological burdens (r_s=0.720 p=0.006). Within the nLBD without cognitive fluctuations a positive relationship was observed with LC Aβ immunopositivity with raphe tau (r_s=0.774, p=0.002) and Aβ (r_s=0.612, p=0.026) pathological burden. No relationships were observed between LC and raphe pathology in the nLBD with and the AD without fluctuations groups (table 5.2).

In the whole cohort, excluding the control group, Spearman's rank correlations revealed associations between pathological burden of α-synuclein, tau and Aβ in the PPN and the pathological burdens in the raphe (table 5.3). Raphe α-synuclein pathology was positively associated with PPN α-synuclein (r_s=0.702, p<0.001). Raphe tau pathological burden was negatively associated with the PPN pathological burden of α-synuclein (r_s=-0.497, p=0.001) and positively associated with PPN tau burden (r_s=0.430, p=0.006). Aβ burden in the raphe was negatively associated with PPN pathological burden of alpha-synuclein (r_s=-0.369, p=0.019) and positively associated with Aβ burden (r_s=0.490, p=0.001).

When the disease groups were assessed independently from each a number of associations observed between raphe pathological burden and LC pathological burden in the whole cohort were lost (table 5.3). Within the DLB with cognitive fluctuations groups a positive association was observed between raphe and PPN α-synuclein pathological burdens (r_s=0.648 p=0.043).

A negative association was identified in the nLBD group with fluctuations between raphe Aβ and PPN α-synuclein (r_s=-0.658, p=0.028). A negative relationship was observed with PPN tau immunopositivity and raphe α-synuclein pathological burden within the nLBD without cognitive fluctuations (r_s=-0.738, p=0.037) and AD without cognitive fluctuation (r_s=-0.748, p=0.033) groups. No further relationships were observed between PPN and raphe pathology in the disease groups (table 5.3).

Table 5.6. Associations between raphe and pedunculopontine nucleus pathological burdens.
Data shown for the Spearman's rank correlation between raphe pathology and PPN pathological burden for α-synuclein, tau and Aβ. Correlations shown for: All-whole cohort minus the control cases, DLB with cognitive fluctuations, nLBD with cognitive fluctuations, nLBD without cognitive fluctuations and AD cases. An Asterix indicates a significant result: * p<0.05 ** p<0.01 ***p<0.001. Abbreviations: AD- Alzheimer's disease; DLB- dementia with Lewy bodies; nLBD- neocortical/limbic Lewy body disease; PPN- pedunculopontine nucleus.

Raphe Pathology	Cognitive fluctuation group	PPN Pathology	Spearman's rank correlation
Alpha-synuclein	All	Alpha-synuclein	r_s=0.702, p<0.001 ***
		Tau	r_s=-0.250, p=0.120
		Amyloid-beta	r_s=-0.171, p=0.292
	DLB with cognitive fluctuations	Alpha-synuclein	r_s=-0.450, p=0.192
		Tau	r_s=0.648, p=0.043 *
		Amyloid-beta	r_s=0.420, p=0.227
	nLBD without cognitive fluctuations	Alpha-synuclein	r_s=0.795, p=0.003 **
		Tau	r_s=0.330, p=0.321
		Amyloid-beta	r_s=-0.102, p=0.766
	nLBD with cognitive fluctuations	Alpha-synuclein	r_s=0.145, p=0.670
		Tau	r_s=-0.273, p=0.417
		Amyloid-beta	r_s=-0.209, p=0.537
	AD without cognitive fluctuations	Alpha-synuclein	r_s=0.387, p=0.355
		Tau	r_s=-0.748, p=0.033 *
		Amyloid-beta	r_s=-0.241, p=0.565
Tau	All	Alpha-synuclein	r_s=-0.497, p=0.001 **
		Tau	r_s=0.430, p=0.006 **
		Amyloid-beta	r_s=0.300, p=0.060
	DLB with cognitive fluctuations	Alpha-synuclein	r_s=-0.565, p=0.089
		Tau	r_s=0.297, p=0.405
		Amyloid-beta	r_s=0.123, p=0.735
	nLBD without cognitive fluctuations	Alpha-synuclein	r_s=-0.200, p=0.555
		Tau	r_s=0.073, p=0.832
		Amyloid-beta	r_s=0.358, p=0.280
	nLBD with cognitive fluctuations	Alpha-synuclein	r_s=-0.273, p=0.417
		Tau	r_s=0.009, p=0.979
		Amyloid-beta	r_s=-0.127, p=0.709
	AD without cognitive fluctuations	Alpha-synuclein	r_s=0.000, p=1.000
		Tau	r_s=0.357, p=0.385
		Amyloid-beta	r_s=-0.048, p=0.911
Amyloid-beta	All	Alpha-synuclein	r_s=-0.369, p=0.019 *
		Tau	r_s=0.409, p=0.054
		Amyloid-beta	r_s=0.490, p=0.001 **
	DLB with cognitive fluctuations	Alpha-synuclein	r_s=-0.217, p=0.547
		Tau	r_s=0.142, p=0.696
		Amyloid-beta	r_s=0.358, p=0.310
	nLBD without cognitive fluctuations	Alpha-synuclein	r_s=-0.211, p=0.533
		Tau	r_s=0.312, p=0.350
		Amyloid-beta	r_s=0.470, p=0.144
	nLBD with cognitive fluctuations	Alpha-synuclein	r_s=-0.568, p=0.028 *
		Tau	r_s=0.153, p=0.654
		Amyloid-beta	r_s=0.505, p=0.113
	AD without cognitive fluctuations	Alpha-synuclein	r_s=0.442, p=0.273
		Tau	r_s=0.195, p=0.643
		Amyloid-beta	r_s=0.464, p=0.247

5.3.1.5 Relationship to cognitive fluctuations

No association was observed between α-synuclein, tau or Aβ raphe pathological burden or TPH2 immunoreactivity with the severity of cognitive fluctuations as measured by CAF score, in all cases that during life possessed cognitive fluctuations (table 5.4). When fluctuation groups were separated into DLB and nLBD with fluctuations, a positive association was observed between last CAF and raphe Aβ immunopositivity in the DLB group (r_s=0.833, p=0.010). No further associations were observed between raphe pathological burden and any of the measures of cognitive fluctuation severity in the separated groups (table 5.4).

Table 5.9. Associations between cognitive fluctuation severity and raphe pathology.
Spearman's rank correlations between raphe pathology and severity of cognitive fluctuations as measure by last, average and maximum CAF score. Associations were undertaken in DLB and nLBD both combined and individually. Abbreviations: CAF- clinical assessment of fluctuation: DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease; r_s- spearman's rank coefficient.

Measurement of cognitive fluctuation severity	Cognitive fluctuation group	Pathology	Cognitive fluctuation severity
Last CAF	nLBD and DLB with cognitive fluctuations	Alpha-synuclein	r_s=-0.301, p=0.240
		Tau	r_s=0.335, p=0.188
		Amyloid-beta	r_s=0.231, p=0.372
	DLB with cognitive fluctuations	Alpha-synuclein	r_s=-0.509, p=0.197
		Tau	r_s=0.036, p=0.932
		Amyloid-beta	r_s=0.833, p=0.010 *
	nLBD with cognitive fluctuations	Alpha-synuclein	r_s=-0.264, p=0.493
		Tau	r_s=0.527, p=0.145
		Amyloid-beta	r_s=-0.266, p=0.489
Average CAF	nLBD and DLB with cognitive fluctuations	Alpha-synuclein	r_s=-0.068, p=0.794
		Tau	r_s=0.406, p=0.106
		Amyloid-beta	r_s=-0.028, p=0.916
	DLB with cognitive fluctuations	Alpha-synuclein	r_s=-0.122, p=0.774
		Tau	r_s=0.171, p=0.686
		Amyloid-beta	r_s=0.531, p=0.176
	nLBD with cognitive fluctuations	Alpha-synuclein	r_s=-0.042, p=0.915
		Tau	r_s=0.513, p=0.158
		Amyloid-beta	r_s=-0.394, p=0.294
Maximum CAF	nLBD and DLB with cognitive fluctuations	Alpha-synuclein	r_s=0.038, p=0.885
		Tau	r_s=0.084, p=0.749
		Amyloid-beta	r_s=0.032, p=0.902
	DLB with cognitive fluctuations	Alpha-synuclein	r_s=-0.100, p=0.814
		Tau	r_s=-0.200, p=0.634
		Amyloid-beta	r_s=0.516, p=0.190
	nLBD with cognitive fluctuations	Alpha-synuclein	r_s=0.156, p=0.689
		Tau	r_s=0.202, p=0.603
		Amyloid-beta	r_s=-0.322, p=0.399

5.3.1.6 Relationship to depression

In the cases that were pathologically classified as LBD, nLBD or DLB groups, which had GDS, no associations were observed between the average GDS score and raphe pathological burden of α-synuclein (p=0.274), tau (p=0.167) and Aβ (p=0.716) or with TPH2 immunopositivity (p=0.174).

5.3.2 Immunofluorescence

5.3.2.1 Demographics

No significant differences were observed in *post-mortem* delay, fixation duration, and age between the study groups. There was no significant difference observed in disease duration between the neurodegenerative disease groups. Between the groups a significant difference was found in the proportion of males to females, with the DLB group having a significantly higher proportion of males compared to AD without fluctuations ($\chi(1)=4.408$, p=0.036) and nLBD with fluctuations ($\chi(1)=5.495$, p=0.019).

CAF scores were available for 22/47 cases. 18/20 of the cases with cognitive fluctuations possessing at least one CAF score. No significant difference was observed between the last (p=0.927), maximal (p=1.000) or average (p=0.789) CAF score between the DLB with fluctuations or the nLBD with fluctuations groups.

Final MMSE scores were available for 40/47 cases: 10 DLB cases with fluctuations, 10 nLBD with fluctuations, 8 nLBD without fluctuations, 7 AD and 5 Controls. No significant difference in the interval between the last MMSE and death was observed between the groups. Significantly lower last MMSE scores were observed between all disease groups and controls: DLB cases with fluctuations (z=-14.900, p=0.017), nLBD with fluctuations (z=20.950, p=0.001), nLBD without fluctuations (z=-19.412, p=0.003) and AD cases (z=-18.600, p=0.005). No significant differences in last MMSE score were observed between the 4 disease groups.

At least one GDS score was available for 25/47 cases: 9 DLB cases with fluctuations, 7 nLBD with fluctuations, 1 nLBD without fluctuations, 3 AD and 5 Controls. No significant difference in average GDS score was observed across the groups within the study.

5.3.2.2 Number of TPH2 neurons assessed

The number of TPH2 neurons that were assessed for corrected TPH2 intensity ranged from 16-688. No significant effect of disease fluctuations groups was observed on the number of TPH2 neurons assessed (χ^2=5.575, p=0.233).

5.3.2.3 Corrected TPH2 intensity

No significant effect of disease group was observed on TPH2 corrected TPH2 intensity adjusted for area (χ^2=1.014, p=0.908) (figure 5.5). The level of raphe pathological burden of α-synuclein (p=0.270), tau (p=0.676), Aβ (p=0.379) or TPH2 immunopositivity (p=0.348) was not found to relate to the TPH2 corrected TPH2 intensity adjusted for area values.

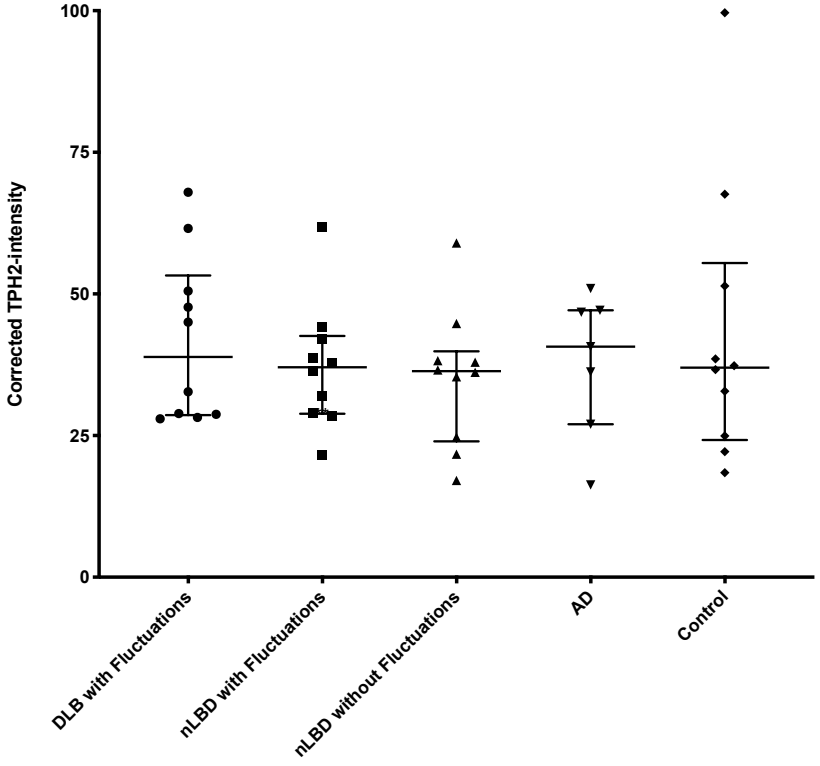


Figure 5.5. Corrected tryptophan hydroxylase 2 intensity in the raphe.
Corrected tryptophan hydroxylase 2 (TPH2)-intensity data. Data is shown as median with interquartile range. Abbreviations: AD- Alzheimer's disease; DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease.

Corrected TPH2 intensity values were calculated for TPH2-positive neurons both with and without Lewy bodies, for those that bore Lewy bodies the corrected TPH2 intensity value was additionally calculated for the cytoplasmic part of the neuron. A ratio was calculated to measure the magnitude of difference in median corrected TPH2 intensity value for the TPH2-postive neurons without Lewy bodies and TPH2-postive neurons with Lewy bodies; as well as a further ratio between the median corrected TPH2 intensity value for the TPH2-

postive neurons without Lewy bodies and the median corrected TPH2 intensity value for the cytoplasmic portion in Lewy body bearing TPH2-postive neurons. A one-sample Wilcoxon signed rank test was undertaken to assess whether the observed median for the two deviated from a hypothetical median of 1. A hypothetical median of 1 represents the value at which there would be no difference between the corrected TPH2 intensity value in the TPH2-postive neurons without Lewy bodies and those with Lewy bodies. A ratio value of >1 would be suggestive that the corrected TPH2 intensity was higher in the TPH2 neurons without Lewy bodies and a ratio <1 suggestive that the corrected TPH2 intensity was higher in the TPH2 neurons with Lewy bodies. All cases which had ratios calculated in DLB and nLBD groups were analysed collectively. No significant deviation from a median of 1 was observed for the magnitude of difference between TPH2 neurons with Lewy bodies and those without, median 0.88, (z=39.000, p=0.650) (figure 5.6A). A significant deviation from a hypothesised median of 1 was observed for the magnitude of difference in corrected TPH2 intensity between the cytoplasmic portion of Lewy body bearing neurons and those without Lewy bodies, median 0.73, (z=3.000, p=0.005) (figure 5.6B). No significant effect of disease group was observed for the magnitude of difference in corrected TPH2 intensity between

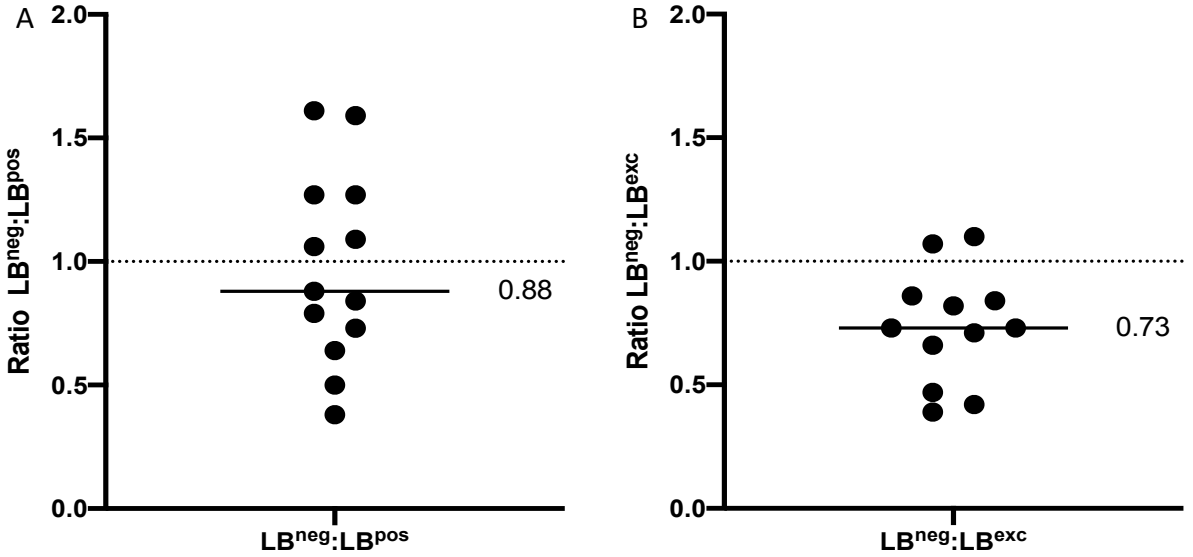


Figure 5.6. Ratios for corrected tryptophan hydroxylase 2 intensity in tryptophan hydroxylase 2-positive neurons with and without Lewy bodies.
Ratios for corrected tryptophan hydroxylase 2 (TPH2) intensity in TPH2 positive neurons without Lewy bodies (LB^{neg}) with corrected TPH2 intensity in TPH2 positive neurons containing Lewy bodies (LB^{pos}) (A) and the corrected TPH2 intensity value for the part of the neuron that the Lewy body did not occupy in Lewy body bearing TPH2-postive neurons (LB^{exc}) (B). Dotted line represents the hypothetical median of 1. Observed median stated.

neurons without Lewy bodies and those with Lewy bodies, inclusive of the Lewy body (χ^2=0.440, p=0.803) or only the cytoplasmic portion (χ^2=1.186, p=0.553).

5.3.2.4 Location of pathology

The percentage of TPH2-postive neurons bearing Lewy bodies was calculated for 6 DLB with fluctuations, 4 nLBD with fluctuations and 3 nLBD without fluctuation cases. A significant effect of disease group was observed on the percentage of TPH2-positive neurons bearing Lewy bodies (χ^2=6.242, p=0.044) (figure 5.7). DLB with fluctuation cases were observed to have a significantly higher proportion of Lewy body bearing TPH2-positive neurons than nLBD without cognitive fluctuation cases (z=6.667, p=0.015). No significant difference in proportion of Lewy body bearing neurons was observed between the nLBD with cognitive fluctuation group and the DLB with (p=0.145) and nLBD without (p=0.313) fluctuations groups.

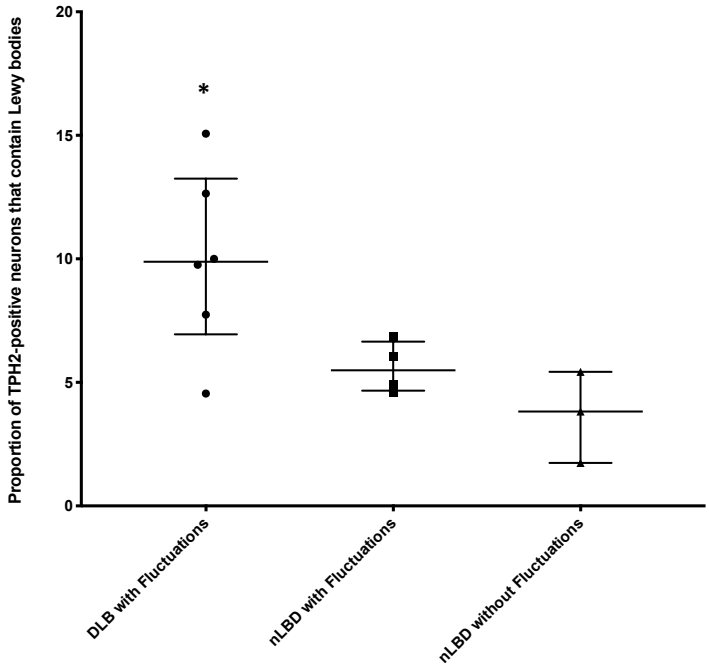


Figure 5.7. Proportion of tryptophan hydroxylase 2-positive neurons bearing Lewy bodies.
Proportion of tryptophan hydroxylase 2 (TPH2)-positive neurons bearing Lewy bodies. Data is shown as median with interquartile range. * p<0.05 shown to nLBD without fluctuations. Abbreviations: DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease.

5.3.2.5 Variability in TPH2 corrected TPH2 intensity

z-scores were calculated to assess variability within the corrected TPH2 intensity values across all the disease groups. The proportion of neurons for each case that had a z-score less than -1 and less than -2 was calculated. No significant effect of disease group was observed in the proportion of neurons with a z-score less than -1 (χ^2=1.554, p=0.817) or a z-score less than -2 (χ^2=1.431, p=0.839) (figure 5.8).

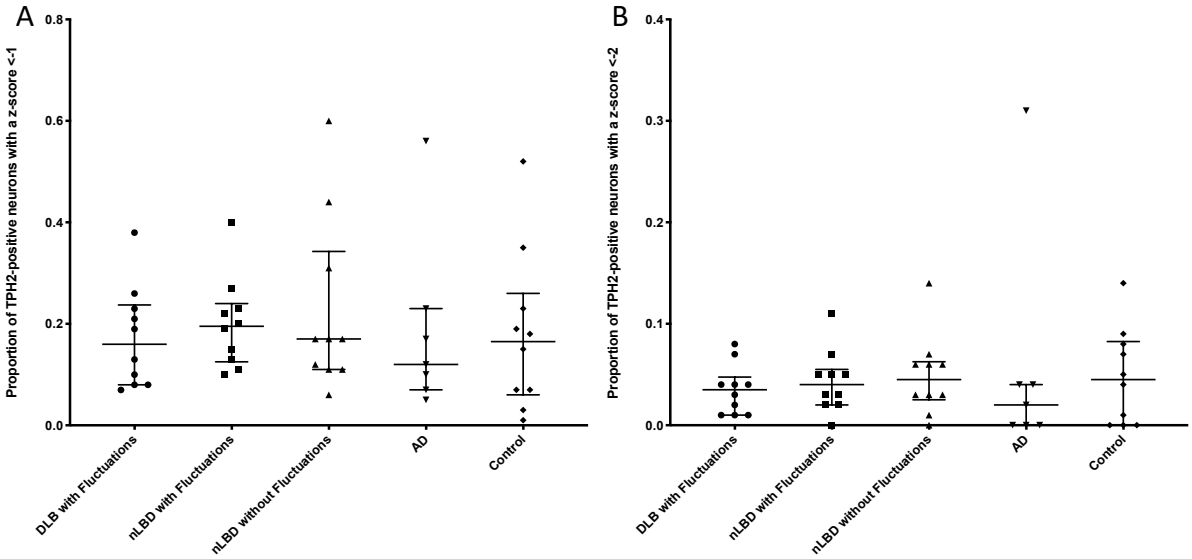


Figure 5.8. Variability of corrected tryptophan hydroxylase 2-intensity.
Proportion of tryptophan hydroxylase 2 (TPH2)-positive neurons with a z-score of less than -1 (A) or less than -2 (B). Abbreviations: AD- Alzheimer's disease; DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease.

5.3.2.6 Relationship to cognitive fluctuations

No association was observed between the proportion of TPH2-postive neurons bearing Lewy bodies and the severity of cognitive fluctuations as measured by CAF score, in all cases that during life possessed cognitive fluctuations (table 5.5). However, a trend towards significance was observed between average CAF score and the proportion of TPH2-neurons bearing Lewy bodies. When the fluctuation groups were separated into DLB and nLBD with

Table 5.11. Associations between cognitive fluctuation severity and the proportion of tryptophan hydroxylase 2 positive neurons bearing Lewy bodies.
Spearman's rank correlations between tryptophan hydroxylase 2 positive neurons bearing Lewy bodies and severity of cognitive fluctuations as measure by last, average and maximum CAF score. Associations were undertaken in DLB and nLBD both combined and individually. Abbreviations: CAF- clinical assessment of fluctuation: DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease; r_s- spearman's rank coefficient.

Measurement of cognitive fluctuation severity	Cognitive fluctuation group	Spearman's rank correlation
Last CAF	nLBD and DLB with cognitive fluctuations	r_s=-0.412, p=0.237
	DLB with cognitive fluctuations	r_s=-0.736, p=0.096
	nLBD with cognitive fluctuations	r_s=0.738, p=0.262
Average CAF	nLBD and DLB with cognitive fluctuations	r_s=-0.615, p=0.059
	DLB with cognitive fluctuations	r_s=-0.899, p=0.015 *
	nLBD with cognitive fluctuations	r_s=0.738, p=0.262
Maximum CAF	nLBD and DLB with cognitive fluctuations	r_s=-0.315, p=0.375
	DLB with cognitive fluctuations	r_s=-0.741, p=0.092
	nLBD with cognitive fluctuations	r_s=0.775, p=0.225

fluctuations, a negative association was observed between average CAF score and the proportion of TPH2-neurons bearing Lewy bodies in the DLB with fluctuations group (r_s=-0.899, p=0.015). No further associations were observed between the proportion of TPH2-neurons bearing Lewy bodies and any of the measures of cognitive fluctuation severity (table 5.5).

The two ratios generated described the magnitude of difference in the corrected TPH2 intensity between neurons without Lewy bodies and either the cytoplasmic portion or the whole neuron of those that do contain Lewy bodies. A value <1 suggests that the corrected TPH2 intensity is higher in the neurons without Lewy bodies and a value >1 that it is created in the neurons with Lewy bodies. A positive association was observed between the magnitude of difference in corrected TPH2 intensity between neurons with and without Lewy bodies and the severity of cognitive fluctuations as measured by last (r_s=0.792,

Table 5.14. Associations between the ratio of corrected tryptophan hydroxylase 2-intensity in tryptophan hydroxylase 2-positive neurons with and without Lewy bodies.
Spearman's rank correlations between corrected tryptophan hydroxylase 2 (TPH2)-intensity in TPH2 positive neurons without Lewy bodies (LB^{neg}) with corrected TPH2 intensity in TPH2 positive neurons containing Lewy bodies (LB^{pos}) and for the part of the neuron that the Lewy body did not occupy in Lewy body bearing TPH2-postive neurons (LB^{exc}) and severity of cognitive fluctuations as measure by last, average and maximum CAF score. Associations were undertaken in DLB and nLBD both combined and individually. Abbreviations: CAF- clinical assessment of fluctuation: DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease; r_s- spearman's rank coefficient.

Measurement of cognitive fluctuation severity	Cognitive fluctuation group	Corrected TPH2-intensity ratios	Cognitive fluctuation severity
Last CAF	nLBD and DLB with cognitive fluctuations	LB^{neg}:LB^{pos}	r_s=0.792, p=0.006 **
		LB^{neg}:LB^{exc}	r_s=0.697, p=0.025 *
	DLB with cognitive fluctuations	LB^{neg}:LB^{pos}	r_s=0.500, p=0.312
		LB^{neg}:LB^{exc}	r_s=0.618, p=0.191
	nLBD with cognitive fluctuations	LB^{neg}:LB^{pos}	r_s=0.949, p=0.051
		LB^{neg}:LB^{exc}	r_s=0.632, p=0.368
Average CAF	nLBD and DLB with cognitive fluctuations	LB^{neg}:LB^{pos}	r_s=0.684, p=0.029 *
		LB^{neg}:LB^{exc}	r_s=0.558, p=0.093
	DLB with cognitive fluctuations	LB^{neg}:LB^{pos}	r_s=0.493, p=0.321
		LB^{neg}:LB^{exc}	r_s=0.609, p=0.200
	nLBD with cognitive fluctuations	LB^{neg}:LB^{pos}	r_s=0.738, p=0.262
		LB^{neg}:LB^{exc}	r_s=0.211, p=0.789
Maximum CAF	nLBD and DLB with cognitive fluctuations	LB^{neg}:LB^{pos}	r_s=0.391, p=0.264
		LB^{neg}:LB^{exc}	r_s=0.027, p=0.940
	DLB with cognitive fluctuations	LB^{neg}:LB^{pos}	r_s=0.800, p=0.055
		LB^{neg}:LB^{exc}	r_s=0.062, p=0.908
	nLBD with cognitive fluctuations	LB^{neg}:LB^{pos}	r_s=0.258, p=0.742
		LB^{neg}:LB^{exc}	r_s=-0.258, p=0.742

p=0.006) and average (r_s=0.684, p=0.029) CAF score, in all cases that during life possessed cognitive fluctuations (table 5.6). A positive association between the magnitude of difference in corrected TPH2 intensity between the cytoplasmic portion of neurons with and without Lewy bodies and severity of cognitive fluctuations as measured by last CAF score was observed (r_s=0.697, p=0.025), in all cases that during life possessed cognitive fluctuations. When the fluctuation groups were separated into DLB and nLBD with fluctuations no associations were observed between two ratios calculated and any of the measures of cognitive fluctuation severity (table 5.6).

5.3.2.7 Relationship to depression

No associations were observed between the average GDS score and TPH2 corrected TPH2 intensity adjusted for area (p=0.961).

5.4 Discussion

The raphe has been implicated in the maintenance of arousal states (Monti, 2011). Alterations to the raphe and its serotonergic projections have been identified in a number of neurodegenerative diseases, including AD and DLB (Chen *et al.*, 2000; Kovacs *et al.*, 2003; Benarroch *et al.*, 2007; Grinberg *et al.*, 2009). The role of the raphe and the serotonergic system in depression within dementia has been examined (Hendricksen *et al.*, 2004; Michelsen *et al.*, 2008), however, the role of any changes in relation to cognitive fluctuations has yet to be investigated.

5.4.1 Relationship between raphe pathology and cognitive fluctuations

The present study identified α-synuclein pathological burden was the highest in the DLB with fluctuations group. However, no specific pattern was observed between raphe pathological burden, α-synuclein, tau and Aβ, on the presence of cognitive fluctuations. No associations were found between pathological burden and severity of cognitive fluctuations, in the combined with fluctuation group. Although, a positive association was observed with Aβ pathological burden and severity of cognitive fluctuation was observed when the DLB with fluctuations group was analysed separately. The study also identified associations between pathological burdens in raphe and those in the LC and PPN within the whole cohort, excluding controls. However, the majority of these associations were lost when the cohort was split into individual disease groups, with no associations present in both groups with cognitive fluctuations.

5.4.1.1 Pathology alone in the raphe does not distinguish fluctuating from non-fluctuating groups

The present study aimed to investigate the involvement of raphe pathology, α-synuclein, tau and Aβ, on the presence and severity of cognitive fluctuations in DLB. Previous studies have investigated the vulnerability of the raphe to accumulation of pathological proteins and neuronal loss in a number of neurodegenerative diseases, including DLB, AD and PD (Braak *et al.*, 2003; Kovacs *et al.*, 2003; Benarroch *et al.*, 2007; Grinberg *et al.*, 2009; Seidel *et al.*, 2015). The results from the current study are in agreement with previous findings that the raphe is vulnerable to α-synuclein and AD-type pathology; along with α-synuclein being identified within TPH2-positive neurons (Kovacs *et al.*, 2003). However, within the present

study no differences in the pathological burdens were related to the presence or absence of cognitive fluctuations.

The raphe and serotonergic system have been strongly implicated to play a role in idiopathic depression and anxiety (Hale and Lowry, 2011). Clinically, depression and anxiety are supportive features of DLB and they are more persistent than those observed in AD (Fritze *et al.*, 2011). Therefore, the presence of depression in DLB and other neurodegenerative dementia disorders may suggest impairment to the serotonergic system. The majority of studies examining the raphe nucleus and the serotonergic system in dementia have centred on the examination of the possible relationship to depressive symptoms, with a studies primarily investigating changes in AD (Chen *et al.*, 2000; Ballard *et al.*, 2002a; Hendricksen *et al.*, 2004; Thomas *et al.*, 2006; Vermeiren *et al.*, 2015). Studies have investigated the raphe and serotonergic system in relation to clinical features of DLB, including visual hallucinations (Perry *et al.*, 1990a; Perry *et al.*, 1993). However, the majority of studies in DLB have been in relation to general alterations of the serotonergic system (Azmitia and Nixon, 2008; Mace *et al.*, 2016) and comparison of pathological burdens to other neurodegenerative diseases (Benarroch *et al.*, 2007). The present study is the first to my knowledge that has investigated the involvement of the raphe in relation to cognitive fluctuations.

The raphe has been implicated to play a complex role in the maintenance of arousal states through its widespread serotonergic projections to regions within the ARAS, including the PPN, LC and hypothalamus, as well as cortical regions (Vertes, 1991; Jones, 2005b; Michelsen *et al.*, 2007). DR neurons have been identified to fire maximally during waking (Trulson and Jacobs, 1979; Mahaffey and Garcia-Rill, 2015), however, lesioning of the nucleus in cats can lead to permanent states of wakefulness (Jouvet, 1968). The raphe's role in the maintenance of arousal systems is believed to occur through a complex network of projections and receptor expression patterns (Mihailescu *et al.*, 2002; Jones, 2003; McDevitt and Neumaier, 2011; Mahaffey and Garcia-Rill, 2015). However, the present study identified no differences in pathological burden specific to those with cognitive fluctuations, with similar lack of associations having been identified with raphe pathology and depression in AD (Chen *et al.*, 2000). Studies have identified alterations to serotonergic system in DLB, including alterations to serotonergic fibre morphology and reductions in serotonin levels in the striatum, neocortex and frontal cortex (Azmitia and Nixon, 2008), although, these were

not explored in relationship to clinical features. Furthermore, reductions to cortical levels of serotonin have been further identified in DLB cases with depression (Vermeiren *et al.*, 2015). With the raphe's control of arousal likely through a complex system of projection and receptor expression patterns it is likely that a complex set of alterations to the raphe and serotonergic system would relate to the clinical manifestations of arousal disturbances, and these changes may be independent of the pathological protein accumulation within the raphe. Analysis of serotonergic receptor population levels and integrity of serotonergic projections were not undertaken within the current study and thus further experiments investigating these changes would be required to explore the role of the raphe and serotonergic system in cognitive fluctuations.

5.4.1.2 Relationship of raphe pathology to pathology in the LC and PPN

When the whole cohort, excluding the controls, was analysed a number of relationships between the level of pathological proteins in the raphe and those in the PPN and LC were identified. However, upon examination of the disease groups individually the majority of these relationships were lost. The likely cause of the associations observed in the whole cohort are group effects due construction of the cohort, with pathologically AD cases having higher AD-type pathology in both the LC and PPN compared to DLB cases. These group differences would drive an apparent association which was then lost upon examination of the groups separately. Although associations were observed between raphe pathology and pathological burden in the LC and raphe when the fluctuations groups were analysed individually, no associations were observed in both the DLB and nLBD groups with fluctuations.

The ARAS is a diffuse complex system comprising of a number of nuclei and neurotransmitter systems, with studies having identified a degree of redundancy with the system (Kovalzon, 2016b). Due to the level of redundancy within the ARAS it is possible to suggest all three key brainstem structures would need to be affected or that one region or neurotransmitter system is severely affected and the parts of the ARAS are not able consistently undertake the role of the affected region, in order to elicit fluctuations in consciousness. If all three key brainstem regions were to be affected creating a vulnerability towards fluctuating cognition, it could be that regions contain a specific pattern of pathology. However, data from the present study and that present in chapter 4, show a lack

of associations between the pathological burdens of the three ARAS nuclei, specific to the groups with cognitive fluctuations. The lack of conserved associations in the fluctuation groups suggests that there is no topographical pattern of pathological burden that is specific for the presence of cognitive fluctuations.

5.4.2 Corrected TPH2 intensity

The present study identified no relationship between the intensity of TPH2 and the presence or severity of cognitive fluctuations. Pathological burden in the raphe was also found not to relate to TPH2 intensity, across the whole cohort including controls. DLB cases were also observed to have a higher proportion of TPH2-positive neurons that contained Lewy bodies compared to the nLBD group without cognitive fluctuations, with a negative association observed between the proportion and fluctuation severity. In the pathologically LBD cases, a difference in TPH2 intensity was observed between the TPH2-positive neurons that do not bear Lewy bodies and the cytoplasm of TPH2-positive neurons that do contain Lewy bodies. Within the fluctuating cognition groups, the magnitude of difference in the corrected TPH2 intensity between neurons without Lewy bodies and either the cytoplasmic portion or the whole neuron of those that do contain Lewy bodies were found to have a positive association with the severity of cognitive fluctuations, however, no association was observed when the fluctuating cognition groups were split into DLB and nLBD.

5.4.2.1 Alterations to the serotonergic system relate to the severity of cognitive fluctuations

The current study identified changes to the serotonergic system which could play a role in the serotonergic dysfunction in LBDs. A limited number of previous studies have investigated the levels of rate-limiting catalytic enzymes in transmitter systems known to be dysfunctional in LBDs, with the main focus being on tyrosine hydroxylase (Whitehouse *et al.*, 1983; Nakashima and Ikuta, 1984; Mori *et al.*, 2006) and none to my knowledge investigating TPH2. The observed difference in TPH2 level between neurons without Lewy bodies and the cytosolic part of those bearing Lewy bodies is in agreement with a study by Dugger *et al.* (2010) who identified a reduction in cytoplasmic levels of ChAT and tyrosine hydroxylase in neurons bearing Lewy bodies compared to neurons without. The observed reduction in cytoplasmic ChAT and tyrosine hydroxylase levels were thought to be due to sequestration of the enzymes into the Lewy body present within the neurons, with the study further investigating where the enzyme was within the Lewy body structure (Dugger

and Dickson, 2010). The current study identified no difference between the magnitude of difference in the corrected TPH2 intensity between neurons without Lewy bodies and the whole neuron of those that do contain Lewy bodies and a hypothesised median of 1; whereas a difference was observed for the magnitude of difference in the corrected TPH2 intensity between neurons without Lewy bodies and the cytoplasmic portion of those that do contain Lewy bodies, which could suggest that TPH2 is sequestered into Lewy bodies in TPH2-positive neurons, although further investigations are required to validate this. The sequestration of enzyme into the Lewy body could lead to a reduction in catalytic enzyme available to synthesis the neurotransmitter, leading to a reduction in serotonergic transmission. However, neither the current study, nor the study by Dugger *et al.* (2010), investigated neurotransmitter levels, so were unable to verify whether the sequestration of catalytic enzyme into Lewy bodies led to a reduction in transmitter level. Although investigations of neurotransmitter levels would verify whether sequestration of catalytic enzymes reduced their levels, at present there is no available method to do so in human *post-mortem* tissues.

Dysfunction to the serotonergic system has been implicated to play a role in a number of clinical features of DLB, including depression (Vermeiren *et al.*, 2015) and visual hallucinations (Perry *et al.*, 1990a). In the current study no difference was observed for TPH2-intensity in relation to the presence of cognitive fluctuations; although utilising IHC TPH2-levels in the raphe both in neuronal soma and fibres was trending towards a significant reduction compared to controls. However, a number of associations were observed with the between the severity of cognitive fluctuations and the proportion of TPH2-positive neurons containing Lewy bodies, and the magnitude of difference in the corrected TPH2 intensity between neurons without Lewy bodies and either the cytoplasmic portion or the whole neuron of those that do contain Lewy bodies.

Cognitive fluctuations are a transient clinical phenomenon, suggestive of a flexible underlying mechanism, rather than a permanent change (O'Dowd *et al.*, 2019). Permanent changes such as the loss of serotonergic neurons or the presence of pathology, measured through antibodies as in the current study, are likely not to underly the more flexible changes required to elicit a transient clinical feature. In the current study a positive association with last CAF score was observed between the magnitude of difference in the

corrected TPH2 intensity between neurons without Lewy bodies and the cytoplasmic portion of those that do contain Lewy bodies, as well as the magnitude of difference in the corrected TPH2 intensity between neurons without Lewy bodies and the whole neuron of those that do contain Lewy bodies. The positive association shows that when the difference for the corrected TPH2 levels in the whole neuron and cytoplasmic fraction of those with Lewy bodies compared to those without Lewy bodies is smaller, closer to 1, more severe cognitive fluctuations are present. A further positive association observed with average CAF score and the magnitude of difference in the corrected TPH2 intensity between neurons without Lewy bodies and the whole neuron of those that do contain Lewy bodies when the fluctuation groups were analysed together. As the ratio values increase this suggests that the TPH2-positive neurons with Lewy bodies have the TPH2-intensity value similar if not higher than the TPH2-positive neurons without. The results obtained from the current study are in support of the hypothesis, with the associations indicating that a more intact, although not necessarily fully functional, serotonergic system is associated with more severe cognitive fluctuations. To verify this, studies would be required to ascertain whether the levels of serotonergic neurotransmitter are altered between neurons with and without Lewy bodies, however currently no technique is capable of doing this in human *post-mortem* tissue. The data from the current study could suggest that to counteract dysfunction to the system the neurons upregulate serotonergic markers in the neuronal soma to try and normalise serotonergic transmission levels. A study examining the serotonergic system in regard to depression in DLB identified the preservation of SERT sites in the parietal neocortex of DLB patients with depression compared to non-affected cases, suggesting a reduction in synaptic plasticity of the serotonergic neurons rather than cell loss within the raphe, which could lead to a more dynamic dysfunction rather than a permanent one (Ballard *et al.*, 2002a).

Although a positive relationship was observed between the magnitude of difference in TPH2 intensity between neurons without and either the cytoplasmic or whole neuron of those bearing Lewy bodies and severity of cognitive fluctuations when the fluctuation groups were combined, a negative association was observed between the proportion of TPH2-positive neurons bearing Lewy bodies and the severity of cognitive fluctuations in the DLB-only group. Therefore, in DLB group the higher the proportion of TPH2-positive neurons that did

not possess Lewy bodies the more severe the cognitive fluctuations experienced. This data combined suggests that there may be alterations to the non-Lewy body bearing TPH2-positive neurons that were not measure within the current study that relate to the vulnerability towards cognitive fluctuations. Further studies are required to ascertain what pathological changes are related to the severity of cognitive fluctuation, including whether the changes could relate to forms of α-synuclein not measured within the current study, such as α-synuclein levels within the synaptic compartments. In the current study colocalization between small TPH2-positive punctate staining and α-synuclein was observed. Although not analysed, α-synuclein in these structures could be more pathogenic to serotonergic dysfunction. Previous studies in dopaminergic neurons have identified disruption to mitochondrial function (Sarafian *et al.*, 2019) and synaptic vesicles (Plotegher et al., 2017) due to interactions between dopaminergic catabolites and α-synuclein; recent studies have identified similar interactions with a serotonergic catabolite (Falsone *et al.*, 2011; Jinsmaa *et al.*, 2015). Disruption to synaptic compartments could alter synaptic plasticity and lead to a more dynamic dysfunction of the system which could underly a variable clinical feature such as cognitive fluctuations. Further research into α-synuclein in serotonergic synaptic compartments is required to understand how this could affect serotonergic transmission.

5.4.4 Limitations

The most influential limiting factor of this study was the small number of cases that underwent analysis for the colocalization of α-synuclein and TPH2, only 6 DLB with fluctuations, 4 nLBD with fluctuations and 3 nLBD without fluctuation cases. All cases in the immunofluorescent cohort underwent dual labelling with pS129, however due to a global pandemic, priority was given to collection of the TPH2 data. Therefore, the small group numbers that meant that the correlations undertaken on the cognitive fluctuation groups, specifically when analysed separately, are likely to be under powered due to the low group numbers and thus any conclusion should be treated with caution until further cases can be added.

The study did not measure the levels α-synuclein present in the TPH2-positive synapses or the degree of neuronal loss within the nucleus. Further clarification of these are required to understand the extent of serotonergic dysfunction in relation to cognitive fluctuations.

The raphe is known to be a heterogeneous structure with different subnuclei, including the DR and MnR known to project to different anatomical locations (Hornung, 2003). Within the current study there was a lack of consistent level analysed and the DR and MnR were analysed together. The variability within the level sampled and combination of the DR and MnR could have affected the relationships observed.

5.4.4 Conclusions

The study aimed to investigate the neuropathological differences in the raphe between cases that clinically presented with cognitive fluctuations and those that did not, along with investigating whether there was an association between neuropathological changes and cognitive fluctuation severity.

The study identified pathological differences in the raphe related to the pathological diagnosis of the cases, with no changes specifically related to the presence or absence of cognitive fluctuations. However, alterations to the level of the serotonin catalytic enzyme, TPH2, in neurons with Lewy bodies did relate to the severity of cognitive fluctuations. This data suggests that cognitive fluctuations could be the result of dynamic serotonergic dysfunction, with further studies required to identify the full extent and causes of the dysfunction.

Chapter 6: Biochemical studies in the medial prefrontal cortex

6.1 Introduction

The ARAS projects from various brainstem nuclei, including the LC, raphe and PPN, to the cortex, thalamus and basal forebrain to mediate wakefulness and arousal through cortical activation, suppression of slow rhythms in EEG and synchronisation of faster frequency rhythms (Sarter, 2006; Edlow *et al.*, 2012; Kovalzon, 2016a). The mPFC is one of the cortical regions to which the nuclei of the ARAS project, both directly and indirectly (Paus, 2000; Jones, 2003). Projections from the LC (Chandler *et al.*, 2014) and raphe (Martín-Ruiz *et al.*, 2001) are known to innervate the mPFC both directly and indirectly via the cholinergic neurons of the basal forebrain (Bloem *et al.*, 2014); with the PPN sending indirect projections via the thalamus (Jang *et al.*, 2020). The mPFC has also been shown to be reciprocally connected with ARAS nuclei, including the raphe (Celada *et al.*, 2001) and PPN (Clark *et al.*, 2018), in order to maintain control cortical states. From the previous studies in the brainstem, it was discovered that pathological protein burden in the ARAS nuclei do not relate to the presence or absence of cognitive fluctuations. Therefore, examination of a cortical ARAS region that the ARAS brainstem nuclei project to, both directly and indirectly would enable the further investigation into whether the projections from the brainstem regions are still innervating the cortical region, as well as whether cortical changes to the ARAS are involved in the underlying pathophysiology of cognitive fluctuations.

The mPFC is a cortical region that integrates information from numerous cortical and subcortical areas (Xu *et al.*, 2019). Dysfunction of the mPFC has been identified in various neurodegenerative disorders, including AD (Xu *et al.*, 2019) and DLB (Chabran *et al.*, 2020). Studies have implicated the mPFC in arousal (Zhang *et al.*, 2014) and attention (Riga *et al.*, 2014), with alterations having been identified in cognitive fluctuations in DLB (Bliwise *et al.*, 2014), as well as the region forming part of the default mode network (Li *et al.*, 2014). In DLB, MRI studies have identified alterations to connectivity within attentional networks, including the default mode network involving the mPFC suggesting that these pathways could be altered (Galvin *et al.*, 2011; Franciotti *et al.*, 2013; Peraza *et al.*, 2014). Furthermore, atrophy has been observed in the basal forebrain which sends cholinergic projections to the mPFC (Grothe *et al.*, 2014; Schumacher *et al.*, 2020). Severity of the loss of cholinergic neurons in the basal forebrain was associated with alterations to arousal

levels and maintenance (Kasanuki *et al.*, 2018). Studies have investigated the role of the mPFC in disorders of consciousness, including alterations to consciousness post-stroke (Liu *et al.*, 2017), as well as where alterations to the default mode network has been suggested. Furthermore, studies of post-stroke alterations to consciousness, have identified an increase in thalamocortical activity to the mPFC, after the initial stroke, was associated with recovery from a vegetative state to a normal conscious state (Jang *et al.*, 2020). Therefore, the present study aimed to determine whether cognitive fluctuations are associated with local pathological protein deposits and/or synaptosomal changes, as a proxy of innervation to the mPFC.

6.1.1 Aims

Using frozen *post-mortem* tissue containing the mPFC from DLB cases with fluctuations, nLBD cases, both with and without cognitive fluctuations, AD cases without cognitive fluctuations and aged cognitively normal controls, this study aims to:

1. Evaluate synaptosomes as a proxy of innervation of mPFC from the ARAS, utilising immunoblots
2. Quantify the burden and identify the neuronal localisation of neuropathological protein lesions, utilising immunoblots
3. Assess whether the neurodegenerative changes seen are related to the presence or severity of cognitive fluctuations, utilising neuropsychiatric data obtain *intra vitam*

6.2 Methods

6.2.1 Study Cohort

Five groups were included in the mPFC study with a total of 49 cases (table 2.1). 10 DLB cases that had been recorded to have experienced cognitive fluctuations during life; 10 nLBD cases whom did not experience cognitive fluctuations during life, mixed AD/DLB cases; 9 AD cases with no recorded experience of cognitive fluctuations during life; 10 nLBD cases whom did experience cognitive fluctuations during life, mixed AD/DLB; and 10 aged matched controls with no history of cognitive impairment. Cases which during life were uncooperative with neuropsychiatric testing, were postulated to have cognitive fluctuations due to medicine regimes and those who had brainstem infarcts were excluded from the study. Within the study cohort a number of those with a history of cognitive fluctuations had the severity of these measured via CAF, 9 DLB with cognitive fluctuations and 9 nLBD with cognitive fluctuations, allowing associations with severity of fluctuations to be assessed.

6.2.2 Tissue Acquisition

Frozen tissue was acquired from the mPFC, BA12 (figure 6.1). BA12 was selected for frozen tissue analysis due to a study by Jang et al (2020) which identified the mPFC as a region involved in post-stroke recovery of consciousness, with the study stating the mPFC to be comprised of BA12 and BA32. However, the literature surrounding the region which BA32 is functionally part of is divided between the anterior cingulate cortex and the mPFC, due to this disparity between functional grouping, only BA12 was selected for the present study.

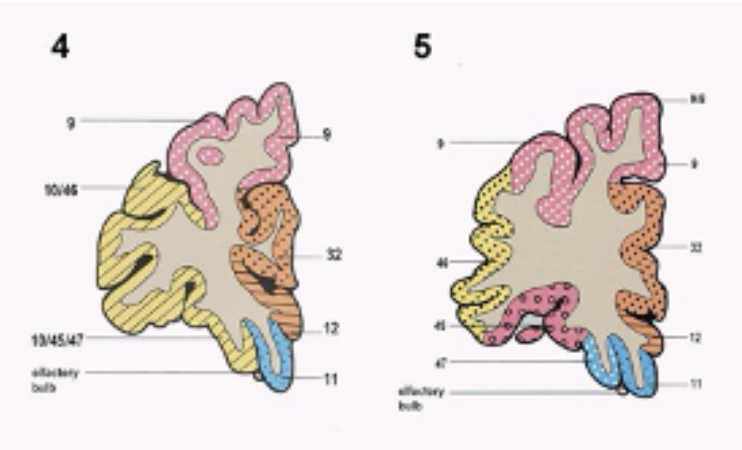


Figure 6.1. Newcastle Brain Tissue Resource frozen tissue dissection protocol.
Brodmann area 12 relating to the medial prefrontal cortex was sampled from block 4 or 5 shown.

Approximately 250mg of tissue was dounce homogenised in cold lysis buffer, as described in 2.9.1, before undergoing synaptosomal fragmentation.

6.2.3 Synaptosomal fragmentation

Homogenised aliquots underwent synaptosomal fragmentation by sequential centrifugation (figure 6.2) as described in 2.9.2, previously described by Wirths (2017), immediately after tissue homogenisation. Sequential fragmentation produced: P1- nuclei, S2-microsomes and soluble enzymes, P2-crude synaptosomal and mitochondrial fraction. All fractions were stored at -80°C until further use.

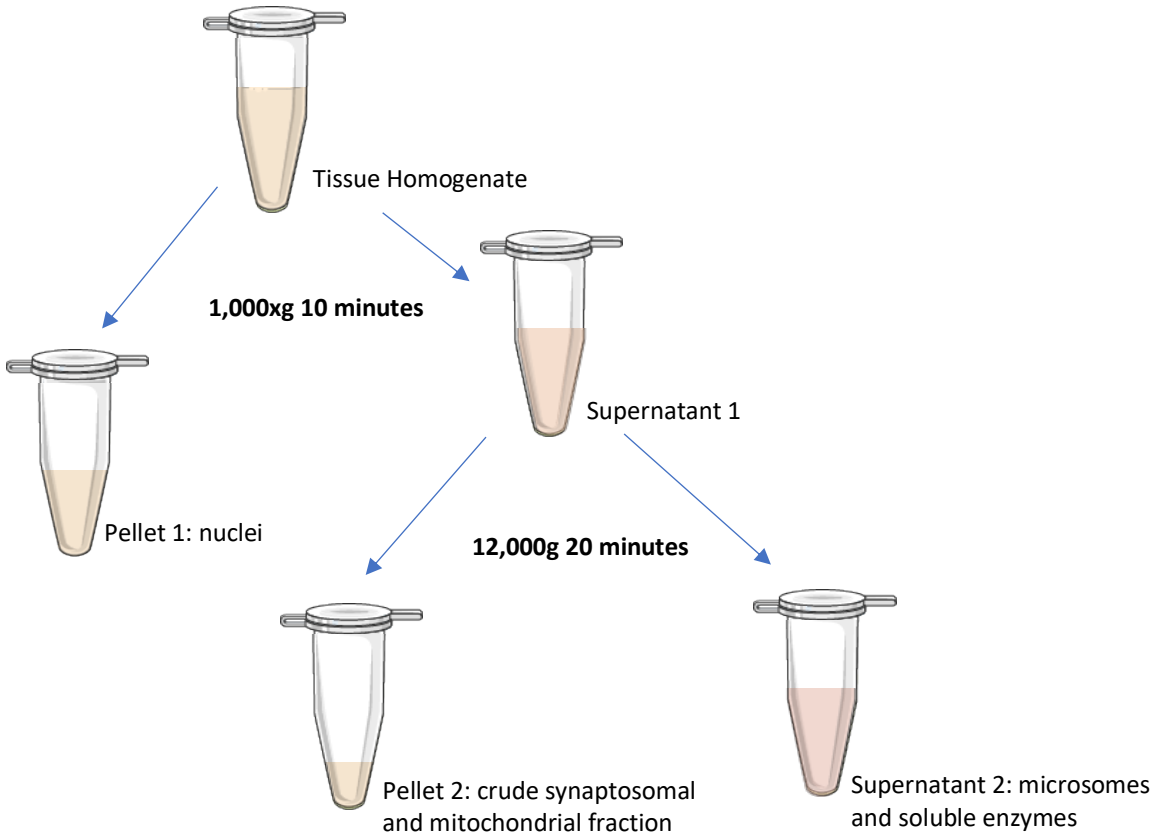


Figure 6.2. Synaptosomal fragmentation protocol.
Flow chart describing the sequential centrifugation for the preparation of a crude synaptosomal fraction.

6.2.4 SDS-PAGE and Western blotting

The three fractions obtained from the sequential fractionation underwent western blotting, as described in 2.9.2.1, to assess the levels of nuclear (H3), mitochondrial (VDAC1) and synaptic markers (SNAP25) in the three fractions. The three fractions were assessed for their respective levels of H3, VDAC1 and SNAP25 in order to validate the fractionation procedure. The three markers assessed were expressed relative to GAPDH, a house-keeping

protein that has a stable and ubiquitous expression, to control for variation in protein loading. Briefly, total protein concentration for each fraction was determined using a Bradford assay, as described in 2.9.2.1. 10µg of 0.5µg/µl total protein was loaded into each well of a NuPAGE 4-12% Bis-Tris gel (Invitrogen, Waltham, MA, USA). The samples were separated using SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and probed with primary and secondary antibodies (table 6.1). Size and intensity of the protein bands on the blots was quantified using FIJI (Schindelin *et al.*, 2012) with the target protein expression normalised to GAPDH expression within the fractions, as described in 2.9.2.1.

Table 6.1. Antibodies utilised to validate the synaptosomal fractional protocol.
Optimised dilutions and species of the antibodies utilised for the synaptosomal fractionation validation via SDS-PAGE and Western blotting.

Antibody	Manufacturer	Dilution	Species
H3, Histone H3	Cell Signalling, UK	1:5000	Mouse
SNAP25, Synaptosome Associated Protein 25	ThermoFisher, MA, USA	1:1000	Rabbit
VDAC, Voltage-dependent anion-selective channel 1	Abcam, UK	1:1000	Mouse
GAPDH, Glyceraldehyde 3-phosphate dehydrogenase	Cell Signalling, UK	1:5000	Rabbit

6.2.5 Immunoblotting

Dot blots were undertaken to assess the levels of a number of different proteins within the P2 and S2 fractions, as described in 2.9.3. Briefly, total protein concentration for each fraction was determined using a Bradford assay, as described in 2.9.2.1. Samples were adjusted to 1µg/µl with distilled water and directly dotted in duplicate onto a 0.2µm nitrocellulose membrane (10µl per sample, 10µg per dot). Two blots were run in parallel for all antibodies: AD, DLB with fluctuations and controls, and nLBD with and without fluctuations and controls. Two blots were utilised to ensure the groups which needed to be directly compared AD and DLB, and nLBD with and without fluctuations could be done directly without pooling across blots as well as to ensure minimal variability across the samples loaded onto the blot which would occur if a larger blot had been used. All 10 control cases were run on each blot to reduce variation in analysis. One antibody, NDUFB8, required sample pre-treatment, incubation at 100^{o}C for 5 minutes, before being dotted onto the blot (table 6.2). Noradrenaline reuptake transporter (NET) antibody underwent a

series of different pre-treatment steps, including addition of various SDS concentrations, incubation at 100^{o}C and 70^{o}C and addition of a reducing agent, in order to try and optimise an experimental protocol (figure 6.5). Blots were blocked in 10% milk powder before being incubated in primary antibody (table 6.2) overnight at 4°C. Blots were then incubated with an appropriate, goat anti-mouse or goat anti-rabbit horseradish peroxidase conjugated secondary antibody. Immunoblots were visualised with enhanced chemiluminescence, total protein was measured utilising ponceau. Images were captured using a Fuji LAS 4000 with imaging software (Fuji LAS Image, Raytek, Sheffield, UK).

To ensure specificity of the detected signal, appropriate non-primary antibody control dot blots were undertaken. For heat treated and soluble enzyme fraction immunoblots a small detectable immunoreactive signal was observed, following an exposure time comparable to the primary treated immunoblot. Non-specific immunoreactive signal was subtracted from the signal detected on the primary incubated immunoblot. No other immunoblots possessed a detectable non-specific immunoreactive signal at an exposure time comparable to the primary incubated blot.

6.2.6 Blot analysis

Immunoreactivity was quantified from 16-bit digitised images utilising FIJI (Schindelin *et al.*, 2012) based on area under curve measurements normalised to total protein load established via area under the curve for ponceau processed blots, as previously described by Koss *et al* (2016). Immunoblot intensity data were normalised within blot using total protein adjusted values and expressed relative to the control cases, prior to being pooled across blots.

Ratios were generated between the levels of 5G4 and pS129 in the P2 and S2 fractions; with values over 1 suggestive that the levels of the α-synuclein species are higher in the P2 fraction, and values under 1 suggestive that the levels of the α-synuclein species are higher the S2 fraction. A ratio was also generated between the level of NDUFB8, a mitochondrial complex I subunit, and TOM20, an outer mitochondrial membrane transporter, (NDUFB8:TOM20). NDUFB8:TOM20 allows examination of differences in Complex I expression relative to the total number of mitochondria, therefore reducing the possibility of differences solely due to alterations in mitochondrial number.

Table 6.4. Antibodies utilised in the medial prefrontal cortex study.
Optimised dilutions, species and sample pre-treatment protocols for the antibodies utilised for the immunoblots in the medial prefrontal cortex study. *various pre-treatments utilised are outlined in 6.3.4. ** three phosphorylated tau antibodies, PHF-1, AT8 and CP-13 were utilised together creating a phospho-tau 'cocktail'. Abbreviations: Aβ- Aβ; Ser-Serine; Thr-Threonine.

Antibody	Manufacturer	Dilution	Species	Sample pre-treatment
SERT, serotonin reuptake transporter	MAb Technologies, WI, USA	1:1000	Mouse	NA
VMAT2, vesicular monoamine transporter 2	Abcam, UK	1:500	Rabbit	NA
NET, noradrenaline reuptake transporter	Atlas antibodies, Bromma, Sweden	1:1000/ 1:500	Mouse	Various pre-treatments*
ChAT, choline acetyltransferase	Atlas antibodies, Bromma, Sweden	1:500	Rabbit	NA
pS129, α-synuclein phosphorylated at Ser129	Abcam, UK	1:1000	Rabbit	NA
5G4, aggregated α-synuclein	Analytik Jena, Germany	1:2500	Mouse	NA
**PHF-1, tau phosphorylated at Ser396 and 404	Peter Davies Lab	1:1000	Mouse	NA
**AT8, tau phosphorylated at Ser199 and 202, and Thr404	Autogen, MA, USA	1:1000	Mouse	NA
**CP-13, tau phosphorylate at Ser202	Peter Davies Lab	1:1000	Mouse	NA
MOAB, Aβ peptide 40/42	Biosensis, SA, Australia	1:1000	Mouse	NA
NDUFB8, Mitochondrial complex I subunit (NDUFB8)	Mitosciences, Abcam, UK	1:1000	Mouse	5 minutes at 100°C
TOM20, TOM subunit (TOM20)	Santa Cruz, Heidelberg, Germany	1:1000	Rabbit	NA

6.2.7 Statistical analysis

Statistical analysis was conducted using SPSS v.26 (IBM). Variables were assessed for normality by the Shapiro-Wilk test and inspection of histograms and Q-Q plots. All immunoblot data, analysed with values expressed relative to control cases within blot, was found to be non-normal leading to non-parametric tests being employed. To determine differences in the non-normally distributed data Kruskal-Wallis and post-hoc Mann-Whitney

were undertaken. Blots were analysed within blot when the disease groups were compared to controls: AD and DLB with fluctuations with controls, and nLBD with and without fluctuations with controls. As all control cases were used on both blots, two values for each control cases were produced, which precluded the analysis of control cases when analysing across blots. A one-sample Wilcoxon signed rank test was utilised to determine whether the ratios between α-synuclein levels in the P2 and S2 fractions calculated deviated from a median of 1.

To determine whether there was an association between any immunoblot measures and neuropsychological data obtained *intra vitam,* Spearman's rank correlations were conducted. Corrections such as Bonferroni were not applied due to the fact that there is not a true null hypothesis because of the classification of neurogenerative diseases, for example AD will have higher levels of AD-type pathology than DLB, therefore a p-value of <0.05 was considered significant.

6.3 Results

6.3.1 Demographics

No significant differences were observed in *post-mortem* delay, fixation duration, and age between the study groups. There was no significant difference observed in disease duration between the neurodegenerative disease groups. Between the groups, no difference was found in the proportion of males to females.

CAF scores were available for 22/49 cases. 17/20 of the cases with cognitive fluctuations had at least one CAF score. No significant difference was observed between the last ($p=0.842$), maximal ($p=0.815$) or average ($p=0.884$) CAF score between the DLB with fluctuations or the nLBD with fluctuation groups.

Final MMSE scores were available for 40/49 cases: 10 DLB cases with fluctuations, 10 nLBD with fluctuations, 8 nLBD without fluctuations, 8 AD and 4 Controls. No significant difference in the interval between the last MMSE and death was observed between the groups. In comparison to controls, significantly lower last MMSE scores were observed in DLB cases with fluctuations ($z=-18.250$, $p=0.014$), nLBD with fluctuations ($z=19.150$, $p=0.005$), nLBD without fluctuations ($z=-20.625$, $p=0.003$) and AD cases ($z=-18.250$, $p=0.009$). No significant differences in last MMSE score were observed between the disease groups.

6.3.2 Fractionation Validation

Western blot analysis of synaptosomal fractions, undertaken in a control case, validated the fractionation with P2 enriched for SNAP25 (figure 6.3). A single band at approximately 15 kDa was demonstrated for Histone H3, in the P1 fraction only. A band for SNAP25, at approximately 23 kDa was observed in all fractions. Two bands were observed for VDAC, at approximately 31 kDa, in the P1 and P2 fractions. When protein levels were expressed relative to GAPDH, fraction P2 was found to be have a two-fold higher relative level of SNAP25 suggestive of an enrichment for synaptosomes.

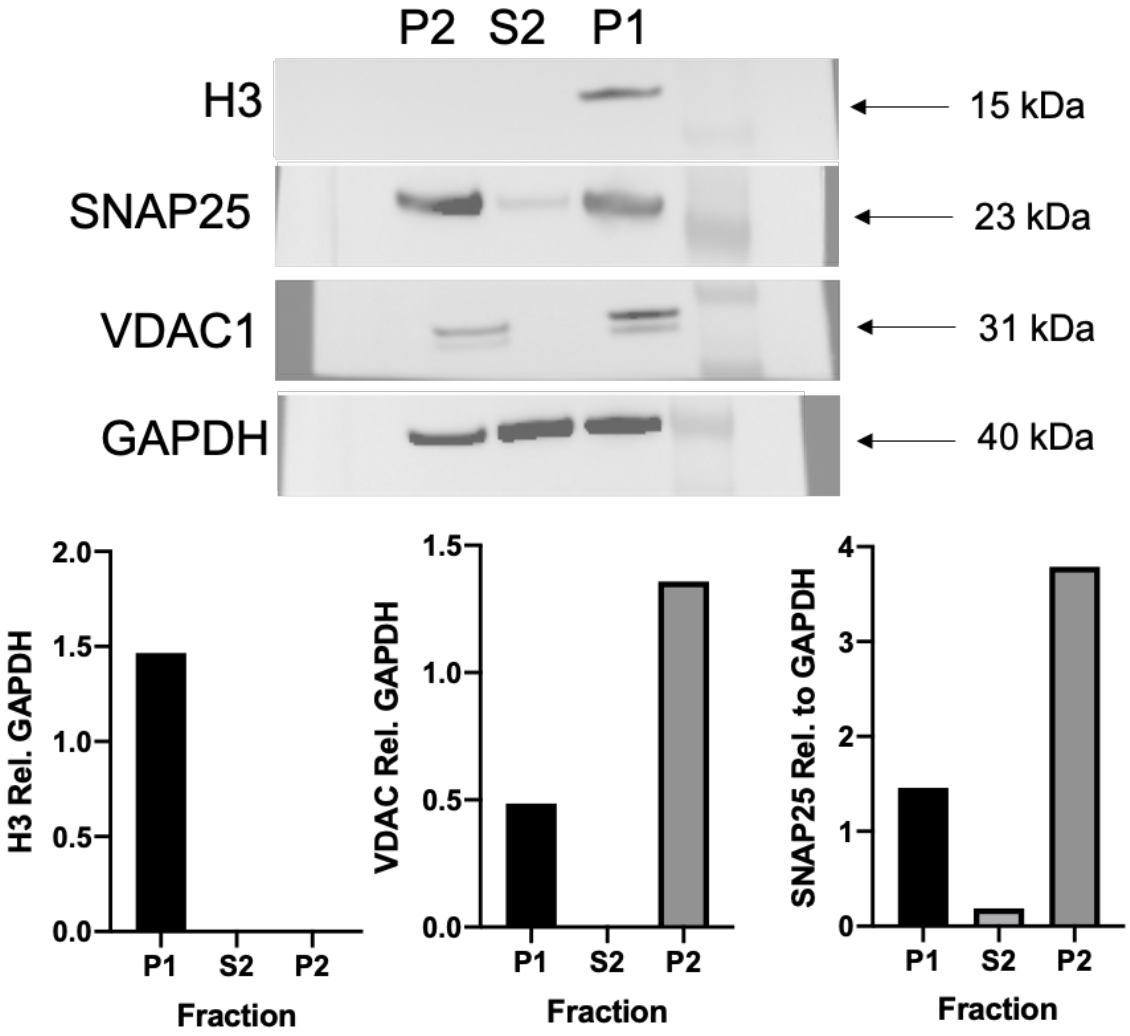


Figure 6.3. Fractionation validations by western blotting.
The three fractions, P1, S2 and P2 were western blotted for nuclear (H3), synaptic (SNAP25) and mitochondrial (VDAC1) markers, with levels of these proteins were expressed relative to GAPDH, a ubiquitous housekeeping protein. Blotting identified that the fragmentation process has produced a crude synaptosomal fragment (P2), however, this fragment did not contain all the synaptosomes from the original tissue fraction due to the presence of SNAP25, albeit at a lower level in the P2 and S2 fractions.

6.3.3 Transporter Blots

Native state dot blots of transporter proteins showed that in the synaptosomal fraction, P2, the disease groups did not differ in comparison to controls. For SERT no difference in SERT level across the blot was observed for AD and DLB (p=0.777) or nLBD with and without fluctuations (p=0.860), no differences were observed between the disease groups (figure 6.4A). For the vesicular monoamine transporter 2 (VMAT2) no difference was observed for AD and DLB (p=0.785) or nLBD with and without fluctuations (p=0.492) and controls, no differences were observed between the disease groups (figure 6.4B). A trend towards a relationship was observed between the synaptosomal level of SERT and the level of VMAT2 (r_s=0.305, p=0.059) in the disease groups.

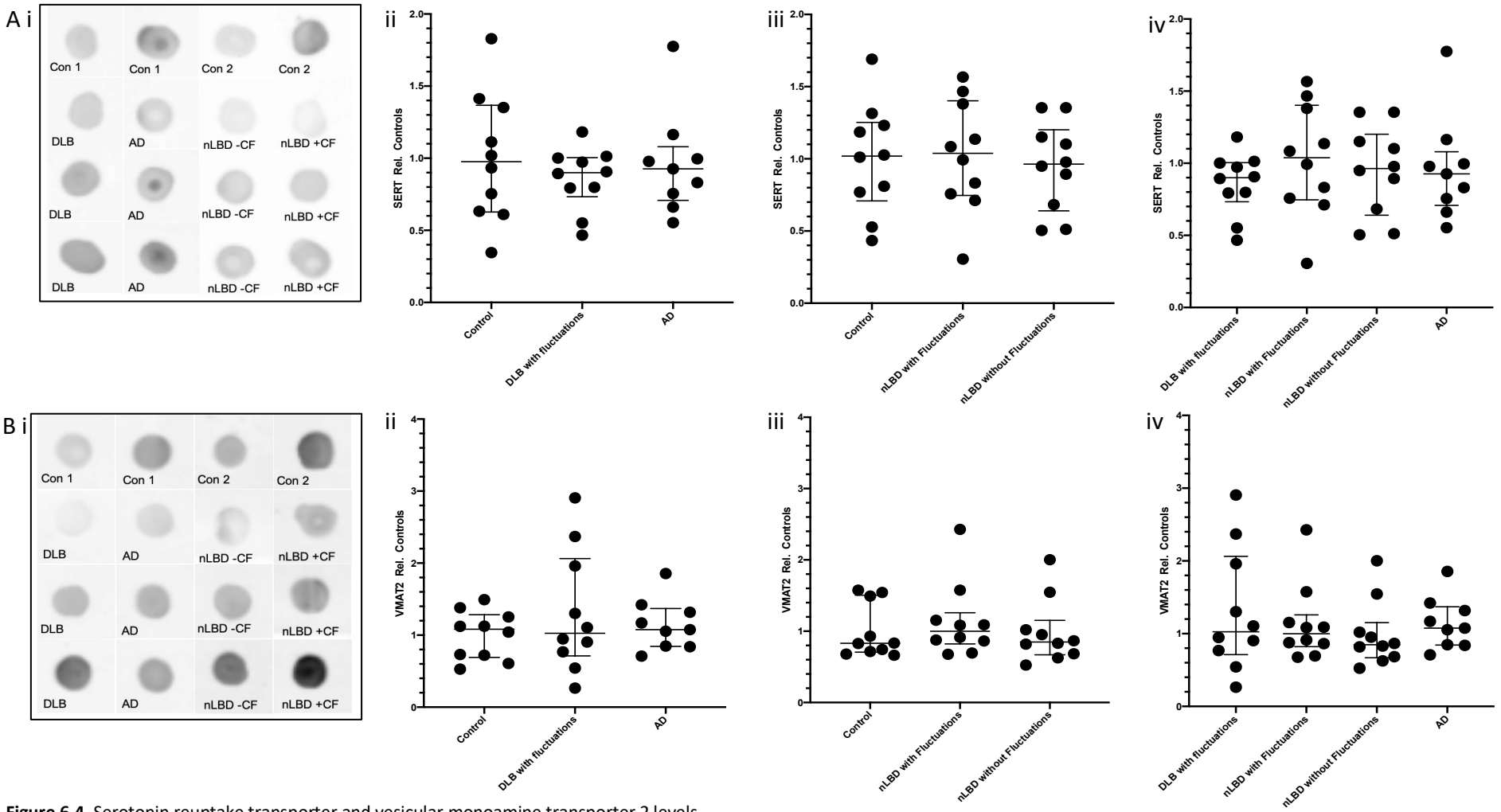


Figure 6.4. Serotonin reuptake transporter and vesicular monoamine transporter 2 levels.
Dot blots for A- serotonin reuptake transporter (SERT) and B- vesicular monoaminergic transporter 2 (VMAT2). i representative dots from both blots. The two left columns are dots from the AD and DLB blot; the right two columns are dots from the nLBD with (+CF) and without (-CF) fluctuations blot. Dots representing the minimum, median and maximum are shown for the four fluctuation groups. Control (Con1 and Con2) show the cases with minimum (left) and maximum level (right) for the two blots, the dots representing the minimum and maximum are the same case for both blots. Analyses ii (AD, DLB and control) and iii (nLBD with and without fluctuations and controls) within blot iv fluctuation groups across blots. Data from both within and across blots shows no difference between the synaptosomal levels of SERT or VMAT2 across any of the groups. Data is shown relative to controls with median and interquartile range.
Abbreviations: AD- Alzheimer's disease; CF- cognitive fluctuations; DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease.

No associations were found between disease duration across the disease groups and synaptosomal SERT (p=0.304) or VMAT2 (p=0.946).

6.3.4 NET optimisation

Noradrenergic projections from the LC are known to project to the mPFC (Samuels and Szabadi, 2008a) and are speculated to play a key role in attentional and cognitive functions

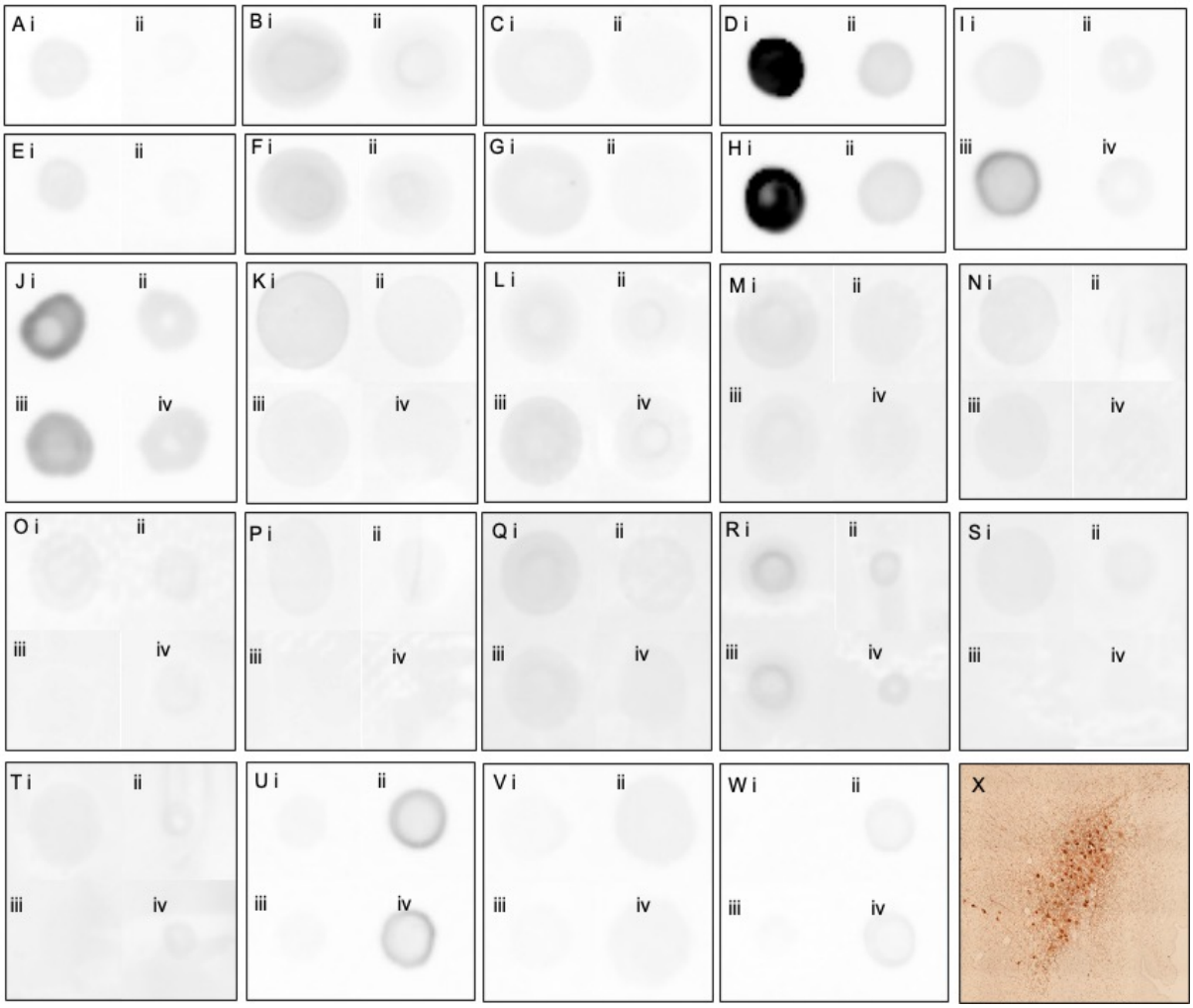


Figure 6.5. Optimisation of noradrenergic transporter dot blot.
Images represent the progression of optimisation experiments for the noradrenergic transporter (NET) antibody. **A**- native state blot NET 1:1000 i pons control (PC) ii synaptosomal fraction from a control case (P2). **B**- 2% SDS, NET 1:1000 i PC ii P2. **C**- 2% SDS with 5 minutes at 100°C, NET 1:1000 i PC ii P2. **D**- 5 minutes at 100°C, NET 1:1000 i PC ii P2. **E**- native state blot NET 1:500 i PC ii P2. **F**- 2% SDS, NET 1:500 i PC ii P2. **G**- 2% SDS with 5 minutes at 100°C, NET 1:500 i PC ii P2. **H**- 5 minutes at 100°C, NET 1:500 i PC ii P2. **I**- Native state NET 1:1000 i PC ii P2, no primary iii PC iv P2. **J**- 5 minutes at 70°C NET 1:1000 i PC ii P2, no primary iii PC iv P2. **K**-0.05% SDS with 5 minutes at 70°C, NET 1:500 i PC ii P2 no primary iii PC iv P2. **L**- 0.05% SDS, NET 1:500 i PC ii P2 no primary iii PC iv. **M**- 0.1% SDS with 5 minutes at 70°C, NET 1:500 i PC ii P2 no primary iii PC iv P2. **N**- 0.1% SDS with 5 minutes at 70°C, NET 1:500 i PC ii P2 no primary iii PC iv P2. **O**- 0.1% SDS, 5 minutes heat at 70°C, Reducing agent 1:10, NET 1:500 i PC ii P2 no primary iii PC iv P2. **P**- 0.05% SDS, 5 minutes heat at 70°C, Reducing agent 1:10, NET 1:500 i PC ii P2 no primary iii PC iv P2. **Q**- 0.1% SDS, 10 minutes heat at 70°C, NET 1:500 i PC ii P2 no primary iii PC iv P2. **R**- 0.05% SDS, 10 minutes heat at 70°C, NET 1:500 i PC ii P2 no primary iii PC iv P2. **S**-0.1% SDS, 10 minutes heat at 70°C, Reducing agent 1:10, NET 1:500 i PC ii P2 no primary iii PC iv P2. **T**- 0.05% SDS, 10 minutes heat at 70°C, Reducing agent 1:10, NET 1:500 i PC ii P2 no primary iii PC iv P2. **U**- 10 minutes heat at 70°C, NET 1:500 i P2 ii soluble enzyme fraction from the same case as P2 (S2) no primary iii P2 iv S2. **V**- native state blot with a 4-fold increase in protein loaded (40µg), NET 1:500, i P2 ii S2 no primary iii P2 iv S2. **W**- reducing agent 1:10, 10 minutes at 70°C, NET 1:500 i P2 ii S2 no primary iii P2 iv S2. **X**- NET immunohistochemical staining 1:50, section is from the fixed hemisphere of the PC.

(Samuels and Szabadi, 2008b). To assess noradrenergic innervation in the mPFC in the present study dot it was aimed to look at the levels of NET via dot blot. The experimental protocol for the NET dot blot underwent a series of optimisations (figure 6.5).

Briefly, the antibody was tested in various conditions on a number of fractions from both the mPFC and the pons. The fractions utilised were from the same control case. mPFC P2 and S2 fractions were examined, the S2 fraction was examined to determine that NET was not present in another fraction. A further S1 fraction, only undergoing the first centrifugation, from the pons containing the LC was also utilised. Conditions included preheating the sample, addition of SDS, in order to denature and linearize the protein, and altering the dilution of NET antibody. However, although the antibody used has been previously validated on human cingulate cortex via immunoblotting (Lina Pattinson, personal communication, 2020), a working experimental protocol could not be established for mPFC tissue. Upon further examination of the antibody in fixed pons tissue containing the LC, where a working dilution of 1:5000 had been previously shown, a dilution of 1:50 was required to obtain positive staining.

6.3.5 ChAT Blots

Native state dot blots of ChAT showed that in the synaptosomal fraction, P2, the disease groups did not differ in comparison to controls. No significant difference was observed for AD and DLB with fluctuations (p=0.959) or nLBD with and without fluctuations (p=0.618) with controls, no differences were observed between the disease groups (p=0.799) (figure 6.6).

No association was found for disease duration across the disease groups for synaptosomal ChAT (p=0.585).

6.3.5.1 Relationship of ChAT with VMAT2 and SERT levels

A positive relationship was observed between synaptosomal ChAT levels and VMAT2 levels (r_s=0.809, p<0.001) (figure 6.7), when the disease groups were analysed together. When analysed individually synaptosomal ChAT and VMAT2 levels were identified to be positively associated in the DLB with fluctuations (r_s=0.903, p<0.001), nLBD without fluctuations (r_s=0.806, p=0.005) and AD (r_s=0.783, p=0.013) groups. A trend towards an association was observed between ChAT and VMAT2, synaptosomal fraction levels in the nLBD with fluctuations group (r_s=0.612, p=0.060).

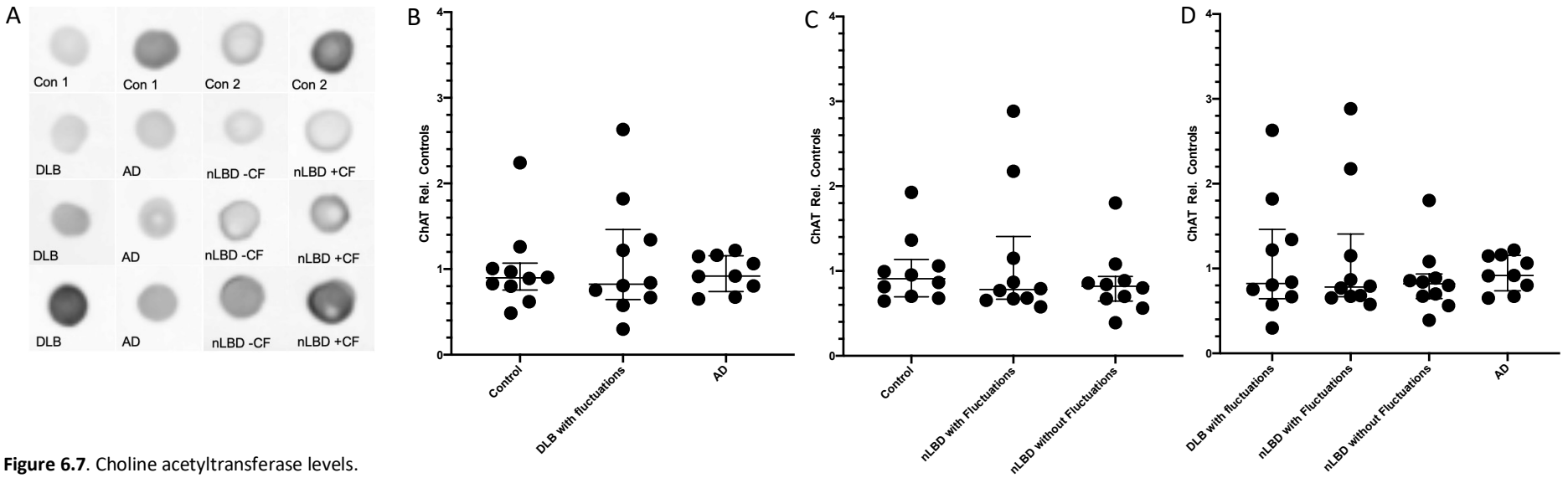


Figure 6.7. Choline acetyltransferase levels.
Dot blot for choline acetyltransferase (ChAT). A- representative dots from both blots. The two left columns are dots from the AD and DLB blot; the right two columns are dots from the nLBD with (+CF) and without (-CF) fluctuations blot. Dots representing the minimum, median and maximum are shown for the four fluctuation groups. Control (Con1 and Con2) show the cases with minimum (left) and maximum level (right) for the two blots, the dots representing the minimum and maximum are the same case for both blots. Analyses B and C within blot; D fluctuation groups across blots. Data is shown relative to controls with median and interquartile range. Abbreviations: AD- Alzheimer's disease; CF- cognitive fluctuations; DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease.

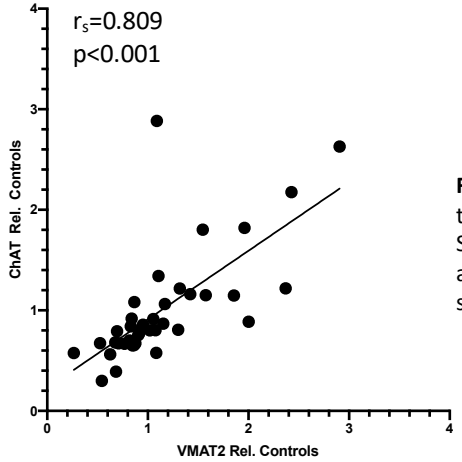


Figure 6.6. Correlation between choline acetyltransferase and vesicular monoamine transporter 2 levels.
Scatterplot illustrating the significant correlation between choline acetyltransferase (ChAT) and vesicular monoamine transporter 2 (VMAT2) levels in all cases apart from controls. Data shown relative to controls.

No relationship was observed for ChAT and SERT levels (p=0.147), when the disease groups were jointly analysed. Synaptosomal fraction ChAT levels were not associated with SERT levels in the disease groups when analysed individually.

6.3.6 mPFC α-synuclein Pathology

α-synuclein pathology was measured utilising two antibodies 5G4, (figure 6.8) which recognises aggregated α-synuclein, and pS129 (figure 6.9) recognising α-synuclein phosphorylated at serine 129, within the synaptosomal, P2, and the soluble enzyme, S2, fractions. 5G4 recognises a conformation that is believed to be pathogenic and pS129 recognises a post-translationally modified form which is upregulated in disease states

No significant effect of disease group was observed between AD and DLB compared to control for 5G4 in the P2 fraction (p=0.276) (figure 6.8A). No effect of disease group was identified for nLBD with and without fluctuations compared to controls for 5G4 in the P2 fraction (p=0.178) (figure 6.8A). When 5G4 levels were analysed across blots a significant effect of disease group was observed in the P2 fraction (χ^2=8.073, p=0.045) (figure 6.8A); AD had a significantly lower level of 5G4 than nLBD with fluctuations (z=-12.056, p=0.021) and nLBD without fluctuations (z=13.656, p=0.009). No differences were observed between the nLBD with and without fluctuation groups (p=0.754) or between the DLB with fluctuations and the nLBD with (p=0.388) or without fluctuations (p=0.239). No further differences were identified.

In the S2 fraction a trend towards a significant effect of disease group on the level of 5G4 was observed between AD and DLB compared to controls (p=0.078) (figure 6.8B). A significant effect of disease group for nLBD with and without fluctuations compared to controls was observed for 5G4 in the S2 fraction (χ^2=11.585, p=0.003) (figure 6.8B). In the S2 fraction controls had a significantly lower level of 5G4 than nLBD with fluctuations (z=-12.200, p=0.002) and nLBD without fluctuations (z=10.900, p=0.006), no difference was observed between the nLBD groups (p=0.741). No effect of disease group was observed when analysed across blots within the S2 fraction (p=0.283) (figure 6.8B).

A significant effect of disease group was observed for AD and DLB with control for pS129 in the P2 fraction (χ^2=13.880, p=0.001) (figure 6.9A). DLB with fluctuations had a significantly higher level of pS129 in the P2 fraction than AD (z=13.556, p<0.001) and controls (z=9.778, p=0.010). A significant effect of disease group for nLBD with and without fluctuations with

controls was observed for pS129 in the P2 fraction (χ^2=14.532, p=0.001) (figure 6.9A). Controls had a significantly lower level of pS129 in the P2 fraction in comparison to nLBD with fluctuations (z=-14.278, p<0.001) and nLBD without fluctuations (z=11.178, p=0.004); no difference was observed between the nLBD groups with and without fluctuations (p=0.416). When analysed across blots a significant effect of disease group was observed for pS129 P2 levels (χ^2=19.432, p<0.001) (figure 6.9A). Within the P2 fraction, pS129 levels were significantly higher in DLB with fluctuations (z=15.711, p=0.003), nLBD with fluctuations (z=-21.811, p<0.001) and nLBD without fluctuations (z=17.511, p=0.001) in comparison to AD. No difference in P2 pS129 levels were observed between the DLB with fluctuation and nLBD with (p=0.232) or without fluctuation groups (p=0.724). No further differences were observed.

Within the S2 fraction a significant effect of disease group was observed for AD and DLB with control for pS129 (χ^2=14.678, p=0.001) (figure 6.9B). DLB with fluctuations had a significantly higher level of pS129 in the S2 fraction than AD (z=11.033, p=0.005) and controls (z=13.850, p<0.001). In the S2 fraction a significant effect of disease group for nLBD with and without fluctuations with controls was observed for pS129 (χ^2=18.715, p<0.001) (figure 6.9B). Control cases had a significantly lower S2 pS129 level than nLBD with fluctuations (z=-15.400, p<0.001) and nLBD without fluctuations (z=14.000, p<0.001); no difference was observed between the two nLBD groups (p=0.722). pS129 levels in the S2 fraction when analysed across blots identified a significant effect of disease group (χ^2=15.305, p=0.002) (figure 6.9B). Within the S2 fraction, pS129 levels were significantly higher in DLB with fluctuations (z=15.389, p=0.003), nLBD with fluctuations (z=-18.389, p<0.001) and nLBD without fluctuations (z=16.489, p=0.002) in comparison to AD. No difference in S2 pS129 levels were observed between the DLB with fluctuations and nLBD with (p=0.556) or without fluctuations groups (p=0.829). No further significant differences between the level of pS129 in the S2 fraction were identified.

In summary, 5G4 was higher in the nLBD with and without fluctuations groups compared to AD, in the P2 fraction and compared to controls in the S2 fraction. pS129 was higher in the DLB with fluctuations, nLBD with and without fluctuations groups in comparison to both AD and controls, in both the P2 and S2 fractions. No further differences were observed.

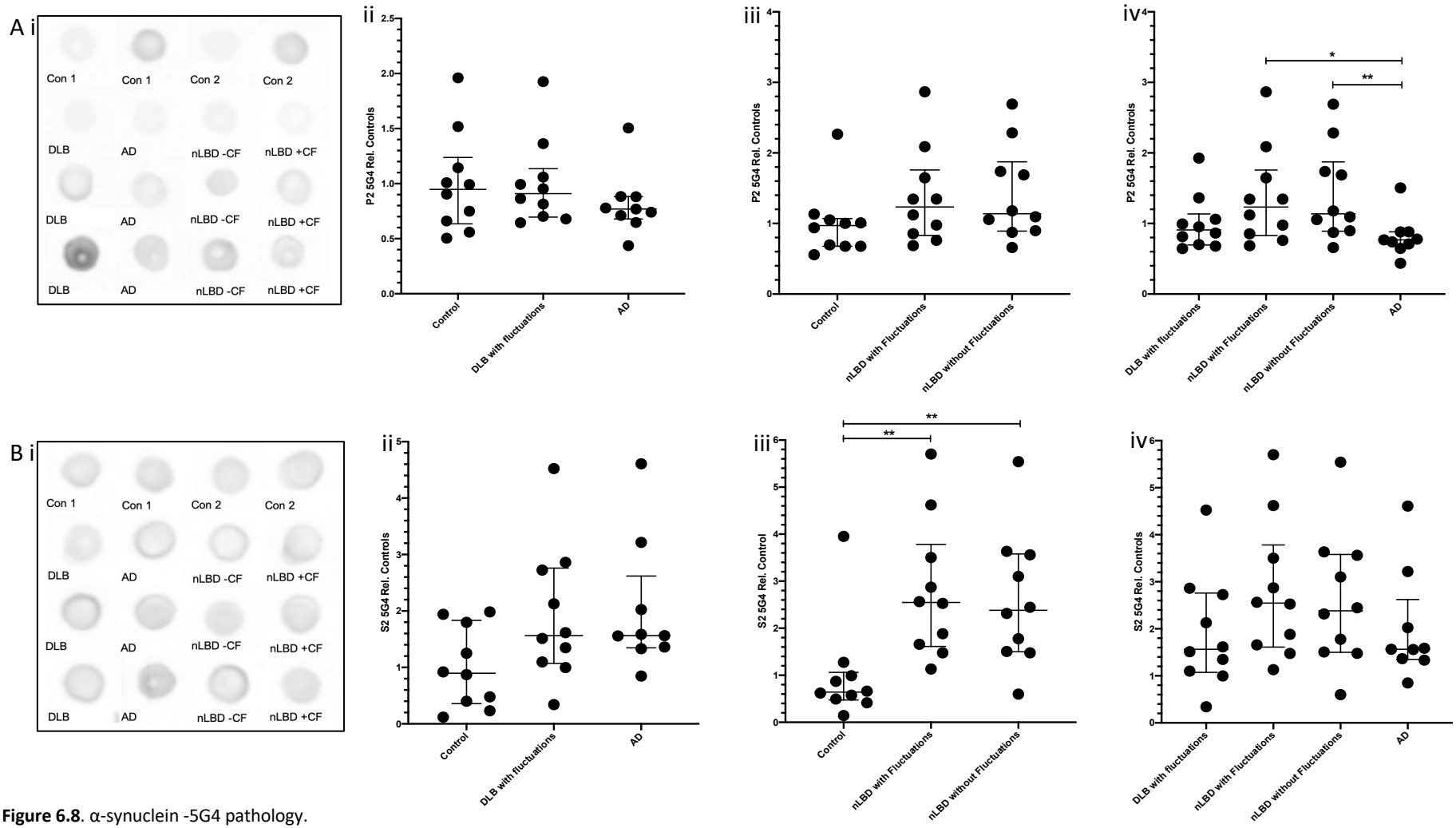


Figure 6.8. α-synuclein -5G4 pathology.

Dot blots for A- Synaptosomal fraction, P2, and B- Soluble enzyme fraction, S2, 5G4. i representative dots from both blots. The two left columns are dots from the AD and DLB blot; the right two columns are dots from the nLBD with (+CF) and without (-CF) fluctuations blot. Dots representing the minimum, median and maximum are shown for the four fluctuation groups. Control (Con1 and Con2) show the cases with minimum (left) and maximum level (right) for the two blots, the dots representing the minimum and maximum are the same case for both blots. Analyses ii and iii within blot iv fluctuation groups across blots. Data is shown relative to controls with median and interquartile range *p<0.05 **p<0.01. Abbreviations: AD- Alzheimer's disease; CF- cognitive fluctuations; DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease.

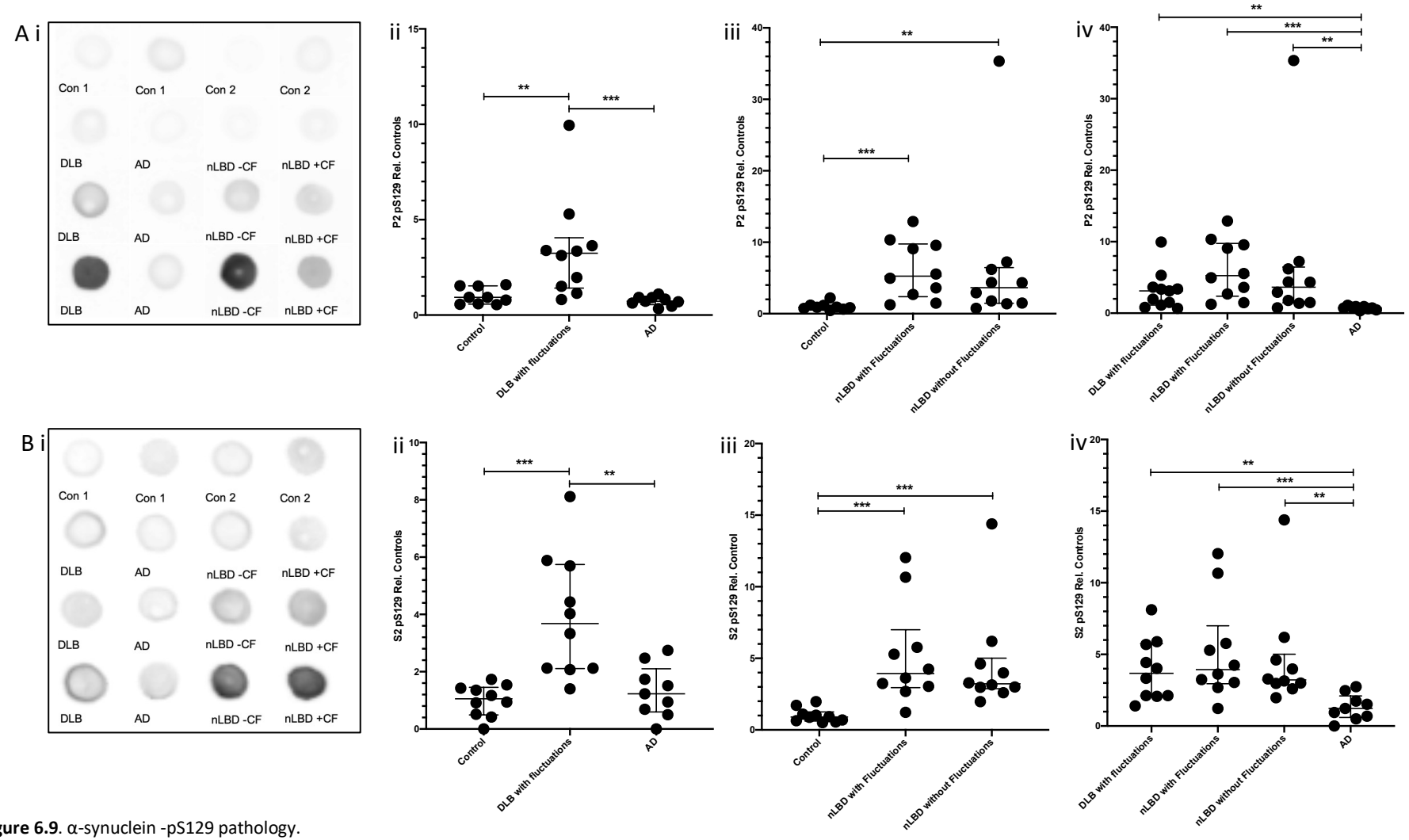


Figure 6.9. α-synuclein -pS129 pathology.

Dot blots for A- Synaptosomal fraction, P2, and B- Soluble enzyme fraction, S2, pS129. i representative dots from both blots. The two left columns are dots from the AD and DLB blot; the right two columns are dots from the nLBD with (+CF) and without (-CF) fluctuations blot. Dots representing the minimum, median and maximum are shown for the four fluctuation groups. Control (Con1 and Con2) show the cases with minimum (left) and maximum level (right) for the two blots, the dots representing the minimum and maximum are the same case for both blots. Analyses ii and iii within blot iv fluctuation groups across blots. Data is shown relative to controls with median and interquartile range *p<0.05 **p<0.01 ***p<0.001. Abbreviations: AD- Alzheimer's disease; CF- cognitive fluctuations; DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease.

6.3.6.1 Relationship between α-synuclein in different cellular locations

A positive association was observed between 5G4 levels in the P2 and S2 fractions, when analysed across the disease groups (r_s=0.417, p=0.008). When analysed separately based upon disease group a positive association was only observed between 5G4 P2 and S2 levels in the nLBD without fluctuations group (r_s=0.673, p=0.033). The level of pS129 was found to have a positive association, when analysed across all disease groups, between the P2 and S2 fraction (r_s=0.869, p<0.001). When the disease groups were analysed individually positive relationships between the pS129 level in the P2 and S2 fraction were observed in the DLB with fluctuations (r_s=0.685, p=0.029), nLBD with fluctuations (r_s=0.891, p=0.001) and the nLBD without fluctuations groups (r_s=0.842, p=0.002).

pS129 and 5G4 are markers of species of α-synuclein, both forms of α-synuclein are thought to be important in LBD. When the disease groups were analysed together no association was observed between the synaptosomal level of 5G4 with the P2 fraction (p=0.252) or S2 fraction (p=0.222) level of pS129. No relationships between the level of 5G4 in the P2 fraction and the level of pS129 in the P2 or S2 fraction were observed when the disease groups were analysed individually. The soluble enzyme, S2 fraction level of 5G4 was not associated with the P2 fraction (p=0.341) or S2 fraction (p=0.098) level of pS129. No relationships between the level of 5G4 in the S2 fraction and the level of pS129 in the P2 or S2 fraction were observed when the disease groups were analysed individually.

6.3.6.2 Cellular location of α-synuclein species

Different species of α-synuclein have been speculated to be preferentially located in different subcellular compartments, for example, the synaptic cleft and the neuronal soma (Miraglia *et al.*, 2018). Ratios were generated between the levels of 5G4 and pS129 in the P2 and S2 fractions; with values over 1 suggestive that the levels of the α-synuclein species are higher in the P2 fraction and those under 1 the S2 fraction.

A one-sample Wilcoxon signed rank test was undertaken to assess whether the observed median for the ratios between P2 and S2 fractions deviated from a hypothetical median of 1, indicative that the species were equally present in both fractions. A significant deviation from the hypothetical median of 1 was observed, in all disease groups combined, for 5G4 levels, actual median 0.53 (z=74.000, p<0.001) (figure 6.10A). When the disease groups were analysed individually a significant deviation from the hypothetical median of 1 was

observed in nLBD with fluctuations, actual median 0.58 (z=3.000, p=0.013) nLBD without fluctuations, actual median 0.53 (z=5.000, p=0.022) and AD, actual median 0.47 (z=0.000, p=0.008) groups for the ratio of 5G4 levels between the P2 and S2 fractions (figure 6.10B). No deviation for the 5G4 ratio was observed for the DLB group, actual median 0.62 (p=0.139). The pS129 ratio between levels in the P2 and S2 fraction did not deviate from the hypothetical median, actual median 0.86 (p=0.446) (figure 6.10A). When the disease groups were analysed individually only the AD group had a pS129 ratio that significantly deviated from 1, actual median 0.49 (z=4.000, p=0.050). The pS129 ratios in the DLB with fluctuations, actual median 0.80 (p=0.386), nLBD with fluctuations, actual median 1.13 (p=0.285) and nLBD without fluctuations, actual median 0.84 (p=0.799) did not deviate from the hypothetical median (figure 6.10C).

To summarise, the data suggests that 5G4 is observed preferentially in the S2 in comparison to the P2 fraction, however when analysed individually based upon disease group DLB with fluctuation cases were not seen to have 5G4 preferentially in either fraction. In comparison, pS129 was observed not to be preferentially located in either fraction, although when analysed individually based upon disease group AD cases were found to have pS129 preferentially in the S2 fraction.

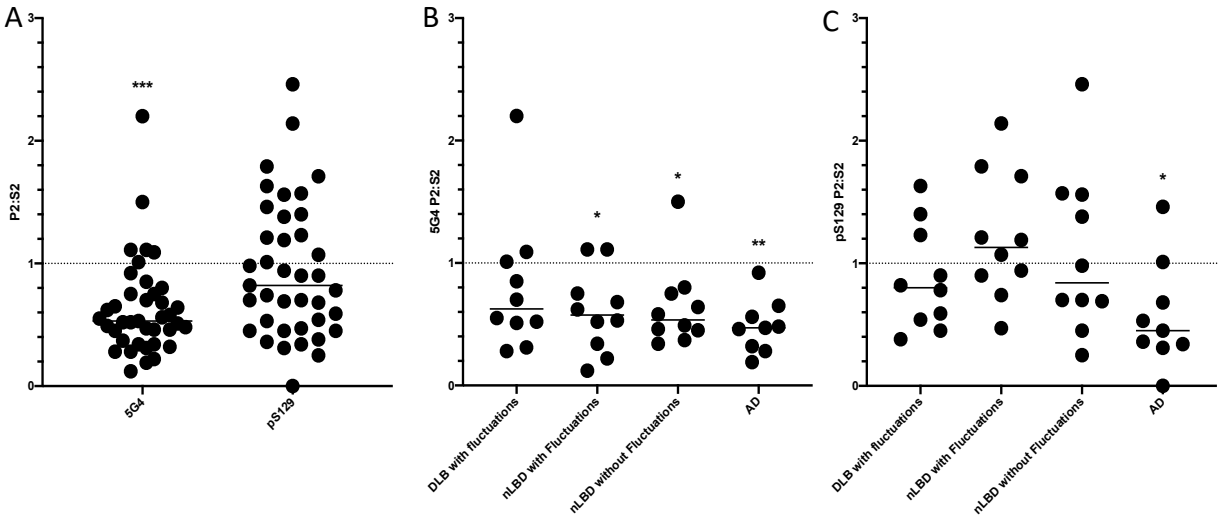


Figure 6.10. Ratios for level of 5G4 and pS129 between the synaptosomal and soluble enzyme fractions.
Ratios for level of 5G4 and pS129 between the synaptosomal, P2, and soluble enzyme, S2 fractions. A- all fluctuation groups, median: 5G4-0.53, pS129-0.86; B- individual fluctuation groups for 5G4 ratio, median: DLB with fluctuations-0.62, nLBD with fluctuations-0.58, nLBD without fluctuations- 0.53, AD- 0.47 ; C- individual fluctuation groups for pS129 ratio, median, DLB with fluctuations- 0.80, nLBD with fluctuations-1.13, nLBD without fluctuations- 0.84, AD- 0.49. Dotted line represents the hypothetical median of 1. *p<0.05 **p<0.01 ***p<0.001. Abbreviations: AD- Alzheimer's disease; DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease.

6.3.6.3 Relationship with cognitive fluctuations

No association was observed between the level of pS129 or 5G4 in the P2 or S2 fraction and the severity of cognitive fluctuations as measured by CAF score, when the DLB and nLBD with fluctuations groups were analysed together (table 6.3). When the fluctuation groups

Table 6.5. Associations between cognitive fluctuation severity and α-synuclein pathology.
Spearman's rank correlations between α-synuclein, 5G4 and pS129, in the synaptosomal, P2, and soluble enzyme, S2, fractions, and severity of cognitive fluctuations as measured by last, average and maximum CAF score. Associations were undertaken in DLB and nLBD both combined and individually. *p<0.05 Abbreviations: CAF- clinical assessment of fluctuation: DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease; r_s- spearman's rank coefficient.

Measurement of cognitive fluctuation severity	Cognitive fluctuation group	Pathology	Cognitive fluctuation severity
Last CAF	nLBD and DLB with cognitive fluctuations	5G4 P2	r_s=0.239, p=0.355
		5G4 S2	r_s=0.051, p=0.847
		pS129 P2	r_s=-0.247, p=0.340
		pS129 S2	r_s=-0.019, p=0.942
	DLB with cognitive fluctuations	5G4 P2	r_s=0.658, p=0.054
		5G4 S2	r_s=0.308, p=0.420
		pS129 P2	r_s=-0.359, p=0.343
		pS129 S2	r_s=0.060, p=0.878
	nLBD with cognitive fluctuations	5G4 P2	r_s=-0.136, p=0.748
		5G4 S2	r_s=-0.111, p=0.793
		pS129 P2	r_s=-0.161, p=0.704
		pS129 S2	r_s=-0.037, p=0.931
Average CAF	nLBD and DLB with cognitive fluctuations	5G4 P2	r_s=0.036, p=0.891
		5G4 S2	r_s=0.284, p=0.269
		pS129 P2	r_s=-0.209, p=0.422
		pS129 S2	r_s=0.101, p=0.701
	DLB with cognitive fluctuations	5G4 P2	r_s=0.542, p=0.131
		5G4 S2	r_s=0.407, p=0.277
		pS129 P2	r_s=-0.492, p=0.179
		pS129 S2	r_s=0.102, p=0.795
	nLBD with cognitive fluctuations	5G4 P2	r_s=-0.335, p=0.417
		5G4 S2	r_s=0.275, p=0.509
		pS129 P2	r_s=-0.072, p=0.866
		pS129 S2	r_s=0.036, p=0.933
Maximum CAF	nLBD and DLB with cognitive fluctuations	5G4 P2	r_s=-0.200, p=0.443
		5G4 S2	r_s=0.197, p=0.449
		pS129 P2	r_s=-0.381, p=0.132
		pS129 S2	r_s=-0.341, p=0.180
	DLB with cognitive fluctuations	5G4 P2	r_s=0.195, p=0.615
		5G4 S2	r_s=0.151, p=0.699
		pS129 P2	r_s=-0.789, p=0.011 *
		pS129 S2	r_s=-0.505, p=0.165
	nLBD with cognitive fluctuations	5G4 P2	r_s=-0.498, p=0.209
		5G4 S2	r_s=0.358, p=0.385
		pS129 P2	r_s=-0.051, p=0.904
		pS129 S2	r_s=-0.217, p=0.606

were analysed separately a negative association was observed with the level of pS129 in the P2 fraction and the severity of cognitive fluctuations as measured by the maximal CAF score (r_s=-0.789, p=0.011) in the DLB with fluctuation group. No further associations were observed between the level of pS129 and 5G4 in the P2 or S2 fraction with the severity of cognitive fluctuations as measured by CAF score in the DLB and nLBD with fluctuations groups when analysed individually (table 6.3).

6.3.7 mPFC Phosphorylated tau Pathology

Tau pathology was measured utilising a 'cocktail' of three antibodies which recognise different phosphorylated forms of tau: PHF-1, recognises tau phosphorylated at serine 396 and serine 404; AT8, recognises tau phosphorylated at serine 199 and 202, and threonine 205; CP-13 recognises tau phosphorylated at serine 202 only. Phosphorylated tau levels were measured in the soluble enzyme, S2 (figure 6.11B) and synaptosomal, P2 (figure 6.11A) fractions.

In the P2 fraction, a significant effect of disease group was observed between AD and DLB compared to control with phosphorylated tau (χ^2=18.237, p<0.001) (figure 6.11A). The level of phosphorylated tau was significantly higher in the AD group in comparison to DLB with fluctuations (z=-12.700, p=0.001) and control (z=15.444, p<0.001). No difference was observed between the level of phosphorylated tau between the DLB with fluctuation group and the control cases (p=0.468). A significant main effect of disease group on the level of phosphorylated tau for nLBD with and without fluctuations in comparison to controls (χ^2=17.906, p<0.001) (figure 6.11A). Phosphorylated tau levels were significantly lower in controls compared with nLBD with fluctuations (z=-13.289, p=0.001) and nLBD without fluctuations (z=15.389, p<0.001). No difference in phosphorylated tau in the P2 fraction was observed between both nLBD fluctuation groups (p=0.581). When analysed across blots there was a significant main effect of disease group on the level of phosphorylated tau in the P2 fraction (χ^2=20.885, p<0.001) (figure 6.11A). P2 fraction phosphorylated tau levels were lower in DLB with fluctuations compared to nLBD with fluctuations (z=-15.000, p=0.003), nLBD without fluctuations (z=-18.300, p<0.001) and AD (z=-21.933, p<0.001). No differences were observed in the level of phosphorylated tau in the P2 fraction between both nLBD fluctuation groups and between the AD cases and the nLBD with (p=0.186) and without (p=0.488) fluctuations.

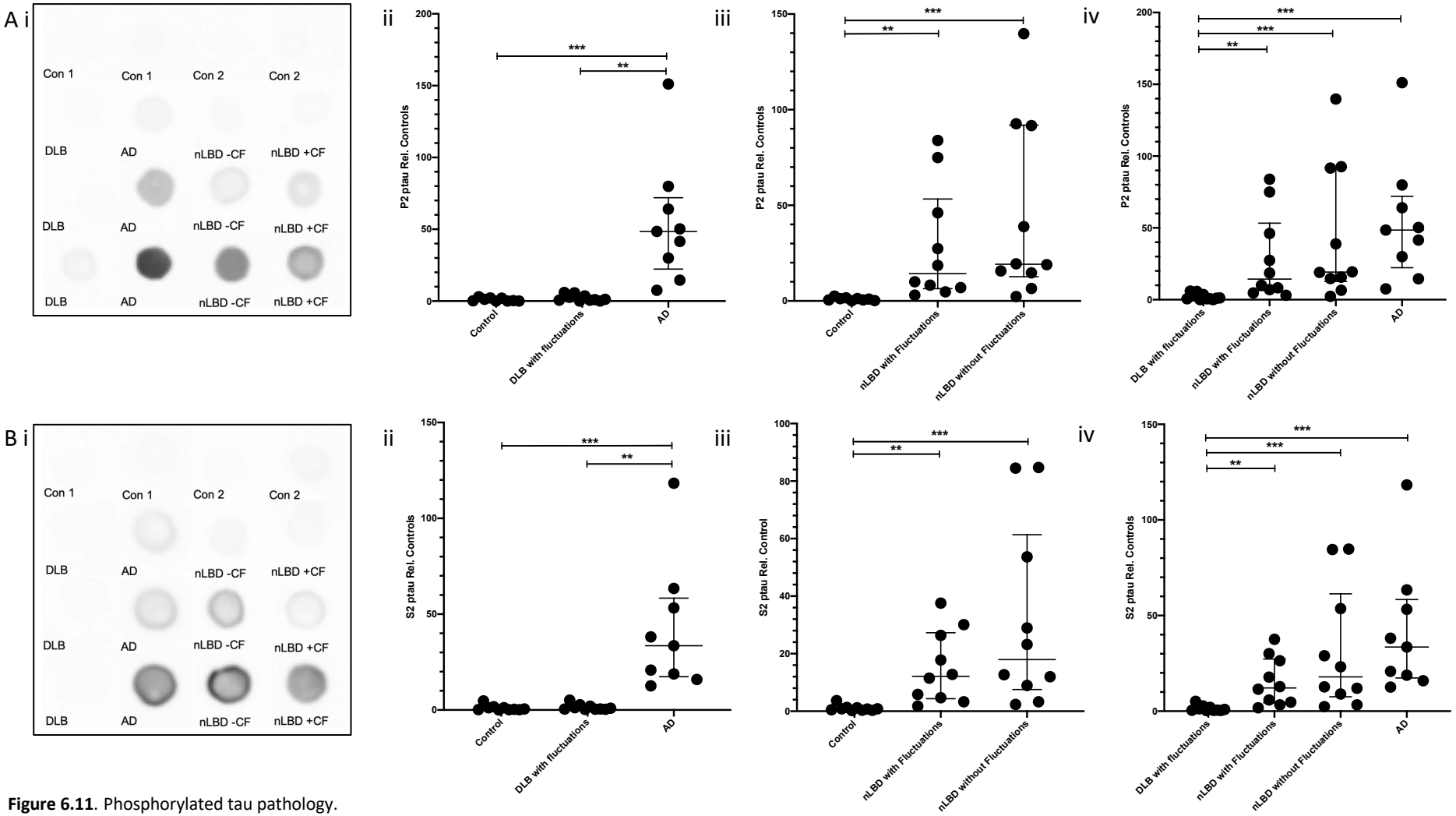


Figure 6.11. Phosphorylated tau pathology.
Dot blots for A- Synaptosomal fraction, P2, and B- Soluble enzyme fraction, S2, phosphorylated tau (ptau). i representative dots from both blots. The two left columns are dots from the AD and DLB blot; the right two columns are dots from the nLBD with (+CF) and without (-CF) fluctuations blot. Dots representing the minimum, median and maximum are shown for the four fluctuation groups. Control (Con1 and Con2) show the cases with minimum (left) and maximum level (right) for the two blots, the dots representing the minimum and maximum are the same case for both blots. Analyses ii and iii within blot iv fluctuation groups across blots. Data is shown relative to controls with median and interquartile range *p<0.05 **p<0.01 ***p<0.001.
Abbreviations: AD- Alzheimer's disease; CF- cognitive fluctuations; DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease.

Within the S2 fraction a significant effect of disease group on phosphorylated tau level was observed for AD and DLB in comparison to controls (χ^2=18.797, p<0.001) (figure 6.11B). The level of phosphorylated tau was significantly higher in the AD group in comparison to DLB with fluctuations (z=-12.800, p=0.001) and control (z=16.200, p<0.001). No difference was observed between the level of phosphorylated tau between the DLB with fluctuations group and the control cases (p=0.372). A significant main effect of disease group on level of phosphorylated tau was observed nLBD with and without fluctuations (χ^2=18.209, p<0.001) in comparison to controls, in the S2 fraction (figure 6.11B). Phosphorylated tau levels were significantly lower in controls compared with nLBD with fluctuations (z=-13.200, p=0.001) and nLBD without fluctuations (z=15.600, p<0.001). No difference in phosphorylated tau in the S2 fraction was observed between both nLBD fluctuation groups (p=0.542). When analysed across blots there was a significant main effect of disease group on the level of phosphorylated tau in the S2 fraction (χ^2=22.547, p<0.001) (figure 6.11B). S2 fraction phosphorylated tau levels were lower in DLB with fluctuations compared to nLBD with fluctuations (z=-14.100, p=0.006), nLBD without fluctuations (z=-18.300, p<0.001) and AD (z=-23.367, p<0.001). No differences were observed in the level of phosphorylated tau in the S2 fraction between both nLBD fluctuation groups and between the AD cases and the nLBD without (p=0.333) fluctuations group, although a trend towards a significant difference was observed between AD and nLBD with fluctuations (p=0.077).

In summary, phosphorylated tau was higher in the AD and nLBD with and without fluctuations groups compared to controls and DLB in both the P2 and S2 fractions. No further differences were observed.

6.3.7.1 Relationship between neuronal sublocations

A positive association was observed between phosphorylated tau levels in the P2 and S2 fractions, when analysed across the disease groups (r_s=0.964, p<0.001). When the disease groups were analysed individually, positive relationships between the phosphorylated tau level in the P2 and S2 fractions were observed in all the groups: DLB with fluctuations (r_s=0.648, p=0.043), nLBD with fluctuations (r_s=0.988, p<0.001), nLBD without fluctuations (r_s=0.939, p<0.001) and AD (r_s=0.967, p<0.001).

6.3.7.2 Relationship with α-synuclein species

Phosphorylated tau levels in the synaptosomal, P2 fraction, when analysed across all disease groups were found not to be associated with the P2 (p=0.883) or S2 (p=0.717) level of 5G4 and the P2 (p=0.407) or S2 (p=0.137) level of pS129. When the disease groups were analysed individually a positive relationship was observed between the level of phosphorylated tau in the P2 fraction and the level of pS129 in the P2 fraction within the DLB with fluctuations group (r_s=0.661, p=0.038). No further associations were observed for the level of phosphorylated tau in the P2 fraction and the level of 5G4 and pS129 in the P2 and S2 fractions in the individual disease groups.

In the soluble enzyme, S2 fraction, the level of phosphorylated tau was identified to not associated with the level of 5G4 in the P2 (p=0.169) or S2 (p=0.488) fraction and the level of pS129 in the P2 (p=0.487) or S2 (p=0.359) fraction, when the disease groups were jointly analysed. When the disease groups were analysed individually a positive relationship was observed between the level of phosphorylated tau in the S2 fraction and the level of pS129 in the P2 fraction within the DLB with fluctuations group (r_s=0.806, p=0.005). No further associations were observed for the level of phosphorylated tau in the S2 fraction and the level of 5G4 and pS129 in the P2 and S2 fractions in the individual disease groups.

6.3.7.3 Relationship with cognitive fluctuations

No association was observed between the level of phosphorylated tau in the P2 or S2 fraction with the severity of cognitive fluctuations as measured by CAF score, when the DLB and nLBD with fluctuations groups were analysed together (table 6.4). When the fluctuation groups were analysed separately a negative association was observed with the level of phosphorylated tau in the S2 fraction and the severity of cognitive fluctuations as measured by the last CAF score (r_s=-0.778, p=0.014) in the DLB with fluctuation group. No further associations were observed between the level of phosphorylated tau in the P2 or S2 fraction with the severity of cognitive fluctuations as measured by CAF score in the DLB and nLBD with fluctuations groups were analysed individually, (table 6.4).

Table 6.7. Associations between cognitive fluctuation severity and phosphorylated tau.
Spearman's rank correlations between phosphorylated tau (ptau), in the synaptosomal, P2, and soluble enzyme, S2, fractions, and severity of cognitive fluctuations as measured by last, average and maximum CAF score. Associations were undertaken in DLB and nLBD both combined and individually. *p<0.05 Abbreviations: CAF- clinical assessment of fluctuation: DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease; r_s- spearman's rank coefficient.

Measurement of cognitive fluctuation severity	Cognitive fluctuation group	Pathology	Cognitive fluctuation severity
Last CAF	nLBD and DLB with cognitive fluctuations	ptau P2	r_s=-0.114, p=0.663
		ptau S2	r_s=-0.354, p=0.163
	DLB with cognitive fluctuations	ptau P2	r_s=-0.248, p=0.520
		ptau S2	r_s=-0.778, p=0.014 *
	nLBD with cognitive fluctuations	ptau P2	r_s=-0.074, p=0.862
		ptau S2	r_s=-0.136, p=0.748
Average CAF	nLBD and DLB with cognitive fluctuations	ptau P2	r_s=-0.046, p=0.861
		ptau S2	r_s=-0.370, p=0.144
	DLB with cognitive fluctuations	ptau P2	r_s=0.068, p=0.862
		ptau S2	r_s=-0.661, p=0.053
	nLBD with cognitive fluctuations	ptau P2	r_s=-0.204, p=0.629
		ptau S2	r_s=-0.287, p=0.490
Maximum CAF	nLBD and DLB with cognitive fluctuations	ptau P2	r_s=-0.018, p=0.944
		ptau S2	r_s=-0.246, p=0.342
	DLB with cognitive fluctuations	ptau P2	r_s=-0115., p=0.768
		ptau S2	r_s=-0.567, p=0.111
	nLBD with cognitive fluctuations	ptau P2	r_s=0.051, p=0.904
		ptau S2	r_s=-0.064, p=0.881

6.3.8 mPFC Aβ pathology

Aβ pathology was measured utilising an antibody that recognises unaggregated, oligomeric and fibrillar forms of Aß42, and unaggregated Aß40 and not APP. Aβ levels were measured in the soluble enzyme, S2 (figure 6.12B), and synaptosomal, P2 (figure 6.12A), fractions.

In the P2 fraction, a significant effect of disease group was observed for AD and DLB compared to controls with the level of Aβ (χ^2=6.808, p=0.033) (figure 6.12A). A significantly higher level of Aβ was observed in the AD group compared to controls (z=10.111, p=0.009). No difference was observed between the DLB with fluctuation group and control (p=0.209) or AD groups (p=0.156). A significant effect of disease group on Aβ level was observed nLBD with and without fluctuations (χ^2=6.806, p=0.033) in comparison to controls, in the P2

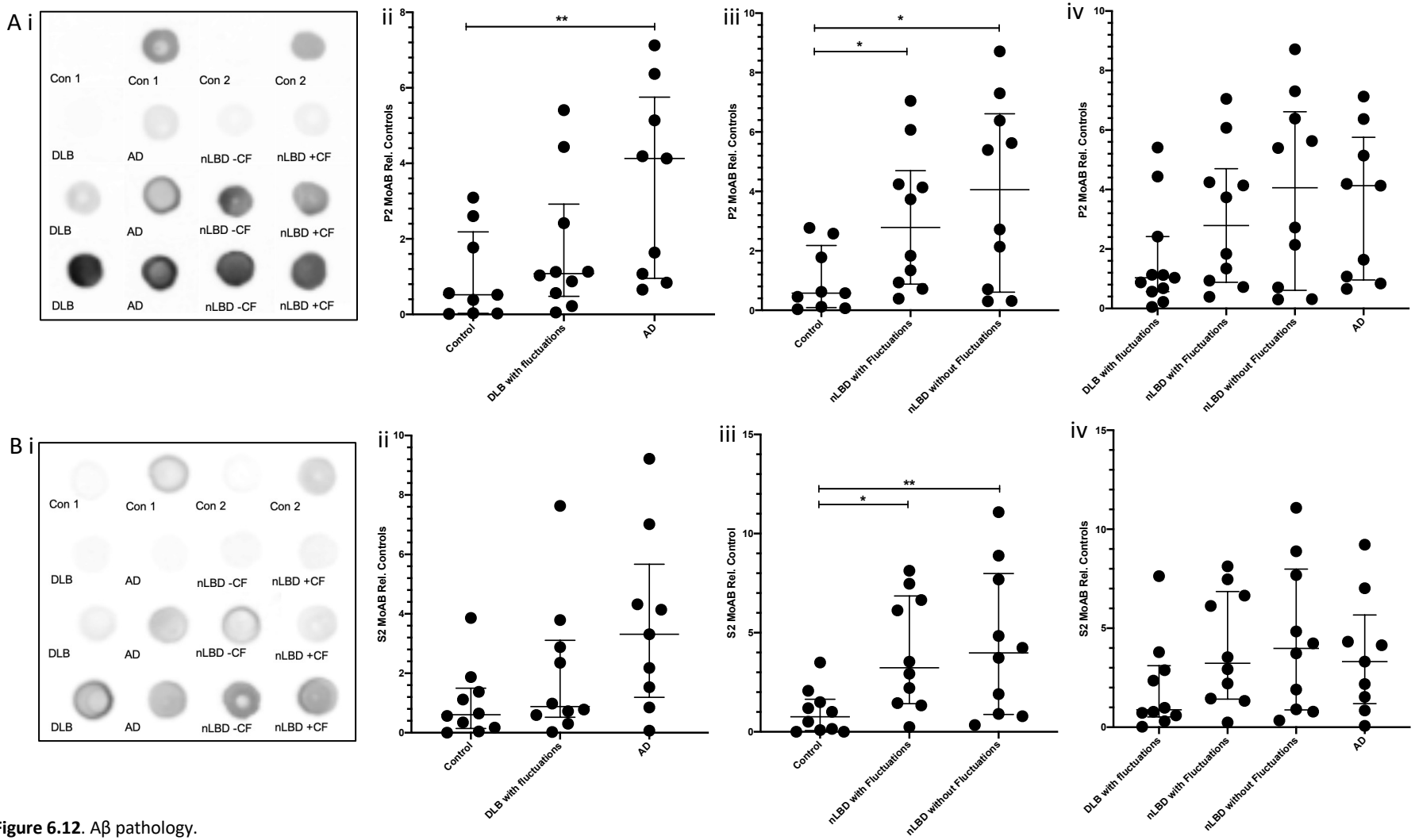


Figure 6.12. Aβ pathology.

Dot blots for A- Synaptosomal fraction, P2, and B- Soluble enzyme fraction, S2, Aβ (MoAB). i representative dots from both blots. The two left columns are dots from the AD and DLB blot; the right two columns are dots from the nLBD with (+CF) and without (-CF) fluctuations blot. Dots representing the minimum, median and maximum are shown for the four fluctuation groups. Control (Con1 and Con2) show the cases with minimum (left) and maximum level (right) for the two blots, the dots representing the minimum and maximum are the same case for both blots. Analyses ii and iii within blot iv fluctuation groups across blots. Data is shown relative to controls with median and interquartile range *p<0.05 **p<0.01. Abbreviations: AD- Alzheimer's disease; CF- cognitive fluctuations; DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease.

fraction (figure 6.12A). P2 fraction Aβ levels were significantly higher in the nLBD with fluctuation group (z=-8.311, p=0.034) and nLBD without fluctuation (z=9.411, p=0.016) groups compared to control cases. No difference was observed in P2 fraction Aβ levels between both nLBD fluctuation groups (p=0.773). When analysed across blots no effect of disease group was observed on the level of Aβ in the P2 fraction (p=0.322) (figure 6.12A).

In the S2 fraction, a trend towards a significant effect of disease group was observed for AD and DLB with control for the level of Aβ (p=0.051) (figure 6.12B). A significant effect of disease group was observed for Aβ level was observed nLBD with and without fluctuations (χ^2=8.954, p=0.011) in comparison to controls, in the S2 fraction (figure 6.12B). S2 fraction Aβ levels were significantly higher in the nLBD with fluctuation group (z=-10.100, p=0.010) and nLBD without fluctuation (z=10.300, p=0.009) groups compared to control cases. No difference was observed in S2 fraction Aβ levels between both nLBD fluctuation groups (p=0.959). When analysed across blots no effect of disease group was observed on the level of Aβ in the S2 fraction (p=0.222) (figure 6.12B).

In summary, Aβ was higher in the AD and nLBD with and without fluctuations groups compared to controls in the P2 fraction and in the S2 fraction Aβ was higher in the nLBD with and without fluctuations groups compared to controls. No further differences were observed.

6.3.8.1 Relationship between neuronal sublocations

A positive association was observed between Aβ levels in the P2 and S2 fractions, when analysed across the fluctuation groups (r_s=0.905, p<0.001). When the fluctuation groups were analysed individually, positive relationships between the Aβ level in the P2 and S2 fractions were observed in all the groups: DLB with fluctuations (r_s=0.927, p<0.001), nLBD with fluctuations (r_s=0.952, p<0.001), nLBD without fluctuations (r_s=0.733, p=0.016) and AD (r_s=0.917, p=0.001).

6.3.8.2 Relationship with α-synuclein species

Aβ in the synaptosomal, P2 fraction, when analysed across all disease groups was not associated with the P2 (p=0.572) or S2 (p=0.449) level of 5G4 and the P2 (p=0.928) or S2 (p=0.457) level of pS129. When the disease groups were analysed individually no associations were observed for the level of Aβ in the P2 fraction and the level of 5G4 and pS129 in the P2 and S2 fractions within the four disease groups.

In the soluble enzyme, S2 fraction, the level of Aβ was not associated with the level of 5G4 in the P2 (p=0.772) or S2 (p=0.644) fraction and the level of pS129 in the P2 (p=0.580) or S2 (p=0.395) fraction, when the disease groups were jointly analysed. When the disease groups were analysed individually no relationships were observed between the level of Aβ in the S2 fraction and the level of 5G4 and pS129 in the P2 and S2 fractions.

6.3.8.3 Relationship with phosphorylated tau levels

The level of Aβ in the synaptosomal fraction was not associated with the level of phosphorylated tau in the P2 (p=0.438) and S2 (p=0.628) fractions, when analysed across all disease groups. When the disease groups were analysed individually a negative relationship was observed between the level of Aβ in the P2 fraction and the level of phosphorylated tau in the S2 fraction in the nLBD with fluctuations group (r_s=-0.648, p=0.043). No further associations were observed between the level of Aβ in the P2 fraction and the P2 or S2 fraction level of phosphorylated tau in the individual disease groups.

Aβ in the S2 fraction, when analysed across all disease groups, was not associated with the level of phosphorylated tau in the P2 (p=0.325) or S2 (p=0.436) fractions. When the disease groups were analysed individually, the level of Aβ in the S2 fraction, and the level of phosphorylated tau in the S2 fraction in the nLBD with fluctuations group negatively associated (r_s=-0.697, p=0.025), and the level of phosphorylate tau in the P2 fraction in the DLB with fluctuations group positively associated (r_s=0.733, p=0.016). No further associations were observed between the level of Aβ in the S2 fraction and the P2 or S2 fraction level of phosphorylated tau in the individual disease groups.

6.3.8.4 Relationship with cognitive fluctuations

No association was observed between the level of Aβ in the P2 or S2 fraction with the severity of cognitive fluctuations as measured by CAF score, when the DLB and nLBD with fluctuations groups were analysed together (table 6.5). When the fluctuation groups were analysed separately no associations were observed between the level of Aβ in the P2 or S2 fraction with the severity of cognitive fluctuations as measured by CAF score in the DLB and nLBD with fluctuations groups (table 6.5).

Table 6.9. Associations between cognitive fluctuation severity and Aβ.
Spearman's rank correlations between Aβ (MoAB), in the synaptosomal, P2, and soluble enzyme, S2, fractions, and severity of cognitive fluctuations as measured by last, average and maximum CAF score. Associations were undertaken in DLB and nLBD both combined and individually. Abbreviations: CAF- clinical assessment of fluctuation: DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease; r_s- spearman's rank coefficient.

Measurement of cognitive fluctuation severity	Cognitive fluctuation group	Pathology	Cognitive fluctuation severity
Last CAF	nLBD and DLB with cognitive fluctuations	MoAB P2	r_s=-0.166, p=0.525
		MoAB S2	r_s=-0.301, p=0.240
	DLB with cognitive fluctuations	MoAB P2	r_s=-0.197, p=0.612
		MoAB S2	r_s=-0.402, p=0.284
	nLBD with cognitive fluctuations	MoAB P2	r_s=-0.148, p=0.726
		MoAB S2	r_s=-0.086, p=0.839
Average CAF	nLBD and DLB with cognitive fluctuations	MoAB P2	r_s=0.215, p=0.408
		MoAB S2	r_s=0.027, p=0.917
	DLB with cognitive fluctuations	MoAB P2	r_s=0.119, p=0.761
		MoAB S2	r_s=-0.119, p=0.761
	nLBD with cognitive fluctuations	MoAB P2	r_s=0.204, p=0.629
		MoAB S2	r_s=0.252, p=0.548
Maximum CAF	nLBD and DLB with cognitive fluctuations	MoAB P2	r_s=0.176, p=0.499
		MoAB S2	r_s=0.051, p=0.845
	DLB with cognitive fluctuations	MoAB P2	r_s=0.186, p=0.631
		MoAB S2	r_s=-0.044, p=0.910
	nLBD with cognitive fluctuations	MoAB P2	r_s=0.217, p=0.606
		MoAB S2	r_s=0.294, p=0.480

6.3.9 Mitochondrial Blots

The P2 fraction, as well as being enriched for synaptosomes, is enriched for mitochondria. The levels of NDUFB8, a subunit of complex I in the electron transport chain (figure 6.13B), and TOM20, an outer mitochondrial membrane transporter protein (figure 6.13A), were examined in the P2 fraction. The level of TOM20 is representative of the number of mitochondria present within the fraction, with the NDUFB8:TOM20 representative of the level of complex I relative to the level of mitochondria.

No effect of disease group was identified on TOM20 for AD and DLB (χ^2=1.117, p=0.572) or nLBD with and without fluctuations (χ^2=0.101, p=0.951) with controls (figure 6.13A). When analysed across blots no effect of disease group was observed for TOM20 (χ^2=2.336, p=0.506) (figure 6.13A). No effect of disease group on NDFUB8:TOM20 was observed for AD and DLB (χ^2=0.727, p=0.695) or nLBD with and without fluctuations (χ^2=2.476, p=0.290) with

controls (figure 6.13B). When analysed across blots no effect of disease group was observed for NDUFB8:TOM20 (χ^2=2.125, p=0.547) (figure 6.13B).

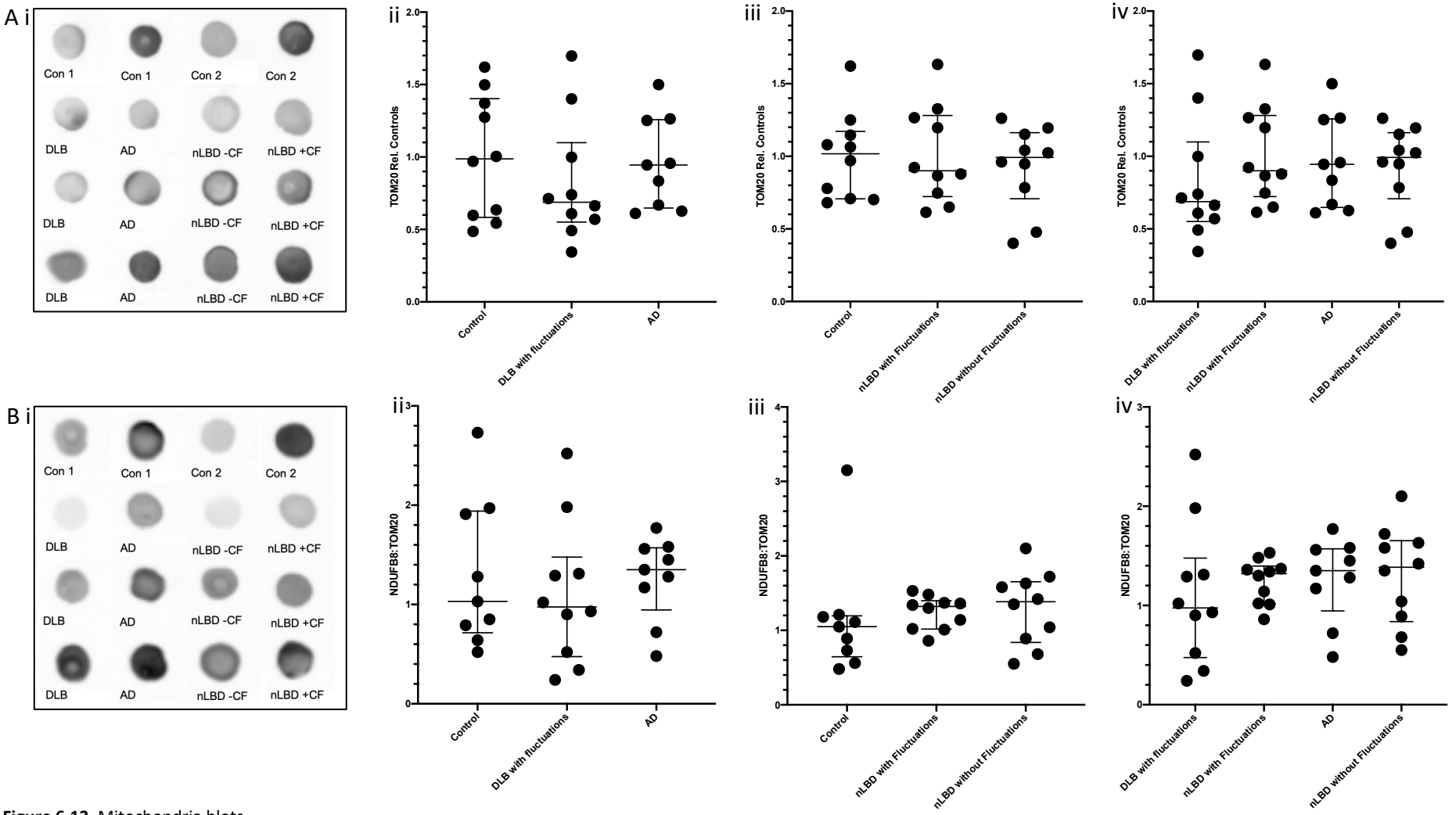


Figure 6.13. Mitochondria blots.

Dot blots for synaptosomal, P2 fraction A- TOM20 and B- NDUFB8:TOM20. i representative dots from A-B-TOM20 and NDUFB8 blots. The two left columns are dots from the AD and DLB blot; the right two columns are dots from the nLBD with (+CF) and without (-CF) fluctuations blot. Dots representing the minimum, median and maximum are shown for the four fluctuation groups. Control (Con1 and Con2) show the cases with minimum (left) and maximum level (right) for the two blots, the dots representing the minimum and maximum are the same case for both blots. Analyses ii and iii within blot iv fluctuation groups across blots. Data is shown relative to controls with median and interquartile range. Abbreviations: AD- Alzheimer's disease; CF- cognitive fluctuations; DLB- dementia with Lewy bodies; nLBD- neocortical/limbic pathologically Lewy body disease.

6.4 Discussion

The present study aimed to examine synaptosomes in a cortical target region of the ARAS, in order to assess the integrity of the projections from the nuclei investigated in the previous chapters, in addition to whether changes to the cortical regions could play a role in the presence or severity of cognitive fluctuations in DLB. Previous studies have identified changes to mPFC and its innervation in neurodegenerative dementias (Galvin *et al.*, 2011; Chabran *et al.*, 2020), with changes to input from the thalamus having been shown to play a role in the recovery from a vegetative state (Jang *et al.*, 2020). The present study identified that in a synaptosomal-enriched fraction neurotransmitter marker levels did not differ from control levels and were not related to the presence of cognitive fluctuations or the level of pathological burden in their source nuclei. Pathological analysis also identified that the burden of pathological lesions in the mPFC were not to related with the presence or severity of cognitive fluctuations. However, differences in the neuronal sublocation of different α-synuclein species was observed. Furthermore, the present study identified that in the synaptosomally enriched fraction there were no differences in the level of mitochondria or the level of complex I.

6.4.1 Neurotransmitter markers do not differ from controls in the synaptosomal fraction

The present study aimed to examine the integrity of the projections both directly and indirectly, via the basal forebrain, from the brainstem nuclei of the ARAS. Analysis of VMAT2 and SERT, markers of direct projections, and ChAT, indirect projections via the basal forebrain, in the synaptosomal fraction identified no difference in neurotransmitter marker levels in the disease groups compared to the control cases.

SERT is located on the presynaptic terminals of serotonergic neurons originating from the raphe nucleus. Alterations to the serotonin system have been observed in a number of neurodegenerative dementias including AD and DLB (Chen *et al.*, 2000; Azmitia and Nixon, 2008; Roselli *et al.*, 2010). In AD a previous study identified a reduction in SERT density compared to controls utilising whole tissue analysis in the prefrontal cortex (Thomas *et al.*, 2006). A similar finding of reduced SERT level has been observed in DLB compared to controls in the parietal cortex (Ballard *et al.*, 2002a; Francis, 2009). The current study, in comparison to previous studies, examined the level of SERT in a synaptosomal fraction rather than a whole tissue fraction. A crude synaptosomal fraction was utilised in order to

specifically measure the levels of functional transporters, those at the synapse, compared to those that were stored in other neuronal compartments. The differences observed between the current study and those previously could be a reflection of the synaptosomal methodology used. The tissue fractionation was undertaken to select for the 'functional' transporters and receptors, those present at the synapse. If the crude synaptosomal fraction represented the levels of 'functional' SERT, our results, in combination with previous studies that have identified a loss of SERT in whole tissue analysis, could suggest that there is a loss of non-synaptically located SERT. This non-synaptically located SERT could represent reserve pool levels of the transporter, located outside of the synaptic compartment. A loss of non-synaptically localised SERT could impact upon dynamics in the serotonergic system, meaning that it is less able to cope with any changes; studies investigating the dopamine transporter have identified alterations to neuronal plasticity when the transporter wans inactivated or reduced (Jones *et al.*, 1998). A serotonergic system that is less dynamic could lead to alterations in arousal levels due to subsequent changes to cortical activation and feedback to the raphe nucleus, which could clinically manifest as cognitive fluctuations. Alterations to levels in non-synaptically located SERT, which were not measured in the current study, may explain why no relationships were observed with severity of cognitive fluctuations.

ChAT is the synthesis enzyme for acetylcholine, where it is the sole mechanism for the generation of acetylcholine in cholinergic neurons. In the mPFC the main cholinergic input originates from the basal forebrain (Bloem *et al.*, 2014), which is the main relay for the monoaminergic nuclei of the ARAS (Mahaffey and Garcia-Rill, 2015). Cortical reductions in ChAT level and cholinergic degeneration in basal forebrain have been widely reported in both AD and DLB (Tiraboschi *et al.*, 2000; Grothe *et al.*, 2014; Taylor *et al.*, 2017). Dysfunction of the cholinergic system has been implicated in visual hallucinations and cognitive fluctuations in DLB (Marra *et al.*, 2012; Chabran *et al.*, 2020). As with the previous studies investigating SERT, the studies investigating levels of ChAT within the brain have examined whole tissue fractions, compared to the fractionated analysis undertaken in the current study. The conflicting data obtained from the current study could be a reflection on level of synaptosomes within the crude synaptosomal fraction. It could be possible that within the disease groups a higher number of synaptosomes were required to produce the

same level of total protein as that in the control fractions. Therefore, although the same level of total protein was loaded onto the blots, a higher number of synaptosomes were loaded in the disease groups, which could lead to the appearance of similar ChAT levels in the synaptosomal fraction. As the immunoblots were not normalized to SNAP25, and validation of the synaptosomal fractionation validation was only examined in a control case it was not possible to assess either the number of synaptosomes present in the crude synaptosomal fraction in the disease groups or, express the levels of ChAT relative to the level of synaptosomes present.

Both a hypothetical loss of the reserve pool of the transporter and/or a difference in the number of synaptosomes within the synaptosomal fraction would need to be validated by examining the levels of SERT, ChAT and SNAP25 within all the fractions, as well as examination of a whole tissue fraction. Furthermore, analysing the levels of SNAP25 present within the crude synaptosomal fraction could allow for the levels of SERT and ChAT to be expressed relative to the synaptosomal number, similar to the NDUFB8:TOM20.

6.4.2 Pathology alone does not distinguish fluctuating from non-fluctuating groups

The present study aimed to investigate the involvement of neuropathological changes in the mPFC, including the burden of α-synuclein, tau and Aβ, on the presence and severity of cognitive fluctuations in DLB. Previous studies have identified neuropathological changes to the mPFC, including neuronal cell loss and accumulation of pathological proteins, in various neurodegenerative diseases including AD and DLB (Lindberg *et al.*, 2012; Xu *et al.*, 2019). Alterations to this region have also been identified to underly aspects of the cognitive impairment observed in AD and DLB (Kobeleva *et al.*, 2017; Berron *et al.*, 2020). The present study is in agreement with previous findings that mPFC is vulnerable to α-synuclein and AD-type pathology. However, the present study did not identify differences in the pathological burdens in relation to the presence or absence of cognitive fluctuations.

The mPFC is a highly interconnected brain region which is involved in a number of large-scale networks, including the default mode network (Li *et al.*, 2014). Through the involvement of the mPFC in large-scale networks, and its implication in arousal (Zhang *et al.*, 2014) and attention (Riga *et al.*, 2014), studies have investigated the role of the mPFC, and the networks it is part of, in alterations to consciousness (Franciotti *et al.*, 2013; Lowther *et al.*, 2014; Peraza *et al.*, 2014; Schumacher *et al.*, 2018; O'Dowd *et al.*, 2019; Jang *et al.*,

2020). Although alterations to attentional networks, including reductions in default mode connectivity, involving the mPFC have been identified in DLB, the studies have not identified an association with cognitive fluctuations, although their role has not been dismissed (O'Dowd *et al.*, 2019). Alterations to mPFC innervation has been observed in AD and DLB, with atrophy of the basal forebrain, which provides cholinergic innervations to the mPFC (Grothe *et al.*, 2014), and more severe atrophy of the cholinergic neurons associated with alterations to arousal levels and maintenance (Kasanuki *et al.*, 2018). Studies examining the mPFC in disorders of consciousness have also identified increases to thalamocortical connectivity to the mPFC is associated with recovery from a vegetative state (Jang *et al.*, 2020), with similar alterations reported for thalamocortical projections to the mPFC in DLB (Chabran *et al.*, 2020).

Although neuropathological and connectivity changes to the mPFC are hypothesised to play a role in alterations to arousal and awareness, that could clinically manifest as cognitive fluctuations, the data from the current study suggests that any alterations are not due to the direct presence of pathological accumulations within the synaptosomal or soluble enzyme fraction of the region. The present study identified no differences in the burden of pathology specific to the presence or absence of cognitive fluctuations in either of the two tissue fractions examined. Analysis further identified that no relationships between pathological protein sublocation and pathological burden were specific for the presence or absence of cognitive fluctuations. Together this data suggests that the presence of pathology within the mPFC is not directly related to the presence or absence of cognitive fluctuations. However, it is not possible to fully conclude that the accumulation of proteins in the mPFC have no role in cognitive fluctuations as it is possible that they indirectly influence alterations to arousal and attention, for example through altered trafficking of receptors or vesicles, or the sequestering of catalytic enzymes, as has been previously observed (Dugger and Dickson, 2010). Furthermore, it could be possible that there may be species of pathological protein that possess more pathological relevance than those examined in the present study.

6.4.3 Neuronal sub-localisation of α-synuclein species

Physiologically α-synuclein is thought to be in a dynamic equilibrium between a free, cytosolic, and membrane bound state (Colla *et al.*, 2018). Historically, α-synuclein toxic

species have been thought to be cytoplasmic, however, recent evidence of α-synuclein's membrane binding abilities has raised the question of whether cellular sub-localisation of pathology impacts upon aggregation and disease progression (Miraglia *et al.*, 2018). Recently, studies have begun to investigate whether α-synuclein species in different subcellular localisations possess different conformations and toxic effects, utilising both *in vivo* (Colla *et al.*, 2018) and *post-mortem* methods (Sanderson *et al.*, 2020).

Data from the present study identified that aggregated α-synuclein, measured by 5G4 antibody, was present preferentially in the S2, microsome and soluble enzyme, fraction, in the nLBD with and without fluctuations and AD disease groups. The levels in the P2, crude synaptosomal and mitochondrial fraction, were observed not to differ from controls in all disease groups. The observations from the current study are in agreement with research by Colla *et al*. (2012a) who identified α-synuclein aggregates and oligomers were associated with microsomes *in vivo.* Further investigations by Colla *et al*. (2012b), identified that this microsomal accumulation of aggregated and oligomeric species preceded neurodegeneration in mouse models of synucleinopathies. Due to the sequential centrifugation of the tissue in order to produce a crude synaptosomal fraction, it is possible that larger α-synuclein aggregates, for example Lewy bodies, would be present in the P1, nuclei fraction, as they would sediment at lower spin speeds. As P1 was not analysed for the levels of α-synuclein burden, it is not possible to comment on all sizes of α-synuclein aggregates. The data collected from the current study in combination with previous findings could suggest that aggregated forms of α-synuclein are not present as readily in synapses compared to cytosolic or microsomal-associated locations, however it is beyond the scope of the current study to assess why or the effect that this distribution has on neuronal health.

pS129 levels were identified not to be preferentially located in with the S2 or P2 fractions in the pathologically LBD groups, however, in the AD group pS129 was preferentially identified in the S2 fraction. The level of pS129 was also observed to be significantly higher in the pathologically LBD groups than both controls and AD, in both the P2 and S2 fractions. Differences in subcellular distribution, as observed between 5G4 and pS129, have been previously observed in DLB post-mortem tissue (Sanderson *et al.*, 2020). In post-mortem DLB tissue a redistribution of membrane associated α-synuclein to cytosolic has been identified compared to control tissue, the study further identified that α-synuclein species in

the different cellular compartments possessed different neurodegenerative effects (Sanderson *et al.*, 2020). However, in the present study we could not compare the levels to controls due to the nature of the analysis where levels were expressed relative to controls. pS129 has been shown *in vivo* to bind to both mitochondria (Wang *et al.*, 2019) and microsomes (Colla *et al.*, 2018), which could suggest why pS129 was not preferentially located in one of the two fractions examined.

Further research is required into the different toxic species of α-synuclein and their subcellular localisation to assess whether there is a specific pattern of redistribution that could relate to clinical features.

6.4.4 Mitochondrial levels do not differ from controls in the synaptosomal fraction

Mitochondrial impairment and the association of α-synuclein with mitochondrial membranes has been widely reported in synucleinopathies (Perier and Vila, 2012; Spano *et al.*, 2015); mitochondrial dysfunction has also been reported in AD (Hawking, 2016). In the current study it was observed that in the P2, crude synaptosomal and mitochondrial, fraction there was no difference in the level of TOM20, a mitochondrial outer membrane marker, or the level of complex I marker relative to the level of mitochondria, NDUFB8:TOM20, in any of the experimental groups compared to controls. Previous studies have reported evidence for a decrease in the level of mitochondria in DLB, with a reduction in the level of complex I that is not merely a consequence of the loss of mitochondria (Garcia-Esparcia *et al.*, 2017; Fløtnes *et al.*, 2018). However, a study by Reeve *et al*. (2018) identified an increase in mitochondrial levels, which was hypothesised due to be impaired mitophagy. α-synuclein species have been observed to interact with membranes, including mitochondria (Wang *et al.*, 2019), and have been shown to increase their permeability (Sanderson *et al.*, 2020). Within the three fractions generated by the sequential centrifugations, VDAC a marker of mitochondria, was located in both the P1 and P2 fractions. Alterations to mitochondrial membrane integrity, possibly through interaction with α-synuclein, could lead to the selection of only 'healthy' mitochondria within the P2 pellet. Furthermore, membrane integrity in general has been proposed to be impaired in NDDs (Yu and Zhong, 2018), which could have further led to the selection of only healthy synaptosomes, supporting the lack of difference observed with the neurotransmitter markers in the present study. As with the neurotransmitter markers, examination of

mitochondrial markers in the other fractions is required to assess levels of mitochondrial impairment and to validate whether the sequential centrifugations selected for healthy mitochondria.

6.4.5 Limitations

The most influential limiting factors of the current study were the lack of analysis of SNAP25 levels in all three fractions across the disease groups, as well as no analysis of a whole tissue fraction. Although, the crude synaptosomal fraction provides a technique to examine transporter and catalytic enzyme levels at the synapse, it is possible that due to alterations in synapses in neurodegenerative dementias, including alterations to membrane integrity (Yu and Zhong, 2018), the levels of synaptosomes in the different fractions are altered. Without analysis of the other fractions or a whole tissue fraction it is not possible to fully conclude how the results in the present study differ from previous studies. Further experiments are required to investigate the levels of synaptosomes within the different fractions in the different disease groups, this could help identify whether the number of synaptosomes in the disease groups is higher or lower than controls and provide a relative value for the protein analysis. Examination of a whole tissue fraction could help identify if reductions to protein levels that have been widely reported, for example ChAT, are also identified within the whole tissue fraction of the mPFC to assess whether a different method would have identified neurotransmitter reductions not identified by isolating synaptosomes.

The study aimed to examine the monoaminergic ARAS projections into the mPFC, however, it was not possible to optimise the experimental protocol to examine the level of NET within this region. Further studies are required to investigate the levels of NET in the region. Also, to understand the full extent of monoaminergic dysfunction within the mPFC examination of receptor levels for both serotonin and noradrenaline would be required.

The mPFC is a large heterogeneous region, with subregions possessing distinct and often opposing roles in cognitive processing, as well as different cellular compositions and neuronal connectivity (Bzdok *et al.*, 2013; Marusak *et al.*, 2016). In the current study only BA12 was examined. The differences in tissue level and subregion of the mPFC obtained could have influenced the data obtained. Further examinations into all regions of the mPFC are warranted to understand the role that the region has in cognitive fluctuations.

6.4.6 Conclusions

The present study identified pathological changes related to the pathological diagnosis rather than the presence or absence of cognitive fluctuations. Although not specific for cognitive fluctuations the changes could contribute vulnerability to fluctuations, although not be directly causative. The absence of alterations to mitochondrial and neurotransmitter markers in the current study contradicted previous studies that had identified a reduction in the markers in neurodegenerative dementias. Further research is required into the synaptosomal fragmentation to assess whether 'healthy' synaptosomes and mitochondria were preferentially selected. This in turn could help examine the differences between 'healthy' and 'non-healthy' mitochondria and synaptosomes in the mPFC in neurodegenerative dementias.

Chapter 7: General Discussion

7.1 Introduction

DLB is the second most common neurodegenerative dementia after AD, characterised by the presence of four core clinical features: cognitive fluctuations, parkinsonian extra-pyramidal symptoms, RBD, and complex visual hallucinations (McKeith *et al.*, 2017). DLB is neuropathologically characterised by the presence of Lewy body pathology, described by the extent of its distribution throughout the brain as neocortical, limbic, brainstem, amygdala-predominant or olfactory bulb only (McKeith *et al.*, 2005). Although DLB is characterised by the presence of α-synuclein aggregates, 50-80% of patients have concomitant AD type pathology (Halliday *et al.*, 2011; McKhann *et al.*, 2011; Attems, 2017). 80-90% of DLB patients experience cognitive fluctuations, although they are thought to be the least well characterised and understood of the four core clinical features (McKeith *et al.*, 2000b; Ballard *et al.*, 2001; Matar *et al.*, 2019). Fluctuating cognition has a profound negative impact on the patient's quality of life and ability to function day to day (Gibb *et al.*, 1987; Yamamoto and Imai, 1988; Byrne *et al.*, 1989; McKeith *et al.*, 1992b; Zweig and Galvin, 2014).

The ARAS, comprising various nuclei within the pons and midbrain, that project to the cortex, thalamus and basal forebrain, has been conceptually linked to cognitive fluctuations (Matar *et al.*, 2019). Dysfunction to arousal/awareness, and attention have been hypothesised to underlie fluctuating cognition. Alterations to regions involved in the maintenance of arousal and awareness could lead to moments where patients appear to be unreceptive to external stimuli (Matar *et al.*, 2019; O'Dowd *et al.*, 2019). The LC, PPN, DR and mPFC have been implicated in these hypotheses, as constituent parts of the ARAS and therefore alterations to the regions could underlie the vulnerability to cognitive fluctuations.

7.2 Pathology in the locus coeruleus does not relate to the presence or absence of cognitive fluctuations

Investigation of the LC identified differences in the burden of alpha-synuclein, tau and amyloid-beta across the disease groups. However, the differences identified were related to pathological diagnosis and not to the presence or absence of cognitive fluctuations. Pathologically LBD groups possessed the highest levels of alpha-synuclein pathology and the

pathologically AD cases the highest tau and amyloid-beta levels. Further analysis in the groups that possessed cognitive fluctuations revealed an association between tau burden in the LC and severity of cognitive fluctuations in the nLBD, but not DLB group. The association observed in the nLBD group could be suggestive that tau likely marks dysfunction and neuronal stress, and dysfunction and neuronal changes of the LC may be necessary but not in itself sufficient to elicit cognitive fluctuations.

The lack of a specific pattern of pathological protein accumulation within the LC suggests a lack of involvement of LC protein inclusions in cognitive fluctuations in DLB. Although, investigation into possible functional impairments of the LC and noradrenergic system was beyond the remit of the study. There are a number of theories that could suggest why the data from the present study suggested a lack of involvement for LC protein inclusions in cognitive fluctuations in DLB. Firstly, it could be possible that alterations in other brain regions, excluding the LC, but including other components of the ARAS are required to predispose towards fluctuations in cognition. Secondly, it could be possible that pathological changes to the LC or the noradrenergic system as a whole predispose to cognitive fluctuations, with the mechanism that elicits the cognitive fluctuations caused by alterations, either pathological or neurochemical, in other brain regions. Cognitive fluctuations are variable by nature, thus it is likely that a combination of more subtle changes to networks, for example alterations to receptor levels or impaired synaptic neurotransmitter release, are related to cognitive fluctuations rather than static accumulations of pathology in the neuronal soma. Therefore, a third explanation could be that a collection of pathological or neurochemical changes in a brain network, such as the ARAS, creates a compromised system that may only manifest a clinically observable feature when a particular level of internal or external stress is placed upon it.

Although, no specific pattern of accumulation of protein aggregates was observed for the presence or absence of cognitive fluctuations, a positive relationship was observed between the burden of tau in the LC and the severity of cognitive fluctuations. However, the relationship with severity of cognitive fluctuations was observed in only the nLBD and not the DLB with fluctuation group. Increased levels of tau within neurons of the LC could be an indirect measure of increased cellular dysfunction, including alterations to vesicular trafficking and alterations to synaptic regions; however, analysis of cellular stress markers

was not undertaken in the study. Alterations to the functioning of the noradrenergic system could partially underly the vulnerability for fluctuations by disrupting the ability of the nucleus to increase awareness and attention. As the LC is affected by tau at the early stages of pathological progression, it could be suggested that higher tau levels in the LC are suggestive of globally higher tau levels and thus higher global dysfunction within the diffuse networks hypothesised to underly cognitive fluctuations. It is unlikely that tau in the LC is the sole factor driving cognitive fluctuation severity as it would be expected that an association to be observed in the DLB group also if this was the case.

7.3 Pedunculopontine nucleus pathological burden does not relate to the presence or absence of cognitive fluctuations

Similarly, to the LC, differences were observed in the burden of pathological proteins in the PPN that were related to the pathological diagnosis and not the presence or absence of cognitive fluctuations. In addition, no associations were observed between the pathological burden in the PPN and severity of cognitive fluctuations in either the DLB or nLBD with fluctuation groups. Furthermore, the results demonstrated that there was no specific pattern related to the presence or absence of cognitive fluctuations seen for the relationship between PPN and LC pathology burden.

The PPN has been described as a key centre in the ARAS, and is most active during waking and REM sleep (Jenkinson *et al.*, 2009; Benarroch, 2013). Impairments to the arousal maintenance networks have been hypothesised to underlie a vulnerability that can lead to fluctuating cognition (Matar *et al.*, 2019; O'Dowd *et al.*, 2019). The PPN is thought to mediate its role in arousal through cholinergic projections to the thalamus (Benarroch, 2013), studies have noted that loss of cholinergic neurons in the PPN leads to a decrease in cholinergic innervation to the thalamus in PD (Francis and Perry, 2007). Studies have also observed alterations to the cholinergic system in the thalamus relating to cognitive fluctuations; including preservation of thalamic nicotinic receptors in those with fluctuations compared to those without (Pimlott *et al.*, 2006) and increased levels of acetylcholine precursors which were closely related to the presence and severity of fluctuations (Delli Pizzi *et al.*, 2015). However, these changes could be independent from the pathological protein accumulation within the region, which would suggest why the present study did not identify a relationship with PPN pathology and cognitive fluctuations. Specific examination

of the cholinergic neurons in the PPN could aid in the understanding of whether neurotransmitter/neuron specific alterations are related to cognitive fluctuations.

Although the PPN is a key component within the systems maintaining the high frequency EEG required for arousal, it is possible that the clinical phenotype of cognitive fluctuations could be a product of a network dysfunction. Dysfunction to the PPN could play a role, but not be the sole factor, within a dysfunctional ARAS network, suggesting why no associations were observed between PPN pathological burdens and the presence of cognitive fluctuations. This is supported by animal studies where lesions of the PPN did not have the expected effect on arousal levels, suggesting redundancy or flexibility to compensate within the system (Mahaffey and Garcia-Rill, 2015). Generation of fluctuations in cognition could rely upon a collection of alterations to a number of different brain regions, including those identified in the PPN, that in tandem elicit fluctuations but not in isolation. Changes to the thalamus, the main relay for PPN cholinergic neurons mediating arousal, could contribute to arousal-promoting signals not being able to elicit their desired effects. Furthermore, molecular and connectivity changes have been observed in the thalamus that have been related to cognitive fluctuations (Pimlott *et al.*, 2006; Delli Pizzi *et al.*, 2015), consistent with the suggestion that signals from the PPN may not be able to elicit their arousal effects on cortical regions.

7.4 Cognitive fluctuations could be the result of dynamic serotonergic dysfunction

In a similar manner to the results from the LC and PPN, differences in the burden of pathological proteins in the raphe were related to pathological diagnosis and not the presence or absence of cognitive fluctuations. Furthermore, analogously to the PPN no relationships were observed between the pathological burden in the raphe and the severity of cognitive fluctuations. However, analysis of TPH2 intensity in neurons with and without Lewy bodies identified differences that could relate to the severity of cognitive fluctuations. Together with the observation that a higher proportion of TPH2-positive neurons without Lewy bodies was associated with more severe cognitive fluctuations, dysfunction to the serotonergic system could underly vulnerability to cognitive fluctuations independent of local neurodegenerative pathology burden.

The raphe forms part of the ventral ARAS pathway (Zeman, 2001), and has been implicated to play a complex role in the maintenance of arousal states (Brodie *et al.*, 1955). Fluctuating

cognition has been hypothesised to reflect disturbed sleep-wake homeostasis leading to alterations in arousal (O'Dowd *et al.*, 2019), consistent with alterations to sleep-wake architecture previously described in DLB (Pao *et al.*, 2013). The raphe conveys its effect on arousal through serotonergic projections to various cortical and subcortical regions, including the basal forebrain and hypothalamus. Studies have identified atrophy of the basal forebrain (Grothe *et al.*, 2014; Schumacher *et al.*, 2020) and volumetric reductions in the hypothalamus associated with changes to arousal (Whitwell *et al.*, 2007), in DLB. Furthermore, clinical features of DLB include depression and anxiety, suggestive of serotonergic dysfunction (McKeith *et al.*, 2017). The present findings of variations in TPH2 intensity in neurons with and without Lewy bodies, and the proportion of TPH2-posiitve neurons bearing Lewy bodies that related to the severity of cognitive fluctuations, likely indicates that serotonergic dysfunction could elicit vulnerability to cognitive fluctuations. Alterations to the serotonergic system could force other neurotransmitter systems in the ARAS, including the noradrenergic and cholinergic pathways, to compensate. Alterations to the noradrenergic pathways and cholinergic pathways have previously been identified in DLB, possibly indicating that these pathways may not be able to fully compensate for serotonergic dysfunction. The inability for the ARAS to reliably compensate for dysfunction in one of its constituent neurotransmitter systems could lead to a less dynamic modulation, which is not capable of handling variability in the load it is placed under, which could lead to lapses in cortical excitement, observed as clinical fluctuations.

Similarly, to the PPN and LC the described alterations to the raphe are not likely to be exclusively responsible for cognitive fluctuations in DLB. The alterations to the raphe are likely to occur in unison with other changes in the ARAS. For example, imbalances between the monoaminergic and cholinergic systems would likely reduce the ability for the brain to dynamically control large scale networks that are required for the basis of consciousness (Matar *et al.*, 2019). Fluctuations may be dependent on various changes that act in unison with those identified in the raphe, such as altered connectivity with higher order ARAS pathway structures. Alterations to the basal forebrain and hypothalamus for example, could further exacerbate serotonergic dysfunction, consequently leading to an ARAS system vulnerable to fluctuating cognition. Studies in the basal forebrain (Grothe *et al.*, 2014; Schumacher *et al.*, 2020) and hypothalamus (Galvin *et al.*, 2011) have identified atrophy,

connectivity and volumetric reductions, that are consistent with the suggestion that numerous ARAS regions are impaired within DLB.

7.5 Synaptosomal levels of neurotransmitter markers and pathology in the medial prefrontal cortex do not relate to the presence or absence of cognitive fluctuations

Immunoblot assessment of synaptosomal and soluble enzyme fractions in the mPFC identified the burden of pathological proteins related to the pathological diagnosis rather than the presence or absence of cognitive fluctuations. Furthermore, analysis of synaptosomal levels of neurotransmitter markers, SERT, VMAT2 and ChAT, along with mitochondrial markers did not identify any differences in the experimental groups in comparison to controls.

Previous studies have identified reductions to the neurotransmitter markers, SERT and ChAT, as well as mitochondrial markers in neurodegenerative disorders including DLB and AD (Tiraboschi *et al.*, 2000; Ballard *et al.*, 2002a; Fløtnes *et al.*, 2018). The apparent preservation of neurotransmitter and mitochondrial markers could be through a preferential selection of 'healthy' synaptosomes and mitochondria in the fraction examined, as well as alterations to reserve pools of transporter proteins. However, even though there may be a preferential selection for 'healthy' synaptosomes, in the synaptosomal fraction analysed in the current study there were no alterations identified in the markers of ARAS projections in the disease groups compared to the controls. A lack of difference compared to the controls could suggest that there is no selective damage to ARAS-projecting synaptosomes. If there was selective damage or loss of ARAS-projecting synaptosomes it could be postulated that there would be a decrease in the ARAS projection markers, as a proportion of the total synaptosome pool and therefore would be an increase in 'non-healthy' synaptosomes in the disease groups. Therefore, as analyses were measured relative to total protein content a decrease in the ARAS projection markers could have been expected. A further suggestion could be that all synaptosomes are equally vulnerable, not just those that are ARAS projecting. If all synaptosomes were reduced at the same rate, this could present as there being no difference between the disease groups and controls, due to the fact analysis was not quantified relative to the synaptosomal number. Examination of the other fractions, or the crude tissue homogenate, is required to fully assess whether 'healthy' synaptosomes and mitochondria were preferentially selected. It is also unlikely

that alterations in the mPFC solely contribute to the cortical vulnerability towards cognitive fluctuations and further alterations, including deafferentation of cholinergic and monoaminergic projections would be necessary in further cortical regions to create a propensity towards fluctuating cognition. Along with possible alterations to reserve pools of transporters, it is likely that subtle changes to receptors, including number, relative subtype expression and topographical location, both globally and neuronally, in the mPFC and other cortical regions may also be necessary to create a system vulnerable to cognitive fluctuations.

7.6 Vulnerability across the ascending reticular activating system and its relationship to cognitive fluctuations

The present series of studies has demonstrated that the presence of pathological protein accumulation within the ARAS is more specific for pathological diagnosis than the presence or absence of cognitive fluctuations. This is consistent with the idea that pathology in a single component of a distributed network may not be sufficient to account for the phenomenon, and that a collection of changes throughout the system could be necessary (Matar *et al.*, 2019). The present series of studies have also identified alterations to the serotonergic neurotransmitter system that could relate to or create vulnerability to the presence of cognitive fluctuations. The raphe plays a complex role in the maintenance of arousal states, with lesions leading to permanent states of arousal and cortical depletion of serotonin having a sedative effect. Therefore, the present results could suggest that alterations that allow for a dynamic system, rather than a permanent diminished state, including alterations to neurotransmitter catalytic enzymes, could be more representative of the alterations required to create a vulnerability towards a transient clinical feature like cognitive fluctuations.

Alpha-synuclein pathology was identified, in the pathologically LBD cases, in all regions examined. The regions investigated have all been implicated in modulating arousal through their connectivity with cortical regions and impairments to these nuclei have been speculated to play a role in the vulnerability towards cognitive fluctuations. However, levels of alpha-synuclein, and AD-type pathology were not different between the nLBD cases both with and without cognitive fluctuations. If cognitive fluctuations were to be purely the results in differences in the deposition of pathological aggregates, then a difference

between the nLBD with and without cognitive fluctuations groups would have been observed. Therefore, the data from the histological studies of the three brainstem nuclei could suggest that perhaps different forms of pathological alterations, including presence and level of different alpha-synuclein species not specifically examined in the present studies or alterations to receptors may be necessary for the manifestation of cognitive fluctuations.

The ARAS is a diffuse complex system comprised of a number of nuclei and neurotransmitter systems, with studies having identified a degree of redundancy with the system (Kovalzon, 2016b). Due to the level of redundancy within the ARAS it is suggested that all three key brainstem structures would need to be affected or that one region or neurotransmitter system would need to be severely affected so that parts of the ARAS are not able consistently undertake the role of the affected region, in order to elicit fluctuations in consciousness. If all three key brainstem regions were to be affected creating a vulnerability towards fluctuating cognition, it could be that regions contain a specific pattern of pathology. However, data from the present studies show a lack of associations between the pathological burdens of the three ARAS nuclei, specific to the groups with cognitive fluctuations. The lack of conserved associations in the fluctuation groups suggests that there is no topographical pattern of pathological burden that is specific for the presence of cognitive fluctuations.

Two main hypotheses have been suggested to explain the aetiology of cognitive fluctuations; fluctuations as a disorder of attention and fluctuations as a disorder of arousal (Matar *et al.*, 2019; O'Dowd *et al.*, 2019). However, studies have suggested that cognitive fluctuations could be a disorder of both attention and arousal (Bliwise *et al.*, 2014; Ferman *et al.*, 2014). Data from the current studies could contribute new insights into the hypotheses surrounding cognitive fluctuations, as the current studies identified no relationships between the accumulation of pathological proteins and the presence or severity of cognitive fluctuations in the ARAS regions examined. This data could suggest that the presence of pathological aggregates alone in the regions is not sufficient to elicit cognitive fluctuations. It should be emphasised however that the lack of association with pathological burden does not suggest that the regions are not involved in the vulnerability towards cognitive fluctuations; further alterations not investigated including functional

changes could relate to the presence of cognitive fluctuations. However, the present series of studies primarily examined the alterations to arousal systems in relation to cognitive fluctuations. Although, the mPFC has been implicated in attentional as well as arousal systems. Therefore, it is possible that in order to elicit cognitive fluctuations impairments to both systems must occur in concert and alterations to the ARAS might represent only part of the vulnerability towards cognitive fluctuations. It is also plausible that a compromised system enables cognitive fluctuations to occur but additional dynamic factors, such as an increased load on the systems or variations to neurotransmitter vesicle pools, are required for a clinical feature to present.

Cognitive fluctuations have already been postulated to be a product of alterations to multiple sites within a system, including the ARAS. It is possible that cognitive fluctuations are elicited by variable involvement in the different nodes of the affected network differentially in each individual, but that produces the same clinical phenotype, as Dugger *et al*. (2012) has postulated for RBD. This hypothesised individual variability within the same network could explain the difference observed clinically between the duration and frequency of cognitive fluctuations. Matar *et al.* (2019) hypothesised that cases that fluctuated frequently and for a short duration, compared to those who fluctuated infrequently but for a longer duration, could be representative of two different neuropathological aetiologies, although could elicit the same score for clinical severity via CAF. Variable involvement of arousal and attentional systems that could elicit fluctuating cognition could explain the lack of relationship to the presence or absence of cognitive fluctuations. Furthermore, results from the present study mainly identified pathological alterations related to the last CAF score, and not the average or maximum values. Within the present cohort a selection of cases possessed last CAF scores that were suggestive of a lack of cognitive fluctuations, although previous scores and clinical notes indicated that cognitive fluctuations had been present previously. The predominance for associations with last CAF score could be suggestive that the alterations that allow fluctuating cognition to occur could vary over the course of disease progression. Those cases which had a last CAF score indicative of a lack of cognitive fluctuation could have 'lost' the dynamic flexibility required to fluctuate, or due to the reversible nature of cognitive fluctuations, although unlikely due to the progression of neurodegenerative pathology, regained the ability to

maintain cortical arousal states. Together this could suggest that there is a high level of variability both between individuals and throughout the duration of disease progression in regard to the underlying neuropathological changes related to cognitive fluctuations. A high degree of variability would have hampered the ability of the present studies to identify specific changes that relate to the presence or absence of cognitive fluctuations.

Overall the data suggests that the ARAS is subject to neurodegenerative changes, including the accumulation of pathological protein aggregates, however it is currently unclear how these could elicit a vulnerability towards cognitive fluctuations. Due to the degree of redundancy within networks, including the ARAS (Kovalzon, 2016b), pathology or structural alterations to individual components of a network are unlikely to relate to the presence of cognitive fluctuations. It is probable that a collection of pathological alterations, molecular and degenerative, to a number of network components contributes to the vulnerability to fluctuations.

7.7 General strengths and limitations

7.7.1 Strengths

The primary strength of this study was the analysis of the same cases across all the ARAS regions examined. As there are multiple neurotransmitter systems that comprise the ARAS, it suggests that there is some level of redundancy within the system (Kovalzon, 2016b), further supported by animal lesion models, show that no pathway is essential for the maintenance of arousal (Jones, 2005b). Therefore, analysis of all regions in the same cases enabled the examination of the ARAS system as a whole in relation to cognitive fluctuations in DLB.

A further strength of the study was that the cases included were clinically and pathologically well-characterised, with a consensus diagnosis based upon clinical and neuropathological features. The clinicopathological diagnosis ensured that all cases were not atypical in their clinical or neuropathological presentation and allowed the correct experimental fluctuation group categorisation that would not lead to anomalous results.

Furthermore, due to the level of clinical information available from the NBTR, it was possible to ensure only DLB and nLBD cases with a history of cognitive fluctuations were included. The NBTR clinical information also meant it was possible to exclude cases that, although, were classified as possessing fluctuating cognition during their disease duration,

these fluctuations were believed to be the result of or induced by medication changes. The extent of the clinical data further allowed for the inclusion of the AD and nLBD cases without a history of cognitive fluctuations. Additionally, the quantity of clinical data available allowed the assessment of the severity of cognitive fluctuations via maximum, average and last CAF score. As cognitive fluctuations are variable in clinical appearance the ability to use more than one measure of severity allows the capture the full range of variation of the clinical phenotype.

The use of two disease control groups, nLBD and AD without cognitive fluctuations, is a further strength of the current studies. The inclusion of a nLBD without fluctuation group enabled specific comparisons to be undertaken in relation to cognitive fluctuations with the DLB and nLBD with fluctuations groups. The AD without cognitive fluctuations group further controlled for the effect of AD-type pathology in the regions analysed in the mixed AD/DLB cases. The inclusion of the non-fluctuating groups enabled the possible identification of a specific pattern or severity of pathological protein deposition, that could lead to a vulnerability towards cognitive fluctuations.

The biochemical analysis of the mPFC utilised synaptosomal fragmentation, in order to determine the 'functional' levels of neurotransmitter markers. A number of neurotransmitter markers, including vesicular transporters can be located at the synapse as well as in other neuronal compartments, therefore the fragmentation allowed the investigation of marker levels at the synaptic level compared with the whole tissue level.

7.7.2 Limitations

The present studies were limited by small cohort numbers, with a maximum of 14 per experimental fluctuation group. The specific inclusion of cases with well documented and assessed cognitive fluctuations meant that a number of possible cases were excluded from analysis; this included the exclusion of cases where fluctuations in cognition appeared to be related to medication and those that were uncooperative with neuropsychiatric testing *intra vitam*. A larger cohort size may have been better powered to identify relationships related to cognitive fluctuations. However, the study by Duggar *et al.* (2012) which undertook a similar analysis of the LC and PPN in relation to RBD utilised a maximum of 10 cases in the LBD with and without RBD groups.

As the present series of studies were retrospective, not all cases utilised had been assessed for cognitive fluctuations. This limited the number of cases that could be utilised to test associations with severity of cognitive fluctuations, using CAF score. Although the number of cases is a limiting factor, a more prominent caveat is the CAF score itself. Some cases, which although had been recorded to clinically have cognitive fluctuations, had CAF scores of 0, which would suggest no fluctuations were present. The CAF consists of a series of informant-directed questionnaires regarding fluctuating confusion and impaired consciousness in the month prior to the assessment (Mainland, 2015). The cases that possessed CAF scores tended to come from clinical studies undertaken in conjunction with the NBTR, these cases were assessed annually with a number of neuropsychiatric including CAF. With CAF only being recorded annually, if a patient did not noticeably fluctuate within the month prior to the assessment, the presence of cognitive fluctuations would not be recorded within the score even if during the year severe and noticeable fluctuations had occurred. Along with CAF scores only being assessed annually, another caveat of the score is that due to the nature of the CAF scoring system, short duration but frequent fluctuations would receive the same score as longer duration, but more infrequent fluctuations; it is thought that these two different fluctuation patterns may be due to differing underlying pathological changes (Matar *et al.*, 2019). A difference in fluctuation pattern could occur between the nLBD and DLB cases with fluctuations which could explain the association with tau in one group but not the other, however, until a more robust and frequent clinical assessment for clinical fluctuations is adopted into practice it will be difficult to fully probe into the pathological correlates for cognitive fluctuations.

A further limitation of the histological studies was the lack of consistent level utilised to assess the three brainstem regions of the ARAS. Although the tissue analysed was obtained from the same NBTR tissue block for each of the three nuclei examined, the levels obtained for analysis could have possessed within block variations to the to the tissue section level. Previous studies have identified that different levels within the LC, PPN and raphe project to different anatomical regions, and have different effects on cognition and arousal (Ward and Gunn, 1976; Hornung, 2003; Hamani *et al.*, 2016). As the blocks containing the LC, raphe and PPN are used for routine diagnostics sections had already been taken from the blocks further limiting the possibility of obtaining a consistent level for each of the nuclei.

A related limitation was that in the histological studies only one marker of alpha-synuclein was assessed. As research has suggested that Lewy bodies may be protective (Tompkins and Hill, 1997; Olanow *et al.*, 2004; Tanaka *et al.*, 2004; Shults, 2006) and more evidence suggests that different species of alpha-synuclein may play more of a role in the proteins neurotoxic effects (Lashuel *et al.*, 2013), a pan alpha-synuclein marker (KM51) may not have assessed the levels of disease relevant alpha-synuclein. Human tissue is a valuable resource, therefore, examination of a wide number of alpha-synuclein species histologically could require exhaustive amounts of tissue, this is especially influential for the LC which is utilised for routine diagnostics where tissue is already limited for research use.

nLBD and AD cases were utilised as non-fluctuating disease control groups. Although the use of nLBD allowed for examination of a pathologically high-grade Lewy body group that did not have fluctuations, its direct comparison to DLB was confounded by the presence of high-grade AD-type pathology. Examination of nLBD with and without cognitive fluctuations allowed for comparison between the groups specifically in relation to cognitive fluctuations. However, the present study assumed that the cognitive fluctuations observed in mixed AD/DLB and DLB had the same aetiology, as changes specific to both fluctuating groups and not the two non-fluctuating groups would represent a fluctuation specific alteration. A more appropriate comparator would have been DLB cases without fluctuations, however as fluctuations are a core features identified in 80-90% of cases it would not be possible to obtain enough cases for meaningful analysis.

7.8 Future directions

7.8.1 Short-term studies

Synaptosomal levels of ChAT and SERT assessed in the mPFC, were observed not to be significantly altered compared to control cases. The results obtained in Chapter 6 were in contradiction to a number of previously reported findings that have identified reduction in global cortical ChAT levels in both AD and DLB (Lippa *et al.*, 1999; Tiraboschi *et al.*, 2000). The lack of difference between the experimental fluctuation and control groups was hypothesised to be due to preferentially selection of 'healthy' synaptosomes during the fractionation processes. Therefore, to validate this hypothesis examination of SNAP25, as well as other synaptic markers, in all the fractions would be required to assess if whether in the experimental fluctuation groups there was a consistent increase in the level of synaptic

markers in the non-synaptosomal fraction. Furthermore, assessment of the other fractions for SERT and ChAT levels could identify whether the different experimental fluctuation groups have differing levels of the neurotransmitter markers in the 'unhealthy' synapses. An additional assessment of NET levels within the fractions should be undertaken to examine the integrity of noradrenergic projections to the mPFC, as no assessment could be undertaken in the current study due to being unable to ascertain a working protocol.

Recent research has suggested that different species, including monomeric, oligomeric and post-translationally modified species of alpha-synuclein exist, with some likely having greater disease relevance that others (Lashuel *et al.*, 2013; Alam *et al.*, 2019; Mahul-Mellier *et al.*, 2020). Therefore, the employment of a pan-alpha-synuclein antibody in the present studies, labelling both pathogenic and less pathogenic species, may have limited the ability to observe relationships between alpha-synuclein and cognitive fluctuations, which is a limitation of the current study These findings in conjunction with the notion that static pathological aggregate may not represent the pathological substrate required to elicit a transient clinical symptom, suggest other species of alpha-synuclein should be investigated. Therefore, quantifying the levels of different alpha-synuclein species, specifically in the synaptosomal fraction of the mPFC, could help identify species that are more relevant to the vulnerability of the ARAS to fluctuating cognition.

The present studies did not include neuronal cell counts, in the LC, PPN or raphe. Therefore, cell counts for noradrenergic, cholinergic and serotonergic neurons in the LC, PPN and raphe respectively should be undertaken. Neuronal cell counts would enable further insight into changes within the ARAS nuclei that could relate to cognitive fluctuations, as suggested in Chapters 3 and 4.

The results from current study of the raphe identified changes to serotonergic neurons containing Lewy bodies which could lead to serotonergic dysfunction that creates a predisposition to the generation of fluctuating cognition. Therefore, further investigations are warranted to examine the specific neuronal localisation of pathology within the LC and PPN. Analysis of whether alpha-synuclein pathology is preferentially located in noradrenergic or cholinergic neurons, and whether the presence of pathology leads to alterations in catalytic enzyme levels, could help further test the hypothesis that dysfunction to ARAS systems is involved in cognitive fluctuations.

7.8.2 Medium and long-term studies

The two main pathways of the ARAS, ventral and dorsal, project from the brainstem through the thalamus, dorsal, and hypothalamus and basal forebrain, ventral (Jones, 2003). Examination of these regions in the cases that have already had the brainstem ARAS nuclei analysed would allow a more comprehensive assessment of the ARAS as a whole. Assessment of pathological changes as well as alterations to neurotransmitter markers could identify whether changes throughout the ARAS are required in order to create a vulnerability towards cognitive fluctuations.

Arousal has been speculated to be mediated by a wide array of different neurotransmitter receptors, including nicotinic, β- and α1-receptors, and 5-HT_{1A} and 5-HT_{1B} receptors (Berridge, 2008; Monti, 2011; Bloem *et al.*, 2014). Examination of receptor levels via autoradiography could allow the identification of further neurotransmitter changes that could lead to the dynamic dysfunction required to elicit a transient clinical feature.

Pathological examination of DLB patients who had undergone extensive clinical assessment of their cognitive fluctuations, for example EEG, MRI, and regular assessment of severity including CAF or DCFS would enable the pathological examination of alterations observed *intra vitam*. Furthermore, utilising cases that had undergone extensive neuropsychiatric testing could enable the examination of whether cases that have cognitive fluctuations that are short in duration but frequent have a similar, or different pathological underpinning to those with longer duration but less frequent fluctuations.

The changes that occur in the different regions that comprise the ARAS are likely to underly a vulnerability to cognitive fluctuations in concert with each other (Matar *et al.*, 2019). Therefore, computational modelling the specific changes observed within the regions examined, including deposition to pathological aggregates, alterations to neurotransmitter markers and receptors for example could provide a general overview of the status of the ARAS in cases with cognitive fluctuations compared to those without. Computational models obtained from pathological data could then be compared with EEG data to further understand the process that elicits cognitive fluctuations.

7.9 Conclusion

This study identified that the severity of protein aggregates in the ARAS was not associated with the presence or absence of cognitive fluctuations. The results may indicate that DLB

patients have a pathologically compromised arousal system, but that this likely acts in concert with other mechanisms to elicit cognitive fluctuations. As cognitive fluctuations are transient, one may speculate that static protein aggregates are unlikely to underlie a clinical feature defined by variable clinical presence and it is likely that cognitive fluctuations are related to a global system failure rather than one specific change. Therefore, research should focus on obtaining a better clinical understanding and, the ability to quantify cognitive fluctuations is required in order to interpret clinico-pathological findings.

Chapter 8: References

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