A clinico-pathological investigation of the human basal ganglia and brainstem in clinical subtypes of Parkinson's disease

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Abstract

Introduction: Parkinson's disease (PD) is a progressive, neurodegenerative disorder with different motor subtypes (tremor-dominant (TD), akinetic-rigid (AR) or mixed) based on variation in the presence and severity of the four main motor signs – tremor, rigidity, akinesia and postural instability/gait difficulty. The exact pathology underlying these presentations is unclear, but responses to dopaminergic and non-dopaminergic medications vary, suggesting that multiple neurotransmitter systems are involved. AR motor subtype appears to have prognostic capacity for predicting cognitive impairment. However, this not well-characterised, and it is unknown whether the relationship between gait and cognition reflects a shared underlying pathophysiology. In this thesis, I set out to delineate this relationship to identify prognostic trends, and to identify the pathology underpinning motor subtype and the decline to cognitive impairment in PD.

Methods: 13 controls, 40 TD, 40 Mixed and 40 AR cases were obtained from the Parkinson's UK Tissue Bank, based on retrospective clinical note evaluation. Tissue sections containing the substantia nigra (SN), locus coeruleus (LC) and striatum were immunostained for tyrosine hydroxylase (TH) to stain for monoaminergic neurons and choline acetyltransferase (ChAT) for cholinergic interneurons (ChIs) in the striatum. Questionnaires from Tissue Bank cases and longitudinal data from the Parkinson's Progression Markers Initiative (PPMI) cohort were used to assess clinical correlations between AR features and cognitive decline in PD.

Results: Loss of TH cell populations and proteinopathy in the SN and LC occurred to the same extent in all motor subtypes compared to controls. In contrast, striatal ChIs showed reduced primary and secondary arborisation and increased tau deposition in AR cases compared to TD and Mixed cases. Tau deposition in the striatum correlated significantly with branching of ChIs. AR features, gait disturbance particularly, were found to be a harbinger of cognitive decline within 3 years. PPMI data showed that this was most significantly associated with measures of attention, verbal learning, visuospatial and executive dysfunction.

Conclusions: The PD motor syndrome in general arises from degeneration of monoaminergic cell populations, but variations in the presence and severity of motor and cognitive symptoms may arise from non-dopaminergic systems such as the cholinergic system. This may explain the differential response of PD motor subtypes to cholinergic medication. Failure of cholinergic neurotransmission, and proteinopathy in regions such as the striatum and the cerebral cortex, may underlie the temporal interaction between gait disturbance and cognitive impairment, with dysfunction of synaptic plasticity playing an important role.

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Declaration of Originality

I declare that the work in this thesis is solely my own work unless otherwise stated in the text and has not been submitted for any other qualification at this or any other university. All other information is appropriately cited in the text, with a complete bibliography supplied at the end of the thesis.

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Tilley BS, Law JR, Patel SR, Goldfinger MH, Pearce RKB, Gentleman SM. (2019). Striatal neuronal tau deposition in cholinergic interneurons in progressive supranuclear palsy. (Manuscript in preparation)

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Oral presentations

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Poster presentations

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Abbreviations

A1 – Dopaminergic cell group 1

A2 – Adenosine class 2 receptor

A2A – Adenosine 2A receptor

A8 – Dopaminergic cell group 8

A9 – Dopaminergic cell group 8

A10 - Dopaminergic cell group 10

Aβ – Amyloid-β

AC – Adenylate cyclase

AChE –Acetylcholinesterase

AD – Alzheimer's disease

AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA – Analysis of variance

AR – Akinetic-rigid

ARTAG – Aging-related tau astrogliopathy

AT8 – Hyperphosphorylated tau

ATP13A2 - ATPase 13A1

BDNF – Brain-derived neurotrophic factor

BJOLT – Benton judgement of line orientation test

BTP – Benign tremulous parkinsonism

CaBP – Calcium binding protein

CAMKII – Calmodulin kinase II

cAMP – cyclic adenosine monophosphate

CBD – Corticobasal degeneration

CCL2 – C-C Motif Chemokine Ligand 2

CD68 – Cluster of differentiation 68

ChAT – Choline acetyltransferase

ChI – Cholinergic interneuron

CLARITY – Clear lipid-exchanged acrylamide-hybridized rigid imaging/immunostaining/in situ hybridization-compatible Tissue-hydrogel

CLi – Caudal linear nucleus

CNS – Central nervous system

CNV – Copy number variant

CRP – C-reactive protein

CSF – Cerebrospinal fluid

CTE – Chronic traumatic Encephalopathy

D1 – Dopamine receptor D1

D2 – Dopamine receptor D2

D3 – Dopamine receptor D3

D4 – Dopamine receptor D4

D5 – Dopamine receptor D5

DAB – 3,3'-diaminobenzidine

DAPI - 4',6-diamidino-2-phenylindole

DARRP32 – dopamine- and cyclic-AMP-regulated phosphoprotein of molecular weight 32,000

DAT – Dopamine transporter

DATscan – Dopamine transporter scan

dbGaP – Database of genotypes and phenotypes

dH_2O – Distilled water

DJ-1 – Protein deglycase DJ-1

DLB – Dementia with Lewy Bodies

dmX – Dorsal motor nucleus of vagus nerve

DNA – deoxyribose nucleic acid

DNAJC6 – Auxilin 1 gene

DNAJC13- DnaJ heat shock protein family member C13

DPX – Distrene plasticiser xylene

DRD1 – Dopamine receptor D1 gene

DRD2 – Dopamine receptor D2 gene

DRD3 – Dopamine receptor D3 gene

DRD4 – Dopamine receptor D4 gene

DRD5 – Dopamine receptor D5 gene

DSM-V – Diagnostic and statistical manual of mental disorders 5

EDO – Early disease onset

EIF4G1 - Eukaryotic Translation Initiation Factor 4 γ 1

FASTclear – Free of acrylamide SDS-based tissue clearing

FBXO7 – F-box only 7

FC – Fluctuating cognition

FFPE – Formalin-fixed paraffin embedded

fMRI – Functional magnetic resonance imaging

FOG – Freezing of gait

FP-CIT – Ioflupane (DATscan ligand)

$G_{\alpha i}$ – G protein inhibitory

$G_{\alpha o}$ – G protein olfactory

$G_{\alpha q}$ – G protein modulatory

$G_{\alpha s}$ – G protein stimulatory

GABA – γ-amino butyric acid

GBA – Glucocerebrosidase

GI – GABAergic interneuron

GP – Globus pallidus

GPe – Globus pallidus externus

GPi – Globus pallidus internus

HRP – Horseradish peroxidase

HTRA2 - Serine protease HTRA2

HVLT – Hopkins verbal learning test

H&E – Haematoxylin & Eosin

H&Y – Hoehn & Yahr

Iba1 – Ionised calcium-binding adaptor 1

ICC – Inter-class correlation

ICD – International classification of diseases

IF – Interfascicular nucleus

IHC – Immunohistochemistry

IL-1β – Interleukin 1β

IL-6 – Interleukin 6

IL-8 – Interleukin 8

iLBD – incident Lewy Body disorder

IMS – Industrial methylated spirit

$KMnO_4$ – Potassium permanganate

LB – Lewy body

LBD – Lewy Body Dementia

LC – Locus coeruleus

LFB – Luxol-fast blue

LID – L-dopa-induced dyskinesia

LiN – Linear nucleus

LRRK2 – Leucine-rich repeat kinase 2

LN – Lewy neurite

LNS – Letter-number sequencing

LTD – Long-term depression

LTP – Long-term potentiation

M1 – Muscarinic receptor M1

M4 – Muscarinic receptor M4

MAO-B – Monoamine oxidase B

MAPT – Microtubule-associated protein tau

MCI – Mild cognitive impairment

MCRS – Motoric cognitive risk syndrome

MDS – Movement Disorder Society

MLF – Medial longitudinal fasciculus

MoCA – Montreal cognitive assessment

MPTP – 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine

MRI – Magnetic resonance imaging

mRNA – Messenger RNA

MSA – Multiple system atrophy

MSN – Medium spiny neuron

nbM – Nucleus basalis of Meynert

NFT – Neurofibrillary tangle

NLR – Neutrophil:lymphocyte ratio

NMDA – N-methyl D-aspartate

NMS – Non-motor symptom

NSAID – Non-steroidal anti-inflammatory drug

NT – Neuropil thread

OPTIclear – Optical properties-adjusting tissue-clearing agent

OR – Odds ratio

PBP – Parabrachial pigmented nucleus

PBS – Phosphate buffered saline

PBS-T – Phosphate buffered saline-Triton X-100

PD – Parkinson's disease

PDD – Parkinson's disease dementia

PDD-DLB – PDD with DLB-like symptoms

PET – Positron emission tomography

PIGD – Postural instability/gait difficulty

PINK1 – PTEN-induced putative kinase 1

PKA – Protein kinase A

PLA – Proximity Ligation Assay

PLA2 – Phospholipase A2

PMI – Post-mortem interval

PPN – Pedunculopontine nucleus

PPMI – Parkinson's Progression Markers Initiative

PN – Paranigral nucleus

PSP – Progressive supranuclear palsy

RBD – REM-sleep behaviour disorder

RLi – Rostral linear nucleus

RNA – ribose nucleic acid

ROI – Region of interest

RSWA – REM-sleep without atonia

SAA – Serum amyloid A

SDM – Symbol-digit modalities

SDS – Sodium dodecyl sulphate

SN – Substantia nigra

SNARE – Soluble NSF attachment protein receptor

SNc – Substantia nigra pars compacta

SNCA - α-synuclein gene

SNP – Single nucleotide polymorphism

SNr – Substantia nigra pars reticulate

SPECT – single photon emission computerised tomography

SPR – Sepiapterin reductase

STN – Subthalamic nucleus

SVF – Semantic Verbal Fluency

SYNJ1 – Synaptojanin 1

TAN – Tonically active neuron

TD – Tremor-dominant

TDP43 – Tar-DNA binding protein 43

TH –Tyrosine hydroxylase

TLR2 – Toll-like receptor 2

TNFα – Tumour necrosis factor α

UCHL2 – Ubiquitin carboxyl-terminal hydrolase like-2

UPDRS – Unified PD rating system

VAMP2 – Vesicular-associated membrane protein 2

VH – Visual hallucinations

VPS13C – Vacuolar protein sorting 13C

VPS35 – Vacuolar protein sorting 35

VTA – Ventral tegmental area

YKL-40 – Chitinase-3-like protein 1

3R – 3-repeat tau

4G8 – Amyloid-β 17-24

4R – 4-repeat tau

5-HT – 5-hydroxytryptophan

Chapter 1 - Introduction

1.1. The history of the clinical syndrome of Parkinson's disease

1.1.1. James Parkinson

In his 1817 *An Essay on the Shaking Palsy* (Parkinson, 2002), James Parkinson described a clinical picture of tremor at rest, subsiding sometimes during voluntary movement; festinant gait and flexed posture; dysarthria and dysphagia; insomnia and severe constipation. Herein, Parkinson reflected on the term Shaking Palsy, or *Paralysis agitans*, employed vaguely by previous writers such as Galen to represent a group of disorders characterised by shaking of the limbs and perceived weakness of said limb. Typical of Parkinson's meticulous nature was his analysis of the previous medical literature on the subject of Shaking Palsy, in which he notes that the term had been previously ascribed to disorders characterised either by tremor but not palsy, or palsy without characteristic tremor, but by "slight tremblings" that may result from the palsy itself. From this Parkinson derived the main argument of his Essay, namely, that certain features were common to one disorder, or strictly speaking to a closely related group of disorders – parkinsonism is a reproducible clinical syndrome (Marks, 1974).

In his *Essay,* Parkinson described six cases which he had witnessed from his Shoreditch clinic, each displaying characteristic symptoms of the parkinsonian syndrome. The first case, of a fifty-year-old male gardener, presented with "trembling" of the left hand and arm and had come on without previous risk factors. The second case was of a sixty-two-year-old gentleman who developed trembling in all four limbs, flexed posture and dysarthria on a background of chronic alcoholism. The third case was that of a sixty-five-year-old gentleman who had "agitation" of his entire body which could not be designated as "trembling", severely bowed posture and festinant gait. The fourth was a fifty-five-year-old male with trembling of both arms for five years and severe inflammation of the skin over left lower rib cage. Upon relieving the inflammation, no change was noted in the tremblings of the upper limbs thus revealing the idiopathic nature of his symptoms. The fifth was a case seen from afar with poverty of movement, festination and severe postural change requiring the aid of an attendant. The final case was by far the most detailed study. It concerned a seventy-two-year-old gentleman with a twenty-year history of lumbago and trembling of the left arm started eight years after. The trembling progressed to affect the right arm and then all limbs and this was followed by constipation and increased mucus production with the stool. Interestingly, the tremor ceased when at rest in an armchair.

On treatment of the malady, Parkinson crucially highlighted the importance of evidence-based attempts at treatment driven by study of the causes of disease, but also accepted that anecdotal treatments may be warranted. Parkinson stressed the importance of the treatment of the progressive symptoms of the disease. He states that “particular circumstances indeed must arise in different cases, in which the aid of medicine may be demanded” and that particular attention must be paid to the progressive symptoms as a means of prolonging life in the final stages of the disease. Parkinson had described cases of varying severities and presentations, also referencing some of the non-motor symptoms (NMS) we today know to be part of the Parkinsonian syndrome. Moreover, he made reference to the timing of symptoms, noting that *scelotyrbe festinans* (festinant gait) may not occur until the later stages of the disease, thus creating a rudimentary staging system for the clinical progression of the disorder. Thus, even from the earliest clinical descriptions, it is clear that parkinsonism is a heterogeneous syndrome with different presentations and variations in symptoms over the clinical course, the nature and causes of which I hope to uncover in this thesis.

1.1.2. Jean-Martin Charcot

Several critical elements of parkinsonism were missed in *An Essay on the Shaking Palsy*. These include muscular rigidity, slowness of movement initiation (bradykinesia) and facial immobility. This brings to bear a misnomer in Shaking Palsy that was highlighted by Jean-Martin Charcot in 1872 in his lectures at La Salpêtrière, Paris. Charcot proved that routine clinical examination of the muscular power of the limbs would reveal a lack of muscular weakness, thus invalidating the term ‘Palsy’ as a descriptor of the disease (Goetz, 2011). Indeed, Charcot went on to suggest that the weakness seen by Parkinson and other authors before him was in fact bradykinesia – slowness in the initiation of voluntary movement (Goetz, 2011). Parkinson asserted in his Essay that the senses and the intellect remain uninjured. The latter descriptions of cognitive impairment and sensory phenomena such as chronic pain by Charcot and others have shown Parkinson’s assessment to not be accurate. Charcot noted “enfeeblement of the intellects” in the terminal phases of the disease and “perversion of sensibilities” that can occur at any point of the disorder. Moreover, Charcot alluded to some of the now recognised non-motor features of *paralysis agitans*. These included descriptions of fatigue, neuropsychiatric abnormalities, “need of frequent

changes of position", perturbation of temperature sensation including excess heat, and convulsions which may include seizures or dyskinesias – abnormal involuntary movements.

Charcot was also a pioneer in detailing clinical characteristics of tremor and thus being able to differentiate diseases based on the type of tremor evident in a particular case. He noted that the tremor present in *paralysis agitans* was one at rest, which ceased on voluntary movement whereas the tremor of disseminated sclerosis (multiple sclerosis) was only present with intention. Fascinatingly, this discovery lends support to the notion that the sixth of Parkinson's six clinical cases, who presented with lumbago tremor that dissipated at rest, may not have been *paralysis agitans* at all. Charcot was able to trace the nature of these two different tremors and this is depicted in Figure 1.1.

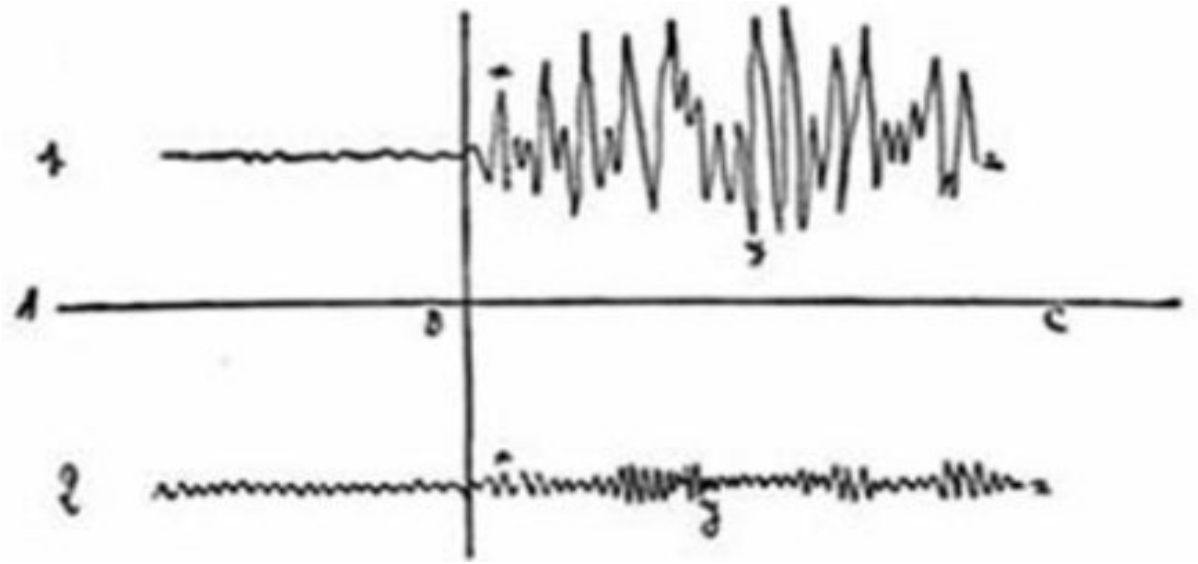


Figure 1.1: "Myographic" curves of intention tremor of disseminated sclerosis and of *paralysis agitans* (Goetz, 2011). Phase A-B indicates the rest period and B-C the period of action. Trace 1 is of a patient with disseminated sclerosis in which the tremor is barely perceptible during the rest phase but is stark and present in a coarse and irregular fashion during the action phase. Trace 2 is of a patient with *paralysis agitans* in which the tremor is present at rest but has postural and re-emergent qualities in the action phase. The method of recording of these traces is unknown, but in other traces Charcot used various pneumatic tambour mechanisms (Goetz, 2011).(Reproduced with permission)

Under Charcot's tutelage, several students, including Leopold Ordenstein, Charles Babinski and Gilles de la Tourette, made important contributions to the understanding

of the field of Neurology especially in the study of *paralysis agitans*. Charcot, with the help of his students, described the clinical syndrome in a vast number of cases, and isolated prototypes of the disease. Charcot designated the condition as *la maladie de Parkinson* (Parkinson's disease (PD)), in honour of James Parkinson, having noted that *paralysis agitans* was an inaccurate description of the syndrome: patients are not markedly weak and they do not necessarily have tremor. Therefore, the disease could be differentiated into those who had tremor and those who did not and thus had marked rigidity and bradykinesia. Ordenstein divided the disease course into three phases: a non-specific prodromal phase, followed by the onset of characteristic motor symptoms such as tremor and gait disorders and then a later phase characterised by rigidity and palsy (Lehmann, Hartung and Kieseier, 2007). Ordenstein and Charcot also pioneered treatment of the disorder using belladonna alkaloids such as hyoscyamine for tremor and rye-based ergot products. In so doing, they had uncovered a crucial aspect of the pathophysiology of PD: the dopaminergic: cholinergic imbalance in the striata of sufferers of PD which could be amended by blocking cholinergic transmission using hyoscyamine, or by stimulating dopaminergic transmission using ergot products. Understanding the neurochemical basis for symptomatic variations in PD is of crucial importance for efficacious treatment. Using hyoscyamine as a targeted therapy for tremor specifically poses a tantalising question: does aberrant cholinergic transmission differ in the striata of tremulous PD cases compared to those who do not experience tremor? Investigation of this question, in the context of the basal ganglia motor system, and in the hope of deriving new knowledge to inform new treatment strategies, will be one of the primary aims of this thesis.

1.1.3. William Gowers

William Gowers in his 1892 Manual of Diseases of the Nervous System (Gowers, 1892) correctly noted the significant male predominance of cases of *paralysis agitans* in which he noted a five to three proportion of male to female sufferers. At that time, Gowers' account was among the most complete appraisals of the epidemiology and natural history of the disease. In the 80 cases he studied, he confirmed the most common age of onset as 50-60 years (which still holds true today) and that rarely cases may present before the age of 30. His analysis of the prevalence of late-onset cases was confounded by the lower average life expectancy of the time.

After establishing the epidemiology of the condition, Gowers provided some of the more prosaic descriptions of the symptoms. Most noteworthy was his analysis of the tremor of *paralysis agitans*:

> *The tremor is an alternating contraction in opposing muscles causing a rhythmical movement of the parts to which they are attached… causes a movement of the fingers at the metacarpo-phalangeal joints similar to that by which Orientals beat their small drums… chiefly in the thumb and forefinger, which may move as in the act of rolling a small object between their tips.*

This description formed the basis for the classical *pill-rolling* description of parkinsonian resting tremor. Moreover, Gowers provided the first account of one of the main characteristics of this symptom: parkinsonian tremor is *re-emergent* in that "a voluntary movement may stop the tremor for a few seconds, sometimes for many, but it recommences and accompanies the movement". Gowers also provided one of the most illuminating illustrations of the muscular rigidity of parkinsonism and the consequences of the associated dystonias. He provides wonderful sketches of some of the common hand postures and the contractures found in *paralysis agitans* with dystonia (Figure 1.2).

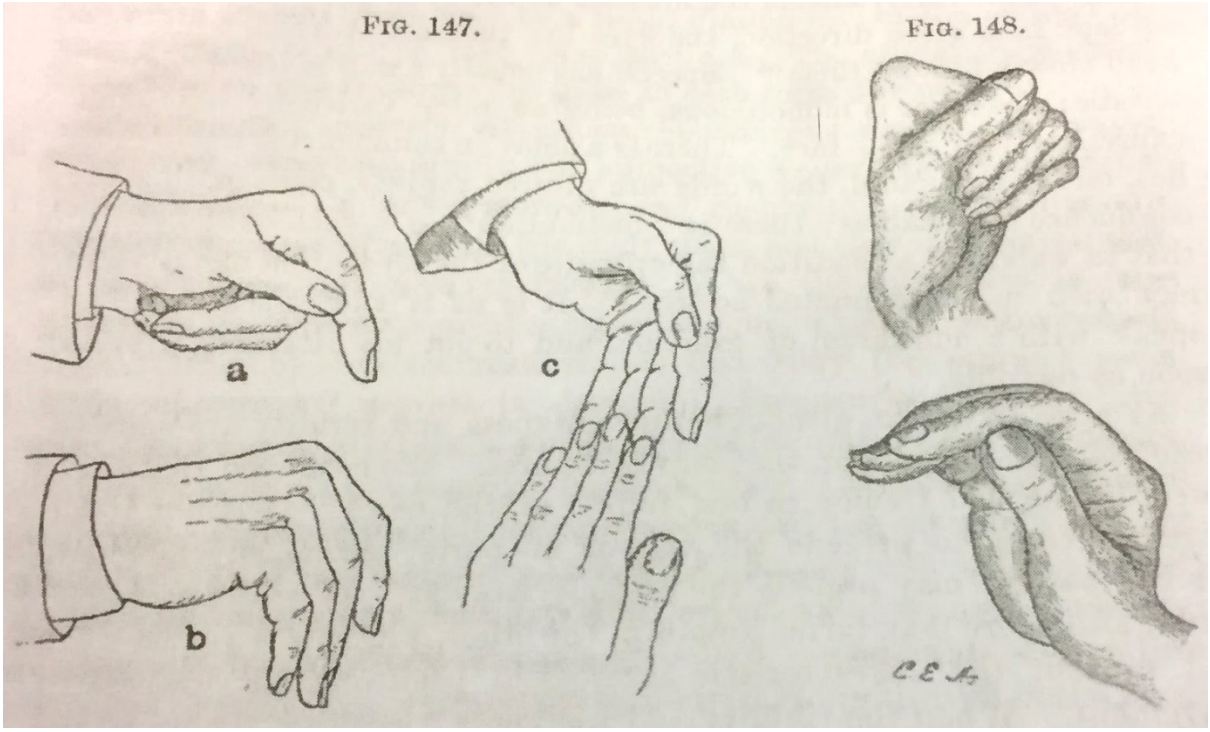


Figure 1.2: Posture of the hands in *paralysis agitans* taken from Gowers' A Manual of Diseases of the Nervous System (Gowers, 1892). Fig.147 A-C are depictions of contracture of the interossei whereas Fig. 148 are depictions of contracture of the fingers.

Although Gowers incorrectly associates the poverty of movement in *paralysis agitans* to muscle weakness, he does correctly identify that muscle atrophy can take place in the later stages of the disease. Perhaps the most impressive aspect of the *Manual* was Gowers' analysis of the progression of *paralysis agitans*. Other contemporary authors such as Marshall Hall had attempted to distinguish varieties of *paralysis agitans,* separating into "hemiplegic" and "general" forms (Hall, 1841) and Gowers references a later addition by Berger which is the "monoplegic" form in which the syndrome starts in one limb (Gowers, 1892). Gowers asserted that these are not varieties but merely stages of the disease. In fact, in so doing, Gowers had provided the basis for the most widely used staging system for the clinical staging of Parkinson's disease – the Hoehn & Yahr system (Hoehn and Yahr, 1967). This system asserts that the syndrome usually starts in one limb, becomes bilateral and then generalised, eventually causing severe disability and this is reflected well in Gowers thoughts on the staging of the disease.

Gowers also remarked that the subtypes of the disease are separable by the presence and severity of each of the three main motor symptoms: tremor, rigidity and weakness. Moreover, Gowers, like Charcot had appreciated an alternative presentation of

paralysis agitans whereby “rigidity, position of limb, and muscular weakness may be conspicuous before the tremor is perceptible. It is this feature that gives rise to the best-marked variety of the disease”. Gowers also noted one of the potential complications which he terms “an attack of ordinary hemiplegia” – this may be the first clinical description of freezing episodes in PD.

After Charcot had first described some of the NMS of *paralysis agitans*, Gowers elaborated with highly detailed descriptions of these phenomena. These included abnormalities of temperature sensation, either manifesting as excessive heat or excessive cold, or alternations of the two. Also, excessive perspiration or excessive dryness of the skin may have often be seen in Gowers’ case histories. Interestingly Gowers observed regional variability in the temperature regulation and sweat production, often finding that one side would be hot and wet, and the other dry and cold. Gowers also alluded to urinary symptoms in *paralysis agitans*. He remarked that “the urine is sometimes increased in quantity” and that, according to other contemporary authors, “contains an excess of phosphates”.

Gowers also noted that *paralysis agitans* also affected the cognitive and neuropsychiatric states of certain individuals, though not all. He described the frequent “mental depression” that can occur throughout the disease but also the “mental weakness and loss of memory… sometimes accompanied by the tendency to delusions”.

On treatment, Gowers summarised the medicaments recommended by previous authors such as hyoscyamine, morphia, conium, tonics of iron carbonate and barium chloride, and strychnine to be of little use through extended trial in his patients. Instead, he recommended oral administration of arsenic and Indian hemp (*Cannabis sativa*), the latter seeming to reduce tremor to only occasional bouts in a case example. Acute administration of Cannabis was later shown to promote dopamine release in the striatum (Ton *et al.*, 1988; Bloomfield *et al.*, 2016), thus reducing parkinsonian symptoms.

1.1.4. Parkinson's disease clinical syndrome: from historical perspectives to viewpoints in the modern era

1.1.4.1. The Hoehn & Yahr staging system

Today Parkinson's disease is widely recognised as a progressive multisystem disorder with a heterogeneous symptom profile that changes as the disease progresses. In an attempt to characterise the clinical progression of the disease, Margaret Hoehn & Melvin Yahr developed the most commonly used staging system in the field (Hoehn and Yahr, 1967). Based on case analysis of a vast number of Parkinson's disease patients, Hoehn & Yahr analysed the chronic progression of the condition, noting past medical history, disease associations, neurological signs and symptoms, and levels of disability to create a five-stage system. Thus "**Stage I**: unilateral involvement only, usually with minimal or no functional impairment. **Stage II**: bilateral or midline involvement, without impairment of balance. **Stage III**: First sign of impaired righting reflexes. This is evident by unsteadiness as the patient turns or is demonstrated when he is pushed from standing equilibrium with the feet together and eyes closed. Functionally the patient is somewhat restricted in his activities but may have some work potential depending upon the type of employment. Patients are physically capable of leading independent lives, and their disability is mild to moderate. **Stage IV**: Fully developed, severely disabling disease; the patient is still able to walk and stand unassisted but is markedly incapacitated. **Stage V**: Confinement to bed or wheelchair unless aided" (Hoehn and Yahr, 1967). They also found that different subtypes can occur. They discovered that the different clinical signs – tremor, akinesia and rigidity – often progress at different rates and that one sign or another may predominate. Those with predominant resting tremor often had a better prognosis than those with an akinetic-rigid (AR) picture.

The Hoehn & Yahr staging system has been critiqued on the basis of a number of different factors (Goetz *et al.*, 2004). Firstly, the increase in disability is not equitably distributed between stages, i.e. the difference between stage I and II is not the same as between II and III. This means that the potential for analysis is limited to rudimentary non-parametric analyses (Goetz *et al.*, 2004). Secondly, comorbid causes of disability can result in inaccurate Hoehn & Yahr stage and relief of that comorbid cause would cause cyclical variations of the staging. Finally, the stages do not reflect clinical progression in all cases. There are two prominent examples that illustrate this

argument. Firstly, the case of a patient with severe unilateral disease may be more disabled than another with mild bilateral disease yet would be assigned a lower Hoehn & Yahr stage. Secondly, patients with postural-instability/gait-difficulty (PIGD)-predominant presentation would enter the staging system at stage II or stage III at disease onset.

However, Hoehn & Yahr stage does correlate significantly with the severity of dopaminergic deficit present on [123I]FP-CIT SPECT imaging (Staffen *et al.*, 2000). Moreover, it is widely used and accepted due to its simplicity and its ease of identification of the defined clinical landmarks that form the basis of the staging system. Further, it has proved to be a useful tool for physicians in informing treatment strategies in their clinical practice (Goetz *et al.*, 2004).

1.1.4.2. The Unified Parkinson's disease Rating Scale (UPDRS)

In the quest to develop a rating system which could be used to assess a wide variety of clinical signs and symptoms with more clinometric benefits than the Hoehn & Yahr system, the Unified Parkinson's disease Rating System (UPDRS) was created by Stanley Fahn and colleagues in the 1980s (Fahn, Elton and Members, 1987). This system incorporated the strengths of a wide variety of pre-existing rating scales, including the Webster (Webster, 1968), Columbia (Duvoisin, 1971), King's College (Parkes *et al.*, 1970), Columbia (Canter, De la Torre and Mier, 1961), Northwestern University (Lieberman, Le Brun and Boal, 1973), New York University and University of California Los Angeles (Diamond, Markham and Treciokas, 1978) scales, to provide an all-encompassing and easily-used system to monitor PD progression (Goetz *et al.*, 2003).

After multiple trials and revisions, the UPDRS in its final published format comprised four main sections: **I** – Assessment of mentation, behaviour and mood, **II** – Assessment of activities of daily living, **III** – assessment of motor severity and **IV** – assessment of PD-related complications (Fahn, Elton and Members, 1987). Completion of the scale is performed by clinical interview and observation of the clinical signs that the patient displays. Occasionally, the scale is presented with two additional sections concerning Hoehn & Yahr staging and Schwab & England but these were not part of the original UPDRS system (Hoehn and Yahr, 1967; Schwab and England, 1968).

Though the scale was criticised for ambiguities in the text, inadequate instructions for raters and the absence of screening questions for several important non-motor features of PD, it was complimented on the comprehensive analysis of motor symptoms, its clinometric properties (reliability and validity) and its wide application across the spectrum of PD and atypical causes of parkinsonism (Goetz *et al.*, 2003). The Movement Disorder Society (MDS) Task Force on Rating Scales for Parkinson's Disease produced a sponsored revision (Goetz *et al.*, 2008) of the UPDRS (MDS-UPDRS) based on the recommendations set out in its original assessment of the UPDRS system (Goetz *et al.*, 2003). This iteration differed from the original by clarifying items to make them more patient-rated. For example in section I, "symptomatic orthostasis" (Fahn, Elton and Members, 1987) in the original version was changed to "lightheadedness on standing" (Goetz *et al.*, 2008), and "sleep disturbances" (Fahn, Elton and Members, 1987) was changed to "daytime sleepiness" and "night-time sleep problems" (Goetz *et al.*, 2008). The new format encompassed four sections as the original did, but the sections were renamed **I** – "non-motor experiences of daily living," **II** – ''motor experiences of daily living,'' **III** – "motor examination," and **IV** – "motor complications" (Goetz *et al.*, 2008).

The UPDRS has be widely used in studies of a number of different types. It has been used in clinical longitudinal studies (Levy *et al.*, 2002; Burn, Rowan, Allan, Molloy, O 'brien, *et al.*, 2006; Burn *et al.*, 2012), cluster analysis studies (Lewis, 2005; Reijnders *et al.*, 2009; Lawton *et al.*, 2015), association studies (Van Der Hoek *et al.*, 2011; Leroi *et al.*, 2012) and neuroimaging studies (Spiegel *et al.*, 2007; Wang *et al.*, 2007; Moccia *et al.*, 2014; Song *et al.*, 2014; Dirkx *et al.*, 2017). Given the wide expanse of literature that has utilised the UPDRS, perhaps the most important quality of the tool is that is reproducible across studies and so can be used for meta-analyses (Pickering *et al.*, 2007). To date, it is among the most invaluable tools to clinicians and researchers alike in the field of movement disorders.

1.2. The symptoms of Parkinson's disease

1.2.1. Motor symptoms

Parkinson's disease is a progressive neurodegenerative disorder that primarily affects the motor system causing an extrapyramidal syndrome. Clinically, PD is characterised by a classical quartet of motor symptoms: bradykinesia, muscle rigidity, tremor and

postural and gait instability (Fahn, 2003). The Movement Disorder Society have defined the diagnostic criteria for PD (Postuma *et al.*, 2015). The diagnostic criteria for parkinsonism are bradykinesia plus one of rigidity or tremor. Once parkinsonism is diagnosed, the certainty of PD diagnosis is ascertained by ruling out exclusion criteria such as cerebellar symptoms, supranuclear gaze palsy, history of dopamine receptor blocker use, cortical sensory loss and normal neuroimaging findings of the presynaptic dopaminergic system (Postuma *et al.*, 2015). Further, inclusion of supportive features such as an unequivocal response to dopaminergic treatment, presence of levodopa-induced dyskinesia and olfactory loss, increase the diagnostic certainty of PD (Postuma *et al.*, 2015). Red flag symptoms such as severe autonomic failure, rapid progression of gait disturbance, early bulbar dysfunction and severe inspiratory failure can reduce the diagnostic probability of PD but are not sufficient to completely exclude it. The probability criteria are displayed in Figure 1.3. The clinical motor features of PD are summarised in Figure 1.4.

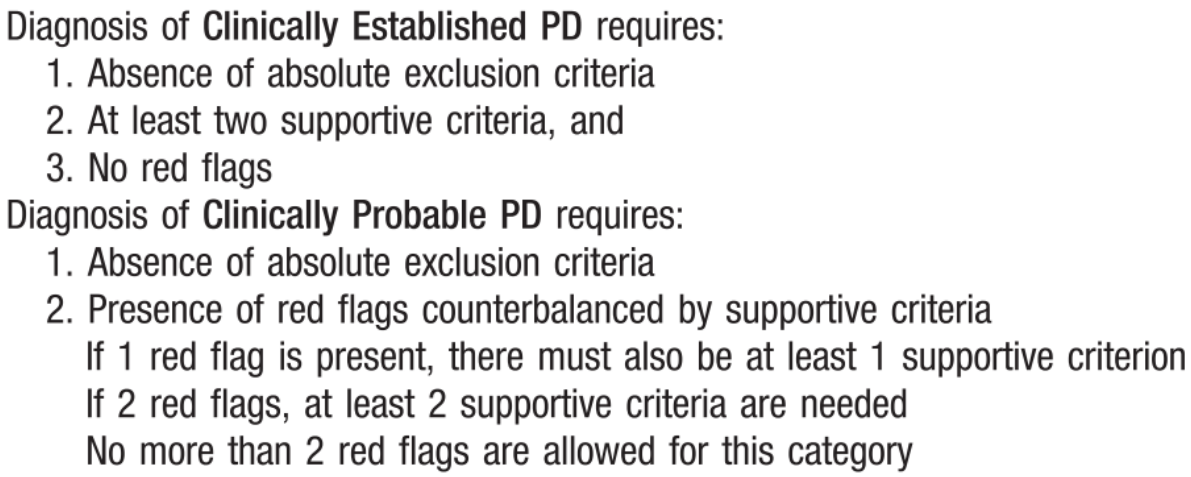


Figure 1.3: Diagnostic probability criteria for PD (Postuma et al., 2015). Reproduced with permission)

1.2.1.1. Bradykinesia

Bradykinesia is the central feature for a diagnosis of parkinsonism. Bradykinesia was first thought to be palsy or weakness by Parkinson and Gowers, but it took Charcot and colleagues to discover, by clinical examination, that PD patients had trouble executing of voluntary movements. Bradykinesia is often used synonymously with terms such as hypokinesia and akinesia but they are all distinct clinical entities: bradykinesia is categorically defined in the Queen Square Brain Bank criteria as

‘slowness of initiation of voluntary movement with progressive reduction in speed and amplitude of repetitive action’ (Gibb and Lees, 1988), hypokinesia as reduced amplitude of voluntary movement (Ling *et al.*, 2012) and akinesia as a poverty of expected voluntary movement, often with slowed reaction time (Golbe and Ohman-Strickland, 2007). While these clinical features can all occur in PD, they are not necessarily well correlated in an individual and thus may have different underlying pathophysiologies (Berardelli *et al.*, 2001). Bradykinesia can be exhibited in the limbs but also axially in the form of slowed, reduced steppage gait. Moreover, poverty of facial expression (facial hypomimia) is caused by reduced amplitude of movement of the facial muscles.

Clinically, bradykinesia is assessed using a repetitive action test such as rapidly pinching one’s index finger and thumb together repeatedly. This has to be performed for between 10 and 20 seconds in order to appreciate the decremental nature of the speed and amplitude of the repetitive action. Initially, patients may start with a normal amplitude test but this will usually dissipate within 20 seconds. This characteristic is unique to PD and can distinguish PD from atypical forms of parkinsonism such as progressive supranuclear palsy (PSP), which is characterised by hypokinesia without decrement (Ling *et al.*, 2012). This is evident not only on finger taps but also on handwriting. PD patients show micrographia (small handwriting) but PSP patients can often have microscopic micrographia whereby the amplitude of the handwriting is even smaller than that of PD patients (Ling *et al.*, 2012).

One of the most fascinating characteristics of akinesia is that it can occasionally be reversed in the presence of reward or significant external sensory stimuli such as danger. This is called *kinesia paradoxica* (Distler *et al.*, 2016). The fact that normal movement can be provoked in PD patients suggests that there is a strong motivational component to the pathogenesis of bradykinesia. Utilisation of the properties of *kinesia paradoxica*, such as response to visual and auditory cueing, may be helpful in treating patients with severe slowness of movement. Indeed, there are reports of patients who experienced *kinesia paradoxica* after an earthquake in Italy that gave a protracted clinical benefit (Bonanni *et al.*, 2010). In some cases, patients have used *kinesia paradoxica* to reduce frequency of off periods, and in other cases, using this technique has been able to provoke dyskinesias (Schlesinger, Erikh and Yarnitsky, 2007):

abnormal movements resulting from the chronic overuse of levodopa (Huot *et al.*, 2013).

Slowed movements can often be found in other neurological disorders such as amyotrophic lateral sclerosis. In these cases, there is no delay in the onset of voluntary movement but slowness in the performance of the movement. This is called *pyramidal slowness*. Occasionally, bradykinesia can also be mistaken for depression and *vice versa*, and it is important to rule out clinical depression in the evaluation of PD. *Obsessional slowness* is a rare phenomenon that can present as part of Obsessive Compulsive Disorder (Hymas *et al.*, 1991). It is characterised by extremely delayed onset of voluntary movement followed by normal speed and amplitude of movement. It is thought to originate from obsessional checking of correct movement and once the necessary checks have been completed, normal movement ensues. In extreme psychological disorders such as post-traumatic stress disorder, complex neurosis can result in a parkinsonian-like syndrome characterised by bradykinesia and occasionally tremor. Careful neurological examination can differentiate these forms of slowness of movement to increase the sensitivity and specificity of PD diagnosis.

1.2.1.2. Rigidity

Muscular rigidity is characterised by increased resistance to movement (Jankovic, 2008). The MDS define rigidity as “velocity-independent resistance to passive movement not solely reflecting failure to relax (i.e., distinct from spasticity or paratonia)” (Postuma *et al.*, 2015). PD rigidity is usually found in two forms: *lead-pipe* rigidity and *cogwheel* rigidity. *Lead-pipe* rigidity occurs when the rigidity is smooth and present throughout the whole range of movement of a limb. Occasionally, the tremor evident in PD can be ‘superimposed’ on top of the lead-pipe rigidity causing a *cogwheel* rigidity phenotype. Notably, *cogwheeling* in the absence of *lead-pipe* rigidity is insufficient for a confirmation of rigidity in PD (Postuma *et al.*, 2015). Rigidity can be found proximally (head, neck, shoulders, hips) or distally (wrists, ankles) (Jankovic, 2008). Axial rigidity concerns hypertonicity of the muscles of the trunk. Often rigidity can be found as part of a prodromal phase of PD and is sometimes mistaken in this phase as rotator cuff injuries, frozen shoulders, and myalgia.

Clinically, rigidity is assessed by examination of the passive movement of fully relaxed muscles. Increased muscle tone will manifest as increased resistance to passive

movement with *cogwheeling* in some cases. *Cogwheeling refers to mild tremor* superimposed on the rigidity in a limb. Where rigidity may be difficult to elicit on clinical examination, exacerbating manoeuvres such as the Froment manoeuvre (repeated movement of the contralateral limb to elicit rigidity in the ipsilateral limb) can be used. Rigidity in PD must not be confused for spasticity in upper motor neuron lesions such as stroke, multiple sclerosis, traumatic brain injury, spinal cord injury and amyotrophic lateral sclerosis. Spasticity is characterised by increased muscle tone with a pathognomonic *clasp-knife* trait: at the extremes of the range of movement of a muscle, the increased tone suddenly decreases.

1.2.1.3. Tremor

The most clinically noticeable symptom of PD is the tremor, which is defined as a tremor evident in a fully resting limb (Postuma *et al.*, 2015). It is generally a slow, coarse tremor that oscillates at a rate of 4-6Hz. Typically, in PD it presents at first as a tremor in the fingers and wrist (with classical, but rare, *pill-rolling* character) of an upper limb unilaterally and later moves to the proximal muscles of that limb or becomes bilateral. Occasionally tremor can present in the lower limbs first, but this is rare. Tremor presenting bilaterally may be an indication of an alternative diagnosis such as senile tremor, though Gowers in his case descriptions indicated there is no “sharp demarcation between the two affections and it is difficult to say in which category some cases should be placed” (Gowers, 1892). In order to meet Movement Disorder Society diagnostic criteria (Postuma *et al.*, 2015), the tremor must be observed at rest. However, in some patients without tremor at rest, the tremor can be elicited by prolonged maintenance of an outstretched posture of the hands, known as *re-emergent tremor* (Jankovic, Schwartz and Ondo, 1999). While the tremor in PD is usually found in the limbs, in some cases patients complain of tremor of the lips, chin and jaw (Jankovic, 2008), and of ‘feelings of internal tremor’ (Shulman *et al.*, 1996). Occasionally features of tremulous voice can be exhibited in PD (Perez *et al.*, 1996), though this is much more common in patients with essential tremor (Jankovic, 2008).

Tremor can also be found in other neurological and some non-neurological conditions that can confound clinical diagnosis. Action tremor, which is a feature more common in essential tremor than PD, is where tremor is present throughout the action of voluntary movement. Postural tremor is an action tremor that is present when holding a limb motionless against gravity (Thenganatt and Louis, 2012) – for example, when

holding an arm outstretched. Intention tremor can be present in a cerebellar syndrome, such as cerebellar strokes, tumours and multiple sclerosis, alongside signs of dysdiadochokinesis, ataxia, nystagmus and dysarthria (Diener and Dichgans, 1992). Intention tremor and action tremor are separable in that action tremor is not necessarily goal-directed (Thenganatt and Louis, 2012). Senile tremor is likely a type of essential tremor (though it can present with parkinsonian-like features) that has a peak incidence in the 6th decade of life (Bhagwath, 2001).

1.2.1.4. Postural and gait instability

Postural instability is one of the key clinical landmarks cited by Hoehn & Yahr in their staging system of PD (Hoehn and Yahr, 1967). Characteristic of stage III of their system, Hoehn & Yahr identified it as impaired righting reflexes, unsteadiness on turning or when pushed from a position of equilibrium (Hoehn and Yahr, 1967). It is said to mark the onset of severe disability (Bloem, 1992). Gait disturbance in PD was identified by the earliest scholars as festinant, with reduced steppage distance, stooped posture and reduced arm swing. This is thought to reflect a reduction in the amplitude, or hypokinesia, of the gait-associated movements (Jankovic, 2008). Moreover, the gait can be particularly slow, with marked initiation difficulties. Postural and gait instability are among the best characterised progressive features of PD – its prevalence increases with disease duration and it tends to deteriorate over time (Evans *et al.*, 2011).

Postural instability is tested for clinically using the *push test* or *pull test* but can also be visualised when the patient turns. The *push* or *pull test* is performed by pushing or pulling the patient backwards or forwards from their equilibrium standing position. A scoring system, which forms part of the UPDRS has been assigned to assess the degree of postural instability (PD workbook, 2006). A normal test result would reveal a patient's ability to rebalance against the force without taking more than two steps, yielding a score of 0. A test where more than two steps is taken, but the patient is able to 'self-right' would yield a score of 1. If assistance is needed to prevent a fall, this would give a score of 2. Scores of 3 and 4 reflect spontaneous instability and an inability to stand, respectively. Gait disturbance is evaluated on a similar scoring scale from the UPDRS where increasing festination and difficulty in mobilising independently yields increasing scores. Likewise, posture and axial bradykinesia (e.g. reduced arm swing) are also rated on a 4 point scale (PD workbook, 2006).

Gait disturbance of other aetiologies need to be ruled out in the assessment of PD. Gait disturbance can be prevalent as gait ataxia in cerebellar disease, alcoholism and multiple sclerosis. More subtly, severe postural and gait instability, with features like anterocollis, might arouse suspicion of an atypical parkinsonian syndrome such as PSP (Golbe and Ohman-Strickland, 2007). Other causes of 'postural instability' or 'disequilibrium', such as inner ear conditions (benign paroxysmal positional vertigo, non-positional vertigo, and labyrinthitis) and systemic neurological conditions (tumours, multiple sclerosis, and infarction) also need to be ruled out. Even within PD, other symptoms such as tremor, dyskinesia or orthostatic hypotension and reduced muscular power can cause instability that is confused for postural instability (Bloem, 1992).

1.2.1.5. Freezing of gait

Freezing phenomena may have first been described by Gowers as transient episodes of hemiplegia (Gowers, 1892). It is one of the most disabling features of PD (Giladi *et al.*, 2001). Freezing typically manifests as a transient inability to move. In a survey of 6620 PD patients, almost half experienced freezing episodes and it was more common in male patients than in females (Macht *et al.*, 2007). Freezing affects the legs most commonly but it has also been documented in the arms and eyelids (Boghen, 1997). Freezing of gait (FOG) can manifest as start hesitation, sudden inability to move the feet during certain tasks or situations (Jankovic, 2008). Schaafsma and colleagues characterised these transient phenomena by subdividing them in to 5 subtypes: I – start hesitation, II – turning hesitation, III – hesitation in tight quarters, IV – destination hesitation and V – open space hesitation (Schaafsma *et al.*, 2003). Freezing of gait is more associated with bradykinesia, rigidity, postural instability and longer disease duration (Giladi *et al.*, 2001). Tremor at disease onset appears to reduce the risk of future gait freezing in PD.

Freezing episodes are more common in the OFF state, i.e. while the patient is not under the influence of anti-parkinsonian medication, and are mitigated by levodopa therapy (Jankovic, 2008). Interestingly, freezing of gait can be overridden using sensory cueing such as auditory commands, music or visual obstacles. This suggests that freezing of gait is a phenomenon rooted in the association of sensory stimuli and motor programmes.

1.2.1.6. Other motor manifestations

Bradykinesia, rigidity and tremor are also known to affect other muscle groups such as the bulbar muscles. Bulbar dysfunction, in the forms of dysphagia (difficulty swallowing), dysarthria (difficulty enunciating words), dysphonia (abnormal speech production/voice hoarseness) and hypophonia (reduced speech volume) is thought to be due to hypokinesia and rigidity of the oro-facial-laryngeal muscle system (Hunker, Abbs and Barlow, 1982; Jankovic, 2008). Sialorrhoea (increased saliva production) may occur because of delayed swallow, though it may also be related to autonomic disturbances causing increased salivary gland activity. Motor deficits may also be prevalent as the loss of primitive reflexes such as the glabellar tap reflex, respiratory disturbances and eye-movement abnormalities (Jankovic, 2008).

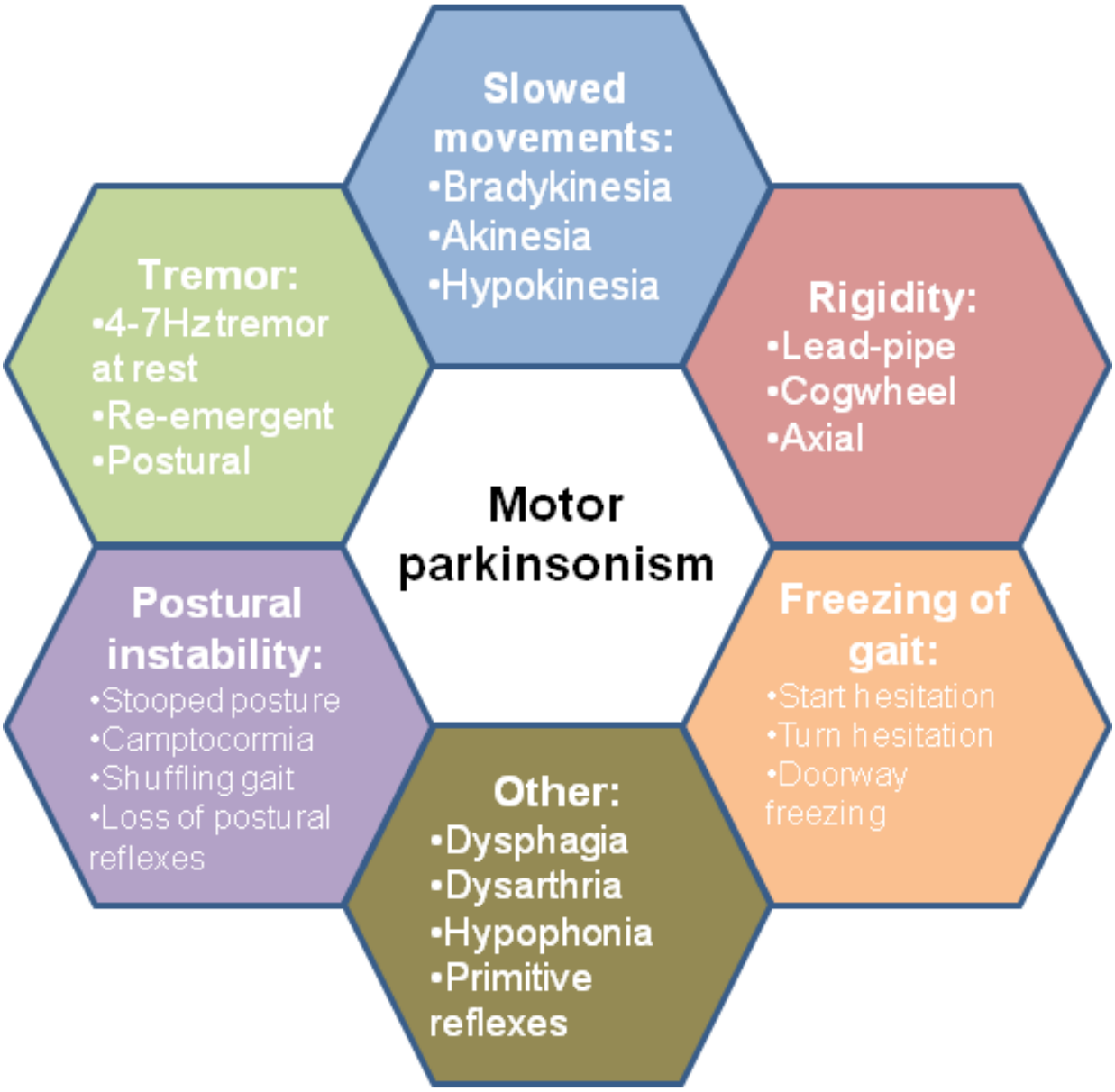


Figure 1.4:Selected symptoms of the Parkinsonian motor syndrome

1.2.2. Non-motor symptoms

NMS are among the most disabling features of PD and are often under-reported and under-diagnosed because of the embarrassment and stigma that can sometimes be attached to them, or because their relationship to PD is simply unknown to patients and carers (Chaudhuri *et al.*, 2006; Chaudhuri and Schapira, 2009). In a recent survey of PD patients and their caregivers aiming to set priorities for PD-related research, 5 of the top 10 priorities concerned the diagnosis, pathophysiology and treatment of non-motor symptoms such as mood disorder, sleep pathology, cognitive impairment and urinary disturbances (Deane *et al.*, 2014). In this section, I will review the clinical

characteristics of the key non-motor features of PD, including cognitive impairment and dementia, neuropsychiatric symptoms, sleep disorders, dysautonomia and sensory abnormalities.

1.2.2.1. Cognitive impairment

Cognitive impairment is a common complaint in PD. 80% of patients complain of cognitive issues, with 10-40% of cases, across a variety of studies, developing dementia as a complication of their disease (Bosboom, Stoffers and Wolters, 2004; Aarsland and Kurz, 2010; Aarsland and Bernadotte, 2015). While the point prevalence of dementia in PD is approximately 30%, the cumulative prevalence increases dramatically over the disease course: cases that enter the second decade of the disease have a cumulative prevalence of dementia of 75% (Aarsland and Kurz, 2010). In the Sydney Multicentre Study of Parkinson's Disease, it was found that dementia was an inevitability in patients who survived more than 20 years of the disease course (Hely *et al.*, 2008).

Dementia (now known by the DSM-V as major neurocognitive disorder) is defined as impairment in multiple cognitive domains sufficient to impair activities of daily living, according to the DSM-V (American Psychiatric Association and others, 2013). The dementia syndrome associated with PD affects different cognitive domains to Alzheimer's disease (AD) – the most common cause of dementia worldwide (Bronnick *et al.*, 2007). In 2007, the Movement Disorder Society Task Force systematically reviewed the literature on the epidemiology, clinical features and pathology of dementia associated with PD, to form a consensus on the diagnostic criteria for the disorder (Emre *et al.*, 2007). They held that deficits in at least two of four main cognitive domains (attention, executive function, visuospatial perception and memory recall), and at least one of five main behavioural symptoms (apathy, depression/anxiety, delusions, hallucinations and excessive daytime somnolence) qualified a case for a diagnosis of Lewy Body Dementia (LBD). In general, the timing of dementia diagnosis relative to the onset of parkinsonism separates the two main subtypes of LBD – PD dementia (PDD) and Dementia with Lewy Bodies – whereby if dementia precedes or occurs within one year of parkinsonism, the diagnosis is DLB, and if it occurs thereafter, the diagnosis is PDD (McKeith, Dickson, Lowe, M. Emre, *et al.*, 2005). However, the most recent DLB consortium diagnostic criteria for DLB have outlined more specific criteria. On top of a diagnosis of dementia, DLB cases have to express

two of four core features: fluctuations of cognition (FC), REM-sleep behaviour disorder (RBD), well-formed visual hallucinations (VH) and spontaneous parkinsonism (McKeith *et al.*, 2017). These symptoms are associated with, but not necessary for, a diagnosis of PDD (Emre *et al.*, 2007).

Subtler cognitive impairment, insufficient for a diagnosis of dementia, can also be found in PD patients. Mild cognitive impairment (MCI) is defined as significant cognitive decline from age-matched norms, but not sufficient to interfere with activities of daily living. This phenomenon is common in PD: a critical review by the MDS Task Force found that across 8 studies, the mean prevalence of MCI in non-demented PD patients was 26.7% (range of 18.9-38.2%) (Litvan *et al.*, 2011). PD patients tend towards major cognitive impairment in the long run through an MCI phase. Cross-sectional and longitudinal studies have found that PD-MCI was associated with progression to dementia (Litvan *et al.*, 2011; Goldman *et al.*, 2014), whereas patients who had no cognitive impairment at baseline were less likely to have developed dementia than those with MCI by 3 year (Pedersen *et al.*, 2013) and 4 year follow-up (Janvin *et al.*, 2006).

1.2.2.2. Neuropsychiatric symptoms

Neuropsychiatric symptoms such as depression, anxiety, apathy and psychosis are common in PD and will affect the vast majority of patients at some point in their disease course (Aarsland *et al.*, 1999). At any one time, the prevalence of significant depressive symptoms in PD patients is roughly 30-40% (Aarsland, Marsh and Schrag, 2009), and is associated with a considerable burden to patients, families and caregivers. Developing new treatments for neuropsychiatric symptoms in PD has accordingly been assigned a high research priority in a survey of carers and patients (Deane *et al.*, 2014).

Neuropsychiatric symptoms such as depression can predate motor symptoms as part of a prodromal non-motor syndrome associated with PD (Ishihara and Brayne, 2006). The key characteristics of depression in PD are classically anhedonia, anorexia, sleep disturbance, decreased libido, fatigue and psychomotor retardation (Aarsland, Marsh and Schrag, 2009) – though these symptoms are found in classifications for most forms of depression. Moreover, it is often clinically difficult to separate symptoms such as sleep disturbance, fatigue and sexual dysfunction as related to depression as

opposed to intrinsic PD non-motor syndrome – disturbances to the sleep-wake cycle, for example, are a well-recognised feature of PD independent of a diagnosis of depression (Fahn, 2003; Jankovic, 2008). Depression in PD can also fluctuate with motor performance, becoming significantly worse in OFF periods (Nissenbaum *et al.*, 1987) and therefore may also be responsive to dopaminergic therapy (Maricle *et al.*, 1995).

The most prevalent manifestations of anxiety in PD are generalised anxiety disorder, social phobias and panic attacks, particularly during OFF periods (Aarsland, Marsh and Schrag, 2009) and these phenomena are thought to affect approximately 40% of PD patients (Aarsland, Marsh and Schrag, 2009). Apathy in PD has a reported prevalence of between 17 and 70% depending on the presence of dementia and depression in the sample population, and the raters and the rating scales used in the assessment (Levy *et al.*, 1998; Aarsland *et al.*, 1999; Shulman, 2000; Pluck and Brown, 2002; Dujardin *et al.*, 2007). Though it can form part of a depressive syndrome, apathy often occurs independently of depression in PD, thus forming the notion that apathy is a distinct neuropsychiatric symptom (Levy *et al.*, 1998; Kirsch-Darrow *et al.*, 2006).

Psychosis and psychotic symptoms such as hallucinations, delusions and illusions, are common, affecting up to 50% of cases (Soulas *et al.*, 2008). Once present, these symptoms tend to be progressive and persist despite attempted treatment (Factor *et al.*, 2003). Of the various psychotic symptoms, VH are most common, but hallucinations are also prevalent in other sensory domains such as auditory phenomena (Aarsland, Marsh and Schrag, 2009). VH are of central importance to the diagnosis of DLB (McKeith *et al.*, 2017).

1.2.2.3. Sleep disorders

Almost all PD patients will experience disturbances of their sleep (Lees, Blackburn and Campbell, 1988) and this usually starts early in their disease course (Chaudhuri *et al.*, 2006). This includes primary and secondary causes of disturbed sleep. Primary causes include insomnia, parasomnias and hypersomnia, whereas secondary causes are mainly due to immobility which may preclude a patient from arising in the night if they experience nocturia (frequent waking in the night to micturate). This may occur

due to loss of circadian rhythm in the secretion of anti-diuretic hormone, which is regulated by dopamine.

Insomnia is the most common sleep disturbance in the general population (Gjerstad, Aarsland and Larsen, 2007). It takes three main forms: 1) difficulty in sleep initiation, 2) sleep fragmentation and 3) early awakening. In PD the burden of insomnia appears to be in the domains of sleep fragmentation and early awakening compared to healthy age-matched controls, whereas difficulties of sleep are equally prevalent in PD and the general population (Tandberg, Larsen and Karlsen, 1998). The origins of insomnia in PD are usually multifactorial: female gender, advanced disease and depression questionnaire scores are all significantly related to insomnia in PD (Gjerstad, Aarsland and Larsen, 2007). Patients with PD frequently have a fragmented, superficial sleeping pattern on polysomnographic investigation which significantly and adversely affects quality of life (Poewe and Högl, 2000).

Hypersomnia during waking hours may result directly from poor sleep pattern during the night, or may be related to dopaminergic therapy for motor symptoms (Adler, 2005). Hypersomnia and sleep attacks (narcolepsy) are common in PD and may result from abnormalities in the diurnal pattern of arousal.

Parasomnias including RBD and REM-Sleep without atonia (RSWA) are a highly prevalent feature in PD and may form part of a prodromal syndrome. RBD is characterised by thrashing out or calling out during the deep stages of sleep (REM sleep) when, in normal physiology, the body is atonic. Frequently this is accompanied by abnormal dream states which are ‘acted out’ by patients in which they often describe vivid dreams which are usually unpleasant. RBD is a well characterised harbinger of neurodegenerative disease, frequently associated with synucleinopathies such as idiopathic PD, LBD and multiple system atrophy (MSA). RBD and RSWA form parts of the diagnostic criteria for DLB in which RBD is assigned core importance while RSWA is an associated feature.

1.2.2.4. Autonomic symptoms

Autonomic symptoms in PD include constipation, orthostatic hypotension, sexual dysfunction, hyperhidrosis and bladder dysfunction (Chaudhuri *et al.*, 2006). These symptoms also form a key feature of multiple system atrophy and pure autonomic failure. Whereas in early PD autonomic features may be rare, their prevalence

increases as the disease advances with erectile dysfunction, blood pressure disturbances and bladder dysfunction rated the most common features in surveys and longitudinal studies of patients with advanced PD (Visser *et al.*, 2004; Hely *et al.*, 2008). Orthostatic hypotension, defined as a drop of 20mmHg or more of systolic blood pressure on standing, is a significant predictor of both falls and cognitive impairment in PD.

1.2.2.5. Sensory disturbances

The best-known sensory disturbance in PD is anosmia. Olfactory disturbances are very common in PD with a prevalence of 90%. This can occur several years before the onset of motor symptoms. Olfactory disturbance therefore forms an important part of the prodromal syndrome of PD. In a longitudinal follow-up study of a large cohort of males, those who scored lowest in objective tests of olfactory function had a greater than 5-fold increase in odds of developing PD (Ross *et al.*, 2008).

1.3. Subtypes of Parkinson's disease

1.3.1. Motor subtypes

The PD motor syndrome exists as distinct motor subtypes such as the tremor-dominant (TD) or AR syndromes (Jankovic *et al.*, 1990; Paulus and Jellinger, 1991, 1992; Selikhova *et al.*, 2009). Parkinsonian patients rarely present to the physician in the same manner, and the current diagnostic criteria for clinical PD requires only two positive motor symptoms – bradykinesia plus one of rest tremor or rigidity - to be present (Postuma *et al.*, 2015). This paradigm allows for the isolation of motor subtypes and for the stratification of patients into these subtypes for clinical studies. These motor phenotypes show different associations to non-motor symptomatology, with the AR subtype generally thought of as more severe in its association to cognitive, psychological and autonomic factors (Burn, Rowan, Allan, Molloy, O 'brien, *et al.*, 2006; Selikhova *et al.*, 2009; Van Rooden *et al.*, 2010; Burn *et al.*, 2012; Lawton *et al.*, 2015).

Motor subtypes of PD rarely remain stable over time, meaning that a single patient can exhibit multiple phases, characterised by a unique clinical phenotype in each phase (Von Coelln *et al.*, 2015). In practice, this produces a significant obstacle to the evaluation of clinical subtype. In previous studies, subtypes have been defined at a number of different time points: the motor presentation at diagnosis (Selikhova *et al.*,

2009; Pascale *et al.*, 2016), the motor picture at the time of the study (Lewis *et al.*, 2011; L. Zhang *et al.*, 2016), and the late-stage phenotype (Paulus and Jellinger, 1991). Each method has merits and disadvantages. For example, using the motor phenotype at the time of study is of particular use for imaging studies because it ties the current symptoms into the studies of neural circuitry.

However, for evaluation in post-mortem pathological studies, the paradigm that should be used is less clear. For post-mortem studies, the logical tool to use is the end-stage phenotype as this is closest clinical picture to the time of death, temporally. However, using this paradigm poses significant issues. At the end-stage, the presence of comorbidity (Martignoni *et al.*, 2004), anti-parkinsonian medication and pathologies such as Alzheimer's-type change can significantly cloud the motor phenotype making an accurate diagnosis for use in studies challenging. Moreover, significant proportions of PD patients become lost to follow-up as they progress into their end-stage, meaning any study using this paradigm may be more susceptible to a selection bias.

Using the presenting motor phenotype carries with it the risk of being temporally inconsistent with the pathological features present in post-mortem specimens. However, it is the most organic clinical picture one can obtain of the patient because it is in their *drug-naïve* state. Further, the lack of temporal consistency with the post-mortem picture could be argued to be irrelevant, given that presenting subtypes carry with them significant associations with late-stage features of the disease such as cognitive decline, falls and autonomic dysfunction – the pathological correlates between these subtypes may provide evidence for this disparity.

1.3.2. Cognitive subtypes

Cognitive impairment in PD is a progressive issue with distinct phases, following on from each other. 80% of PD patients will develop cognitive impairment during their disease course and this will range from MCI to frank LBD. The dementia syndrome in LBD is characterised by prominent early visuo-spatial and executive dysfunctions, attentional deficits and occasional memory deficits (Emre *et al.*, 2007; Friedman, 2017). LBD comprises two main phenotypes of dementia which are separated by an arbitrary 'one-year-rule'. DLB is defined by dementia preceding or occurring within one year of the onset of Parkinsonian motor features (McKeith *et al.*, 2017), whereas PDD is characterised by dementia occurring over a year after the onset of parkinsonism

(Emre *et al.*, 2007). Though some authors argue that DLB and PDD are essentially similar conditions (Friedman, 2017), their diagnostic criteria differ in critical ways (Emre *et al.*, 2007; McKeith *et al.*, 2017). The diagnostic criteria for DLB assign FC, VH and RBD central importance, whereas the PDD criteria specify these as associated but not core features (Emre *et al.*, 2007; McKeith *et al.*, 2017). Core features for a PDD diagnosis are dementia, developing within the context of parkinsonism, with impairment in more than one cognitive domain (Emre *et al.*, 2007). The discrepancy in these diagnostic criteria appears to be with good reason: FC has been reported in up to 80% of DLB patients (Ferman *et al.*, 2004) compared to 29% of PDD (Noe *et al.*, 2004); RBD is associated significantly with DLB (McKeith *et al.*, 2017) but is not a confirmed risk factor for PDD (Emre *et al.*, 2007); VH, though common in PDD, are even more common in DLB (Fénelon *et al.*, 2000; Aarsland, Ballard, *et al.*, 2001; Aarsland *et al.*, 2007). However, recent studies have elucidated the fact that PDD in certain forms can mimic DLB. Varanese and colleagues separated PDD patients into those that have FC and those who do not, and found that fluctuating PDD patients show a more similar pattern of cognitive dysfunction to DLB than those PDD patients without FC (Varanese *et al.*, 2010). PDD patients without FC showed more mild deficits in attention and memory, and lower prevalence of hallucinosis and delusion. Similarly, Dugger and colleagues split LBD cases by presence of RBD, finding more aggressive locus coeruleus noradrenergic cell loss in both compared to controls and Alzheimer's disease (Dugger *et al.*, 2012). PDD and DLB are most likely to be opposing ends of a clinical dementia spectrum based on the presence or absence of DLB-like symptoms: where these are present in PDD, this may represent a middle ground on the continuum.

1.4. Parkinson's disease – what causes it?

Parkinson had originally proposed the medulla oblongata to be the neuroanatomical origin of *paralysis agitans* in his *Essay* (Parkinson, 2002). However, other scholars, such as Blocq and Marinesco, had also reported on a case of hemiparkinsonism with tremor associated with a tuberculoma in the right cerebral peduncle (Okun and Koehler, 2007). Brissaud, and then Tretiakoff, localised the lesion to the substantia nigra (Okun and Koehler, 2007; Lees, Hardy and Revesz, 2009). The former, Brissaud, was the first to hypothesise that a nigral lesion may underpin the Parkinsonian syndrome. Tretiakoff in his medical thesis described depigmentation,

neuronal loss and gliosis in the substantia nigra in post-mortem PD brains. The pathologic hallmark was discovered by Friedrich Lewy who described hyaline-rich inclusions in neurons of the dorsal motor nucleus of the vagus nerve in the medulla oblongata (Lewy, 1913). Tretiakoff also noticed the presence of these structures in the substantia nigra, naming them 'Lewy Bodies' after Lewy (Lees, Hardy and Revesz, 2009). 84 years after their discovery in 1913 by Lewy (Lewy, 1913), Spillantini and colleagues finally uncovered the chemical composition of the Lewy body as ubiquitinylated α-synuclein (Spillantini *et al.*, 1997). Hence, PD is principally a disorder of α-synuclein and thus a *synucleinopathy*. α-synuclein is a synaptic protein, functions of which include promoting synaptic organisation, regulating synaptic plasticity and organising vesicular transportation and release (Bendor, Logan and Edwards, 2013).

Accumulation of α-synuclein as Lewy bodies (LB), and of Alzheimer's-associated proteins such as microtubule-associated protein tau and amyloid-β are likely to contribute synergistically to the progression of PD and the development of LBD (Compta *et al.*, 2011, 2014).

1.4.1. The neuropathology of Parkinson's disease

1.4.1.1. The Braak α-synuclein staging system

The Braak staging system for α-synuclein pathology in PD, developed in 2003, sought to define the temporal spread of LB pathology through the CNS, originating from the dorsal motor nucleus of the vagus nerve (dmX) before spreading rostrally through the brainstem, basal forebrain, subcortical and temporal mesocortical regions, followed by the higher order cerebral cortices (Braak, Tredici, *et al.*, 2003).

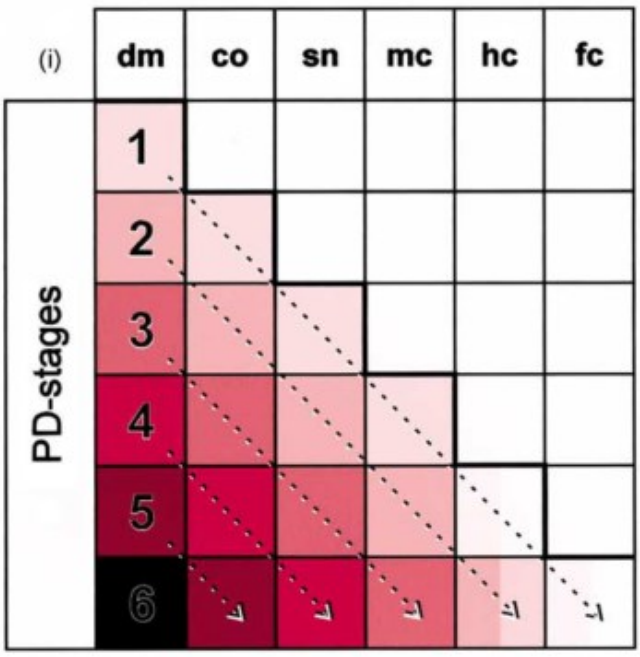


Figure 1.5: The Braak α-synuclein staging system. dm = dorsal motor nucleus of the Vagus nerve, co = locus coeruleus, sn = substantia nigra, mc = temporal mesocortex, hc = higher cortical regions, fc = frontal cortex. Reproduced with permission from (Braak, Del Tredici, *et al.*, 2003)

This hypothesis of PD pathology posits two entry sites for Lewy Bodies into the CNS – the first being the dmX and the second being via the olfactory bulb and anterior olfactory nucleus (Braak, Tredici, *et al.*, 2003). These two affected regions share one common feature: they are both contact points with the extra-cranial environment and so raise the possibility of environmental causes of sporadic PD.

The Braak hypothesis, however, works on the assumption that the first affected nuclei in the schema of spread will also be the most severely affected by the last stages of the disease (see colour scheme in Figure 1.5). This does not hold true in a variety of circumstances, including amygdala-predominant PD (Alafuzoff *et al.*, 2009), but also in Braak-stereotypical cases in which mild caudal brainstem pathology is found in cases with significant rostral brainstem, prosencephalic and neocortical pathology. Therefore, alternative conceptions of the staging of PD pathology were needed. McKieth and colleagues established a novel staging system based on the predominance of pathology in either brainstem, limbic (transitional) or neocortical regions (McKeith, Dickson, Lowe, M. Emre, *et al.*, 2005). Later, Leverenz and colleagues proposed an additional pathologic entity known as the amygdala-

predominant subtype (Leverenz *et al.*, 2008; Alafuzoff *et al.*, 2009), based on observations of up to 20% of cases in two pathologic series, and 29% in a third series (Zaccai *et al.*, 2008), that had predominant amygdala pathology with minimal brainstem and neocortical α-synuclein deposition. Whilst some authors have argued that amygdala-predominant pathology is evidence of a new pathologic entity (Uchikado *et al.*, 2006), it could be hypothesised that the amygdala is another potential entry site of α-synuclein pathology in the central nervous system (CNS). However, up to 49% of cases are unclassifiable according to this version of the McKeith classification, and thus a further "mixed" classification was proposed (Leverenz *et al.*, 2008; Jellinger, 2009). These data, taken together, suggest that there is no consistent and stereotypical hierarchy of spread of α-synuclein pathology in PD. This paves the way for explanations of clinical heterogeneity based on variations of the distribution of pathology throughout the CNS, perhaps influenced by differential regional susceptibility (Sulzer and Surmeier, 2013). For example, Selikhova and colleagues found increased global LB load in NTD cases compared to TD, especially in the neocortices (Selikhova *et al.*, 2009). More cases with neocortical LBs conformed to the diagnosis of NTD subtype. A quantitative approach within each brain region is warranted to enhance comparisons between clinical subtypes of PD. This should not only encompass the α-synuclein pathology found in the CNS, but the presence of comorbid pathologies too such as tau, amyloid-β (Aβ) and vascular pathology.

1.4.1.2. The influence of comorbid pathologies

Kalaitzakis and colleagues found that clinical phenotype in PD is not fully explained by α-synuclein pathology, but is more likely to be modulated by the combination of α-synuclein, tau and Aβ pathology (Kalaitzakis and Pearce, 2009). For example, they found that the striatal Aβ load was heightened in PDD and DLB cases compared to that of non-cognitively-impaired PD cases. This correlated with PET imaging findings, in the case of *cored* Aβ plaques (Figure 1.6B) in the striata of DLB cases (Kalaitzakis *et al.*, 2011). Aβ lesions are also increased in PDD cases compared to non-impaired PD in the entorhinal cortex (Kalaitzakis, Gentleman and Pearce, 2013). Aβ can also be found around blood vessels in a phenomenon called cerebral amyloid angiopathy (CAA; Figure 1.6C) and as diffuse plaques (Figure 1.6A). This presence of CAA was found to be increased, alongside Aβ plaque load in NTD-PD cases compared to TD by Selikhova and colleagues (Selikhova *et al.*, 2009).

With regards to tau protein and its contributions to PD pathology, it has been shown that PD patients with prominent sleep disturbance have significant increases in tau neurofibrillary tangle (NFT; Figure 1.6D) burden in the amygdala, CA2 section of the hippocampus and the entorhinal cortex (Kalaitzakis, Gentleman and Pearce, 2013). Further, tau burden was also found to be significantly increased in demented PD patients compared to non-demented cases, alongside higher Aβ pathology (Compta *et al.*, 2011).

Like the Braak α-synuclein staging system, staging systems exist for Aβ and tau proteins. Thal phases denote the stereotypical spread of Aβ plaque pathology through the CNS, starting in the neocortex, spreading to the allocortex, striatum and brainstem sequentially (Thal *et al.*, 2002). Braak tau staging tracks the stereotypical spread of tau NT pathology originating from the temporal mesocortex, spreading to the limbic cortices towards the striate and peristriate cortices in the occipital lobe (Braak *et al.*, 2006).

Tau pathology also can take the form of aging-related changes such as primary age-related tauopathy (PART) or aging-related tau astrogliopathy (ARTAG) (Kovacs *et al.*, 2016; Liu *et al.*, 2016; Kovacs, Lee and Trojanowski, 2017). ARTAG is found primarily in association with the blood-brain-barrier in perivascular, sub-pial, sub-ependymal and sulcal depth distributions. Hyperphosphorylated tau protein becomes deposited in astrocytes, which causes a *thorn-shaped* (Fig 1.6E) or granular/fuzzy astrocytic morphology. The exact meaning of ARTAG as a pathological entity is as yet unknown – while common in patients of advanced age with dementia, it is also common in age-matched non-demented individuals and in control brains (Liu *et al.*, 2016). That being said, small vessel changes, and small vessel disease has been correlated with Parkinsonian signs in a number of studies (van der Holst *et al.*, 2015; Hatate *et al.*, 2016; Shirzadi *et al.*, 2018), and vascular risk factors have been shown to contribute to the clinical phenotype in PD, with PIGD cases showing higher prevalences of vascular risk factors such as diabetes mellitus, obesity, hypertension, previous stroke or transient ischaemic attack history (N Malek *et al.*, 2016). Thus small vessel pathology can be a significant contributor to morbidity in PD.

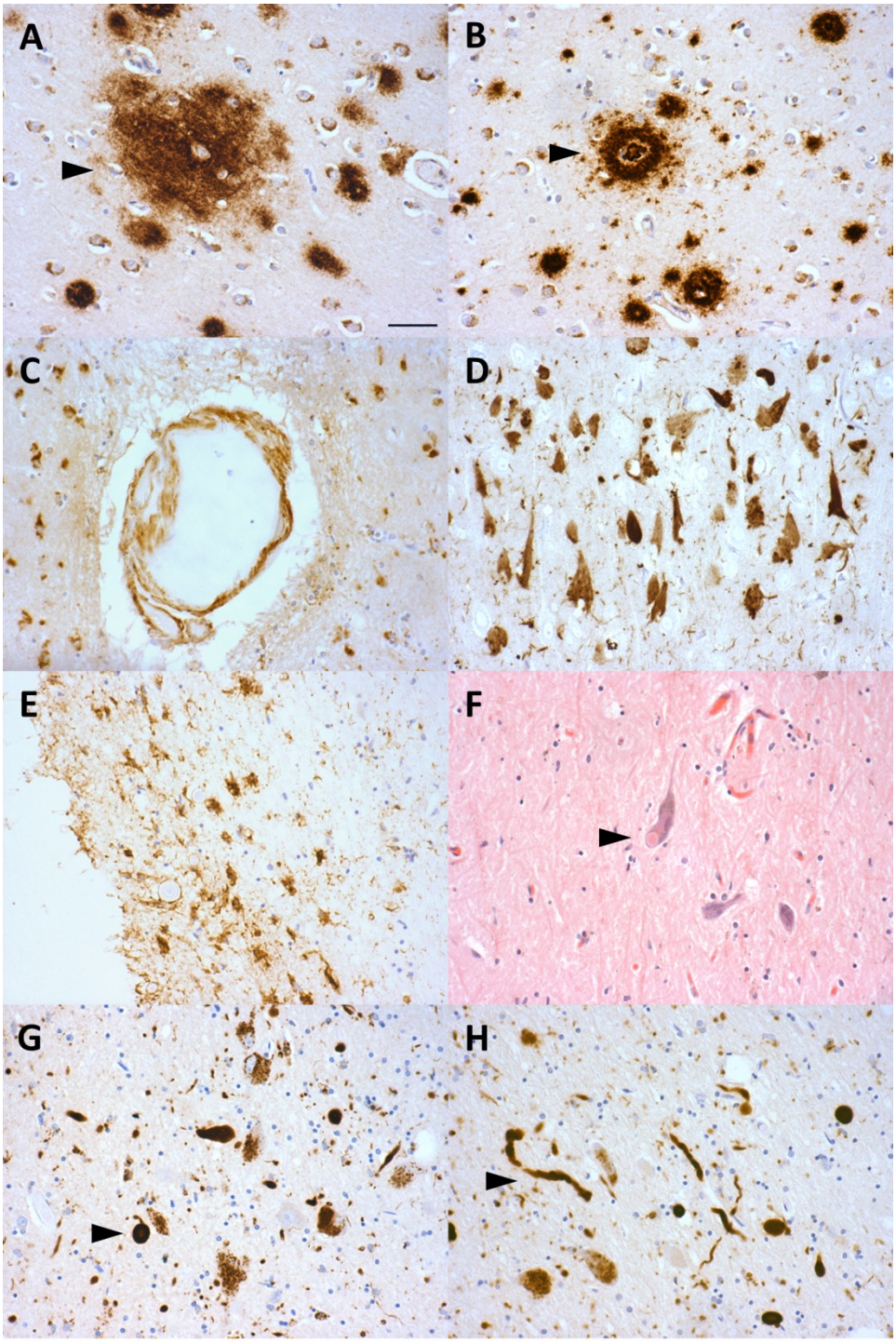


Figure 1.6: Pathologies in the PD brain. A) Diffuse Aβ plaque. B) Cored Aβ plaque. C) Cerebral Amyloid Angiopathy in a small arteriole. D) Tau neurofibrillary tangle. E) Subpial aging-related tau astrogliopathy. F) Small vessel disease with perivascular oedema and corpora amylacea. G) Subcortical α-synuclein Lewy Bodies and H) Lewy Neurites. Images taken under a 20x objective lens. Scale bar in A) = 100μm

1.4.2. Genetics of PD

In 1997, the SNCA gene was the first genetic association with autosomal dominant PD that was discovered. In the current PARK genes schema (find a complete list of the features of each of the 23 PARK genes in Table 1.1), SNCA is responsible for two of the PARK associations whereby missense mutations (A53T (Polymeropoulos *et al.*, 1997), A30P (Krüger *et al.*, 1998), E46K (Zarranz *et al.*, 2004) and H50Q (Appel-Cresswell *et al.*, 2013); PARK1) or changes in gene quantity production (duplications or triplications; PARK4) underlie autosomal dominant PD. SPR (sepiapterin reductase), UCHL1 (ubiquitin c-terminal hydroxylase 1), LRRK2 (leucine-rich repeat kinase 2), HTRA2 (HTRA serine peptidase 2), VPS35 (vacuolar protein sorting 35), EIF4G1 (Eukaryotic translation initiation factor 4 gamma 1), DNAJC13 (DNAJ/HSP40 homolog subfamily C member 13) and CHCHD2 (coiled-coil helix coiled-coil helix domain 2) – a novel locus, PARK22 – are also associated with autosomal dominant PD; whereas Parkin, PINK1 (PTEN-induced putative kinase 1), ATP13A (Lysosomal type 5 ATPase), PLA2 (phospholipase A2), FBX07 (F-box only protein 7), DNAJC6 (DNAJ/HSP40 homolog subfamily C member 6), SYNJ1 (synaptojanin 1) and VPS13C (vacuolar protein sorting 13C) – a novel locus, PARK23 – all cause autosomal recessive PD.

10% of PD cases have a known familial component (Klein and Westenberger, 2012), where monogenic causes of PD account for 30% of familial PD and 3-5% of sporadic cases. Ethnicity is an important determinant of genetic risk of PD. LRRK2 mutations (G2019S in particular), the most common cause of monogenic PD, show strong prevalence in Ashkenazi Jewish (Ozelius *et al.*, 2006) and Arab patients (Lesage *et al.*, 2006). Genetic PD cases show a large degree of clinico-pathological heterogeneity with differences in the typical ages of onset, clinical symptoms, tendency to dementia, presence of Lewy pathology and distribution of neuronal loss.

There are also a number of genetic risk factors that are not associated with monogenic PD such as GBA (lysosomal enzyme β-glucocerebrosidase) which is related to the lysosomal storage disorder, Gaucher's disease (Neumann *et al.*, 2009). GBA mutation is the most common genetic risk factor for PD (Lesage *et al.*, 2011), found in 8-14% of all PD cases (Klein, Schneider and Lang, 2009). GBA has also been linked with DLB and was shown to carry a significant association with the presence of cortical Lewy Bodies, even after correction for sex, age of death and APOE4 status (Clark *et*

al., 2009). Further, MAPT (microtubule associated protein tau), which is normally associated with AD and atypical Parkinsonian syndromes such as PSP, CBD and Parkinson's dementia complex of Guam, has also shown relationships to particular subtypes of PD. The MAPT gene has two haplotypes – H1 and H2 – that show varied associations with different neurodegenerative diseases. In contrast to H2, the H1 haplotype is evolutionarily dynamic and contains a number of single nucleotide polymorphisms that are highly correlated with one another (Pittman *et al.*, 2005). H1 haplotype has been associated with tauopathy in a number of studies, but also synucleinopathies such as PD (Refenes *et al.*, 2009; Pascale *et al.*, 2016) – specifically NTD subtype where the allele frequency of H1 was found to be significantly increased compared to TD by a number of studies (Di Battista *et al.*, 2014; Pascale *et al.*, 2016). Importantly, there were no significant differences found between TD and control groups, perhaps highlighting differential genetic modulation of the PD phenotype.

PARK	Gene locus	Gene	Protein	Inheritance	LB presence	Neuropathology	Clinical phenotype	Dementia	Mechanism	References
PARK1	4q21-22	SNCA	α-synuclein	Autosomal dominant	Yes	Neuronal loss in SN and LC; gliosis	Early onset (A53T, G51D) or late onset (A30P, E46K, H50Q), aggressive	Yes	Gain of function: protein self-aggregation	(Polymeropoulos *et al.*, 1997; Krüger *et al.*, 1998; Zarranz *et al.*, 2004; Appel-Cresswell *et al.*, 2013; Lesage *et al.*, 2013; Proukakis *et al.*, 2013)
PARK2	6q25.2-27	PARK2	Parkin	Autosomal recessive	Yes (not all cases)	Neuronal loss in SN and LC	Early onset parkinsonism	No	Loss of function: reduced ubiquitinylation	(Matsumine *et al.*, 1997; Hattori *et al.*, 1998; Kitada *et al.*, 1998; Lücking *et al.*, 1998)
PARK3	2p13	SPR	Sepiapterin reductase	Autosomal dominant	Yes	Neuronal loss in SN and LC; gliosis, tau NFTs and senile plaques	Classical parkinsonism	Yes	Unknown	(Gasser *et al.*, 1998; Cardona and Perez-Tur, 2017)
PARK4	4q21	SNCA(x3)	α-synuclein	Autosomal dominant	Yes	Typical LB features	Early onset parkinsonism	Yes	Gain of function: protein self-aggregation	(Singleton *et al.*, 2003)
PARK5	4p13	UCHL1	Ubiquitin c-terminal hydrolase	Autosomal dominant	Yes	Typical LB features	Classical parkinsonism	Unknown	Aberrant proteolysis, protein aggregation	(Leroy *et al.*, 1998)
PARK6	1p35-36	PINK1	PTEN-induced putative kinase 1	Autosomal recessive	Yes	SN neuronal loss, LB and LN in SN, nbM, brainstem reticular formation	Early onset parkinsonism	No	Loss of function: reduced mitochondrial activity	(Valente *et al.*, 2004; Samaranch *et al.*, 2010)
PARK7	1p36.23	PARK7	DJ-1	Autosomal recessive	Yes	DJ-1-positive LBs in SN	Early onset parkinsonism	Rarely	Loss of function: oxidative stress	(Bonifati *et al.*, 2003; Bandopadhyay *et al.*, 2004; Taipa *et al.*, 2016)
PARK8	12q12	LRRK2	Leucine-rich repeat kinase 2	Autosomal dominant	50% of cases	Severe SN degeneration, tau, LBs, TDP-43	Classical parkinsonism	Variable	Gain of function: reduced autophagy; increased kinase activity	(Funayama *et al.*, 2002; Paisán-Ruíz *et al.*, 2004; Zimprich *et al.*, 2004)
PARK9	1p36	ATP13A2	Lysosomal type 5 ATPase	Autosomal recessive	Yes	Neuronal loss, LBs Aβ, tau NFTs	Kufor-Rakeb syndrome; atypical parkinsonism with dementia, spasticity, supranuclear gaze palsy	Yes	Lysosomal dysfunction	(Ramirez *et al.*, 2006)
PARK10	1p32	Unknown	Unknown	Risk locus	Yes	Typical LB features	Classical parkinsonism	Yes	N/A	(Hicks *et al.*, 2002; Beecham *et al.*, 2015)
PARK11	2q36-37	Unknown	Unknown	Autosomal dominant	Undefined	Undefined	Late onset parkinsonism	Unknown	Unknown	(Pankratz, William C Nichols, *et al.*, 2003)
PARK12	Xq21-25	Unknown	Unknown	X-linked	Undefined	Undefined	Classical parkinsonism	Unknown	Unknown	(Pankratz, William C. Nichols, *et al.*, 2003)
PARK13	2p12	HTRA2	HTRA serine peptidase 2	Autosomal dominant	Yes (LBs are positive for Omi/HTRA2)	LB pathology	Classical parkinsonism	Undefined	Loss of function; mitochondrial dysfunction	(Strauss *et al.*, 2005)
PARK14	22q13.1	PLA2G6	Phospholipase A2	Autosomal recessive	Yes	LB pathology	Early-onset dystonia-parkinsonism; rapid cognitive decline	Yes	Loss of function: impaired autophagy,	(Paisan-Ruiz *et al.*, 2009; Zhou *et al.*, 2016)
PARK15	22q12-13	FBX07	F-box only protein 7	Autosomal recessive	Yes (LBs are positive for FBX07)	Typical LB features	Early-onset Parkinsonian-pyramidal syndrome	Yes	Loss of function: mitochondrial dysfunction, proteosome dysfunction	(Di Fonzo *et al.*, 2009; Paisán-Ruiz *et al.*, 2010; T. Zhao *et al.*, 2013; Joseph, Schulz and Stegmüller, 2018)
PARK16	1q32	Unknown	Unknown	Risk locus	Unknown	Unknown	Classical parkinsonism	Unknown	Unknown	(Satake *et al.*, 2009)

PARK17	16q11.2	VPS35	Vacuolar protein sorting 35	Autosomal dominant	Undefined	Undefined	Late onset tremor-dominant parkinsonism	No	Reduced lysosomal trafficking	(Vilariño-Güell *et al.*, 2011; Zimprich *et al.*, 2011)
PARK18	3q27.1	EIF4G1	Eukaryotic translation initiation factor 4 gamma 1	Autosomal dominant	Yes	LBs in SN & LC	Classical parkinsonism	No	Impaired mRNA translation initiation	(Chartier-Harlin *et al.*, 2011)
PARK19	1p31.3	DNAJC6	DNAJ/HSP40 homolog subfamily C member 6	Autosomal recessive	Undefined	Undefined	Juveline atypical parkinsonism	In one family	Impaired clarthin-mediated endocytosis	(Edvardson *et al.*, 2012; Olgiati *et al.*, 2016)
PARK20	21q22.11	SYNJ1	Synaptojanin 1	Autosomal recessive	Undefined	Undefined	Juveline atypical parkinsonism, epilepsy, developmental delay	Yes	Impaired clarthin-mediated endocytosis	(Quadri *et al.*, 2013)
PARK21	3q22.1	DNAJC13	DNAJ/HSP40 homolog subfamily C member 13	Autosomal dominant	Yes	LBs in SN & LC, tauopathy	Classical/Late onset parkinsonism	Yes	Impaired vesicular transport, endosomal recycling, endolysosomal degradative pathway	(Vilariño-Güell *et al.*, 2014)
PARK22	7p11.2	CHCHD2	Coiled-coil helix coiled-coil helix domain 2	Autosomal dominant	Yes	Typical LB features	Classical parkinsonism	Yes	Loss of function: mitochondrial dysfunction, cytochrome C disruption	(Ogaki *et al.*, 2015; Meng *et al.*, 2017)
PARK23	15q22.2	VPS13C	Vacuolar protein sorting 13 homolog C	Autosomal recessive	Yes	undefined	Young-adult-onset parkinsonism, dysautonomia	Yes	Loss of function: mitochondrial dysfunction and increased mitophagy	(Lesage *et al.*, 2016; Poewe *et al.*, 2017)

Table 1.1: Genetic associations with Parkinson's disease. LB = Lewy body, SN = substantia nigra, LC = locus coeruleus, NFT = neurofibrillary tangles, nbM = nucleus basalis of Meynert, TDP43 = tar-DNA binding protein 43. This table has been adapted and updated from the PhD Thesis of AKL Liu, (Liu, 2016)

1.4.3. Environmental factors

In 1983, a group of young drug addicts attempted to synthesise meperidine (synthetic heroin), which they injected intravenously. These individuals then developed incidental parkinsonism, which was found to be caused by contamination of the meperidine with the neurotoxicant 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) which is a by-product of meperidine synthesis (Langston *et al.*, 1983). This has provoked the use of MPTP in experimental models of PD (Bezard *et al.*, 1997; Przedborski *et al.*, 2001; Petryszyn *et al.*, 2016), but has also stimulated the investigation of environmental risk factors of PD. Herbicides and pesticides such as paraquat and rotenone share a number of similarities of chemical structures with MPTP and these have all been used in animal models of PD. This hints at a significant risk of PD with rural living, exposure to herbicides, pesticides and the consumption of water from rural wells (de Lau and Breteler, 2006). In a meta-analysis, the greatest risk of PD was carried by exposure to pesticides and herbicides (OR = 1.78; 95% CI = 1.50-2.10), followed by rural living (OR = 1.43; 95% CI = 1.04-1.40), farming and drinking well water (OR = 1.21; 95% CI = 1.04-1.40) (Noyce *et al.*, 2012).

Accumulation of heavy metals in the brain such as iron in the SN, provokes auto-oxidation of dopamine, the subsequent production of hydrogen peroxide and the resultant depletion of glutathione stores (an antioxidant). This results in increased oxidative stress (Zecca *et al.*, 2004) and neuronal loss. Dietary consumption of iron carries a trend increase in risk of PD (OR = 1.7; 95% CI = 1.0-2.7) (Powers *et al.*, 2003). Copper is another significant metal to consider, given that parkinsonism is a distinct neurological presentation of the disorder of copper, Wilson's disease (Bandmann, Weiss and Kaler, 2015). Cases of parkinsonism in Wilson's disease tend to have dystonic tremor but can have typical Parkinsonian rest tremor. The α-synuclein protein contains two copper binding sites and formation of the α-synuclein-copper complex enhances the aggregation of α-synuclein (Binolfi *et al.*, 2010). However, the levels of copper in the SN and serum of PD patients is actually reduced compared to controls (Dexter *et al.*, 1991; H. W. Zhao *et al.*, 2013). The role of calcium in PD is still controversial.

1.4.4. Diet and lifestyle factors

Many dietary factors have been associated with risk of PD and protection against PD. High levels of dietary fat has been linked with increased PD risk due to oxidative stress

caused by lipid peroxidation (Farooqui and Horrocks, 1998). However, this association has not been realised in longitudinal, population-based studies. These studies, including the impressive Honolulu-Asia Aging Study (Abbott *et al.*, 2003), attempted to correlate several lifestyle factors including characteristics of dietary fat such as total caloric intake, saturated, monounsaturated and polyunsaturated fat intake with the incidence of PD. The Honolulu study found that poly-unsaturates appeared to be protective, while the other lipid factors showed no association at all. This has been replicated by other studies in diverse populations (Chen, Zhang, Miguel A Hernán, *et al.*, 2003; de Lau *et al.*, 2005). Vitamins C and E, which are dietary antioxidants, have also been associated with reduced oxidative stress in PD. But yet again, these factors showed no relationship with longitudinal risk of PD (Abbott *et al.*, 2003; de Lau and Breteler, 2006). Diet is, however, incredibly difficult to study objectively and quantitatively, and relies on self-reported diet questionnaires and diaries.

The study of lifestyle modification of PD risk has centred on the consumption of cigarettes, alcohol, caffeine and exercise and the inverse relationship between these and PD risk. In a large meta-analysis on the subject, it was found that the greatest reduction of risk was carried by being a current smoker (vs non-smoker; relative risk, RR = 0.44, 95% CI = 0.39-0.50), followed by caffeine consumption (OR = 0.67, 95% CI = 0.58-0.76) and alcohol consumption (OR = 0.90, 95% CI = 0.84-0.96) (Noyce *et al.*, 2012). Smoking may be neuroprotective against PD due to nicotine's action on nicotinic acetylcholine receptors, α4β2 receptors in particular, prevalent on striatal dopaminergic terminals, which makes dopamine more readily available in the striatum (Chang *et al.*, 2014). Further, nicotine has been purported to reduce the activity of monoamine oxidase B (MAO-B) enzyme which breaks down dopamine in the synaptic cleft (Fowler *et al.*, 1996). Chronic nicotine intake has been shown to inhibit nigral cell loss in a rodent PD model (Prasad *et al.*, 1994), and also blocks α-synuclein fibril formation (Hong, Fink and Uversky, 2009), which may prevent LB production and subsequent neuronal loss in PD. Studies have consistently shown an inverse, and dose-dependent (Ross *et al.*, 2000), relationship between caffeine intake and risk of PD (de Lau and Breteler, 2006). However, this association differs between sexes (Ascherio *et al.*, 2001), possibly due to the administration of oestrogen-replacement therapy in females (Ascherio *et al.*, 2004). Caffeine acts on adenosine A2 (A2A) receptors in an antagonistic fashion which has been shown to protect striatal

dopaminergic terminals from degeneration and to improve motor function in rodent and primate models of PD (Richardson, Kase and Jenner, 1997). Further, A2A receptor agonists have been shown to increase acetylcholine production from striatal preparations (Brown *et al.*, 1990; Kirk and Richardson, 1994; Kurokawa *et al.*, 1994), thus blocking the A2A receptor should correct dopamine: acetylcholine imbalance in the striatum in PD.

The benefits of exercise for a wide variety of systemic medical conditions is well-documented (Pate *et al.*, 1995). Vigorous exercise has also been shown to have benefits in preventing AD (Hamer and Chida, 2009) and in reducing the loss of striatal dopaminergic innervation in rodent toxin models of PD (Ahlskog, 2011). In humans, exercise has been shown to improve quality of life and physical functionality for patients with PD (Goodwin *et al.*, 2008). However, the benefit of the activity is dependent on the exact nature of the exercise. Salgado and colleagues reviewed the literature to determine an exercise regime for PD patients and concluded the following was beneficial for PD: 1) cardiovascular exercise such as treadmill running or cycling has been shown to reduce bradykinesia and gait disturbance, 2) moderate volume resistance training for muscle strength, 3) balance training with Tai Chi for maintenance of postural stability, 4) rehabilitation exercises with a physiotherapist for patients with advanced PD (Salgado *et al.*, 2013). While there are obvious benefits of exercise for the motor symptoms of PD, there are less obvious benefits for some of the NMS, especially cognitive impairment. A systematic review from 2018 reviewed clinical trials of the effect of exercise on cognitive impairment in PD (Da Silva *et al.*, 2018). They found that exercise programs promote global improvement of cognitive function, especially in sustained attention, processing speed and mental flexibility in PD patients. However, the strongest effect seen was with cardiovascular exercise such as treadmill running, when performed for an hour, 3 times a week for a long period of time (Da Silva *et al.*, 2018). This shows that the best exercises for promoting gait integrity and reducing bradykinesia are also best for cognitive impairment. Cholinergic deficits are present in both cognitive impairment and gait disturbance in PD, and there is evidence that aerobic activity boosts cholinergic and dopaminergic neurotransmission in rodents (Fordyce and Farrar, 1991; Poulton and Muir, 2005). Beneficial effects of exercise may also arise from improved cerebral blood flow.

1.4.5. Miscellaneous risk factors for PD: infectious, inflammatory, traumatic

Post-encephalitic parkinsonism (also known as Economo's disease or encephalitis lethargica) established a link between infection and PD after the outbreak of cases during the 'Spanish Flu' pandemic in 1918 (Ravenholt and Foege, 1982). However, as of yet, there has been no conclusive epidemiological evidence regarding a link between a specific infectious aetiological agent and risk of PD (Kasten, Chade and Tanner, 2007). It is not clear whether the risk of PD is carried by a particular agent itself or more generally by the resulting inflammation that is associated with neurodegeneration in PD. In post-mortem PD brains (particularly the nigrostriatal pathway), blood and CSF, the concentrations of pro-inflammatory cytokines such as tumour necrosis factor (TNFα), interleukin (IL)-1β and IL-6 are increased compared to controls (Nagatsu and Sawada, 2005; Sawada, Imamura and Nagatsu, 2006). Furthermore, these studies also found reduced neurotrophic factors such as brain-derived neurotrophic factor (BDNF) in the nigrostriatal system and CSF. Taken together, the authors hypothesise that increased inflammatory cytokine levels and reduced neurotrophic factor levels are caused by the presence of activated microglial cells which promote apoptosis and phagocytosis of nigrostriatal dopaminergic neurons (Nagatsu and Sawada, 2005; Sawada, Imamura and Nagatsu, 2006). The link between inflammation and PD is boosted by the fact that non-steroidal anti-inflammatory drugs (NSAIDs) are protective against PD risk in longitudinal studies (Abbott *et al.*, 2003; Chen, Zhang, Miguel A. Hernán, *et al.*, 2003). However, a more recent meta-analysis has not found a similar association with aspirin or paracetamol (Noyce *et al.*, 2012).

Head trauma has been linked with risk of developing PD in case-control studies, with a meta-analysis of these studies finding a 1.6-fold increase in PD risk with head trauma regardless of whether there was associated loss of consciousness (Noyce *et al.*, 2012). Repetitive mild head injury increases the risk of developing a recently-defined tauopathy called chronic traumatic encephalopathy (CTE) in which hyperphosphorylated tau is found deposited in neurons and astroglia around blood vessels at the depths of cortical sulci (McKee *et al.*, 2013, 2015, 2016). We recently carried out a clinico-pathological study of an archival series of retired boxers who had presented with various neurological deficits (Goldfinger *et al.*, 2018). Half of these boxers (7 of 14) were found to contain pathognomonic CTE-tau lesions at the sulcal

depths, some of whom showed parkinsonism and 'subcortical' dementia symptoms, but without LB pathology under the microscope. Other boxers had developed atypical Parkinsonian syndromes such as PSP with pathognomonic 4R tau lesions in tufted astrocytes. This series illustrates the general risk of parkinsonism associated with head trauma, despite the heterogeneous nature of the pathologies underlying the clinical syndrome.

1.5. The physiology of the basal ganglia

The basal ganglia comprise a group of functionally and neuroanatomically interconnected nuclei found in the core of the brain and rostral brainstem. These structures are the striatum, SN pars compacta (SNc) and pars reticula (SNr), subthalamic nucleus (STN), globus pallidus (GP) external (GPe) and internal segments (GPi). Here, the physiology of the basal ganglia at gross pathway and cellular levels are reviewed.

1.5.1. The direct and indirect pathways

The central hypothesis of basal ganglia function concerns two pathways that regulate the thalamo-cortical axis of the central motor circuitry. The direct pathway has been said to upregulate movement via activation (reduced inhibition) of thalamo-cortical glutamatergic projections, whereas indirect pathway activity increases the inhibition of this axis (Figure 1.7). This involves the recruitment of various basal ganglia centres including the GPe and GPi, the STN and SN. The pathways utilise GABAergic inhibitory and glutamatergic stimulatory signals to ultimately influence the activity of a SNr/GPi recurrent inhibitory loop. This activity of this complex, determined by afferent connections, either inhibits or does not inhibit the ventral anterior and ventral lateral thalamic nuclei. Thus, if the SNr/GPi complex is active, this inhibits the ventral thalamus, and thereby reduces thalamo-cortical signals.

Dopamine from the SN modulates these pathways via dopamine receptors (Table 1.2), particularly D1 and D2 receptors. D1 receptors localise to striato-nigral, substance-P-positive medium spiny neurons (MSNs) which form the direct pathway striatal output to the SNr, whereas D2 receptors localise to the striato-pallidal, enkephalin-positive MSNs of the indirect pathway (Calabresi *et al.*, 2014). This means that the action of dopamine in the striatum is to increase direct pathway activity and reduce indirect pathway activity, to promote movement. D1 and D2-positive MSNs have different

levels of baseline excitability whereby D2 MSNs show higher frequency of firing and therefore have a more excitable predisposition to D1 MSNs (Cepeda *et al.*, 2008). This is largely controlled by baseline membrane potential and input resistance but means that the pro-kinetic properties of dopamine are mainly a consequence of indirect pathway inhibition rather than direct pathway stimulation. This may explain why normal subjects are not hyperkinetic.

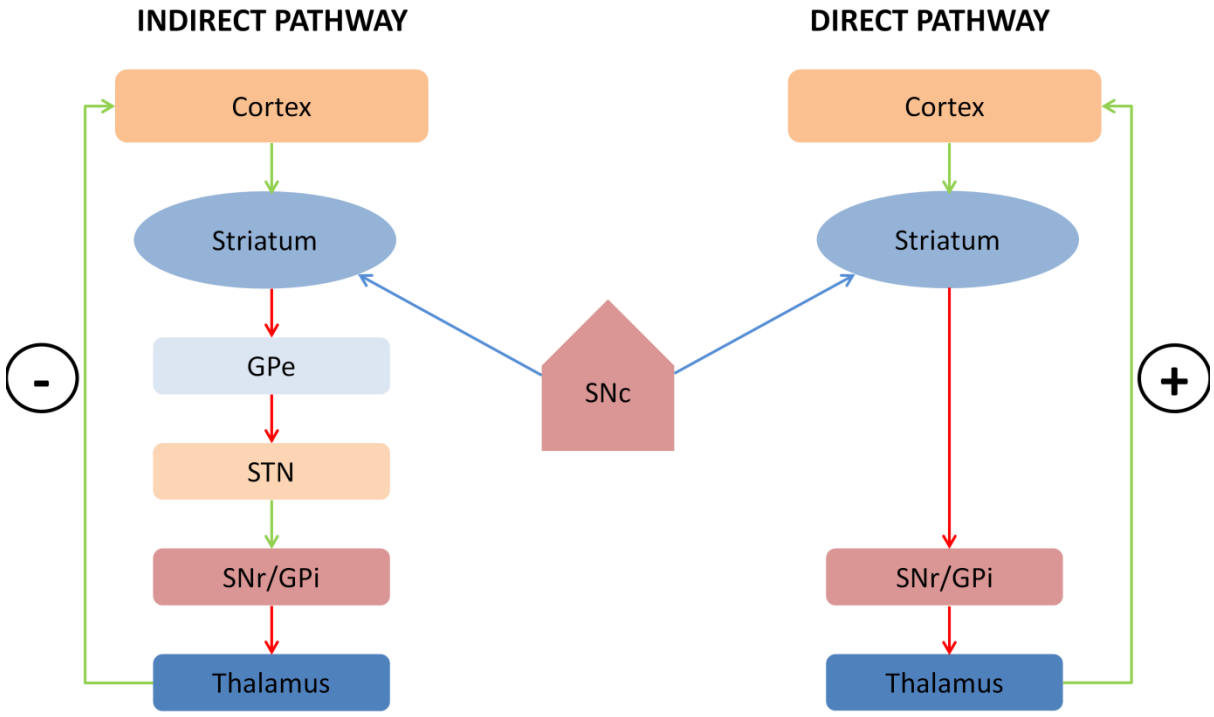


Figure 1.7: The direct and indirect pathways of the basal ganglia. GPe = Globus pallidus externus, GPi = Globus pallidus internus, SNc = Substantia nigra pars compacta, SNr = Substantia nigra pars reticulata. Blue arrows – dopaminergic modulatory efferents. Red arrows – GABAergic inhibitory efferents. Green arrows – glutamatergic stimulatory efferents.

This model of basal ganglia function is not without criticism, however. Frequently this model is criticized for being an oversimplification of basal ganglia function. For example, a study from Cui and colleagues, who undertook to study these pathways *in vivo* in rodents found that both showed transient increases in activity during voluntary movement, where there was no activity found in these pathways when the rodents were not moving (Cui *et al.*, 2013). The model itself is based on the assumptions that the pathways are both functionally opposing and anatomically segregated. However, recent data has showed that 60% of striato-nigral MSNs also send projections to the

GPe, which have been named *bridging collaterals* due to their ability to bridge the direct and indirect pathways (Cazorla *et al.*, 2014). This is corroborated by data from earlier studies of the distribution of substance-P and enkephalin in the basal ganglia which found that there is substance-P staining present in the GPe, though less than that in the GPi (Mai *et al.*, 1986). It has also been shown that a large number of striatal MSNs show plasticity and co-expression of both D1 and D2 receptors, further reducing the anatomical segregation of the two pathways (Nadjar, 2006). Furthermore, the postulation of a *hyperdirect* pathway from the cortex to subthalamic nucleus, bypassing the striatum and pallidum altogether (Nambu, Tokuno and Takada, 2002), has added yet more complexity to the modelling of basal ganglia function.

1.5.2. The role of striatal interneurons

To reduce the role of the striatum to the release of dopamine by nigrostriatal neuron terminals and the subsequent modulation of the activity of the direct and indirect pathways would be to ignore the complex and important roles of striatal interneurons. These include the striatal GABAergic, and striatal cholinergic interneurons (ChIs).

1.5.2.1. Striatal GABAergic interneurons

There are multiple subtypes of striatal GABAergic interneurons (GIs), including parvalbumin-positive, neuropeptide-Y-positive, calretinin-positive and, recently defined, TH-positive GIs (Huot, Lévesque and Parent, 2007; Tepper *et al.*, 2010, 2018a). These all have different morphologic, electrophysiological and connective properties as well as different relationships with other striatal neurons (Tepper *et al.*, 2018b). For example, the parvalbumin-positive GIs are fast-spiking, medium to large interneurons with wide dendritic fields and wider axonal fields with many branches that show 80% connectivity with MSNs. In contrast, the low-threshold-spiking neuropeptide-Y-positive GIs are medium sized interneurons with narrow, aspiny dendrites but long sparsely-branched axons that extend up to 1mm from the soma and connect much more with ChIs than MSNs. Neuropeptide-Y-positive GIs can also bear neurogliaform morphology with a very dense and highly compact dendritic field. This neuronal type connects heavily with striatal MSNs.

Each GI subpopulation also differs in terms of their roles in striatal physiology. For example, the low-threshold-spiking parvalbumin-positive GIs express α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl D-aspartate

(NMDA) glutamate receptors, whereas the action of glutamate on fast-spiking GIs is mediated solely by AMPA receptors, which explains their fast-spiking nature (Gittis *et al.*, 2010). Fast-spiking interneurons also seem to preferentially act on MSNs of the direct pathway over indirect pathway MSNs suggesting a role for GIs in the rapid-pathway specific regulation of striatal output (Gittis *et al.*, 2010). The main mechanism for this regulation is feed-forward inhibition in order to precisely time the action potentials of the MSNs (Tepper and Bolam, 2004).

1.5.2.2. Striatal cholinergic interneurons

Striatal ChIs are fascinating neurons with a large cell soma, up to 50μm in diameter (Sullivan, Chen and Morikawa, 2008), with an aspiny multipolar morphology. The fact that these neurons are aspiny means that they do not form structurally defined synapses, allowing them to release acetylcholine volumetrically into the striatal milieu (Pisani *et al.*, 2007). The striatal ChIs exhibit autonomous pacemaker activity (Bennett and Wilson, 1999) which occurs at a rate of approximately 5Hz (Zhou, Wilson and Dani, 2002). Intrinsic to this activity is the expression of muscarinic autoreceptors of the M4 subtype which rapidly inhibit ChI firing by increasing K^+ efflux and reducing Ca^{2+} influx in response to increased striatal acetylcholine concentration (Zhou, Wilson and Dani, 2002; Pisani *et al.*, 2007). The striatal ChIs are mainly regulated by dopamine at D2 receptors which reduce autonomous pacemaker activity by reducing Na^+ and Ca^{2+} currents at afferent synaptic inputs (Yan, Song and Surmeier, 1997; Pisani *et al.*, 2000; Maurice *et al.*, 2004). ChIs also express ionotropic (AMDA and NMDA) and metabotropic glutamate receptors, which allow cortico-striatal and thalamo-striatal glutamatergic neurons to regulate ChI activity (Pisani *et al.*, 2007).

Striatal ChIs are critical regulators of the striatal outputs to the basal ganglia pathways. A large variety of striatal neuronal populations express nicotinic and muscarinic acetylcholine receptors. For example, MSNs predominantly express M1 and M4 muscarinic receptors (Howe and Surmeier, 1995; Galarraga *et al.*, 1999). M1 agonists have been shown to depolarize MSNs by restricting K^+ efflux, and positively modulate NMDA-receptor-mediated currents across cortico-striatal synapses, inducing long-term potentiation (LTP) (Calabresi *et al.*, 2000). Similarly, a reduction in acetylcholine production induces long-term depression (LTD) across these synapses. M4 receptors, expressed on MSNs, GIs and nigrostriatal dopaminergic terminals, inhibits membrane depolarisation and action potentials leading to reduced GABA and dopamine release

(Koós and Tepper, 2002; Rice and Cragg, 2004). Conversely, nicotinic receptors have been shown to stimulate the release of GABA and dopamine.

While the effects of ChIs on cortico-striatal synaptic plasticity point to a clear role in motor learning and associative memory processes, there are other interesting physiological properties of these neurons that show further potential roles of ChIs. Unlike other striatal neuron populations which are inactive at rest, the striatal ChIs are tonically active (hence they were previously referred to as tonically active neurons(TANs; Aosaki *et al.*, 1994)). TAN activity is modulated by incentivised stimuli, inducing a pause response, followed by an increased rate of firing (Kimura, 1990). In this regard, ChIs may be involved in linking goal-directed stimuli to motor program selection (Kawagoe, Takikawa and Hikosaka, 1998).

1.5.3. The role of other neurotransmitters

The presence of serotonin, histamine, noradrenaline and adenosine receptors in the striatum shows that there are roles for these neurotransmitters in basal ganglia physiology. Serotonin, for example, induces membrane depolarization in striatal ChIs via 5-HT2C, 5-HT6 and 5-HT7 receptors (Pisani *et al.*, 2007). Similarly, histamine (via H1 receptors) and noradrenaline (via β1 receptors) also induce membrane depolarization. Adenosine has a dual effect on striatal ChIs: 1) inhibitory due to A1 receptors 2) stimulatory due to A2 receptors.

Thus, the physiology of the striatum is highly complex and relies on several key players including interneurons, cortical signalling, dopaminergic activity from the SN, and differing downstream targets in the basal ganglia pathways. It is not surprising therefore, that pathology in these regions is able to produce a wide range of clinical syndromes including Huntington's disease, Tourette's syndrome, ballismus and hemiballismus, PSP, CBD, dystonia, dyskinesia, psychomotor disorders and, importantly, PD and the wide spectrum of motor phenomena therein.

	D1	D2	D3	D4	D5	References
Location	Dorsal striatum	Dorsal striatum and ventral striatum	Ventral striatum	Rare in striatum	Not in striatum	(Meador-Woodruff *et al.*, 1996)
Gene (locus)	DRD1 (5q35.2)	DRD2 (11q23.1)	DRD3 (3q13.31)	DRD4 (11p15.5)	DRD5 (4p16.1)	-
Function	Stimulatory ($G_{\alpha s}$, $G_{\alpha olf}$)	Inhibitory ($G_{\alpha i}$, $G_{\alpha o}$) Short form = pre-synaptic autoreceptor Long form = post-synaptic classical receptor	Inhibitory ($G_{\alpha i}$, $G_{\alpha o}$)	Inhibitory ($G_{\alpha i}$, $G_{\alpha o}$)	Stimulatory ($G_{\alpha s}$, $G_{\alpha q}$)	(Beaulieu and Gainetdinov, 2011)
Signalling mechanism	Stimulates AC, ↑cAMP, ↑Ca^{2+}, ↑PKA, ↑DARPP32	Inhibits AC, ↓cAMP	Inhibits AC, ↓cAMP	Inhibits AC, ↓cAMP	Stimulates AC, ↑cAMP, ↑Ca^{2+}, ↑PKA, ↑DARPP32	(Calabresi *et al.*, 2007; Beaulieu and Gainetdinov, 2011)
Role in normal striatal physiology	Promotes ACh release from striatal ChIs Expressed on MSNs of the direct (striatonigral) pathway Regulates cortico-striatal synaptic plasticity	Short form inhibits DA release Long form inhibits post-synaptic depolarization by reduced Ca^{2+} influx and increased K^+ efflux Inhibits ACh release from striatal ChIs Reduces corticostriatal glutamatergic influence on striatal ChIs Expressed on MSNs of the indirect (striatopallidal) pathway Regulates cortico-striatal synaptic plasticity	Only found in ventral striatum Autoreceptor that inhibits DA release	Unknown	None	(Aosaki, Kiuchi and Kawaguchi, 1998; Calabresi *et al.*, 2000; Surmeier *et al.*, 2007; Beaulieu and Gainetdinov, 2011)
Fate in PD	Increased signalling in LID primate models compared to non-LID MPTP primates Reduced D1-mRNA and D1 binding in MPTP compared to controls L-DOPA upregulates D1	Increased D2-mRNA and D2 binding in MPTP primates Comparable with controls in LID primate models	Decreased binding correlates with motor deficit and low mood in drug-naïve early-PD	Unknown	None	(Aubert *et al.*, 2005; Hisahara and Shimohama, 2011)

Table 1.2: Dopamine receptors in health and Parkinson's disease. $G_{\alpha s}$ = Stimulatory G protein, $G_{\alpha olf,}$ = olfactory G protein, $G_{\alpha i}$ = inhibitory G protein, $G_{\alpha o}$ = o alpha G protein subunit, $G_{\alpha q}$ = q alpha G protein subunit. AC = adenylyl cyclase. cAMP = cyclic adenosine monophosphate, PKA = protein kinase A, DARPP32 = dopamine and cAMP-related neuronal phosphoprotein-32, DA = dopamine. Ach = acetylcholine, ChI = striatal cholinergic interneuron, MSN = medium spiny neuron, LID = L-dopa induced dyskinesia, MPTP = 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine.

1.6. The pathophysiology of PD

PD involves the degeneration of several key brain systems. This section will review each of these systems and their contributions to different aspects of the Parkinsonian syndrome including the motor, autonomic and cognitive symptoms associated with PD. PD is unique among neurodegenerative diseases because of the sheer diversity of neurotransmitters that are implicated, primarily involving dopamine, noradrenaline and acetylcholine, but also serotonergic, glutamatergic and GABAergic systems. It is likely that varying severity of pathology in each of these systems underlies clinical heterogeneity in PD and articulating these changes may pave the way for targeted therapy for individual PD symptoms.

1.6.1. Pathophysiology of PD motor symptoms

The constellation of PD motor symptoms is traditionally ascribed to the loss of dopaminergic neurons in the SN and the resultant loss of dopamine innervation at the level of the dorsal striatum. This has formed the rationale for treatment of PD with dopamine replacement therapies such as L-dopa, dopamine receptor agonists, monoamine oxidase inhibitors and catechol-O-methyl transferase inhibitors. However, this unified model of pathophysiology does not explain the diversity in the clinical presentations of the disease (Jankovic *et al.*, 1990; Thenganatt and Jankovic, 2014), and how the disease varies over time in a single individual (Von Coelln *et al.*, 2015). The cardinal motor features of the disease (bradykinesia, rest tremor, rigidity and postural instability) are not always co-expressed by patients and almost certainly progress differently in different individuals. Therefore, to understand the clinical symptomatology of PD, we need to adapt the classical model of nigral degeneration to account for the variability of clinical symptoms. This will involve different neurotransmitter systems and thus different brain regions such as the striatum, motor cortices and cerebellum.

1.6.1.1. Bradykinesia

Bradykinesia refers to reduced speed of movement, though amplitude is usually affected too. However, speed appears to be disproportionately more affected than amplitude in the OFF state and is less normalized by L-dopa than amplitude which implies they may occur by separate mechanisms (Espay *et al.*, 2009; Magrinelli *et al.*, 2016). This symptom, of all cardinal PD features, fits best with the classical model of indirect pathway (Figure 1.8B) predominance over the direct pathway (Figure 1.8A)

after dopaminergic denervation (Figure 1.8C) (Obeso *et al.*, 2000). This model suggests that basal ganglia output failure leads to reduced reinforcement of cortical signals which would otherwise be involved in the preparation and execution of movement (Obeso *et al.*, 2000).

A second potential novel hypothesis of bradykinesia and akinesia involves the regulation of corticostriatal synaptic plasticity. Cortex-derived glutamatergic axons form synaptic connections with a wide range of striatal neuronal populations. The post-synaptic terminals at these synapses express dopamine receptors in a variety of combinations that allow dopamine to modulate synaptic plasticity (Calabresi *et al.*, 2007). When these neurons express D1 receptors, the effect of dopamine is to induce long-term potentiation (LTP), whereas co-expression of D1 and D2 receptors induces long-term depression (LTD; Calabresi *et al.*, 2007). LTP and LTD are the principal mechanisms involved in synaptic plasticity, thus if there is a reduction in dopaminergic tone in the striatum, plasticity across these synapses becomes disrupted which may affect the efficiency of signal transduction in the selection of motor programs. This is supported by evidence from Lewis and colleagues who found that increased overall activation of the basal ganglia circuitry was needed in AR PD cases compared to TD to perform the same motor task, reflecting underlying inefficiency of signal conduction in AR cases (Lewis *et al.*, 2011). The disruption of these synapses may be severe enough to manifest as synaptic loss in PD. Stephens and colleagues found that the dendritic arbor of medium spiny striatal projection neurons was not only shorter in PD patients, but also had a lower dendritic spine density in PD compared to controls (Stephens *et al.*, 2005). This lends support to a novel hypothesis that PD is characterised by a reduction in the capacity for signal transduction in the striatum and that slower transduction may underlie slowed movements. ChIs are also critical regulators of cortico-striatal plasticity and dopamine has been shown to indirectly influence this via regulation of ChIs (Calabresi *et al.*, 2000, 2007; Deffains and Bergman, 2015). Interestingly, the role of ChIs in goal-directed behaviour (see Chapter 1.5.2.2) lends support to this hypothesis when one considers *kinesia paradoxica* – the rare phenomenon that incentivised stimuli, such as reward or fear, are able to produce faster movements in patients who are normally bradykinetic. Increased speed of movements in these circumstances may be attributable to increased cholinergic transmission in the striatum.

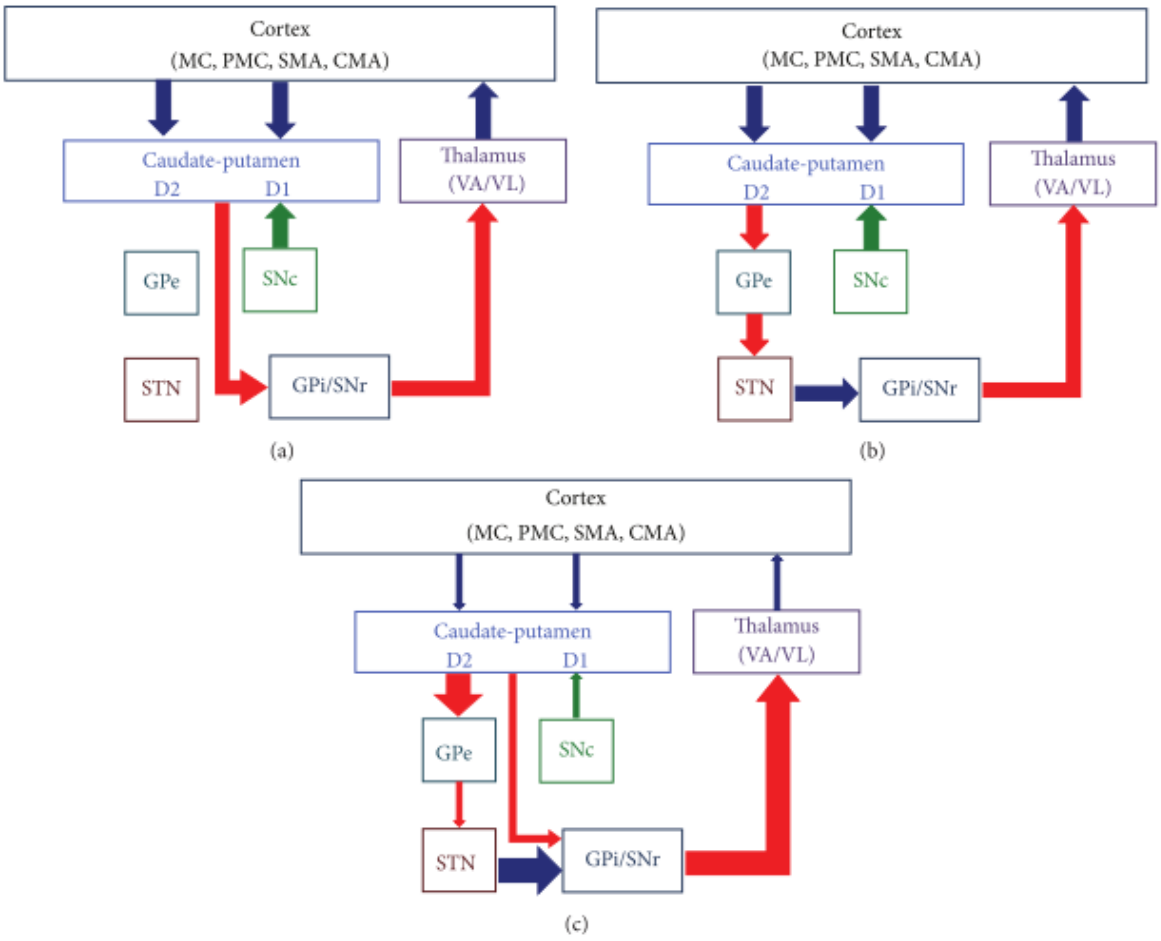


Figure 1.8: Classical concepts of the basal ganglia motor system in PD. A) The direct pathway. B) The indirect pathway. C) The basal ganglia system in PD with predominance of indirect pathway over direct pathway. Blue arrows = excitatory glutamatergic pathways, green arrows = modulatory dopaminergic pathways, red arrows = inhibitory GABAergic pathways. CMA = cingulate motor area, GPe = globus pallidus externus, GPi = globus pallidus internus, MC = motor cortex, PMC = premotor cortex, SMA = supplementary motor area, SNc = substantia nigra pars compacta, SNr = substantia nigra pars reticulata, STN = subthalamic nucleus, VA = ventral anterior nucleus of the thalamus, VL = ventral lateral nucleus of the thalamus. Figure taken from Magrinelli and colleagues, 2016 (Magrinelli et al., 2016).

1.6.1.2. Rest tremor – a tale of two circuits

The pathophysiology of tremor has up until now been largely unknown, but there is good evidence to suggest that it differs to the pathophysiology of bradykinesia (Helmich *et al.*, 2011). Tremor progresses at a different rate and with a different trajectory when compared to other PD symptoms (Louis *et al.*, 1999). Furthermore, tremor severity does not correlate with the severity of these other symptoms (Louis *et al.*, 2001). The current hypotheses of parkinsonian rest tremor centre on two distinct

neuroanatomical circuits that interact via relays in the thalamus (Helmich *et al.*, 2012). Helmich and colleagues studied the neurocircuitry of tremor using a joint magnetoencephalographic and electromyographic approach to assess cerebro-muscular coherence in tremor (Helmich *et al.*, 2011). The first of these circuits is a striato-pallidal axis which regulates activity in the ventral thalamus. The significance of this is that activity in this circuit drives tremor onset but shows no relationship with tremor amplitude (Figure 1.9C). Tremor amplitude is modulated by activity in the second, cerebello-thalamo-cortical circuit (Figure 1.9D). The interaction of these two circuits under pathological circumstances resembles that of a dimmer-switch, in which the tremor is switched on by one circuit, and the intensity of the tremor by the second. These circuits control tremor at rest, but the cerebello-thalamo-cortical circuit is also involved in action tremors such as those of essential tremor. The driving force behind activity in this circuit in essential tremor is dysfunction of cerebellar GABAergic neurons of the dentate nucleus, which fail to regulate the oscillations of the cerebello-thalamo-cortical circuit (Helmich *et al.*, 2013).

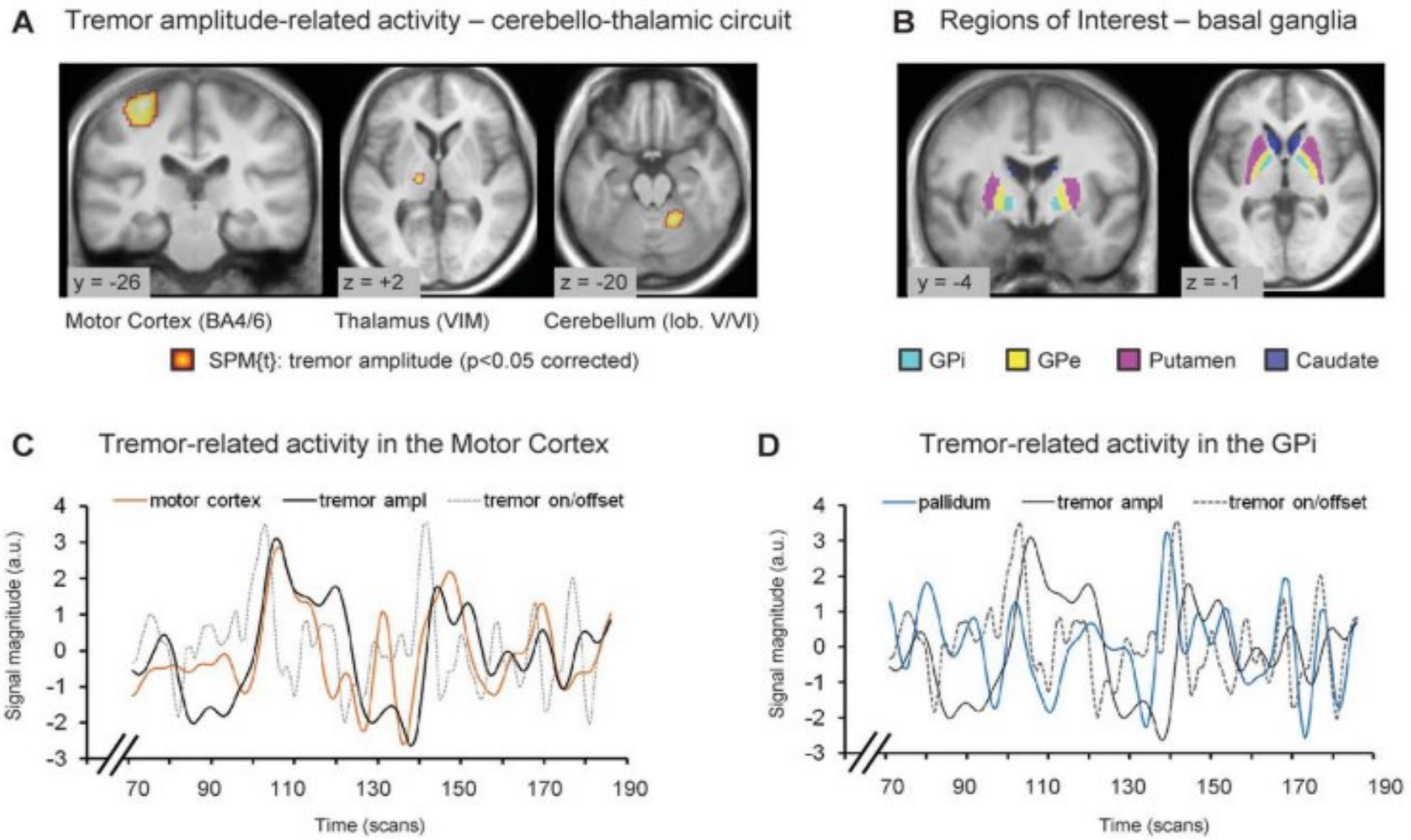


Figure 1.9: Tremor on/offset is related to activity in striatopallidal circuits whereas tremor amplitude is regulated by activity in the cerebello-thalamo-cortical circuit. Figure reproduced from (Helmich et al., 2011).

There are other proposed models for the onset of PD tremor such as the thalamic filter hypothesis (Pare *et al.*, 1990), which suggests that high frequency (12-15Hz) oscillations in the basal ganglia are transformed into lower frequency (4-6Hz) oscillations in thalamic relay neurons in the ventral lateral thalamic nucleus. While supported by the finding that TD parkinsonian vervet monkeys exhibit 10Hz pallidal oscillations when NTD parkinsonian macaque monkeys do not (Rivlin-Etzion *et al.*, 2010), this hypothesis is weakened by data that shows that high frequency stimulation of the pallidum is unable to generate tremor activity (Rivlin-Etzion *et al.*, 2008). Furthermore, reducing high frequency oscillatory activity in the STN in PD patients with dopaminergic therapy only correlated with reduction in akinesia and rigidity, suggesting that basal ganglia oscillations may relate more to other PD symptoms (Kühn *et al.*, 2006). Another proposed model for PD tremor is the loss-of-segregation hypothesis which is based on the finding in normal primates that pallidal neurons show unsynchronised activity to one another (Bar-Gad *et al.*, 2003), whereas in parkinsonian primates, remotely situated pallidal neurons show markedly increased correlation in activity (Bergman *et al.*, 1998). This may cause excessive synchronization in the basal ganglia, potentially because inhibitory collaterals in the pallidum are affected by dopaminergic denervation (Bevan *et al.*, 1998), resulting in tremor (Deuschl *et al.*, 2000). This places the basal ganglia as the main tremor pacemaker in PD. However, as with the thalamic filter hypothesis, there is a lack of coherence between basal ganglia oscillations and tremor oscillatory activity *in vivo* in PD. Thus, the best model for explaining tremor is the dimmer-switch hypothesis as mentioned previously.

What are least well known about the pathophysiology of rest tremor are the underpinnings of aberrant tremor circuit activity at a cellular level.As discussed in Chapter 1.1.2. Jean-Martin Charcot sought to treat Parkinsonian rest tremor with anticholinergic drugs such as hyoscyamine. The fact that the pathognomonic characteristic of PD tremor is that it occurs at rest means that it would be logical to analyse the cellular activity in the basal ganglia at rest. As alluded to in Section 1.5.2.2. the only cell type in the striatum that is active at rest is the striatal ChI (Aosaki *et al.*, 1994). It is therefore plausible that dysregulation of these neurons, as exhibited by loss of dopaminergic innervation in PD, may underlie aberrant activation of striato-pallidal circuitry to trigger PD rest tremor in the dimmer-switch model of tremor pathophysiology.

1.6.1.3. Rigidity

Rigidity in PD is perhaps the symptom where the pathophysiology is the most unclear (Magrinelli *et al.*, 2016). It has been hypothesised that the pathogenesis of rigidity in PD is related to changes in the passive mechanical properties of the musculoskeletal system, the enhancement of stretch reflexes and abnormal peripheral sensory inputs into the stretch reflexes, all influence the response to muscle stretch in PD resulting in rigidity (Andrews, Burke and Lance, 1972; Rothwell *et al.*, 1983; Delwaide, Pepin and Maertens de Noordhout, 1991; Valls-Solé, 2000). How these changes and others relate to dopaminergic denervation in the basal ganglia in the classical models of PD is unclear as of yet (Rodriguez-Oroz *et al.*, 2009). However, we can ascertain clues based on some of the unique characteristics of PD rigidity such as exacerbation by contralateral limb movement, known as the Froment manoeuvre. This suggests that rigidity involves a network of brain regions on both ipsilateral and contralateral sides of the cerebrum. This was highlighted by a study from Baradaran and colleagues who found an fMRI pattern associated with rigidity that involved bilateral cortical and subcortical regions including the basal ganglia, primary motor cortex and pre-motor cortex (Baradaran *et al.*, 2013).

1.6.1.4. Postural instability

Several different hypotheses have been posited to explain postural instability in PD, but up until now, the true pathophysiology has remained elusive. One such hypothesis is that asymmetry of aberrant basal ganglia outflow and other PD-related phenomena such as rigidity and dystonia cause postural instability (Magrinelli *et al.*, 2016). However, some patients with postural instability exhibit combinations of directions of instability. For example, some exhibit both propulsion and retropulsion. Others exhibit lateropulsion in both directions. This suggests a mechanism other than asymmetric PD phenomena is responsible for postural instability. Other hypotheses include abnormal processing of proprioceptive afferents or abnormal spatial cognition. These suggestions, although still unproven, make more sense for two reasons: i) testing for postural instability involves assessing self-righting mechanisms in response to a postural challenge, which relies on proprioceptive function, ii) the onset of postural instability is associated with increased temporal risk of incident cognitive impairment in PD (Alves *et al.*, 2006). However, work by Bekkers et al., has shown that proprioceptive deficits do not contribute to postural instability in PD any more than they

do for age-matched non-PD fallers (Bekkers *et al.*, 2014). Thus, they concluded that postural instability in PD may be more a motor manifestation rather than a sensory phenomenon. However, cognitive deficits may also still play a role. For example, deficits in attention may contribute to a lack of postural control during dual-walking tasks (Yarnall, Rochester and Burn, 2011). An excellent review on the topic (Park, Kang and Horak, 2015) synthesised the argument that postural instability is a consequence of a number of different factors: 1) rigidity affecting biomechanics of balance, 2) bradykinesia of righting movements and anticipatory postural adjustment movements, 3) impaired kinaesthesia (awareness of position and movement of body parts) for sensory integration, 4) reduced automaticity of gait, 5) bradykinesia of gait.

1.6.1.5. Gait disturbance and freezing of gait

Shuffling gait, festination, reduced arm swing and freezing of gait, alongside postural instability, constitute the clinical features associated with PIGD phenotype. However, the pathophysiology of these features may be different depending on whether these are early-stage or end-stage occurrences. In early stage PD, the gait disorder shows bradykinetic, reduced-amplitude steppage, but with nearly normal cadence (Magrinelli *et al.*, 2016). This probably relates to underlying bradykinesia meaning it may be managed by dopaminergic therapy. On the other hand, end-stage gait impairment is likely to be more complex in its nature, probably due to involvement of cognitive, sensory and motor systems in the dysfunction of gait (Magrinelli *et al.*, 2016). To this end, different neurotransmitters have been implicated in progressive gait disturbance in PD. The current hypothesis of gait disturbance in PD centres on the pedunculopontine nucleus (PPN)-thalamus cholinergic pathway which functions in normal physiology to promote locomotion (Pahapill and Lozano, 2000). Located in the mesopontine reticular formation, and a key part of the mesencephalic locomotor region (Magrinelli *et al.*, 2016), the PPN has been shown to be a site of significant neurodegeneration in a number of neurodegenerative diseases including PD, AD and PSP (Jellinger, 1988b). Karachi and colleagues carried out a post-mortem quantitative analysis of the cholinergic neuron population of the PPN in human PD cases (Karachi *et al.*, 2010). They found that a significantly reduced PPN cholinergic neuron count associated with falls in PD when compared to non-faller PD cases. This data has been supported by in vivo PET imaging using cholinergic markers, which found significantly reduced thalamic acetylcholinesterase (AChE) PET signal in PD fallers compared to

those who do not have falls (Bohnen *et al.*, 2009). The PPN has also been a target of clinical trials for DBS to treat gait issues (Nandi *et al.*, 2002). Thus far, there is a published dataset of fewer than 100 cases in which DBS has been attempted in the PPN, and review of this dataset has shown that DBS has mild benefit for medication-refractory gait freezing and falls (Thevathasan *et al.*, 2018). It is likely that gait disturbance in PD is also related to dysfunction in other brain regions. One study has found reduced grey matter volume on MRI in motor, limbic, cognitive and associative brain regions in PIGD cases compared to TD PD (Rosenberg-Katz *et al.*, 2013). This data can be interpreted in two ways. Firstly, the involvement of cortical and sub-cortical motor regions suggests that gait disturbance is characterised by inefficient transmission of neural signals through the motor system, in a similar mechanism to that discussed in Section 1.6.1.1. Secondly, the pathophysiology of gait disturbance may involve a cognitive component, mediated by neurodegeneration of cognitive, limbic and associative brain regions.

FOG commonly occurs in the response to challenging or provocative situations such as crowded spaces and narrow doorways. This manifests as either start hesitation (a failure of gait ignition) or paroxysmal episodes of motor block during gait (Magrinelli *et al.*, 2016). Emotional factors such as anxiety may also contribute to FOG episodes. The above circumstances all require complex dynamic changes to gait motor programming due to increased cognitive load (Giladi and Hausdorff, 2006). The neurochemical basis for FOG is poorly understood, but can be present in early and late PD, suggesting that several different neurotransmitter systems may be involved. At a cellular level, it has been proposed that loss of cholinergic neurons in the PPN may be involved in FOG, similarly to general gait disturbance in PD (Benarroch, 2013). Furthermore, loss of cholinergic innervation of the cerebral cortex from the nbM, along with increased Aβ plaque deposition has also been proposed to contribute to FOG episodes (Bohnen *et al.*, 2014). FOG has also been linked with grey matter atrophy of the inferior parietal lobe and angular gyrus (Herman *et al.*, 2014).

The contribution of cognition in the pathophysiology of FOG has been the subject of recent research. For example, the association of visual stimuli with FOG has provoked the theory that FOG resultants from impaired visuo-spatial function (Almeida and Lebold, 2010). In support of this theory, Nantel and colleagues found that visuo-spatial tests were able to discriminate between freezers and non-freezers (Nantel *et al.*,

2012). However, other studies have showed that the nature of the contribution of visuo-spatial ability is more to do with motor imagery and mismatch of this to motor programming as opposed to visuo-spatial misjudgment in FOG (Cohen *et al.*, 2011). FOG is now thought to be more of a multisystem symptom. Where previously, gait was deemed to be a low-level automated motor task requiring minimal higher cortical input, it is now well-recognised to involve complex cognitive functions including attention and executive function (Heremans *et al.*, 2013). Dysfunction of these two aspects of cognition are key components of LBD (Emre *et al.*, 2007; McKeith *et al.*, 2017). Given the previous association of gait disorder and incident cognitive impairment in PD (Alves *et al.*, 2006), it is becoming more obvious that PD symptoms should not be grouped independently in motor or cognitive domains. In fact, the growing corpus of literature suggests that cognitive dysfunction may play a significant role in the onset of certain motor phenomena of PD. This may occur for various reasons, including impairment of higher cortical functions critical to the execution of motor programmes, or that there is a pathophysiological process that is common to both motor symptoms and cognitive impairment. For example, both cognitive impairment and bradykinesia have been hypothesised to involve impairment of processes controlling synaptic plasticity, potentially involving the presynaptic protein α-synuclein (Schulz-Schaeffer, 2010), in motor and cognitive brain regions. Dysfunction of α-synuclein in the cerebral cortex may therefore bring out motor and cognitive phenomena.

1.6.2. Pathophysiology of PD cognitive symptoms

It has long been known that α-synuclein pathology in the cholinergic nuclei of the basal forebrain, with corresponding cell loss, is a significant contributor to the pathogenesis of cognitive impairment in PD (Whitehouse *et al.*, 1983; Whitehouse, 1986; Perry *et al.*, 1993). The basal forebrain cholinergic nuclei, Ch1-4, innervate the cortex and other sub-cortical regions thereby regulating cognitive activities in the brain. The organisation of the cholinergic basal forebrain region was recently reviewed in an excellent publication (Liu *et al.*, 2015). The medial septal nucleus (Ch1) and vertical limb of the diagonal band of Broca (Ch2) both project to the hippocampus. The horizontal limb of the diagonal band of Broca (Ch3) innervates the olfactory bulb, whereas the nbM (Ch4) innervates the cerebral cortex and the amygdala. The Ch4 can be further subdivided into anterior (Ch4a), intermediate (Ch4i) and posterior (Ch4p) segments which innervate different cortical regions topographically i.e. Ch4a

innervates the frontal and cingulate cortices, Ch4i innervates the parietal and occipital cortices and Ch4p innervates the temporal lobe structures, including the amygdala (Liu *et al.*, 2015). It was hypothesised in this study that the spread of α-synuclein pathology through the Ch4 occurs in an antero-posterior direction and that this corresponds to the temporal sequence of clinical deficits in LBD. For example, pathology of Ch4a is associated with executive dysfunction and personality change, pathology of Ch4i, is linked to visuospatial impairment and hallucinosis, and pathology of Ch4p correlates with memory loss.

Executive dysfunction may encompass more than just Ch4a and frontal lobe pathology. Timing plays an important role in the production of executive functions. A role for the striatum has been proposed because the striatum is a key site in the incorporation of signals from prefrontal networks and internally generated timers to generate decisions and decision responses via motor circuits (Parker *et al.*, 2013a). In this model of executive function, it is proposed that the striatal MSNs act as timers and striatal-prefrontal networks therefore allow timing of executive function related to memories. Deficits in timing of behaviours is mediated by loss of striatal dopamine (Meck, 2006; Parker *et al.*, 2013b). There is a growing body of evidence that a key factor in the pathogenesis of LBD is the deposition of Aβ pathology in the striatum (Kalaitzakis *et al.*, 2008, 2011; Kalaitzakis and Pearce, 2009). This probably acts in tandem with reduced activation of prefrontal cortical networks by PD patients compared to controls (Dirnberger, Frith and Jahanshahi, 2005). Reduced medial frontal activity correlates with executive dysfunction in PD (Huang *et al.*, 2007). Thus, it is pathology in several key brain regions that underpins executive dysfunction in LBD.

Visuo-spatial impairment can often be one of the earliest presentations of cognitive impairment in PD and is sometimes perceivable at a subclinical level. fMRI studies have shown that subclinical visuo-spatial impairment is associated with reduced brain activity in the insula, striatum and hippocampus compared to controls and overactivity of the dorsolateral prefrontal cortex and posterior parietal lobe (Caproni *et al.*, 2014). This does not tally with the topography of spread of pathology proposed by Liu et al., (Liu *et al.*, 2015) suggesting that the pathology behind cognitive impairment in PD is heterogenous and complex. Overactivity of the dorsolateral prefrontal cortex and posterior parietal lobe suggests that there is reduced efficiency of signal transduction

which results in compensatory brain activity when faced with visuo-spatial task performance (Caproni *et al.*, 2014).

Memory impairment in PD tends to manifest as memory retrieval deficits – the so-called “tip of the tongue phenomenon” (Fahn, 2003) – rather than overt loss of memory. This suggests that memory impairment in PD is not due to frank neuronal loss, resulting in reduced memory storage, rather reduced efficiency of signal transduction during memory retrieval. This may reflect synaptic dysfunction mediated by α-synuclein in LBD (Schulz-Schaeffer, 2010). α-synuclein is critical to the regulation of synaptic plasticity by controlling synaptic shape, vesicular trafficking and the induction of plastic proteins (Bendor, Logan and Edwards, 2013). Therefore, the loss of α-synuclein, manifesting in LB pathology in the cortex could be directly linked with reduced efficiency of signal transduction in memory retrieval. This is supported by some of the other cognitive symptoms of PD such as bradyphrenia (slowed thought (Fahn, 2003)) which also highlights slowed signal transduction. Other memory deficits, such as amnesia, may reflect comorbid AD-type pathology. It is likely that overall, the cognitive syndrome in PD is underpinned by a confluence of pathologies including α-synuclein, tau and Aβ protein deposition (Compta *et al.*, 2014).

As discussed in Section 1.3.2, FC, VH and RBD are also of central importance, particularly in DLB (McKeith *et al.*, 2017). These symptoms highlight sleep-wake-cycle dysfunction, and disturbance of arousal and attention in LBD. The LC is the major source of noradrenaline in the central nervous system, innervating cortical, subcortical and limbic structures (Dahlstroem and Fuxe, 1964; Foote, Bloom and Aston-Jones, 1983; Halliday, 2004). In so doing, the main LC functions are to maintain sleep-wake-cycles, concentration, alertness and attention, thereby promoting the steady cognitive state (Benarroch, 2009). It is well known that the LC succumbs to α-synuclein pathology early in the current pathologic schemes of PD (Braak, Tredici, *et al.*, 2003; McKeith, Dickson, Lowe, M Emre, *et al.*, 2005; Alafuzoff *et al.*, 2009). Ponto-geniculo-occipital (PGO) waves are electroencephalogram correlates of hallucinosis but are also involved in the initiation of REM-sleep (Steriade *et al.*, 1990). They originate from the coeruleus/subcoeruleus complex, thereby implicating pathology in these regions specifically in DLB (Manford and Andermann, 1998). DLB features therefore appear to show deficits in the functions of the LC. LC cell loss has been shown in PDD and Diffuse LBD (now known as DLB), (Perry *et al.*, 1990) but these analyses were based

on outdated clinical criteria, very small numbers of cases and non-immunohistochemical staining methods such as Cresyl violet. There is therefore a need to reappraise the association between the LC and LBD to build knowledge of the multiple neurotransmitter systems that become dysfunctional in PD-related cognitive impairment.

1.7. Hypotheses and aims of this thesis

From this comprehensive review, it is clear that PD is not just a movement disorder, but a heterogeneous disorder with symptoms in various neurological domains including cognition. It is also apparent that deficits in different clinical domains are linked in the natural history of the disease, and that this may aid clinicians in assessing prognosis. For example, the literature has highlighted the associated between AR motor phenotype and cognitive decline in PD. I hypothesised that features of the AR motor phenotype will be able to predict cognitive decline in PD and will therefore have prognostic value. Therefore, the first aim of this thesis was:

1) To examine the link between features of the AR phenotype and cognitive decline, in comparison to other clinical features such as tremor and other non-motor features such as sleep disturbance, to establish the utility of AR phenotype as a predictor of cognitive decline in PD (Chapter 3).

I also hypothesised that a neurodegenerative disease with such a varied symptom profile must reflect differential pathology in several different neurotransmitter systems over and above the nigrostriatal dopaminergic system. To address this hypothesis:

1) I aimed to develop a novel midbrain atlas, based on 2D and 3D-immunohistochemical methods, as opposed to traditional histochemical methods, to address definitional issues of the SN anatomy in the previous literature (Chapter 4).
2) I aimed to carry out a clinicopathological analysis of the SN dopaminergic system in motor subtypes of PD. The dopaminergic neuronal populations of the SN were quantified and pathologic protein deposition quantitatively assessed in PD motor subtypes (Chapter 5).
3) With the results of Chapter 5 in hand, I aimed to assess the contribution of striatal pathology, with particular focus on the striatal ChIs to motor subtype in PD (Chapter 6).

4) I aimed to carry out clinicopathological correlations with LBD phenotype in the LC system by quantifying monoaminergic neurons in the LC and monoaminergic fibres in LC cortical and sub-cortical target regions. Pathologic protein deposition was also quantified in these regions. (Chapter 7)

Chapter 2 - Materials and Methods

2.1. Human brain tissue

Human post-mortem brain tissue samples were obtained from the Parkinson's UK Tissue Bank at Imperial College London (PD and age-matched control cases) for use in this study. Formalin-fixed paraffin-embedded (FFPE) tissue samples containing the dorsal striatum, rostral midbrain, caudal midbrain, rostral pons, amygdala, hippocampus, superior frontal gyrus, cingulate cortex, hypothalamus and thalamus were selected for use in this study.

Cases from the Parkinson's UK Tissue Bank with available clinical and neuropathological assessments completed at dates up to and including 18th December 2015 were assessed for suitability for this study.

2.1.1. Neuropathological assessment of cases

Cases from the Parkinson's UK Tissue Bank were assessed neuropathologically by tissue bank neuropathologists: Prof. Steve Gentleman, Dr. Federico Roncaroli, Dr. Ilaria Bravi and Prof. Manuel Graeber.

A standard sampling regimen was completed of 20 brain regions (Table 2.1) for each case. Haematoxylin & Eosin (H&E) staining and immunohistochemistry for α-synuclein, tau and amyloid β protein was performed for each tissue block. Based on the presence of α-synuclein-immunopositive inclusions in diagnostic brain region slides, cases were diagnosed with PD. The staging of PD-related pathology was performed in accordance with the BrainNet Europe protocol (Alafuzoff *et al.*, 2009) with the Braak α-synuclein staging system (0-6) (Braak, Tredici, *et al.*, 2003) and McKeith type staging system (brainstem, limbic, neocortical) (McKeith, Dickson, Lowe, M Emre, *et al.*, 2005). PD is diagnosed neuropathologically according to established criteria (Ince *et al.*, 2008), including the depletion of mesopontine pigmented neurons in the substantia nigra and locus coereleus and the presence of at least one Lewy body in surviving neurons with no concomitant pathology that could explain a parkinsonian motor presentation (Hughes *et al.*, 1992; Gelb, Oliver and Gilman, 1999).

The presence of hyperphosphorylated tau protein deposition was graded using the modified Braak staging system (0-6) (Braak and Braak, 1995) based on neuritic tau pathology in immunohistochemically stained sections in accordance with the BrainNet Europe diagnostic protocol (Alafuzoff *et al.*, 2008).

Aβ pathology (plaques and CAA) was assessed on 4G8 immunostained slides. Vascular pathology (microinfarcts, macroinfarcts, small vessel disease) demyelinating plaques and malignant neoplastic lesions were assessed for on H&E sections.

2.1.2. Clinical assessment of cases

Clinical assessment of cases was carried out on clinical notes collated retrospectively using records from movement disorder neurologists, neurosurgeons, psychiatrists, geriatricians, PD nurse specialists and general practitioners. Clinical summaries (an example is provided in Appendix 1) were then compiled by movement disorder specialists and neurologists (using a template created by Dr Ronald Pearce). Only cases with last clinical visit within 2 years of death were used for this study.

Clinical parkinsonism was defined using the current MDS task force criteria (Postuma *et al.*, 2015), whereby the presence of 2 of the 4 cardinal features of PD – bradykinesia plus one of resting tremor, rigidity or postural instability – was sufficient for a clinical diagnosis of parkinsonism.

2.1.2.1. Motor subtype diagnosis

Cases were analysed further and assigned a motor subtype based on the initial presenting clinical symptoms and initial disease course. Some donors prospectively filled out the official Parkinson's UK Tissue Bank Donor Lifestyle and Medical History Questionnaire (Appendix 2) and Donor Follow-up Questionnaire (Appendix 3). Where possible, these were used to aid motor subtype diagnosis. A range of different diagnostic criteria had been used in previous literature on PD subtypes for diagnosis in their cohorts (Table 2.2).

Table 2.1: Parkinson's UK Tissue Bank standard 20 block tissue sampling protocol with corresponding brain areas

Brain Area number	Region of interest	Coronal level of sampling
1	Anterior frontal cortex	Just rostral to temporal pole
2	Cingulate cortex	Nucleus accumbens
3	Nucleus accumbens	Nucleus accumbens
4	Temporal cortex	Mammillary body
5	Middle basal ganglia (lentiform nucleus, nucleus basalis of Meynert)	Decussation of anterior commisure
6	Posterior basal ganglia	Mammillary body
7	Amygdaloid complex	-
8	Thalamus	Subthalamic nucleus
9	Anterior hippocampus	Mammillary body
10	Posterior hippocampus	Lateral geniculate body
11	Posterior frontal cortex (primary motor cortex)	Pulvinar nucleus of thalamus
12	Occipital cortex (primary visual cortex)	Striate cortex
13	Parietal cortex	1cm caudal to splenium
14	Cerebellum (dentate nucleus)	
15	Rostral midbrain (substantia nigra)	Red nucleus
15a	Caudal midbrain	Superior cerebellar decussation
16	Rostral pons (locus coereleus)	Locus coereleus
16a	Caudal pons	-
17	Medulla oblongata (rostral and caudal)	-
18	Spinal cord (cervical, thoracic, lumbar)	-
19	Olfactory bulb and tract	-
20	Optic chiasm and tract	-

Table 2.2: Motor subtypes used by previous studies with corresponding clinical descriptions

PD motor subtype	Descriptions	References
Tremor-dominant	"Rest tremor as sole initial symptom or sustained dominance of tremor over bradykinesia and rigidity" [1] Marked resting tremor with mild bradykinesia/rigidity" [2] "[UPDRS] mean tremor score/mean PIGD score less than or equal to 1.5" [3, 4] "akinesia and rigidity were less pronounced than tremor… only minimal head and trunk tremor" [5]	[1] - (Selikhova *et al.*, 2009) [2] - (Paulus and Jellinger, 1991) [3] - (Jankovic *et al.*, 1990) [4] - (H. M. Chen *et al.*, 2015) [5] - (Timmermann *et al.*, 2003)
Mixed	"When the [UPDRS] tremor/PIGD ratio was more than 1.0 and less than 1.5, patients were classified to be in the indeterminate class"[1] [UPDRS] Scheiss ratio of 0.78-0.97 [2, 3] [UPDRS] tremor/PIGD ratio more than 1.0 and less than 1.5 [4] Same MDCS[+] tremor, bradykinesia or rigidity grade [5]	[1] - (Alves *et al.*, 2006) [2] - (Schiess *et al.*, 2000) [3] - (Fereshtehnejad and Postuma, 2017) [4] - (Jankovic *et al.*, 1990) [5] - (Rajput *et al.*, 2017) [+] - Movement Disorders Clinic Seskatchewan
Non- tremor-dominant	"predominantly bradykinetic motor features with no or only mild rest tremor" [1] "patients with predominant rigidity and bradykinesia but no tremor or only mild tremor at rest" [2]	[1] - (Selikhova *et al.*, 2009) [2] - (Pascale *et al.*, 2016)
Akinetic-rigid	"primary rigidity and bradykinesia with minimal tremor" [1] "[UPDRS] nontremor score was at least twice the tremor score" [2] "prominent bradykinesia and rigidity, but no visible tremor" [3] Schiess ratio of <0.78 [4] "marked akinesia/bradykinesia and rigidity with no or only mild tremor" [5]	[1] - (Lewis *et al.*, 2011) [2] - (Eggers *et al.*, 2011) [3] - (Rajput *et al.*, 2008) [4] - (Schiess *et al.*, 2000) [5] - (Paulus and Jellinger, 1991)
Postural instability/gait difficulty	"Bradykinesia, and postural instability and gait difficulty" [1] [UPDRS] tremor/PIGD ratio <1 [2, 3, 4] [UPDRS] tremor/PIGD ratio <0.9 [5]	[1] - (Jankovic *et al.*, 1990) [2] - (H. M. Chen *et al.*, 2015) [3] - (Alves *et al.*, 2006) [4] - (Burn, Rowan, Allan, Molloy, O 'brien, *et al.*, 2006) [5] - (Stebbins *et al.*, 2013)

Across all previous studies of PD motor subtypes, there is more consensus on the TD subtype compared to others. As demonstrated in Table 2.2, there is less consensus on the non-tremulous subtype with three variations, each with subtly different diagnostic criteria. The non-tremor dominant and AR subtypes have very similar diagnostic criteria and these differences between them are purely semantic. The term non-tremor dominant is non-specific and relies purely on the absence of significant tremor. This means that any combination of PD symptoms with the exclusion of tremor

may be fit to be characterised in this subtype and thus early-onset cases with prominent dyskinesia and dystonia may be included within the NTD subtype. The AR subtype has a much more specific definition, with prominent inclusion criteria and exclusion criteria. In a recent review article, PIGD as a subtype was questioned by the author who noted that PIGD is more likely to be a progressive feature on a continuum rather than a discrete subgroup (Kotagal, 2016). This is because postural instability does not usually occur until later in the disease course (Hoehn and Yahr, 1967) and becomes progressively worse, causing significant disability by the late stages. This is reflected by the finding that PIGD subtype is not reproducible using cluster analyses which consistently fail to isolate a cluster of cases that are non-tremor dominant or PIGD to compare with TD subgroups that cluster very significantly (Van Rooden *et al.*, 2010). Moreover, the propensity to develop PIGD trait is strongly influenced by risk factors including advanced age and increased number of medical comorbidities. In the CamPaIGN study, an incident PD cohort study based at Cambridge, United Kingdom, the level of medical comorbidity present at baseline significantly predicted earlier postural instability. Further, comorbidity at baseline is a significant risk factor for the development of gait disturbance and mobility impairment (Parashos *et al.*, 2013; King *et al.*, 2014). Therefore, PIGD is not a reliable subtype to be used in retrospectively characterised cohorts. Because of the aforementioned factors, cases were characterised as TD, mixed or AR.

2.1.2.2. Assessment of cognitive status

By DSM-IV and ICD-10 criteria, cognitive impairment significant enough to interfere with activities of daily living is classified as ‘dementia’. If PD patients met these criteria along with those defined by Emre and colleagues in the Movement Disorder Society Task Force diagnostic criteria they were classified as PDD (Emre *et al.*, 2007) . These criteria are “impairment in attention, memory, executive and visuo-spatial functions, behavioral symptoms such as affective changes, hallucinations and apathy are frequent”.

The one-year rule was applied to distinguish between PDD and DLB (McKeith, Dickson, Lowe, M. Emre, *et al.*, 2005): if parkinsonism predates the onset of dementia by at least one year then the dementia is of PDD; if dementia occurs before or less than one year after the onset of parkinsonism then the dementia is of DLB. Further, the most recent iterations of the diagnostic criteria for PDD (Emre *et al.*, 2007) and

DLB (McKeith *et al.*, 2017) were applied to these cases. DLB was characterised, alongside the one-year rule, by the presence of the four core clinical features ascribed to it in the clinical diagnostic criteria: FC, recurrent well-formed VH, RBD and spontaneous parkinsonism. If two or more of these core features were present, a diagnosis of probable DLB was made retrospectively and confirmed based on the pathology present (McKeith *et al.*, 2017). It has been well documented that these features may also occur in PDD (Emre *et al.*, 2007; Varanese *et al.*, 2010), thus where these were present in PDD cases, the cases were assigned the designation 'PDD-DLB' to reflect a potential clinical middle-ground to be explored pathologically.

If cognitive deficits were present in an individual without reaching the threshold for dementia, i.e. not enough to impair performance of activities of daily living, then a classification of PD-MCI was given. These deficits can occur in any cognitive domain including memory, visuospatial function, executive function, attention and language. Sometimes these symptoms are accompanied by psychotic features and hallucinations.

2.1.3. Inclusion and exclusion criteria

Primarily, cases with adequate tissue fixation and tissue quality were included for this study. Cases with inadequate tissue condition were excluded. For all cases that met these primary criteria, an assessment of the quality of clinical notes was performed. If the clinical notes for a case were of poor enough quality to not be able to distinguish presenting and late disease features, they were excluded. Hence, cases without documentation of presenting symptoms, or those with last clinical description more than 48 months prior to death were excluded. Atypical causes of parkinsonism such as multiple system atrophy, progressive supranuclear palsy, corticobasal degeneration (CBD), motor neurone disease and vascular parkinsonism were excluded. Furthermore, cases with significant vascular lesions such as cerebral infarcts, haemorrhage, CAA and small vessel disease were excluded alongside cases with other comorbid neuropathologic findings such as AD (Braak stage > IV), demyelinating disease, frontotemporal lobar dementia and Creutzfeldt-Jakob disease.

Control cases were selected based on clinical case note and neuropathology examinations. Cases with clinical or neuropathology findings of neurological disease such as dementia, parkinsonism or demyelinating disease were excluded.

2.1.4. Summary of cases included for this study

I analysed clinicopathological reports from 273 cases and 45 controls from the Parkinson's UK Tissue Bank according to the above selection criteria. 84 cases and 25 controls were excluded by these criteria. In total, 189 cases and 20 controls met the inclusion criteria for this study. The expanded cohort of 189 PD cases was used for analysis of the relationships between different clinical features and cognitive impairment in PD. For pathological studies, The 219 cases and controls were narrowed into two manageable cohorts: the first was for the study of PD motor subtypes and utilised 120 PD cases (40 TD, 40 Mixed and 40 AR; Appendix 4 – PD cohort of 40 TD, 40 Mixed and 40 AR cases for this thesis) were used; the second for the study of the different subtypes of LBD utilised 108 cases (38 PD without cognitive impairment, 56 PDD and 14 DLB; Appendix 6 – LBD cohort for Chapter 7 of this thesis). 13 age-matched controls were recruited for comparison (Appendix 5 – Control cohort used in this thesis).

2.1.5. Preparation of tissue for histopathology

Tissue for histopathology was prepared according to the Standard Operating Protocol of the Parkinson's UK Tissue Bank. Fresh full brain, brainstem and cerebellum samples were hemisected through the sagittal plane (if post-mortem delay was less than 48 hours; if more than 48 hours, intact samples were placed into formalin) and placed into neutralised buffered 10% formalin for at least 4 weeks. A first coronal cut was made at the level of the mammillary bodies followed by further coronal slices using standard 0.5cm and 1cm guides. After this, 20 diagnostic tissue blocks were sampled (Table 2.1). These blocks were then dehydrated in increasing concentrations of ethanol followed by xylenes. Dehydrated blocks were then embedded in paraffin wax with a tissue processor and then allowed to cool in moulds on an ice block before 7μm thick sections were cut on a Leica RM2145 microtome. Tissue sections were laid out on a 40-43°C water bath before being mounted onto SuperFrost (VWR) microscope slides and dried in a 37°C oven overnight prior to staining.

2.1.6. The Parkinson's Progression Markers Initiative

In addition to data collected from the Parkinson's UK Tissue Bank, I sought and gained access to the Parkinson's Progression Markers Initiative (PPMI) database via http://www.ppmi-info.org/. PPMI collects baseline and regular follow-up data from a

vast number of cases including prodromal PD, PD, genetic PD, SWEDD and healthy control cases. UPDRS and Montreal cognitive assessment (MoCA) data alongside demographics were collected for all PPMI cases. Cases were then filtered for a diagnosis of PD. In total, 1067 cases met this criterion and were analysed in this study. Details of the specific analyses can be found in Chapter 3.3.3.

2.2. Histological staining

For all histological and immunohistochemical staining procedures, tissue sections needed to be dewaxed and rehydrated before staining: tissue sections were dewaxed in two changes of xylene (10 mins then 5 mins) before being rehydrated in descending concentrations of alcohol (100% IMS for 2 changes of 5 mins, 90% IMS for 5 mins and 70% IMS for 5 mins) and then distilled water.

After the specific staining protocol was carried out, slides were dehydrated again in increasing concentrations of alcohol (70% IMS for 5 mins, 90% IMS for 5 mins and 100% IMS for 2 changes of 5 mins) and cleared in two changes of xylene (5 mins each) before being mounted and coverslipped with Distrene-Plasticiser-Xylene (DPX). Slides were then allowed to dry and excess DPX cleaned off before visualisation under a microscope.

2.2.1. Haematoxylin & Eosin staining

After deparaffinisation and rehydration, slides were immersed in Mayer's Haematoxylin (TCS Biosciences) for 2 mins and then washed in running tap water for 'blueing' effect enhancement. Slides were then immersed in 1% eosin for 5 mins before being briefly dipped in distilled water, 70% IMS and 90% IMS followed by two 5mins washes in 100% IMS. Slides were then cleared in two changes of xylene before being mounted with DPX and coverslipped.

2.2.2. Luxol Fast Blue (LFB) with Periodic acid-Schiff staining

After deparaffinisation slides were washed in 100% alcohol twice for 5 mins each and immersed in LFB (0.1% wt/vol LFB, 0.05% vol/vol acetic acid in 100% IMS) in a sealed container in a 60°C oven overnight. The next day, slides were briefly washed in 100% IMS twice followed by running tap water. Saturated lithium carbonate solution immersion was then used to differentiate the grey and white matter (visible macroscopically) after which slides were washed in running tap water. They were then immersed in 1% periodic acid solution for 5 minutes, rinsed in distilled water and then

immersed in Schiff's reagent (VWR, UK) for 15 mins before being washed in running tap water for 30 mins. Mayer's Haematoxylin was used to counterstain the sections for 1 minute and immersion in running tap water was used for 'blueing' contrast enhancement. Slides were then dehydrated by briefly rinsing in 70% IMS, 90% IMS and twice in 100% IMS before clearing in 3 changes of xylene. Slides were then mounted with DPX and coverslipped.

2.3. Immunohistochemistry

As referred to previously, tissue sections were deparaffinised and rehydrated. Following this, endogenous peroxidase enzyme quenching was performed by immersing slides in 1% H_2O_2/1x PBS (pH 7.4) for 30 minutes before washing in distilled water for 5 minutes. If antigen retrieval was required, this was performed at this stage as per the protocols outlined in Table 2.3. Samples were then rinsed in distilled water for 5 minutes before being rinsed in PBS (once for ImmPRESS secondary detection system (Vector Labs, UK), three times for Super Sensitive polymer-HRP immunohistochemistry detection system (Biogenex, UK)). For ImmPRESS system, 2% horse serum was added to slides for serum blocking for 20 minutes. For Supersensitive Kit and ImmPRESS system post-serum blocking, antibody solution diluted in primary diluent (PBS, 0.3% Triton-X100) was added to slides which were then stored in a humified chamber at 4°C overnight.

For ImmPRESS secondary detection system, slides were rinsed in PBS for 5 minutes and then incubated with polymer-horse anti-goat IgG secondary antibody for 30 minutes before being rinsed gain in 3 changes of PBS for 5 minutes each. Slides were then immersed with 3-3' diaminobenzidine (DAB) for 5 minutes to visualise staining.

For Super Sensitive polymer-HRP detection system, the producer's protocol was used. Thus, slides were first rinsed twice in PBS for 5 minutes each and then incubated with SS Super Enhancer Reagent for 20 minutes. Slides were then washed briefly in PBS twice for 5 minutes each before being treated with a tertiary layer reagent consisting of polymer-HRP for 30 minutes. After this, slides were washed again in three changes of PBS before being visualised with DAB included in the kit for 10 minutes.

For both secondary methods, slides were then rinsed in distilled water and counterstained for 1 minute in Mayer's haematoxylin before being rinsed in tap water

for ‘blueing’ contrast enhancement. Slides were then dehydrated in increasing concentration of IMS and cleared in two changes of xylene before being mounted with DPX and coverslipped.

For all staining protocols, omission of primary antibodies was used as a negative control to test for non-specific staining by the secondary and tertiary layer reagents in immunohistochemistry.

Table 2.3: Primary antibodies used in this thesis. ImmPRESS: ImmPRESS anti-goat (Vector, USA; MP-7405); Super Sensitive: Super Sensitive Link-Label Polymer HRP Detection System (Biogenex, UK; QD410-YAXE)

Antigen	Host	Clonality	Producer	Catalogue number	Immunogen	Antigen retrieval	IHC Dilution	IF dilution	Secondary detection method
Choline acetyltransferase (ChAT)	Goat	Polyclonal	EMD Millipore	AB144p	Human placental ChAT	Autoclave in 0.01M sodium citrate buffer (pH 6.0) for 25 mins	1:100	1:50	ImmPRESS
Tyrosine Hydroxylase (TH)	Rabbit	Polyclonal	EMD Millipore	AB152	Rat phaeochromocytoma TH	Autoclave in 0.01M sodium citrate buffer (pH 6.0) for 25 mins	1:2000	1:200	Super Sensitive
Substance P	Mouse	Monoclonal	Abcam	Ab14184	Human Substance P aa 58-68	Steam in 0.01M sodium citrate buffer (pH 6.0) for 20 mins	1:4000	1:200	Super Sensitive
α-synuclein, 42	Mouse	Monoclonal	BD Biosciences	610787	Rat Synuclein-1 aa. 15-123	80% Formic acid immersion for 15 mins	1:4000		Super Sensitive
Paired helical filament tau (AT8)	Mouse	Monoclonal	Thermofisher	MN1020	Partially purified human PHF tau	No pre-treatment required	1:1600	1:200	Super Sensitive
RD3 (3R tau clone 8E6/C11)	Mouse	Monoclonal	EMD Millipore	05-803	amino acids 209-224 of human 3-repeat tau isoform RD4	Autoclave in 0.01M sodium citrate buffer (pH 6.0) for 25 mins followed by 10 mins $KMnO_4$ + 2 mins oxalic acid	1:3000	1:200	Super Sensitive
RD4 (4R tau clone 1E1/A6)	Mouse	Monoclonal	EMD Millipore	05-804	amino acids 275-291 of human 4-repeat tau isoform RD4	Autoclave in 0.01M sodium citrate buffer (pH 6.0) for 25 mins followed by 10 mins $KMnO_4$ + 2 mins oxalic acid	1:1000	1:200	Super Sensitive
Amyloid-β,17-24 (4G8)	Mouse	Monoclonal	Biolegend	SIG-39220	17-24 of β amyloid	80% Formic acid immersion for 15 mins	1:15000		Super Sensitive

2.4. Immunofluorescent staining

As before, tissue sections were deparaffinised and rehydrated before being pre-treated using an autoclave. After cooling, slides were washed in distilled water and PBS and were incubated with primary antibodies in a donkey serum diluent (0.2% donkey serum in 1x PBS with 0.1% Triton X-100 and 0.001% sodium azide) overnight at 4ºC. In the case of multiple primary antibodies raised in the same host animal, the second of these primary antibodies was added separately to the others after incubation in secondary antibodies and thorough washing. Thereafter, the primary antibody was added, followed by its own secondary.

After washing in PBS, fluorescent secondary antibodies (AlexaFluor, Invitrogen, UK) specific to the host of each primary antibody were added in the concentration of 1:100 for one hour to the samples in darkness before slides were washed again in PBS in darkness. Slides were then incubated with filtered sudan black to block endogenous auto-fluorescence and then washed in running distilled water. Finally, slides were mounted and coverslipped with a nuclear counterstain of Vectashield 4',6-diamidino-2-phenylindole (DAPI) mounting medium (Vector, USA). Mounted slides were stored in darkness until visualisation to avoid bleaching of the fluorophores.

2.5. Tissue clearing

Larger tissue blocks were sampled for use in tissue clearing experiments. This involved the application of a protocol called FASTclear – an adaptation of CLARITY – as per the methodology optimised by our group (Hei Ming Lai *et al.*, 2018). According to this methodology, formalin-fixed tissue blocks were incubated in 4% sodium dodecyl sulphate in boric acid buffer (SDS; pH 8.5) at 54ºC to remove lipid from the sample to achieve optical transparency. Desired clarity was defined by the ability to read text through the sample (Figure 2.1). Blocks were then washed sequentially in PBS with 0.1% triton-X 100 (PBS-T) to remove excess SDS. A defined volume of PBS-T was used as diluent for primary antibodies which were incubated with the sample at 37ºC. These were titrated up to a concentration of 1:200 over the course of three days in a stepwise manner: on day 1, 1:500 was added; on day 2, 1:300 was added; and on day 3 1:200 was added. Fluorescent secondary antibodies specific to the primary antibodies used were added in darkness in the same series after primary was removed with sequential PBS-T washes. Refractive index matching using a refractive index

homogenisation reagent, OPTIClear (for Optical Properties-adjusting Tissue-Clearing agent) was performed overnight at room temperature.

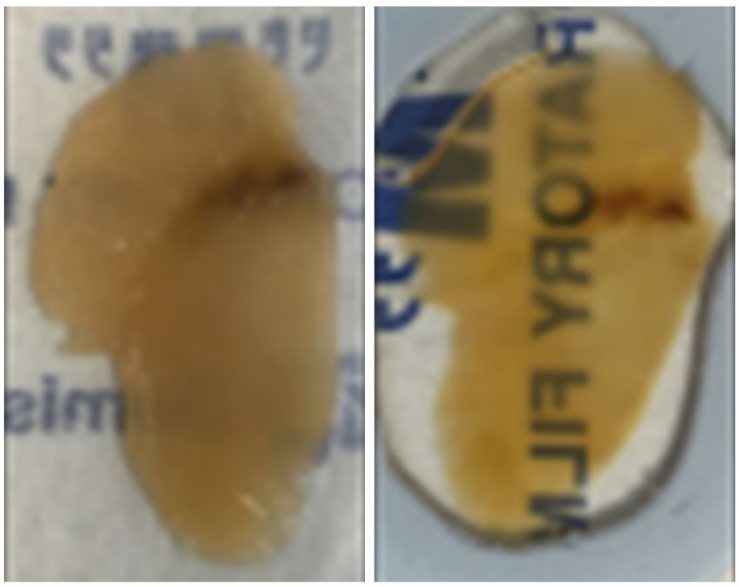


Figure 2.1. A human midbrain tissue-clearing block, before (left) and after (right) SDS treatment. Note the ability to visualise the text through the cleared sample after SDS treatment. Image adapted from our published manuscript (Hei Ming Lai et al., 2018). Reproduced under the Creative Commons License http://creativecommons.org/licenses/by/4.0/.

2.6. Microscopic image analysis

All image analysis of histochemically and immunohistochemically-stained slides was performed using an Olympus VANOX AHBT3 attached to a Micropublisher 3.0 camera. Images were visualised using Image-Pro Plus 7.0 (Media Cybernetics, USA). These analyses were performed blinded to the clinical subtype diagnosis. Immunofluorescence slides and tissue clearing samples were imaged with Leica RMi8 or Carl Zeiss LSM-780 inverted confocal microscopes, using Leica Application Suite-X (LAS-X) or Carl Zeiss Zen Black for the Leica and Carl Zeiss confocal microscopes, respectively.

2.6.1. Slide scanning

For the creation of macroscopic figures, microscope slides were scanned using a Leica Aperio AT2 slide scanner with Leica Aperio Scanscope software at 20x magnification.

2.6.2. Neuronal counts

The region of interest (ROI) for each nucleus analysed was defined as the area of maximal neuronal density as most nuclei have compact and diffuse divisions which make them too large to fit under a single objective lens field. Immunoreactive structures were included in the count if they exhibited a clear neuronal morphology with a defined nucleus and nucleolus. Two potential methods for counting neurons were experimented with in this study: 1) manual tagging and 2) threshold-based quantification. Manual tagging involves tagging each neuron using the 'manual tag' function of Image-Pro Plus 7.0 (Figure 2.2). This method allowed for accurate evaluation of each immunoreactive structure according to the previously outlined inclusion criteria for a neuron but was subject to human bias. Threshold-based quantification involved extracting the blue channel from the image and then calibrating the threshold manually to capture the entire area of an immunoreactive structure (

Figure 2.3). This could remove human bias from the quantification but was unable to differentiate between structures that did or did not exhibit the aforementioned neuronal morphology criteria. Therefore, quantification of neuronal counts was performed using the 'manual tag' function of Image-Pro Plus 7.0.

2.6.3. Morphologic analysis of ChIs

10 ChIs from the caudate nucleus and 10 ChIs from each of the medial, intermediate and lateral portions of the dorsal putamen were sampled using a 'lateral sweeping' motion for each subregion and the 10 sampled ChIs were then analysed morphologically. ChIs were identified by their distinctly large somata (Sullivan, Chen and Morikawa, 2008) and immunoreactivity to anti-ChAT antibodies. To be selected for branching quantification, the neuronal soma had to contain a clearly defined nucleus and nucleolus. Primary branches were defined as branches that originated from the cell soma of the ChI. Secondary branches were defined as any branch that arises directly from a primary branch.

2.6.4. Quantification of pathology

α-synuclein, tau and amyloid pathology were assessed with %-coverage of staining in a 20x objective lens field. This was performed using a threshold-based quantification method like the one outlined in Chapter 2.6.2. First, the blue channel was extracted and then the threshold was calibrated manually to capture the entire area of the

immunoreactive structures in the field. Structures not deemed to be ‘real’ staining, or pigmented neurons were removed from the count using the ‘Toggle on/off’ function. Then, using the ‘Per area (obj/total)’ function, an area coverage figure (expressed as a number between 0 and 1) was multiplied by 100 to derive percentage coverage.

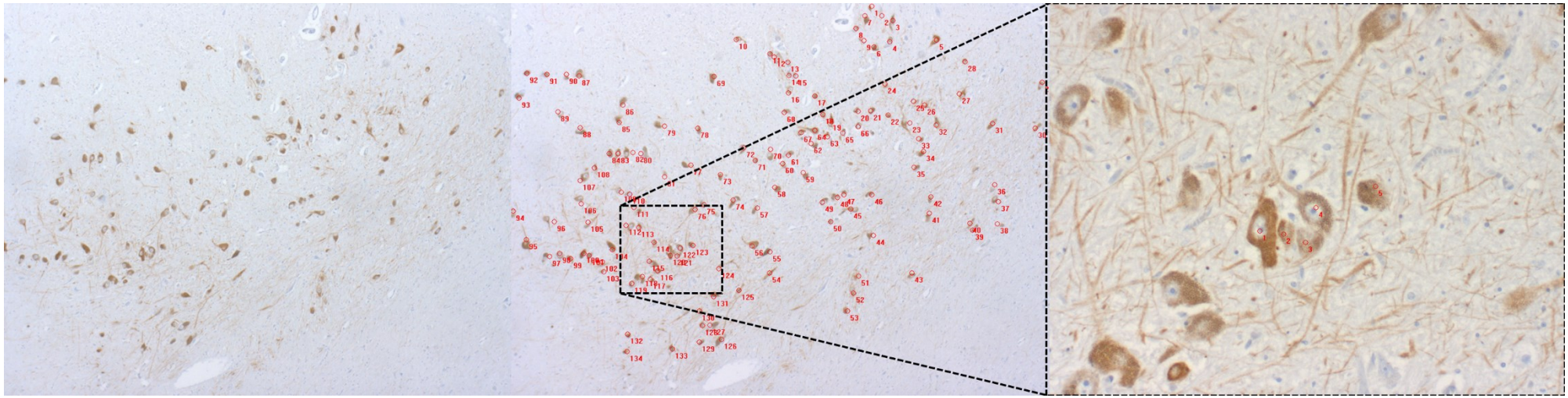


Figure 2.2: Neuronal quantification by manual tagging method. First (left) an image is captured under a 4x objective lens field. Second (middle), immunoreactive structures that meet criteria for neuronal count (neuronal morphology and clear nucleus and nucleolus) were tagged manually using the manual tag function of Image-Pro Plus 7.0. Inset right: illustration of the benefit of being able to distinguish cell number in a cluster of cells by tagging each one individually. This method yielded a neuron count of 134 in this image.

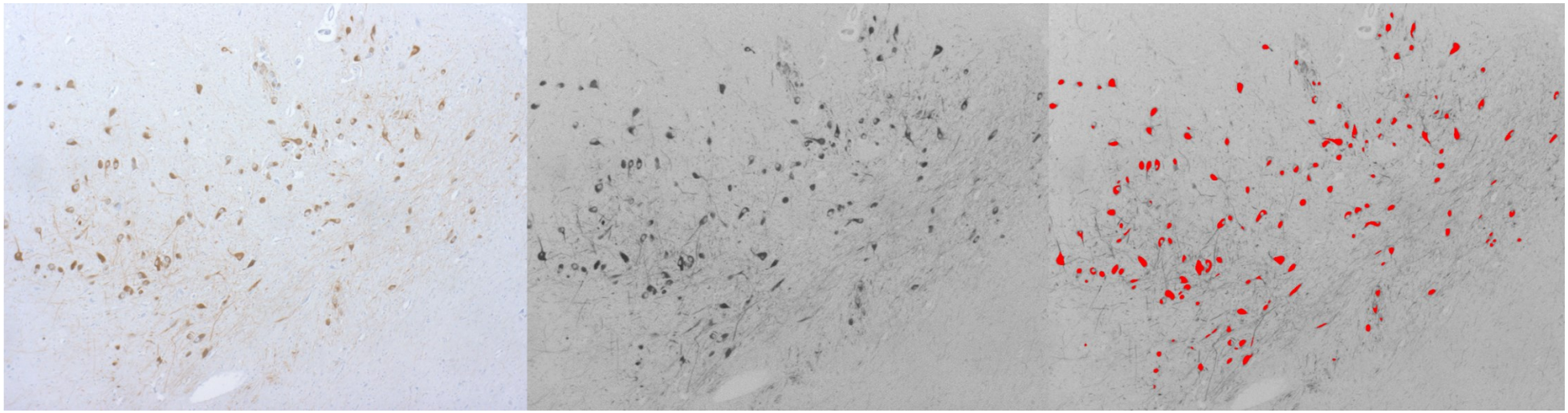


Figure 2.3: Neuronal quantification by threshold-count method. First (left) an image is captured under a 4x objective lens field. Second (middle), the blue channel is extracted, yielding a black and white image. Thirdly (right), a threshold is applied (red) to the image. This process yielded a neuronal count of 158 in this image.

2.7. Statistical analysis

A specific account of the statistical analyses performed will be outlined in each chapter. In general, all statistical analyses were carried out using QuickCalcs software on GraphPad Prism 8.0.0 and MATLAB 2019a. All data sets were assessed for normality of distribution using a Shapiro-Wilk normality test with the null hypothesis that the data set is normally distributed ($p>0.05$). For normally distributed data sets, parametric one-way analysis of variance tests (ANOVA) and t-tests were used to compare means between groups. For non-parametric data, Kruskal-Wallis and Mann-Whitney U tests were used. Post-hoc pairwise corrections for multiple comparisons were performed (Dunn's corrections).

Linear regression analyses were employed in three circumstances. Firstly, to assess the contributions of various disease and demographic factors to decline in PD (Chapter 3); secondly to assess the contributions of these factors to the degree of neuronal loss and protein pathology exhibited by cases; and thirdly to control for these variables when comparing pathology across disease subtypes.

Intra and inter-rater reliabilities were calculated to assess the degree of consistency in quantifications of neuronal number and pathologic burden, using intra-class correlation (ICC; two-way mixed; absolute agreement). ICC coefficient (Cronbach's α) ranges from 0-1 (no agreement to perfect agreement). A coefficient >0.7 indicates good agreement.

2.8. Ethical considerations

The work carried out on human post-mortem brain tissue was performed under ethical approval held by the Parkinson's UK Tissue Bank at Imperial College London (Research Ethics Committee Ref: 07/MRE09/72). Parkinson's UK is a registered charity in England and Wales (258197), and in Scotland (SC037554). The Parkinson's UK Tissue Bank is an approved Research Tissue Bank by the Wales Research Ethics Committee (Ref. No. 08/MRE09/31+5). An additional case (utilised in Chapter 4) was taken from the Corsellis Collection – an archival collection run by the West London Mental Health Trust (http://www.wlmht.nhs.uk/) Ethical approval was granted through BRAIN UK Ref 15/019, by the South Central- Hampshire B ethics committee.

Chapter 3 - Predicting current and future cognitive impairment in PD

3.1. Introduction

PD is the most common neurodegenerative movement disorder, characterised classically by a quartet of motor symptoms including bradykinesia, muscle rigidity, resting tremor and postural/gait instability (Fahn, 2003). Whilst these symptoms can be distressing to patients and loved ones and cause disruption to daily life, some of the most cumbersome and worrying phenomena in this disease are in the domain of NMS. In a large survey of PD patients and their caregivers, the study of NMS in PD were identified as among the most important ten research priorities for PD research (Deane *et al.*, 2014). Of the NMS in PD, symptoms of cognitive impairment, falls and depression contributed the most significantly to loss of quality of life for PD patients (Shibley *et al.*, 2008). Cognitive dysfunction in PD is common, with a prevalence of approximately 40% and a relative risk of 6 times that of the general population (Emre, 2003). Lewy Body Dementia (LBD) is responsible for a large caregiver burden and increased risk of mortality (Aarsland *et al.*, 2000; Levy *et al.*, 2002).

LBD tends to present with 'subcortical' type cognitive impairments such as executive dysfunction, attentional deficits and visuospatial impairment. This is in contrast with the 'cortical' type deficits prevalent in AD, which presents with amnestic features and language impairment (Emre *et al.*, 2007). Dementia in PD can take one of two forms – PDD or DLB – depending on the temporal relationship with the onset of parkinsonian motor features. The diagnostic 'one year rule' (McKeith, Dickson, Lowe, M Emre, *et al.*, 2005) is applied to distinguish between these two similar dementias: if dementia is diagnosed before or within one year of the onset of motor symptoms, the dementia is termed DLB whereas PDD is diagnosed as dementia that occurs over one year post-onset of motor parkinsonism. This arbitrary separation has remained controversial (Lippa *et al.*, 2007; Friedman, 2017; Weil *et al.*, 2017).

Previous literature has highlighted the importance of motor phenotype in assessing the risk for cognitive impairment in patients. Generally, PD patients tend to exhibit any of three main motor phenotypes, TD, AR or a mixed presentation, depending on the dominant symptoms present. Burn and colleagues found that most PDD and DLB cases were of PIGD-subtype at their baseline assessment and that for those who were non-demented at baseline, only PIGD cases then developed PDD (Burn, Rowan, Allan, Molloy, O 'brien, *et al.*, 2006). However, this study only used a two-year study

period and recruited patients who had already had PD for a number of years. What this study was able show was that PIGD was associated with an increased risk of PDD within two years, compared to TD which showed no change. Therefore, there is a substantial temporal risk of cognitive impairment associated with PIGD subtype. Williams-Gray performed a similar study, the CamPaIGN study, using a 3.5 year follow up and also found that PIGD was more associated with dementia than TD (Williams-Gray *et al.*, 2007). In contrast to Burn, Williams-Gray found that some baseline TD cases demented after 3.5 years. The finding that even TD cases will develop PDD if the study period is long enough raises suspicion that baseline motor phenotype is not a sufficiently strong marker for predicting dementia risk. Motor phenotypes of PD rarely remain stable over time (Von Coelln *et al.*, 2015), with TD patients tending to develop PIGD over time. This raises the possibility that subtype progression is related to dementia risk in PD. This idea was highlighted by Alves and colleagues who not only found that baseline-PIGD patients were more likely to develop PDD, but that TD patients only developed dementia once their subtype switched to PIGD (Alves *et al.*, 2006). Other studies have showed that PIGD phenotype was a precondition of the development of LBD, and that this was true of patients whether they had an initial PIGD presentation or had developed PIGD-dominant PD secondary to a TD presentation (Aarsland and Kurz, 2010; Aarsland and Bernadotte, 2015). This shows that the paradigm of baseline motor subtype might be insufficient for investigating dementia risk in PD. Further, if TD cases only develop dementia once progressive PIGD features set in, then there is merit in investigating the timing of PIGD onset and cognitive decline in PD, and whether at a single time point there is a relationship between PIGD symptoms and cognitive impairment.

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3.2. Hypothesis and aims

Given the previous associations highlighted between gate disturbance and cognitive dysfunction in PD, I hypothesised that there will be a temporal and symptomatologic association between these phenomena and that gait disturbance onset will be predictive of future cognitive impairment. The aims of this study were 1) to test association between baseline motor subtype and cognitive onset in PD; 2) to assess the predictive value of gait disturbance as a longitudinal *in vivo* marker for dementia to compare with baseline motor subtype; 3) to attempt to predict dementia risk using multivariate regression analyses based on factors including gait disturbance in combination with other clinical and demographic features and 4) to assess the link between gait and cognitive impairment severity at a cross-sectional level using a single time point analysis.

3.3. Methods

3.3.1. Longitudinal clinical record selection

Clinical case notes, clinical summaries and neuropathology reports were obtained from the Parkinson's UK Tissue Bank for 253 cases. Selection and exclusion criteria (outlined in Chapter 2.1.3) were applied to these cases. In summary, 189 cases of pathologically confirmed PD, without significant clinical comorbidity or concomitant pathology, were included in this study based on the availability and quality of clinical case notes. This included 59 PD cases without cognitive impairment, 35 cases with mild cognitive impairment (MCI), 85 cases with PDD and 8 with DLB. PD cases were also sub-classified by motor phenotype (TD, AR, and Mixed) at presentation as described previously in Chapter 2.1.2.1. Demographic data, including sex, disease duration and onset were also documented for each case.

3.3.2. Case selection for cross-sectional analysis

Cases were selected based on the same selection and exclusion criteria used for longitudinal clinical record selection. However, not all of the cases used in the longitudinal analysis had cross-sectional, single timepoint data available, depending on whether they had filled out the Parkinson's UK Tissue Bank Donor Lifestyle and Medical History Questionnaire and Donor Follow-up Questionnaire. Therefore, the two cohorts, though comparable in size, and chosen using the same criteria, do not represent the same cases. In total, 160 PD cases were included for the cross-sectional analysis.

3.3.3. Cases from the PPMI study

In total, over 2000 cases have been enrolled in the PPMI study. Included within this number are 1067 cases that meet the criteria for a clinical diagnosis of PD, either idiopathic or genetic (LRRK2, SNCA or GBA mutations only). Demographic, UPDRS parts I and III, MoCA, Hopkins Verbal Learning Test (HVLT), Benton Judgement of Line test (BJOLT), semantic verbal fluency (SVF), Letter Number Sequencing (LNS) test and Symbol Digit Modalities (SDM) test data from baseline screening visits and longitudinal follow-up visits were collated. The MoCA served as a global measure of cognitive impairment, whereas the HVLT tested verbal learning and memory ability, the BJOLT tested visuospatial function, SVF tested speech fluency, LNS tested memory and executive function and SDM tested attention. Additionally, DATscan

uptake ratio data from baseline DATscans were collected for cases in which this was performed.

3.3.4. Longitudinal determination of clinical milestones

The clinical notes of PD cases from the Parkinson's UK Tissue Bank were analysed retrospectively to determine the year of onset of various clinical milestones such as gait disturbance, the incidence the onset of cognitive decline and the incidence of dementia. The year of onset of each feature was defined as the calendar year in which it took place minus the year of onset of PD to control for differences in age of onset and disease duration. To determine the onset of gait disturbance, case notes were analysed for evidence of postural instability, stooped posture, shuffling or festinant gait, gait freezing and reduced arm swing. If two or more of these features were present simultaneously then this was determined to be the gait disturbance onset.

Cognitive impairment in PD encompasses a number of different symptoms including executive dysfunction, memory loss, hallucinosis, visuospatial deficits and attentional deficits (Watson and Leverenz, 2010). The onset of cognitive impairment was defined as the clinically confirmed evolution of one or more of these features. Further, if any cognitive deficit was elicited as an adverse effect of pharmacologic therapy and was relieved on discontinuation of the causative agent, a definition of cognitive onset was not applicable. Thus, only organic and clinically evaluated cognitive impairment was sufficient to diagnose the onset of cognitive impairment. Additionally, the onset of dementia was timed according to the time point of clinical diagnosis of dementia. For comparisons between categorical baseline variables, this data was expressed as years without dementia, i.e. the year of dementia diagnosis minus the year of PD diagnosis.

3.3.5. Cross-sectional single time point analysis

Using the prospectively completed Parkinson's UK Tissue Bank Donor Lifestyle and Medical History Questionnaires (Appendix 2 – The Parkinson's UK Tissue Bank Donor Lifestyle and Medical History Questionnaire) and Donor Follow-up Questionnaires (Appendix 3 – The Parkinson's UK Tissue Bank Donor Follow-up Questionnaire), cross-sectional data was collected from 272 donors, of which 160 exhibited pathologically confirmed PD. The other 112 cases had diagnoses of AD, MSA, PSP, pure nigral degeneration, showed no definite neuropathology, or had not yet had

complete pathology work up. Only the donors with pathologically confirmed PD were used for this study. In the Donor Follow-Up Questionnaire, 100 questions are asked and symptoms are thus rated on a verbal ordinal scale using 'none', 'a little', 'some' and 'a lot' to represent increasing severity of a symptom. Numerical values were assigned to each of these stages on the ordinal scale whereby 0 = 'none', 1 = 'a little', 2 = 'some', 3 = 'a lot'. Each symptom domain was assessed with a variable number of questions. For example, 3 questions concerned the nature and severity of tremor, 6 questions concerned the nature and severity of gait disturbance, and 11 questions concerned that nature and severity of cognitive impairment. Based on the answers given in these Questionnaires and their corresponding numerical values, individual composite scores were calculated for tremor, gait disturbance, cognitive impairment, neuropsychiatric symptoms, autonomic dysfunction and others. These composite scores, and the ratios between them were used to calculate motor subtype (TD, AR or mixed), and motor severity (mild, moderate or severe). Furthermore, demographic data such as age of onset, age at Questionnaire completion and sex, alongside pathologic data, such as McKeith stage (McKeith *et al.*, 2017).

3.3.6. Data analysis

3.3.6.1. Longitudinal cohort

Using the baseline motor characteristics present in cases in the longitudinal cohort, the relationship between baseline motor subtype and decline to cognitive impairment was assessed using different statistical methods. Firstly, the time taken in years to develop first cognitive symptoms, and latterly dementia, wascompared in baseline motor subtypes using Kruskal-Wallis tests with Dunn's corrections for multiple comparisons. Secondly, deterioration to the aforementioned cognitive end-points will be compared using Kaplan-Meier survival analysis, with Gehan-Breslow-Wilcoxon tests to compare survival curves for cognitive impairment and dementia respectively between baseline motor subtype groups and log-rank tests for trends. This particular analysis was chosen because it weights earlier time points more strongly than later time points which is appropriate for this dataset because the clear majority of data points elapsed in early years following PD diagnosis. Further, with this chapter, I wanted to understand predictors for early cognitive risk, as it is well known already that advanced disease duration is a significant risk factor for dementia (Hely *et al.*, 2008). X^2 analysis was used to analyse statistical differences between the groups.

All factors measured were input into a correlation matrix and the correlations between these were assessed using Pearson's R tests. Factors were then assessed for homogeneity of variance, multicollinearity, outliers and normal distribution of residuals. Based on the results of the analysis of these key assumptions, all factors that met the assumptions and that were significantly correlated with cognitive decline were entered into multivariate regression analyses using the least squares regression method. In the longitudinal, cohort, time to cognitive impairment and dementia were used as dependant variables for multivariate analyses. The potential predictors were age of onset, sex, year of tremor onset, year of gait impairment onset, year of rigidity onset and year of hallucinosis. Statistical analyses were carried out using GraphPad Prism 8.0.0.

3.3.6.2. Cross-sectional cohort

Kruskal-Wallis tests were employed to compare the cognitive impairment severity scores between PD motor subtypes. Thereafter, these results were compared with individual motor symptom scores to assess which motor symptom sorting technique best predicted cognitive impairment in PD. Composite motor symptom scores, calculated as the total score for all of the questions pertaining to a particular symptom, were used alongside other factors such as hallucinations, anosmia, swallow/speech impairment, demographic and pathologic factors, as predictor variables in multivariate regression analyses using the least squares regression analysis technique with cognitive impairment score as a dependent variable. Statistical analyses were carried out using GraphPad Prism 8.0.0.

3.3.6.3. PPMI cohort

In the PPMI cohort, demographic, UPDRS part III, MoCA, HVLT, BJOLT, LNS, SVF and SDM test data from baseline screening visits and longitudinal follow-up visits were collated. Cognitive outcomes, i.e. MoCA score out of 30, HVLT average score out of 12 across three trials, BJOLT score out of 30, LNS score out of 20, total SVF score for naming objects in three verbal categories in one minute, SDM score out of 110, were used as dependent variables. UPDRS part III was used to compile aggregate scores for motor symptom severity, whereby all items pertaining to one symptom were totalled to give an overall score. Symptom categories were defined as rigidity, bradykinesia, gait impairment, postural instability and tremor. To assess the value of motor features as predictors of cognitive impairment, these factors, alongside demographic data such

as sex and age of onset, were used as predictor variables. Like the cognitive score data, these were expressed as raw scores whereby a high score in a symptom domain indicates increased severity of the symptom.

As the data from this cohort was not normally distributed, canonical correlation analysis (CCA) was used instead of multivariate regression analysis for statistical comparisons. This is an accepted and rigorous technique that uses a multiple matrix approach to assess maximal cross-correlations between canonical modes in different matrices (Smith *et al.*, 2015). In this case, UPDRS part III data was stored in a motor matrix, cognitive test data in a cognitive matrix, demographic data in a demographic matrix and DATscan data in a DATscan matrix. The technique assumes that there is cross-covariance between the matrices because each of row of each matrix pertains to the same case and time point. A single *vector* is created for each matrix and the CCA attempts to find maximal correlations to vectors in the second matrix. Each combination of cross-correlated vectors is known as a *mode*. If the mode reveals a statistically significant correlation, it is back-projected onto the constituent matrices to assess the exact combinations of variables that produced the mode, and the correlation coefficients for each. By iterating this between combinations of all matrices, a network of cross-correlated variables can be determined. Statistical analysis was performed using MATLAB 2019a.

3.4. Results

3.4.1. Motor subtype as a determinant of cognitive risk

Comparisons between baseline motor subtype groups in the longitudinal cohort show that TD-presenting cases take significantly longer on average (9.08 years) to develop cognitive symptoms than both Mixed (5.68 years) or AR (5.82 years) cases (Figure 3.1A). Further to this, TD cases also took significantly longer (10.97 years) to develop dementia than AR (7.17 years) cases, with Mixed (8.96 years) cases representing a mid-point (Figure 3.1B). Having a TD presentation did not preclude cases from having cognitive impairment present at baseline, even though this was an extremely small proportion of the TD cohort (2/64 cases). However, the proportion of TD cases that eventually developed PDD was statistically comparable to mixed and AR cases ($p=0.2328$). The progression from cognitive impairment onset to dementia onset also did not differ between subtypes. Furthermore, at the cross-sectional level, TD cases had an average self-rated cognitive impairment severity score of 5.64/30, compared to 6.79/30 for Mixed and 7.28/30 for AR cases. There were no significant differences between these motor subtype groups.

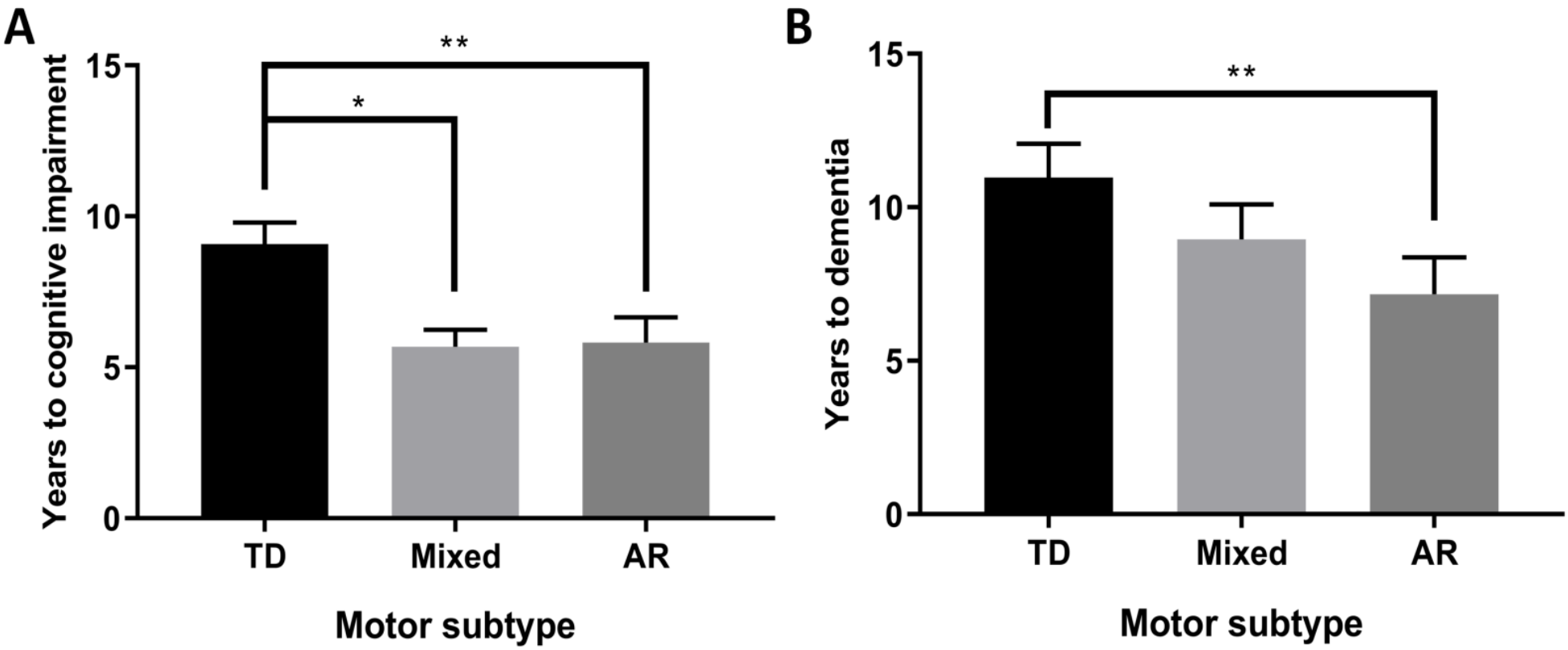


Figure 3.1: Baseline motor subtype is associated with differential development of cognitive impairment (A) and dementia (B). Kruskal-Wallis tests for Dunn's corrections for multiple comparisons were used for statistical analysis. * = $p<0.05$, ** = $p<0.01$.

Kaplan-Meier survival analysis revealed that the rate of progression longitudinally to cognitive impairment in baseline motor subtype groups is significantly different (Figure 3.2A). Cases with a TD motor onset progressed more slowly to cognitive impairment than cases with a mixed or AR presentation. However, by the end of the disease course, the proportions of cases of each clinical subgroup that had not developed some form of cognitive impairment were comparable. Thus, even TD cases who certainly progress more slowly to cognitive impairment than AR cases, are equally susceptible in the final analysis, but take longer to develop these symptoms. When the baseline subgroups were analysed for progression to dementia diagnosis, there were no significant differences found over the whole disease course, though in the early phases of the disease, AR cases progressed more aggressively to dementia than TD or mixed cases. By the end of disease course, 58% of TD-presenting cases had not developed dementia compared to 44% of AR cases and 51% of Mixed cases. By 10 years, the differences between the survival curves of each presentation had maximised with approximately a 20% difference between the survival of TD cases and AR cases. However, the majority of data points fell at early time intervals. Further, at later stages of the disease, multiple co-morbidities accumulate which hinders the accuracy of data collection on cognitive impairment timing. Also, as Hely and colleagues (Hely *et al.*, 2008) suggest in their research, dementia is an inevitability at 20 years. Therefore, the same analysis was performed using a *15 years of disease* cut off for cognitive impairment data, and 20 years of disease cut off for dementia diagnosis in order to account for diagnostic record inaccuracy and to assess the contribution of motor subtype to dementia diagnosis in the stages where it is not inevitable. Survival without cognitive impairment was significantly different between presenting motor subtype groups with TD showing a significantly increased proportion of survivors relative to Mixed and AR cases (Figure 3.3A). By 15 years, 30% of TD patients had not developed signs of cognitive impairment compared to 15% of Mixed and 19% of AR cases. Furthermore, TD presentation was also protective against the development of dementia by 20 years, such that 62% had survived without dementia at this time point, compared with 55% of Mixed and 46% of AR cases (Figure 3.3B).

To summarise, the data presented thus far suggest that motor subtype is associated, weakly, with longitudinal progression to dementia, but that current motor subtype has no relationship with the severity of cognitive impairment at the cross-sectional level.

This data also refutes the data from Hely et al., who suggested that dementia is inevitable by 20 years – in this cohort, approximately half of all cases had not yet developed dementia, with significantly fewer cases coming from the TD-presenting group.

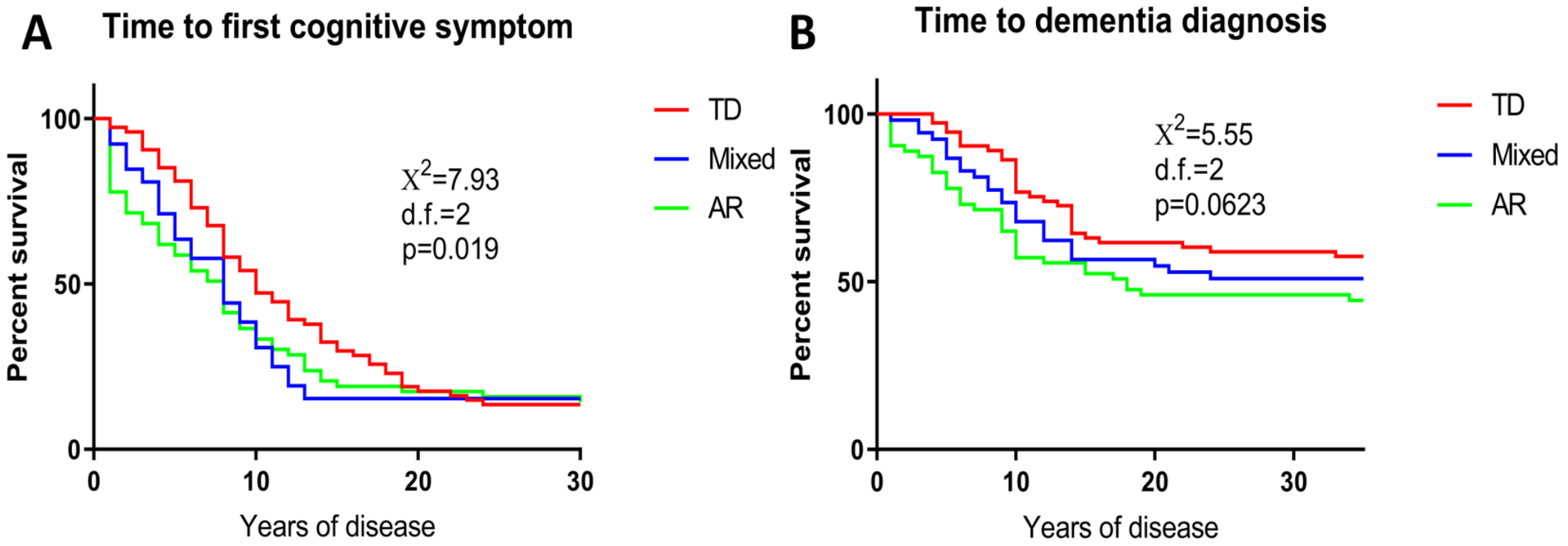


Figure 3.2: Baseline motor subtype is associated with differential rates of progression to cognitive impairment in PD. A) Kaplan-Meier survival curves to first sign of cognitive impairment for TD, Mixed and AR cases. B) Kaplan-Meier survival curves to dementia diagnosis for TD, Mixed and AR cases. Gehan-Breslow-Wilcoxon tests were used for statistical analysis. Log-rank tests for trends were also carried out. In B) Long-rank test for trend showed a statistically significant difference between survival curves (p=0.0477).

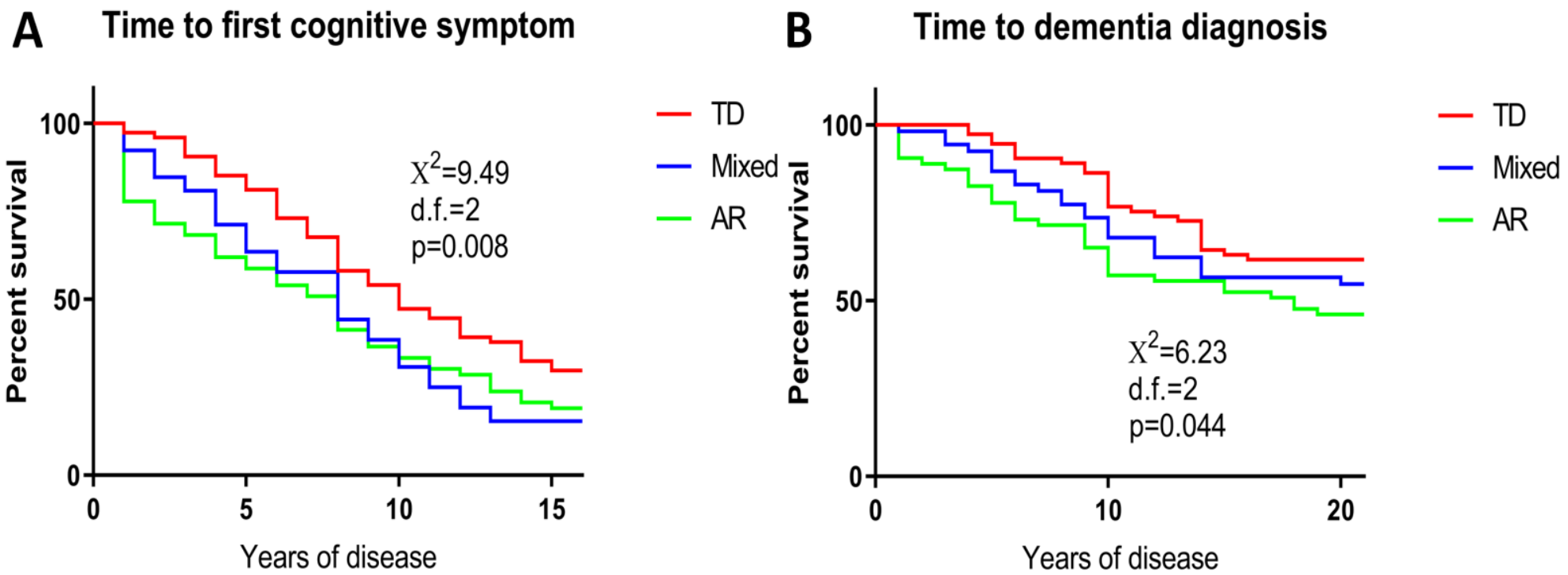


Figure 3.3: Baseline motor subtype is associated with differential rates of progression to cognitive impairment in PD in earlier stages of the disease. A) Kaplan-Meier survival curves to first sign of cognitive impairment for TD, Mixed and AR cases, with 15 years of disease cut off. B) Kaplan-Meier survival curves to dementia diagnosis for TD, Mixed and AR cases with 20 years of disease cut off. Gehan-Breslow-Wilcoxon tests were used for statistical analysis.

3.4.2. Individual symptom onset as a longitudinal predictor of cognitive impairment

Individual symptom onsets in the longitudinal cohort, such as gait disturbance, tremor, rigidity, sleep disturbance, cognitive symptoms, orthostatic hypotension and hallucinations were noted as the year of disease in which they took place. All factors were entered in to a correlation matrix (Table 3.1: Multivariate regression analysis with cognitive impairment onset as dependent variable.). Factors that were associated with cognitive symptom onset were gait disturbance, age of onset, orthostatic hypotension and hallucinations. These factors were entered a multivariate regression analysis with cognitive impairment onset as the dependent variable. With all factors controlled for, gait disturbance onset, age of onset and hallucinations onset predicted cognitive symptoms onset (Table 3.2). Early cognitive decline was predicted by early gait disturbance onset, older age at diagnosis, and early hallucinations onset.

Table 3.1: Multivariate regression analysis with cognitive impairment onset as dependent variable. F = 41.12, P < 0.0001, R^2 = 0.5264. All significant predictor variables displayed.

Predictor	β co-efficient (95% CI)	P Value	Significance
Constant	9.667 (4.134 – 15.20)	0.0008	***
Gait disturbance onset	0.3189 (0.1539 – 0.4839)	0.0002	***
Age of onset	-0.1098 (-0.1878 – -0.0317)	0.0063	**
Hallucinations onset	0.4468 (0.3163 – 0.5773)	<0.0001	****

However, these factors were predictive of cognitive impairment onset whether it predated or occurred after. For example, hallucinations were often found to develop secondarily to executive, memory and visuospatial dysfunction, confusion or inattention. In contrast, gait disturbance was found to predate cognitive impairment onset in all but 15 of 189 cases. The difference in timing between cognitive impairment and gait disturbance was calculated by subtracting the year of gait disturbance from year of cognitive impairment – a positive result would indicate that gait disturbance

predated cognitive impairment. The same formula was used to calculate the differences between cognitive impairment onset and other motor symptoms such as tremor and rigidity (bradykinesia is an omnipresent symptom in the diagnostic schema for PD (Postuma *et al.*, 2015) and so is inappropriate in this analysis). Given that gait disturbance onset is predictive of cognitive impairment onset, an understanding of the timescale of predictive capacity of this symptom – e.g. whether the onset of gait disturbance significantly associated with a risk of cognitive impairment within 5 years – was sought. On average, the interval between gait and cognitive impairment was 3.64 years compared to 8.14 years for tremor and 9.06 years for rigidity. Cases were categorised into two groups depending on whether cognitive impairment occurred within 5 years of gait impairment, tremor or rigidity respectively, or not. X^2 analysis revealed that gait disturbance onset was significantly more associated with cognitive impairment within 5 years than either tremor or rigidity onset ($X^2 = 24.32$, $p < 0.0001$).

3.4.3. Cross-sectional predictors of self-rated cognitive impairment severity

Using the Parkinson's UK Tissue Bank Donor Follow-up Questionnaire, composite scores were created for tremor, bradykinesia, rigidity, gait disturbance, postural instability, neuropsychiatric symptoms, sleep disturbance, anosmia, cognitive impairment and others. The composite motor scores were used to determine current motor subtype (TD, Mixed or AR) in these cases, and the cognitive scores in each of these subgroups were compared. I found no significant differences between the cognitive impairment scores for each of these motor subtypes ($p = 0.2773$). Though AR cases showed the highest average cognitive impairment score and TD the lowest average score, the variance of the data was wide, meaning that motor subtype was not an explanatory variable for cognitive impairment. To ascertain which, if any, variables in the data set explained the variance in cognitive impairment severity score, the composite scores for each symptom were entered into a correlation matrix to assess correlation with cognitive impairment and to remove collinear variables. Variables significantly correlated with cognitive impairment were gait disturbance, sleep disturbance, neuropsychiatric symptoms, speech/swallow impairment, orthostatic symptoms, anosmia and hallucinations. These were entered into a multivariate regression analysis. Of these factors, gait disturbance, neuropsychiatric symptoms, anosmia and hallucinations were all significantly associated with cognitive impairment in the regression model, when all other variables were controlled for (Table

3.2). Higher scores in each of these symptom domains was strongly and significantly associated with higher scores in the cognitive impairment domain. Together, the entire model explained 38.11% of the variance in cognitive score data, suggesting that, though the model captures a significant proportion of the spread of data, there are still significant predictor variables not covered.

Table 3.2: Multivariate regression analysis of the cross-sectional cohort with cognitive impairment score as the dependent variable. F = 29.44, P < 0.0001, R^2 = 0.3811. All significant predictor variables are displayed in the table.

Predictor	β co-efficient (95% CI)	P Value	Significance
Constant	0.6875 (-0.6560 – 2.403)	0.2613	NS
Gait disturbance score	0.4025 (0.1880 – 0.6170)	0.0003	***
Neuropsychiatric symptom score	0.2952 (0.1218 – 0.4686)	0.0010	***
Anosmia score	0.6270 (0.2835 – 0.9705)	0.0004	***
Hallucinations score	0.7586 (0.4666 – 1.051)	<0.0001	****

3.4.4. Cross-sectional relationships of cognitive impairment in the PPMI cohort

CCA was performed to assess the cross-sectional relationships of cognitive impairment severity with motor, demographic and DATscan features in the PPMI cohort (Figure 3.4). The most statistically significant mode between the cognitive and motor matrices (R = 0.3386, p < 0.0001) was back-projected onto both matrices to characterise the nature of the mode in terms of correlations between motor and cognitive features. The characteristics of this mode in the cognitive matrix were worse scores on the HVLT, BJOLT, SVF, LNS and SDM and no change in MoCA. Thus, there existed a significant positive correlation between all cognitive tests aside from total MoCA score. The mode, when projected onto the motor matrix, correlated most significantly with the motor mode characterised by high scores on bradykinesia, rigidity, gait disturbance and postural instability items, but low scores on tremor items. Therefore, there existed a significant canonical correlation between cognitive test scores and severity of AR motor features and low TD features of PD. The canonical mode was correlated with matched data from the demographic and DATscan matrices.

This revealed that cognitive side of mode 1 of the CCA between motor features and cognitive impairment was correlated with increased age and age at onset in the demographic dataset, and reduced radioligand uptake in the caudate nucleus bilaterally, but not the putamen. The motor side of the mode was associated with similar demographics but reduced uptake in all striatal regions, suggesting that dopaminergic denervation in the striatum is particularly related to bradykinesia, rigidity and gait impairment.

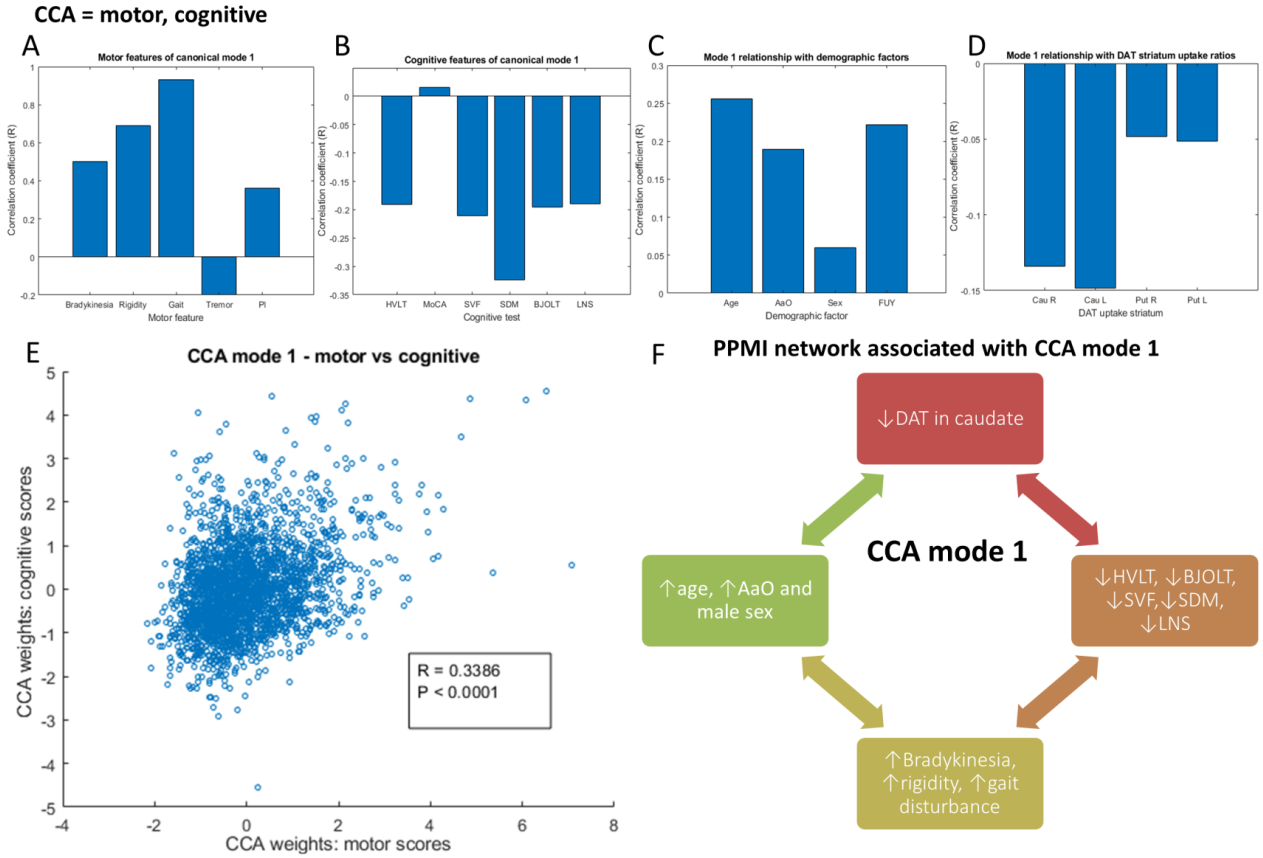


Figure 3.4: Canonical correlation analysis between motor and cognitive features in the PPMI cohort. A) motor features of canonical mode 1, B) cognitive features of canonical mode 1, C) canonical mode 1 relationship with demographic features, D) canonical mode 1 relationship with DATscan features, E) scatter plot of CCA mode 1 weights of motor and cognitive measures, F) Network of CCA mode 1. AaO = age at onset, BJOLT = Benton Judgement of Line Orientation test, Cau = caudate, CCA = canonical correlation analysis, DAT = dopamine transporter, HVLT = Hopkins Verbal Learning test, LNS = Letter-number sequencing, PI = postural instability, PPMI = Parkinson's Progression Markers Initiative, Put = putamen, SDM = symbol-digit modalities, SVF = semantic verbal fluency. Analysis carried out on MATLAB 2019a.

3.5. Discussion

In this chapter, I have been able to provide unique insights into the temporal relationship between AR features and cognitive impairment. The previous literature has focused on baseline features as predictors of progression to cognitive impairment (Burn, Rowan, Allan, Molloy, O 'brien, *et al.*, 2006; Williams-Gray *et al.*, 2007, 2013; Anang *et al.*, 2014; Johnson *et al.*, 2016; Lawson *et al.*, 2016; Rajput *et al.*, 2017). For example, a number of studies have shown that non-tremulous phenotype at baseline is significantly associated with faster decline to cognitive impairment and dementia (Williams-Gray *et al.*, 2007, 2013; Johnson *et al.*, 2016). However, it is well-known that the progression of PD is highly dynamic, in that motor features do not remain stable over time, such that tremulous subtype gives way with advanced disease to akinetic features. This transition has been noted in one study to give rise to increased risk of cognitive impairment. One of the aims of my study was to determine the temporal relationship of the transition to AR phenotype with incidence of cognitive impairment. I have been able to show that evolution of AR subtype is significantly associated with the incidence of cognitive impairment in PD, within 5 years, with the vast majority of cases developing cognitive impairment within 3 years of the onset of cognitive impairment. By comparison, no such relationship exists for tremor or rigidity (bradykinesia in an omnipresent symptom in PD according to the current diagnostic criteria and therefore was not analysed). Our findings were similar to that of Velseboer and colleagues who found that gait disturbance features were associated with unfavourable outcomes at 5 year follow-up (Velseboer *et al.*, 2016). Similarly, though not an overt conclusion of the study, Prange and colleagues found that dementia tended to occur between 3 to 5 years after the onset of FOG in PD. These data raise the question of how useful motor subtype is for predicting PD-related cognitive decline, as bradykinesia, rigidity and gait disturbance and grouped together in one subtype, yet only one of these symptoms is predictive of impending cognitive decline. Moreover, the motor features of PD seem to have independent relationships to cognitive decline. For example, gait disturbance is predictive of cognitive impairment whether tremor is present (Mixed subtype) or not (AR subtype). This is highlighted by the data presented in Figure 3.1, Figure 3.2 and Figure 3.3 which outlines how Mixed cases have a similar association with cognitive impairment to AR cases. This agrees with the previous literature where Mixed cases were grouped together with AR cases as opposed to TD cases when trying to predict cognitive decline (Williams-Gray *et al.*, 2007).

To investigate further the link between gait disturbance and cognition, it was sought to determine whether gait disturbance severity, as opposed to presence or absence, was related to the severity of cognitive impairment at a cross-sectional, single time point level. We found that gait disturbance severity, along with hallucinations and anosmia severities was able to predict the severity of cognitive impairment. This meant that severe gait disturbance was likely to coexist with severe cognitive impairment scores. One study has been performed relating postural stability and cognitive impairment severity at a single time-point, finding that worsened postural stability shows a strong correlation with cognitive impairment, measured by Mini Mental State Examination (MMSE) score (Lee *et al.*, 2012). To my knowledge, this is the first study to attempt to link gait disturbance severity with cognitive impairment severity at a cross-sectional level. This, in combination with the longitudinal data, suggests that gait and cognition in PD are inextricably linked phenomena.

Previous studies on this topic have employed interesting techniques to refine the regression models used to predict cognitive impairment in PD, such as backwards stepwise regression. The backwards stepwise regression method removes the most insignificant variables one at a time to leave just the most significant factors (Williams-Gray *et al.*, 2007). However, using this method risks being an oversimplification of real life given that, in a patient, you cannot remove characteristics to distil down to a few significant predictors. Thus, if a factor is predictive when all measured characteristics are present, this is the most accurate representation of real life. I have applied this reasoning to the statistical methods in this study. All factors considered, the strongest predictors of cognitive impairment, both longitudinally and at the cross-sectional level, were gait disturbance, anosmia, hallucinations. Older age of onset was also associated with earlier cognitive decline in the longitudinal study.

There were several weaknesses in this study such as the retrospective nature of clinical case note evaluation. This reduces the accuracy of the analysis as the data collection was heavily dependent on various factors: 1) the detail included by the physician in the clinical notes, 2) the time interval between clinic follow-up appointments, 3) the completeness of the set of clinical case notes sent to the Tissue Bank, upon request, from the donor's General Practitioner. Variability of these three factors was evident when appraising the cases. This also meant that critical details

such as the laterality of PD symptoms – whether they were unilateral or bilateral and symmetric – were inconsistently documented. The lack of data on these details is a weakness of the statistical models that I have developed in this chapter. Incomplete clinical documentation is also a source of bias in the data collection from retrospective clinical case note evaluation. To protect against these biases, longitudinal data from the PPMI cohort was employed. I found that the main motor mode, characterised by gait disturbance, bradykinesia and rigiditiy, along with age of onset and sex, displayed an intimate relationship with most measures of cognitive impairment, except for overall MoCA score. Therefore, this analysis provided validation for the conclusions I made based on the data from the Parkinson's UK Tissue Bank cases. Further work will attempt to refine the predictive model from the PPMI dataset further. The study is longitudinal which means that the CCA can be adapted to include a longitudinal component. Additional variables such as genetic status and previous medical history of vascular disease and metabolic syndrome may help to capture more of this variation. My analysis of cross-sectional questionnaire data attempted to link the severities of different types of PD symptoms. However, the severity of each symptom was rated subjectively from each patient, which means that there is inbuilt variability in the data caused by differences in opinion from patient to patient. The subjective ratings of symptom severity may bear no resemblance to a clinician-rated assessment based on neurological examination. Moreover, the association of gait impairment with incipient cognitive decline could be confounded by the presence of normal pressure hydrocephalus: the accumulation of CSF with little or no increase in intracranial pressure.This phenomenon can cause reversible cognitive impairment, gait difficulties and bladder issues among others. It is also significantly underdiagnosed, and is rarely noted in retrospective clinical case notes.

A recent trend in the evaluation of motor function in PD is wearable technology. Toosizadeh and colleagues used wearable technology to compare functional measures, such as a timed up-and-go test in the clinic compared with spontaneous physical activity at home (Toosizadeh *et al.*, 2015). While the findings of this study revealed a lack of correlation between these measures in PD cases, they did find a strong correlation in healthy controls. Function in PD is variable over time. Perhaps a longer time-period for measurement of function is needed for the assessment of PD symptoms with wearable technology. Wearable technology could be employed for

long-term assessment of PD patients, in a similar way to the use of 24-hour ECG Holter monitoring for cardiac arrhythmias, as an adjunct to examination in the clinic. The development of technology has the potential to greatly increase our accuracy of detection of PD symptoms. Some studies have used accelerometry on mobile phones among other measures to assess postural stability (Alberts *et al.*, 2015; Ozinga *et al.*, 2017; Ozinga, Linder and Alberts, 2017). The use of smartphone technology and tablets can extend to the use of cognitive 'games' as another way of assessing the cognitive function of patients in the long-run. Nombela and colleagues, in the ICICLE-PD study, used tasks such as the tower of London test, spatial rotations and memory encoding tasks to test executive function, visuospatial function and memory function respectively (Nombela *et al.*, 2014). Though these tests were employed in an MRI scanner to compare with neuroimaging findings, they would be equally applicable on their own as a means of testing cognitive function in PD. Novel technologies are an exciting new way of assessing patients at home where they cannot be thoroughly assessed in clinic due to time or funding constraints.

Studies on the natural history and evolution of phenomena associated with PD are vital to understand the aetiology, pathophysiology and clinical phenotypes of PD. Such studies may provide exciting new insights and opportunities for novel therapeutic approaches to slowing physical and mental decline in PD.

3.6. Conclusion of chapter

In this chapter, I have shown that PD motor subtype at diagnosis is associated with differences in longitudinal progression to cognitive impairment in PD. However, as motor subtype varies significantly over time, the incidence of akinetic features such as gait disturbance was investigated as a temporal predictor of cognitive decline as a replacement for motor subtype. In doing so, I have added a more nuanced perspective on the inextricable link between gait disturbance and cognition, which has been highlighted in previous studies.Crucially, I have validated these conclusions using data from the PPMI cohort study. This data has the potential to yield significant utility clinically in the prediction of cognitive impairment in PD cases – a phenomenon that is associated with significant mortality in PD and is of grave concern to patients and family members. Clinical cohort studies with long follow-up periods and rigorous experimental protocols are best-placed to validate the data in this chapter and are vital to understanding the pathophysiology of the PD clinical syndrome. To summarise, I have been able to confirm my initial hypothesis that there is a temporal relationship between gait disturbance features and the incidence of cognitive impairment.

Chapter 4 - Mapping the substantia nigra – a novel midbrain tegmentum atlas

4.1. Introduction

The midbrain is the most densely populated dopaminergic region in the human and primate brain. It contains three main dopaminergic cell populations: 1) The A8 group of the retrorubral field 2) the A9 group of the SN and 3) the A10 group of the medial and dorsal tegmentum (Dahlstroem and Fuxe, 1964; McRitchie, Hardman and Halliday, 1996). Each of these mesencephalic subregions performs different functions. The functions of the A9 are the best characterised out of all of the aforementioned populations: it shares reciprocal connections with the dorsal striatum to exert control over motor function via dopaminergic manipulation of cholinergic, glutamatergic and GABAergic neurons (Pisani *et al.*, 2000; Centonze *et al.*, 2003; Calabresi *et al.*, 2007). In contrast the functions of the A8 and A10 are much less well known. The A8 is thought to project to the dorsolateral striatum and the substantia nigra pars reticulata-thalamus-prefrontal cortex pathway (Deutch *et al.*, 1988; Percheron *et al.*, 1994). There is some evidence to suggest that loss of neurons in the A8 region plays a role in the pathophysiology of PD rest tremor (Hirsch *et al.*, 1992). It has also been shown to project to the hippocampal formation in the rat (Gasbarri *et al.*, 1996). The A10, which is independent of striatal influence, shows considerable projections to limbic brain regions such as the amygdala, hippocampus, medial striatum and cingulate gyrus as well as to the motor and premotor cortices in the primate brain (Gerfen, Baimbridge and Miller, 1985; Gerfen, 1992; Lynd-Balta and Haber, 1994). Domesick summarised her group's study of the connections A10 in the rat, citing the main projections as reciprocal to limbic regions such as the central nucleus of the amygdala and the reciprocity of connections between these regions and the VTA (Domesick, 1988). These studies in rodents and primates have formed the presumed basis of the human anatomy of these pathways, though similar studies in humans are unfortunately lacking. Thus each dopaminergic cell region is functionally distinct despite sharing close anatomical proximity to one another (McRitchie, Hardman and Halliday, 1996). Moreover, the dopaminergic cell groups concerned show differential susceptibility in neurodegenerative conditions such as Parkinson's disease (PD) (McRitchie, Cartwright and Halliday, 1997; Hall *et al.*, 2014). It remains to be understood why neighbouring nuclei are targeted in such a distinctive pattern. A precise knowledge of the anatomy of this small but densely packed area is required to ensure the correct targeting of specific regions for study, as loss of neurons in different regions should have differential pathophysiological consequences.

A number of previous attempts have been made to define the anatomy of the region in atlases. The first characterisation of the A10 region came from Dahlstrom & Fuxe who studied the mesencephalon of the rat (Dahlstroem and Fuxe, 1964). They created a subregional atlas of the A10 by subdividing it into 6 constituent nuclei: the parabrachial pigmented nucleus (PBP), the caudal and rostral regions of the linear nucleus (CLi and RLi), the oculomotor nucleus, Edinger-Westphal nucleus and the interstitial nucleus of the ventral tegmental decussation in between the fasciculi retroflexus. Saper and Petito aimed to define the human analogue to the A10 focussing on melanin-pigmented neurons as the human equivalents of the catecholaminergic neurons found in the rat (Saper and Petito, 1982). They defined the A10 as a region of pigmented neurons extending ventrally from the ventrolateral aspect of the medial longitudinal fasciculus (MLF) to the dorsomedial aspect of the substantia nigra. Fearnley & Lees defined tiers of the SNc such as the dorsal and ventral tiers, which they used as the basis for clinicopathological investigations of the SN in PD and controls (Fearnley and Lees, 1991). McRitchie and colleagues defined the region both with tyrosine hydroxylase (TH) immunohistochemistry and with calcium binding proteins (McRitchie, Hardman and Halliday, 1996) as well as using substance P immunohistochemistry to highlight the SN (Figure 4.1). In so doing, the study redefined nuclei that were originally included in the A9 as A10 nuclei: the paranigral nucleus (PN) and PBP. However, this atlas used distance in cm along the rostrocaudal midbrain length as a means of defining anatomical landmarks for the isolation of nuclei. The length of the human midbrain differs significantly in brains of different sizes and thus the atlas is difficult to apply to individual cases. Further, the population of nuclei at different rostrocaudal levels was not quantified to find the maximal density. This would be beneficial for research aiming to target specific nuclei for study. More recent literature has attempted a simplified atlas by subdividing the A10 into medial and lateral divisions (Hall *et al.*, 2014) but this does not allow for the study of individual nuclei and their connections.

Accurate brain atlases are important for conducting reliable clinicopathological studies. Before the publication of the best midbrain atlas to date (McRitchie, Hardman and Halliday, 1996; McRitchie, Cartwright and Halliday, 1997), a number of clinicopathological correlative studies were carried out on the SN in PD, including those of Paulus and Jellinger who posited that PD motor subtypes arose due to

differential pigmented neuron loss in the SN (Paulus and Jellinger, 1991, 1992). The advent of good atlases showed that the methodology in the studies of Paulus and Jellinger was not optimal. There is a need to reassess their results with a novel midbrain atlas.

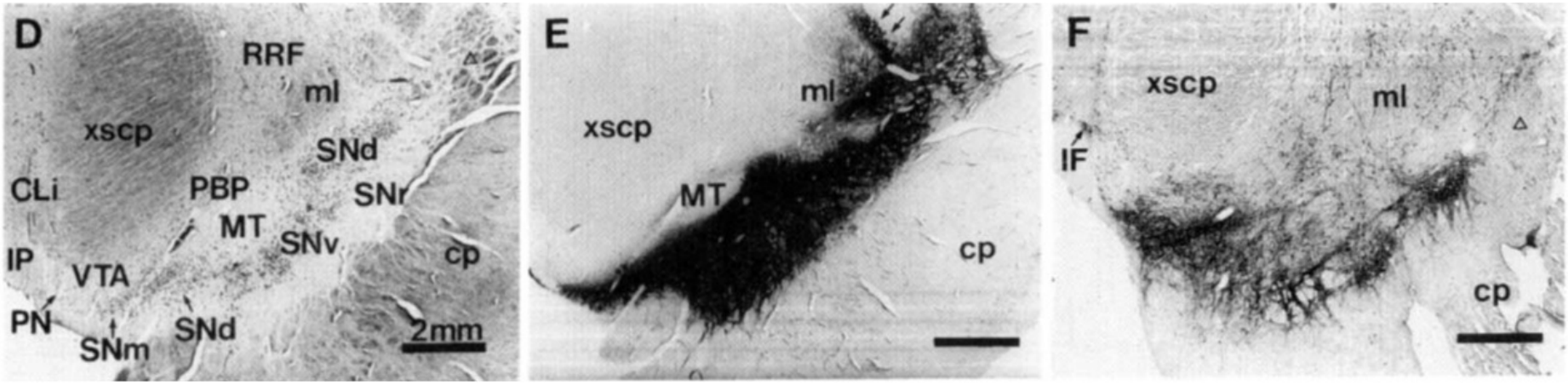


Figure 4.1: Midbrain atlas created by McRitchie and colleagues using SP and TH immunohistochemistry. Images adapted with permission from (McRitchie, Hardman and Halliday, 1996). CLi = caudal linear nucleus, cp = cerebral peduncle, D) Cresyl violet staining of the midbrain, E) SP staining of the midbrain, F) TH staining of the midbrain. Scale bar in all = 2mm.

4.2. Hypothesis and aims of Chapter

In this Chapter, I hypothesised that the dopaminergic populations of the midbrain tegmentum will be more readily segregated with a novel midbrain atlas that utilises the strengths of previous atlases while improving on their weaknesses. I aimed to address this hypothesis by: 1) defining rostro-caudal anatomical landmarks as atlas reference points instead of measuring by midbrain length (McRitchie, Hardman and Halliday, 1996); 2) defining TH positive populations and their distributions relative to these reference points using TH and SP immunohistochemistry; 3) investigating other methods for distinguishing tegmental dopaminergic populations such as microscopic morphologic analysis.

4.3. Methods

4.3.1. Human midbrain tissue

Post-mortem human brain tissue was collected with prospective consent as part of the Parkinson's UK Tissue Bank and the archival Corsellis Collection at Imperial College London. Whole brain samples were processed as described in Chapter 2.1.5. One certified control case, i.e. with no documented neurological or neuropathological evidence of disease was used to create the atlas. This was a 75 year-old female, with no evidence of neurological disease, who died of a myocardial infarction.

4.3.2. Histology and immunohistochemistry

For assessment of anatomical landmarks in midbrain sections, LFB staining was performed according to the protocol outlined in Chapter 2.2.2 using the periodic acid-Schiff staining method. Serial sections were then immunostained against TH and SP according to the protocols outlined in Chapter 2.3 and Table 2.3.

4.3.3. Creating the atlas

One in every ten 7μm midbrain sections were stained for TH, SP and LFB in series. Thus, a complete set of slides were stained at 70 μm intervals throughout the midbrain. LFB samples were then assessed for the presence of defined landmarks (colliculi, oculomotor nucleus, oculomotor nerve exit point, trochlear nucleus, emergence of LC) in the rostrocaudal plane.

SP stains the striatonigral GABAergic fibres that innervate the A9 neurons but not A10 neurons, thereby separating the A9 and A10 cell groups definitively. The SP-stained area was superimposed on the TH stained slides to demarcate the A9 and A10

A10 nuclei (PN, PBP, IF, VTA, RLi, CLi) were defined according to the subdivision stated in the atlas of McRitchie and colleagues (McRitchie, Cartwright and Halliday, 1997). For simplicity, the caudal and rostral linear nuclei were treated as one entity and entitled 'linear nucleus'. At each rostrocaudal level, quantification of neurons in each A9 and A10 nucleus was performed.

4.3.4. Microscopy and quantification

All atlas slides were scanned using a Leica Aperio AT2 slide scanner, as outlined in Chapter 2.6.1. Microscopically, slides were assessed on an Olympus Vanox AHBT3 with a 4x objective lens and images captured using with a Q-Imaging Micropublisher

3.3 camera. Images were quantitatively analysed by manual tagging in defined regions of interest for each nucleus. For longitudinal nuclei such as the linear nucleus or SN, multiple regions of interest were sampled by capturing multiple adjacent images. Each nucleus was then assessed for the area of maximal neuronal density for the atlas.

4.4. Results

4.4.1. Defining rostro-caudal atlas levels

Anatomical landmarks that are present in all human midbrains and appear in a consistent rostro-caudal series were audited for this study. These included, from rostral to caudal, the bodies of the superior colliculi, the exit of the oculomotor nerves, the bodies of the oculomotor nuclei, the rostral emergence of the trochlear nuclei, the bodies of the trochlear nuclei, the bodies of the central nuclei of the inferior colliculi and the rostral emergences of the LC (Haines, 2015). For use in this study, evenly spaced landmarks were selected based on their ease of identification on standard histological LFB staining. These were, from rostral to caudal: the bodies of the oculomotor nuclei (Figure 4.2A), the rostral emergences of the trochlear nuclei (Figure 4.2C), the bodies of the trochlear nuclei (Figure 4.2E), the bodies of the central nuclei of the inferior colliculi (Figure 4.2G) and the rostral emergences of the LC (Figure 4.2I).

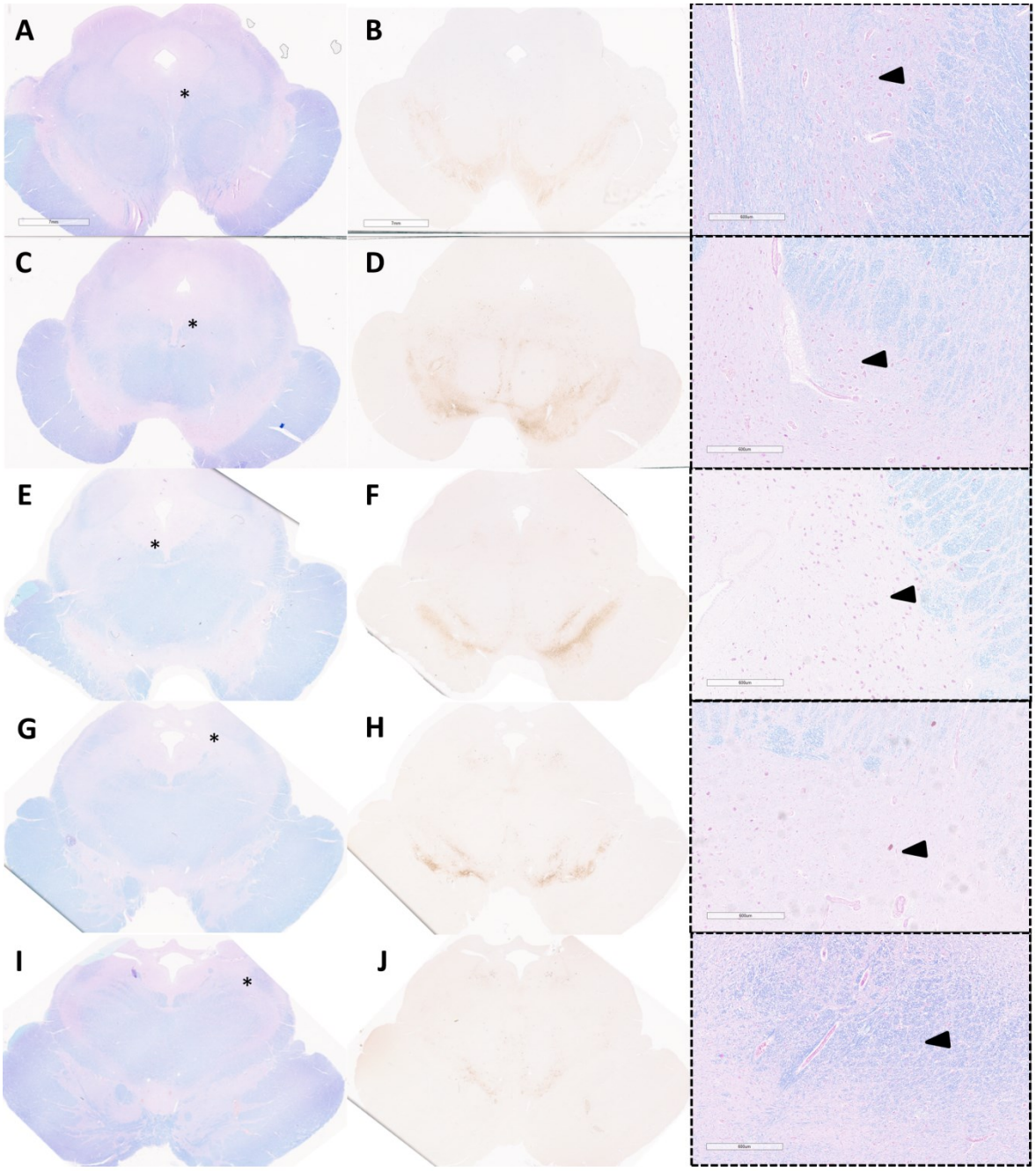


Figure 4.2: The five rostro-caudal levels of the midbrain atlas. A) LFB section of the midbrain at the level of the oculomotor nucleus body, B) TH stained section at the level of the oculomotor nucleus body. C) LFB section of the midbrain at the level of the rostral emergence of the trochlear nucleus, D) TH stained section at the level of the rostral emergence of the trochlear nucleus. E) LFB section of the midbrain at the level of the trochlear nucleus body, F) TH stained section at the level of the trochlear nucleus body, G) LFB section of the midbrain at the level of the rostral emergence of the locus coeruleus, H) TH stained section at the level of the rostral emergence of the loci coeruleus, I) LFB section of the midbrain at the level of the body of the inferior colliculus, J) TH stained section at the level of the body of the inferior

colliculus. * denotes location of anatomical landmark and position of 4x objective image (in boxes). Scale bar in A & B denotes 7mm, 600μm in boxes.

4.4.2. The relationship of TH and SP staining

TH stained a large number of neurons and neuronal processes in the tegmentum at all rostro-caudal levels (Figure 4.2B, D, F, H & J), in the A9 and A10 regions, as well as some smaller neurons in the periaqueductal grey matter, and the trochlear nucleus. SP labelled fine fibres in a dense neuropil in the A9 but considerably less staining was present in the A10 (Figure 4.3). Thus, where dense SP staining was found in the tegmentum, this was used to delineate the A9 from the A10. SP staining was also detected in the ventral periaqueductal grey matter, trochlear nuclei, LC and around the border of the central nuclei of the superior and inferior colliculus. Applied to a case with pathologically confirmed PD, this technique revealed severe degeneration of the SN, with more mild loss in the A10 nuclei (Figure 4.4).

4.4.3. TH populations vary through the extent of the midbrain

Populations of TH-neurons of the A9 and A10 were distributed differently throughout the rostro-caudal extent of the atlas. As demonstrated in Figure 4.2, the pattern of TH staining was noticeably different at a macroscopic level. At rostral levels, densely TH-stained populations tracked the circumference of the red nucleus. Extending ventrally from the red nucleus, TH neurons were organised in laminar distribution, which could be delineated using SP immunohistochemistry. The first layer is not SP stained, and so forms part of the A10 and was believed to be the PBP, as highlighted by previous atlases (McRitchie, Hardman and Halliday, 1996; McRitchie, Cartwright and Halliday, 1997). Further SP-negative populations were located medially to this nucleus, which were adjudged to be the VTA and PN nuclei. At the midline, linearly-arranged, reticular neurons were located bilaterally, and adjudged to be the LiN. In previous atlases, a distinction was made between rostral and caudal LiN, but this appeared to be arbitrary, and no clear distinction could be made in my atlas. Therefore, these were grouped as one nucleus. The SN spanned the dorsal border of the cerebral peduncle, extending dorsolaterally from the ventrolateral borders of the VTA and PN. Moving caudally through the midbrain, further SP-negative TH populations arose in the tegmentum. These were located in the midline, ventral to the bodies of the LiN and dorsomedial to the PN and VTA. Upon inspection of the area on LFB stained slides, the nuclei were interspersed among myelinated fascicles spanning transversely across the midline

between the decussations of the superior cerebellar peduncle. These nuclei were determined to be the IF nuclei of the A10. Approaching the mesopontine junction, at the level of the rostral emergences of the LC, A10 populations in the midline began to dissipate. The PBP and SN were still densely stained at this level. At the level of the inferior colliculi, only SNm populations remained, as revealed by SP immunostaining.

The distributions of these nuclei was mapped to determine their points of maximal density and rostral and caudal boundaries (Figure 4.5). Generally, the maximal densities of A10 populations were found rostrally to that of the A9, between the oculomotor nucleus body and rostral emergence of the trochlear nucleus. The VTA and LiN found their maximal densities more rostral to that of the PBP, PN and IF, whose maximal density was best located at the rostral emergence of the trochlear nucleus. The SNm and SNl showed maximal densities at the same rostro-caudal level which was the body of the trochlear nucleus. The SNm and SNl extended caudally to the most caudal boundary of the atlas, whereas nuclei of the A10 reach their caudal boundary at a more rostral level. The VTA and PN extend as far as the rostral emergence of the LC and the IF, PBP and LiN extend no further than the central nucleus of the inferior colliculus.

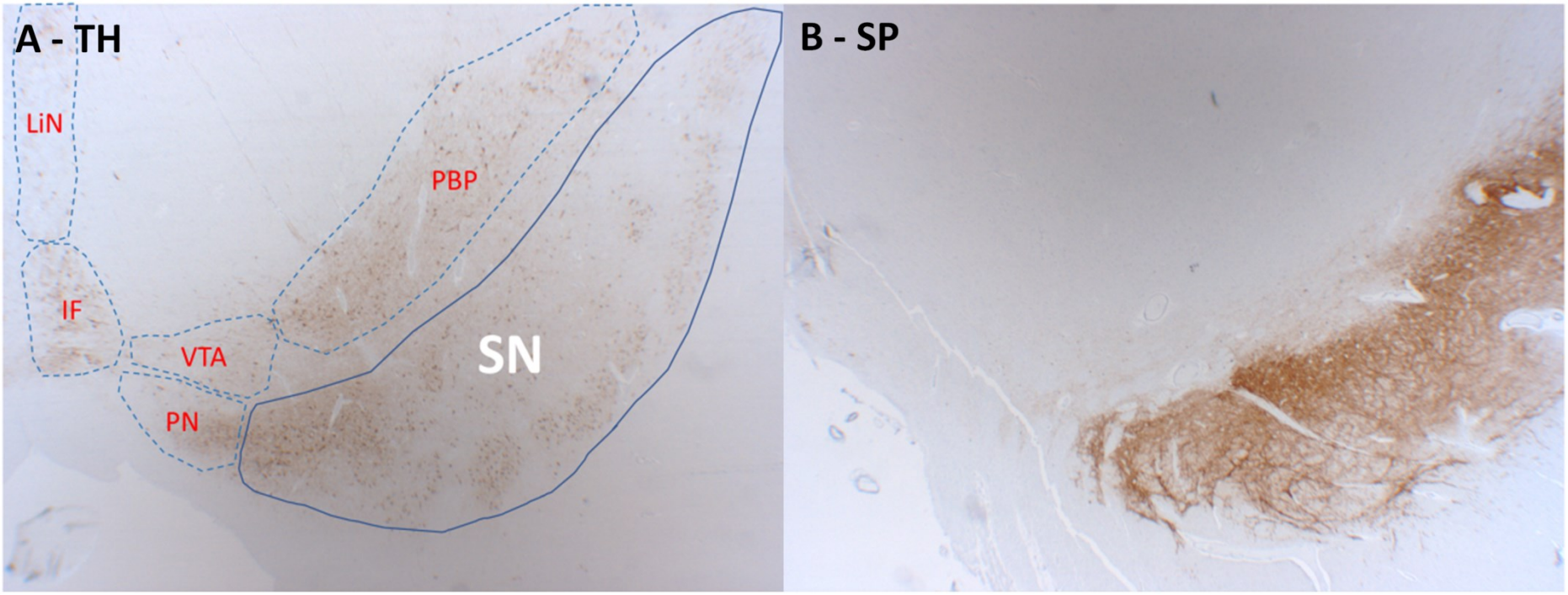


Figure 4.3: TH and SP staining in the tegmentum in the midbrain atlas IF = interfascicular nucleus, LiN = linear nucleus, PBP = parabrachial pigmented nucleus, PN = paranigral nucleus, SN = substantia nigra, SP = substance P, TH = tyrosine hydroxylase, VTA = ventral tegmental nucleus. SP colocates with TH in the SN but not in the A10 nuclei (VTA, PBP, PN, IF, LiN) .

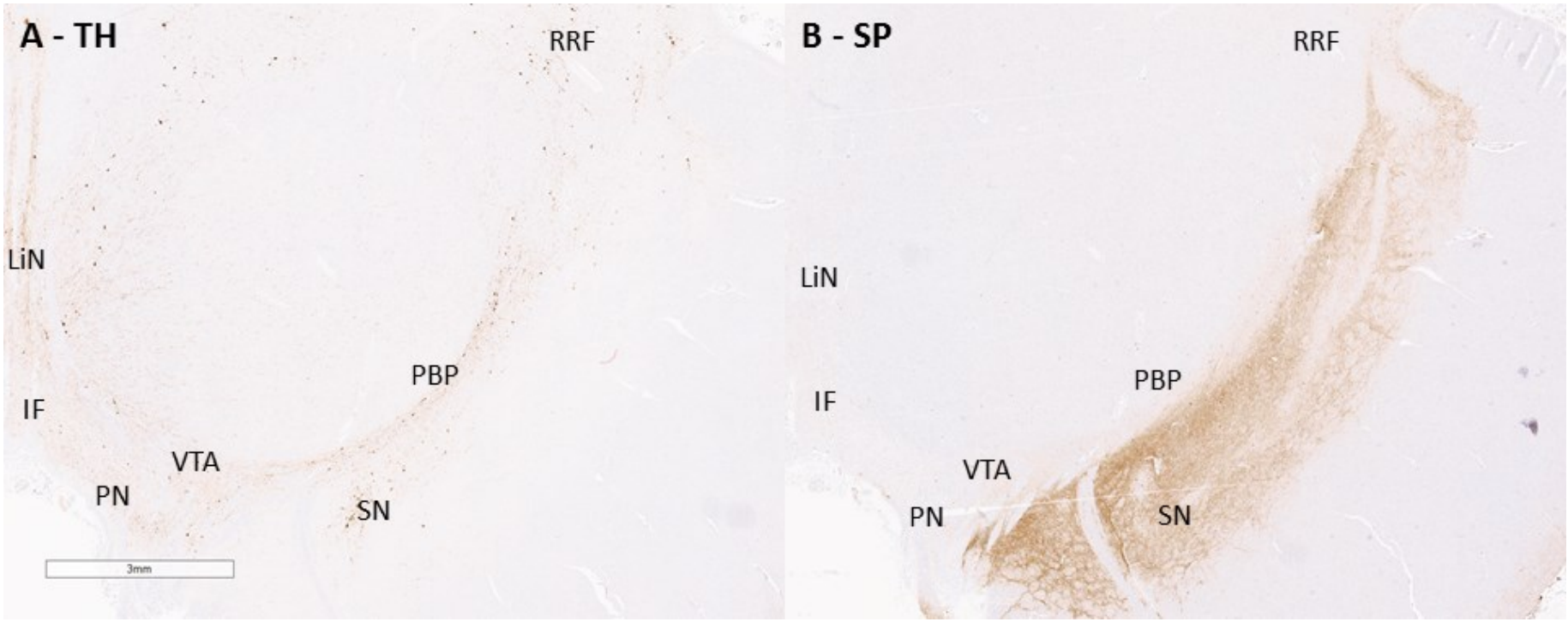


Figure 4.4: SP and TH immunohistochemistry applied to a PD case. A) TH staining in a PD case, B) SP staining in a serial section. TH and SP colocate in the SN but not in the A10. IF = interfascicular nucleus, LiN = linear nucleus, PBP = parabrachial pigmented nucleus, PN = paranigral nucleus, RRF = retrorubral field, SN = substantia nigra, VTA = ventral tegmental area. Scale bar denotes 3mm for all panels.

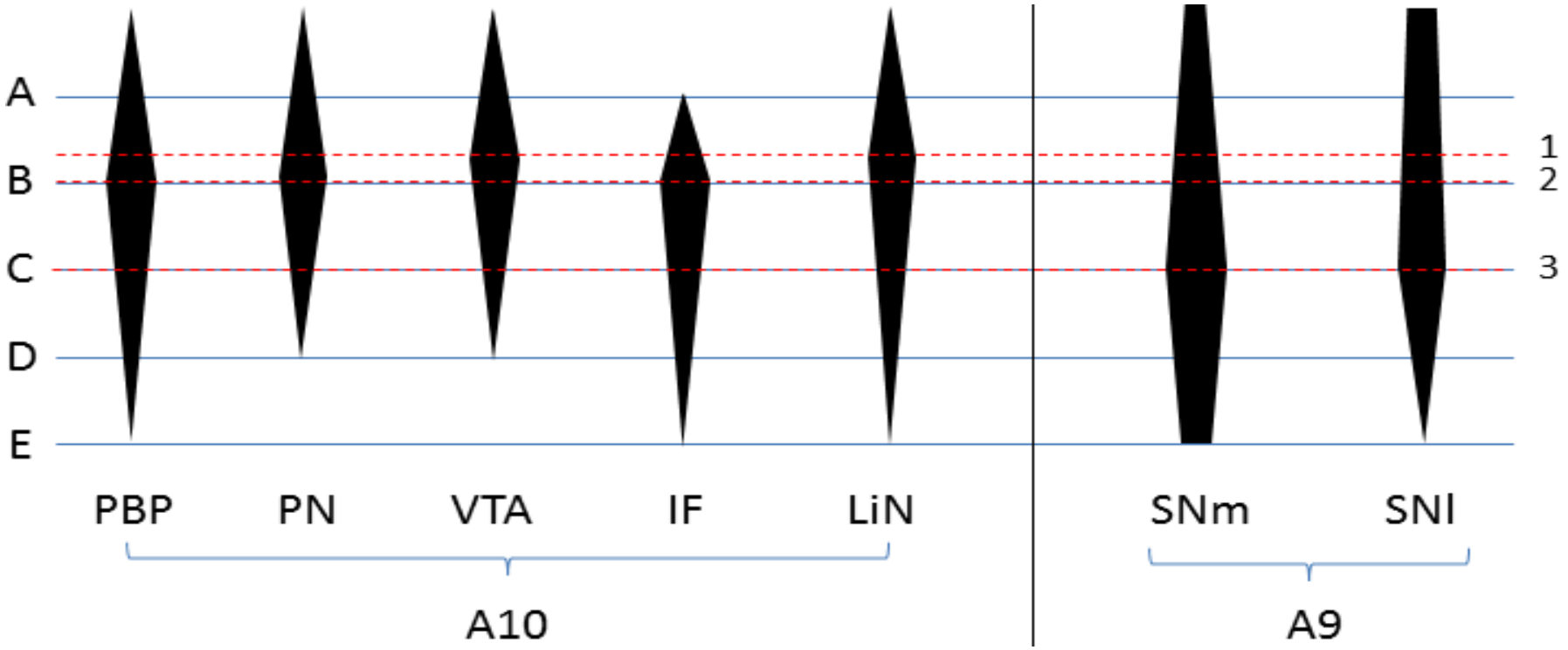


Figure 4.5: The distribution and maximal densities of nuclei of the A9 and A10 in the midbrain. A = body of the oculomotor nucleus, = rostral emergence of the trochlear nucleus, C = body of the trochlear nucleus, D = rostral emergence of the locus coeruleus, E = body of the central nucleus of the inferior colliculus. PBP = parabrachial pigmented nucleus, PN = paranigral nucleus, VTA = ventral tegmental area, IF = interfascicular nucleus, LiN = linear nucleus, SNm = medial substantia nigra pars compacta, SNl = lateral substantia nigra pars compacta. 1 = maximal density of the VTA and LiN, 2 = maximal density of the PN, PBP and IF, 3 = maximal density of the SNm and SNl.

4.4.4. Differentiating the A9 and A10 based on TH microarchitecture

Though it was possible to delineate the TH populations of the tegmentum using SP immunohistochemistry, this was not the only differentiating factor between the A9 and A10. In fact, at the microscopic level, there were many distinguishing features between populations of the A9 and A10 (Figure 4.6); including differences in the size, orientation and arrangement of neurons in the nuclei, and the size, density and organisation of TH stained fibres in the neuropil. The SN exhibited the largest neurons, with an approximate average diameter of 30-40μm (Figure 4.6A). The orientation of these neurons appeared random in nature, with many large projecting fibres extending in all directions. The neuropil was characterised by many large and medium-calibre fibres, with comparatively few fine fibres. This suggests that these fibres target structures in a different brain region, rather than projecting locally. There were no appreciable differences in this arrangement between the SNm and SNl.

The most similar A10 nucleus to the SN in terms of microarchitecture was the VTA (Figure 4.6E). The neurons appeared marginally smaller than the SN –25-30μm in diameter – but the orientation and organization of the neurons and fibres also appeared to be random. In contrast to the SN, the size of the TH fibres in the VTA appeared to be more mixed, with many more fine fibres staining a dense neuropil. This suggests that there is complexity in the projections of the VTA, perhaps extending to distant brain regions but also involved in local circuitry. The commonalities of the VTA and SN may highlight a shared developmental origin, which seems intuitive given that they both project to regions of the striatum – dorsal striatum for the SN and the ventral striatum/ nucleus accumbens for the VTA. Conversely, the microstructures of the LiN (Figure 4.6B), PBP (Figure 4.6C), PN (Figure 4.6D) and IF (Figure 4.6F) appeared to share more features with each other than with the VTA or SN. These nuclei displayed small (15-25μm in diameter) reticular neurons with fibres arranged linearly in parallel bundles. The exception to this was the IF where a mix of small reticular neurons and even smaller neurons (5-10μm in diameter) was found. In general, neurons in the PBP and LiN were larger than those of the PN and IF. In contrast to the VTA, fine fibres were seldom located in the neuropil of the LiN and PBP and the clear majority of fibres were large. However, the IF and PN displayed a mix of coarse and fine fibres. In a similar manner to the LiN and PBP, coarse fibres in the IF and PN tended to arrange in parallel. Taken together, this provides evidence of a potential separation between

different nuclei of the A10, reflected in morphological variations. This may highlight functional differences in the contributions of each to the meso-limbic circuitry whereby nuclei with fine fibres in the neuropil and comparatively small neurons (PN and IF) are involved in local regulation, whereas nuclei with larger neurons and fibres may project to distant sites such as the nucleus accumbens or other limbic regions.

4.4.5. The anatomy of the retrorubral field

The RRF was characterised by sparse, large TH-positive neurons. The region was not SP positive which allowed it to be delineated from the adjacent SN pars lateralis. However, the RRF showed no signs of being a nucleus, i.e. a compact core and diffuse exterior, and large neurons were scattered in an indistinct pattern throughout the region bounded by the SN ventrally, red nucleus ventromedially, medial lemniscus dorsomedially. No clear distinction could be made between TH neurons in this region and those scattered in the reticular formation. Further research is needed to understand the anatomy of this region.

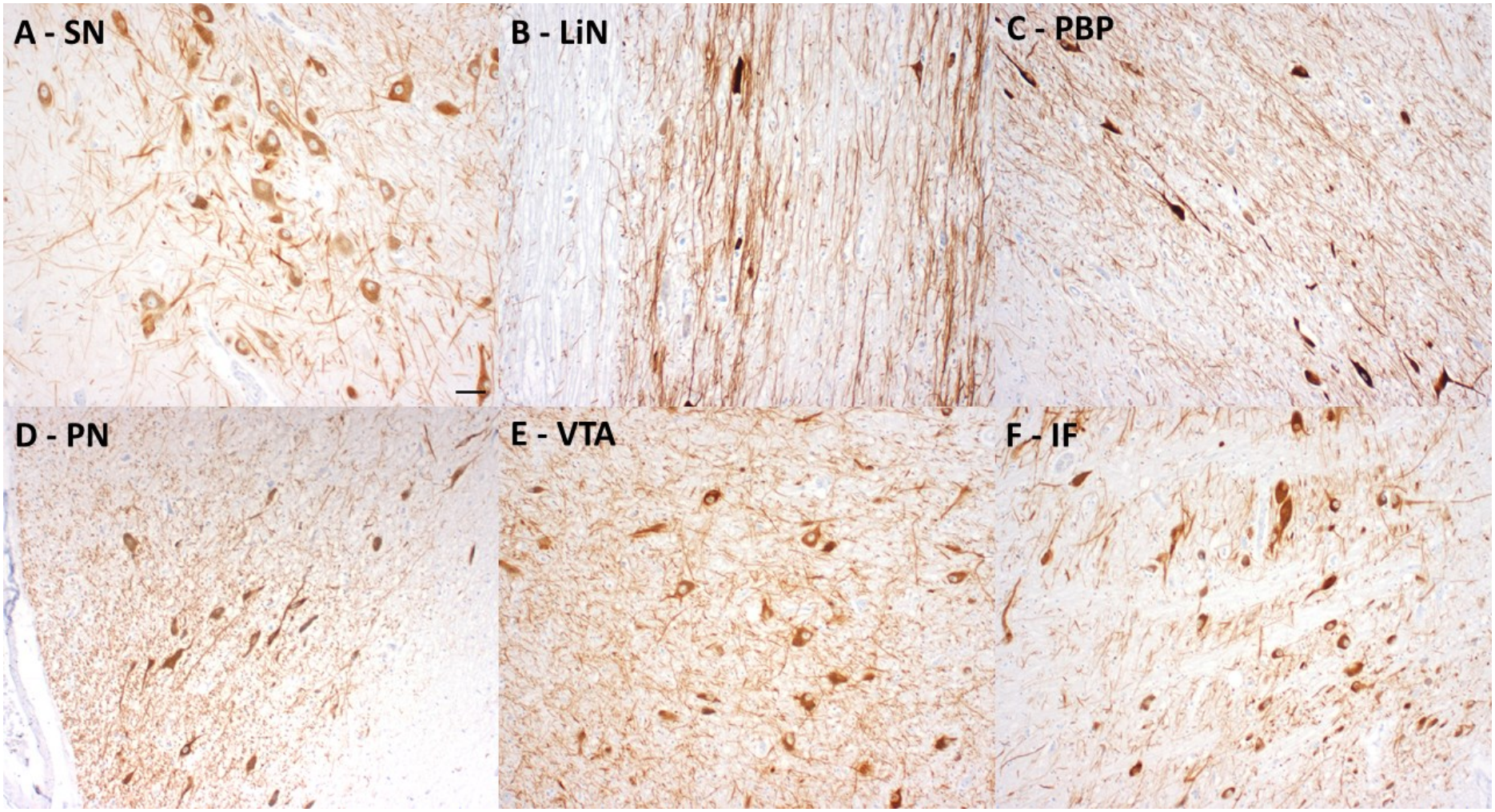


Figure 4.6: Microarchitectural differences in TH staining between nuclei of the A9 and A10. A) TH staining in the SN, B) TH staining in the LiN, C) TH staining in the PBP, D) TH staining in the PN, E) TH staining in the VTA, F) TH staining in the IF. Images captured under 10x objective lens. Scale bar in A represents 50μm for all panels.

4.5. Discussion

This chapter is the first midbrain atlas, to my knowledge, to define the distribution of the TH-positive neuronal populations of the midbrain tegmentum relative to a series of defined rostro-caudal anatomical landmarks. This is a useful technique for assessing these populations because the landmarks used are easy to locate and consistently occur in series to one another. For example, to access the SN, the best rostro-caudal level to sample at would be the body of the trochlear nucleus. Though at this level, other nuclei haven't been avoided, an additional feature of this atlas is that I have been able to differentiate the A9 and A10 nuclei based on the microarchitecture of the neurons and neuropil in TH stained sections. For example, neurons of the LiN, PBP and PN have a reticular morphology with projections arranged in parallel bundles. The exception to this rule is the VTA, which shows a more random pattern of organisation in a manner similar to the SN. The SN and VTA projections are of similar lengths, and extend to brain regions that are anatomically, functionally and developmentally linked. These commonalities suggest that the nigrostriatal and VTA-accumbens pathways develop together. The variations in the morphologies of these nuclei may reflect key differences in the projections and local regulation of each. For example, the VTA, though similar to the SN, displays more fine fibres which may indicate more complex local regulation by individual VTA neurons than that of the SN. In contrast, the fibres of the PBP and LiN are large and arranged in parallel, suggesting that there is very little local regulation and that these nuclei are exclusively projection nuclei.

There have been a number of previous atlases of the region which I have attempted to improve upon in creating this atlas. General brain atlases, such as that created by Mai and colleagues, are excellent for gross anatomical inquiries because they use histological stains such as LFB to distinguish white and grey matter (Mai, Paxinos and Voss, 2008). However, these types of atlases are far less useful for microscopic anatomy, as they are only able to give general locations of some of the more commonly sought regions, for example, the SN. This particular atlas makes no mention of any of the A10 nuclei, much less how to delineate them from the A9. This is only achievable by microscopic atlases. Saper and Petito used histological stains to delineate nuclei of melanin-pigmented neurons as the human equivalents of the catecholaminergic neurons found in the rat (Saper and Petito, 1982). Using this technique, they defined the borders of the A10 region. However, in this atlas I have

improved on this using immunohistochemistry to distinguish between A9 and A10 regions but also between sub-regions of the A10. Furthermore, I have employed these techniques at multiple levels in the midbrain, whereas Saper and Petito employed one hypothalamic level, one midbrain level, one pontine level and one medullary level. Hall and colleagues attempted to subdivide the A10 into medial and lateral portions (Hall *et al.*, 2014). While this is an easy and reproducible methodology for appraising the A10, it fails to distinguish between individual A10 nuclei, which I have done, importantly, in this atlas. The best atlas to date was created by McRitchie and colleagues (McRitchie, Hardman and Halliday, 1996; McRitchie, Cartwright and Halliday, 1997). They used immunohistochemical methods, with antibodies against TH and SP to differentiate the A9 and A10 and between different nuclei of the A10. Moreover, they used multiple midbrain levels to define their atlas. However, the position of the atlas level was defined by midbrain length, which is a curious method given that the midbrain differs in size from person to person. Further, in some neurological diseases, where such an atlas may prove useful, such as PSP, the midbrain is subject to considerable atrophy, thus reducing the volume and dimensions of the midbrain in all planes. Thus, the atlas created here, using anatomical landmarks as atlas reference points, provides a more realistic and applicable atlas to all cases. The advantage of their atlas is that they also appraised other nuclei such as the retrorubral field (A8) using calbindin D28K immunohistochemistry. They also compared the integrity of the nuclei between controls and PD cases, finding degeneration in the PBP and the parapeduncular nucleus, a region of the A10 that exists above the rostral border of my atlas. A combination of my approach, utilising anatomical reference points for defining the atlas and detailed microscopic differentiation of A10 nuclei based on TH immunohistochemistry, and the approach of McRitchie and colleagues (McRitchie, Hardman and Halliday, 1996; McRitchie, Cartwright and Halliday, 1997), assessing more nuclei in more cases would provide an excellent midbrain atlas for use in clinicopathological studies.

Yet, without these techniques, clinicopathological studies on the SN in PD have been carried out. Paulus and Jellinger, for example, found that cases of AR PD had more aggressive pigmented neuron loss in the SN and LC than TD cases (Paulus and Jellinger, 1991, 1992). However, in these studies, there was no clear methodology on how they separated the SN from surrounding A10 nuclei. We have established that it

is difficult to differentiate these nuclei using histological stains such as cresyl-violet or H&E – methods employed by Paulus and Jellinger (Paulus and Jellinger, 1991, 1992) – and that the gold standard is using TH and SP immunohistochemistry. There is therefore a need to re-appraise the findings of these studies using updated methodologies. This is what I will attempt to do in Chapter 5.

4.5.1. Limitations

This study is limited by the fact that I only used one control case for the atlas. It remains to be assessed whether different cases show a different distribution of TH-positive neuron populations relative to defined rostro-caudal anatomical landmarks. Further, this case, though a neurological control, was an aged individual, and it is well-known that nigral neurons are lost with age. Moreover, various disease processes may selectively degenerate neuronal populations at different anatomical levels, which would lead to skewed population distribution charts. Plotting these distributions in different diseases may reveal new clinicopathological relationships between brain regions and symptom onset in different neurodegenerative disorders. Future work should attempt this atlas technique in multiple control cases, and, where possible, it should be replicated in various disease cohorts. There are benefits to using melanised neuronal counts in pathological studies. These include the fact that TH levels may fluctuate over time or may be overtly affected by disease processes where the quantity of neuromelanin remains consistent. Comparisons between TH and neuromelanin-positive neuron counts would provide useful insights into this process, and will shed further light on the efficacy of using an immunohistochemical atlas over a histological atlas.

4.6. Conclusions of chapter

In this Chapter I have created a midbrain atlas based on immunohistochemical techniques from previous atlases (McRitchie, Hardman and Halliday, 1996; McRitchie, Cartwright and Halliday, 1997). However, I have improved on their methodology by defining anatomical landmarks as atlas references. The most novel finding of this study is that the nuclei of the SN and A10 can also be differentiated at the microscopic level based on the 'texture' of the neuronal morphology of TH-staining. The morphological differences in these nuclei may also highlight different roles for each in the circuitry of these regions. To summarise, I have been able to confirm my initial hypothesis that the tegmental dopaminergic populations can be reproducibly separated using a novel midbrain atlas. Clinicopathological studies on the tegmentum should be re-appraised with the novel methodologies presented in this Chapter. Chapter 5 will attempt to do just that by re-analysing the findings of Paulus and Jellinger on the SN and LC in PD motor subtypes (Paulus and Jellinger, 1991, 1992).

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Chapter 5 - ‘The neuropathologic basis of clinical subgroups of Parkinson’s disease’ revisited – are the SN and LC really to blame for PD motor subtypes?

5.1. Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder with prominent motor symptomatology. These motor symptoms include bradykinesia, resting tremor, muscular rigidity and postural instability (Fahn, 2003), the presence of bradykinesia in addition to at least one other symptom forming the diagnostic basis for clinical parkinsonism (Postuma *et al.*, 2015). However, the spectrum of symptoms in PD exhibits a high degree of heterogeneity and it is well recognised that various subtypes of PD can be grouped to reflect differences in motor symptoms, onset and the types of initial presenting symptoms. Traditionally, motor subtypes of PD have been known as TD or AR, though other names have been used such as PIGD subtype or NTD (Paulus and Jellinger, 1991; Selikhova *et al.*, 2009; H. M. Chen *et al.*, 2015). The presence of distinct subtype groups allows us a unique opportunity to explore the pathophysiology of individual clusters of PD motor symptoms, adding a more detailed understanding of the complexity of the brain motor circuitry in the basal ganglia and other key motor regions.

The neuropathology of PD includes degeneration of key brainstem structures early on in the disorder including the SN and LC where neuronal loss, gliosis and the accumulation of aggregated α-synuclein as Lewy bodies and neurites are found (Braak, Del Tredici, *et al.*, 2003). Previous studies on the neuropathology of PD motor subtypes have therefore focused on these areas. Among these studies, those of Paulus & Jellinger are the most prominent. Their first papers on the issue, in 1991 and 1992, found increased neuronal loss in the SN and LC in AR cases compared to TD (Paulus and Jellinger, 1991, 1992), albeit in a restricted number of cases. These studies however lacked immunohistochemical analysis of their cases. Instead, common histological techniques such as H&E and cresyl violet staining were used. This means that their methodology will also sample monoaminergic neurons that no longer express TH and could therefore be considered to be 'hypofunctioning'. As I have shown with a novel midbrain atlas in Chapter 4 of this thesis, in agreement with previous atlases of the region, the anatomy of the dopaminergic cell groups of the midbrain tegmentum is complex, with neuronal populations of the SN and A10 (the mesolimbic dopaminergic groups) lying in close proximity to one another (McRitchie, Hardman and Halliday, 1996). These nuclei are all pigmented, leading to difficulty in separating the neuronal populations for quantification in disease states. In PD,

neurons of the A9 and A10 are both susceptible to pathology and undergo degeneration (McRitchie, Cartwright and Halliday, 1997). Therefore, standard histological staining, such as those employed by Paulus & Jellinger, might be deemed insufficient to assess and differentiate the tegmental populations for studies of subtypes of PD. I have derived a methodology using TH and SP immunohistochemistry to delineate these neuronal populations (Chapter 4). This technique will be employed in this chapter to assess the SN.

Motor subtypes pose a substantial challenge to physicians and researchers for a number of reasons: Firstly, PD subtypes rarely remain stable over time, with a tendency toward increasing akinesia evident as the disease progresses (Von Coelln *et al.*, 2015). This is reflected in the most commonly used staging system for PD – the H&Y staging system (Figure 5.1) – where later stages of the disease are assigned based on the loss of mobility and, crucially, the onset of postural instability and gait disturbance as key landmarks in the clinical progression of the malady (Hoehn and Yahr, 1967). However, the response to dopamine replacement for treating individual symptom clusters such as those related to tremor or akinesia is variable among cases, and in general is less efficacious in later stages of the disease. This suggests that the latest clinical stages of the disease are characterised by pathology of several different neurotransmitter systems, and – with advancing age – multiple comorbidities. Hence, another drawback of the aforementioned studies is the use of end-stage clinical phenotype to separate patients into subtype groups (Paulus and Jellinger, 1991, 1992). We know from more recent clinical studies that older patients may have worse motor function due to causes unrelated to their PD and that this, combined with pharmacologic interventions, has the potential to mask the clinical phenotype (Lawton *et al.*, 2015). There is therefore a need to revisit the findings of Paulus & Jellinger using updated techniques to separate the SN from the A10, and to subtype cases clinically.

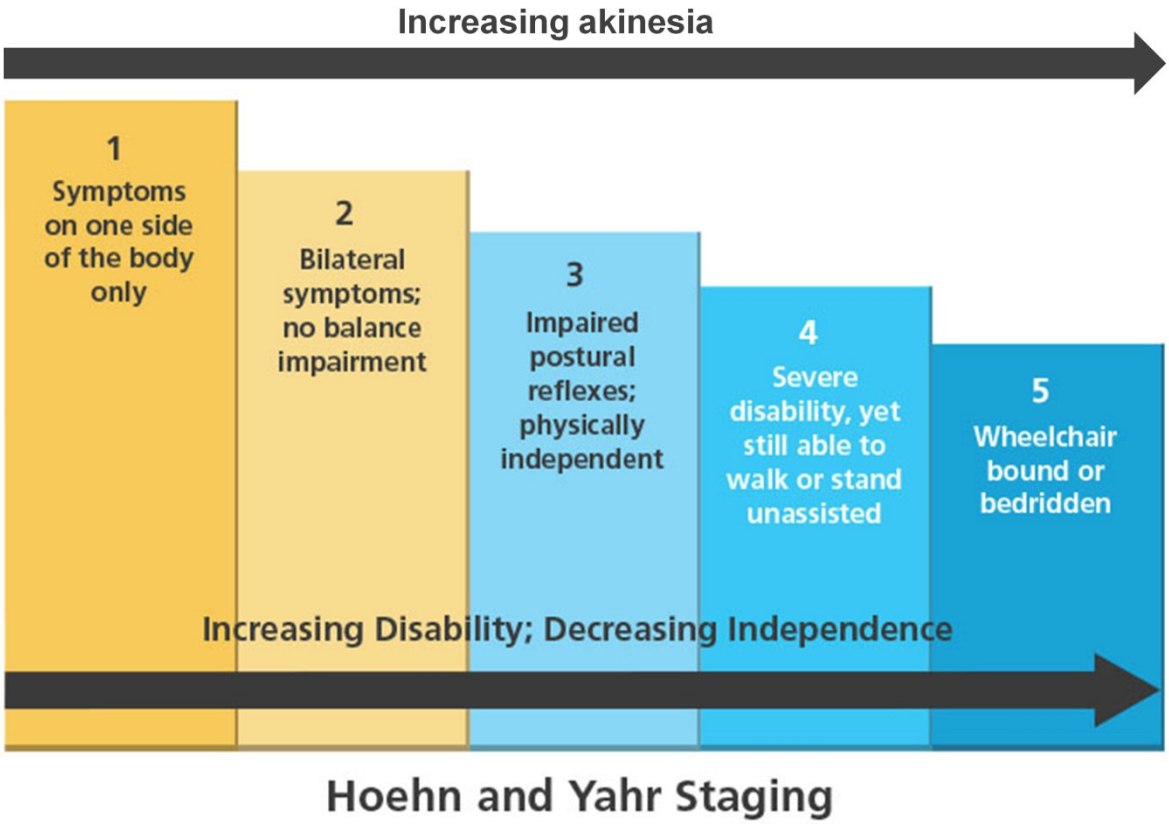


Figure 5.1: The Hoehn & Yahr staging system for clinical parkinsonism. Increasing stages reflect increasing akinesia and resultant disability.

From recent in vivo studies, there is still insufficient evidence to confirm the hypothesis of dopaminergic tone discrepancy as a cause for differential motor subtype (Thenganatt and Jankovic, 2014; Magrinelli *et al.*, 2016). It is also unclear how the different PD motor symptoms respond to levodopa therapy. Seemingly, bradykinesia, rigidity and tremor all have positive responses to levodopa whereas postural instability, gait disturbance and falls do not respond to levodopa and may be more related to cholinergic deficits (Tedeschi *et al.*, 1990; Jankovic and Aguilar, 2008; Bohnen *et al.*, 2009; Magrinelli *et al.*, 2016). Similarly, studies of the noradrenergic contribution to motor symptoms in PD in rodents have shown that reserpine, a vesicular monoamine transporter inhibitor that reduces noradrenaline release, provokes bradykinesia, tremor and rigidity (Colpaert, 1987). Yohimbine, an α2-adrenoceptor blocker, was able to resolve reserpine-induced tremor and rigidity. Further, Wilbur et al., have proposed that a noradrenergic mechanism, modulating the basal ganglia, contributes to parkinsonian tremor in humans and that this is reducible by treating with clonidine, a presynaptic α2-adrenoceptor agonist, and β-adrenoceptor blockers (Wilbur, Kulik and

Kulik, 1988). This data suggests that noradrenergic denervation does not particularly predispose to tremulous parkinsonism or non-tremulous parkinsonism to a greater degree over the other. Katz and colleagues performed a retrospective analysis of a randomised controlled study of deep brain stimulation (DBS) in which the responses of patients who had received DBS of the GPi or STN to the treatment was reclassified by motor subtype (Katz *et al.*, 2015). It was found that TD patients overall had a better response to DBS, regardless of location, than PIGD patients, who constituted 60% of the cohort, and that gait impairment was curiously improved more in the TD cohort than the PIGD group. This suggests that akinetic features are not easily treatable by manipulation of basal ganglia circuit function in comparison to tremor. It could therefore be hypothesised that the involvement of the basal ganglia is of a different character in these two disease states.

Since the early studies of motor subtype neuropathology, there have been enormous advances in the understanding of the pathophysiology of idiopathic PD, including the discovery of phosphorylated α-synuclein as the major constituent of Lewy bodies by Spillantini and colleagues (Spillantini *et al.*, 1997). This revolutionised the neuropathologic assessment of PD cases at autopsy and thus allowed for the introduction of a staging system – the Braak system - based on the stereotypical rostro-ventral spread of α-synuclein pathology through the CNS from the dorsal motor nucleus of the vagus nerve up through the pons and midbrain and limbic structures, finally inculcating itself in various areas of the cerebral cortex (Braak, Del Tredici, *et al.*, 2003). Further, it has now been recognised that comorbid hyperphosphorylated tau and Aβ contribute to the clinical phenotype of PD cases and it is likely that clinical progression is related to a confluence and interplay of these three key pathologies (Jellinger, 2012; Compta *et al.*, 2014). Selikhova and colleagues found increased burden of Lewy pathology and greater levels of CAA and Aβ plaque deposition in the cerebral cortex AR cases compared to TD, though this study did not include an analysis of tau deposition (Selikhova *et al.*, 2009). In this study, I will compare the SN and LC in presenting motor subtypes of PD combined with a quantitative analysis of α-synuclein, tau and Aβ deposition.

5.2. Hypothesis and aims

Given the flaws in previous clinicopathological studies of subtypes in PD and the lack of clear evidence of discrepancies in dopaminergic or noradrenergic tone between subtypes, I hypothesised that there will be no significant difference in the SN and LC to distinguish motor subtypes of PD. The aims of this Chapter were: 1) To compare and contrast the techniques of previous studies to the novel, enhanced technique outlined in Chapter 4; 2) to quantify neuronal loss in these regions to compare between motor subtypes; 3) to quantitatively assess pathological deposition of α-synuclein, tau and Aβ in each subtype.

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5.3. Materials and Methods

5.3.1. Cohort selection and clinical assessment of cases

Post-mortem human brain tissue containing the SN and LC was obtained from the Parkinson's UK Tissue Bank in accordance with the selection and exclusion criteria set out in Chapter 2.1.3. To summarise, 273 cases were analysed and 189 cases met clinicopathological diagnostic criteria for PD. Cases were subtyped based on the presence and severity of the four main PD motor symptoms – bradykinesia, tremor, rigidity and postural instability. TD cases were characterised by predominant rest tremor at presentation with mild or absent other motor features. AR cases were defined by a lack of tremor at onset with significant akinesia and rigidity. Mixed cases were notable for having equally dominant features of both subtypes. Control cases had no evidence of clinical neurological deficits. Sex, age of onset, disease duration, and age of death was noted for each case. 40 TD, 40 Mixed and 40 AR PD cases (Appendix 4 – PD cohort of 40 TD, 40 Mixed and 40 AR cases for this thesis, along with 12 age-matched controls were selected for this study.

5.3.2. Neuropathological assessment

Neuropathological diagnosis was based on α-synuclein, tau and Aβ immunohistochemistry. PD cases were confirmed neuropathologically using an established protocol for the neuropathologic diagnosis of PD (Alafuzoff *et al.*, 2009). Cases were excluded based on the criteria outlined in Chapter 2.1.3. Control cases were selected based on a lack of clinical neurological diagnoses and a relative lack of neuropathological findings on microscopic examination. Braak α-synuclein stage, Braak tau stage and Thal Aβ phase were calculated for each case based on routine sampling of recommended brain regions for staging these pathologies.

5.3.3. Post-mortem human brain tissue

Human brain tissue containing the SN was sampled between the levels of the exiting oculomotor nerve fibres, rostrally, and the decussation of the superior cerebellar penduncle, caudally. The LC was sampled at the rostral pontine level because of marked cell loss in this region in LBD (German *et al.*, 1992). Sections containing the dorsal striatum were also sampled to assess general correlations between SN degeneration and loss of striatal innervation. Samples were fixed for 4 weeks in 10% buffered formaldehyde and then dehydrated in 70% ethanol, 90% ethanol, 100%

ethanol and xylene before being embedded in paraffin wax. 7µm sections were cut by microtome, mounted onto microscope slides using a 40°C water bath, and dried for 24 hours in a 37°C oven in preparation for immunohistochemistry.

5.3.4. Histology and immunohistochemistry

All sections of midbrain and pons were stained histologically with H&E and LFB in series to assess pigmented neuron populations and for anatomical localisation according to the protocols outlined in Chapter 2.2.1 and 2.2.2. Serial sections were immunostained according to the protocols laid out in Chapter 2.3with anti-TH, anti-α-synuclein, anti-AT8 tau and anti-Aβ (4G8) antibodies, according to the conditions and protocols outlined in Table 2.3. SN midbrain slides were also stained with anti-SP antibodies. In short, slides were dewaxed in xylene, rehydrated in descending concentrations of ethanol before being rinsed in distilled H_2O (dH_2O). Endogenous peroxidase quenching was carried out using a bath of 1% H_2O_2, following which, slides were rinsed in dH_2O. After any necessary pretreatments were carried out, slides were rinsed in PBS twice before being incubated in primary antibody in a humid chamber at 4°C, overnight.

After primary antibody incubation, slides were rinsed twice in PBS. TH slides utilised the BioGenex Super Sensitive Kit secondary detection system according to the manufacturer's protocol, were visualised using 3,3'-diaminobenzidine and counterstained with Mayer's Haematoxylin (VWR) for 1 min, rinsed in running tap H_2O, dehydrated in ascending concentrations of ethanol, cleared in xylene and coverslipped using distrene-plasticiser-xylene.

5.3.5. Determination of the SN location in the midbrain tegmentum

Based on the atlas and methodology outlined in Chapter 4 - , midbrain sections containing the tegmentum were assessed based on SP and TH immunohistochemistry to isolate the SN from surrounding A10 neuronal populations. Where SP staining and TH staining overlapped, this area was defined as SN and other TH structures defined as part of the A10. The SN as a ROI was also demarcated on the H&E stained slide, using P staining as a template, to compare the pigmented neuron count to the TH-neuron count in the SN and to pigmented neuron counts conducted without demarcation based on SP staining.

5.3.6. Quantification of neurons and pathology in the SN and LC

Image capture of SN and LC sections was carried out using an Olympus Vanox microscope attached to a Micropublisher 3.0 camera under a 4x objective lens. TH-positive neuronal counts were performed, blinded to the clinical diagnosis, using the manual tag function on Image Pro Plus 7.0. For the SN, two adjacent, non-overlapping 4x objective images of TH staining were used for each nigral subregion, e.g. medial SN (SNm) or lateral SN (SNl). The SNm and SNl were subdivided according to the method of Paulus and Jellinger, who plotted a straight line between the ipsilateral colliculus and the midpoint of the cerebral peduncle, thus dividing the SN into SNm, medially, and SNl, laterally (Paulus and Jellinger, 1991). For the LC, a single 4x objective lens image focused on the area of maximal cell density was used for counting. Structures were included in the count if they exhibited a clear neuronal morphology with a defined nucleus and nucleolus. α-synuclein and tau pathologic burdens were assessed with %-coverage of staining under a 20x objective lens field. Aβ plaque pathology was assessed for presence or absence.

5.3.7. Statistical analysis

Statistical analyses were carried out using GraphPad Prism 8.0. Comparisons of TH, α-synuclein and tau were performed using Mann-Whitney tests for single comparisons, or Kruskal-Wallis tests with Dunn's post hoc corrections for multiple comparisons. Statistical significance was set at $p<0.05$. Intra-class correlations (Cronbach's α test) for intra-rater reliability of the %-coverage analyses, and multivariate regression analyses for contributions of clinical and demographic factors, were also performed.

5.4. Results

5.4.1. Cohort demographics

Table 5.1: Cohort demographics. TD = tremor-dominant, AR = akinetic-rigid, αSN = α-synuclein, Aβ = amyloid-β protein, PMI = post-mortem interval. ^{X} = χ^2 analysis performed, ^{K} = Kruskal-Wallis test performed.

	Control	TD	Mixed	AR	P value
Sex (M:F)	5:7	23:17	22:18	25:15	0.7866^{X}
Age of onset (yrs)	-	65.15	68.45	66.45	0.2314^{K}
Disease duration (yrs)	-	13.83	11.88	11.2	0.2390^{K}
Age of death (yrs)	79.25	78.98	80.33	77.65	0.2358^{K}
Braak αSN (mean)	-	5.65	5.57	5.69	0.5356^{K}
Braak tau (mean)	1.54	1.45	2.00	1.68	0.1028^{K}
Thal Aβ phase (mean)	2.00	2.07	1.96	2.18	0.9842^{K}
PMI (mean)	31.50	24.40	23.11	25.10	0.3350^{K}
Brain weight (g)	1309.25	1267.27	1267.97	1256.72	0.8916^{K}

Statistical analysis of the cohort demographics reveal that all PD motor subgroups and controls are well matched in all respects including age of death, sex of donors, and staging of AD-type pathology (Table 5.1). The PD baseline motor subtypes are well matched also in terms of age of onset, disease duration and Braak αSN stage.

5.4.2. Pigmented and TH-positive neuron counts – a comparison of techniques

It was first sought to compare the methodology of Paulus & Jellinger (Paulus and Jellinger, 1991, 1992) to an enhanced methodology using presenting motor subtype instead of end-stage motor subtype, immunohistochemical techniques and a novel midbrain tegmentum atlas (see Chapter 4). Neuromelanin-positive neuron counts on H&E sections, and immunoreactive neuron counts on TH-stained slides were

performed in the SNm and SNl (Figure 5.2A-F) on serial slides based on substance-P immunohistochemistry. The counts were compared statistically using Wilcoxon matched-pairs signed rank tests. This analysis revealed no statistically significant difference between H&E and TH-stained SN neuron counts in the SNm ($p = 0.2408$) but significantly increased counts in the SNl in TH-stained sections ($p = 0.0397$). Across both subregions of the SN, TH+ neuron counts were slightly higher than H&E pigmented neuron counts by approximately 5 neurons per frame. This may indicate that some nigral dopaminergic neurons do not contain neuromelanin deposits. However, this is not supported by the literature, in which it is widely reported that PD cases exhibit neuromelanin-positive neurons that are TH-negative, or that have depleted TH levels (Mori *et al.*, 2006). A more likely explanation is that it is more difficult to identify neuronal morphologies on H&E sections than on TH-stained sections, as TH immunohistochemistry fully stains a neuron, giving a distinctive morphology. On the other hand, H&E staining has the potential to stain the neuromelanin, but yields a cytoplasmic stain that is similar in colour to the surrounding parenchyma which makes identification of neurons more challenging. However, the data shows that TH-neuron count is not consistently higher in all cases and that there is considerable variation in the rank differences between H&E and TH counts (Figure 5.2G & H). It appears that the quantification of neurons is more consistent between H&E and TH in cases where the total neuron count is low. Therefore, it could be argued that quantification is more difficult in more intact neuronal populations because cells reside in clusters. If this is true, the technique that most facilitates the identification of individual neurons is the most appropriate. Thus, it can be concluded that TH immunohistochemistry is a superior technique for this experiment because it facilitates neuronal identification, but also because it is able to delineate the SN from neighbouring meso-limbic dopaminergic populations, in conjunction with substance-P immunohistochemistry (Chapter 4).

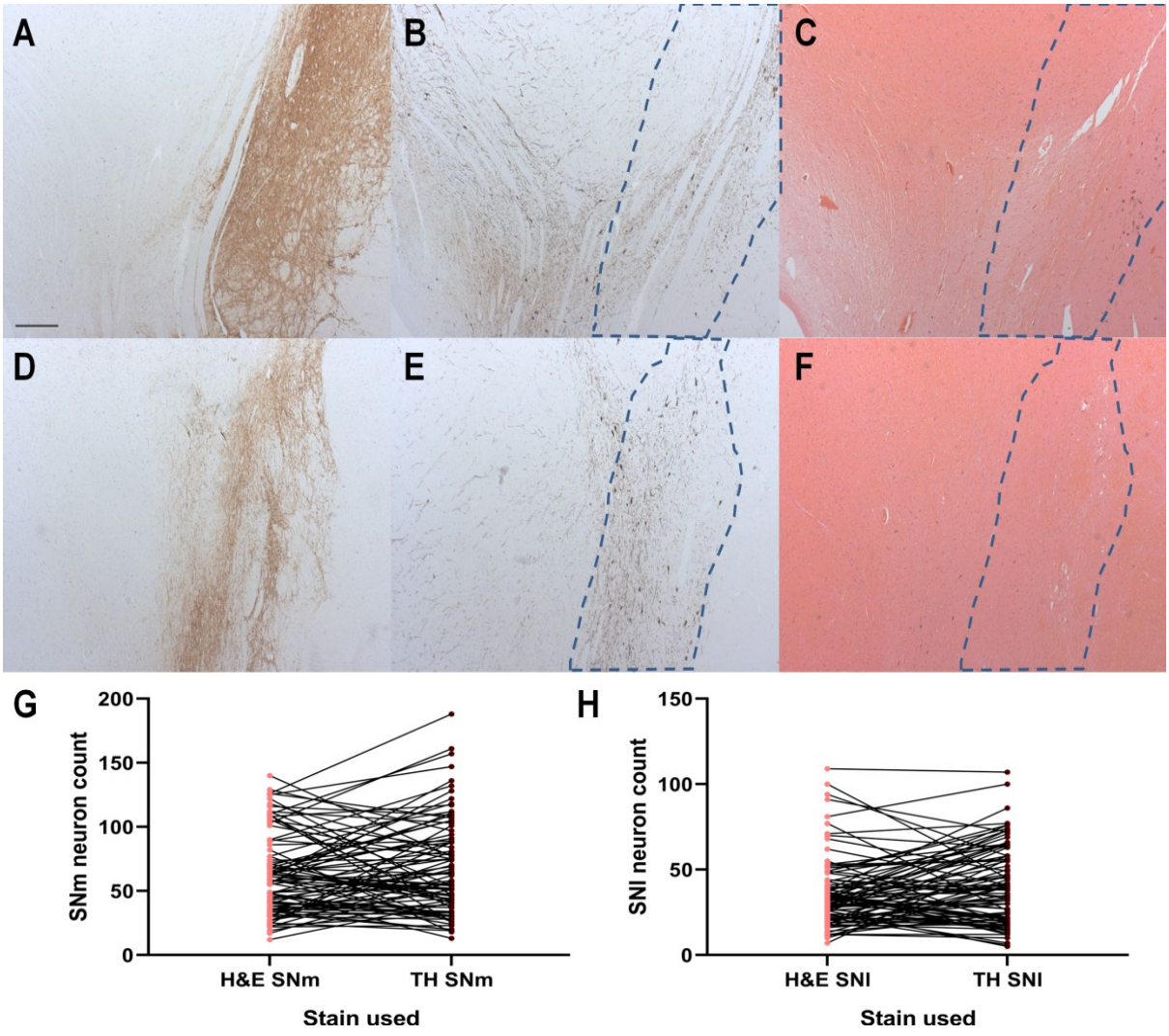


Figure 5.2: Comparisons of H&E-based method of neuronal quantification with an immunohistochemical methodology with TH and substance-P in PD cases. A-C) SNm, D-F) SNl. A & D) substance-P staining, B & E) TH staining, C & F) H&E staining. G) Pairwise comparison of SNm neuron counts in H&E and TH stained serial sections ($p = 0.2408$). H) Pairwise comparison of SNm neuron counts in H&E and TH stained serial sections ($p = 0.0397$). Scale bar in A) = 250µm. Dashed lines represent substance-P stain superimposed onto TH and H&E stained slides to delineate the SN. Paired statistical analysis was performed using two-tailed Wilcoxon signed rank tests.

5.4.3. Clinicopathological correlations with neuron counts in the SN

Despite the conclusions drawn in Chapter 5.4.2, the findings of Paulus & Jellinger (Paulus and Jellinger, 1991, 1992) in their cohort had to be compared like-for-like with this cohort, with and without the enhanced methodology outlined in Chapter 4 - . Therefore, H&E neuromelanin-positive and TH-stained SN neurons were quantified in the SNm and SNl in all cases and compared between controls and PD cases, and

between PD presenting motor subtypes (Figure 5.3). Compared to controls which averaged a neuron count of 149 in the SNm and 129 in the SNl, PD cases had significantly lower neuron counts (64 in SNm ($p < 0.0001$) and 36 in SNl ($p < 0.0001$). Across the vast majority of cases, the SNl was more severely degenerated than the SNm. When PD cases were split into presenting motor subtype groups, TD cases had an average of 69 SNm neuromelanin-positive neurons, compared with 56 neurons in Mixed cases, and 66 in AR cases (Figure 5.3A). In the SNl, TD cases had an average of 39 neuromelanin-positive neurons, Mixed had 33 neurons, and AR had 34 (Figure 5.3C). On TH-immunostained sections, TD cases averaged 70 SNm TH-positive neurons compared with 64 for Mixed and 71 for AR cases (Figure 5.3B). In the SNl, TD and Mixed cases exhibited an average of 44 SNl TH-positive neurons each, compared to 39 for AR cases (Figure 5.3D). There were no significant differences between baseline motor subtype groups, either on H&E or TH sections, in the SNm and SNl, though the SNl had significantly lower neuron counts than the SNm in PD cases.

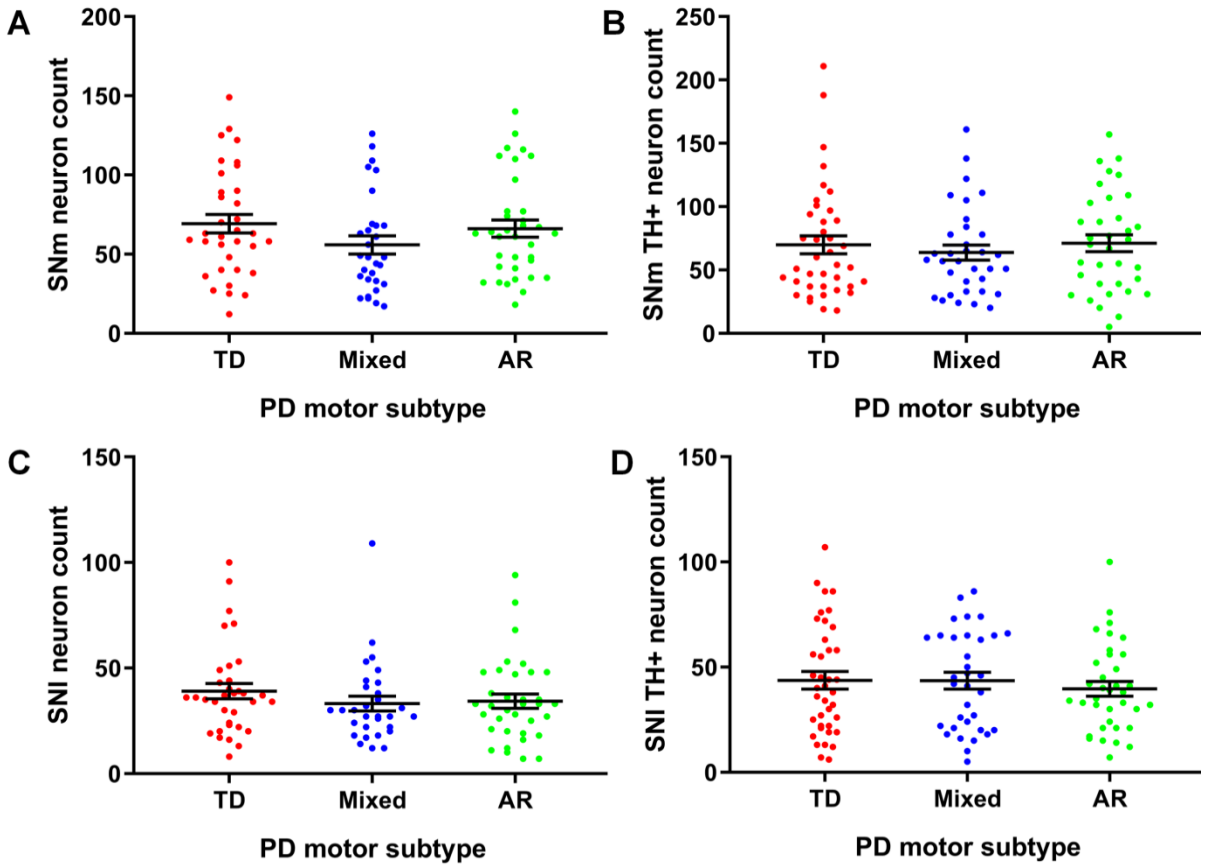


Figure 5.3: Quantification of SN neuronal number in PD motor subtypes using H&E and TH staining. A-C) Neuromelanin-positive neuron counts using H&E sections. B & D) TH+ neuron counts. A & B) SNm counts, C & D) SNI counts. Statistical analysis was performed using Kruskal-Wallis tests with Dunn's post-hoc correction for multiple comparisons. A) p = 0.2022, B) p = 0.7292, C) p = 0.3372, D) p = 0.7754.

Motor subtype, sex, age of onset, disease duration, cognitive status and Braak α-synuclein stage were entered into multivariate regression models with SNm (

Table 5.2) and SNI (Table 5.3) TH-positive neuron counts as dependent variables. This analysis revealed that the clinical characteristics of PD cases are only weakly predictive of SN neuron counts, and that the only significant predictor was disease duration – longer disease duration predicted lower SN TH-positive neuron counts, indicative of more severe neurodegeneration. Thus, adjusted for other clinical factors, SN TH-positive neuron count is not associated with variations of PD motor subtype.

Table 5.2: Least squares multivariate regression analysis table with SNm TH+ neuron count as dependent variable. R^2 = 0.1207, F = 3.242, p = 0.0062.

Factor	β-coefficient (95% CI)	P value	Significance
Constant	108.9 (21.22 – 196.6)	0.0155	*
Motor subtype	-3.249 (-12.49 – 5.995)	0.4869	NS
Disease duration	-2.436 (-3.687 – -1.184)	0.0002	***
Age of onset	0.2681 (-0.5224 – 1.059)	0.5023	NS
Sex	10.13 (-6.590 – 26.85)	0.2320	NS
Dementia status	-1.553 (-11.35 – 8.245)	0.7537	NS
Braak α-synuclein stage	-6.007 (-17.77 – 5.760)	0.3133	NS

Table 5.3: Least squares multivariate regression analysis table with SNl TH+ neuron count as dependent variable. R^2 = 0.09873, F = 2.807, p = 0.0148.

Factor	β-coefficient (95% CI)	P value	Significance
Constant	64.51 (14.09 – 114.9)	0.0127	*
Motor subtype	-2.799 (-8.078 – 2.481)	0.2952	NS
Disease duration	-1.343 (-2.062 – -0.6237)	0.0004	***
Age of onset	-0.03779 (-0.4912 – 4.156)	0.8689	NS
Sex	7.786 (-1.815 – 17.39)	0.1107	NS
Dementia status	-1.022 (-6.652 – 4.609)	0.7193	NS
Braak α-synuclein stage	-1.515 (-8.278 – 5.249)	0.6576	NS

5.4.4. The role of pathologic protein deposition in the SN

The levels of α-synuclein and tau protein deposition in the LC were quantified using a threshold-based %-coverage methodology. α-synuclein existed in the forms of LBs

(Figure 5.4A) and LNs (Figure 5.4B). hyperphosphorylated tau was found as NFTs (Figure 5.4C) and NTs (Figure 5.4D).

There were no significant differences in the deposition of α-synuclein (Figure 5.4E & F) and hyperphosphorylated tau protein (Figure 5.4G & H) in the SNm and SNl between the PD motor subtypes. This effect prevailed when α-synuclein and tau %-coverages were entered into multivariate regression analyses with other factors such as SN neuron count, sex, age of onset, disease duration, cognitive status and Braak α-synuclein stage. Pathologic protein deposition in the SN was unrelated to any measured clinical parameter.

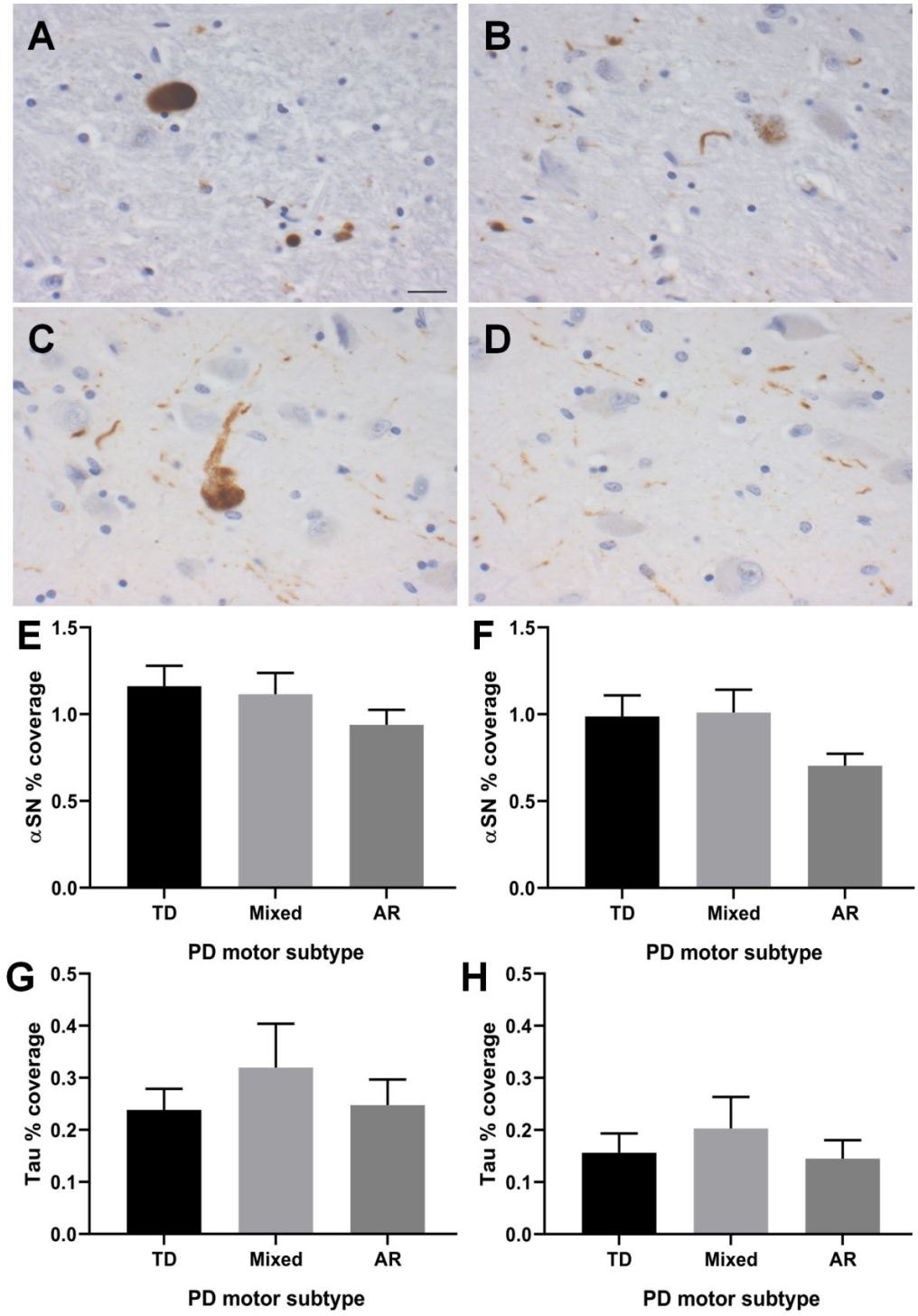


Figure 5.4: Contributions of pathologic protein deposition in the SN in PD motor subtypes. A) α-synuclein LB in the SN of a PD patient, B) α-synuclein LN in the SN, C) tau NFT in the SN, D) Tau NT in the SN, E) α-synuclein % coverage in SNm ($p = 0.3294$), F) α-synuclein % coverage in SNl ($p = 0.1030$), G) Tau % coverage in SNm ($p = 0.6033$), H) Tau % coverage in SNl ($p = 0.7843$). Scale bar in A) = 20μm for all histology panels. Statistical analysis was performed using Kruskal-Wallis tests with post-hoc Dunn's corrections for multiple comparisons.

5.4.5. Striatal TH fibres in PD motor subtypes

To assess whether degeneration at the level of the SN is proportional to loss of resultant TH+ innervation of the striatum, TH-immunostained striatal sections were analysed qualitatively for TH+ fibres in the striatum. In control cases (Figure 5.5), TH strongly immunostained the entire striatum. At the microscopic level, staining was present in large and fine fibres and occasional small neurons. Fibre staining was also observed in the lenticulo-striate bridging fibres that span the internal capsule. TH staining was found only in the matrix compartment of the striatum, whereas TH fibres circumnavigated the striosome compartment. Large TH fibres were also located in the external capsule, nbMand GP. The largest concentration of TH fibres in the GP was found spanning the borders of the GPe and GPi, though TH fibres were also exhibited in the GP itself. Fine fibres were also occasionally located in the insula and thalamus. TH-positive neuron populations were located in the hypothalamus (cell group A1) and the supraoptic nucleus.

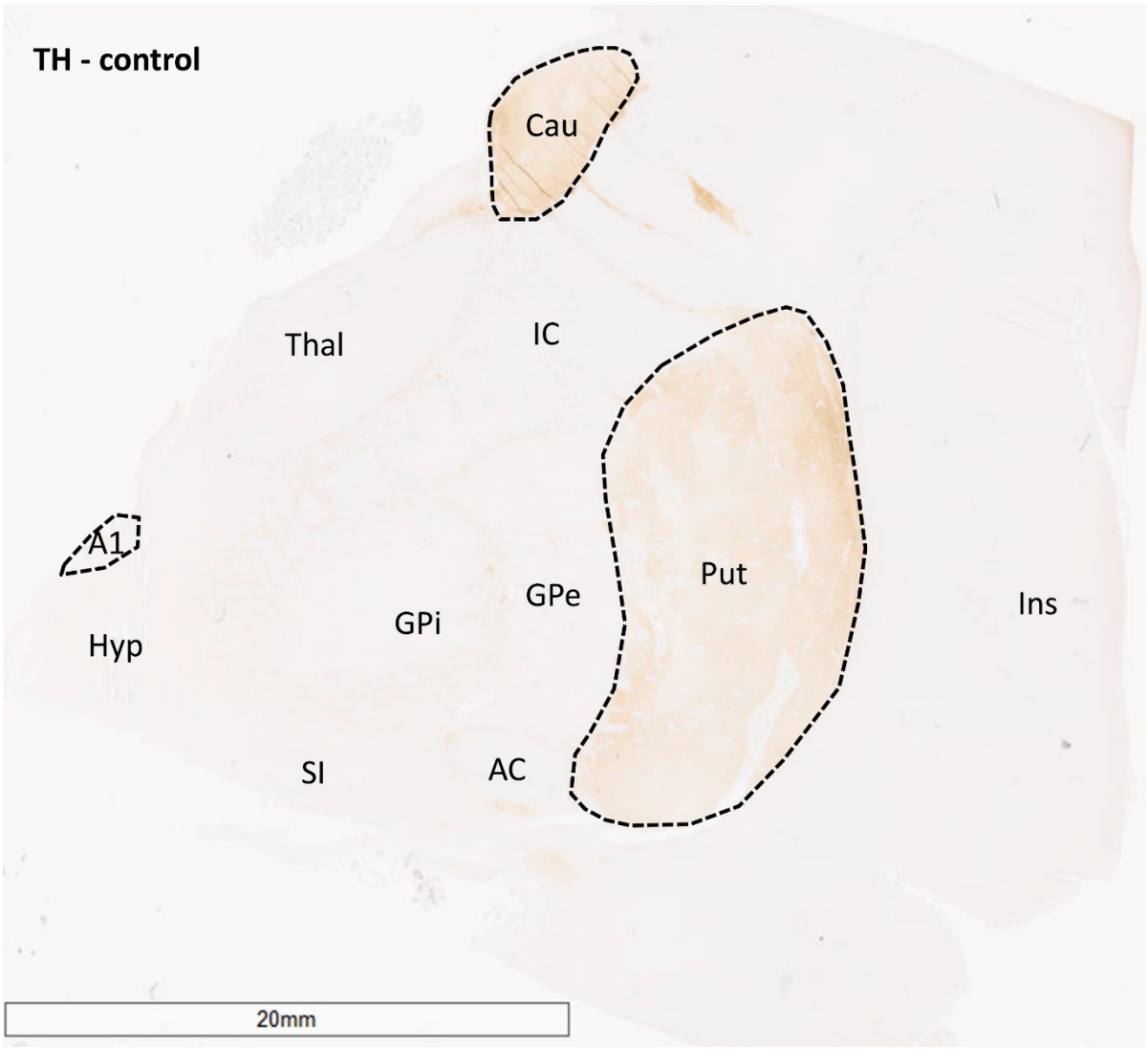


Figure 5.5: TH immunostaining in the basal ganglia in a control case. A1 = monoaminergic cell group A1, AC = anterior commissure, Cau = caudate nucleus, GPe = globus pallidus externus, GPi = globus pallidus internus, Hyp = hypothalamus, IC = Internal capsule, Ins = insula cortex, Put = putamen, SI = substantia innominata, Thal = thalamus.

Several trends were perceptible in the patterns of TH loss in PD cases. In general, the loss of fibres in the striatum affects the putamen first, followed by the caudate. This was established by the observation that caudate TH staining was preserved in short disease duration PD cases where putaminal staining was lost, whereas caudate staining was lost only in advanced disease duration cases. Thus, the caudate is, until the late stages of the disease, the least affected sub-region of the striatum. There was a typical dorso-ventral gradient of loss of TH where dorsal regions were more severely affected earlier than ventral regions. There is also a clear medio-lateral gradient of TH loss whereby lateral regions exhibit the earliest and greatest degeneration. This seemed to topographically match the pattern of degeneration in the SN in which the SNl is more severely affected than the SNm (Figure 5.3).

TD, Mixed and AR cases were grouped into cases with short (approximately 6 years), medium (approximately 13 years) and long (approximately 20 years) disease durations and striatal sections compared qualitatively, blind to the diagnosis. The extent of degeneration, in general, was proportional to disease duration. However, wild variability existed in the integrity of the TH+ innervation within a disease duration length category, and between categories whereby some short duration cases exhibited markedly reduced staining compared to medium cases (Figure 5.6). TD cases appeared to show a more intact pattern of TH staining by 20 years than Mixed or AR cases, though there were no differences between the subtypes early in the clinical phase of the disease. Cases were defined by whether the caudate or putamen exhibited high, medium or low TH staining and multivariate regression analysis was used to assess which factors of sex, disease duration, presenting motor subtype and SNm and SNl count, was best predictive of TH staining in the striatum. In the putamen, all cases exhibited extensive degeneration of TH such that there were no cases in the high staining bracket. There were no significant predictors of TH staining in the putamen. By comparison, in the caudate, a larger range of degrees of staining was present. In general, the caudate displayed more intact TH staining than the putamen, suggesting that putamen degeneration precedes or occurs more severely than the caudate in PD. SNm TH neuron count, but not SNl TH neuron count, showed a weak but significant association with TH staining in the caudate ($\beta = 0.005$, $p = 0.017$), suggesting a topographic innervation of the striatum from the SN. SNl counts were

significantly lower than SNm counts in the vast majority of cases, which mirrors TH findings at the level of the striatum.

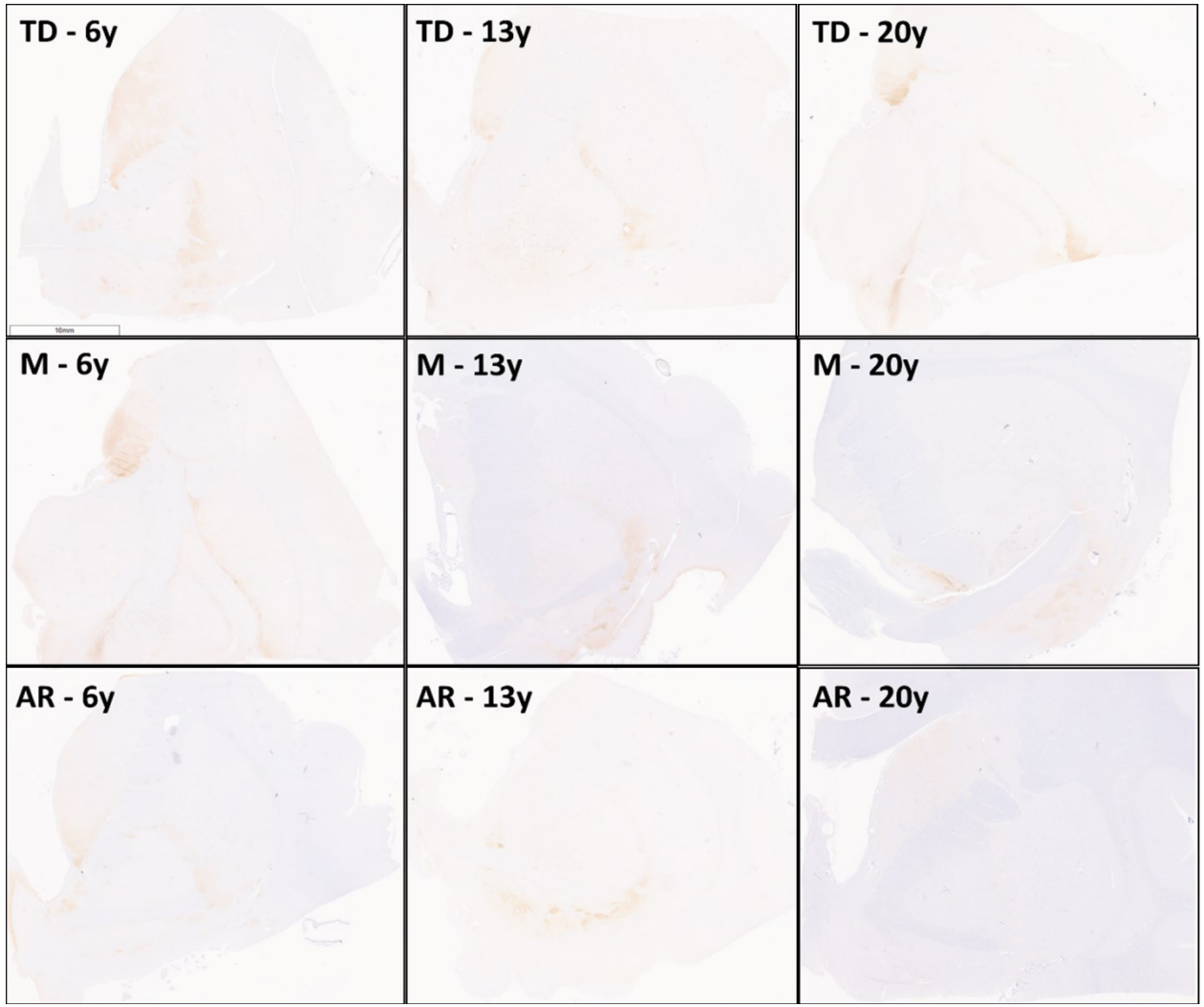


Figure 5.6: TH staining in the striatum in PD motor subtypes at different disease durations. AR = akinetic-rigid, M = Mixed, TD = tremor-dominant.

5.4.6. Clinicopathological correlations with neuron counts in the LC

TH+ neuron counts in the LC were quantified and compared between controls and PD and between PD motor subtypes (Figure 5.7A). PD cases exhibited a statistically significant reduction in LC TH-positive neurons compared to controls, with controls averaging 65 neurons compared to 31 ($p < 0.0001$). When PD cases were divided into baseline motor subtype groups, there were no statistically significant differences in TH-positive neuron count ($p = 0.5693$). TD and Mixed cases displayed an average of 29 TH-positive LC neurons each compared to 33 in AR cases.

To account for other clinical factors, sex, age of onset, disease duration, motor subtype, cognitive status and Braak α-synuclein stage for each case were entered into a multivariate regression analysis with LC TH-positive neuron count as the dependent variable. Fascinatingly, the only predictive factors of LC TH-positive neuron count were cognitive status and Braak α-synuclein stage, whereby the presence of dementia, and advanced Braak stages, predicted lower neuron counts in the LC. Dementia diagnosis was positively correlated, and so collinear, with Braak stage suggesting a potential relationship between cognitive impairment, encroachment of Lewy pathology into the cerebral cortex, and degeneration of the LC. This topic will be addressed further by this thesis, in Chapter 7.

5.4.7. The contribution of pathologic protein deposition in the LC

The levels of α-synuclein and tau protein deposition in the LC were quantified using a threshold-based %-coverage methodology. AR cases displayed heightened α-synuclein deposition in the LC compared to TD cases ($p =0.0101$) and mixed cases displayed a level of deposition intermediate to this (Figure 5.7B). However, this effect disappeared when controlled for other clinical factors such as sex, age of onset, disease duration, cognitive status and Braak α-synuclein stage. In fact, there were no significantly predictive factors for LC α-synuclein protein deposition.

There were no significant differences in deposition of hyperphosphorylated tau protein in the LC between the PD motor subtypes (Figure 5.7C). Interestingly, when tau %-coverage was input into a multivariate regression analysis with the aforementioned factors, the only significant predictive factor was cognitive status, whereby increased

tau protein deposition was predicted by increased cognitive impairment. This will also be covered further by this thesis, in Chapter 7.

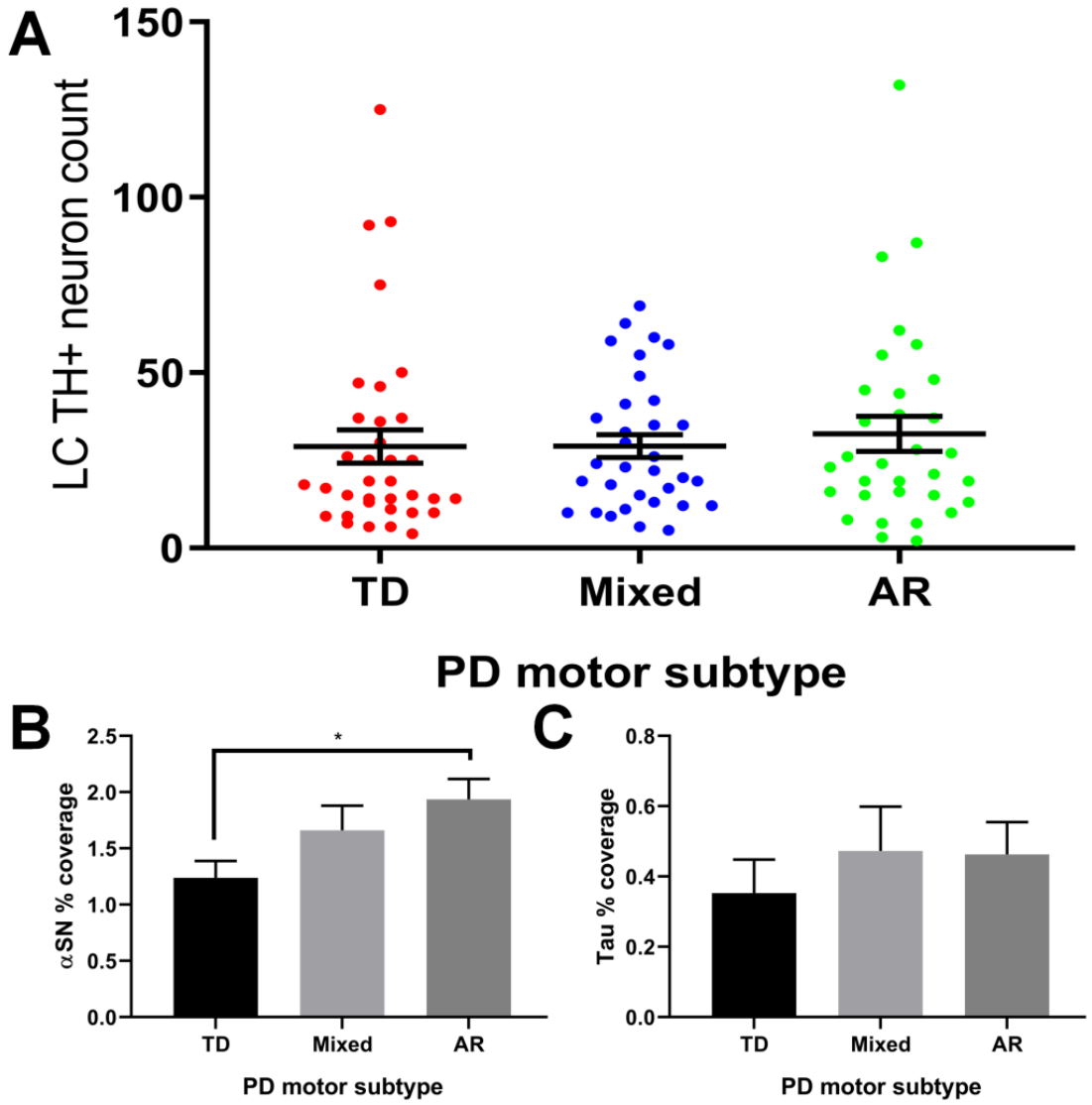


Figure 5.7. Clinicopathological correlations in the LC with PD motor subtypes. A) LC TH+ neuron counts in PD motor subtype ($p = 0.5693$), B) α-synuclein % coverage in LC ($p = 0.0136$) C) Tau % coverage in LC ($p = 0.4430$). Statistical analysis was performed using Kruskal-Wallis tests with post-hoc Dunn's corrections for multiple comparisons.

5.5. Discussion

This chapter set out to reappraise the results of previous renowned clinic-pathological studies on the neuropathology of PD motor subtypes. In order to do this, a novel methodology was employed, using an immunohistochemical atlas based on substance-P and TH staining to delineate the SN from neighbouring meso-limbic nuclei, using a much larger cohort. Motor subtype at presentation was used to reduce the influence of end-stage comorbidity and to attempt to understand the basis of differential motor presentations. With this enhanced method, the results presented in this chapter disagree entirely with those of Paulus & Jellinger, as I found that there is no difference in the number of SNc neurons between PD motor subtype (Paulus and Jellinger, 1991, 1992). None of the methods employed could differentiate the PD motor subtypes. In fact, the extent of degeneration in the SN was associated only with advanced disease duration. Even given this statistical association, wild variability still exists in the extent of SN degeneration, and resultant TH+ fibre loss at the level of the striatum. Our findings regarding TH staining in the striatum in PD neatly mirror the findings of Kordower and colleagues (Kordower *et al.*, 2013) who performed a similar analysis, though only in relation to disease duration and not clinical subtype. They found similar patterns of degeneration of the nigrostriatal systems to the present study, but also significant variation between cases. It is likely that the level of degeneration is highly individual, and a function of a number of factors including genetic, epigenetic, environmental and demographic factors (Chapter 1.4). There is no evidence currently to suggest that having a high level of risk factors in these categories predisposes a person to developing either TD or AR phenotype.

The idea that differential pathology in the SN might elicit different motor presentations is intuitive at first glance – the SN is known to produce the PD motor syndrome (Fahn, 2003; Jankovic, 2008). However, differential degeneration in a single nucleus is logically unable to produce two distinct motor presentations. If AR subtype was caused by more neurodegeneration in the SN, then all cases would follow a stereotypical progression with an initial TD presentation followed by AR as the degeneration worsens. The fact that cases can present with AR motor subtype directly disproves this hypothesis. Therefore, to produce two different motor presentations, differential pathology in at least one other brain region is required. Paulus & Jellinger's study also found differential degeneration of the LC, whereby AR cases showed more aggressive

neuronal loss (Paulus and Jellinger, 1991). In fact, they described the LC as the main locus for PD motor subtypes. However, our results show otherwise. In fact, LC neuron count was associated only with cognitive decline and not with motor symptoms in PD. This is in agreement with some authors who have linked the LC with dementia in PD (Zweig *et al.*, 1993; Brunnström *et al.*, 2011; Haglund *et al.*, 2016)

In vivo imaging studies of PD motor subtypes have highlighted reduced dopamine transporter (DAT) availability using FP-CIT single photon emission computerised tomography (SPECT) in AR cases compared to TD (Spiegel *et al.*, 2007; Eggers *et al.*, 2011; Schillaci *et al.*, 2011; Moccia *et al.*, 2014). Interestingly, data from Schillaci and colleagues included a mixed phenotype which showed a pattern of loss of DAT availability similar to AR cases. This suggests that nigrostriatal degeneration correlates with akinesia features but not tremor, given that tremor is present in both TD and mixed cases. This may indicate that the locus of tremor as a symptom resides outside of the nigrostriatal dopaminergic system. However, it has been shown that DAT signal does not correlate with neuronal number in the SN (Saari *et al.*, 2017). This leads to questions as to whether there is upregulation or downregulation of DAT levels relative to TH+ axons, whether SN TH is correlated with striatal TH and whether the interplay between SN TH, striatal TH and striatal DAT is what varies across different PD motor subtypes. Further, while the overall subtype cohorts are well-matched in these imaging studies, it would be interesting to see whether TD and AR cases matched on an individual basis for disease duration or other demographic factors in paired comparisons would still show the same pattern of DAT loss across subtypes.

SN dopaminergic neurons innervate a number of target neurons in the striatum including MSNs and ChIs. There is evidence for differential cholinergic tone in PD motor subtypes, given that anticholinergics are useful for treating tremor (Manyam and Sanchez-Ramos, 1999; Goetz *et al.*, 2002; Katzenschlager *et al.*, 2003), and that falls and gait impairment are associated with cholinergic deficits (Bohnen *et al.*, 2009; Henderson *et al.*, 2016). Therefore, analysing the role of cholinergic brain systems such as that of the striatum may be crucial to understanding the pathophysiology of PD motor subtypes.

5.5.1. Limitations

This study was limited by a variety of factors. Firstly, only one tissue section per case was used for immunohistochemical analysis and subsequent neuronal quantification. This was based on limited or inconsistent tissue availability for each case, which made stereological estimation of the total population of neurons impossible in this cohort. However, a previous study has demonstrated strong concordance between single section quantification and stereological estimates (Ma *et al.*, 1995). Furthermore, a meta-analysis comparing stereological methods to non-stereological methods in the nbM, SN and LC found no significant differences in the resultant neuronal counts in comparisons between these methods (Lyness, Zarow and Chui, 2003). Secondly, although every effort was taken to sample the midbrain at a consistent rostro-caudal level, it was not always possible to do this because of post-mortem artefacts such as distorted fixation positions or removal damage. Given that the population density of SN TH-positive neurons varies across the rostro-caudal axis of the midbrain, this may reduce the reliability of our results. However, as I have shown in Chapter 4, the variability of SN neuronal counts in the rostro-caudal plane of the midbrain is not as profound as that of the A10 populations, meaning we can be reasonably confident that the sampling of the SN was representative enough to make informed conclusions based on the results.

5.6. Conclusions of Chapter

In this chapter, I have showed that the SN and LC are not able to distinguish between PD motor subtypes as was first posited by Paulus & Jellinger. Using a larger cohort, a novel midbrain atlas, immunohistochemical methods and pathologic protein deposition quantification, I have improved on the methodology used by early studies on the SN and LC in PD motor subtypes in critical ways. There were no significant differences found in neuronal number and pathologic burden between PD motor subtypes in the SN and LC when controlled for clinic-pathological and demographic factors such as Braak stage, dementia status and disease duration. Pathology in the LC may be more related to cognitive decline in PD – this topic will be covered more extensively in Chapter 7 - .

Chapter 6 - Clinicopathological relations of the striatal cholinergic system to motor subtypes of Parkinson's disease

6.1. Introduction

PD is a progressive neurodegenerative movement disorder with four main clinical motor symptoms – bradykinesia, rigidity, tremor and postural instability (Fahn, 2003; Jankovic, 2008). As discussed, the motor syndrome of PD affects patients heterogeneously, with some patients experiencing a TD presentation of disease, whereas others present with an AR phenotype or a mix of both akinesia and rest tremor (Mixed).

Studies of the neuropathology underpinning the differential presentations of PD have analysed post-mortem donor tissue in the domains of neuronal loss and pathologic protein accumulation. Paulus and Jellinger found more aggressive neuronal loss in the substantia nigra of AR cases compared to TD, thus suggesting a more significant dopaminergic deficit in those cases affected by significant akinesia (Paulus and Jellinger, 1991, 1992). However, due to reasons discussed in Chapter 4 - , and supported by data in Chapter 5 - , I have not been able to reproduce these results in our cohort. Selikhova and colleagues reviewed the neuropathology of a large number of cases of different PD clinical subtypes, finding that AR cases had more aggressive LB deposition in the cerebral cortices, greater cortical Aβ plaque and CAA pathology, and an association between cortical α-synuclein load, bradykinetic onset and cognitive decline (Selikhova *et al.*, 2009). However, they were unable to distinguish between other subtypes neuropathologically. Moreover, their assessment of Lewy pathology neglected to investigate clinicopathological relations between α-synuclein deposition in the critical basal ganglia movement centres and motor phenotype. Additionally, no assessment of tau NFT, NT or neuritic plaque pathology was carried out. In later studies by the same group, the interplay between tau, α-synuclein and Aβ in determining clinical phenotype and progression of PD was analysed, finding that higher levels of these pathologies in the neocortical regions was associated with poor prognosis, non-tremor dominant phenotype and dementia (Compta *et al.*, 2011, 2014).

Among the earliest used treatments for PD were the anticholinergics (Manyam and Sanchez-Ramos, 1999), used originally to control sialorrhoea but were also found to be efficacious at treating Parkinsonian rest tremor, which was considered the diagnostic feature for *paralysis agitans* during the 19th century. Leopold Ordenstein and Jean-Martin Charcot were responsible for the introduction of the belladonna alkaloids, such as hyoscyamine and atropine, to the arsenal of treatments for

physicians treating *paralysis agitans* (Lehmann, Hartung and Kieseier, 2007; Goetz, 2011). Later, these compounds were found to act at muscarinic acetylcholine receptors. At the level of the striatum, dopaminergic denervation is believed to result in an increase in cholinergic tone – dopamine/acetylcholine antagonism theory dictates that dopamine, acting primarily via the inhibitory D2 receptor, inhibits the tonic release of acetylcholine by intrinsic striatal cholinergic interneurons (ChIs). Thus, a reduction of dopamine would result in higher intra-striatal acetylcholine production. Anticholinergics such as the belladonna alkaloids are believed to redress this imbalance. However, given the specific targeting of rest tremor by anticholinergics, combined with evidence of rest tremor as an adverse effect of cholinesterase inhibition, a question arises as to whether TD and AR presentations of the disease share the same dopamine/acetylcholine imbalance.

Striatal ChIs are the principal source of striatal acetylcholine and make up approximately 1-2% of the striatal neuronal population, alongside other neuronal types including GABAergic interneurons, GABAergic spiny projection neurons, axons of cortico-striatal glutamatergic neurons. Striatal ChIs are highly ramified structures, each with an extensive dendritic tree (Bolam, Wainer and Smith, 1984; Contant *et al.*, 1996). ChIs have a rotund, multipolar morphology and an extensive arborisation that extends symmetrically around their large somata to form a dense neuropil (Arendt *et al.*, 1995). Some studies estimate that they form up to 500,000 varicosities which act as neurotransmitter release sites for volumetric transmission (Bolam, Wainer and Smith, 1984; Contant *et al.*, 1996). These unique morphological characteristics suggest that the roles of these cells in the motor striatum are both complex and crucial to the execution of movement. Alterations of this morphology or disruption of function by accumulation of pathologic protein in and around these neurons may underlie potential differences in cholinergic tone in TD and AR PD.

6.2. Hypotheses and aims of chapter

Given the apparent discrepancy in cholinergic tone in TD and AR cases of PD, I hypothesised that: i) there will be morphological differences in striatal ChIs that distinguish between PD motor subtypes and ii) that morphological disruption will be associated with greater protein pathology in AR cases. Firstly, I aimed to morphometrically analyse ChIs by quantifying primary and secondary arborisation from the cell soma using striatal slides immunostained with anti-ChAT antibodies (Table 2.3). Secondly, I aimed to quantify the burden of α-synuclein, tau and Aβ pathologies in the striatum by using a %-coverage methodology and using double-immunofluorescent staining to assess the degree of colocalisation of pathology in ChIs. Thirdly, I aimed to employ novel tissue clearing techniques to validate findings from slide-based microscopic analysis in 3-dimensions (3D).

6.3. Materials and Methods

6.3.1. Case selection and human brain tissue

Case selection was performed based on the selection and exclusion criteria outlined previously (Chapter 2.1.3). In summary, 120 PD cases (Appendix 4 – PD cohort of 40 TD, 40 Mixed and 40 AR cases for this thesis were included and subdivided by presenting motor subtype which was assigned based on the clinical criteria described in Chapter 2.1.2.1.

Post-mortem human brain tissue blocks containing the basal ganglia were collected from the Parkinson's UK tissue bank. The basal ganglia were sampled at two antero-posterior levels: 1) at the level of the anterior commisure and 2) at the level of the mammillary bodies. ChIs are differentially distributed in the sagittal and coronal planes in the human striatum, meaning that accurate and thorough sampling required sections to be acquired at multiple coronal levels. Further, the striatum at the two antero-posterior levels subserves different functions: at the pre-commissural level, the striatum performs associative functions compared to the sensorimotor functions of the post-commissural striatum. Tissue sections were cut at 7μm using a Leica Microtome, and then dried in a 37°C oven overnight.

6.3.2. Immunohistochemistry, immunofluorescence and tissue clearing

Serial sections were immunostained with anti-ChAT, anti-α-synuclein, anti-AT8 tau, anti-3R tau, anti-4R tau and anti-Aβ using the optimised protocols outlined in Chapter 2.3 2.3 and Table 2.3 using DAB as the chromogen.

Immunofluorescence staining was carried out to assess the relationship of pathologic protein deposition and striatal ChIs. The staining was carried out according to the protocols outlined in 2.4 and Table 2.3.

Given that ChIs arborise symmetrically in 3D around their cell soma, it is necessary to develop a protocol that allows for 3D morphometric analysis. In our lab, we have developed a novel tissue clearing method for the visualisation of post-mortem brain tissue in 3D (H.M. Lai *et al.*, 2018). The protocol for this technique is discussed extensively in Chapter 2.5. 2mm thick formalin-fixed tissue blocks containing the striatum were dissected and incubated with anti-ChAT antibodies after a period of delipidation using SDS. The tissue blocks were then incubated with AlexaFluor-488

donkey anti-goat secondary antibodies and cleared in a novel refractive index matching solution, OPTIclear ®, developed by our lab.

6.3.3. Microscopic analysis of ChAT staining

ChAT-immunostained basal ganglia slides were macroscopically assessed for the location of the striatum (caudate and putamen). These areas were demarcated as the regions of interest (ROIs) macroscopically. The putamen was divided into medial, intermediate and lateral portions, as demonstrated in Figure 6.1, to ensure that ChI sampling took place throughout the extent of the putamen. Further to this, a lateral-sweeping technique was used to sample ChIs within a sub-region to ensure reproducibility across all cases. This is demonstrated in Figure 6.1. This is because ChIs are differentially distributed across the striatum (Bernácer, Prensa and Giménez-Amaya, 2007). In total, 10 ChIs per sub-region were sampled, meaning that 40 were sampled in each section. ChI arborisation was assessed microscopically by quantifying the number of primary and secondary branches, as outlined in Chapter 2.6.2. Primary branches were defined as branches that arise directly from the cell soma. Secondary branches were therefore branches that arise from a primary branch. ChI neuron counts were also performed in each of the aforementioned striatal subregions using manual tag functions on Image Pro Plus 7.0 under a 10x objective lens.

6.3.4. Assessment of pathologic protein deposition in the striatum

Pathologic deposition of α-synuclein, tau and Aβ proteins was assessed in each of the striatal subregions. To sample more completely throughout the striatum, each region was split in two via a straight line plotted longitudinally through the length of the striatum, making 8 sampling ROIs in total. Analysis was performed using the %-coverage methodology outlined in Chapter 2.6.3 under a 20x objective lens for α-synuclein and tau proteins, and under a 10x lens for Aβ as these deposits are much larger. More generally, the nature of the deposits, for example, neuronal or glial tau, LBs or LNs, was noted qualitatively.

6.3.5. Analysis of immunofluorescence and tissue clearing

Immunofluorescent slides were analysed on a Leica RMi8 confocal microscope. Slides that were stained with ChAT and pathologic markers were analysed qualitatively to assess whether a pathologic protein was present in striatal ChIs. Tissue blocks

prepared for 3D-analysis using tissue clearing (FASTclear) were mounted onto imaging dishes, suspended in OPTIclear clearing solution, to be imaged on imaged on a Carl Zeiss LSM-780 inverted confocal microscope.

6.3.6. Statistical analysis

All statistical analyses were carried out using GraphPad Prism 8.0. Comparisons between PD motor subtypes were performed with Kruskal-Wallis tests with post-hoc Dunn's corrections for multiple comparisons for one-way analyses, whereas ordinary two-way ANOVA tests with Tukey's corrections for multiple comparisons where used to compare striatal subregions and PD motor subtypes simultaneously. X^2 tests were performed to compare categorical variables, for example, the presence or absence of neuronal tau in cases of different PD motor subtypes.

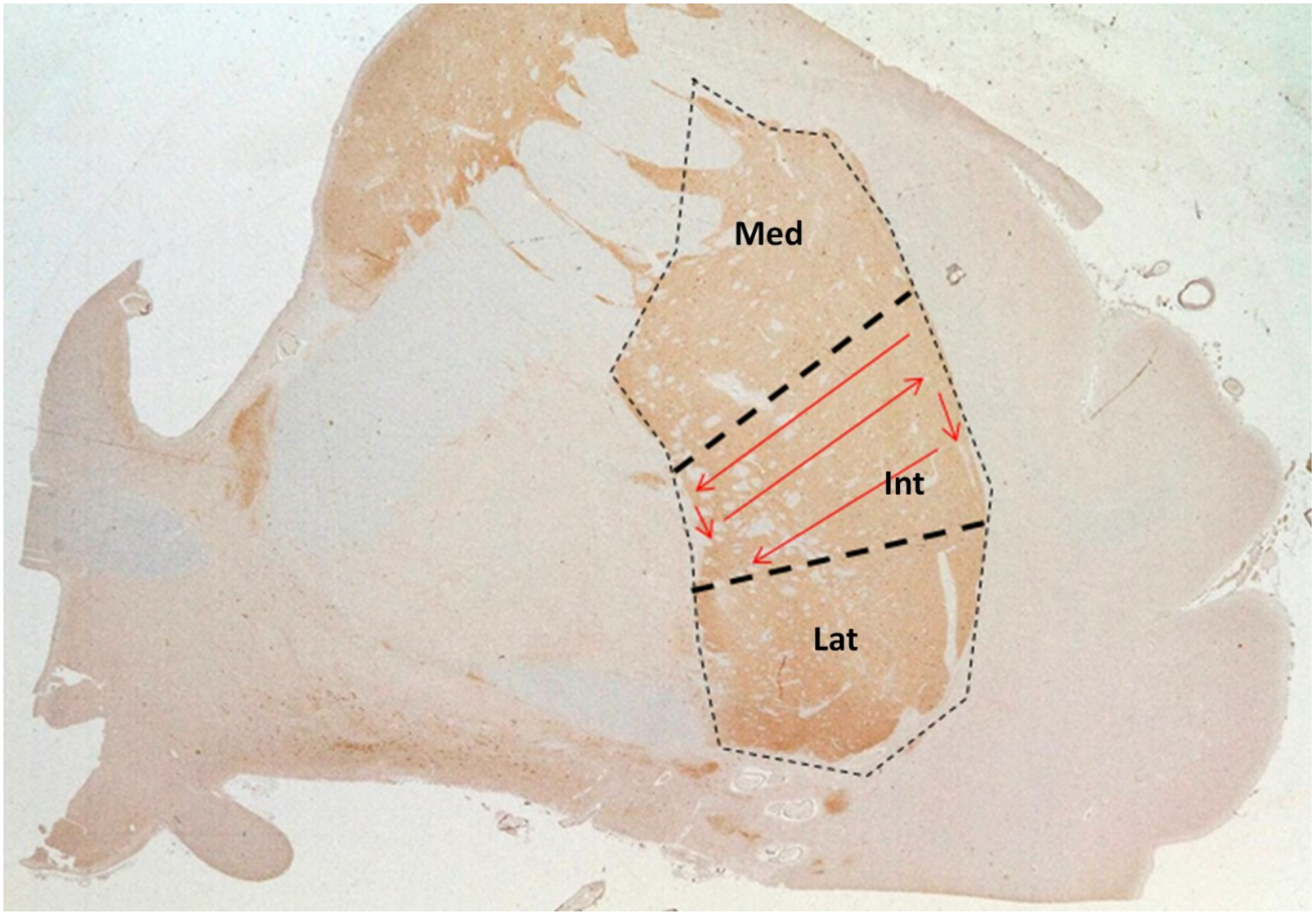


Figure 6.1: Sub-regions of the putamen on a ChAT-immunostained basal ganglia section. Med = medial, Int = intermediate, Lat = lateral. Red arrows demonstrate a lateral sweeping motion for sampling ChIs for morphometric analysis.

6.4. Results

6.4.1. Cohort demographics

See Chapter 5.4.1 and Table 5.1 for the cohort demographics in this study. Statistical analysis reveals a well-matched cohort in all regards including demographic details and disease-related and pathologic factors.

6.4.2. The pattern of ChAT immunostaining in the basal ganglia

Immunohistochemical staining of the basal ganglia with anti-ChAT antibodies is displayed in

Figure 6.2. ChAT strongly immunostained the striatum (caudate, putamen and lenticulostriate bridging fibres) but ChAT immunoreactivity was also found in the nucleus basalis of Meynert, the bed nucleus of the stria terminalis, the medial septal nucleus and the nucleus subputaminalis (nucleus of Ayala). Fainter staining was found in the globus pallidus. Microscopically, large ChAT-positive aspiny multipolar neurons and a densely stained cholinergic neuropil were visible in the striatum (

Figure 6.3A). These neurons bear the morphology of the striatal ChIs. ChIs were found in the matrix compartment, but also occasionally in the striosome compartment (

Figure 6.3B), or spanning the matrix-striosome border (

Figure 6.3C). Faint, aspiny cholinergic fibres were observed in the globus pallidus (

Figure 6.3D) but no ChAT-positive neurons were visualised, suggesting the origin of these fibres is elsewhere in the brain. Large reticular neurons were found in the cholinergic basal forebrain region. This was observed in the nucleus basalis of Meynert (

Figure 6.3E), medial septal nucleus and nucleus of Ayala (

Figure 6.3F). These were accompanied by fibrous neuropil staining with fibres arranged longitudinally in parallel bundles in the nucleus basalis of Meynert, but were associated with fine neuropil staining in the nucleus of Ayala. Similar fine neuropil staining was observed in the bed nucleus of the stria terminalis without discernible neuronal morphologies (

Figure 6.3G). Fibres with punctate spines were visualised in the insular cortex (

Figure 6.3H) and are found in all cortical layers. These morphologies are also noted in most other isocortical and allocortical regions and are arranged perpendicular to the pial surface, i.e. they span between superficial and deep cortical layers. Occasional small ChAT-positive neurons are noted in layers III/IV.

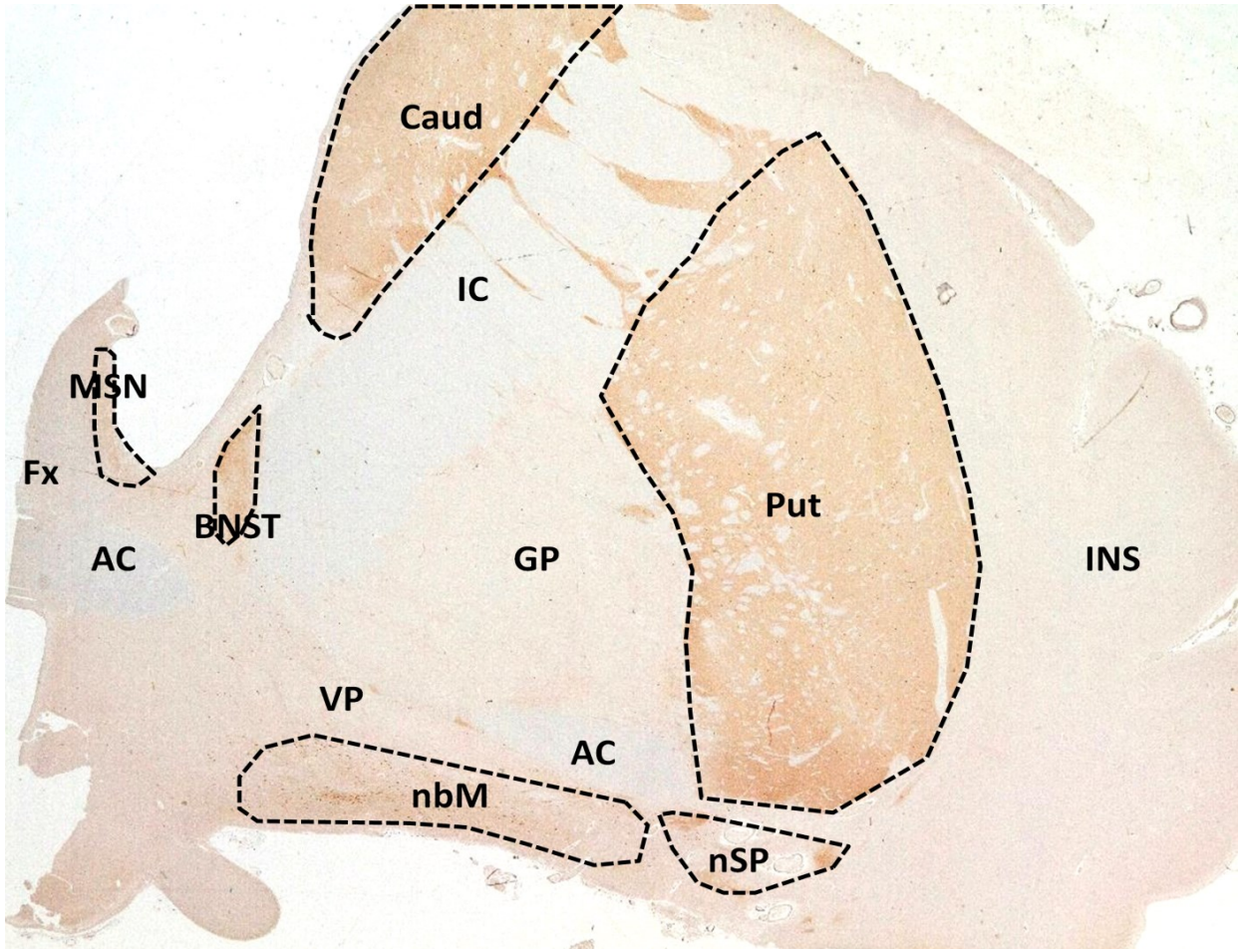


Figure 6.2: Macroscopic pattern of ChAT immunostaining in the basal ganglia. Regions with dashed line boundaries represent ChAT stained regions. AC = anterior commisure, BNST = bed nucleus of stria terminalus, Caud = caudate, Fx = fornix, GP = globus pallidus, IC = internal capsule, INS = insula, MSN = medial septal nucleus, nbM = nucleus basalis of Meynert, nSP = nucleus subputaminalis (nucleus of Ayala), Put = putamen, VP = central pallidum.

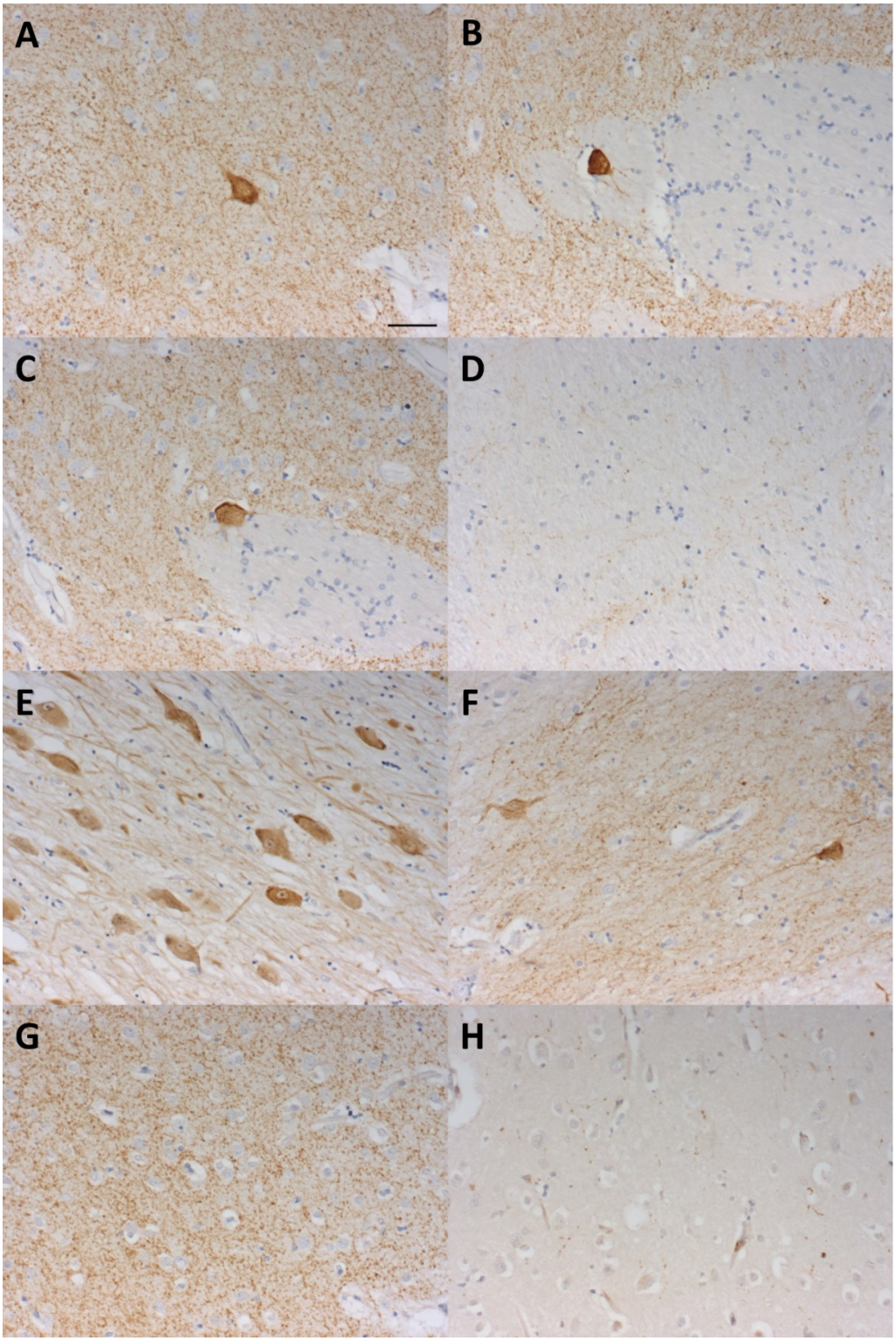


Figure 6.3: Microscopic pattern of ChAT immunostaining on slides containing the basal ganglia. A) ChI in striatal matrix, B) ChI in striosome, C) ChI at matrix-striosome border, D) Fibre staining in the globus pallidus, E) Large neurons in nucleus basalis of Meynert, F) large neurons in nucleus subputaminalis (nucleus of Ayala), G) Fibre staining in the bed nucleus of the stria terminalis, H) punctuate fibre staining in the insula. Images taken under a 20x objective lens. Scale bar in A = 50μm for all panels.

6.4.3. ChI numbers differ between PD motor subtypes

ChAT-immunostained striata were subdivided into caudate, medial, intermediate and lateral putamen sub-regions and neurons counted in two non-overlapping frames in the superior and inferior parts of each sub-region. The highest density of cells was found in the caudate and lateral subregion of the putamen (Figure 6.4). In TD cases, there were significantly higher numbers of ChIs in the lateral putamen than medial and intermediate subregions. This pattern was not exhibited in Mixed or AR cases. In the lateral putamen, AR cases showed a significant reduction in ChI cell numbers compared to TD cases ($p = 0.0384$). Also, a trend reduction was noted in Mixed cases compared to TD ($p = 0.0972$). This data suggests that the locus of pathology that differentiates TD and AR cases may be the lateral subregion of the putamen.



Figure 6.4: ChAT neuron counts in striatal sub-regions in PD motor subtypes. TD = tremor-dominant, AR = akinetic-rigid, Med Put = medial putamen, Int Put = intermediate putamen, Lat Put = lateral putamen. Statistical analysis was performed with ordinary two-way ANOVAs with Tukey's corrections for multiple comparisons. * = $p < 0.05$.

6.4.4. ChIs appear atrophic in AR PD

ChIs in the striatum displayed ramified aspiny morphologies with large nuclei and nucleoli. Accompanying this structure was a dense cholinergic neuropil. However, the appearance of these features was notably different between PD motor subtypes (Figure 6.5). TD and Mixed cases showed preservation of ramified morphology and dense cholinergic neuropil, whereas ChIs in some AR cases showed pallor of the ChI, a rounded morphology with sparse branching, and a pale neuropil. This suggests that striatal ChIs are somewhat atrophic in AR cases when compared to TD and Mixed cases.

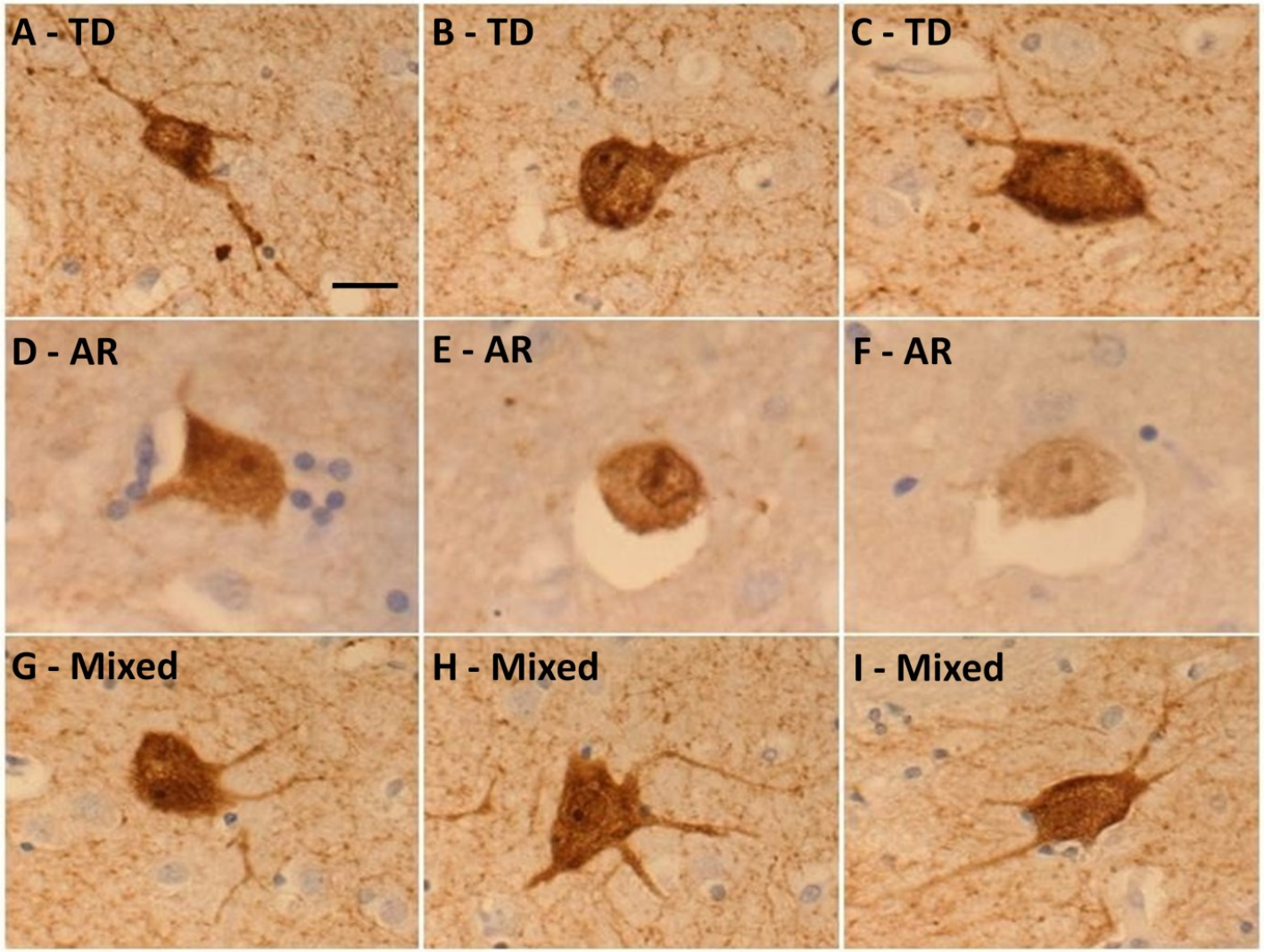


Figure 6.5: Morphological differences between striatal ChIs in PD motor subtypes. TD = tremor-dominant, AR = akinetic-rigid. A-C) ChIs in TD cases, D-F) ChIs in AR cases, G-I) ChIs in Mixed cases. Images taken under 40x objective lens. Scale bar in A = 50μm.

6.4.5. ChIs display reduced primary branching in AR PD cases throughout the striatum

Primary dendrites, defined as those arising from the cell soma, were quantified in 40 ChIs per slide per case, 10 in each striatal subregion in the pre-commissural and post-commissural striatal levels. At the pre-commissural level, AR cases were found to have significantly reduced numbers of primary branches from caudate ChIs compared to TD cases ($p = 0.0126$) and Mixed cases ($p = 0.0284$) (Figure 6.6A). A starker difference in ChI branching between motor subtypes was found in the putamen whereby AR cases showed particularly significantly reduced numbers of primary branches compared to TD cases ($p = 0.0002$) and Mixed cases ($p = 0.0151$) (Figure 6.6B).

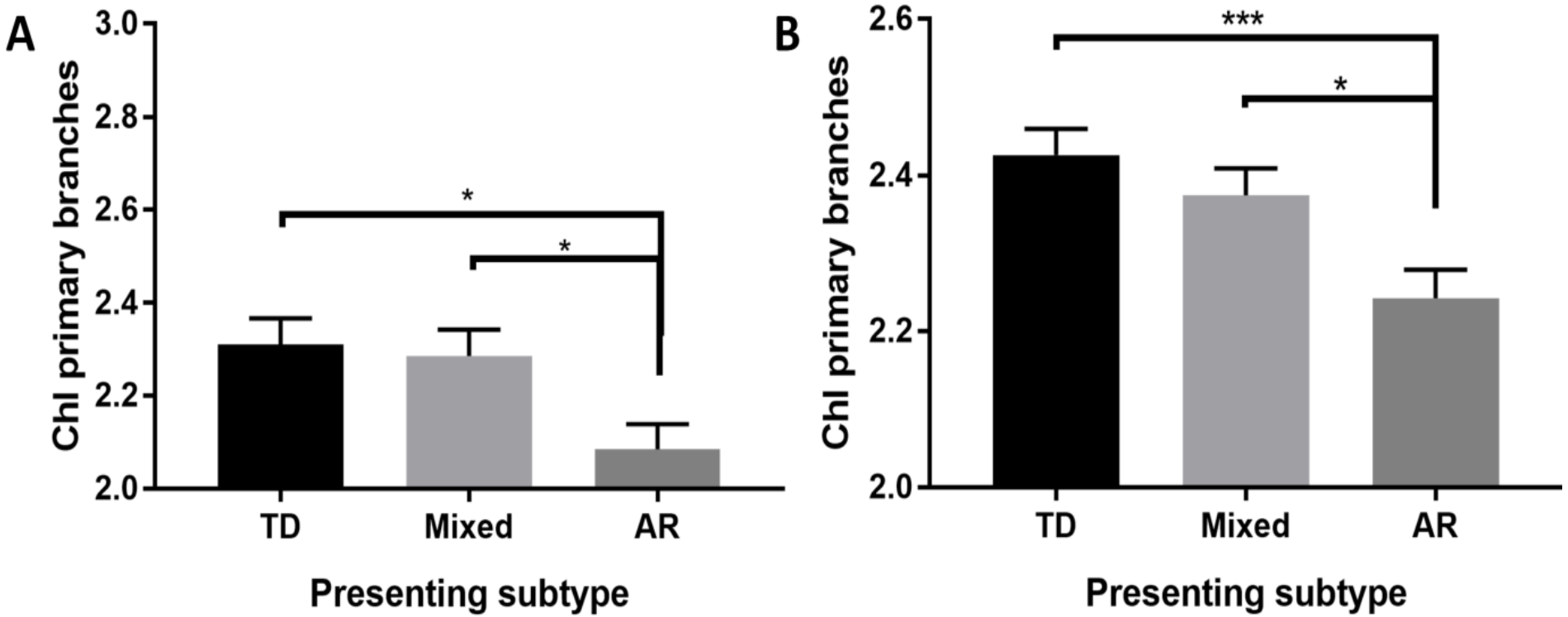


Figure 6.6: Loss of primary branches in AR cases at the pre-commissural level. A) Comparison of primary ChI branching in the caudate nucleus in PD motor subtypes, B) Comparison of primary ChI branching in the putamen in PD motor subtypes. Analysis based on a sample size of 1600 ChIs per motor subtype, 4800 in total. Statistical analysis performed with Kruskal-Wallis tests with post-hoc Dunn's corrections for multiple comparisons. * = $p < 0.05$, *** = $p < 0.001$.

At the post-commissural level, the same pattern of branching loss was noted, significantly in both the caudate (Figure 6.7A) and putamen (Figure 6.7B). However, the differences between AR cases and non-AR cases were more severe than at the pre-commissural level. Thus, AR cases are characterised by atrophic ChIs throughout the striatum, when compared to TD and Mixed cases. AR cases in general were more likely to display atrophic ChI (ChIs with no primary branches) than TD cases. TD cases also displayed more ‘hypertrophic’ ChIs (ChIs with over 5 primary branches).

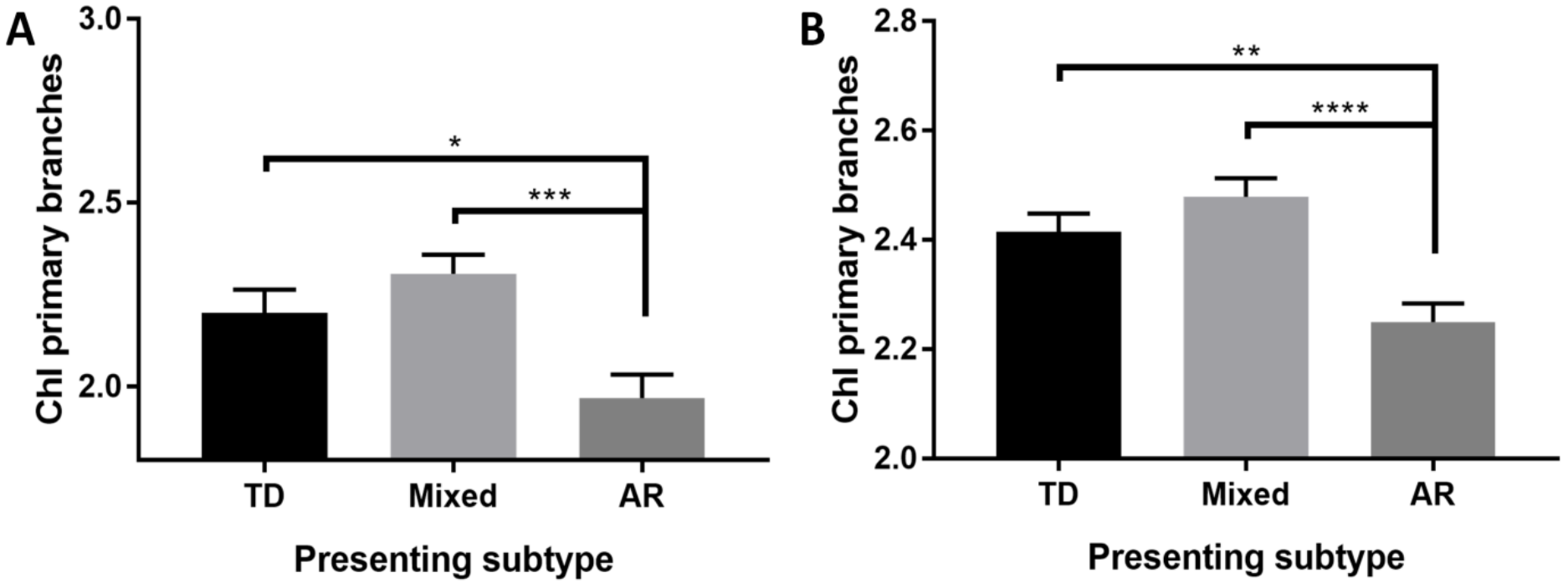


Figure 6.7: Loss of primary branches in AR cases at the post-commissural level. A) Comparison of primary ChI branching in the caudate nucleus in PD motor subtypes, B) Comparison of primary ChI branching in the putamen in PD motor subtypes. Analysis based on a sample size of 1600 ChIs per motor subtype, 4800 in total.Statistical analysis performed with Kruskal-Wallis tests with post-hoc Dunn's corrections for multiple comparisons. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

In order to assess which putamenal sub-regions were responsible for the significant differences observed between putamenal ChIs in PD motor subtypes, the mean ChI branching counts per case were divided into medial, intermediate and lateral regions and compared between motor subtypes. AR cases consistently showed reduced branching compared to Mixed and TD cases throughout all striatal sub-regions, but most severely in the lateral putamen ($p = 0.036$). Thus, the cholinergic system of the lateral putamen, especially, appears to be a major discerning factor in terms of PD motor subtypes.

6.4.6. Secondary branching reduction in ChIs is proportional to loss of primary branches

Secondary branches from ChIs, defined as branches arising from a primary branch, were quantified in the striatal sub-regions at the pre-commissural and post-commissural levels. The pattern of loss of secondary branching from ChI mirrors that of primary branches. To this end, AR cases showed significantly reduced secondary branching from ChIs in the caudate (Figure 6.8A) at the pre-commissural level compared to TD ($p = 0.0048$) and Mixed cases ($p = 0.0003$), whereas a trend reduction ($p < 0.1$) of secondary branching was observed in AR cases compared to TD in the putamen (Figure 6.8B).

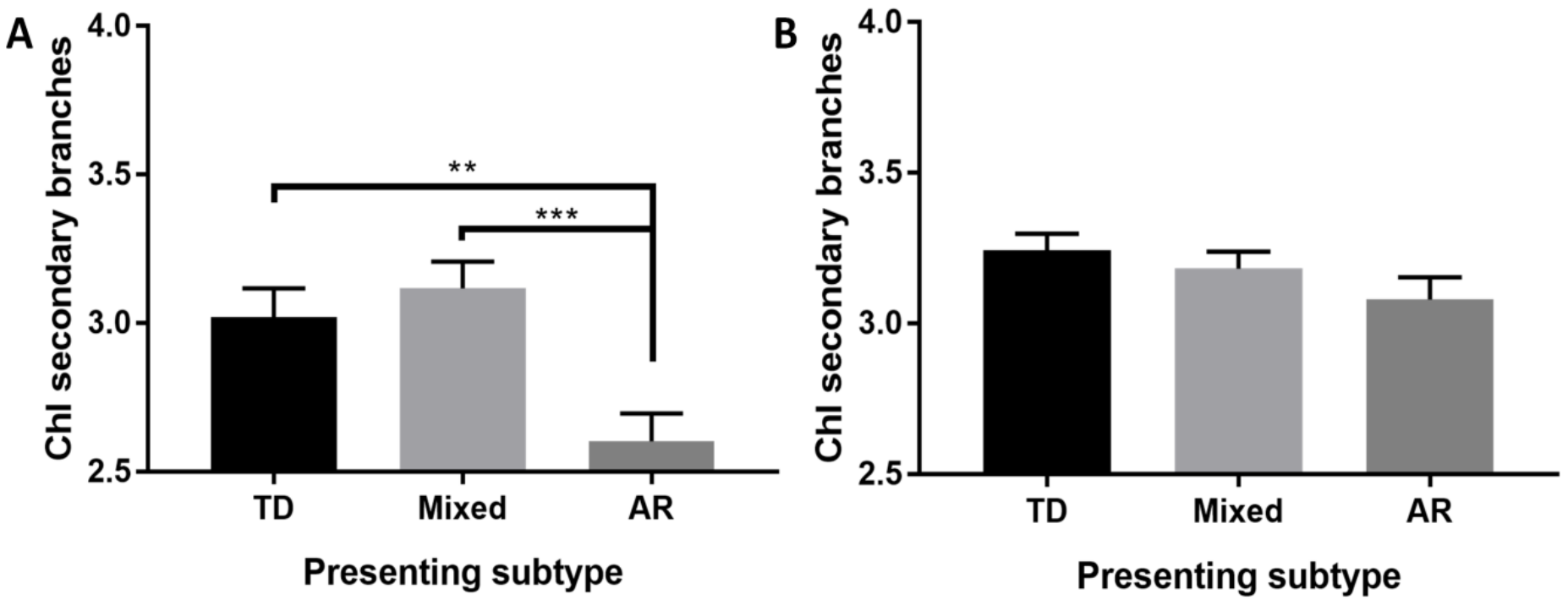


Figure 6.8: Loss of secondary branches in AR cases at the pre-commissural level. A) Comparison of secondary ChI branching in the caudate nucleus in PD motor subtypes, B) Comparison of secondary ChI branching in the putamen in PD motor subtypes. Analysis based on a sample size of 1600 ChIs per motor subtype, 4800 in total.Statistical analysis performed with Kruskal-Wallis tests with post-hoc Dunn's corrections for multiple comparisons. ** = $p < 0.01$, *** = $p < 0.001$.

At the post-commissural level, AR cases also showed reduced secondary branching, particularly compared to Mixed cases, in both the caudate (Figure 6.9A; $p = 0.0022$) and putamen (Figure 6.9B; $p = 0.0007$). In order to determine whether reduced secondary branch numbers were caused by reduced primary numbers, secondary: primary branch ratios were calculated in each case and compared between PD motor subtypes. A consistent ratio of 1.4 secondary branches per primary branch was noted in most cases and was comparable between PD motor subtypes. Therefore, it was inferred that secondary branching loss resulted from a loss of primary branches, not due to a specific loss of secondary branches only.

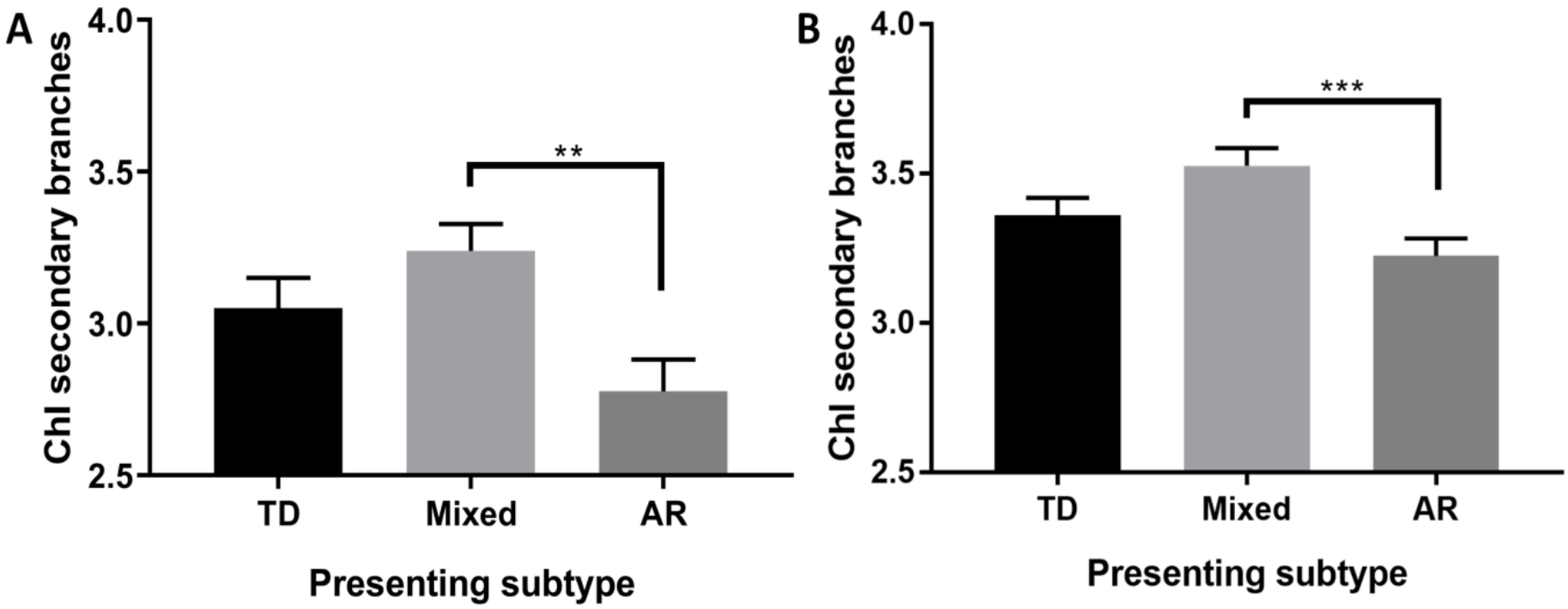


Figure 6.9: Loss of secondary branches in AR cases at the post-commissural level. A) Comparison of secondary ChI branching in the caudate nucleus in PD motor subtypes, B) Comparison of secondary ChI branching in the putamen in PD motor subtypes. Analysis based on a sample size of 1600 ChIs per motor subtype, 4800 in total. Statistical analysis performed with Kruskal-Wallis tests with post-hoc Dunn's corrections for multiple comparisons. ** = $p < 0.01$, *** = $p < 0.001$.

6.4.7. Tissue clearing to visualise striatal ChIs in 3D

To determine if branching quantification could be carried out in 3D, tissue clearing, according to the FASTclear protocol outlined in Chapter 2.5 was carried out. Unfortunately, ChAT antibody staining yielded non-specific staining, even in samples where co-staining with other markers such as TH was successful Figure 6.10. Further work is needed to optimise the FASTclear protocol for use with anti-ChAT antibodies to visualise striatal ChIs.

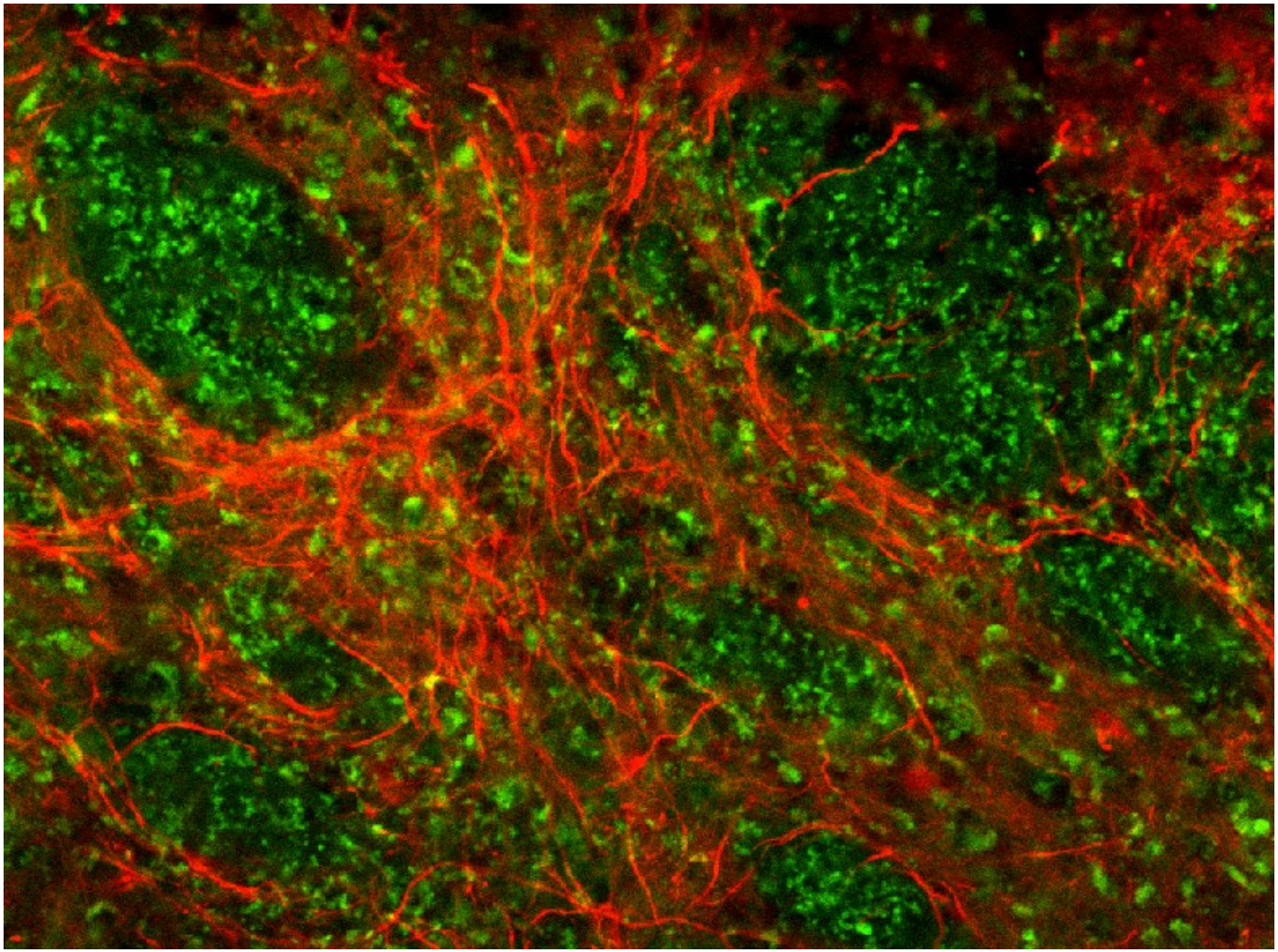


Figure 6.10: FASTclear control putamen immunostained with anti-ChAT and anti-TH antibodies. ChAT = green, TH = red. Image taken on a Leica DMi8 confocal microscope under a 20x objective lens. Maximal intensity projection of a 200µm z-stack.

6.4.8. Striatal α-synuclein pathology in PD motor subtypes

Striatal α-synuclein pathology took a number of forms in PD. As well as classical LBs (Figure 6.11A) and LNs (Figure 6.11B), glial α-synuclein deposition was seen in some cases (Figure 6.11C), most likely in microglia. The α-synuclein deposition was highly variable between cases with some exhibiting substantial deposition and some exhibiting none, irrespective of Braak α-synuclein stage. Nevertheless, the burden of α-synuclein deposition was quantified using a threshold-based %-coverage methodology. AR cases exhibited the highest α-synuclein deposition of all the subtypes in all striatal sub-regions (Figure 6.12). However, there were no statistically significant differences between the groups.

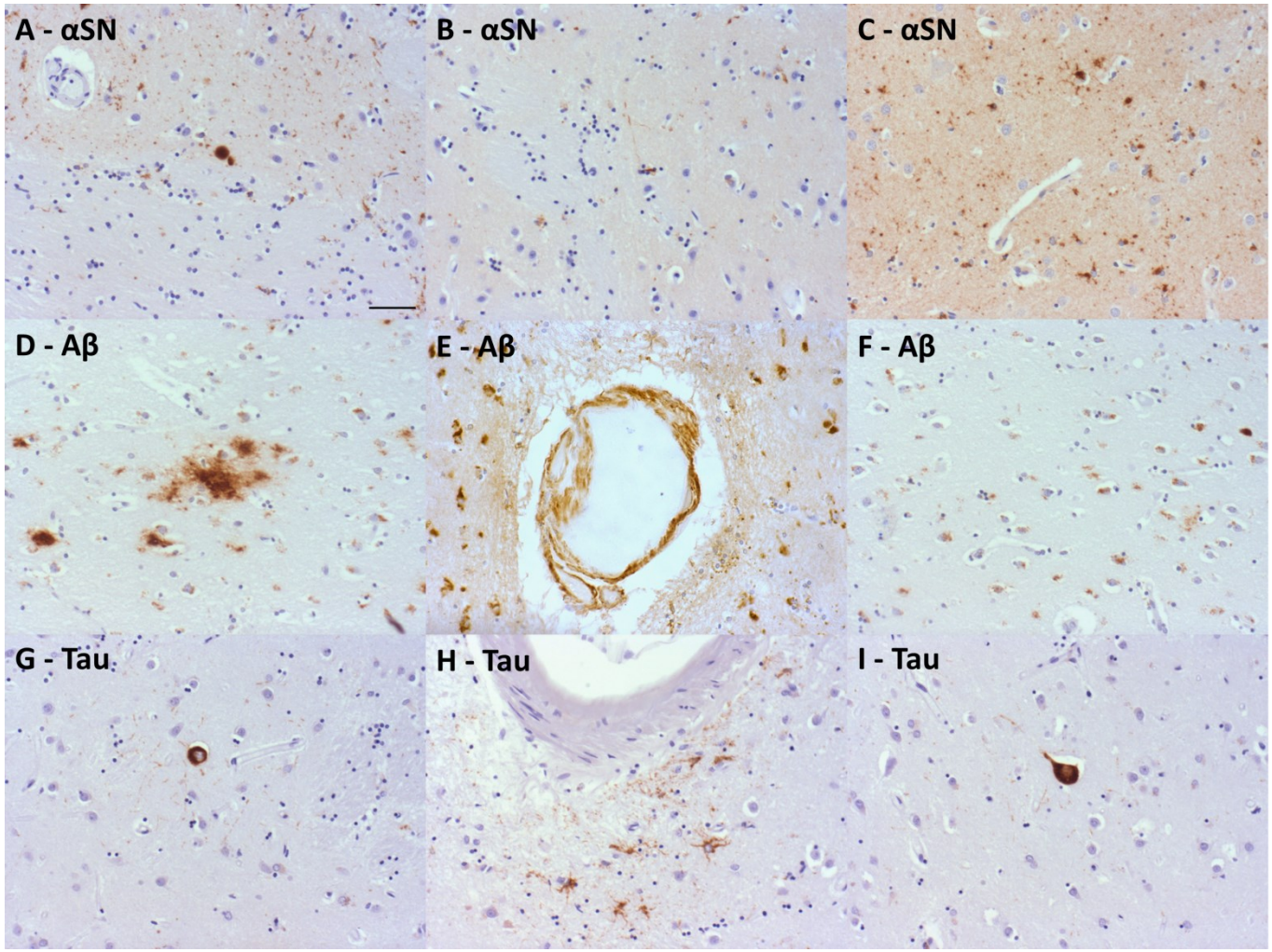


Figure 6.11: Pathologies found in the striatum in PD cases. A) α-synuclein Lewy Bodies, B) α-synuclein Lewy Neurites, C) α-synuclein in glial cells, D) Aβ plaque, E) mild cerebral amyloid angiopathy, F) granular cytoplasmic Aβ staining, G) tau in NTs and NFT, H) aging-related tau astrogliopathy, I) Tau in globose NFT (morphology of striatal ChI). Images taken under 20x objective lens. Scale bar in A = 50μm in all panels

To assess whether α-synuclein pathology was associated with differences in ChI arborisation, correlation between α-synuclein %-coverage and ChI branching was carried out. It was found that α-synuclein pathology only correlated weakly with ChI morphology in the caudate nucleus ($r = -0.2566$, $p = 0.0208$), whereby higher α-synuclein pathology was associated with reduced ChI arborisation. No significant correlation was noted in the medial, intermediate or lateral putamen subregions.

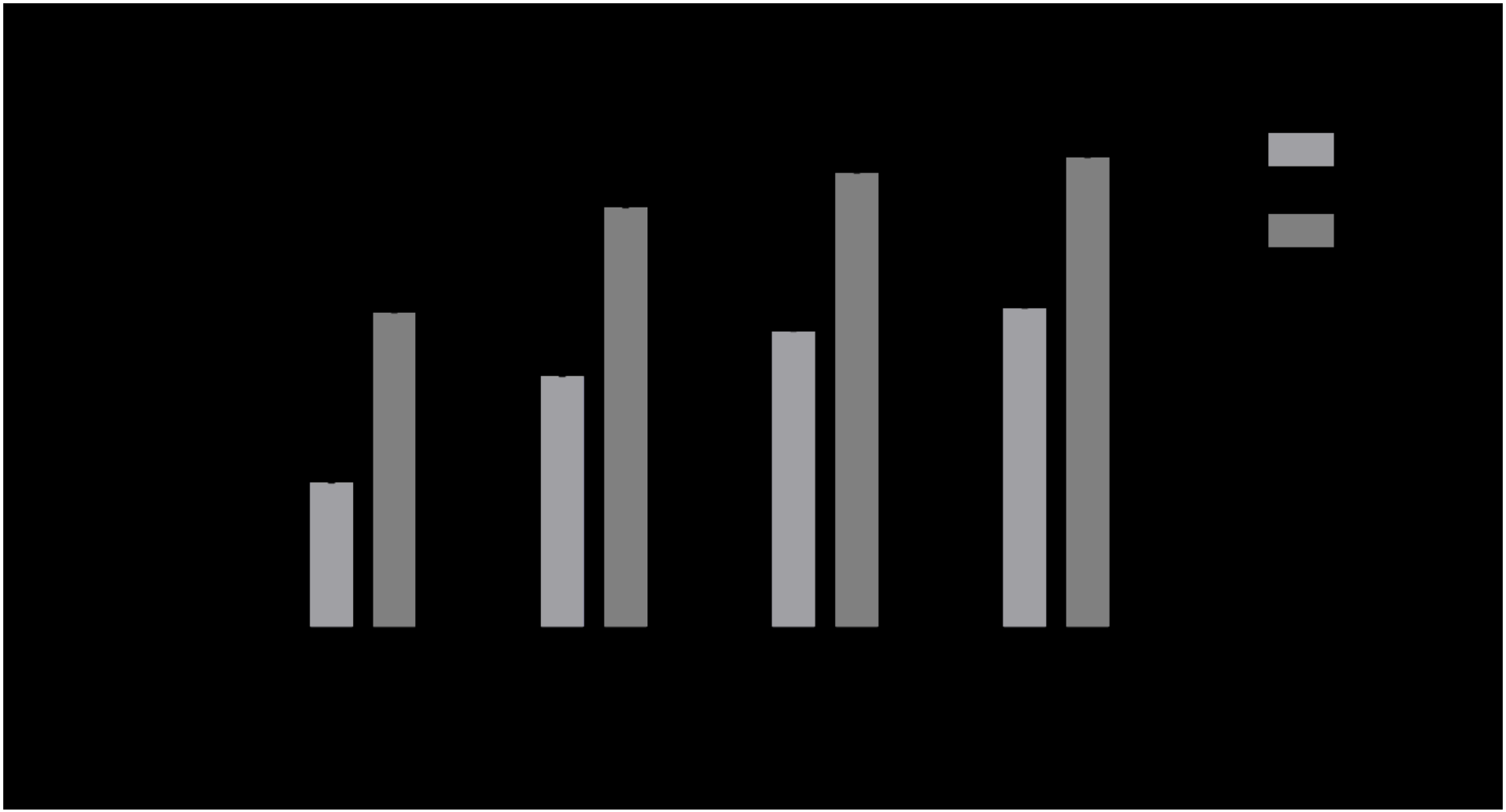


Figure 6.12: Comparisons of striatal α-synuclein deposition in different striatal sub-regions. TD = tremor-dominant, AR = akinetic-rigid, αSn = α-synuclein, Med Put = medial putamen, Int Put = intermediate putamen, Lat Put = lateral putamen. Statiscial analysis was performed using ordinary two-way ANOVAs with post-hoc Tukey's corrections for multiple comparisons.

6.4.9. Striatal Aβ pathology in PD motor subtypes

Amyloid pathology in the striatum took the forms of diffuse or cored Aβ plaques (Figure 6.11D), cerebral amyloid angiopathy (CAA; Figure 6.11E) and granular cytoplasmic staining (Figure 6.11F). Aβ pathology was measured using the %-coverage methodology. Though there were no significant differences between groups, TD cases consistently showed the highest level of Aβ deposition throughout the striatum (Figure 6.13). In the medial putamen, TD cases showed a trend increase in deposition of Aβ compared to Mixed cases ($p = 0.091$). Most of the deposition took the form of granular cytoplasmic deposits. These were present in all cell types, including large aspiny interneurons, likely ChIs. The significance of this pattern of staining is not clear. It is likely that this staining represents Aβ epitopes in physiological amyloid precursor protein molecules.



Figure 6.13: Comparisons of striatal amyloid deposition in different striatal sub-regions. TD = tremor-dominant, AR = akinetic-rigid, Aβ = amyloid-β, Med Put = medial putamen, Int Put = intermediate putamen, Lat Put = lateral putamen. Statiscial analysis was performed using ordinary two-way ANOVAs with post-hoc Tukey's corrections for multiple comparisons. Med put, TD vs Mixed, p = 0.091.

6.4.10. Striatal tau pathology in PD motor subtypes

6.4.10.1. Are there differences in overall tau burden between PD motor subtypes?

Striatal tau deposition took many different forms. NTs (Figure 6.11G), NFTs (Figure 6.11G & I) and ARTAG (Figure 6.11H) were all prevalent in the striatum. Occasional large aspiny globose NFTs/pretangles were also found (Figure 6.11G & I). These structures bore a similar morphology to the striatal ChIs. Tau deposition overall was also quantified with the %-coverage methodology. Like α-synuclein, tau pathology was highly variable between cases. Tau burden in the striatum was weakly correlated with Braak tau stage ($R = 0.2319$, $p = 0.0338$). When tau burden was compared between PD motor subtypes, AR cases displayed consistently higher tau than TD or Mixed cases (Figure 6.14). However, the only region in which there was a statistically significant increase in tau deposition in AR was the lateral putamen (vs Mixed, $p = 0.0109$; vs TD, $p = 0.0559$).

To assess the contribution of tau to ChI pathology in PD, correlations between tau %-coverage and ChI arborisation were carried out in each striatal sub-region. No significant correlation was noted in the caudate but there were significant correlations between tau pathology and ChI arborisation in all putamenal subregions whereby higher tau pathology was associated with a reduction in ChI arborisation (overall correlation in putamen: $R = -0.2438$, $p = 0.0002$).

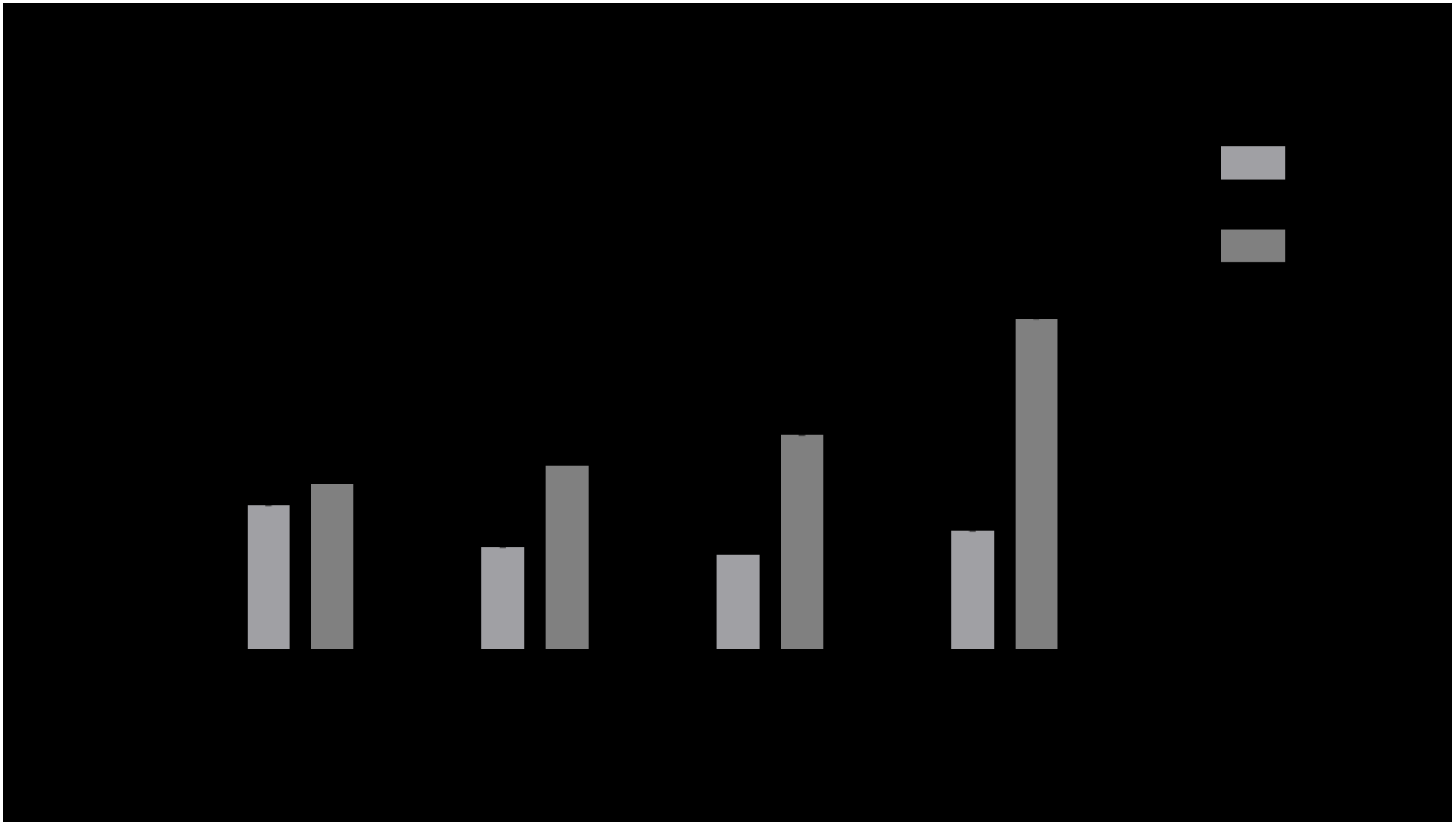


Figure 6.14: Comparisons of striatal tau deposition in different striatal sub-regions. TD = tremor-dominant, AR = akinetic-rigid, Med Put = medial putamen, Int Put = intermediate putamen, Lat Put = lateral putamen. Statistical analysis was performed using ordinary two-way ANOVAs with post-hoc Tukey's corrections for multiple comparisons.* = $p < 0.05$.

6.4.10.2. The nature of striatal tau lesions in ARPD

As mentioned in Chapter 6.4.10.1, tau depositions frequently took the form of large globose NFTs/pretangles in the striatum. This phenomenon was more frequently found in AR cases than either TD or Mixed cases. These large tau-positive structures bore a striking resemblance to the striatal ChIs. ChAT, TH and AT8 tau triple immunofluorescence staining was carried out to determine the localization of tau pathology in the striatum. In the large globose tangles, AT8 tau colocalised consistently with ChAT (as shown in Figure 6.16) suggesting that tau deposition takes place in striatal ChIs, most frequently in ARPD cases.Tau pathology in ChIs was also found in the vast majority of PSP cases in a pathological audit of PSP cases at the Parkinson's UK Tissue Bank (Tilley, in preparation). In both PD and PSP, this deposition was 4R tau positive – no 3R tau deposition was noted in the striatum in these diseases. In PSP, 4R and AT8 tau staining was found in pathognomonic tufted astrocytes alongside the large globose tangles. Double immunofluorescence staining with ChAT and AT8, 3R or 4R tau demonstrated that AT8 tau was consistently found in ChIs. This tau was 4R-positive and there was no 3R deposition was found in the striatum. This section highlights three completely novel findings of this study: 1) that tau deposition takes place in ChIs, 2) that ChI tau deposition is linked to the onset of AR phenotype in PD, 3) that tau deposition in the ChIs is a pathophysiologic factor linking AR PD with PSP, which are both diseases characterised by AR syndrome and gait impairment.

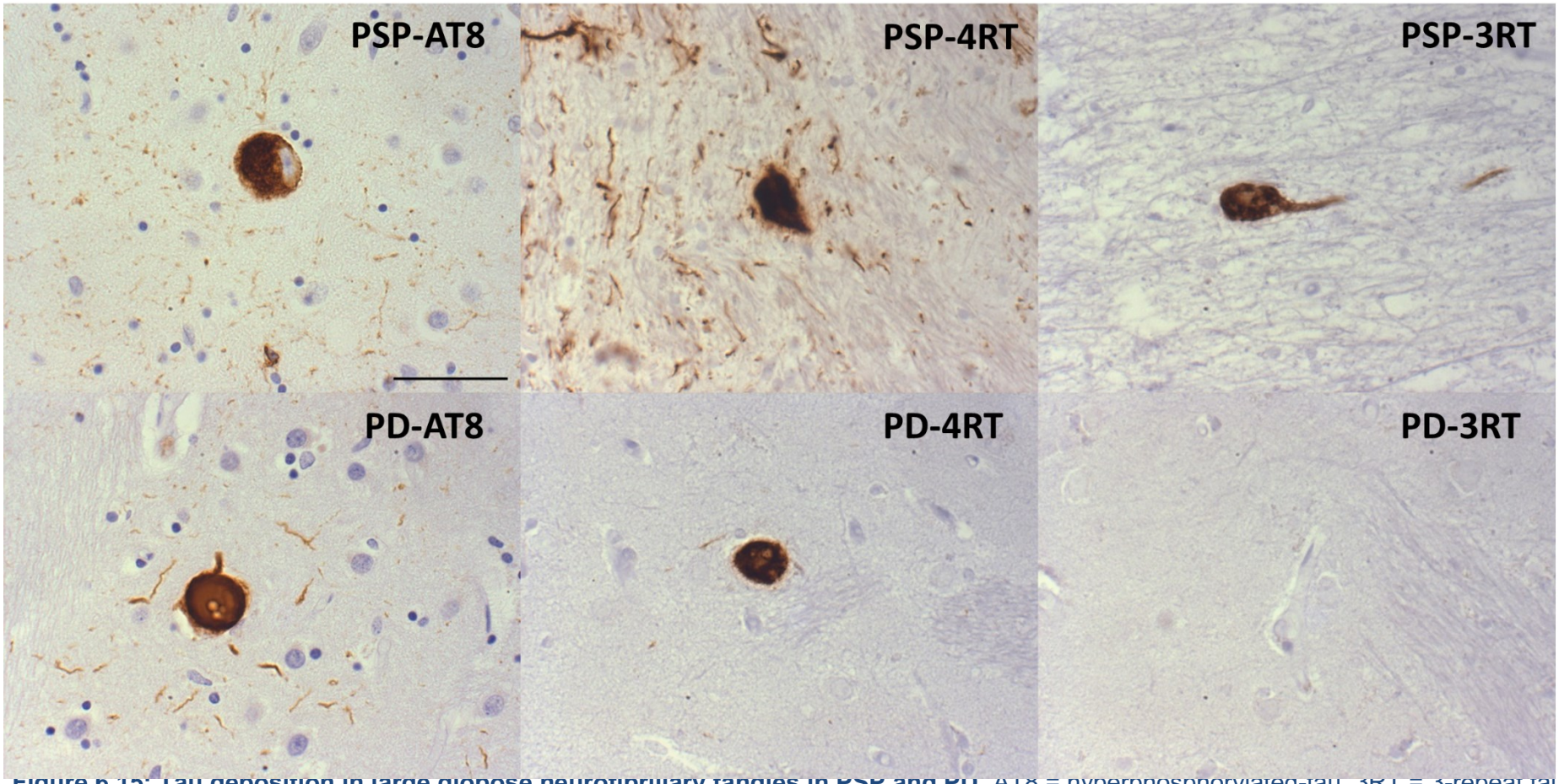


Figure 6.15: Tau deposition in large globose neurofibrillary tangles in PSP and PD. AT8 = hyperphosphorylated-tau, 3RT = 3-repeat tau, 4RT = 4-repeat tau. PSP-3RT is a tangle in the nbM. All other images are of striatum. There were no 3R tau deposits in the striatum in PSP or PD. Images taken under 40x objective lens. Scale bar = 50μm for all panels.

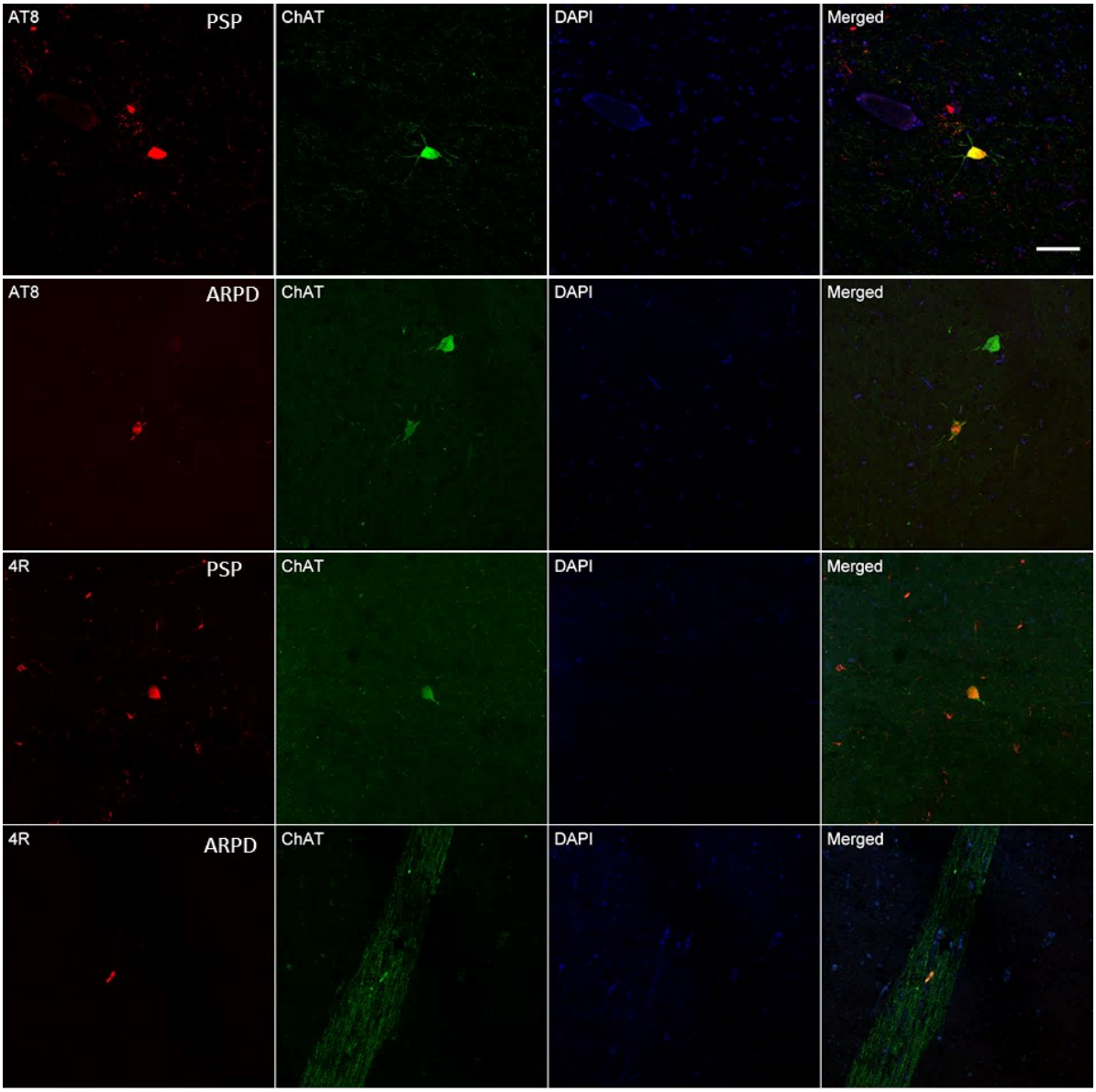


Figure 6.16: AT8 and 4R tau deposition in striatal ChIs in PSP and ARPD. AT8 = hyperphosphorylated tau, ChAT = choline acetyltransferase, DAPI = 4',6-diamidino-2-phenylindole, 4R = 4-repeat tau. No 3R tau is found in the striatum. Scale bar = 50μm in all panels.

6.4.11. α-synuclein, tau and Aβ pathologies show synergism in the striatum in PD

Correlation tests were carried out between the three pathologies measured in the striatum, and then with the degree of ChI branching. The burdens of α-synuclein, tau and Aβ pathologies showed remarkably high correlations with one another in the striatum. These correlations were positive and statistically significant in all cases. The strongest correlation came between Aβ and α-synuclein ($R = 0.5224$, $p < 0.0001$). However, the only protein pathology that correlated with ChI branching was tau. Higher levels of tau pathology were significantly associated with reduced ChI branching ($R = -0.2438$, $p = 0.0002$). α-synuclein showed a trend association with ChI branching in the striatum ($R = -0.2045$, $p = 0.073$). In some cases, extremely high levels of deposition of these pathologic proteins were noted. In these cases, the striatal cholinergic system showed signs of severe degeneration. A case where this was true is demonstrated in Figure 6.17. Not only do the ChIs show reduced branching, but the normally densely ChAT-stained striatal neuropil shows a severe reduction in ChAT-immunoreactivity. In these cases, the clinical phenotype was characterised by severe akinesia and a lack of tremor. One of these cases was later confirmed to have a parkin mutation, suggesting a possible role for mutant parkin protein in this specific aspect of PD pathophysiology.

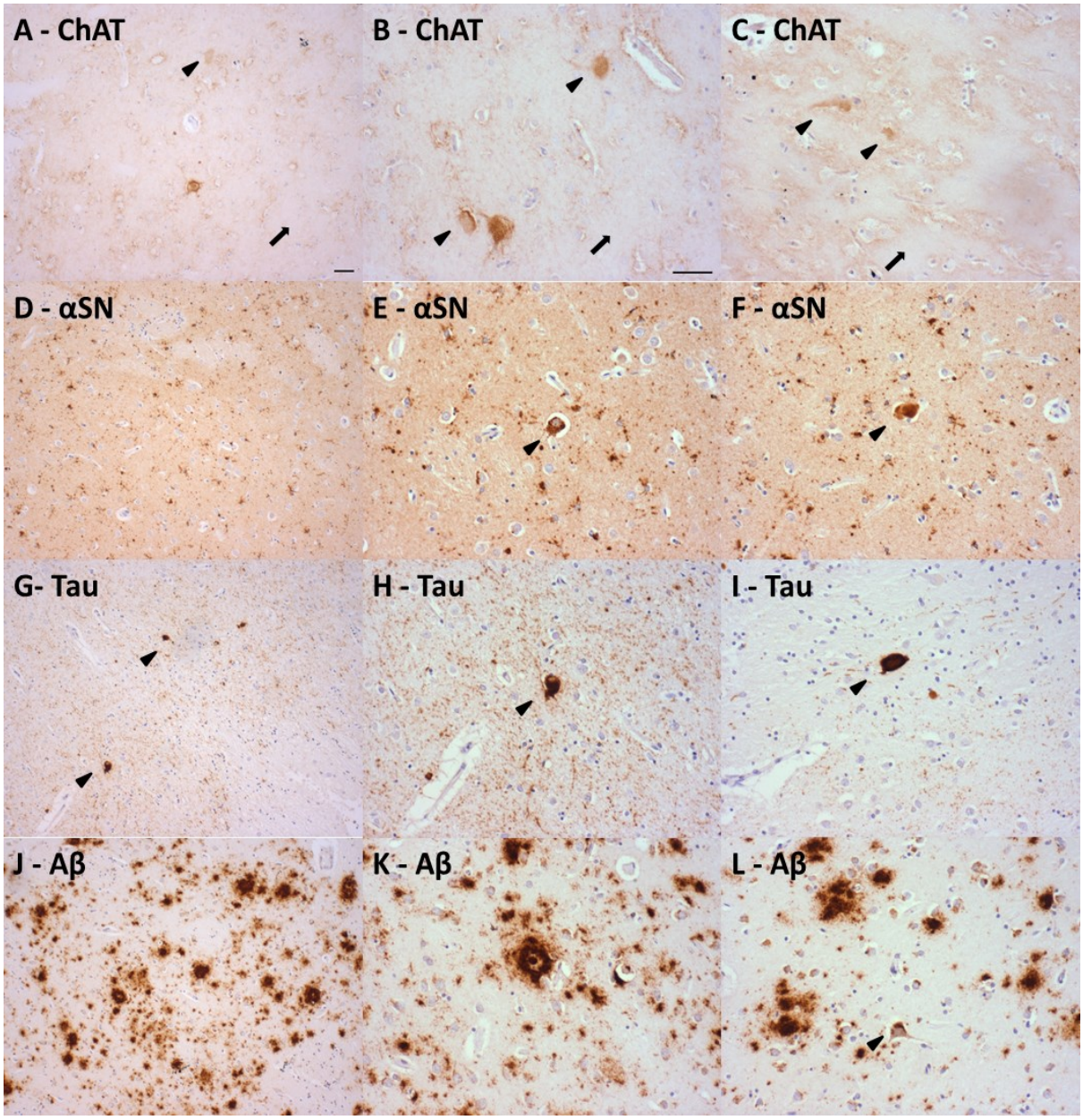


Figure 6.17: Severely degenerated striatal cholinergic system in an AR PD case with significant pathologic protein deposition. A-C) Degenerative striatal ChIs, D-F) Severe α-synuclein pathology, including deposition in striatal ChIs (E & F), G-I) Severe tau deposition in striatal ChIs and neuropil, J-L) Severe Aβ pathology including intracellular staining in striatal ChIs (L). Scale bar in A = 50μm for A, D, G and J (10x objective images). Scale bar in B = 50μm for B, C, E, F, H, I, K and L (20x objective images). Arrows indicate areas where ChAT staining is lost, arrowheads indicate degenerative striatal ChIs.

6.5. Discussion

This is the first study to implicate the striatal cholinergic system in the pathogenesis of PD motor subtypes. In fact, previous studies have gone as far as to say that striatal ChI are not involved in PD pathogenesis (Sulzer and Surmeier, 2013). The data presented here has shown that striatal ChIs are atrophic in AR cases but ramified in cases with tremor. These neurons have also been implicated in the pathogenesis of tardive dyskinesia, LID following chronic neuroleptic treatment. A study by Miller & Chouinard hypothesised that chronic blockade of dopamine receptors on striatal ChIs causes them to degenerate (Miller and Chouinard, 1993). It has also been shown that pathologic protein deposition in the striatum is linked to atrophy of the ChIs, most notably related to tau. Tau protein, for the first time, was demonstrated to be deposited in large aspiny neurons in the striatum, which had been proved to be ChIs. Given the relationship between tau burden and ChI arborisation, whereby higher tau deposition correlated with reduced branching, one can hypothesise that hyperphosphorylated tau is linked to morphologic alterations of striatal ChIs. These tau-positive structures are also found in atypical parkinsonism cases such as PSP and CBD in the striatum. These disorders are also characterised by significant AR syndrome alongside severe postural instability and supranuclear gaze palsy in PSP, and apraxia and *alien limb* phenomena in CBD. Pathologically, these disorders are defined by the presence of 4R tau in particular astrocytic morphologies such as tufted astrocytes in PSP and astrocytic plaques in CBD. However, while these pathologies are obviously important, the neuronal pathology in these disorders is largely ignored. I have found large, aspiny, tau-positive neurons in almost all PSP cases in an audit of PSP pathology at the Parkinson's UK Tissue Bank (Tilley, unpublished data), and this may contribute to cholinergic degeneration in the striatum in PSP (Warren *et al.*, 2005).

Tau protein has a crucial role in the physiology of microtubules within neurons. It distributes itself in axons in adult neurons where it promotes microtubule stability and axonal outgrowth, facilitates axonal transport and vesicular trafficking (Wang and Mandelkow, 2016). When tau is found in the soma, there is some evidence to suggest that it is involved in the maintenance and repair of DNA (Violet *et al.*, 2014). When tau becomes pathological, it may undergo several post-translational modifications which reduce its capacity to bind microtubules. Therefore, pathological tau has deleterious effects including microtubule disassembly, axonal spine loss and DNA damage.

Pathological tau also gains the capacity to aggregate and seed further aggregation. It may also trespass into the presynaptic terminal region where it disrupts synaptic function and plasiticity, and causes synaptic loss (Decker *et al.*, 2015). Fascinatingly, this process appeared to occur early in the pathologic process and was dependent on calcium dysfunction. Petryszyn and colleagues performed MPTP lesions in Macaque monkeys and found significant upregulation of calretinin, a calcium binding protein, in striatal ChIs (Petryszyn *et al.*, 2016). This suggests that ChI dysfunction in PD is mediated by calcium. Disruption of calcium homeostasis in these cells may promote tau and α-synuclein pathology. These pathologies are synergistic and enhance the deposition of each other (Compta *et al.*, 2014). This synergism may involve the proline-rich regions and microtubule-binding domains of tau protein that have been shown interact with other pathologic proteins such as α-synuclein (Guo, Noble and Hanger, 2017). This may underlie the correlations that were found in this study between tau and α-synuclein pathologic burden. To close the loop, where these pathologic proteins were found in high density in the striatum, this contributed to loss of branching of striatal ChIs. Analysis of neuronal morphology in the striatum in PD is not new. McNeill and colleagues assessed MSN dendritic morphologic alterations in patients with advanced PD and found significant atrophy of this morphology (McNeill *et al.*, 1988). More recently, the morphologies of ChIs in different striatal regions were assessed, albeit qualitatively, in a number of primate and non-primate species (Gonzales and Smith, 2016). Here, a new technique for morphometric analysis of ChIs has been employed on striatal ChAT-stained sections. The data collected using this technique was then validated using our novel tissue clearing technique, FASTclear (H.M. Lai *et al.*, 2018), to assess the morphology of these neurons in 3D.

Ramified (highly branched) morphology of striatal ChIs, as opposed to atrophic (sparsely branched) morphology, may predispose to increased cholinergic neurotransmission in the striatum. This may underlie the responsiveness of tremor, and not akinesia, to anticholinergic medication. The result of increased cholinergic neurotransmission in the striatum is an increase in striatal output (Maurice *et al.*, 2015). This is because MSNs of the direct and indirect pathways express stimulatory M1 muscarinic receptors (Lim, Kang and McGehee, 2014). Direct pathway MSNs may also express inhibitory M4 receptors (Howe and Surmeier, 1995; Galarraga *et al.*, 1999). Muscarinic receptors are also found on presynaptic terminals of cortico-striatal

glutamatergic neurons where they facilitate glutamate release onto striatal MSNs and promote LTP (Calabresi *et al.*, 2000). A lack of acetylcholine may therefore cause LTD resulting in slowed neurotransmission through the basal ganglia circuitry. Thus acetylcholine in the striatum has the potential to modulate both striatal output streams (Lim, Kang and McGehee, 2014). This could highlight a novel model of basal ganglia dysfunction in PD whereby tremor results from increased acetylcholine production in the striatum which would increase the speed and frequency of transmission of signals through the basal ganglia circuitry. Conversely, a lack of acetylcholine, say in AR cases, causes slowed neurotransmission through pathologic protein deposition, leading to reduced acetylcholine release from ChIs and synaptic dysfunction. This slowing of neurotransmission may be reflected phenotypically by significant reduction in the, amount, speed and amplitude of voluntary movement – akinesia, bradykinesia and hypokinesia, respectively.

This hypothesis is supported by the current thinking on the pathophysiology of tremor in PD at the circuit level. Helmich and colleagues proposed that tremor results from a dysfunction of two interdependent circuits – the striato-pallidal and cerebello-thalamo-cortical circuits (Helmich *et al.*, 2012). They posited that the interaction of these circuits in tremor is akin to that of a *dimmer-switch* in that one of the circuits, the striato-pallidal circuit drives tremor onset (hence acting a switch) and the cerebello-thalamo-cortical drives tremor amplitude (acting as the dimmer). In vivo recordings of synchronous EMG and fMRI activity in tremulous limbs demonstrated that increased aberrant activity of the striato-pallidal axis drives tremor onset and offset (Helmich *et al.*, 2011). Further, data from Mure and colleagues, who used fluorodeoxyglucose PET imaging to assess brain metabolic correlates of tremor, found that activity in the lateral putamen played a role in the PD tremor-related activity pattern (Mure *et al.*, 2011). This was extremely interesting given that the data presented in this chapter also highlights the lateral putamen as a locus for tremor-activity as opposed to non-tremulous PD.

6.5.1. Limitations

Though this study highlighted substantially innovative findings, utilising novel analyses of ChI morphology, the caveat is that these analyses were far from perfect. In this study, 7μm sections were used to study the morphology of the striatal ChIs. However, these neurons can be up to 50μm in diameter at the cell soma meaning that the technique used is unlikely to capture all branches. I tried to circumvent this by

employing a novel tissue clearing technique, FASTclear, to visualise these neurons and their branching in 3D. However, incubation of FASTclear samples with ChAT antibodies has been difficult, and I was unable to successfully visualise ChAT-positive neurons with this technique. In contrast, other neuronal markers such as TH can be utilised for visualisation of other neuronal types with considerable success. This has been demonstrated with LC TH-positive neurons in Chapter 7 (Figure 7.6). More work is needed to improve the penetrance of ChAT antibodies into FASTclear tissue samples for the visualisation of striatal ChIs. An alternative way forward would be to use thick sections (e.g. 30μm sections) of striatal tissue, and future work should attempt to correlate findings in these studies with my branching results obtained in 2D.

Further, more studies will be needed to understand the neurobiological consequences of ChI pathology. It is unclear whether morphological alterations of ChIs result in changes to the output of acetylcholine in the striatum, or whether the morphology of the ChI is an appropriate analogue of the health of the neuron. Biochemical analyses such as measuring ChAT expression and activity in tissue homogenates (Ruberg *et al.*, 1985) or by autoradiography (Suzuki *et al.*, 2002). Vesicular acetylcholine transport expression could also be a useful marker of cholinergic axons. These markers have all been used in previous studies on PSP and so would be useful for further study in PD motor subtypes.

6.6. Conclusion of chapter

To conclude, it was found that the striatal cholinergic system shows fascinating morphological discrepancies between TD and AR cases. AR cases show atrophic striatal ChIs when compared to TD, and heightened deposition of pathological tau protein in these neurons, in a strikingly similar pattern to that seen in PSP striata. This may highlight a new aspect of shared pathophysiology that should be explored further. Pathologic deposition of α-synuclein, tau and Aβ proteins showed a synergism whereby these amounts of deposition of each were strongly positively correlated with one another. When the severity of deposition was high, this was accompanied by severe degeneration of the striatal cholinergic system.

I propose a new model of basal ganglia dysfunction in which tremor and AR features result from increased basal ganglia transmission and slowed basal ganglia transmission, respectively, mediated by differential pathology of striatal ChIs.

Chapter 7 - Investigating the role of the LC noradrenergic system in PD-related cognitive impairment

With gratitude to Shivani Patel and Charlie Hession who performed research on this topic under my surpervision, for allowing me to reproduce their results for this thesis chapter. Shivani Patel's MSc thesis covered projections of the LC to the frontal and cingulate cortices in PD and LBD subtypes, and Charlie Hession's BSc thesis analysed LC projections to the amygdala.

7.1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder associated with specific phenotypes of dementia (Emre *et al.*, 2007). The dementia syndrome in PD is characterised by visuo-spatial, executive, attentional and occasional memory deficits (Emre *et al.*, 2007; Friedman, 2017). LBD patients may also experience cognitive fluctuations, hallucinosis, and sleep disorders (Boeve, Silber and Ferman, 2004; Metzler-Baddeley, 2007). LBD comprises two main phenotypes of dementia, separated by an arbitrary 'one-year-rule' (as discussed in Chapter 1.2.2.1). DLB is defined by dementia preceding or occurring within one year of the onset of parkinsonism (McKeith *et al.*, 2017), whereas PDD is dementia occurring over a year after the onset of parkinsonism (Emre *et al.*, 2007).

Though some authors argue that DLB and PDD are similar conditions (Friedman, 2017), their diagnostic criteria differ in critical ways (Emre *et al.*, 2007; McKeith *et al.*, 2017). The core diagnostic criteria for DLB are FC, VH and RBD, whereas PDD criteria specify these as 'associated' features (Emre *et al.*, 2007; McKeith *et al.*, 2017). Core features for PDD diagnosis are dementia, developing within the context of parkinsonism, with impairment in more than one cognitive domain (Emre *et al.*, 2007). The discrepancy in these criteria appears to be well-founded: FC is reported in up to 80% of DLB patients (Ferman *et al.*, 2004) compared to 29% of PDD (Noe *et al.*, 2004); RBD is a risk factor for DLB (McKeith *et al.*, 2017) but is not confirmed for PDD (Emre *et al.*, 2007); VH, though common in PDD, are more prevalent in DLB (Fénelon *et al.*, 2000; Aarsland, Ballard, *et al.*, 2001; Aarsland *et al.*, 2007). However, recent studies shown that some PDD cases can mimic DLB. Varanese and colleagues separated PDD patients by the presence of FC and found that fluctuating PDD showed a more similar pattern of cognitive dysfunction to DLB than non-fluctuating PDD (Varanese *et al.*, 2010). PDD patients without FC showed milder attention and memory deficits, and lower prevalence of hallucinosis.

LBD is characterised by the accumulation of inclusions of aberrant α-synuclein in Lewy bodies and neurites. Whilst LBD is a synucleinopathy, commonly, increased levels of tau and Aβ deposition are found in cases of LBD compared to controls and non-impaired PD (Kalaitzakis and Pearce, 2009; Kalaitzakis *et al.*, 2011). Thus LBD probably reflects a confluence of pathologies. LBD has been shown in previous studies to reflect a later α-synuclein pathologic stage compared to PD without dementia

(Compta *et al.*, 2011, 2014), suggesting an increased level of cortical pathology contributes to LBD pathogenesis. However, the precise neuropathological substrates of LBD and LBD symptomatology have remained controversial (Jellinger, 2012).

The LC is the major source of noradrenaline in the central nervous system, innervating cortical, subcortical and limbic structures (Dahlstroem and Fuxe, 1964; Foote, Bloom and Aston-Jones, 1983; Halliday, 2004). In so doing, the LC functions to maintain sleep-wake-cycles, concentration, alertness and attention, thereby promoting the steady cognitive state (Benarroch, 2009). PGO waves are electroencephalogram correlates of hallucinosis but are also involved in the initiation of REM-sleep (Steriade *et al.*, 1990). They originate from the coeruleus/subcoeruleus complex, thereby implicating these regions in DLB (Manford and Andermann, 1998). DLB features therefore appear to show deficits in the functions of the LC. As discussed in Chapter 5.4.6, LC neuron count is not associated with different PD motor subtypes, but instead was found to be significantly predicted by cognitive status, whereby increasing cognitive impairment predicted greater degeneration of the LC.

7.2. Hypothesis and aims of chapter

I hypothesise that pathology in the LC will differentiate cognitively impaired PD patients from those without cognitive impairment, and between LBD subtypes. In the present study, the pathology and integrity of the LC neuronal population will be compared in PD, DLB and PDD cases, with and without DLB-like features. I will also assess the contribution of the LC to the symptomatology of DLB. TH+ fibres will also be quantified at LC target regions in a subset of cases in each PD clinical subgroup known to be vulnerable in PD. These are: i) the frontal cortex, ii) the cingulate cortex, iii) the amygdala.

7.3. Methods

7.3.1. Cohort selection and clinical assessment of cases

Clinical data of cases from the Parkinson's UK Tissue Bank at Imperial College London were compiled by specialist movement disorder neurologists (including R.K.B.P.). Only subjects evaluated by a clinician within two years prior to death and with complete clinical histories were used for this study. The clinical diagnoses of PD, PDD and DLB were assigned based on published clinical criteria (Emre *et al.*, 2007; McKeith *et al.*, 2017). Only cases with detailed clinical information were used. Retrospective case note evaluation was used to examine cases for pertinent clinical characteristics. This type of analysis is a well-accepted method of case ascertainment and has often been used in clinicopathological studies involving both dementia and parkinsonism (Kalaitzakis *et al.*, 2009, 2011, Selikhova *et al.*, 2009, 2013). While it was not sought to construct *post hoc* formal diagnoses, all cases were analysed for evidence of FC, VH and RBD – the key features of DLB, alongside dementia and spontaneous parkinsonism – thereby creating the three dementia groups in a manner similar to Varanese and colleagues in their study: PDD without DLB features (PDD), PDD with DLB features (PDD-DLB), and DLB (Varanese *et al.*, 2010). Additionally, the presence of depression and anxiety disorders were also noted.

7.3.2. Neuropathological assessment

All human tissue work was carried out under the ethical approval held by the Parkinson's UK Tissue Bank at Imperial College London (REC Ref: 07/MRE09/72). Neuropathological diagnosis was based on α-synuclein, tau and Aβimmunohistochemistry. PD cases were confirmed neuropathologically using an established protocol for the neuropathologic diagnosis of Lewy Body disease (Alafuzoff *et al.*, 2009). Cases were excluded based on the exclusion criteria outlined in Chapter 2.1.3. In total, 38 PD, 56 PDD, 22 DLB and 11 age-matched control cases were included for this study (Appendix 6 – LBD cohort for Chapter 7 of this thesis.

7.3.3. Post-mortem human brain tissue

Human brain tissue from the LC was routinely sampled as part of the diagnostic work-up of cases at the Parkinson's UK Tissue Bank. Samples were prepared for immunohistochemistry by the protocol outlined in Chapter 2.1.5. Frontal cortex, cingulate gyrus and amygdala sections samples were collected for a smaller subset

of cases in each LBD subgroup. 10 PD, 10 PDD, 10 PDD-DLB and 10 DLB cases were recruited for this part of the study.

7.3.4. Histology, immunohistochemistry and tissue clearing

Tissue sections were stained with LFB according to the protocol outlined in Chapter 2.2 immunostained with anti-TH, anti-α-synuclein, anti-AT8 tau and anti-Aβ antibodies according to the protocols outlined in Chapter 2.3 and Table 2.3. Tissue clearing was carried out using our novel FASTclear methodology as described in Chapter 2.5 with anti-TH antibodies for the 3D visualisation of LC neurons. These samples were visualised on a Leica RMi8 or inverted confocal microscope, using Leica Application Suite-X (LAS-X) for image capture.

7.3.5. Microscopic analysis of slides

Image capture of LC sections was carried out using an Olympus Vanox microscope attached to a Micropublisher 3.0 camera at 40x magnification. TH-positive neuronal counts were performed, blinded to the clinical diagnosis, using the manual tag function on Image Pro Plus 7.0 (Chapter 2.6.2). Structures were included in the count if they exhibited a clear neuronal morphology with a defined nucleus and nucleolus. TH+ fibres were also counted using the manual tag function in the frontal cortex, cingulate gyrus and amygdala. For cortical regions, areas of interest were demarcated at sulcal depths and gyral crests, in superficial (II and III) and deep (V and VI) cortical layers in order to sample consistently throughout the cortex. The amygdala was subdivided into centromedial and basolateral sub-regions (Figure 7.1) according to the atlas of Mai and colleagues (Mai, Paxinos and Voss, 2008). α-synuclein, tau and amyloid pathology were assessed with %-coverage of staining in a 200x objective lens field.

7.3.6. Statistical analysis

Statistical analyses were carried out using GraphPad Prism 7.01. Comparisons of TH, α-synuclein and tau were performed using Mann-Whitney tests for single comparisons, or Kruskal-Wallis tests with Dunn's post hoc corrections for multiple comparisons. Statistical significance was set at $p<0.05$. Intra-class correlations for intra-rater reliability of the %-coverage analyses, and multivariate regression analyses for contributions of demographic factors, were calculated using IBM SPSS 24.

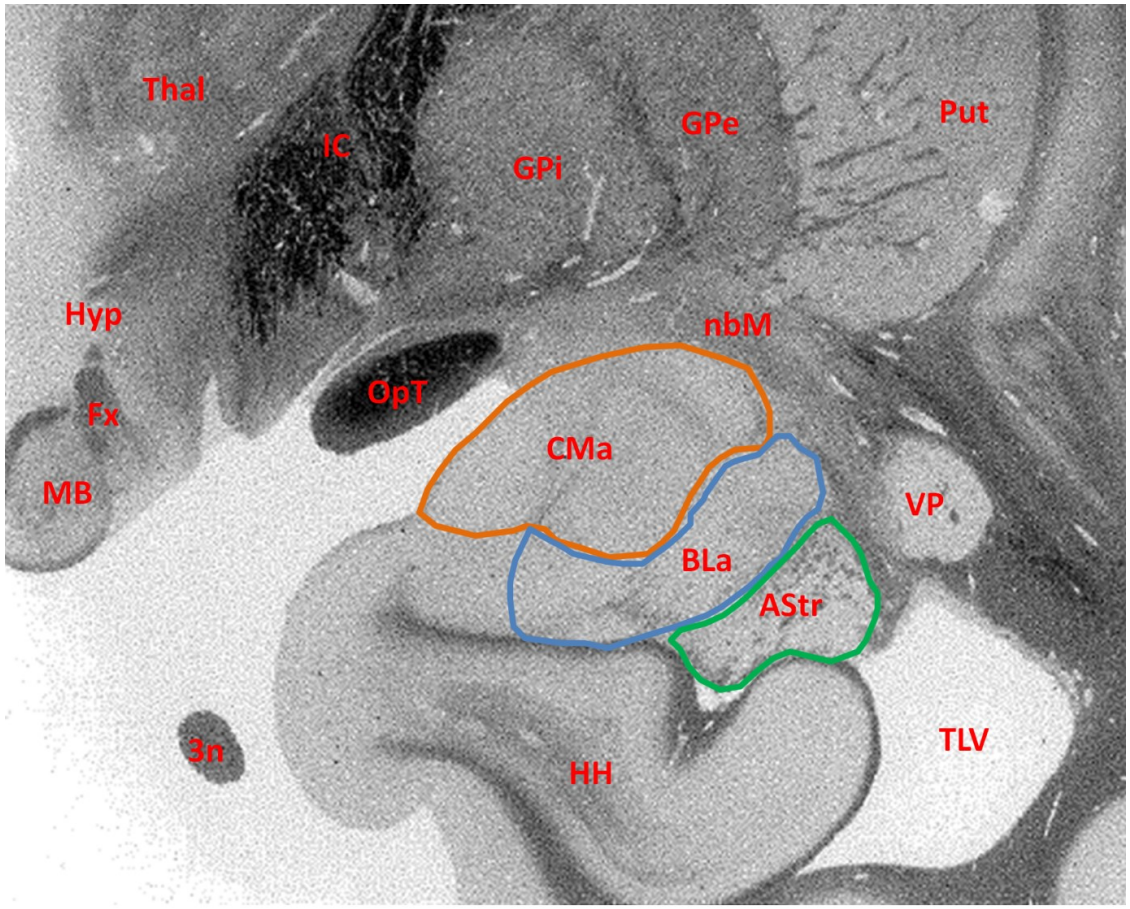


Figure 7.1: The anatomy of the amygdala region at the level of the mammillary body. 3n = oculomotor nerve AStr = amygdalo-striate transition zone, BLa = basolateral amygdala sub-region, CMa = centromedial amygdala sub-region, Fx = fornix, GPe = external segment of globus pallidus, GPi = internal segment of globus pallidus, HH = hippocampus head, Hyp = hypothalamic area, IC = internal capsule, MB = mammillary body, nbM = nucleus basalis of Meynert, OpT = optic tract, Put = dorsal putamen, Thal = thalamic area, TLV = temporal horn of lateral ventricle, VP = ventral putamen. Figure adapted from atlas image derived from Mai et al. (Mai, Paxinos and Voss, 2008).

7.4. Results

7.4.1. Cohort demographics

Demographic analysis of the cohort (

Table 7.1) revealed that the groups were separable by sex, disease duration and Braak α-synuclein stage. The dementia groups had a stark male sex bias compared to the other groups, in line with previous reports (Savica *et al.*, 2013; Gomperts, 2016). DLB is much more likely to affect males than females with a 2.91 odds ratio found in a previous study (Nelson *et al.*, 2010). DLB also had a reduced disease duration which was expected because the PDD group had parkinsonism for approximately 10 years on average before the development of a dementia syndrome. DLB also has a reduced age of death due to dementia being a significant cause of mortality in the population. However, DLB cases had a significantly longer duration of dementia than PDD cases. PD without cognitive impairment had a significantly lower mean Braak α-synuclein stage than PDD and DLB, reflecting the greater neocortical pathology found in dementia cases than non-dementia cases in previous reports (Compta *et al.*, 2011, 2014). Further, Braak tau stage was significantly different between the disease groups with PDD exhibiting the highest mean Braak tau stage. There was comparable tau burden between the other disease groups. Aβ pathology was also highest in PDD, significantly so when compared to PD. In summary, PDD cases showed the highest degree of Alzheimer's pathology, perhaps highlighting DLB as a dementia syndrome more purely associated with α-synuclein.

	Control	PD	PDD	DLB	P value
Sex (M:F)	4:7	16:22	40:16	21:1	<0.0001$^{\chi}$
Disease duration (years)	-	11.75	11.60	6.46	0.0095^{K}
Years of dementia (years)	-	-	3.33	6.46	<0.0001^{M}
Age of death (years)	81.58	78.73	78.91	75.78	0.3370^{K}
Braak αSN	-	5.21	5.78	5.64	0.0099^{K}
Braak Tau	1.54	1.47	1.84	1.33	0.0305^{K}
Thal Aβ phase	2.00	1.48	2.40	1.95	0.0667^{K}

Table 7.1: Cohort demographics for controls, PD without cognitive impairment (PD), PDD and DLB. χ – Chi squared analysis performed. K – Kruskal-Wallis Test performed. M – Mann-Whitney U Test performed. P<0.05 indicates statistically significant differences between groups

7.4.2. LC degeneration in PD and LBD

The quantity of TH+ neurons in the LC was significantly reduced in synucleinopathy cases (PD, PDD and DLB combined) compared to controls (p<0.0001; Figure 7.2). When separated into PD, PDD and DLB, there was a stepwise reduction in LC TH+ neuron count with significant differences found between controls and PDD, controls and DLB, and PD and DLB (Figure 7.3A& B). The effect of disease phenotype on TH+ neuron count prevailed after correction for disease duration, years of dementia, and gender in a multivariate regression analysis (p<0.001). Disease duration was the predominant explanatory variable for the variance of LC TH+ neuron count within groups. This provides evidence that the LC is directly involved in the pathophysiology of PD, and more specifically in the pathogenesis of LBD.

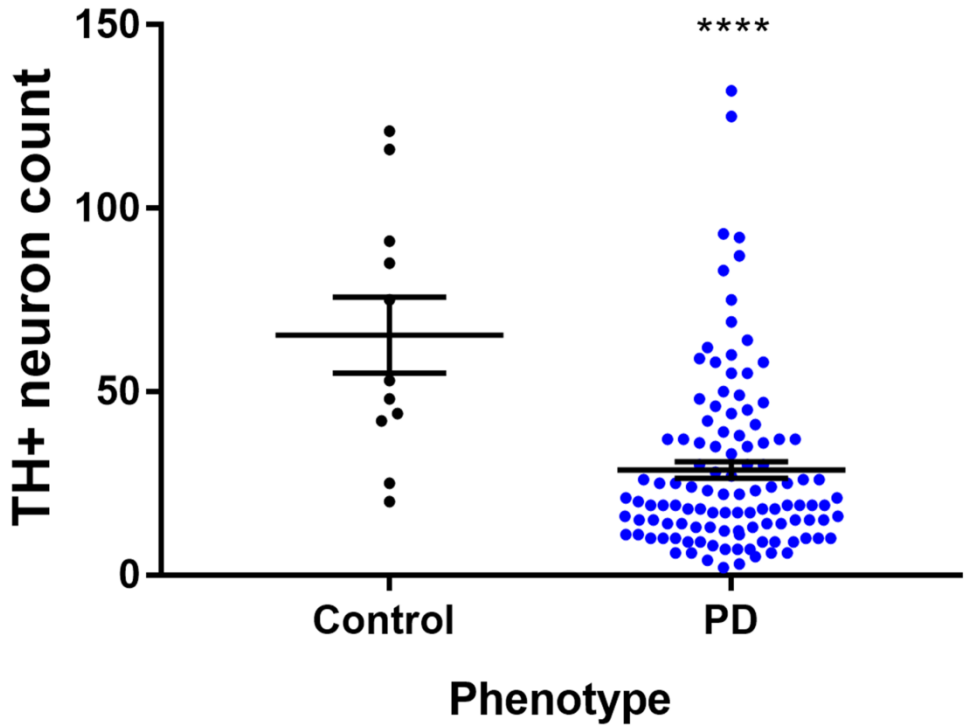


Figure 7.2: TH+ LC neuron count in PD cases compared to controls. Statistical analysis by Mann-Whitney U test, **** = p<0.0001. PD refers to all cases no matter the diagnosis.

7.4.3. Clinical overlap between PDD and DLB, mirrored by degeneration of the LC

A specific subset of PDD cases show FC and RBD and thus exhibit characteristic features of the DLB-related clinical syndrome (PDD-DLB). Of the 56 PDD cases, 26 showed presence of RBD, VH and FC and were assigned to the PDD-DLB group. TH+ neuronal counts in the LC were reanalysed, taking into account the subdivision of LBD cases into PDD, PDD-DLB and DLB (Figure 7.4). This revealed that the PDD-DLB group represented a middle ground between PDD and DLB in terms of LC integrity. The LC was significantly more degenerated in the DLB group compared to PDD which held when controlled for disease duration, gender and years of dementia (p=0.02). These data support the notion in recent literature that LBD represents a continuum with PDD at one end and DLB at the other (Weil *et al.*, 2017).

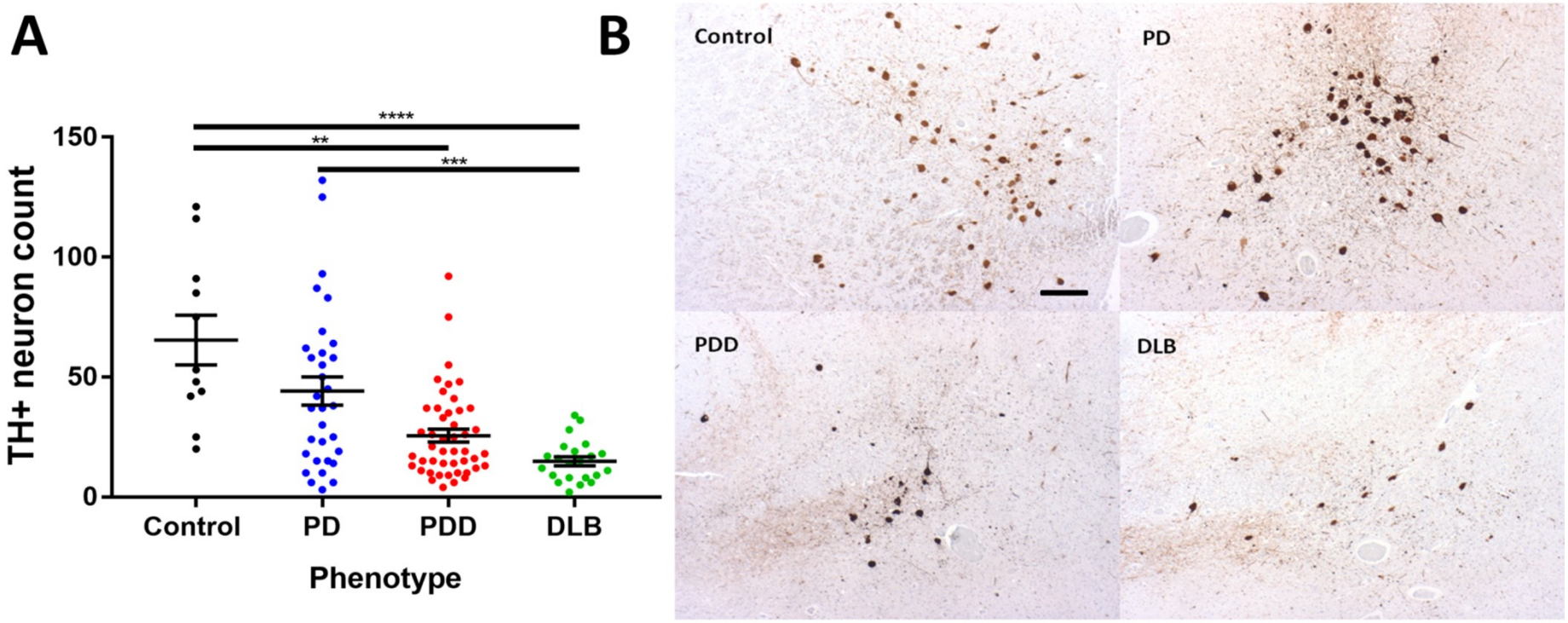


Figure 7.3: TH+ neuron counts in the LC in cognitive phenotypes of PD and controls. A) TH+ neuron counts are signifcantly reduced in LBD compared to PD and controls. Statistical analysis was performed by Kruskal-Wallis Test with post-hoc Dunn's correction for multiple comparisons. ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$. Cronbach's α test =0.903 indicating high inter-rater reliability. B) Representative photomicrographs of TH-immunostained sections containing the LC in cognitive phenotypes of PD and controls. Images captured under a 4x objective lens. Scale bar in Control panel represents 100μm.

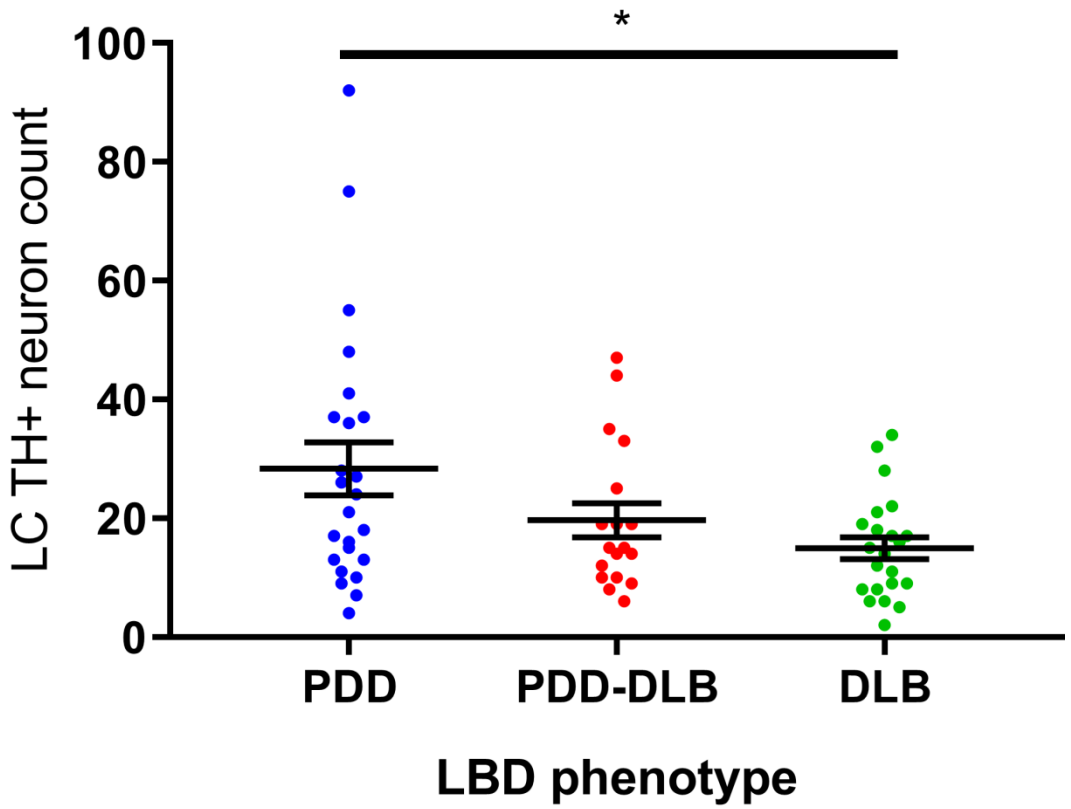


Figure 7.4: LC integrity in PDD, PDD-DLB and DLB cases. Statistical analysis performed by Kruskal-Wallis Test with post-hoc Dunn's correction for multiple comparisons: * = $p<0.05$.

7.4.4. The contribution of LC degeneration to the symptomatology of DLB

Because of the functional role of the LC in controlling the sleep-wake cycle and alert cognitive state, the degeneration of the LC neuron population was assessed for the contribution to each of the three core DLB features – FC, RBD and VH (McKeith *et al.*, 2017). VH and RBD were occasionally present in the PD group. The presence of each of these three clinical features, irrespective of overall diagnosis, was associated with significantly reduced LC TH+ neuron count (Figure 7.5A, B & C), suggesting that the LC is a significant arbiter of the clinical phenotype. There were no significant differences found between depressed and non-depressed groups, and anxious and non-anxious groups.

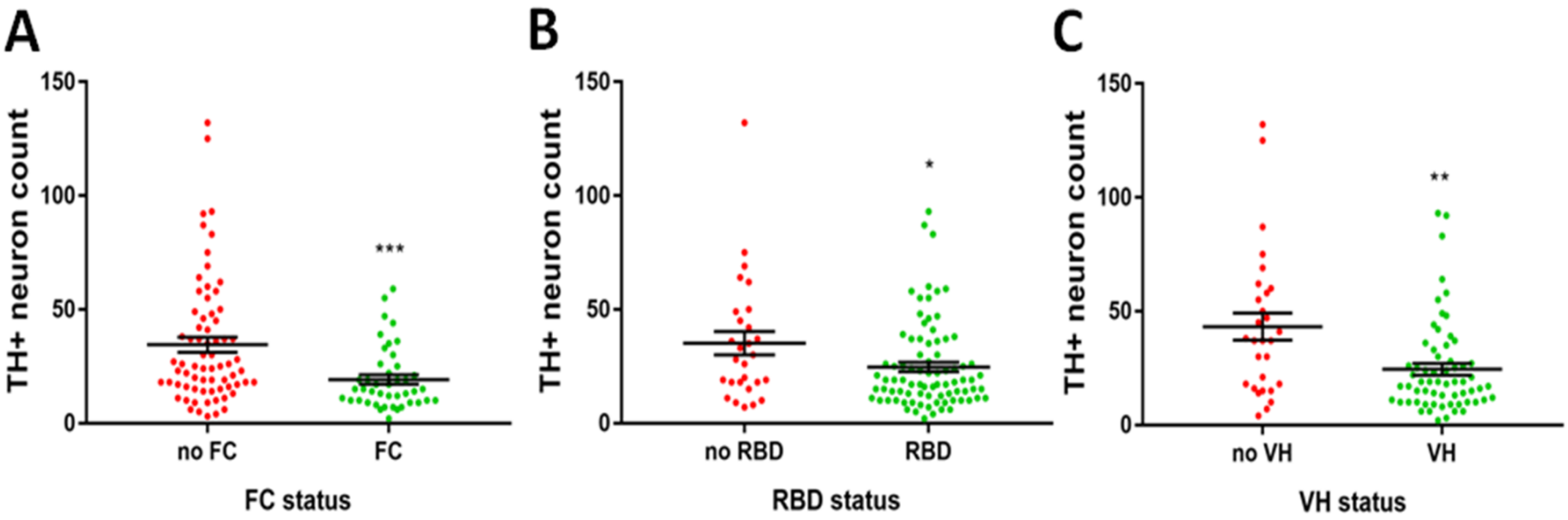


Figure 7.5: LC integrity relating to the different symptoms of DLB: FC, RBD and VH. Cases were separated based on the presence or absence of each symptom in the case history in all cases, irrespective of diagnosis. A) The presence of FC. B) The presence of RBD. C) The presence of VH. Statistical analysis performed by Mann-Whitney U test. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

7.4.5. LC degeneration in LBD is supported by tissue clearing

Tissue clearing was carried out on a sample from a DLB case, PD365, which had an TH-neuron count of 12. This technique demonstrated a few LC TH-positive neurons with extensive arbours projecting around the cell soma (Figure 7.6). The number of neurons found throughout a 120µm z-depth, twenty-times thicker than a single microscope section, in the LC was 25, suggesting that a measurement of neuronal quantity on a single slide is proportional to the cell count in a large block of LC tissue.

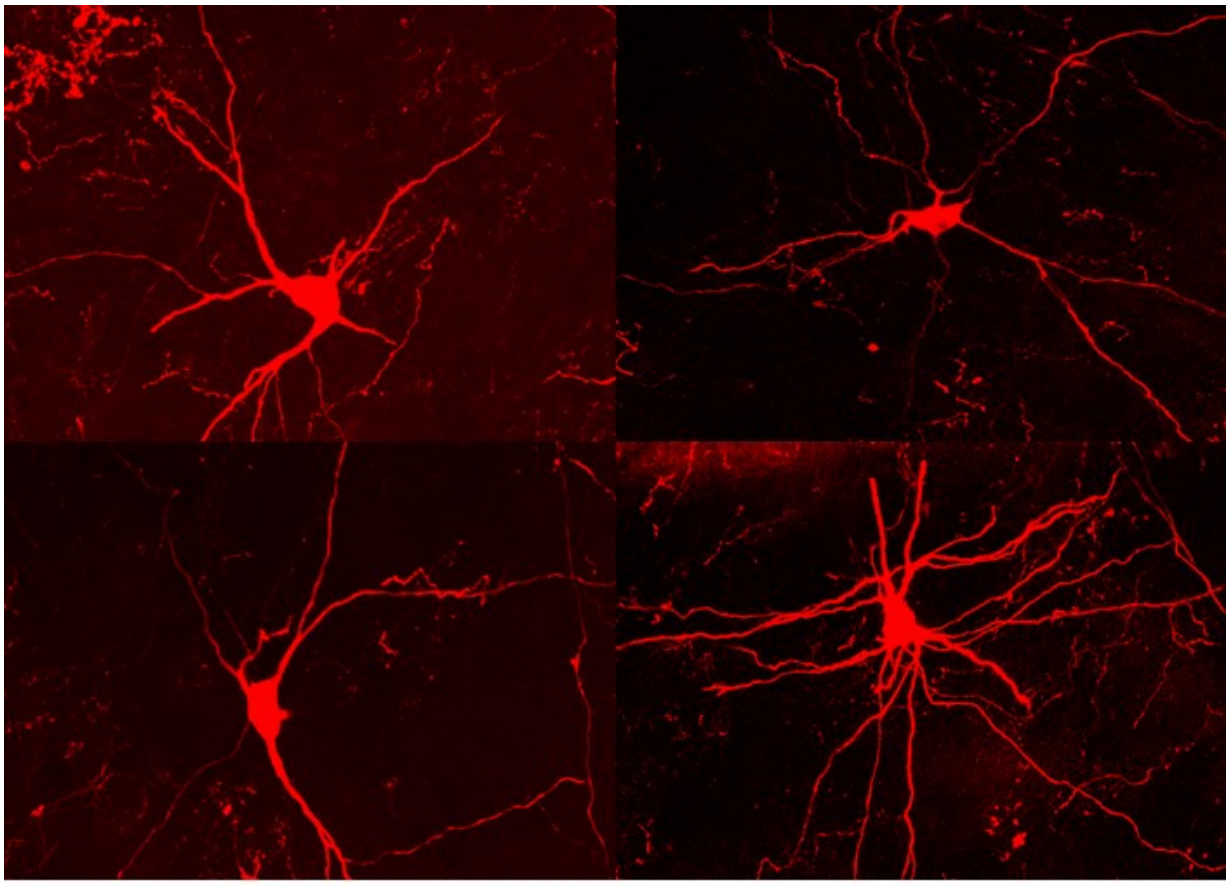


Figure 7.6: TH-immunostained LC neurons in 3D revealed by FASTclear tissue clearing. Imag4es are maximum intensity projections of 120µm z-stack series captured on a Leica DMi-8 confocal microscope under a 10x objective lens.

7.4.6. α-synuclein, tau and amyloid pathology in the LC in LBD

Using our %-coverage methodology, Lewy pathology was found covering the LC field at percentages between 0.3% and 5.3%. Overall however, there were no discernible differences in the %-coverage of α-synuclein pathology in the LC between, PD, PDD and DLB, and between LBD subtypes, though DLB showed a significant reduction compared to PDD which disappeared after correction for multiple comparisons (Figure

7.7A & B). Lewy pathology was predominantly found intracellularly. Therefore, reduced cell count was associated with reduced overall pathologic %-coverage. When adjusted for the neuronal count, a trend increase in α-synuclein pathology was observed per neuron present in DLB cases. On the other hand, PDD and DLB were differentiable by the severity of the presence of tau pathology in the forms of NTs and NFTs. PDD cases were significantly more affected by this pathology than DLB cases (Figure 7.7C & D). The significant difference in tau pathologic burden was maintained when controlled for disease duration, years of dementia, gender and TH+ cell count ($p=0.013$). Disease duration and gender were also able to predict tau pathologic burden. Aβ immunostained intracellular deposits were seen in the majority of cases, without any discernible variation between groups. In summary, DLB cases appear to display less tau pathology than PDD cases. This indicates that DLB represents a purer synucleinopathy in the LC than PDD.

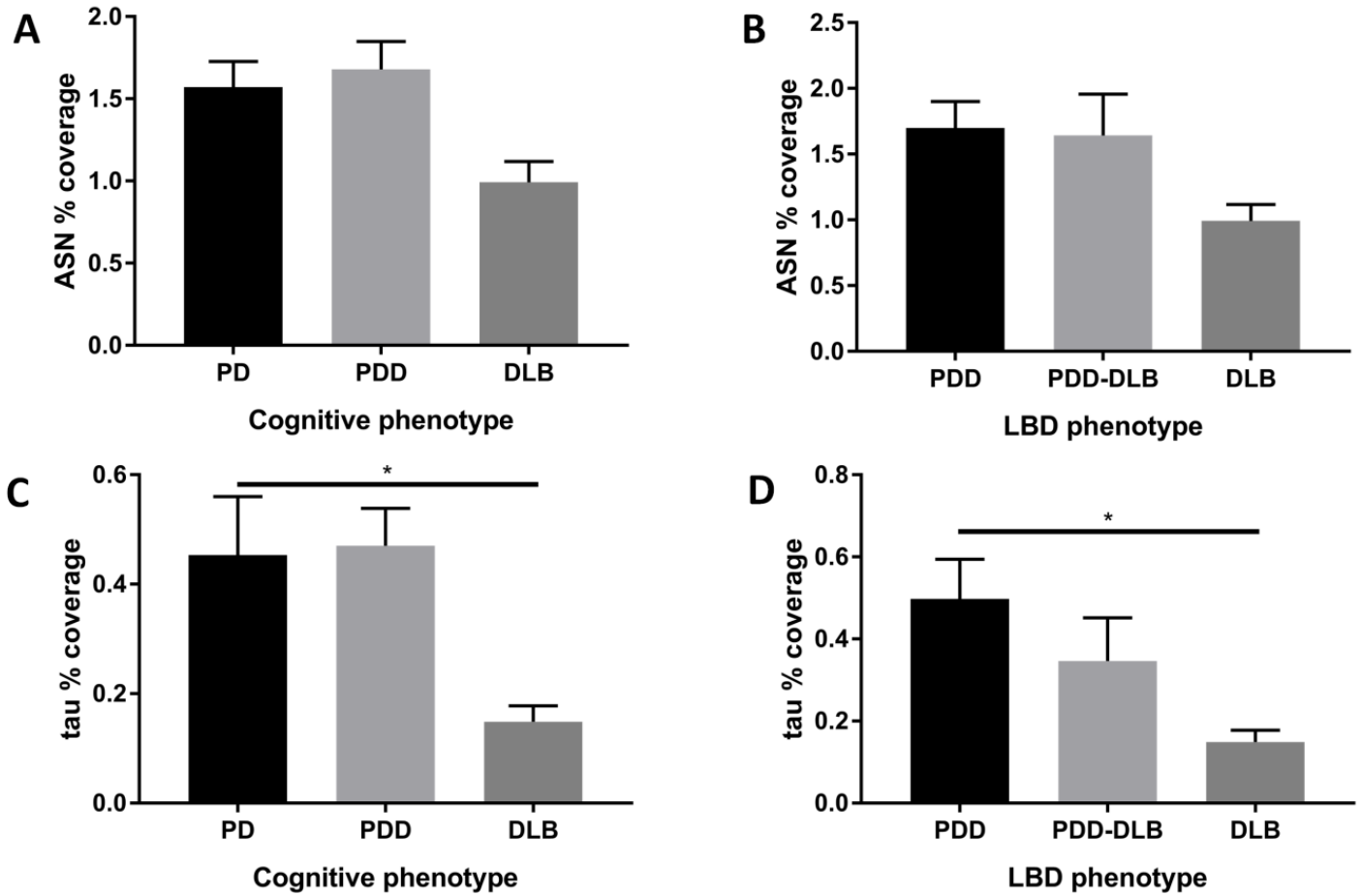


Figure 7.7: α-synuclein (ASN) and tau pathologic burdens in the LC in cognitive phenotypes of PD and LBD. A: ASN pathologic burden in PD cognitive phenotypes. B: ASN pathologic burden in LBD phenotypes. C: Tau pathologic burden in PD cognitive phenotypes. Statistical analysis was performed with Kruskal-Wallis tests with Dunn's corrections for multiple comparisons. * = $p < 0.05$. A) PDD vs DLB, $p = 0.0219$ (before multiple comparisons). B) PDD vs DLB, $p = 0.0237$ (before multiple comparisons).

7.4.7. Neuronal loss in the LC correlates with TH+ fibre density in subcortical LC target regions

TH+ fibre density was quantified in brain regions innervated by the LC that are affected in PD. These were the frontal cortex, cingulate gyrus and amygdala. TH+ fibre density in the superior frontal gyrus was highest in PD cases without cognitive impairment. There was a stepwise reduction in TH+ fibre count in the frontal cortex from PD to DLB whereby PD cases exhibited significantly more TH+ fibres per microscope frame than LBD cases, especially DLB (Figure 7.8A). Compared to PD cases, DLB showed a statistically significant reduction in TH+ fibre density ($p < 0.05$). However, the number of TH+ LC neurons did not correlate with TH+ fibre density in the frontal cortex (Figure 7.8B). The pattern of TH+ fibre loss was similar in the cingulate gyrus (Figure 7.8C), though differences between groups did not reach statistical significance. As opposed to the frontal cortex, LC neuron count also showed a trend correlation with fibre density in the cingulate gyrus ($p=0.0782$; Figure 7.8D). In the amygdala, LFB sections where used to delineate the basolateral and centromedial amygdala subregions based on previous atlases. In both the basolateral and centromedial amygdala, a stepwise reduction in TH+ fibre count was observed from PD to DLB (Figure 7.8E) in a pattern similar to that seen in the frontal and cingulate cortices. The reductions in TH+ fibre count were more severe in the basolateral amygdala than the centromedial sub-region. A statistically significant positive correlation was also found between amygdala TH fibre count and LC TH neuron count (Figure 7.8F). Thus, the effects of LC degeneration were noticeable in several key brain regions, but more so in limbic regions such as the cingulate and amygdala than cortical regions. The expression of noradrenergic loss in DLB implicates the limbic system in the pathophysiology of DLB. α-synuclein pathology in the amygdala and cingulate was also significantly higher in LBD cases (though not between LBD subtypes) compared to PD suggesting that limbic system dysfunction is a significant substrate of dementia in PD. Frontal α-synuclein was significantly higher than PD in PDD cases but not in DLB cases suggesting that the predominance of pathology in DLB is limbic, but that the pathology in PDD is more widespread.

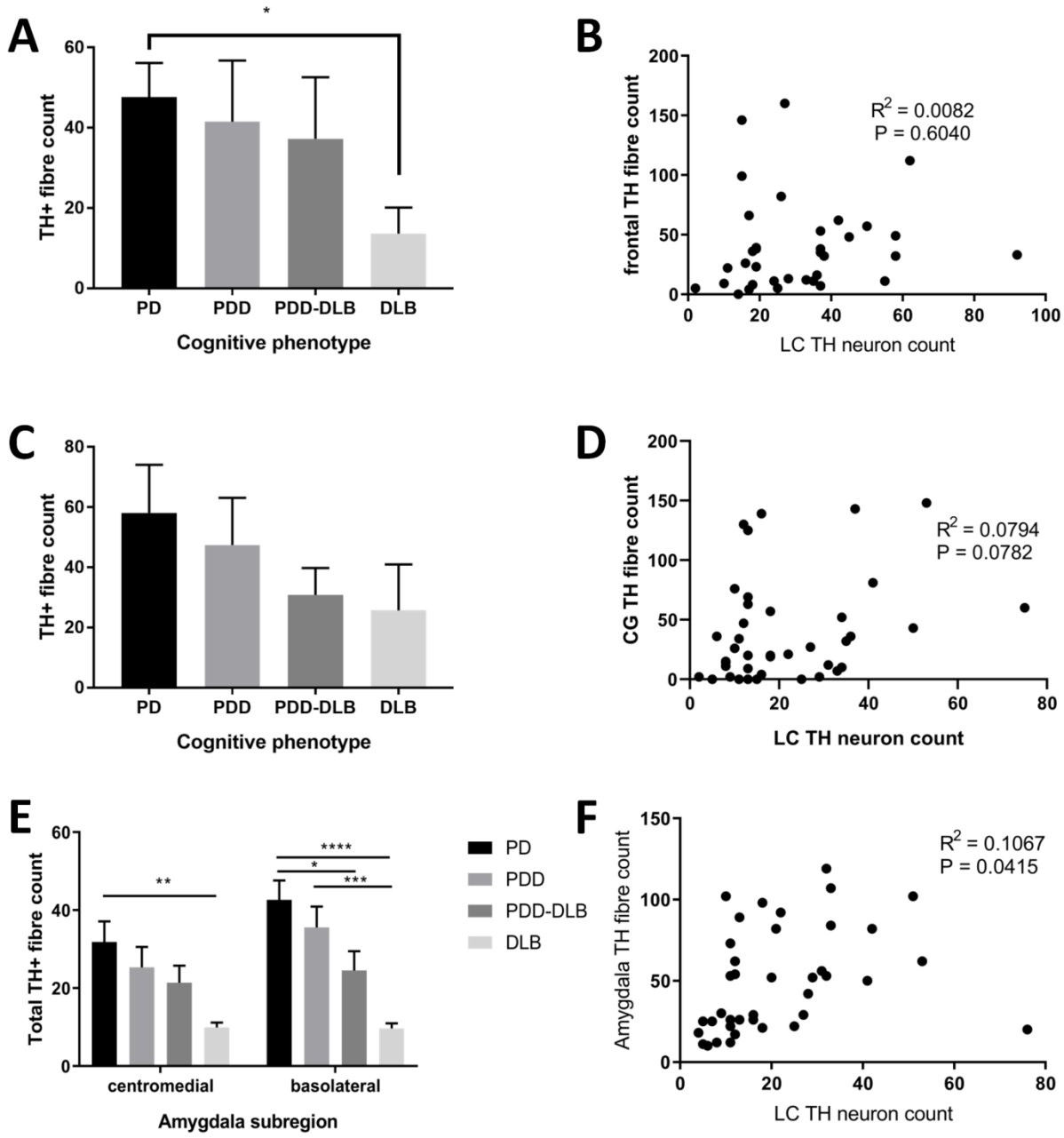


Figure 7.8: TH degeneration in LC target regions. A) TH fibre count in the frontal cortex in clinical subgroups of LBD, B) Correlation of frontal TH fibre count with LC TH neuron count, C) TH fibre count in the frontal cortex in clinical subgroups of LBD, D) Correlation of cingulate TH fibre count with LC TH neuron count, E) TH fibre count in the centromedial and basolateral sub-regions of the amygdale in clinical subgroups of LBD, F) Correlation of total amygdala TH fibre count with LC TH neuron count. For comparisons between subgroups Kruskal-Wallis tests with post-hoc Dunn's corrections for multiple comparisons. For correlations, Pearson's correlations were performed. * - $p < 0.05$, ** - $p < 0.01$, *** – $p < 0.001$, **** = $p < 0.0001$.

7.5. Discussion

The most important finding of the present study is the exhibition of pathological changes in the LC that can differentiate between subtypes of LBD. The differential burden of pathology across LBD cases illustrates a continuum whereby DLB is more severely affected than PDD, and that PDD cases that exhibit DLB-related symptoms show a level of pathology intermediate to PDD and DLB. The contribution of the LC to the dementia syndrome in these cases appears to centre on the presence of DLB-related symptoms such as FC, RBD and VH. I have shown that the presence of these symptoms individually is associated with reduced LC neuron count. Thus, it can be hypothesised that the LC contributes more to the dementia syndrome in LBD than dementias of differing aetiologies. This was highlighted in previous studies where it was found that LBD had significantly more degeneration of the LC than in cases of vascular or frontotemporal lobar dementia (Brunnström *et al.*, 2011; Haglund *et al.*, 2016). However, AD has also been shown to exhibit severe degeneration of the LC, and is the site of Aβ and tau pathology in late stages of the AD pathologic stages (Thal *et al.*, 2002; Braak *et al.*, 2006; Szot *et al.*, 2006; Gannon *et al.*, 2015). Interestingly, the studies by Brunnstrom and Haglund (Brunnström *et al.*, 2011; Haglund *et al.*, 2016) did not investigate the deposition of pathologic proteins in their cohorts, nor did they separate LBD into DLB and PDD as I have in the present study. Furthermore, in this study, reduced LC neuron count has been shown to correlate with reduced resultant fibre counts, particularly in limbic regions. Limbic system pathology may therefore be linked directly to the symptomatology of DLB, where PDD may exhibit a stronger relative neocortical deficit. The relationship between LC neuron count and pathologic burden was the inverse of what might have been expected, with more pathology being present in more intact neuronal populations. Deposition of protein aggregates occurring intracellularly resulted in higher %-coverage of pathology in more intact neuronal populations.

Our results indicate a noradrenergic deficit in PD that particularly affects LBD cases. The role of noradrenaline in the pathophysiology of PD has been the subject of increased focus in recent literature (Vazey and Aston-Jones, 2012; Buddhala *et al.*, 2015; Vermeiren and De Deyn, 2017). I have been able to show that the noradrenergic deficit in LBD might be a distinguishing feature compared to PD cases without cognitive impairment. Moreover, I have shown that noradrenaline has the potential to

control the nature of the dementia phenotype present in LBD cases whereby the presence of FC, RBD and VH are associated with evidence of a stronger noradrenergic deficit than those where these features are not present. *In vivo* evidence for the role of the LC in these symptoms has also been found using T1-weighted neuromelanin-sensitive MR imaging. A study by Garcia-Lorenzo and colleagues found that PD patients with RBD had reduced signal in the LC compared to those without RBD and controls (García-Lorenzo *et al.*, 2013). This lends support to the notion that LBD exists on a clinical and pathological continuum (Gomperts, 2016; Weil *et al.*, 2017), and that LBD pathology should be thought about in terms of the clinical symptoms present rather than the timing of dementia relative to the onset of motor symptoms.

The mesopontine reticular formation and coeruleus/subcoeruleus regions are critical for the regulation of the sleep-wake-cycle. The harbinger of REM-sleep is the onset of specific forms of PGO waves, which typically occurs 30-90 seconds before REM-sleep (Steriade *et al.*, 1990). PGO waves are initiated and regulated by the coeruleus/subcoeruleus region and mesopontine reticular formation (Datta, 1997). These regions have crucial roles in sleep-wake-cycle regulation by performing the switching of thalamic relay nuclei from the waking relay mode, where they transmit sensory signals to the cortex, to the sleeping burst mode where they do not transmit these signals (Manford and Andermann, 1998). Alterations of PGO signals may cause “sleep disturbance and abnormal cortical processing of sensory afferents.” (Manford and Andermann, 1998) Therefore, it is possible to think of hallucinations as abnormal encroachment of sleep characteristics into the waking state, i.e. dreams. The pattern of symptoms prevalent in DLB – VH, RBD and FC – paint a picture of sleep-wake-cycle dysregulation whereby VH represents dreams encroaching on the waking state, RBD represents inappropriate motor activity during ‘deep sleep’ and FC represents variations in arousal. Unresponsive periods, and hypersomnolent episodes are also frequent in DLB and form some of the supportive features of a DLB diagnosis (McKeith *et al.*, 2017). Severe pathology of the coeruleus/subcoeruleus region and mesopontine reticular formation, resulting in dysregulation of the sleep-wake-cycle could plausibly underlie all of these clinical DLB-related phenomena.

Identification of the pathologic substrates of specific dementia symptoms would allow for more tailored treatments of an individual’s unique dementia syndrome. Currently,

the only licensed drug class for dementia is the cholinesterase inhibitors, thought to act by addressing the cortical cholinergic deficit caused by loss of neurons in the cholinergic basal forebrain (Liu *et al.*, 2015). Noradrenergic fibres originating from the LC have been shown to innervate the nucleus basalis of Meynert (Jones, 2004) which is one of the four main basal forebrain cholinergic nuclei and has been shown to exhibit cell loss in LBD (Whitehouse *et al.*, 1983). A loss of noradrenergic innervation in this area early in the disease course may underlie nucleus basalis degeneration and the resultant cortical cholinergic deficit. Noradrenergic medications have also been shown to improve certain aspects of cognition in PD as well as improving other neuropsychiatric symptoms such as impulsivity, anxiety and depression (Vermeiren and De Deyn, 2017). Noradrenaline has been shown to induce long-term potentiation of hippocampal synapses by acting at β-adrenergic receptors (Sara, 2009). Receptor binding studies in this area have shown downregulation of key adrenergic receptors in DLB cases (Szot *et al.*, 2006).

Our study is not without limitations, including the retrospective nature of our case history analysis and sampling the LC at only one rostro-caudal level. Another potential limitation is the use of representative sections of the LC instead of unbiased stereological methods to perform neuron counts. However, previous studies using single representative section counts and stereological estimates have found a high degree of comparability (Ma *et al.*, 1995). Nonetheless, our findings have highlighted an important aspect of the pathophysiology of LBD which may have implications for the administration of noradrenergic medications in clinical practice. Future work will involve studies of the cerebral targets of LC-noradrenergic system, including the amygdala, hippocampus and cerebral cortex. *In vivo* neuroimaging studies should focus on the LC-noradrenergic system to validate our neuropathologic data during life. Clinical trials of the effects of noradrenergic drugs on specific dementia symptoms in LBD are warranted.

7.5.1. Limitations

Similarly to Chapter 5, this study was limited by factors including single section analysis without stereological estimation of the total population of neurons, though I have previously provided a justification for this based on comparisons between stereological and non-stereological estimates in the literature (Ma *et al.*, 1995), (Lyness, Zarow and Chui, 2003).

Another potential limitation of this study was the use of TH as a marker of LC neurons. TH is an enzyme that is involved in the production of all monoamines and so will stain dopaminergic, noradrenergic and (weakly) serotonergic neuron populations in the CNS. Though this is not relevant for the LC, which hosts only noradrenergic neurons in the monoamine class, it will have an impact on the conclusions we can draw from staining in the LC target regions such as the frontal and cingulate gyri. In these regions, there is a dopaminergic input from the meso-limbic and meso-cortical projection regions in the ventral tegmentum. However, it is debatable as to whether the amygdala receives an overt dopaminergic input from these regions. There is some limited evidence to suggest the presence of dopamine receptors in the amygdala (Merlo *et al.*, 2015), but there is also evidence to suggest that noradrenergic fibres innervating medial temporal lobe structures from the LC may co-release dopamine and noradrenaline (Kempadoo *et al.*, 2016). The discrepancies in the monoaminergic innervation of these regions highlight interesting trends in the data from this chapter: there is a stronger correlation between the LC neuron counts and resultant fibre density in the amygdala than in any other region. To address these discrepancies, more specific markers for noradrenergic neurons should be optimised, such as dopamine-β-hydroxylase and the experiments presented in this chapter should be repeated with this marker and compared.

7.6. Conclusion of chapter

In conclusion, the LC-noradrenergic system is significantly affected in PD and LBD specifically. Deficits in this system, resulting from neuronal loss and dysfunction of surviving neurons, may underlie the onset of DLB-related symptoms such as RBD and FC. Based on a symptom-by-symptom approach, I have provided further evidence that LBD is more likely to reflect a continuum caused by different severity of pathology in several key brain regions including the LC. Further work will be needed to validate these data *in vivo,* and to assess the clinical efficacy of noradrenergic drugs as an adjunct or alternative to cholinesterase inhibitors in the treatment of LBD.

Chapter 8 - Discussion

8.1. The link between akinesia and cognition and proposed subtypes of PD

At the beginning of this thesis, I set out to explore the clinical and pathological characteristics of the clinical subtypes of Parkinson's disease. I hypothesised that the link between AR motor subtype and cognitive decline, which had been proposed by the extensive body of previous literature on the topic (Aarsland, Andersen, *et al.*, 2001; Burn, Rowan, Allan, Molloy and McKeith, 2006; Williams-Gray *et al.*, 2007, 2013; Reijnders *et al.*, 2009; Johnson *et al.*, 2016; Velseboer *et al.*, 2016; Fereshtehnejad and Postuma, 2017), would extend further than the prognostic value of baseline clinical subgroup. This hypothesis was provoked, in the main, by the fact that PD motor subtypes do not remain stable over time (Von Coelln *et al.*, 2015), with a tendency towards increasing akinesia with advanced disease duration being noted by even the earliest scholars (Hoehn and Yahr, 1967; Parkinson, 2002; Goetz, 2011), and the fact that changes in motor subtype towards an AR picture is associated with a strongly increased risk of cognitive decline in PD (Alves *et al.*, 2006).

On the prognostic value of presenting motor subtype, I have found that AR phenotype is associated with a significantly faster rate of decline to cognitive impairment and dementia than TD cases, though this effect seemed to disappear over time – the TD cases eventually caught up with the AR population in terms of decline to cognition on a cohort level. This provoked the question of what factors mediated the risk of cognitive impairment in those who presented with TD phenotype, given that this subtype had long been thought to be protected against cognitive decline. I have found that the incidence of akinetic features such as postural instability and gait disturbance in any case of PD, whether it presented with TD or AR phenotype, was robustly associated with the onset of cognitive impairment. Such was the predictive strength of this phenomenon, 95% of cases with incident gait disturbance in PD developed cognitive impairment within 5 years, with the majority exhibiting cognitive decline within 3 years. This was supported significantly by my findings in the cross-sectional cohort of PD cases who had completed self-rated symptom severity questionnaires and by my data from the PPMI cohort which showed that gait disturbance severity at any time point is signifcantly linked to impairment of cognitive function. I was able to demonstrate that high UPDRS gait impairment item scores at the first time point predicted worse scores on a variety of cognitive tests including the BJOLT, HVLT, SF, SDM and MoCA, thereafter. The other significant predictors of these scores were male sex and older

age of onset. The temporal relationship between the incidences of gait disturbance and cognitive impairment is very close. Verghese and colleagues have recently described a similar phenomenon, which they named Motoric Cognitive Risk Syndrome (MCRS; Verghese *et al.*, 2014). This is a syndrome characterised by slow gait and cognitive impairment in cases with or without PD. Similarly, Buracchio et al. found that gait speed and hand tapping speed slowed at a faster rate in those that developed MCI compared to those who did not (Buracchio *et al.*, 2010). However, my study is the first to demonstrate this temporal relationship in PD. This is a key novel finding of my thesis, and one that would logically lead to speculation about a shared aspect of pathophysiology that underpins both, and the extent to which cognition plays a role in normal gait production. One hypothesis that can be derived from this is that the origins of gait and cognitive co-impairment may not involve α-synuclein. This is because the MCRS can occur in non-parkinsonian patients, suggesting either an alternative pathophysiology, or that synucleinopathy can take place in individuals without PD to mediate MCRS.

In the Parkinson's UK Tissue Bank cohort, very few cases presented with cognitive impairment preceding the onset of gait disturbance features, and even fewer cases survived the whole disease duration without cognitive impairment despite the development of AR disease. Interestingly, Mixed subtype cases showed a similar pattern of decline to cognitive impairment to AR cases, whereby cognitive decline showed a tight relationship with the onset of akinetic features, independently of tremor. From this I was able to reject the theory that tremor is a protective feature in PD. Historically, it appears that tremor was mistakenly believed to protect against cognitive impairment. However, my data shows that tremor, as a symptom, is a red herring and that the true effect that was being witnessed by the previous literature was that cases that present with tremor tend to have lower prevalence and severity of akinetic gait features (Selikhova *et al.*, 2009; Konno *et al.*, 2018). Instead there appears to be a positive linear relationship between gait and cognition with no interaction with tremor at all. The only conclusion that can be drawn from the relationship of tremor and cognitive impairment is that tremulous patients, in the main, do not develop cognitive impairment until after they develop significant akinetic symptoms such as gait disturbance, a finding that reproduces the data from Alves and colleagues (Alves *et al.*, 2006). Interestingly, while akinetic features increase in prevalence and severity

over the course of PD, the trend for tremor follows an entirely different course: At first, the prevalence and severity of tremor increase, usually starting unilaterally in one arm, but often spreading bilaterally into the other arm or the ipsilateral leg. Then, usually within 10 years, tremor appears to dissipate before vanishing entirely in the majority of cases. By this point, akinesia, rigidity and gait disturbance have already taken hold. However, in a restricted subset of cases, mild resting and postural tremor is the dominant symptom throughout the entire disease course. This is known as benign tremulous parkinsonism (BTP). This condition shares pathology with PD – in one study, mild-moderate SN neuron loss was found alongside α-synuclein-positive LBs in a distribution akin to PD (Selikhova *et al.*, 2013). It can therefore be argued that there are at least four main prototypes of PD: 1) TD onset with gradual progression to AR disease and cognitive impairment, 2) AR onset with rapid progression and early cognitive decline, 3) Mixed onset with quick progression and early cognitive decline, 4) TD onset with benign progression without major cognitive impairment (BTP). Figure 8.1 outlines my proposition for the natural history of these four prototypes based on the population prevalence and relative severity of tremor, rigidity, gait impairment and dementia symptoms. Based on data from previous studies (Gibb and Lees, 1988; Selikhova *et al.*, 2009; Nelson *et al.*, 2010), it is clear that sex and age of onset provide further variability in the group classifications such that males and old age of onset cases are much more likely to develop early dementia than females or young onset cases.

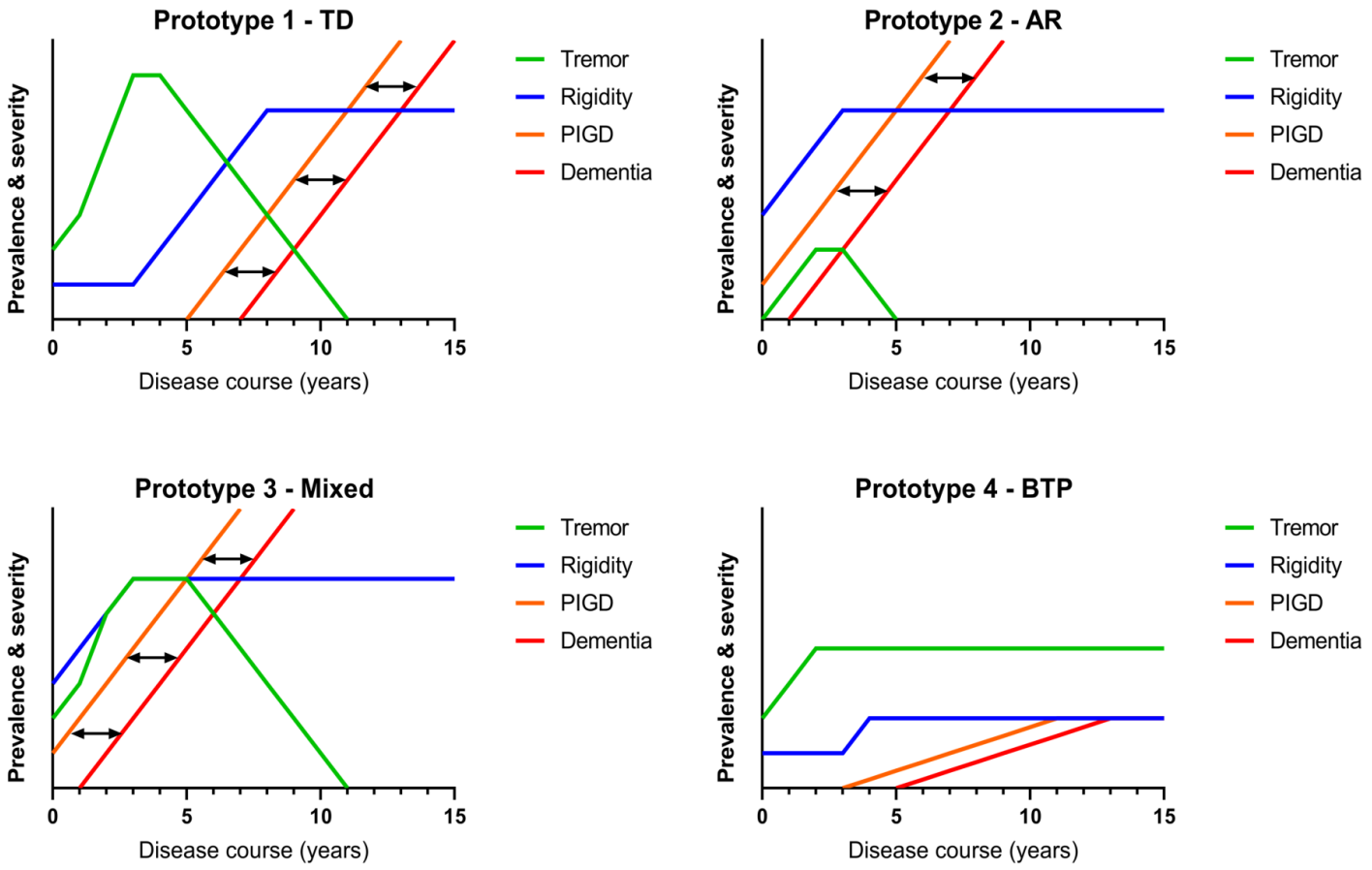


Figure 8.1: Proposed prototypes of PD. AR = akinetic-rigid, BTP = benign tremulous parkinsonism, PIGD = postural instability, gait difficulty, TD = tremor-dominant. 1) Stereotypical TD onset with steady progression to AR subtype within 10 years followed by end-stage dementia, 2) Stereotypical AR onset with minimal tremor, early evolution of gait and cognitive impairment, 3) Mixed onset with tremor and akinesia at diagnosis, with early evolution of gait and cognitive impairment, 4) BTP with consistent mild-moderate tremor and very minimal other features with benign progression. Note the consistent relationship between gait and cognitive impairment.

8.1.1. A potential pathophysiological link between gait disturbance and cognition

Motor programs, such as gait, and cognition share a number of underlying characteristics: i) they are both highly plastic processes in the brain, ii) they both require the input of multiple brain regions, iii) They are both perfected during infant and adolescent development through practice, learning and play. Therefore, it could be hypothesised that because gait and cognition develop together, they would decay together too, and that a pathologic process that caused the dysfunction of one would necessarily cause the decline of the other. Given that both gait and cognition rely on the plastic capacity of the brain, neural plasticity would be a logical starting point in the investigation of a common pathophysiological link between the two.

Plasticity can be thought of at two levels: 1) as a mechanical process at the synaptic level, mediated by the complex interactions of multiple proteins in response to changes in the activity level across that synapse, and 2) as an abstract instrument at the theoretical and philosophical levels, used more generally as an explanation of how, when it comes to human behaviour, the concepts of *practice makes perfect* and *use it or lose it* apply. In this sense, it is possible to think more generally about PD as a disease in which there is a challenge to the integrity of these two life maxims. For example, despite gait and cognition being commonly-used and highly practiced brain functions, in PD, these gradually decline together with advancing disease. Thus, it could be said that the plastic mechanisms underlying the brain's ability to maintain the *practice makes perfect* and *use it or lose it* doctrines malfunction in PD, which leads to the concomitant breakdowns of gait and cognitive function.

At the synaptic level, plasticity, which was first proposed by Ramon y Cajal in 1911 (Ramon Y Cajal, 1911), and then refined into a theoretical model by Hebb (Hebb, 1949), relies on two processes occurring in balance to one another. These are LTP, where increases in activity across a synapse causes a corresponding increase in the *strength* of the synapse, and LTD, where reductions in activity across a synapse causes a corresponding decrease in the *strength* of the synapse. The strength of the synapses can be expressed as a function of the potential change across the post-synaptic membrane needed to conduct an action potential, the size of the synaptic boutons and neurotransmitter receptor upregulation (Matsuzaki *et al.*, 2004; Tokay *et al.*, 2013; Meyer, Bonhoeffer and Scheuss, 2014). In this regard, one would expect a

potentiated synapse to show large boutons, upregulation of receptors and fewer excitatory potentials required to fire an action potential, thereby disposing to increased speed of neurotransmission (Nicoll, 2017a).

The vast majority of work on plasticity was conducted on post-synaptic terminals at glutamatergic synapses, where Ca^{2+} influx through NMDA receptors not only triggers an action potential, but also effects downstream signalling changes through calcium-sensitive molecules such as calmodulin-dependent kinase II (CAMKII). Through molecules like CAMKII, a number of LTP-associated processes, such as engagement of the actin cytoskeleton to promote spine enlargement and scaffold formation for the insertion of newly-produced AMPA receptors, are provoked (Nicoll, 2017b). A long running debate in the years after the discovery of LTP was whether this arose as a result of presynaptic changes, such as the quantity of neurotransmitter release, or post-synaptic changes as detailed above (Nicoll, 2017b). Though there is no doubt that the main locus of plasticity is at the post-synaptic membrane, there have proven to be presynaptic mechanisms of plasticity. These are proposed to occur in four main ways: 1) recurrent activity promotes a long-lasting increase in neurotransmitter release probability (occurs in hippocampal mossy fibre synapses), 2) cross-stimulation of presynaptic receptors by neighbouring presynaptic terminals (in associative cortical inputs to the lateral amygdala), 3) retrograde stimulation of presynaptic terminals by post-synaptic signalling molecules (in somatosensory cortex glutamatergic neurons), 4) retrograde cross-stimulation of presynaptic terminals by neighbouring post-synaptic signalling molecules (in somatosensory cortex glutamatergic neurons) (Castillo, 2012).

As mentioned, both gait and cognition rely on these processes of plasticity for efficient transmission of signals between relevant brain regions. Thus, in a disease like PD in which I have been able to show that there is slowing or dysfunction of gait and cognition in tandem, one would hypothesise that disintegration of plasticity would play a prominent role.

8.1.2. How does PD influence synaptic plasticity?

As PD is known to involve pathology of α-synuclein, a protein that plays an important role in presynaptic terminal physiology, regulating plasticity, vesicular dynamics, neurotransmitter trafficking and neurotransmitter release (Burré, 2015; Calo *et al.*,

2016). It therefore stands to reason that dysfunction of α-synuclein disrupts the normal functioning of synapses leading to disordered plasticity and inefficient transduction of signals from one neuron to the next. Though PD is defined by the accumulation of fibrillar α-synuclein as LBs (Braak, Del Tredici, *et al.*, 2003), this is likely to be a pathological end-state after a period of aggregation of toxic α-synuclein oligomers. In fact, synapses are the primary site of pathology in PD (Calo *et al.*, 2016). Numerous studies have demonstrated that pathological α-synuclein oligomers accumulate at the presynaptic terminal before LB formation (Milber *et al.*, 2012) and that these are more toxic than α-synuclein fibrils (Karpinar *et al.*, 2009; Winner *et al.*, 2011). By using proteinase K digestion to remove non-aggregated protein, one study found that 50-92% of cortical α-synuclein aggregates in DLB are located in the pre-synaptic terminals (Kramer and Schulz-Schaeffer, 2007). These small aggregates of α-synuclein disrupt its interaction with proteins like VAMP2 which compromises SNARE complex, formation and vesicular trafficking, leading to reduced neurotransmitter release and therefore reduced or slowed synaptic transmission (Calo *et al.*, 2016). Reduced neurotransmitter release is therefore an indirect mechanism for α-synuclein to cause disordered synaptic plasticity. Furthermore, data from animal studies suggest that a lack of α-synuclein is associated with reduced vesicular neurotransmitter stores which attenuates synaptic responses to repeated stimulation (Cabin *et al.*, 2002). In these ways, it is clear to see that dysfunctional α-synuclein leads to synaptic depotentiation, which results in slowed neurotransmission in the PD brain.

At the neurotransmitter level, there is a wealth of evidence provided by this thesis and other literature to support strong deficits in several neurotransmitter systems including dopaminergic, noradrenergic and cholinergic to name but a few (Paulus and Jellinger, 1991, 1992; Berridge and Waterhouse, 2003; Bohnen *et al.*, 2003, 2009, 2014; O'Brien *et al.*, 2004; Del Tredici and Braak, 2013). Dopamine at the level of striatum has been shown to modulate different aspects of plasticity in dendritic spines of striatal MSNs (Yan, Song and Surmeier, 1997; Surmeier *et al.*, 2010). Furthermore, dopamine receptors, located on the presynaptic membrane of corticostriatal fibres synapsing MSNs and ChIs allows dopamine to modulate the function of these synapses (Yan, Song and Surmeier, 1997; Maurice *et al.*, 2004; Surmeier *et al.*, 2007). Dopaminergic input to the motor cortex from the meso-cortical dopaminergic neurons of the A10 has also been shown to promote plasticity through activation of D1 and D2 receptors to

promote LTP and motor learning (Rioult-Pedotti *et al.*, 2015). Acetylcholine release from striatal ChIs also plays a critical role in the modulation of striatal synaptic plasticity by modulating cortico-striatal glutamate release and controlling the activity of MSNs (Calabresi *et al.*, 2000; Bonsi *et al.*, 2011; Deffains and Bergman, 2015). Further, cholinergic innervation from the nbM to the cortex, amygdala and hippocampus is a critical modulator of plasticity associated with attention, memory and fear learning (Yarnall, Rochester and Burn, 2011; Liu *et al.*, 2015; Jiang *et al.*, 2016; Wilson, Fadel and Carolina, 2017). Similarly, noradrenaline is also a critical regulator of the polarity of spike-timing-dependent plasticity in the visual cortex via activation of β-adrenergic receptors, and by reducing feed-forward inhibition of neuronal activity (Seol *et al.*, 2007; Kuo and Trussell, 2011). From this it is clear to see that the loss of dopamine, acetylcholine and noradrenaline, which is well established in PD and other related movement disorders from my thesis and the literature (Whitehouse *et al.*, 1983; Jellinger, 1988a; Paulus and Jellinger, 1991, 1992; Warren *et al.*, 2005; Karachi *et al.*, 2010; Liu *et al.*, 2015), will result in dysfunctional synaptic plasticity.

It is conceivable that this aspect of PD pathophysiology could explain several of the key PD clinical features, such as bradykinesia, akinesia, gait disturbance and cognitive impairment, all of which go together with one another in the evolution of the disease (see Chapter 3 -). For example, akinesia, defined as inability of execution of voluntary movement, might result from an increase in the strength of synaptic inputs needed to fire an action potential in a motor neuron. Likewise, bradykinesia and gait disturbance may be caused by a slowness of conduction of action potentials through brain circuits involved in the maintenance of programmed motor behaviours. Slowness of thinking (bradyphrenia) may arise from a similar mechanism, and memory retrieval deficits may be reflective of increased amounts of synaptic inputs needed to fire action potentials in neurons in memory-associated cortical and subcortical regions such as the parietal and temporal cortices, hippocampi and amygdalae. Selikhova and colleagues, in a large pathological series, could find no significant differences in the cortical LB load between non-demented AR cases and PD cases with early dementia, suggesting that cortical α-synuclein may be a unifying pathology in the link between these PD-related phenomena.

8.2. Theories on the neural underpinnings of PD motor subtypes

8.2.1. The origins of tremor

In the dimmer switch hypothesis of tremor (Helmich *et al.*, 2011, 2012), a striato-pallidal circuit drives a cerebello-thalamo-cortical circuit, whereby activity in the first circuit switches tremor on and off, and activity in the second controls the amplitude (Figure 1.9 & Figure 8.2 – Top). The trigger for the tremor-onset-related activity in the striato-pallidal circuit appears to be degeneration of the nigrostriatal dopaminergic pathway. However, as discussed in this thesis, this explanation does not suffice as I have shown that nigrostriatal degeneration occurs equally in TD and AR-presenting PD cases (Chapter 5). Therefore, there must be a second decision point in the PD pathophysiology flowchart. This addressed one of my aims at the beginning of the thesis which was to carry out a clinicopathological analysis of the SN dopaminergic system in motor subtypes of PD. Given that this discerning factor appears not to be in the SN, it might be located in the next region of the PD-related motor circuitry – the striatum. Helmich et al., proposed that increased striatal output to the GP drives the onset of PD tremor (Helmich *et al.*, 2011, 2012).

Pursuant to my aim at the outset of this thesis, which was to conduct Clinicopathological correlations in the striatum with PD motor subtypes, I have provided evidence that there are morphological differences in the striatal cholinergic system and differential proteinopathy between TD, Mixed and AR-presenting cases (Chapter 6 -). I found that striatal ChIs are atrophic, with reduced arborisation and show tau deposition in the majority of AR cases. On the other hand, ChIs appear preserved in TD and Mixed-onset cases with many branches and a densely stained cholinergic neuropil. Given that the arborized morphology and dense neuropil of ChIs is crucial for neurons, so few in number, to exert their effects, changes to the morphological characteristics of these cells may result in functional deficits. Furthermore, ChIs are aspiny, which means that they employ volumetric transmission of acetylcholine in order to modulate a wide range of different cell types in the striatum. Therefore, pathology involving branching of these interneurons may interfere with their ability to engage in volumetric neurotransmission. The effect of dopamine deficiency on intact ChIs should be to increase their activity, given that the predominance of D2 receptors on these interneurons confers an inhibitory effect of dopamine under physiological circumstances. If the striatal cholinergic system is intact, as I propose it

is in TD and Mixed cases, dopamine deficiency should increase the striatal cholinergic drive. This increases the striatal output to the GP, thereby disinhibiting the thalamus to allow tremor onset. Conversely, if there is pathology of the striatal cholinergic system that either predates or occurs simultaneously to nigrostriatal degeneration, then there will be no upregulation of striatal cholinergic transmission, and thus no increase in striatal output to the GP. This would hypothetically lead to a non-tremulous disease phenotype. This model is supported significantly in the literature. fMRI studies comparing PD motor subtypes have found increased signal in the thalamus, putamen, GP, cerebellum and motor cortices – areas that are all included in the dimmer-switch hypothesis of PD tremor posited by Helmich et al. (Helmich *et al.*, 2012) – in TD cases compared to AR (J. Zhang *et al.*, 2016). Interestingly, TD cases also showed increased signal in the parietal cortex, suggesting stronger connectivity in sensory regions in cases with tremor. Furthermore, Chen and colleagues found that TD cases showed increased amplitude of low frequency fluctuations in the cerebellum, putamen, superior temporal gyrus, superior frontal gyrus and pariental gyrus compared to PIGDs (H. M. Chen *et al.*, 2015). In drug naïve early PD cases, TD subtype was associated with increased functional connectivity between the striatum and the motor and visual cortices compared to controls (Hou *et al.*, 2018). These studies highlight increased output from the striatum as a critical mechanism in the onset of tremulous PD but not AR PD. This suggests that striatal output is hindered in AR cases, which might be a a result of pathology of striatal ChIs, as I have shown in this thesis.

The STN is a critical locus in the pathogenesis of tremor and dyskinesia in PD (Rodriguez *et al.*, 1998). It shows synchronous activity at the tremor frequency in PD patients, more so in the dorsal STN than the ventral STN (Amtage *et al.*, 2008), and is also the site for deep brain stimulation in PD to treat intractable tremor (Wong *et al.*, 2018). Thus, STN overactivity plays a role in PD tremor activity. In fMRI studies, it has been shown that there is increased functional connectivity between the STN and cerebellum in TD patients compared to controls and PIGD cases, and decreased functional connectivity between the bilateral putamen and STN in PIGD compared to TD and controls (Wang *et al.*, 2016). Curiously, in this study they also found increased connectivity between the STN and visual cortex in PIGD, suggesting a compensatory mechanism involving increased utilisation of visual stimuli. The afferent influence of the GPe exerts an inhibitory effect on STN neurons. Therefore, if striato-pallidal MSNs

are overactive, this should inhibit the GPe, thus liberating the STN from GPe inhibitory afferents (see Figure 1.7). This is not reflected by electrophysiological studies of basal ganglia neurons before and after MPTP lesions. Deffains and colleagues used chamber recordings of different basal ganglia structures to assess the contributions of the striatum and STN to downstream basal ganglia signalling in normal and parkinsonian conditions. They found that neurons in all basal ganglia regions but the striatum (GPe, SNr, STN) showed activity fluctuations that correlated with downstream basal ganglia output in response to a classical conditioning task (Deffains *et al.*, 2016). Predictably, the story was more nuanced than this. The authors measured the activity of two different striatal neuron types: 1) the striatal MSNs and 2) the striatal TANs (electrophysiological analogues of the ChIs). They found that activity of ChIs showed significant concordance with the activity of neurons in other basal ganglia regions, but that the activity of MSNs showed no relationship. This was fascinating because the activity of ChIs can only affect downstream targets in the basal ganglia by modulating the activity of the striatal output neurons – the MSNs – as ChIs only project locally within the striatum (Lim, Kang and McGehee, 2014). Studies of this kind show that the current models of basal ganglia function, i.e. the direct and indirect pathways, are over-simplifications.

8.2.2. The origins of the AR subtype

It would be too simplistic to define the AR subtype purely by the absence of tremor. By doing so, you capture a cohort of cases that show significant heterogeneity in their clinical symptoms. Therefore, one has to define subtypes by the presence of features rather than their absence. On the other hand, TD cases tend to exhibit less rigidity and akinesia. Using this concept, the movement disorders field has defined AR and PIGD subtypes, though debate remains as to whether the latter reflects an actual subtype or whether PIGD exists on a clinical continuum (Kotagal, 2016). Critical contributors to the pathophysiology of early AR and gait symptoms in PD probably include the cholinergic basal forebrain and brainstem systems. Brainstem cholinergic nuclei such as the PPN show significant pathology in a number of neurodegenerative diseases including AD, PSP and PD (Jellinger, 1988b; Zweig *et al.*, 1989; Pahapill and Lozano, 2000; Karachi *et al.*, 2010). Within PD, clinicopathological correlations in the PPN have been conducted by Karachi and colleagues between those cases with frequent falls and non-fallers, finding greater AChE-positive cell loss in PD fallers (Karachi *et al.*,

2010). These findings have been supported by PET-imaging studies such as those conducted by Bohnen and colleagues, who found that AChE (AChE) PET signal was significantly reduced in the thalamus in PD fallers compared to non-fallers and age-matched controls (Bohnen *et al.*, 2009). I believed that this technique could be applied to the striatal cholinergic system in order to validate my data on this system in PD motor subtypes. However, in conversation with the authors of this study on the topic, they advised me that it is difficult to measure the striatal cholinergic PET signal because it is severely upregulated in PD as a result of dopaminergic denervation. Further optimisation is needed to be able to assess differences between regions of high PET signal in PD motor subtypes. Critically, this study also found no significant differences between fallers and non-fallers in the nigrostriatal dopaminergic system. However, further studies from this group found no differences in thalamic cholinergic PET signal between FOG patients and non-FOG patients, but reduced cortical cholinergic PET signal in those with FOG (Bohnen *et al.*, 2014). This suggests that gait disturbance and FOG have different aetiologies, whereby FOG results from more cortical cholinergic pathology than non-FOG patients. This may explain why FOG appears to have trigger stimuli such as doorways or wide/narrow spaces. The model that I have proposed would suggest that the cortical arrangement of appropriate motor responses to sensory stimuli is slowed and requires increased presynaptic excitation to stimulate a motor program. In both studies, however, there was a reduction of cholinergic PET signal in the cerebral cortex, presumably resulting from reduced cholinergic neuron counts in the basal forebrain. Basal forebrain pathology was shown to be more aggressive in AR cases compared to TD by a previous PhD student in our lab. Reduced cholinergic innervation of the cerebral cortex, particularly the frontal motor areas, may significantly contribute to slowed movement initiation. Magrinelli and colleagues in their review on the pathophysiology of PD symptoms cited slowed reaction times in the pathogenesis of bradykinesia, evidenced by increased premotor excitability in the cortex (Magrinelli *et al.*, 2016). Increased premotor activity shows that stronger presynaptic potentials are needed to provoke neurotransmission in the motor cortex, suggesting a failure in movement preparation. This data highlights slowed cortical neurotransmission as a key mechanism for bradykinesia, perhaps mediated by dysfunctional plasticity caused by the accumulation of toxic deposits of α-synuclein, as discussed in Chapter 8.1.2. Increased premotor activity shows that

stronger potentials are needed to provoke neurotransmission in the cortex, highlighting disordered plasticity as a mechanism.

8.2.3. Why are subtypes unstable over time?

In the previous sections, I have provided a basic hypothetical model for how different motor symptoms develop at PD onset. However, it is well-known that motor subtypes are unstable over time, with a trend towards increasing akinesia with advancing disease and a dissipation of tremulous symptoms (Hoehn and Yahr, 1967; Alves *et al.*, 2006; Von Coelln *et al.*, 2015). Thus, the model that I have proposed, though useful for explaining the genesis of symptoms, is insufficient as an explanation of the degeneration and progression of symptoms over time. There is currently no explanation in the literature for why tremor is overtaken and is replaced by AR subtype and cognitive impairment. One hypothesis that I propose that may help us to understand this process is based on the progression over time of α-synuclein into the cerebral cortex (Braak α-synuclein stage VI (Braak, Del Tredici, *et al.*, 2003); McKeith early and diffuse neocortical type (McKeith, Dickson, Lowe, M. Emre, *et al.*, 2005). This hypothesis suggests that infiltration of α-synuclein and pre-LB toxic α-synuclein oligomers into the cerebral cortex causes slowed neurotransmission and disordered synaptic plasticity. In this way, α-synuclein pathology may act as a resistor in motor circuitry which would then slow neurotransmission from the cortex to the striatum and cerebellum (Figure 8.2 – Middle), leading to akinetic features. This constitutes one of two models I will propose for the degeneration of TD subtype over time. Crucially, the cerebral cortex is not the only locus of pathology at Braak stages V and VI, approximately. There is evidence that α-synuclein pathology begins to accumulate in striatal ChIs and that, critically, this accumulation did not occur before Braak stage V in a series of 25 PD cases (Mori *et al.*, 2008). This was corroborated by data in my case series that found α-synuclein in striatal ChIs in PD, though this deposition showed only a trend correlation with ChI arborisation. If ChI activity is a determinant of tremor production, pathological accumulation of α-synuclein in these neurons may also play a role in the dissipation of tremor as a clinical feature over time. Alongside this, α-synuclein pathology in motor program-associated areas would cause slowed execution of neurotransmission associated programmed movements such as gait and coordinated fine finger movements. This would explain the trend of increasing akinesia with advanced disease and may provide a neuropathologic explanation of the

transition of cases to H&Y stage 5 (end-stage disability) in the long run. This constitutes model 2 as an explanation for tremor dissipation over time (Figure 8.2 – Bottom). As speculated in Chapter 8.1.1, this also has implications for explanations of the evolution of cognitive impairment at this point in the disease course (Alves *et al.*, 2006).

It is not clear which of the two models I have proposed for the instability of PD motor subtypes (if any) is correct, or whether both could be true in different individuals. What is clear from the clinical symptomatology of PD is that we do not have a good appreciation of how the basal ganglia work in health, and how they degenerate in disease to produce different types of extra-pyramidal syndromes such as TD or AR. Further work is required to outline the physiology of the basal ganglia, perhaps based on evidence of how it degenerates in diseases such as PD.

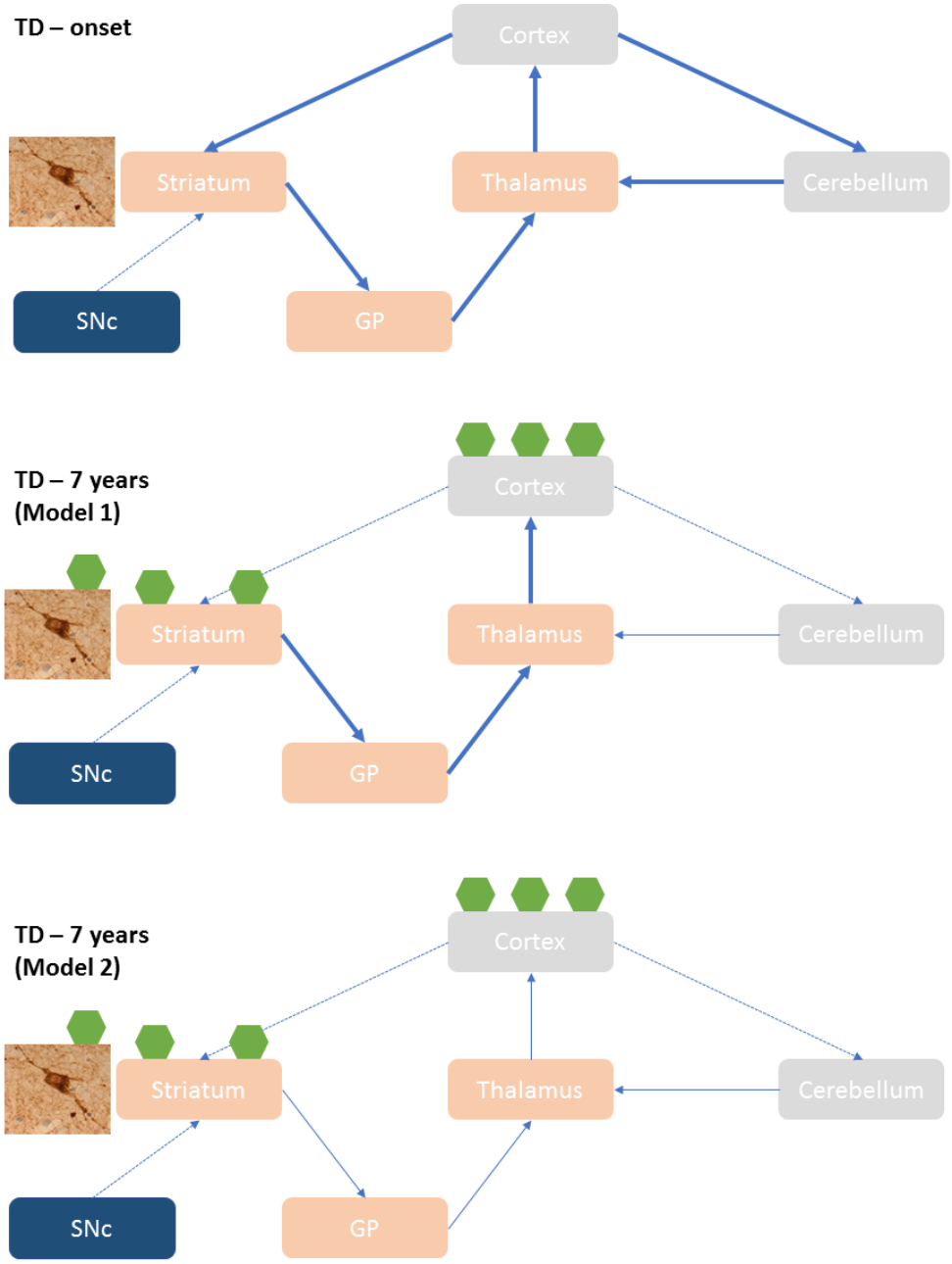


Figure 8.2: Two models for the degeneration of TD subtype over time. Top: Motor circuit activity at TD presentation. Degeneration of the substantia nigra leads to increased striatal output to the globus pallidus when ChIs are arborised and healthy. Middle: Model 1 of TD degeneration whereby accumulation of pathological α-synuclein (green hexagons) in the cortex leads to reduced cortical output to the striatum and cerebellum. Increased basal ganglia output into the thalamocortical axis is preserved. Bottom: Model 2 of TD degeneration whereby accumulation of pathological α-synuclein (green hexagons) in the cortex and striatum leads to reduced basal ganglia output in addition to reduced corticostriatal and corticocerebello activity. GP = globus pallidus, SNc = substantia nigra pars compacta.

8.2.4. Hypotheses for the differing rate of clinical progression in AR and TD cases

Not only do motor subtypes not remain stable over time, but the clinical progression of these presenting subtypes differs considerably. It is clear from the previous literature that AR cases not only demonstrate earlier cognitive impairment (as discussed in Chapter 8.1.1) but also worse neuropsychiatric (Reijnders *et al.*, 2009; Burn *et al.*, 2012) and autonomic symptoms (Marras and Chaudhuri, 2016) and reach end-stage disability sooner (Rajput *et al.*, 2009). The progression to unfavourable clinical outcomes should be related to the rate of progression of PD pathology, and the severity of neurodegeneration. Therefore, it could be hypothesised that factors that predispose to or protect against neurodegeneration in PD will be differentially expressed in PD motor subtypes. In this section, I will review the literature on these factors and speculate as to how these may be expressed in different PD motor subtypes. I will consider five main topics herein: 1) comorbid neuropathologies such as Aβ and tau, 2) neuroinflammation, 3) vascular comorbidity and cerebrovascular disease, 4) calcium homeostasis and 5) genetic factors. For the purposes of this exercise, clinical progression will be defined as the deterioration of patients to unfavourable outcomes such as end-stage disability or dementia.

8.2.4.1. Comorbid neuropathologies

As discussed in Chapter 1.4.1.2, there are a few comorbid neuropathologies that may significantly contribute to the progression of PD and may help to distinguish between the progressions of different motor subtypes. Selikhova and colleagues investigated the burden of amyloid pathology in these subtypes, and found that AR cases had a higher plaque load than TD cases in a large pathological series (Selikhova *et al.*, 2009). In the CSF, PIGD cases were found to have a significantly higher tau concentration and lower Aβ42 levels compared to TD cases, indicating a higher brain burden of tau and Aβ pathology (Jin *et al.*, 2017). This was corroborated by other studies that showed that TD cases had comparable Aβ42, Aβ40 and Aβ38 levels to controls and that AR cases had significantly reduced levels of all three of these amyloid markers, indicating increased brain Aβ plaque load (Alves *et al.*, 2013). However, my investigations in the striatum found trend increases in Aβ plaque load in TD cases compared to AR, which contrasts with this literature. Further work is warranted to assess the global brain amyloid burden in PD motor subtypes, looking especially at

the cerebral cortex. On the other hand, I found significantly increased levels of tau pathology in the striata of AR cases, particularly in the lateral putamen. Tau pathology is also found in other neuropathologic entities such as CTE. CTE is a neurodegenerative disorder characterised by a heterogeneous clinical phenotype that usually involves dementia. In a seminal series of boxer cases from the archival Corsellis collection, we found that 50% of them met the current diagnostic criteria for CTE, which specifies a pathognomonic lesion of tau deposition in astrocytes and neurons around the blood vessels at the depths of cortical sulci (McKee *et al.*, 2016). Approximately two-thirds of the cases that met CTE diagnostic criteria displayed parkinsonism, which was characterised by a moderate-severe syndrome of tremor, rigidity, gait impairment and dementia (Goldfinger *et al.*, 2018). Further, in a series of footballers with associated dementia, a large proportion showed Parkinsonian signs including tremor, gait disturbance, postural instability, dysarthria and features of LBD including hallucinations (Ling *et al.*, 2017). ARTAG, which shows a distribution of pathology similar to CTE is more common in the aged, and shows a predilection for DLB cases (Kovacs *et al.*, 2016; Liu *et al.*, 2016; Kovacs, Lee and Trojanowski, 2017). It is unclear whether ARTAG shows differential prevalence in PD motor subtypes, and further work should attempt to delineate the relationship between PD motor features and perivascular tau pathology.

8.2.4.2. Neuroinflammation as a driver of clinical progression in PD

Neuroinflammation and the role of microglia is an ever-expanding topic in the field of neurodegeneration. Microglial activation has been shown to play a role in the progression of PD. In one study, CD68 and toll-like receptor 2 (TLR2) – markers of activated microglia – and ionised calcium binding adaptor protein 1 (Iba1) – a pan-microglial marker – were studied in PD cases compared to age-matched controls and incidental Lewy Body Disease cases (iLBD) (Doorn *et al.*, 2014a). In this study, iLBD cases were used to represent prodromal PD. CD68-positive amoeboid microglia were found to be more numerous in the SN in PD compared to iLBD and controls and showed positive correlation with α-synuclein burden. Upregulation of activated microglia has been associated with pro-inflammatory cytokine production in the SN (Hirsch and Hunot, 2009). Furthermore, CD68 was also increased in the of the hippocampus – a region which is well known to develop prominent α-synuclein pathology in PD. However, TLR2 was found to be more highly expressed in iLBD

compared to controls and PD, though PD showed significantly higher levels of TLR2 than controls (Doorn *et al.*, 2014b). Iba1 showed no differences between PD and iLBD in the SN, but there was significantly higher Iba1 density in PD compared to controls. There were no differences between the groups in terms of Iba1 density in the hippocampus. This data suggests regional variability in the microglial activation in PD. Also, the study highlights that the regulation of microglial activation is complex and likely reflects a spectrum. This is because, while one activated microglia marker (CD68) increases in PD, another (TLR2) decreases, even though both markers colocalise in activated microglia in PD SN sections (Doorn *et al.*, 2014b). CD68 and TLR2 are likely to perform different functions in activated microglia, and it is not clear whether these differing roles are protective or degenerative. It could be hypothesised that TLR2 is protective, given that it is reduced in PD compared to iLBD, and that CD68 is degenerative given that it is positively correlated with α-synuclein deposition and shows increased levels in PD compared to the other groups. Another route for exploring neuroinflammation is by testing the CSF for inflammatory markers. Hall et al., studied inflammatory markers such as CRP, SAA, IL-6, IL-8, YKL-40 and MCP-1 (CCL2) in PD, PDD, MSA and PSP cases (Hall *et al.*, 2018a). They found that these markers were highest in PDD and MSA cases but that there was a significant positive correlation between the levels of these markers and the severity of motor and cognitive impairment (Hall *et al.*, 2018b). This suggests that neuroinflammation plays a significant role in the progression of PD features. There may be a link between peripheral inflammation and neuroinflammation and so this warrants study in neurodegenerative diseases. Neutrophil to lymphocyte ratio (NLR) is a marker of peripheral inflammation. In one study, this marker was compared between PD motor subtypes and control cases. NLR was shown to correlate with motor severity in TD subtype but not PIGD (Sanjari Moghaddam *et al.*, 2018). Interestingly, NLR showed correlation with striatal DATscan uptake ratios, such that increased peripheral inflammation was associated with reduced putamen DAT-ligand binding bilaterally. However, another study on peripheral inflammatory markers was not able to successfully discriminate between PD motor subtypes on this basis (Yilmaz *et al.*, 2018).

To my knowledge, there have not been any studies comparing neuroinflammation burden in PD motor subtypes using immunohistochemistry, and this is an important

avenue to be explored in explaining the clinical heterogeneity in PD. Based on the aforementioned literature, it could be inferred that microglial activation and neuroinflammation propels the progression of PD. Given that AR features become more prominent in the later stages of the disease, perhaps neuroinflammation has a role in the pathogenesis of AR features in the end-stage of PD. However, this requires confirmation in clinicopathological studies. Further, it remains to be seen what the role of microglia is in the pathophysiology of the onset of AR cases of PD.

8.2.4.3. Vascular comorbidity and cerebrovascular disease

While vascular disease is a well-known and well-studied risk factor for vascular dementia and AD, the role of vascular comorbidity as a risk factor for PD is underappreciated. A multitude of studies have now elucidated the contribution of vasculopathy to the clinical progression of PD. Li *et al*., found that vascular risk factors aggravate clinical progression in PD (Li *et al.*, 2015). This study followed 247 PD patients in an outpatient setting for 5 years. The incidence of vascular risk factors including hypertension, diabetes, hypercholesterolemia, hyperhomocysteinemia and carotid atherosclerotic plaques were analyzed. Patients were dichotomised into those with and without vascular risk factors and the H&Y scores compared at initial and final evaluations. The authors found a significant association between H&Y decline and hypertension and hyperhomocysteinaemia, especially in the young where these risk factors exacerbated dyskinetic decline (Li *et al.*, 2015). Hyperhomocysteinaemia, which is linked to atherosclerosis, vascular disease, depression and dementia, was shown by other studies to be increased in L-dopa-treated PD cases. There is limited evidence that L-dopa-induced hyperhomocysteinaemia causes changes to the structure and function of cerebral blood vessels, which may underlie progression to cognitive impairment and motor disability (Zoccolella *et al.*, 2005, 2006). Cognitive impairment, gait dysfunction and urinary disturbance can also be caused by idiopathic normal pressure hydrocephalus. Patients undergoing shunt surgery for this condition show significant CAA and vascular pathology on biopsies taken intraoperatively (Golomb *et al.*, 2000), raising the possibility that AD pathology can significantly contribute to gait and cognitive issues in PD and other neurodegenerative dementing disorders via impaired drainage of CSF (Chakravarty, 2004). Other papers have studied the cerebral vasculature in various ways, such as Guan et al., who investigated endothelial cell clustering, a marker of vessel disturbance, and found increased

numbers of endothelial cell clusters, reduced vessel branching and reduced vessel length in middle frontal gyrus and SN of PD cases compared to controls (Guan *et al.*, 2013). Other studies on the vasculature have found that macroinfarcts, microinfarcts and cerebral arteriosclerosis are each independently associated with the severity of parkinsonian gait (Buchman *et al.*, 2011). Furthermore, cerebral microbleeds in deep white and grey matter regions such as the thalamus were found to be much more common in PIGD cases compared to TD in a recent study (Chiu *et al.*, 2018). Jellinger and Attems found that cerebrovascular lesions showed a significant association with neuritic tau Braak stage in PDD and DLB but not PD without dementia (Jellinger and Attems, 2008). This highlights the relationship between vessel disease, tau pathology and neurodegeneration, as has been discussed previously in Chapter 8.2.4.1 with regards to ARTAG and CTE. The perivascular nature of the pathology in these conditions means it is possible that vascular compromise of any aetiology may lead to the hyperphosphorylation and aggregation of tau protein which in turn may drive the PD disease process forward (Compta *et al.*, 2014) and aggravate Parkinsonian signs.

Most of the literature has focused on the impact of vascular disease clinical markers and risk factors on the progression of PD. For example, Malek and colleagues found that PD patients with vascular risk factors have higher UPDRS part 3 scores and worse cognition than those without vascular risk factors (N. Malek *et al.*, 2016). Furthermore, the pattern of disease in those with vascular risk was of an AR/PIGD-predominant phenotype (N. Malek *et al.*, 2016). Furthermore, type II diabetes mellitus has been shown to independently increase the risk of rigidity and gait disturbance signs in geriatric patients, but not bradykinesia and tremor (Arvanitakis *et al.*, 2004). This, in conjunction with the study by Buchman and colleagues, suggests a role for vasculopathy in the onset and progression of AR features of PD (Buchman *et al.*, 2011). Though the current burden of literature suggests a clear role for vascular disease in the progression of PD, further work is needed to evaluate these risk factors in PD motor subtypes. There is some evidence to suggest that vascular comorbidity is especially associated with AR features and related dementia. These data need to be supported by data from neuropathologic studies. In particular, pathological studies looking for small vessel disease in PD-associated brain regions such as the LC, SN, striatum and frontal cortex, may be revealing in the quest for understanding PD motor subtypes and their progression.

8.2.4.4. Calcium homeostasis

Calcium homeostasis has been implicated in the pathophysiology of PD. Despite being a crucially important ion, involved in communication across synapses, calcium and its binding proteins are also implicated in neurotoxicity in non-physiological circumstances such as ischaemia (Freund *et al.*, 1990) and ageing (Iacopino, Christakos and Wasserman, 1990). Calcium binding proteins (CaBPs) are highly expressed throughout the brain (Iacopino, Christakos and Wasserman, 1990) where they act as intraneuronal calcium buffers and facilitate calcium transport across cell membranes Although the SN, one of the main loci of pathology in PD, is a region that is rich in CaBP (McRitchie, Hardman and Halliday, 1996) from the striatonigral GABAergic fibres, the dopaminergic neurons themselves are not CaBP-rich, and the topography of the CaBP-positive innervation of the SN is 'patchy' (Damier 1999a). Data shows that the pattern of dopaminergic neuron loss in the SN corresponds to the degree of CaBP-positive innervation, whereby the CaBP-rich regions (matrix) seem to confer neuroprotective effects on the resident nigral neurons, but that this benefit is lost in CaBP-poor regions (nigrosome). Thus, neuronal loss predominates in nigrosome regions (Damier 1999b). In primate models of parkinsonism, in which the SN is lesioned with MPTP, the striatum also shows changes in the distribution of CaBP. Petryszyn and colleagues performed lesion experiments of macaques and found that the number of calretinin-expressing striatal ChIs was significantly increased in the lesioned primates than those not lesioned (Petryszyn *et al.*, 2016). The upregulation of calretinin may protect striatal ChIs from excitotoxic damage. This data shows that CaBPs are implicated in both parts of the nigrostriatal system and may play a role in differential progression of pathology and neuronal loss. It is not clear whether there is a difference in the CaBP response between PD motor subtypes – this question warrants further investigation.

As the pathology spreads, the basal forebrain cholinergic system becomes involved. 72% of cholinergic neurons in the basal forebrain express CaBP in non-aged individuals (Geula 2003). With increasing age, the degree of CaBP expression reduces considerably to approximately 28%. As the basal forebrain is implicated in several age-associated neurodegenerative diseases, such as PDD, DLB and AD, the loss of CaBP in the basal forebrain may underlie age-related risk for these diseases (Geula, 2003). In AD, it has been shown that the loss of CaBP contributes to the

development of NFT pathology, where only 1% of CaBP-positive neurons contained tau deposits, and CaBP protected against the development of late-stage hyperphosphorylated-tau epitopes (Ahmadian, 2015). Cases of Early-disease-onset (EDO) PD (Jankovic *et al.*, 1990; Selikhova *et al.*, 2009) appear to be more benign in progression and more resistant to cognitive impairment than 'classical' cases of PD with onset between 55-65. As well as in AD, degeneration of the basal forebrain cholinergic system contributes to cognitive deterioration in PD (Whitehouse *et al.*, 1983; Liu *et al.*, 2015). It could therefore be hypothesised that EDO cases are more resistant to cognitive impairment due to increased presence of CaBP in the cholinergic basal forebrain at disease onset.

Braak stages 5 and 6 are characterised by the encroachment of Lewy pathology into the cerebral cortices, appearing first in the anterior cingulate gyrus, and thereafter in the frontal cortex and other cortical regions (Braak, Tredici, *et al.*, 2003). The distribution of the pathology in the cortex varies from individual to individual, but predominantly affects the deeper cortical layers of 5 and 6 (Harding and Halliday, 2001). The pathology tends to spread to layers 3 and 2 in advanced disease (Burn, 2004). In studies from non-human primates, the bulk of the distribution of calcium binding proteins tends to be between layers 1-4 with only sparse CaBP-positive neurons in layers 5 & 6 (Condé *et al.*, 1994; Dombrowski, 2001; Zaitsev *et al.*, 2005). Cortical lewy bodies tend to appear intracytoplasmically (Burn, 2004), suggesting that cortical neuronal death may not be as extensive in PD as it is in other neurodegenerative disorders such as AD. This is supported by work from Gómez-Tortosa and colleagues who found that CaBP expression spared cortical neurons in DLB (Gómez-Tortosa *et al.*, 2001). From the above data, it is clear to see that CaBPs play a role in the spread of pathology in PD. Therefore, it should also determine the speed, at which pathology accumulates in the Braak regions, which translates to the severity of progression of the PD syndrome. The distribution of CaBPs in these regions can easily be studied using immunohistochemistry, and mapping this in controls and PD cases of differening progression subtypes may help to answer the question of the role of CaBPs in mediating PD progression over time.

8.2.4.5. Genetic factors

As demonstrated in Table 1.1 and Chapter 1.4.2, there are a large number of genetic associations with PD. However, the influence of genetics and other molecular factors

on the progression of PD is a field that is less well researched in the literature. Tau has been shown as an independent genetic risk factor for PD in a series of pathologically proven PD cases with no PSP pathology (Charlesworth *et al.*, 2012). As discussed previously, MAPT haplotype, i.e. H1 or H2, may mediate the PD risk posed by tau genetics and is differentially expressed in PD motor subtypes (Di Battista *et al.*, 2014; Pascale *et al.*, 2016). MAPT H1 haplotype and H1c subhaplotype – associated with degenerative diseases such as AD, PSP and CBD (Kovacs, 2015) – are found in elevated frequencies in AR compared to TD cases, which show frequencies comparable to controls (Di Battista *et al.*, 2014; Pascale *et al.*, 2016). Moreover, H1 haplotype has been found to be associated with cognitive decline in a longitudinal cohort of PD cases (Williams-Gray *et al.*, 2009). Therefore, it is possible that MAPT genetics make a significant contribution to the prognosis of PD. Furthermore, MAPT haplotype and subhaplotype, particularlu H1c, highlights a potential overlap of genetic susceptibility between ARPD and PSP. Given that these two diseases show similar clinical syndromes and are both characterised by tau pathology in ChIs the striatum (Chapter 6.4.10.2), H1c haplotype, which predisposes to 4R tauopathy, may underlie these two similar conditions. Future work should focus on disentangling this aspect of movement disorder pathophysiology. Interestingly, MAPT H1g subhaplotype, a relatively rare form, has been shown to increase the risk of DLB (Labbé *et al.*, 2016).

Other genetic risk factors for PD progression include GBA mutations. A recent meta-analysis of the clinical symptoms of PD cases with and without GBA mutations found that those with mutations showed significant differences in age of onset (earlier onset), UPDRS-III score (worse motor function), H&Y stage (higher stage), dementia (more cognitive impairment), wearing-off and depression (Zhang *et al.*, 2018). GBA cases have been found to have a greater tendency to dementia (Agosta *et al.*, 2013). Malek and colleagues found that GBA mutation carriers were significantly more likely to present with PIGD-dominant PD, corroborated by other studies (Goker-Alpan *et al.*, 2008) and higher H&Y stage in a series of cases assessed early in their disease course from the PRoBaND study (Malek *et al.*, 2018a). Crucially, there were no differences in the MoCA score of GBA mutation carriers compared to non-carriers at this time point (Malek *et al.*, 2018b), which suggests that cross-sectional analysis is insufficient to assessing cognitive risk with regards to GBA mutations. Furthermore,

another study in a different cohort found that baseline UPDRS-III motor scores were not significantly different between GBA mutation carriers and non-carriers, despite there being a significant difference in hazard ratios for progression from baseline to H&Y stage 3 and dementia (Winder-Rhodes *et al.*, 2013). This is in agreement with other longitudinal studies which have shown that GBA carriers show a more aggressive disease course and reduced survival compared to idiopathic PD cases (Brockmann *et al.*, 2014). Longitudinal studies of GBA-mutation PD natural history are much more informative than cross-sectional studies and should be the focus of future work.

Mutations in the SNCA gene are naturally significant contributors to the risk of PD. They also play a role in determining the clinical syndrome. SNPs in the SNCA gene are associated with an earlier onset syndrome than idiopathic PD cases (Campêlo and Silva, 2017). However, debate exists as to whether these variants influence the motor syndrome and disease progression in PD. Some authors have found that some variants of SNCA are associated with worse motor decline (Ritz *et al.*, 2012), but other authors have found that the same variants are protective (Markopoulou *et al.*, 2014a). Another study found that common SNCA variants are not related to progression of UPDRS-III motor scores (Davis *et al.*, 2016). Other studies have found significant associations of SNCA SNPs and hyposmia in PD (W. Chen *et al.*, 2015) and increased risk of cognitive decline and PDD (Markopoulou *et al.*, 2014b; Guella *et al.*, 2016). An interesting new avenue into the exploration of SNCA mutations is the finding that some somatic mutations – mutations occurring sporadically after the zygotic state – of SNCA occur (Proukakis, Houlden and Schapira, 2013). Some somatic mutations lead to copy number variants (CNVs) in the normal number of copies of the SNCA gene (Mokretar *et al.*, 2018a), whereby mosaicism occurs – where an individual cell is made up of more than one genotype of a single in human brain neurons. In PD cases compared to controls, the proportion of SN pigmented neurons exhibiting CNVs was increased. Also, the proportion of pigmented neurons exhibiting CNVs was negatively correlated with age of onset, but not disease duration or age of death (Mokretar *et al.*, 2018b). However, TD cases displayed a lower proportion of CNVs than AR cases. Petrucci et al., reviewed the literature on the phenotypic differences between the major SNCA mutations and CNVs (Petrucci, Ginevrino and Valente, 2016). They found that the number of SNCA gene copies was significantly associated with younger onset and

shorter disease duration, implying rapid progression. Furthermore, this was also associated with severe sleep, autonomic and cognitive symptoms.

LRRK2 is the most common gene implicated in heritable PD. In a recent large study of Ashkenazi Jewish PD patients with G2019S LRRK2 mutations, the authors found that this mutation was associated with a significantly slower progression of UPDRS part III scores, and a trend association with slower decline in MoCA scores over a 7 year follow-up period (Saunders-Pullman *et al.*, 2018a). Debate exists about whether LRRK2 mutation predisposes to different PD motor subtypes. A seminal study on the clinical phenotype associated with LRRK2 mutation found that these patients were much more likely to develop a TD disease subtype – 63% of LRRK2-PD – acompared to those with pathologically proven idiopathic PD (Healy *et al.*, 2008). However, further studies have demonstrated conflicting findings to these. A series of studies by Alcalay and colleagues found that the motor phenotype significantly associated with G2019S LRRK2 mutations was PIGD, and that tremor scores in the UPDRS part III were reduced in LRRK2 mutation carriers compared to idiopathic PD cases (Alcalay *et al.*, 2009, 2013; Saunders-Pullman *et al.*, 2018b). These findings have been reproduced in diverse range of populations, including early onset PD cases, Ashkenazi Jews, and in a series of Tunisian LRRK2-PD cases (Nabli *et al.*, 2015a). These findings are fascinating given that PIGD cases of idiopathic PD show aggressive disease progression but slowed disease progression in LRRK2 cases. However, Nabli and colleagues found that the minority of LRRK2 cases that showed TD phenotype progressed significantly more slowly than PIGD cases (Nabli *et al.*, 2015b). Thus, PIGD is a risk factor for disease progression in idiopathic and genetic PD cases. The complex genetic environment along with epigenetic regulation, has a key role to play in controlling the phenotype and progression of PD cases. The effects of differential gene expression and mutations on individual neuron populations implicated in PD are currently unknown. For example, does MAPT H1 haplotype produce tau molecules that are less able to stabilise microtubules, leading to structural and morphological alterations in neurons such as striatal ChIs that accumulate tau pathology in neurodegenerative movement disorders? Answering questions like these may unlock new thinking on the cellular basis for phenotypic differences in neurodegenerative disorders and therefore warrant investigation.

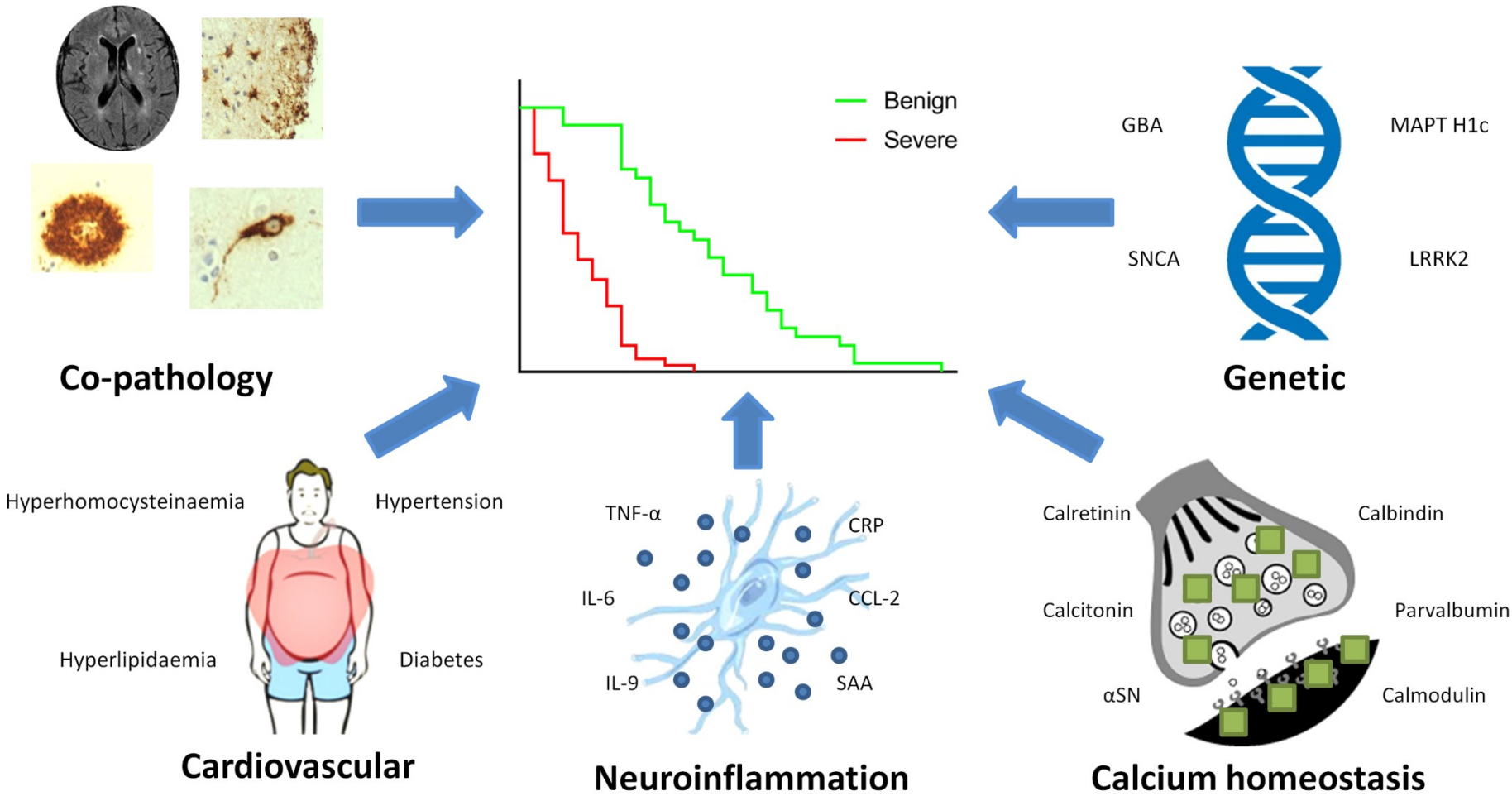


Figure 8.3: Risk factors for aggressive progression of PD. CCL-2 = , CRP = c-reactive protein, GBA = glucocerebrosidase, IL = interleukin, LRRK2 = leucine-rich repeat kinase 2, MAPT = microtubule associated protein tau, SAA = , SNCA = α-synuclein, TNF-α = tumour necrosis factor-α. Co-pathology: top left = small vessel disease, top right = ARTAG and CTE, bottom left = amyloid plaque, bottom right = tau neurofibrillary tangle. Survival curves represent decline to a negative disease outcome, e.g. H&Y stage 5, falls, dementia, death.

8.3. Dementia in PD – are PDD and DLB distinct syndromes?

In Chapter 7 of this thesis, I provided evidence for differential neuropathology between PDD and DLB – the two dementia phenotypes associated with PD. This centred on the noradrenergic neurons of the LC, where a significant reduction in neuronal number was found in DLB cases compared to PDD which resulted in loss of TH staining in subcortical target brain regions such as the amygdala. Despite some authors and groups stipulating that these syndromes are distinct, other authors group them together and assert that these are essentially overlapping and similar conditions. This topic remains controversial, and in this section, I will attempt to summarise the main arguments for and against treating PDD and DLB as different diseases.

8.3.1. Arguments against separating PDD and DLB

8.3.1.1. Clinical similarities between PDD and DLB

Currently, clinicians are using the one-year rule to distinguish between PDD and DLB cases in clinic (McKeith, Dickson, Lowe, M Emre, *et al.*, 2005). However, this rule has come in for criticism recently for several reasons which led Postuma et al., to recommend it be abolished (Berg *et al.*, 2014; Postuma *et al.*, 2015). Firstly, the one-year rule is completely arbirtrary. If a PD patient develops dementia at one year and one day after disease onset, it is defined as PDD not DLB according to the current clinical criteria. Postuma and colleagues argue that 'just as we do not believe that "constipation with Lewy bodies" and "PD constipation" are different diseases, neither should we do the same with dementia or parkinsonism' (Postuma *et al.*, 2016). This argument relies on the fact that the dementia syndromes in PDD and DLB are remarkably similar, characterised by executive dysfunction, visuospatial impairment, inattention, memory retrieval deficits, VH, FC and RBD (Emre *et al.*, 2007; McKeith *et al.*, 2017). There is therefore an argument for defining dementia in PD as DLB at all points. Critical to the notion of DLB as a separate condition is the idea that PDD is something that occurs only after many years an end-stage syndrome in PD, and that parkinsonism is present only in the end-stage of DLB. However, recent evidence shows that this is incorrect. Not only does parkinsonism occur early in DLB, but we now know that cognitive impairment is prevalent in PD throughout the disease course. In fact, the risk of dementia in PD over time is generally constant. There is no natural inflection point in dementia risk in PD – the cognitive risk in PD is constant throughout the disease course (Compta *et al.*, 2011; Postuma *et al.*, 2016). This means to say

that the risk of incident dementia is the same (or even higher) in the early stages of the disease as it is at end-stage (Postuma *et al.*, 2016). Moreover, there is now clear documentation of mild cognitive changes even in prodromal PD (Compta *et al.*, 2011). Moreover, prodrome in PD can also be characterised by RBD without cognitive changes (Berg *et al.*, 2014), suggesting that RBD is far from being specific to DLB. This is in sharp contrast to what would be expected if DLB and PD were mutually exclusive diseases. Most DLB cases have parkinsonism at baseline, which means they may reflect the early dementia risk category in PD. Thus, the crux of the argument for combining PDD and DLB, is that the methodology used to practically distinguish between them are arbirtrary or flawed, or both.

8.3.1.2. Pathological similarities between PDD and DLB

There are various similarities in the neuropathology underlying PDD and DLB. For example, both arise primarily as a result of synucleinopathy and the accumulation of Lewy pathology under the microscope. Both show evidence that they spread stereotypically along the Braak staging system (Braak, Del Tredici, *et al.*, 2003; Jellinger and Korczyn, 2018). Moreover, both are influenced by a confluence of pathology, whereby AD-type proteinopathy and synucleinopathy propogate and drive each other to contribute to the onset of dementia (Clinton *et al.*, 2010; Compta *et al.*, 2011; Kalaitzakis *et al.*, 2011). PDD and DLB are also similar in the degree of cholinergic neuronal loss incurred in the nbM, demonstrated pathologically by my predecessor Alan Liu (unpublished), but also by neuroimaging studies utilising AChE PET. Numerous studies have shown that AChE PET signal is significantly reduced in the neocortex in PDD and DLB patients compared to non-cognitively-impaired PD patients and healthy controls (Shimada *et al.*, 2009; Klein *et al.*, 2010). Fascinatingly, there seems to be a particular deficit in cholinergic PET signal in the medial occipital cortex – the striate region – in LBD (Shimada *et al.*, 2015), which supports Alan Liu's findings that there is more significant atrophy of the nbM posterior division. This may play a role in the pathophysiology of visual hallucinosis in LBD.

8.3.2. Arguments for separating PDD and DLB

8.3.2.1. Clinical differences between PDD and DLB

As stated in Chapter 8.3.1.1, the clinical syndromes of PDD and DLB share many similar characteristics. However, there are also various key differences between them.

DLB is characterised by the presence of three core clinical features alongside parkinsonism and dementia. These are FC, VH and RBD according to the most recent consensus diagnostic criteria (McKeith *et al.*, 2017). Intuitively, the prevalence of DLB symptoms would be, and is, much lower in PDD than it is in DLB (Fénelon *et al.*, 2000; Aarsland, Ballard, *et al.*, 2001; Ferman *et al.*, 2004; Noe *et al.*, 2004; Aarsland *et al.*, 2005, 2007). However, the logical conclusion from this data is that, though DLB symptoms are less prevalent in PDD, they are found, which means that some PDD cases show a DLB-like syndrome (Varanese *et al.*, 2010). This is something that we found in 46% of PDD cases in the PDD cohort used in Chapter 7 - Thus there are two apparent subtypes of PDD, whereby the majority exhibit dementia without fluctuations, hallucinations or RBD but a subset display a syndrome indistinguishable from DLB, if the one-year rule is not applied. If we compare the PDD group without DLB symptoms to a cohort of DLB cases, considerable differences are apparent. Neuropsychological studies have highlighted more severe executive and attentional dysfunction in DLB cases compared to PDD (Gnanalingham *et al.*, 1997; Downes *et al.*, 1998). Moreover, DLB cases have more severe deficits in visual recognition memory (Mondon *et al.*, 2007). Thus, the available evidence suggests that DLB is a more complex, and perhaps a more severe dementia syndrome than PDD. Interestingly, the motor syndrome in PDD and DLB demonstrate differences too in that tremor is far less common in DLB as a presenting motor feature than it is in PDD (Fritz *et al.*, 2016a). Moreover, total UPDRS motor score is lower in DLB compared to PDD, a difference which is much more stark in male patients as opposed to female patients (Fritz *et al.*, 2016b). DLB patients also had slower armswing and worse balance than PDD patients, while showing trend reductions in the stride length during backward walking and fast forward walking (Fritz *et al.*, 2016b).

Numerous neuroimaging studies have been conducted to identify differences between PDD and DLB (for an excellent recent review on the topic, see Yousaf *et al.*, 2019). Mak and colleagues studied MRI images of LBD cases, and found that grey matter atrophy was more pronounced in DLB cases compared to PDD (Mak *et al.*, 2014). However, the distribution of grey matter loss in DLB cases varies between studies. For example, Beyer et al. found that the grey matter atrophy was predominantly localised to the temporal, parietal and occipital lobes using voxel-based morphometry technique (Beyer, Larsen and Aarsland, 2007), whereas Lee et al. found greater occipital and

striatal grey matter loss (Lee *et al.*, 2010). Sanchez-castaneda et al., found greater loss of grey matter volume in the limbic regions of the McKeith DLB diagnostic criteria (McKeith, Dickson, Lowe, M Emre, *et al.*, 2005) such as the anterior cingulate gyrus, right amygdala and right hippocampus, and that this loss correlated with cognitive decline in DLB (Sanchez-Castaneda *et al.*, 2009). In a follow up study, the same group found greater grey matter loss in the left precuneus region and inferior frontal lobe which correlated with VH in DLB, but not in PDD (Sanchez-Castaneda *et al.*, 2010a). This suggests that there is heterogeneous pathophysiology of hallucinations in LBD depending on whether they occur early or late in the disease course (Sanchez-Castaneda *et al.*, 2010b). To date, there has only been one comparative functional neuroimaging study comparing PDD and DLB, conducted by Peraza and colleagues (Peraza *et al.*, 2015). This study utilised functional MRI and voxel-based morphometry, to assess differences in functional connectivity between PDD cases, DLB cases and controls. Greater functional connectivity alterations were noted in the fronto-parietal network in DLB cases compared to PDD and controls, though differences between PDD and DLB didn't reach statistical significance (Peraza *et al.*, 2015). Voxel-based morphometric analysis highlighted more grey matter loss in the motor cortex and precuneus bilaterally in PDD cases compared to DLB and controls (Peraza *et al.*, 2015), which is likely to be because PDD cases have had a longer previous history of parkinsonism compared to DLB cases.

Perhaps the most convincing argument for maintaining the separation between PDD and DLB is that we simply do not have enough data to suggest whether they are the same or not. The data that we in the field really need is that the answers the question of whether DLB, which can be thought of as PDD in reverse based on the timing of symptoms, is the reverse of PD in terms of the sequence brain regions in which α-synclein accumulates. The lack of this data means there is no literature-based endorsement of either camp and until we have the answer to this question I believe we should treat these disorders as different for research purposes. A shift in the accepted dogma towards grouping these as a single LBD entity would reduce the ability of researchers in the field to perform clinicopathological studies on this subject. The counter argument to this is that the differentiation isn't useful clinically and causes confusion to patients (Postuma *et al.*, 2016), especially given that the diagnosis and treatment of these disorders in clinic is overwhelmingly alike. Cholinesterase inhibitors

such as donepezil and rivastigmine are the first line of treatment for both PDD and DLB, meaning that the differentiation of the two LBD phenotypes has no relevance for the patient. My data suggests a more significant noradrenergic deficit in DLB compared to PDD, meaning that cases with a DLB syndrome could benefit from noradrenaline reuptake inhibitors such as atomoxetine. Clinical trials of these drugs are indicated in LBD.

8.3.2.2. Pathological differences between PDD and DLB

Aside from the clinical differences between PDD and DLB summarised in the last section, there are several studies that demonstrate differences in the neuropathology between these diseases too. Given that the main difficulty for the field has been the separation between DLB and AD, the natural tendency has been towards studies of AD-related pathology in PDD and DLB. For example, a large body of literature has now highlighted that DLB cases are characterised by higher striatal Aβdeposition than PDD cases (Jellinger and Attems, 2006; Halliday, Song and Harding, 2011; Kalaitzakis *et al.*, 2011). This means that they occupy a later Thal Aβphase than PDD cases (Thal *et al.*, 2002). This finding is clouded by natur PDD cases can have a more severe burden of AD pathology (Irwin, Lee and Trojanowski, 2013) and a strong correlation between LB and AD pathology exists in the neocortices of these cases (Compta *et al.*, 2011). A high burden of LB and AD pathologies predicts shorter interval between motor and cognitive and shorter survival time in PD cases (Irwin *et al.*, 2017). However, a subset of DLB cases shows low AD pathology (Hely *et al.*, 1996). In the main, this type of DLB case formed the DLB cohort in Chapter 7 - . In addition to these cases, several other DLB cases were audited for use in this study, but ruled out due to excessive tau NFT pathology. This suggests that AD pathology is not a prerequisite for the clinical syndrome of DLB. A neuropathological audit study of clinician's accuracy of DLB diagnosis showed that the ability to diagnose DLB was not affected by Aβload (Tiraboschi *et al.*, 2015). In fact, the study noted an inverse relationship between LB pathology and neuritic AD pathology. Cerebral microbleeds are found more commonly in DLB than PDD (Kim *et al.*, 2015), especially in the occipital lobe (Gungor *et al.*, 2015) and this appears to be independent of CAA burden (De Reuck *et al.*, 2013).

The degree of neuronal loss in LBD-implicated brain regions can also differ between LBD subtypes. For example, the SN is more degenerated in PDD cases compared to

DLB (Tsuboi and Dickson, 2005), which is intuitive given that PDD cases have usually exhibited parkinsonism for a number of years before the development of dementia. Thus, there is a clear disease duration effect in the SN, which is something I have been able to corroborate from the study presented in Chapter 5 - . However, the same trend is not found in the LC. There is no clear disease duration effect on the LC TH+ neuron count. In fact, the only predictive variables for LC neuron count are cognitive status and Braak α-synuclein stage (Chapter 5.4.6). This suggests that DLB cases represent a disease entity where α-synuclein pathology spreads more rapidly through the Braak regions. At the outset of this thesis, I aimed to carry out clinicopathological correlations with LBD phenotype in the LC system by quantifying monoaminergic neurons in the LC and monoaminergic fibres in LC cortical and sub-cortical target regions. LC TH+ neuron counts were lower in DLB cases than PDD cases, an effect that was enhanced when controlled for disease duration, as DLB cases have a much shorter survival period. Interestingly, where PDD cases, showed a DLB-like syndrome, this correlated with reduced LC neuron counts. Therefore, there is significant heterogeneity in the extent of LC degeneration which is modified by clinical presentation and a host of factors that may be hidden behind the scenes, such as those described in Chapter 8.2.4. Some evidence suggests that differential PPN cholinergic cell loss correlates with hallucinations in PDD but not DLB (Hepp *et al.*, 2013). I have also shown that loss correlated with VH, as well as the other DLB symptoms (Chapter 7.4.4). The above data show that grouping PDD and DLB together can be problematic due to the pathological heterogeneity on display. Until such point as we can explain these in a central narrative or hypothesis, there should be a separation between PDD and DLB, which well help us perform the studies that will enable us to make a conclusion.

8.4. Ongoing and future work

There are several ongoing projects relating to the topic of this thesis. For example, we are exploring the genetics of the clinical phenotypes of PD. Unraveling the genetic etiology of neurological disease provides fundamental insights into the cellular pathways underlying neurodegeneration. It also facilitates disease modeling, as well as the design and testing of targeted therapeutics. I, alongside collaborators at the National Institute for Neurological Disorders & Stroke am sequencing the genome of 2,000 LBD cases and 1,500 neurologically normal controls. Genome sequencing is

being performed on an Illumina X10 system, and analysis will be completed on Google Genomics. Cloud-based computing lowers the complexity associated with the proposal. Our primary aim is to identify genetic loci that alter risk of developing LBD. In addition, we will make the data immediately available on the dbGaP repository. Researchers can then access and combine this unique resource with their own data to increase experimental power. Our genome sequencing project represents the next step in unraveling the complex etiology of LBD. We will also be using this genome data to map the genetic susceptibilities to PD motor subtypes, and the prognostic subtypes that I set out in Chapter 8.1. With this, I aim to add more complexity to the modelling of cognitive impairment risk that I performed in Chapter 3 - We are also performing RNA sequencing in a small cohort of PD, PDD and DLB cases to assess differences in the transciptomic microenvironment of these cases in the anterior cingulate gyrus.

Further ongoing work surrounds analysis of the PPMI dataset. In this thesis, I have demonstrated the immense potential for complex statistical analysis and modelling of the PPMI cohort to identify prognostic markers for cognitive decline in PD. To take this project further, I, in collaboration with Adam Hampshire and Amy Jolly, will be incorporating structural neuroimaging data, using the T1 MRI scans of the PPMI participants to assess longitudinal changes to brain region volumes, using an already-optimised analysis pipeline. This will be added to the CCA alongside longitudinal clinical data to create a longitudinal predictive model for cognitive decline in PD. Also, numerous different types of statistical modelling such as permutation analysis will be employed to assess the precision of our predictive model. This is advantageous because permutation tests allow for the precision analysis of datasets with mixed data types such as categorical, ordinal and metric data. The PPMI cohort has data points of all these types. Moreover, assessing the precision of our model allows us to determine its clinical usefulness. Ultimately, we do not want to apply erroneous models to patients, and thereby incur emotional burden to patients and carers, or make ill-informed decisions relating to disease modifying therapy.

Future work relating to the pathology elements of this thesis primarily involves improvement of tissue clearing techniques. FASTclear tissue clearing has the potential to significantly improve the quantification of striatal ChI morphology. However, there has been difficulty in utilising ChAT as a marker in FASTclear tissue, as demonstrated

in Chapter 6.4.7. Further work is needed to adapt the FASTclear methodology to maximise the capability for ChAT antibody penetration and binding, or for investigating other cholinergic markers such as AChE, ChT and VaCHT. I have been able to demonstrate excellent antibody penetrance for other neuronal markers such as TH. Z-stack images of individual neurons can be *flattened*, as shown in Chapter 7.4.5, to allow for analysis of arborisation using techniques such as the Sholl method. This method involves quantification of branches within pre-defined radiuses from the cell-soma. If we can adapt FASTclear for the use of cholinergic neuronal markers, this same methodology could be used to assess the morphology of the striatal ChIs in a more robust, fully quantitative way. This would improve upon the methodology I employed in this thesis.

From the data outlined in Chapter 6.4.10.2, it was clear to see that the burden of AT8 tau pathology was not explained by the burdens of 3R and 4R tau deposition. In all cases, the amount of AT8 tau pathology vastly outweighted the sum of the amounts of 3R and 4R tau. This suggests that there are alternative tau epitopes, which are picked up by AT8 antibody immunostaining but not 3R and 4R. Equally, some epitopes of pathological tau may be not be highlighted by the AT8 antibody, which is specific for tau phosphorylated at serine 202 and threonine 205, but other phosphorylation sites include serine 396 and 404, which the paired-helical-filaments 1 antibody is specific for. Moreover, none of these antibodies detect pre-aggregated hyperphosporylated tau. Courtesy of Dr Javier Alegre Abarrategui and Elisavet Velentza Almpani, we will be attempting to utilise the tau proximity ligation assay that they have developed to immunostain pre-aggregated, self-interacting, hyperphosporylated tau in the basal ganglia of different PD motor subtypes and PSP. This, in addition to staining with antibodies against other hyperphosporylated tau epitopes, will form the basis for futher investigation of the role of tau in the basal ganglia pathology in neurodegenerative movement disorders.

8.5. Conclusions

In this thesis, while I have confirmed that dopaminergic loss is a necessary condition for the pathogenesis of PD, the specific motor and cognitive phenotypes expressed in affected individuals is related more to pathology in extra-dopaminergic systems. In particular, the cholinergic system of the striatum, composed of striatal ChIs, have been shown to be differentially affected in PD motor subtypes, whereby the system appears

intact in TD cases, but atrophic in AR cases, with concomitant hyperphosphorylated tau pathology driving the degeneration of these neurons. I have added significantly to the data outlining the relationship between features of AR subtype and cognitive decline in PD. Specifically, gait disturbance heralds the incidence of cognitive impairment within 3 years, but also shows a single-time point relationship whereby the severities of gait impairment and cognitive dysfunction are positively correlated. I hypothesised that this relationship is underpinned by the encroachment of PD pathology into the cerebral cortex, leading to depotentiation of synapses, culminating in slowed neurotransmission. At the phenotypic level, this may manifest as slowed movement and cognition. The cholinergic pathophysiology of dementia in PD is well-known. However, I have shown that noradrenergic pathology also has a role to play in the onset of the different types of LBD. In DLB cases, there is severe atrophy of the LC, and PDD cases that display DLB-like features show a similar level of degeneration to DLB cases. By comparison, controls and PD cases without cognitive impairment show relative preservation of LC TH-positive neurons. Previous authors have debated whether PDD and DLB should be considered as a single disease entity or should be separated. There is a growing body of literature that I have now added to that provides evidence that these two syndromes are similar because they are characterised by synucleinopathy but are differentiated, at least in part, by their involvement of the LC, their burden of AD and vascular pathology and by different genetic predispositions.

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Appendix 1 – A Parkinson's UK Tissue Bank clinical report from a PD case in the cohort

Clinical report **PD**763

General remarks

Sex: female**DoB:** 01/05/1931**Died:** 19/12/2014
Quality of notes: good
Age of onset: 67y**Date of onset:** 01/01/1999**Disease duration:** 15 yrs
Occupation: Post Mistress
Cause of death: not stated
Health Care professional access:
Neurologist:
Reporting Neurologist: E Fernandez

For the neuropathology report

Presentation: resting tremor **Clinical diagnosis:** idiopathic Parkinson's disease
Clinical History - 2000: presenting with 1y history of resting tremor and deterioration of hand writing. No clear loss of dexterity. No effect of alcohol on symptoms. Father with long history of tremor. Also reporting anosmia for 1 year. O/E bradykinesia, resting tremor, cog wheel rigidity and reduced arm swing on the R. No BP drop. Mild symptoms and medication not started. Had normal CT head - 2001: progression of the symptoms with painful stiffness and dragging R leg. Ropinirole started with good response. Dose gradually increased up to 15mg/d - 2003: using walking stick and 2 falls in part related to orthopaedic problems (knee arthrosis and previous hip replacement) - 2005-2008: progression of the symptoms and Ldopa is started with great improvement. The dose is gradually increased and also entacapone and rasagiline are added - 2009: motor fluctuations with dyskinesias. Amantadine is started and stalevo reduced with partial improvement. - 2010: visual hallucination which initially resolved after medication adjustments. Gait initiation difficulties - 2012: Recurrent falls (wrist fracture). Progressive cognitive decline and bothersome hallucinations requiring admission. Diagnosed of PD dementia and rivastigmine patches and quetiapine are started. CT head showed small vessel disease and minimal atrophy of medial temporal lobes. Discharged to residential home - 2013: gradual deterioration of mobility and cognition. Zimmer frame and wheelchair for long distances. Recurrent falls (fracture of neck of the femur)

- 19/12/2014 Died.
Complications: dyskinesia 2009 and wearing off 2010. Recurrent falls 2012. Wheelchair 2013.
Autonomic: anosmia, constipation
Psychiatric: PD dementia (2012), visual hallucinations (2010).
Premorbid & Risks: non-smoker, alcohol with moderation
Family History: father with long history of tremor
Other medical: HTN, osteoporosis, asymptomatic cebrovascular disease and osteoarthritis (hip and knee replacement)
Neuroimaging: CT head 2000 (normal) and 2012 (small vessel and minimal atrophy medial temporal lobes)
Medication: PD: stalevo 100 qds, ropinirole 15mg/d, rasagiline, rivastigmine patch 4.6mg. Non-PD: alendronate, calcium, aspirin, furosemide, losartan.

Appendix 2 – The Parkinson's UK Tissue Bank Donor Lifestyle and Medical History Questionnaire

Donor Lifestyle and Medical History Questionnaire

Please fill in as much of the questionnaire as possible, with the aid of your carer or PD specialist care nurse for example, if required. Please sign below to confirm that you consent for us to contact your GP if any issues should arise about your health and well-being from the questionnaire information. If you do not wish to consent, please do not complete this part of the questionnaire.
Please note that some of the questions may not be applicable particularly if you don't have Parkinson's.

I consent to the UK PDS Tissue Bank contacting my GP should any issues regarding my health and well-being arise from my answers to this questionnaire.

Signed.....

Date.....

Full Name: .....
Date of Birth: .....
Person with PD ☐
Person with PD related disease ☐
Person without PD ☐
Donor Card Number:_____ (for office use)

A) History of Parkinson's (If you do not have Parkinson's move to section 2c)

Date of onset of Parkinson's:.....

At onset of Parkinson's which side of the body was affected?
Right ☐ Left ☐ Both ☐

Please tick the relevant boxes:

1) Which symptoms at **onset**:

Tremor (limbs)	☐	Slowness of movements/clumsiness	☐
Hallucinations	☐	Swallowing and speech problems	☐
Rigidity/Stiffness	☐	Memory and concentration problems	☐
Imbalance and falls	☐	Bladder and bowel problems	☐
Sleep problems	☐	Dizziness and faints	☐

Was any treatment given at onset, if so please state. If no, state when treatment started:

.....

2) **Development** of symptoms, please state if and when the following symptoms developed:

		Date symptoms started
Tremor (limbs)	☐	.....
Slowness of movements/clumsiness	☐	.....
Hallucinations	☐	.....
Swallowing and speech problems	☐	.....
Rigidity/Stiffness	☐	.....
Memory and concentration problems	☐	.....
Imbalance and falls	☐	.....
Bladder and bowel problems	☐	.....
Sleep problems	☐	.....
Dizziness and faints	☐	.....
Fatigue	☐	.....

B) Medication.

1) Have you been referred to a neurologist?

Yes ☐ No ☐ If so, when? .....

2) Does your General Practitioner manage your medication on a regular basis?

Yes ☐ No ☐

3) Does your specialist (e.g. PD nurse) manage your medication on a regular basis?

Yes ☐ No ☐

If so, who? .....

C) General Information

1) Do you smoke?
a) Now:

Yes ☐ No ☐ If so, how many? .....

When did you start smoking? .....

b) In the past:

Yes ☐ No ☐ If so, how many? .....

When did you start smoking? .....

2) Do you drink alcohol?
a) Now:

Yes ☐ No ☐

What type?..... How often?.....

b) In the past:

Yes ☐ No ☐

What type?..... How often?.....

3) Have you had exposure to drugs of abuse? (If you would like to make a statement on this it will be taken in total confidence)

.....
.....
.....

4a) Before diagnosis how many portions of fruits and vegetables did you consume on average every day? If you are a person without Parkinson's what is your current average intake of fruit and vegetables.

None ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 4+ ☐
Unsure ☐

4b) After diagnosis did you eat the recommended portions of fruits and vegetables every day? If you do not have Parkinson's ignore this question.

None ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 4+ ☐
Unsure ☐

5a) Before diagnosis, did you regularly consume vitamin and mineral supplements? If you do not have Parkinson's do you consume vitamins now?

Yes ☐ Occasionally ☐ No ☐ In the past ☐

If so, what and how often? .....

5b) After diagnosis, did you regularly consume vitamin and mineral supplements? If you do not have Parkinson's ignore this question.

Yes ☐ Occasionally ☐ No ☐

If so, what and how often? .....

6) Where does your drinking water come from?

Mains (tap) ☐ Bottle ☐ Well ☐ Other ☐

Please state: .....

7) Did you have any major childhood illness? (Measles, chicken pox etc)

.....

.....

.....

.....

.....

8) Have you ever had a head injury? Yes ☐ No ☐

If Yes please specify with dates where possible:

.....

.....

.....

.....

.....

9) Please describe your previous medical history, listing all major illnesses, operations and conditions under treatment with dates:

.....

.....

.....

.....

.....

10) Do any illnesses run in your family? (including PD, tremor, falls, dementia etc). If so, please state what and which other family member suffered from them, at what age, age of death and any other details.

.....

.....

.....

.....

.....

.....

.....

.....

11) Please describe your work history i.e. what occupations you have had

.....

.....

.....

.....

.....

.....

12) As part of your working life or personal life have you had any major exposure to certain chemicals? (Please describe)

.....

.....

.....

.....

13) Has your work involved the use of solvents?

Yes ☐ No ☐ If so, how what and for how long? .....

14) Have you ever been involved in farming or rural living?

Yes ☐ No ☐ If so, please describe to what extent and for how long?

.....

.....

15) Where did you attend school and what qualifications did you achieve?

.....

.....

16) What sports have you participated in regularly during your lifetime? (football, cricket, boxing, running, golf, athletics etc)

.....

.....

.....

.....

17) Please describe your medication history including drugs for dizziness, aches and pains, nausea and vomiting, as best you can remember:

Appendix 3 – The Parkinson's UK Tissue Bank Donor Follow-up Questionnaire

Donor Follow-up Questionnaire

Please complete as much as the questionnaire as you can. Your answers should reflect how you have felt in the last month. You will not experience all of the symptoms listed, particularly if you do not have Parkinson's but your responses are important since we need to know the occurrence of such symptoms in the general population as well.
Please sign below to confirm that you consent for us to contact your GP if any issues should arise about your health and well-being from the questionnaire information. If you do not wish to consent, please do not complete this part of the questionnaire.

I consent to the UK PDS Tissue Bank contacting my GP should any issues regarding my health and well-being arise from my answers to this questionnaire.

Signed.....

Date.....

Full Name: .....
Date of Birth: ..... Donor number (see back of your donor card):
Date of Questionnaire completion: .....

When on or off have you in the last month have you ever experienced the following:

	No	a little	some	a lot
1. Do you have shaking(tremor) when your hands are quiet/resting	☐	☐	☐	☐
2. Do you have shaking when your hands are moving	☐	☐	☐	☐
3. Do you have shaking in your legs	☐	☐	☐	☐
4. Do you have slowed down movements	☐	☐	☐	☐
5. Do you have stiffness	☐	☐	☐	☐
6. Do you have weakness	☐	☐	☐	☐
7. Do you have clumsiness	☐	☐	☐	☐
8. Do you have poor balance/unsteadiness	☐	☐	☐	☐
9. Do you trip	☐	☐	☐	☐
10. Do you have falls	☐	☐	☐	☐
11. Do you have difficulty starting to walk	☐	☐	☐	☐
12. Do you have difficulty getting through doorways	☐	☐	☐	☐
13. Do you use a walking stick	☐	☐	☐	☐
14. Do you need assistance to walk	☐	☐	☐	☐
15. Do you have a wheelchair	☐	☐	☐	☐
16. Do you have difficulty getting out of bed	☐	☐	☐	☐
17. Do you have difficulty rolling over in bed	☐	☐	☐	☐
18. Do you have difficulty getting up out of a chair	☐	☐	☐	☐
19. Does your medication give you some improvement in your symptoms	☐	☐	☐	☐

	No	a little	some	a lot
20. Do you get involuntary movements/ fidgets (dyskinesia)	☐	☐	☐	☐
21. Do you find that some doses of medication do not work	☐	☐	☐	☐
22. Do you suddenly turn off or "freeze"	☐	☐	☐	☐
23. Does your medication wear off before the next dose	☐	☐	☐	☐
24. Do you feel restless and need to move about	☐	☐	☐	☐
25. Do you have nausea or a hangover feeling with your tablets	☐	☐	☐	☐
26. Do you have swollen ankles	☐	☐	☐	☐
27. Do you get clawing of your hands and/or feet	☐	☐	☐	☐
28. Does your medication stop working	☐	☐	☐	☐
29. Is your response to medication unpredictable	☐	☐	☐	☐
30. Do you have difficulty washing yourself	☐	☐	☐	☐
31. Do you need assistance to wash	☐	☐	☐	☐
32. Do you have difficulty brushing your teeth	☐	☐	☐	☐
33. Do you have difficulty combing or brushing your hair	☐	☐	☐	☐
34. Do you have difficulty shaving/putting on make-up	☐	☐	☐	☐
35. Do you have difficulty going to the toilet	☐	☐	☐	☐
36. Do you have difficulty getting dressed	☐	☐	☐	☐
37. Do you need help to get dressed	☐	☐	☐	☐
38. Do you have difficulty using a knife & fork	☐	☐	☐	☐
39. Do you need help cutting up your food	☐	☐	☐	☐
40. Do you spill drinks from cups	☐	☐	☐	☐
41. Do you have difficulty getting to sleep	☐	☐	☐	☐
42. Do you have difficulty getting comfortable in bed	☐	☐	☐	☐
43. Do your legs feel restless or fidgety in bed	☐	☐	☐	☐
44. Do you have vivid dreams	☐	☐	☐	☐
45. Do you have nightmares	☐	☐	☐	☐
46. Do you move or thrash about in your sleep	☐	☐	☐	☐
47. Do you talk in your sleep	☐	☐	☐	☐
48. Do you sleep in the day	☐	☐	☐	☐
49. Do you fall asleep suddenly in the day	☐	☐	☐	☐
50. Do you feel low	☐	☐	☐	☐
51. Do you feel anxious or frightened	☐	☐	☐	☐
52. Do you get mood swings	☐	☐	☐	☐

	No	a little	some	a lot
53. Do you feel fed-up or frustrated	☐	☐	☐	☐
54. Do you feel hopeless or pessimistic	☐	☐	☐	☐
55. Do you feel positive and confident	☐	☐	☐	☐
56. Do you get panicky	☐	☐	☐	☐
57. Do you have drooling	☐	☐	☐	☐
58. Do you need to empty your bladder often	☐	☐	☐	☐
59. Do you need to get to the toilet quickly	☐	☐	☐	☐
60. Do you have accidents	☐	☐	☐	☐
61. Do you need to urinate in the night	☐	☐	☐	☐
62. Do you have speech problems	☐	☐	☐	☐
63. Do you have difficulty making yourself understood	☐	☐	☐	☐
64. Do you have swallowing difficulties	☐	☐	☐	☐
65. Do you cough when eating or drinking	☐	☐	☐	☐
66. Do you feel dizzy on standing up	☐	☐	☐	☐
67. Do you have blackouts or faints	☐	☐	☐	☐
68. Do you have problems with sex/erections	☐	☐	☐	☐
69. Do you have a shiny rash on the face	☐	☐	☐	☐
70. Do you have too much sweating	☐	☐	☐	☐
71. Do you have loss of smell	☐	☐	☐	☐
72. Do you have loss of taste	☐	☐	☐	☐
73. Do you get muddled with your clothes	☐	☐	☐	☐
74. Do you get muddled getting about your home	☐	☐	☐	☐
75. Do you get muddled in finding your way about outside	☐	☐	☐	☐
76. Do you get lost	☐	☐	☐	☐
77. Do you have difficulty understanding people	☐	☐	☐	☐
78. Do you have memory problems	☐	☐	☐	☐
79. Do you have poor concentration	☐	☐	☐	☐
80. Do you get confused	☐	☐	☐	☐
81. Do you have difficulty understanding what you read	☐	☐	☐	☐
82. Do you have difficulty following the plot on TV/in a film	☐	☐	☐	☐
83. Do you feel people watch you or stare at you	☐	☐	☐	☐
84. Do you feel some people are against you	☐	☐	☐	☐
85. Do you feel suspicious	☐	☐	☐	☐

	No	a little	some	a lot
86. Do you feel a presence in the room	☐	☐	☐	☐
87. Do you feel a presence behind or beside you	☐	☐	☐	☐
88. Do you see animals or faces or people	☐	☐	☐	☐
89. Do you hear noises or voices	☐	☐	☐	☐
90. Do you get cramps	☐	☐	☐	☐
91. Do you get bad pains in the legs	☐	☐	☐	☐
92. Is your appetite affected	☐	☐	☐	☐
93. Have you lost weight	☐	☐	☐	☐
94. Is your energy level reduced	☐	☐	☐	☐
95. Do you have stiff or painful joints	☐	☐	☐	☐
96. Do you have shortness of breath	☐	☐	☐	☐
97. Do you go out to see friends and family	☐	☐	☐	☐
98. Do you go to the cinema or on other outings	☐	☐	☐	☐
99. Do you eat out in restaurants	☐	☐	☐	☐
100. Do you feel lonely or isolated	☐	☐	☐	☐

Appendix 4 – PD cohort of 40 TD, 40 Mixed and 40 AR cases for this thesis

Abbreviations: αSN = α-synuclein, AR = akinetic-rigid, TD = tremor-dominant, PD = Parkinson's disease without cognitive impairment, PD-MCI = PD with mild cognitive impairment, PDD = PD dementia, DLB = dementia with Lewy Bodies.

Case	Gender	Age at onset	Age at death	Duration of disease	Motor subtype	Cognitive status	Braak aSN	Braak tau
PD255	M	53	79	26	mixed	PDD	6	2
PD264	F	64	84	20	AR	PDD	6	3
PD267	F	72	78	6	mixed	PD	6	3
PD269	M	68	76	8	TD	PDD	5	2
PD272	M	68	84	16	mixed	PD	6	2
PD273	M	68	73	5	TD	PDD	6	2
PD284	F	55	75	20	TD	PD	6	2
PD286	M	64	82	18	AR	PDD	6	1
PD287	M	77	88	11	mixed	PDD	6	2
PD299	M	60	83	23	mixed	PDD	6	2
PD301	F	64	83	19	mixed	PDD	5	1
PD304	F	76	87	11	mixed	PDD	6	1
PD307	F	70	83	13	AR	PDD	6	3
PD310	M	56	62	6	TD	PD	5	0
PD323	M	68	77	9	AR	PDD	6	1
PD324	M	87	94	7	mixed	PDD	6	2
PD331	M	65	76	11	TD	PD-MCI	6	1
PD336	M	73	81	8	AR	PDD	6	2
PD337	M	59	75	16	TD	PDD	6	2
PD341	M	54	68	14	AR	PDD	6	2
PD343	M	80	81	1	AR	DLB	6	1
PD347	F	68	84	16	TD	PD	5	2
PD348	M	72	78	6	AR	PD	6	2
PD356	F	77	86	9	AR	PD	5	2
PD366	M	49	69	20	AR	PDD	6	1
PD370	M	62	66	4	AR	PD	6	0
PD371	M	63	77	14	TD	PDD	6	1
PD372	F	53	71	18	AR	PDD	6	3
PD373	M	72	85	13	AR	PD	6	0
PD374	M	51	74	23	AR	PDD	6	0
PD378	F	54	80	26	TD	PD-MCI	6	1
PD386	M	84	91	7	TD	PD-MCI	6	1
PD391	M	68	79	11	TD	PDD	6	0
PD398	M	50	74	24	TD	PD	5	0
PD399	F	66	77	11	TD	PDD	6	2
PD404	M	76	86	10	AR	DLB	6	1

PD413	F	51	72	21	TD	PD	6	2
PD415	F	68	79	11	TD	PDD	6	2
PD416	F	73	85	12	TD	PD	6	2
PD419	M	75	84	9	mixed	PD	6	1
PD423	F	53	66	13	TD	PD	6	2
PD428	F	73	76	3	TD	PD	6	1
PD433	F	55	74	19	mixed	PD	6	2
PD451	M	35	64	29	TD	PD	6	0
PD465	F	78	86	8	AR	PD-MCI	6	1
PD479	M	75	80	5	TD	PD-MCI	6	3
PD501	F	82	89	7	TD	PDD	6	2
PD503	M	53	75	22	TD	PD-MCI	6	2
PD506	M	59	67	8	AR	PD	6	0
PD511	M	70	83	13	mixed	PD-MCI	5	0
PD513	M	68	74	6	TD	PDD	6	0
PD517	M	54	69	15	mixed	PD-MCI	6	0
PD523	F	68	86	18	mixed	PD	6	2
PD524	F	75	90	15	mixed	PD-MCI	6	4
PD530	M	77	85	8	TD	PDD	6	0
PD531	M	73	80	7	TD	PDD	6	0
PD538	M	67	73	6	TD	PDD	5	0
PD539	M	68	81	13	mixed	PDD	5	2
PD540	F	55	70	15	mixed	PDD	6	2
PD541	M	66	72	6	TD	PD-MCI	5	2
PD542	F	76	81	5	mixed	PD	5	1
PD544	M	73	81	8	mixed	PDD	6	3
PD545	M	51	67	16	mixed	PDD	5	2
PD546	F	71	84	13	mixed	PD	5	3
PD548	F	64	84	20	TD	PDD	6	2
PD550	F	77	84	7	mixed	PD-MCI	6	1
PD561	M	54	76	22	TD	PDD	6	2
PD563	M	63	75	12	AR	PDD	6	2
PD564	M	65	83	18	TD	PDD	6	2
PD565	F	61	80	19	TD	PD	6	1
PD566	M	56	63	7	AR	DLB	6	2
PD568	F	66	80	14	TD	PD-MCI	6	1
PD603	M	62	84	22	TD	PDD	6	2
PD616	M	78	89	11	mixed	PD	6	2
PD618	F	58	77	19	TD	PD-MCI	6	2
PD619	F	82	87	5	mixed	PD-MCI	6	2
PD624	F	65	89	24	TD	PDD	6	2
PD633	F	71	81	10	mixed	PD-MCI	6	2
PD639	F	48	79	31	AR	PDD	6	3
PD640	F	52	67	15	AR	PD-MCI	6	1
PD644	M	61	77	16	mixed	PD-MCI	6	2
PD645	M	68	75	7	AR	PDD	6	2

PD647	F	72	79	7	AR	PD	5	1
PD655	M	67	79	12	AR	PD	6	2
PD656	M	68	77	9	AR	PDD	5	1
PD659	M	49	68	19	mixed	PDD	6	2
PD660	M	71	80	9	AR	PDD	6	2
PD661	M	77	85	8	AR	PDD	6	2
PD662	M	73	78	5	mixed	PDD	6	3
PD666	F	78	93	15	mixed	PD	6	2
PD674	F	75	83	8	AR	PD	6	2
PD675	F	70	81	11	AR	PD	4	1
PD677	M	74	78	4	mixed	PDD	6	2
PD678	M	81	83	2	AR	PDD	6	2
PD679	M	71	79	8	AR	PDD	5	2
PD680	M	72	80	8	mixed	PD-MCI	5	1
PD683	F	68	83	15	AR	PD	4	2
PD690	F	60	81	21	AR	PD-MCI	6	2
PD691	M	70	73	3	mixed	PDD	6	2
PD692	M	62	75	13	mixed	PDD	5	2
PD695	M	70	77	7	mixed	PD	4	2
PD696	F	78	87	9	AR	PDD	6	2
PD699	F	74	81	7	AR	PDD	6	4
PD701	M	61	74	13	TD	PDD	5	2
PD703	M	82	90	8	TD	PD	3	1
PD704	M	57	66	9	AR	PDD	6	3
PD705	M	55	68	13	mixed	PD	4	1
PD708	M	69	83	14	TD	PD	3	1
PD711	F	76	85	9	mixed	PDD	5	2
PD716	F	61	69	8	AR	PD	3	0
PD717	F	60	68	8	mixed	PD	3	2
PD718	M	73	87	14	TD	PD-MCI	5	4
PD722	M	61	70	9	AR	PDD	6	1
PD732	M	69	81	12	AR	PD	5	2
PD735	F	88	92	4	mixed	PD	6	4
PD741	M	69	79	10	AR	PD-MCI	4	3
PD746	F	75	88	13	mixed	PD	4	2
PD747	M	74	83	9	TD	PD	6	2
PD759	F	66	83	17	mixed	PDD	6	3
PD763	F	67	83	16	mixed	PDD	6	3

Appendix 5 – Control cohort used in this thesis

Case	Gender	Age of Death	Cause of death	Braak tau
PDC003	M	84	Pancreatic carcinoma & Gastrointestinal haemorrhage	2
PDC006	F	82	Unknown	1
PDC013	M	77	Unknown	2
PDC022	M	75	Malignant squamous cell carcinoma of the lung	1
PDC024	F	68	Unknown	2
PDC026	F	80	Breast carcinoma with spinal metastasis; carcinosarcoma uterus	2
PDC034	M	90	Respiratory failure secondary to bronchial carcinoma	1
PDC041	M	66	Carcinoma of lung	1
PDC044	F	97	Congestive heart disease, inoperable aortic stenosis	2
PDC053	F	89	Urosepsis; Stroke	2
PDC056	F	70	Sepsis, stage IV peritoneal ca, ischemic heart disease	0
PDC058	F	83	Unknown	2
PDC081	F	92	Heart failure, ischemic heart disease	2

Appendix 6 – LBD cohort for Chapter 7 of this thesis

Abbreviations: αSN = α-synuclein, DLB = dementia with Lewy Bodies, FC = fluctuating cognition, N = no, PD = Parkinson's disease without cognitive impairment, PDD = PD dementia, PDD-DLB = PDD with DLB-like syndrome, RBD = REM-sleep behaviour disorder, VH = visual hallucinations, Y = yes.

Case	Gender	Age of death	Disease duration	Subgroup	FC	VH	RBD	Meets DLB criteria	Braak aSN	Braak tau
PD060	M	84	6	DLB	Y	Y	Y	Y	6	1
PD089	M	70	1	DLB	N	Y	Y	Y	6	1
PD098	M	76	12	DLB	N	Y	Y	Y	5	2
PD120	F	83	5	DLB	N	Y	Y	Y	6	4
PD134	M	74	10	DLB	Y	Y	Y	Y	5	2
PD152	M	77	8	DLB	Y	Y	Y	Y	6	1
PD163	M	76	6	DLB	Y	N	Y	Y	6	2
PD189	M	70	8	DLB	Y	Y	Y	Y	6	2
PD195	M	80	4	DLB	Y	Y	Y	Y	6	2
PD255	M	79	26	PDD-DLB	Y	Y	Y	Y	6	2
PD264	F	84	20	PDD-DLB	Y	Y	Y	Y	6	3
PD267	F	78	6	PD	Y	N	Y	N	6	3
PD269	M	76	8	PDD-DLB	Y	Y	Y	Y	5	2
PD272	M	84	16	PD	N	N	Y	N	6	2
PD273	M	73	5	PDD	N	Y	N	N	6	2
PD284	F	75	20	PD	N	Y	N	N	6	2
PD286	M	82	18	PDD	N	Y	N	N	6	1
PD287	M	88	11	PDD-DLB	Y	Y	Y	Y	6	2
PD294	M	78	6	DLB	Y	Y	N	Y	6	2
PD296	M	71	9	DLB	Y	Y	N	Y	6	2
PD301	F	83	19	PDD	N	N	Y	N	5	2
PD304	F	87	11	PDD-DLB	Y	Y	N	Y	6	1
PD307	F	83	13	PDD-DLB	Y	Y	Y	Y	6	3
PD310	M	62	6	PD	N	N	Y	N	5	0
PD323	M	77	9	PDD	N	N	Y	N	6	1
PD324	M	94	7	PDD	N	Y	N	N	6	2
PD332	M	77	3	DLB	Y	N	Y	Y	6	2
PD336	M	81	8	PDD	N	Y	N	N	6	2
PD337	M	75	16	PDD-DLB	Y	Y	Y	Y	6	2
PD341	M	68	14	PDD	N	Y	N	N	6	2
PD343	M	80	1	DLB	N	Y	Y	Y	6	1
PD347	F	84	16	PD	N	N	Y	N	5	2
PD348	M	78	6	PD	N	N	N	N	6	2
PD356	F	86	9	PD	N	N	N	N	5	2
PD365	M	82	5	DLB	Y	Y	Y	Y	6	4
PD366	M	69	20	PDD	N	Y	Y	N	6	1
PD370	M	66	4	PD	N	N	N	N	6	0
PD371	M	77	14	PDD	N	N	N	N	6	1
PD372	F	71	18	PDD-DLB	Y	Y	Y	Y	6	3
PD374	M	74	23	PDD	Y	N	N	N	6	0
PD385	M	80	5	DLB	Y	Y	Y	Y	6	1
PD391	M	79	11	PDD	N	Y	Y	N	6	0
PD398	M	74	24	PD	N	Y	Y	N	5	0
PD399	F	77	11	PDD-DLB	Y	Y	Y	Y	6	2
PD404	M	87	10	DLB	Y	Y	Y	Y	6	1

PD413	F	72	21	PD	N	N	N	N	6	2
PD415	F	79	11	PDD	N	N	N	N	6	2
PD416	F	85	12	PD	N	N	Y	N	6	2
PD419	M	84	9	PD	N	N	Y	N	6	1
PD422	M	75	5	DLB	Y	Y	Y	Y	6	0
PD423	F	66	13	PD	N	N	N	N	6	2
PD424	M	68	3	DLB	Y	Y	N	Y	6	2
PD428	F	76	3	PD	N	N	Y	N	6	1
PD433	F	74	19	PD	N	Y	Y	N	6	2
PD451	M	64	29	PD	N	Y	N	N	6	0
PD453	M	69	7	DLB	Y	N	N	Y	6	2
PD501	F	89	7	PDD	N	N	Y	N	6	2
PD506	M	67	8	PD	N	Y	N	N	6	0
PD511	M	83	13	PDD-DLB	Y	Y	Y	Y	5	0
PD513	M	74	6	PDD	N	Y	N	N	6	0
PD523	F	86	18	PD	N	N	Y	N	6	2
PD530	M	85	8	PDD-DLB	Y	Y	Y	Y	6	0
Pd531	M	80	7	PDD-DLB	Y	Y	Y	Y	6	0
PD538	M	73	6	PDD	N	Y	N	N	5	0
PD539	M	81	13	PDD-DLB	Y	Y	Y	Y	5	2
PD540	F	70	15	PDD-DLB	Y	Y	Y	Y	6	2
PD542	F	81	5	PD	N	N	Y	N	5	1
PD545	M	67	16	PDD-DLB	Y	Y	Y	Y	5	2
PD546	F	84	13	PD	N	N	N	N	5	3
PD548	F	84	20	PDD-DLB	Y	Y	Y	Y	6	2
PD561	M	76	22	PDD	N	N	Y	N	6	2
PD563	M	75	12	PDD-DLB	Y	Y	Y	Y	6	2
PD564	M	83	18	PDD-DLB	Y	Y	Y	Y	6	2
PD565	F	80	19	PD	N	Y	N	N	6	1
PD566	M	63	7	DLB	Y	Y	Y	Y	6	2
PD603	M	84	22	PDD-DLB	Y	Y	Y	Y	6	2
PD616	M	89	11	PD	N	Y	N	N	6	2
PD624	F	89	24	PDD	Y	N	N	N	6	2
PD639	F	79	31	PDD-DLB	Y	Y	N	Y	6	3
PD645	M	75	7	PDD	N	Y	N	N	6	2
PD647	F	79	7	PD	N	Y	Y	N	5	1
PD655	M	79	12	PD	N	N	Y	N	6	2
PD656	M	77	9	PDD	Y	N	Y	N	5	1
PD659	M	68	19	PDD-DLB	Y	Y	N	Y	6	2
PD660	M	80	9	PDD	N	Y	N	N	6	2
PD661	M	85	8	PDD	Y	N	Y	N	6	2
PD662	M	78	5	PDD	N	N	N	N	6	3
PD666	F	93	15	PD	N	N	Y	N	6	2
PD669	M	65	10	DLB	N	Y	Y	Y	6	2
PD674	F	83	8	PD	N	N	N	N	6	2
PD675	F	81	11	PD	N	Y	Y	N	4	1
PD677	M	78	4	PDD	N	N	N	N	6	2
PD678	M	83	2	PDD	N	Y	N	N	6	2
PD679	M	79	8	PDD	N	Y	N	N	5	2
PD683	F	83	15	PD	N	N	N	N	4	2
PD691	M	73	6	PDD	N	Y	N	N	6	2
PD692	M	75	13	PDD	Y	N	Y	N	5	2
PD695	M	77	7	PD	N	N	Y	N	4	2
PD699	F	81	7	PDD	Y	N	N	N	6	4
PD701	M	74	13	PDD-DLB	Y	Y	Y	Y	5	2
PD703	M	90	8	PD	N	N	N	N	3	1
PD704	M	66	9	PDD	N	N	Y	N	6	3

PD705	M	68	13	PD	N	Y	Y	N	4	1
PD706	M	67	11	DLB	N	Y	Y	Y	5	2
PD708	M	83	14	PD	N	Y	Y	N	3	1
PD711	F	85	9	PDD-DLB	Y	Y	N	Y	5	2
PD716	F	69	8	PD	N	N	Y	N	3	0
PD717	F	68	8	PD	N	N	N	N	3	2
PD718	M	87	14	PDD	Y	N	N	N	5	4
PD732	M	81	12	PD	N	N	N	N	5	2
PD735	F	92	4	PD	N	N	N	N	6	3
PD746	F	88	13	PD	N	N	N	N	4	2
PD747	M	83	9	PD	N	N	Y	N	6	2
PD759	F	83	17	PDD	N	N	N	N	6	3
PD763	F	83	16	PDD	N	Y	N	N	6	3

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