A Clinicopathological Study

of the Neuropsychiatric Features of Parkinson's Disease

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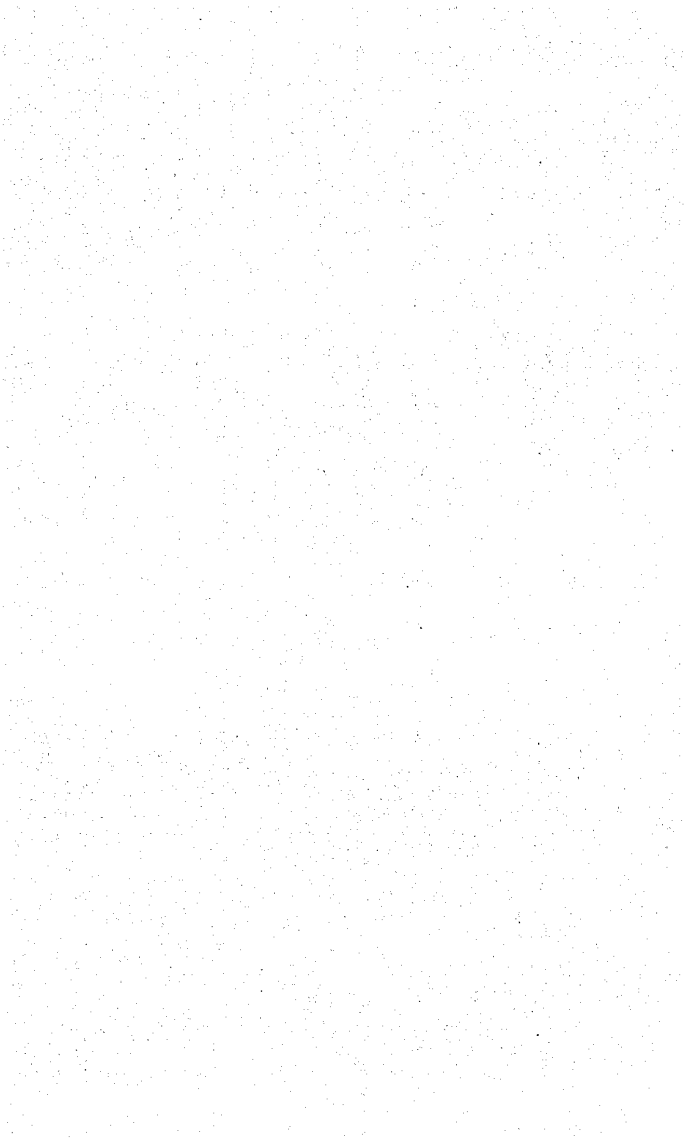
Abstract

Parkinson's disease (PD) has been traditionally viewed as a disease of the substantia nigra with motor deficits as the main clinical symptom. However, non-motor symptoms are also commonly present, with neuropsychiatric disturbances being among the most prominent. Despite extensive research, the pathological basis for neuropsychiatric complications in PD is still unclear. The present thesis addresses the possible anatomical and pathological substrate(s) of neuropsychiatric complications using clinically well characterised post-mortem PD human tissue from the UKPDSTB.

In the first part of the project the extent of α-synuclein (αSN) deposition and Alzheimer's disease (AD)-related pathology was assessed in eight regions of the brain that have been shown to play a role in cognitive function. The aim of the study was to localise the specific pathological and anatomical correlates of dementia and visual hallucinations in PD. The results reveal a strong association between αSN pathology in limbic regions and dementia and also possibly shed some new light on the anatomical basis of visual hallucinations. In the second part of the project I investigated the extent of striatal pathology in PD brains and examined its clinical relevance. There were a number of PD cases with extensive striatal β-amyloid pathology and the presence of this pathology strongly associated with the presence of dementia. The third section of the thesis deals with possible pathological correlates of anxiety, a common psychiatric problem among PD sufferers. In non-demented PD subjects with anxiety, αSN pathology was particularly prevalent in the amygdala and hippocampus. This observation of an apparent limbic origin of anxiety in PD may also have a broader significance in terms of other neuropsychiatric disorders.

Quite apart from the pathological basis of the neuropsychiatric features of PD there is still debate over the origin and progression of αSN pathology in the brain. In the fourth and final part of the thesis I examined the distribution pattern of αSN in the brain and spinal cord of 101 unselected consecutive cases accrued by the UKPDSTB. More than 50% of the confirmed PD cases fitted the staging scheme proposed by Braak and colleagues but in a significant minority of the cases the distribution of pathology did not support a caudal

(medullary) origin. In fact the most consistently affected areas were the substantia nigra and the nucleus basalis of Meynert.



Declaration

I declare that the work presented in this thesis is solely that of the author unless otherwise stated in the text. None of this work has been submitted for any other qualification at this or other university.

Michail E. Kalaitzakis

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Abbreviations

αSN:	Alpha-synuclein
Aβ:	Amyloid beta peptide
Ab:	Antibodies
ANOVA:	Analysis of variance
ADL:	Activities of daily living
AD:	Alzheimer's Disease
AH:	Auditory hallucinations
ALS:	Amyotrophic Lateral Sclerosis
APP:	Amyloid precursor protein
b-C :	b-carboline
βSN:	β-synuclein
BCSG1:	Breast cancer specific gene
CAA:	Cerebral amyloid angiopathy
γSN:	γ-synuclein
CVLs:	Cerebrovascular lesions
CERAD:	The consortium to establish a registry for Alzheimer's Disease
CN:	Caudate nucleus
DLB:	Dementia with Lewy bodies
DAB:	3,3'-diaminobenzidine
DSM:	Diagnostic and Statistical Manual of Mental Disorders
DMV:	Dorsal motor nucleus of the vagus nerve
DRN:	Dorsal raphe nucleus
EDTA:	Ethylenediaminetetraacetic acid
ET:	Essential tremor
FA:	Formic acid
GSK:	GlaxoSmithKline
GABA:	γ-Aminobutyric acid
GCIs:	Glial cytoplasmic inclusions
GFAP:	Glial fibrillary acidic protein
Ht:	High temperature

HCHWA-D: Hereditary cerebral hemorrhage with amyloidosis Dutch type

H&E: Haematoxylin and eosin

HD: Huntington's disease

IHC: Immunohistochemistry

IMS: Industrial methylated spirits

ILBD: Incidental Lewy Body Disease

LB: Lewy bodies

LN: Lewy neurites

L-DOPA: Levodopa

LC: Locus coeruleus

mAb: Monoclonal antibodies

mRNA: Messenger RNA

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MSA: Multiple System Atrophy

NFT: Neurofibrillary tangles

NTs: Neuropil threads

NBM: Nucleus basalis of Meynert

TH: Tyrosine hydroxylase

NPV: Negative predictive value

NAcc: Nucleus accumbens

NFs: Neurofilaments

NAC: Non-amyloid component

NBIA I: Neurodegeneration with brain iron accumulation type 1

pAb: Polyclonal antibodies

PD: Parkinson's Disease

PDD: Parkinson's Disease with dementia

PBS: Phosphate buffered saline

PPV: Positive predictive value

PDD(H-): PD cases with high clinical scores for dementia and no VH

PD(H+): PD cases with high clinical scores for VH and no dementia

PDD(H+): PD cases with high clinical scores for both dementia and VH

PD(H-): Cognitively intact PD cases

PET: Positron emission tomography

Put: Putamen

PD(A+): PD cases with high clinical scores for anxiety

PD(A-): Cognitively intact PD cases

PPN: Pedunculopontine nucleus

PHFs: Paired helical filaments

PPNr: Pedunculopontine nucleus pars compacta

PSP: Progressive Supranuclear Palsy

SPECT: Single-Photon Emission Computerised Tomography

SNc: Substantia nigra pas compacta

TIQ: Tetrahydroisoquinoline

Ub: Ubiquitin

UPP: Ubiquitin-proteasome pathway

UCH-L1: Ubiquitin C-terminal hydrolase L1

UKPDSTB: UK Parkinson's Disease Society Tissue Bank at Imperial College

VH: Visual hallucinations

VTA: Ventral tegmental area

Chapter 1

Introduction

1.1 Parkinson's Disease

1.1.1 History

James Parkinson, a British physician, published his medical classic "An Essay on the Shaking Palsy", in 1817 at the age of 62 on a subject that he called "paralysis agitans". His work is considered a medical classic because of the eloquent and detailed description of the main clinical features of the disease that is now known as Parkinson's Disease (PD). Parkinson based his description solely on six patients of which three were "casually met on the street" or "only seen at a distance". He defined the disease as follows: "Involuntary tremulous motions, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forward, and to pass from a walking to a running pace: the senses and intellects being uninjured". There are several indications that before 1861 the disease had received little attention. It was in 1861 and 1862 that Jean-Martin Charcot with Alfred Vulpian recognised this disease as distinct from other neurologic entities with which it had been previously confused (i.e. multiple sclerosis) and attached the name of James Parkinson to this syndrome. Charcot's descriptions of PD were amplified throughout his career. It was Charcot who identified rigidity as an important sign in PD and differentiated it from spasticity. Following Charcot's seminal contributions to the disease, Bechet in 1891 made the remark that all symptoms of PD were secondary to rigidity, tremor being an exception. Although Charcot and Parkinson pointed out that patients move slowly and with difficulty, they did not see this as a symptom on its own right. The slowness of movement was firstly taken into account by Claveleira in 1872 and became a new element of the definition while Jaccoud in 1873 referred to this symptom as akinesia. In 1886, the neurologist Sir William Richard Gowers, introduced the word bradykinesia and emphasised that this symptom should be mentioned in any definition of PD. Over the years the constellation of symptoms and signs of this clinical entity have come to describe a discrete clinically

recognisable malady that even 190 years after its initial description by James Parkinson has an aetiopathogenesis that is largely unknown. Today PD is viewed as a distinctive progressive disorder characterised clinically by tremor, rigidity, and bradykinesia as well as a variety of non-motor complications including neuropsychiatric and autonomic deficits; and pathologically by nerve cell loss in the substantia nigra and the presence of Lewy intraneuronal inclusion bodies in nigral and extranigral brain regions (Hughes *et al.* 1992). A precise definition of three levels of diagnostic confidence for the diagnosis of PD has been proposed (Hughes *et al.* 1992;Gelb *et al.* 1999). Patient diagnoses are classified as Probable when the patients have clinical features that are absolutely typical of PD, at least 3 years of parkinsonian symptoms without the development of atypical features, and a clear clinical response to dopaminergic treatment. The criteria for Possible PD are less strict: patients are not required to have as many typical features of PD, symptom duration can be less than 3 years, and patients need not have received an adequate trial of dopaminergic therapy. Patients in either category who have autopsy findings consistent with PD are designated as Definite.

1.1.2 Epidemiological Aspects of PD

PD is the second most common neurodegenerative disease after Alzheimer's Disease (AD). According to the United Nations, at least four million people have the disease. It is estimated that the prevalence and incidence rates of PD in Europe is approximately 108 to 257/ 100,000 and 11 to 19/ 100,000 per year, respectively. In the older age groups (i.e. > 60 years) the rates of prevalence and incidence are much higher: 1280 to 1500/ 100,000 and 346/ 100,000, respectively (von Campenhausen *et al.* 2005).

PD is a progressive neurodegenerative disorder which leads to physical disabilities that predispose to deep vein thrombosis, pulmonary embolism, pneumonia, aspiration, falls with resulting complications and a premature death (Mosewich *et al.* 1994). Life expectancy in Western countries has increased over the last several decades due to better treatment strategies (Rajput *et al.* 1990), improved socioeconomic conditions and better health care. PD patients of today share those longevity gains and hence the life expectancy of contemporary patients is longer than that of patients diagnosed 30 or 50 years ago and is not significantly lower than average life expectancy.

1.1.3 PD of Known Cause

The majority (90%) of patients with PD are described as idiopathic/sporadic (i.e. having no specific identified cause). However, although idiopathic PD is the most common cause of parkinsonism, a wide range of other aetiologies can lead to parkinsonian-like symptomatology (see also section 1.2.1.1). There are some PD cases where the symptoms may result from genetic mutations, toxicity, head trauma or other factors.

1.1.3.1 Genetic

There have been two approaches to genetic studies that have yielded very important information with regards to the pathogenesis of PD. The first genetic approach is that of investigating families with parkinsonism following Mendelian inheritance. These studies have identified genes that underlie monogenic PD in particular α-synuclein (αSN; Park 1, and Park 4; triplication of αSN) (Polymeropoulos *et al.* 1997;Singleton *et al.* 2003), LRRK2 (Park 8) (Zimprich *et al.* 2004;Paisan-Ruiz *et al.* 2004) and IGYF2 (Park 11) (Pankratz *et al.* 2003) in dominant families as well as the recessive PD genes parkin (Park 2) (Kitada *et al.* 1998), PINK 1 (Park 6) (Valente *et al.* 2004), DJ-1 (Park 7) (Bonifati *et al.* 2003) and UCH-L1 (Park 5) (Leroy *et al.* 1998) (Table 1.1). There are also three gene loci (Park 3, Park 9 and Park 10) with unknown gene products that have also been identified to confer forms of genetic PD (Gasser *et al.* 1998;Ramirez *et al.* 2006;Hicks *et al.* 2002). These mutations cause PD in a very small subset of families. The clinical and neuropathological features of PD cases with different mutations vary, but individuals with genetic PD consistently respond well to L-DOPA (Mizuno *et al.* 2006).

Table 1.1 Genetics of PD

(Following page) *Abbreviations:* AD: autosomal dominant, AR: autosomal recessive, 3xα-synuclein: α-synuclein triplication, UCH-L1: ubiquitin C-terminal hydrolase L1, PINK 1: PTEN induced kinase 1, DJ 1: oncogene DJ1, LRRK2: leukocine-rich repeat kinase 2,

Locus name	Chromosomal localisation	Gene product	Mode of inheritance	Lewy body pathology	Special clinical features
Park 1	4q21	α-synuclein	AD	Yes	Dementia
Park 2	6q25.2-27	Parkin	AR	No	Early onset, foot dystonia
Park 3	2p13	Unknown	AD	Yes	Dementia
Park 4	4q21	3xα-synuclein	AD	Yes	Dementia, postural tremor
Park 5	4q21	UCH-L1	AD	Unknown	Not described
Park 6	1p35-36	PINK 1	AR	Unknown	Early onset, tremor dominant
Park 7	1p36	DJ 1	AR	Unknown	Early onset, dystonia, psychic disturbances
Park 8	12p11.2-q13.1	LRRK2	AD	Unknown	Unknown
Park 9	1p32	Unknown	AR	Unknown	Late onset
Park 10	1p32	Unknown	Unknown	Unknown	Not described
Park 11	2q36-37	IGYF2	AD	Unknown	Not described

Despite the fruitful insights provided by these previous genetic studies, the contribution of the mechanisms of these and other genes to idiopathic PD as a whole is still poorly defined. In fact, the second genetic approach aims at defining this contribution using association studies or non-paramentric linkage methodology not only in sibling pair families but also in large populations of patients with sporadic PD (DeStefano *et al.* 2002;Pankratz *et al.* 2002;Martinez *et al.* 2004;Paisan-Ruiz *et al.* 2004;Scott *et al.* 2001;Scott *et al.* 2001;Maraganore *et al.* 2005). Although these studies have provided interesting findings, none of them has lead to the discovery of bona fide disease genes.

1.1.3.2 Toxins

There are several studies to suggest that toxins can cause PD or parkinsonism. The toxins most strongly suspected at present are pesticides. Evidence of their role as neurotoxins is provided by clinical and experimental work. Most, though not all, epidemiological studies have found that pesticide exposure is associated with a high risk of PD (Seidler *et al.* 1996;Liou *et al.* 1997;Gorell *et al.* 1998;Petrovitch *et al.* 2002). The results are consistent with a dose dependent effect in agricultural workers, risk is increased with duration of pesticide use (Liou *et al.* 1997;Petrovitch *et al.* 2002). Information about exposure to specific pesticides also suggests that bipyridyl (paraquat), organochlorine and carbamate derivatives could have a permissive or causal role in PD (Liou *et al.* 1997;Seidler *et al.* 1996). For example, in a study in Taiwan, where the herbicide paraquat is commonly sprayed on rice fields, a strong association was reported between paraquat exposure and PD risk: the risk was increased by more than six times in individuals who had been exposed to paraquat for more than 20 years (Liou *et al.* 1997).

Studies of the relation between toxins and the development of PD have also focused on metals. In idiopathic PD, experimental work with metals has mostly focused on the potential role of iron and other transition elements. Iron is unevenly distributed throughout the brain, but it reaches particularly high concentrations in the basal ganglia (Zecca *et al.* 1994). Increased iron in the brains of patients with PD was first reported by Earle (Earle 1968) and subsequent studies have confirmed this accumulation in the pars compacta of the substantia nigra (Sofic *et al.* 1988;Dexter *et al.* 1989). Dexter et al., (Dexter *et al.* 1990) have also measured decreased levels of ferritin in the nigral tissue of patients with PD (ferritin is the primary intracellular protein capable of keeping iron

bound in a non-reactive status). Thus, iron accumulation together with decreased binding capability may enhance the risk for iron-mediated toxic reactions in PD. In particular, the Fenton reaction could generate the highly toxic hydroxyl radical in the presence of iron and hydrogen peroxide, thus leading to oxidative stress and possibly neurodegeneration. In a population-based case-control study, the potential role of metals as risk factors for PD was evaluated in the occupational setting in which the effects of metals would be expected to be magnified by greater and prolonged exposures (Gorell *et al.* 1997). Increased risk for PD was found in workers exposed to metals for more than 20 years, suggesting a long-latency and/or slowly progressive mechanism of accumulation and toxicity. Also, enhanced risk was shown as a result of combined metal exposures, i.e. lead–copper, lead–iron and iron–copper, raising the possibility that metals may act synergistically in contributing to nigrostriatal degeneration. Based on these data, the effects of prolonged exposures and interactions should become a focus of future studies investigating the relationship between metals and PD.

In the early 1980s, parkinsonism in young drug addicts was linked to their exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Langston *et al.* 1983). This unfortunate accident provided a new insight into the link between environmental agents and PD. If MPTP is capable of inducing neurochemical, pathological and clinical features that resemble those of idiopathic PD (Di Monte *et al.* 2002), comparable effects might be caused by other neurotoxins similar to MPTP but naturally occurring in the environment (MPTP analogs). Several classes of heterocyclic molecules structurally related to MPTP have been proposed as candidate endogenous and exogenous parkinsongenic agents. Among these classes of MPTP analogs, tetrahydroisoquinoline (TIQ) and b-carboline (b-C) derivatives have been the most studied. TIQs and b-Cs are naturally occurring alkaloids present, for example, in a variety of foods (Makino *et al.* 1988). They can also be generated, however, within the brain by reactions involving biogenic amines, such as condensation of the amine group of dopamine with the reactive carbonyl groups of aldehydes and a-ketoacids (Collins and Origitano 1983). Interestingly, both TIQs and b-Cs have been detected in the human brain (Niwa *et al.* 1987;Matsubara *et al.* 1993) as well as in the cerebrospinal fluid of patients with PD (Kotake *et al.* 1995;Matsubara *et al.* 1995). They have also been reported to

cause nigrostriatal damage in experimental animals including nonhuman primates (Nagatsu and Yoshida 1988;Neafsey *et al.* 1995).

1.1.4 Clues to the Cause of PD from Epidemiological Studies

1.1.4.1 Increasing Age

Of all factors studied so far, age is the most highly linked to PD. The incidence of PD increases steeply from less than 10 new cases per 100,000 at age 50 to at least 200 new cases per 100,000 at age 80 (Bower *et al.* 2000;Van Den Eeden *et al.* 2003). Although, some studies show a decrease in the very elderly, this most likely reflects poor ascertainment rather than an actual decrease in disease prevalence.

1.1.4.2 Gender

PD is approximately 1.2 to 1.5 times more common in men than in women (Mayeux *et al.* 1992;Van Den Eeden *et al.* 2003;Harada *et al.* 1983). The increased incidence in men over women may result from biological phenomena, for instance, influence of sex hormones or genetic factors (i.e. genes on chromosome X vs. Y) or due to a male associated exposure such as occupational toxicant exposure. In addition, epidemiological studies of oestrogen in women show that women with PD are more likely to have undergone hysterectomy and had earlier menopause than control subjects (Benedetti *et al.* 2001).

1.1.4.3 Race

Several studies have suggested an increased incidence of PD in Caucasian rather than African-American or Asian-American populations, however, this question remains unsettled (Van Den Eeden *et al.* 2003;Schoenberg *et al.* 1985). Across European countries studies have revealed no substantial difference in the prevalence of PD (de Rijk *et al.* 2000;de Rijk *et al.* 1997;Rocca 2000). Nevertheless, more studies are needed to determine whether people of different ethnicity have differing risks of PD.

1.1.4.4 Head Trauma

Several authors have reported a significant association between head trauma and the later development of PD (Bower *et al.* 2003;Goldman *et al.* 2006;Factor and Weiner 1991;Taylor *et al.* 1999;Semchuk *et al.* 1993). A recent study by Bower and colleagues found that those who have experienced a head injury are four times more likely to develop PD than those who have never suffered a head injury (Bower *et al.* 2003). In addition, this study reported that the risk of developing PD increases eightfold for patients who had a head trauma and required hospitalisation and it increases to elevenfold for patients who have experienced severe head trauma. Although the results of this study may be biased by recall Goldman and co-workers found a similar risk of 3.8, with increasing risk associated with more severe injury and hospitalisation (Goldman *et al.* 2006). Interestingly, severe head trauma in mice is associated with age-dependent enhanced immunoreactivity to synucleins, which could be the pathogenetic basis of PD after trauma in humans (Uryu *et al.* 2003). Dementia pugilistica, also known as punch-drunk syndrome, offers another link between head trauma and the later development of PD symptoms. This neurological condition occurs in approximately 20% of professional boxers with risk factors including increased exposure (i.e. duration of career, total number of bouts, age of retirement), poor performance and increased sparring (Jordan 2000). Clinically, boxers present with dementia and parkinsonism (Jordan 2000). Pathologically, dementia pugilistica shares many characteristics with AD (i.e. neurofibrillary changes, diffuse amyloid plaques) (Jordan 2000;Toth *et al.* 2005;Schmidt *et al.* 2001).

1.1.4.5 Tobacco, Coffee and Alcohol

Smoking of cigarettes is among the most studied risk factors for PD and one of the few for which very consistent results have been obtained. Many epidemiological studies have shown a reduced risk of PD among cigarette smokers. The vast majority of these were case-control studies, but some large prospective cohort studies confirmed their results (Grandinetti *et al.* 1994;Paganini-Hill 2001;Hernan *et al.* 2001).

Several studies assessed coffee consumption in relation to PD risk, with fairly consistent results. Again, findings from case-control studies have been confirmed in several large follow-up studies (Ascherio *et al.* 2001). A meta-analysis based on eight

case-control studies and five cohort studies showed a significantly decreased PD risk for coffee drinkers that was not attenuated when analyses were adjusted for smoking (Hernan *et al.* 2001).

The findings on smoking and coffee consumption and the hypothesised role of dopaminergic reward systems have led some researchers to examine the association between alcohol consumption and the risk of PD. However, results of a number of case-control studies and some prospective cohort studies (Hernan *et al.* 2003) have not been very straightforward, with inverse associations in some studies (Fall *et al.* 1999) but no significant association in others (Benedetti *et al.* 2000;Checkoway *et al.* 2002).

1.2 Clinical Picture

1.2.1 Motor Deficits - Cardinal Features of PD

Tremor at rest, rigidity, bradykinesia and abnormalities of posture, gait and balance are the cardinal clinical manifestations of PD.

Tremor is the most conspicuous of the cardinal features of PD and gave rise to the initial name of PD, "shaking palsy". Approximately 70% of patients notice tremor as the first symptom (Calne and Stoessl 1986). Tremors of all behavioural type, and therefore of differing pathophysiological basis, are recognised in PD (Findley 1996;Findley *et al.* 1981) however, PD tremor is classically described to be present at rest rather than a postural or action tremor. Characteristically, tremor is 3-5 Hz rhythmic "pill rolling" movements of the thumb and forefinger and has varying amplitude (Deuschl *et al.* 1998;Hallett 1998). At a later stage of the disease tremor may spread to the proximal joints of the limb. Onset of tremor is usually in one of the hands (Findley and Gresty 1981) and it progresses to involve the other upper limb or ipsilateral lower limb. The jaw, tongue, head or trunk may be affected by tremor although, head tremor or trunkal tremor are not usually associated with early PD. Early in the disease, tremor is usually intermittent for many years and is evident only under stress. Tremor in PD is worsened by nervousness, fatigue and stress and diminished with voluntary movement or sleep. Studies have revealed that PD patients may also have other types of tremor. In particular, in early phases of their disease, many patients report a feeling of tremulousness without having an obvious resting tremor (Lees 1992). PD tremor is produced by alternate activation of agonist and antagonist muscles. In this respect, PD tremor is distinct from essential tremor, which is produced by oscillating co-contractions of agonist and antagonist muscles (Boose *et al.* 1996).

Rigidity (defined as an increased resistance of a joint to passive movement) frequently associated with cogwheeling (palpable tremor), is a plastic, lead-pipe form of hypertonia that affects many muscles of the limbs, neck and trunk and is responsible for the typical stooped posture of the PD patients especially as the disease progresses (Calne *et al.* 1992). In contrast to spasticity, rigidity is constant throughout the range of movement. Rigidity is often asymmetrical in the early stages of the disease. Like tremor,

it may vary during the course of the day and with stress. Although rigidity is considered as one of the cardinal features of PD, it only minimally contributes to the impairment experienced by the patients. The subjective correlate of rigidity is a feeling of stiffness and a reduced ability to relax limb muscles.

Akinesia (literally meaning the absence of movement) is another cardinal symptom of PD however, it is an imprecise term to describe the slowness and poverty of movements characteristic of PD. From a scientific point of view, bradykinesia and hypokinesia is a more appropriate term, since a minority of PD patients experience situations in which movements are completely impossible/absent (Klockgether 2004;Fahn 2003). Bradykinesia/hypokinesia affects all voluntary and involuntary movements and is one of the most disabling components of all the clinical signs of PD especially as the disease progresses (Hallett and Khoshbin 1980). Bradykinesia/hypokinesia in the upper extremities often manifests as micrographia. However, in early stages of PD may manifest as minimal slowness. Bradykinesia/hypokinesia and rigidity usually occur together and in most cases are comparable in severity (Rao *et al.* 2006;Fahn 2003).

Abnormalities of posture, gait and balance usually occur about five years after the onset of PD (Marttila and Rinne 1977), although may sometimes occur earlier (Zetusky *et al.* 1985;Gibb and Lees 1988). The posture may show slight flexion of the neck or trunk or a lean to one side. Abnormalities of gait may include the following: 1) loss of arm swing on one or both sides, 2) slowing, 3) tripping over objects, 4) inability to turn quickly, and 5) difficulty in walking on toes or heels. As the disease progresses gait becomes a problem. Short term arrests of ongoing movements (known as freezing) also occur in advanced PD (Giladi *et al.* 1992).

1.2.1.1 Differential Diagnosis

It is very important for the diagnosis of PD to be differentiated from other recognisable parkinsonian disorders or conditions with parkinsonian-like symptomatology including multiple system atrophy, progressive supranuclear palsy, corticobasal degeneration, dementia with Lewy bodies, vascular parkinsonism, essential tremor, drug-induced parkinsonism, toxin-induced parkinsonism and hereditary/metabolic parkinsonism (e.g. Wilson's disease, juvenile Huntington's disease, Machado-Joseph disease and Hallervorden-Spatz disease) (Oder *et al.* 1993;Adams *et al.* 1988;Barbeau *et*

al. 1984;Swaiman 1991;Poewe and Wenning 2002;Weiner 2005;Facca and Koller 2003;Mark 2001;Hobson 2003). In fact, the triad of cardinal signs described earlier makes up what neurologists perceive to constitute PD and when present a diagnosis should be seriously considered. If, in addition, there is improvement on L-DOPA treatment with the eventual emergence of dyskinesias and motor fluctuations then 80-90% the diagnosis is probable. In the absence of a biological marker for PD the diagnosis can only be made with certainty at autopsy. However, imaging has proved helpful in confirming the diagnosis in some cases. For instance, 2ß-carboxymethoxy-3ß(4-iodophenyl) tropane also known as DOPASCAN™ and Single Photon Emission Computerised Tomography (SPECT) as well as Positron Emission Tomography (PET) using various ligands have been used to help the diagnosis of PD as well as to exclude secondary causes of parkinsonism such as progressive supranuclear palsy, cerebrovascular disease, Huntington's disease, normal pressure hydrocephalus and olivopontocerebellar atrophy (Seibyl *et al.* 1995;Wolfson *et al.* 1985;Martin *et al.* 1984;Garnett *et al.* 1983;Eidelberg *et al.* 1995;Morrish *et al.* 1995;Guttman *et al.* 1997;Rinne *et al.* 1995;Antonini *et al.* 1998;Brooks *et al.* 1990;Brooks 1993;Hobson 2003).

1.2.2 Non-motor Deficits

1.2.2.1 Autonomic

There are several autonomic disorders that occur in PD patients. Although, some patients with parkinsonism and autonomic disorders go on to be diagnosed as multiple system atrophy and Shy Drager syndrome, most idiopathic PD patients develop some degree of autonomic dysfunction over time. These disorders can be divided according to the system involved.

1.2.2.1.1 Gastrointestinal Dysfunction

Among the most common gastrointestinal symptoms in patients with established PD is constipation (Edwards *et al.* 1993). This may occur secondary to the disease itself or may be a side effect of the medications (dopaminergic, anticholinergic) used to treat PD (Sakakibara *et al.* 2003). Constipation in PD may also result from a variety of other

factors (not necessarily related to autonomic nervous system dysfunction) including decreased physical exercise, diminished intake of food and liquid or dysfunction of sphincters.

Swallowing difficulty is another common gastrointestinal problem in PD and because of the risk of aspiration and the impact on nutrition this can be potentially life-threatening. The primary symptoms are drooling and dysphagia, the latter is described as a sensation of food sticking at the level of the thyroid cartilage or below (Edwards *et al.* 1993). Drooling in PD and difficulty with saliva are manifestations of disordered swallowing and not of abnormal salivation. Patients with PD do not secrete excess saliva, indeed salivary output in PD is either normal or decreased (Edwards *et al.* 1992). Drooling reflects a difficulty in transporting saliva to the posterior pharynx (Edwards *et al.* 1991).

1.2.2.1.2 Cardiovascular Dysfunction

Clinically significant cardiovascular dysfunction in PD is rare, with orthostatic hypotension serving as the most notable exception. Orthostatic hypotension is a drop in blood pressure when arising from a sitting or lying position, indeed orthostatic hypotension is very common in PD (Gross *et al.* 1972;Goldstein *et al.* 2002;Goldstein 2003;Bonuccelli *et al.* 2003). The drop in blood pressure most often occurs after a change in posture but can also occur following exercise and after eating when blood is diverted to the splanchnic vasculature (Turkka *et al.* 1987).

1.2.2.1.3 Thermoregulatory Dysfunction

Excessive sweating and less commonly hypohydrosis, may occur in patients with PD and may be related to involvement of the hypothalamus (Swinn *et al.* 2003). Tanner and co-workers found 48% of PD patients to have excessive sweating (Tanner *et al.* 1982). Often the excessive sweating occurs in patients who develop motor fluctuations (Swinn *et al.* 2003;Tanner *et al.* 1982). Of interest, some patients have this in the *on* state, especially with dyskinesias, but most have it in the *off* state when it is often more severe (Witjas *et al.* 2002).

1.2.2.1.4 Urologic Dysfunction

Urinary frequency and urgency are common complications in PD found in both men and women (Olanow *et al.* 2001). This autonomic symptom is probably due to involvement of components of the autonomic nervous system. The pathology in PD targets many brain regions normally involved in detrusor control including pathways of the basal ganglia, hypothalamus, and locus coeruleus (Sotolongo, Jr. 1988). Detrusor hyperactivity occurs in the majority of PD patients (Andersen 1985). Urinary symptoms in PD usually take the form of nocturia, urinary frequency during the day, urinary urgency and urge incontinence.

1.2.2.1.5 Pupillary Changes

Pupillary change is another autonomic change seen in PD patients. Abnormally slow pupillary responses to light and pain in PD patients have been described (Micieli *et al.* 1987). More specifically, studies focusing on pupillary responses in PD have found that while resting diameters are normal the response to changes in light is less (Micieli *et al.* 1991). The results of instillation of pharmacologic agents to the eye have demonstrated that the peripheral autonomic nervous system to be intact in PD subjects. The findings suggest that pupillary abnormalities in PD result from central autonomic dysfunction centred in the parasympathetic Edinger-Westphal nucleus of the midbrain. Nevertheless, clinically significant pupillary changes do not appear to be common.

1.2.2.1.6 Sexual Dysfunction

Sexual dysfunction occurs in many patients with PD and can be due to autonomic dysfunction as well as the effects of the PD medications (Koller *et al.* 1990;Wermuth and Stenager 1995;Bronner *et al.* 2004). Men often develop difficulty with attaining and maintaining erections or ejaculating, whereas women may have difficulty with orgasms. Some patients respond to dopaminergic treatment and in rare instances, both men and women may become hypersexual on these medications (Uitti *et al.* 1989) or after PD surgery (Roane *et al.* 2002).

1.2.3 Neuropsychiatric

Although PD is dominated by motor symptoms during the initial stages, most if not all patients develop additional non-motor manifestations. Among the most common are neuropsychiatric disturbances. Neuropsychiatric symptoms have a major impact on the natural history and prognosis of PD and have been identified as one of the cardinal risk factors for nursing home placement and increased mortality (Goetz and Stebbins 1993).

1.2.3.1 Anxiety and Depression

Anxiety disorders are common in PD. One of the first formal studies of anxiety in PD was conducted by Rubin et al., (Rubin AJ *et al.* 1986). The authors reported that anxiety was greater than that estimated for the general population. Other subsequent studies have also observed that anxiety in patients with PD occurs more frequently than expected. Menza and colleagues studied 104 PD patients and 61 medical controls with similar disability for symptoms of anxiety and depression (Menza and Mark 1994). All patients completed the Zung self rating for depression and the Zung self rating for anxiety scale: the mean anxiety scale was 25.2 for patients with PD and 20.9 in control patients. Thus, patients with PD scored significantly higher than control subjects on this measure of anxiety ($p<0.001$). In another study by Menza et al., 42 patients with PD were compared with 21 matched medical control subjects (patients with chronic debilitating osteoarthritis matched for age and length of illness) using Diagnostic and Statistical Manual of Mental Disorders (DSM)-III-R criteria and a variety of psychiatric scales (Menza *et al.* 1993). Twelve PD patients (29%) but only one medical control subject (5%), had a formal anxiety disorder. Gotham and co-workers assessed patients with PD, patients with arthritis and normal controls using the Beck depression inventory, the Beck hopelessness scale and the Spielberger anxiety index (Gotham *et al.* 1986). Patients with PD scored significantly higher than the normal controls but did not differ from those with arthritis. The prevalence of anxiety in PD patients ranges from 5.3 to 40%. Stein et al., systematically evaluated 24 patients with PD for the presence of DSM-III-R axis syndromes. He found that nine of the twenty-four had clinical important anxiety (Stein *et al.* 1990). Starkstein et al., found that 40% of patient with PD met DSM-III criteria for

anxiety disorders (Starkstein SE *et al.* 1993). While anxiety was associated with depression, some patients showed anxiety without depression. Thus most studies have shown that anxiety occurs commonly in PD at a higher rate than in normal or other disease comparison populations.

The type of anxiety disorder in PD seems to be clustered in the panic disorder, phobic disorder and generalised anxiety disorder areas. However, a variety of other types of anxiety disorder have been identified in patients with PD (see Table 1.2) (Menza *et al.* 1993;Stein *et al.* 1990;Schiffer *et al.* 1988;Lauterbach and Duvoisin 1991). Despite the high incidence of anxiety in PD it is still unclear why PD patients are especially prone to anxiety. Some suggest that anxiety may relate to the psychological and social difficulties of the disease (i.e. social anxiety) (Ellgring *et al.* 1990), yet others have found that depressed PD patients have significantly higher anxiety levels than depressed multiple sclerosis patients (Schiffer *et al.* 1988). Therefore, anxiety in PD may not simply be the result of psychological and social difficulties of the disease but rather may be due to specific neurobiological mechanisms.

Table 1.2 List of different types of anxiety disorder identified in patients with PD

Anxiety Disorders found in PD
• Generalised Anxiety Disorder
• Panic Disorder
• Social Phobia
• Phobic Disorder
• Agoraphobia
• Obsessive-Compulsive Disorder
• Anxiety Disorder not otherwise specifie

Among the most common affective and behavioural symptoms that accompany PD is depression (Cummings 1992;Cummings and Masterman 1999;Poewe and Luginger 1999). Depression in PD has been classified as major depression. Major depression as defined by the DSM IV criteria (American Psychiatric Association 1994) is present if a patient exhibits five or more of the following: 1) depressed mood, 2) decreased interest (apathy) or pleasure in activities (anhedonia), 3) significant weight loss, 4) insomnia or excessive sleep, 5) psychomotor retardation, or agitation, 6) loss of energy (anergia), 7) feelings of inappropriate guilt and 8) recurrent thoughts of death. Indeed, many of these symptoms are seen in PD patients. However, the profile of depressive symptoms observed in PD is not identical to that reported in patients with primary depression (also known as endogenous depression). Distinctive features of depression in PD include elevated levels of dysphoria, irritability, little guilt or feelings of failure and a low suicide rate despite a high frequency of suicidal ideation. Diagnosis of depression in PD is complicated by overlapping symptoms of the two disorders. Symptoms of depression including psychomotor slowing and retardation; reduced mimics and apathy may be indistinguishable from neurological motor deficits in PD. Diagnosis of depression in PD is almost exclusively based on subjectively experienced depressive symptoms including: (1) feeling of emptiness and hopelessness, (2) reduced reactivity to emotional stimuli, (3) loss of the ability to enjoy and feel pleasure (anhedonia).

Given the difficulties in diagnosing depression in PD is not surprising the marked differences in the prevalence of depression in PD as reported in Europe and North America: from 4% to 70% (Leentjens 2004;Burn 2002;Zesiewicz *et al.* 1999;Cummings 1992). The results of prevalence studies are further confounded by the diagnostic instrument used, how depression is defined and the nature of the study population (hospital vs. community-based). In a review of all studies of depression in PD published from 1992 to 1998, a total of 45 studies comprising 5911 PD patients, 31% of patients were at any given time depressed (Brooks and Doder 2001). This compares to a prevalence of depression in the general population of 16% (Kessler *et al.* 2003).

Attempts have been made to correlate depression with disease severity as well as disease duration, young age of onset and age (Leentjens 2004;Burn 2002;Zesiewicz *et al.* 1999;Menza and Mark 1994;Slaughter *et al.* 2001). Some reports show a correlation between depression and disease severity, some with young age of onset, and some show

no correlation between depression and disease severity. The weak correlation between depression and disease severity speaks to depression being not a psychological reaction to PD, but being part of PD related to neurodegeneration in the nervous system. In addition, the relative absence of guilt, shame, or sorrow in depressed PD patients speaks to depression not being a psychological reaction to PD.

Both depression and anxiety may predate the onset of motor features of PD by a number of years. In one series, depressive symptoms preceded the motor disturbance in 12-37% of patients (Taylor *et al.* 1986). Shiba and colleagues studied the association between preceding anxiety, depression and PD using a case control design (Shiba *et al.* 2000). Depressive disorders were significantly associated with PD, although the significance of the association was lost when depressive disorders first recognised within the 5 years preceding onset of PD were excluded. Anxiety disorders were also significantly associated with PD and the association remained significant up to 20 years or more before the onset of motor symptoms of PD. These finding would be consistent with anxiety and depression being early non-motor manifestations of the disease process underlying PD.

It is well known that in psychiatric populations, anxiety and depression commonly coexist. In a review of coexisting anxiety and depression, Lydiard noted that up to 60% of patients with depressive symptoms also had anxiety (Lydiard 1991). Interestingly, there appears to be a relationship between anxiety and depression in PD as well (Gotham *et al.* 1986). Patients with the disease have significantly higher levels of depression and anxiety than normal controls (Gotham *et al.* 1986). Henderson *et al* found that depression in combination with panic and/or anxiety occurred in 38% of parkinsonian patients compared with 8% of healthy spouse controls: depression and anxiety were highly correlated in patients with PD (Henderson *et al.* 1992). Twenty per cent of these patients reported the onset of depression and/or anxiety before the onset of physical symptoms. Menza *et al* found that 92% of patients with PD who had an anxiety disorder diagnosis also had depressive disorders or symptoms and 67% of patients with a depressive disorder had an anxiety disorder diagnosis (Menza *et al.* 1993). A number of other studies have also found that the degree of comorbidity between anxiety and depression in patients with PD is in excess of that found in those without (Cummings 1992). These findings may

suggest that the structures and pathways mediating depression and anxiety may be contiguous and shared explaining, in part, the high comorbidity of both symptoms in PD.

Depression and anxiety are important contributors to caregiver stress (Aarsland *et al.* 1999b). Caregivers of PD patients with psychiatric symptoms including depression and anxiety a greater number of them report tiredness, sadness and less satisfaction with life compared with healthy elderly subjects (Aarsland *et al.* 1999b).

1.2.3.2 Psychosis (Hallucinations and Delusions)

Psychosis is a prominent neuropsychiatric symptom among PD patients. According to DSM-IV the definition of psychosis requires hallucinations and/or delusions with loss of insight. Delusions are false, fixed, idiosyncratic beliefs that are maintained despite evidence to the contrary. Hallucinations are defined as abnormal perceptions without physical stimulus that can involve any sensory modality and may be simple or complex. Visual hallucinations (VH) are the most common psychotic symptoms in PD with some studies reporting more than 90% of all psychotic PD patients having experienced VH (Graham *et al.* 1997). One categorisation of VH in PD is simple vs. complex. Simple VH are characterised by the absence of form and are often photopsias such as flashes or light or colour. On the other hand, complex VH are characterised by visions that are clearly defined, have specific form and can include animals, objects and humans. VH in some cases are confused with illusions which are misperceptions of real stimuli that are often visual in nature.

VH in PD tend to be present intermittently, lasting seconds to minutes at a time unlike schizophrenic hallucinations (Barnes and David 2001). The frequency can vary, but most studies register the presence of hallucinations when they occur at least one time per week. Often, they occur several times per day. VH tend to occur during times of low ambient stimulation, most typically in the evening or when the patient is alone in a quiet environment. VH in PD tend to persist, so that while each VH episode is brief, the hallucinatory state, once established, is a chronic one (de Maindreville *et al.* 2005;Goetz *et al.* 2001). Delusions are commonly paranoid, consisting of beliefs about spousal infidelity or abandonment (Marsh 2004). Grandiose, somatic, persecutory (Factor and Molho 2004) and religious delusions also occur, but are less frequently reported in clinical series (Diederich *et al.* 2003).

Auditory hallucinations (AH), although, less common than VH are generally experienced by PD patients. AH range from indistinct whispers or music to threatening voices (Inzelberg *et al.* 1998b;Marsh 2004;Factor and Molho 2004). AH variably co-occur with visual hallucinations (8%–13%) and are less likely to be seen in isolation (Inzelberg *et al.* 1998b;Marsh 2004). This pattern is in contrast to the AH typical of schizophrenia. Similarly, in PD tactile, olfactory and gustatory hallucinations are relatively uncommon and generally occur with visual hallucinations (Marsh 2004).

VH in PD are classically considered as adverse events of anti-parkinsonian medication (Friedman 1991;Factor *et al.* 1995). This is based on common clinical experience that psychotic symptoms are closely linked to dopaminergic treatment, while dopamine receptor blockers can alleviate these symptoms (Friedman 1991). However, comparisons of hallucinators and non-hallucinators have seldom shown major differences in drug history. Goetz et al., studied five non-demented patients with PD with daily hallucinations who were given high dose of levodopa infusions in a placebo controlled trial (Goetz *et al.* 1998). No hallucinations were provoked. In two other studies no correlation was found between levodopa administration and hallucinations or delusions in patients with PD (Klein *et al.* 1997;Aarsland *et al.* 1999a). Other risk factors for the development of hallucinations in PD have been described including older age and longer duration of disease, cognitive impairment, depression and sleep disturbances (Barnes and David 2001;Graham *et al.* 1997;Aarsland *et al.* 1999a;Fenelon *et al.* 2000).

Psychosis (hallucinations and delusions) is associated with increased caregiver burden (Aarsland *et al.* 1999b), nursing home placement and increased mortality (Factor *et al.* 2003). A case-control study examined PD patients who entered nursing homes and compared each of them with 2 community-dwelling PD subjects who were matched for age, gender and PD duration. The investigators compared motor disability, dementia and hallucinations as putative risk factors for nursing home placement. The only statistically differentiating features were the presence and severity of hallucinations, with 82% of the nursing home subjects having this behaviour compared to 5% of the subjects living in the community (Goetz and Stebbins 1995).

1.2.3.3 Dementia

Cognitive impairment is commonly associated with PD, although deficits may be relatively subtle and not clinically apparent or not overtly affect daily functioning. In fact, cognitive dysfunction in PD can be classified as domain-specific cognitive impairments not extensive or severe enough to fulfil the DSM-IV criteria for the diagnosis of dementia or severe enough to qualify as dementia.

The prevalence of dementia in PD varies considerably among studies, possibly due to differences in patient population, study design and criteria for diagnosing PD with dementia. In cross-sectional studies, prevalence of dementia in PD ranges from 10% to over 40% (Aarsland *et al.* 1996;Mayeux *et al.* 1988;Mayeux *et al.* 1992). Features associated with prevalence of dementia includes age (Mayeux *et al.* 1988;Aarsland *et al.* 1996), age at onset of PD (Aarsland *et al.* 1996;Mayeux *et al.* 1988;Mayeux *et al.* 1992), disease severity (Mayeux *et al.* 1988), disease duration (Aarsland *et al.* 1996), depression (Aarsland *et al.* 1996) and presence of atypical parkinsonian symptoms such as early occurrence of autonomic failure, symmetrical disease presentation and moderate response to dopaminergic treatment (Aarsland *et al.* 1996). The association with older age is of particular importance. The ApoE 4 allele, which has been consistently shown to be associated with a higher risk of AD does not constitute a risk factor for dementia in PD (Inzelberg *et al.* 1998a). Prospective studies have reported a cumulative incidence of 19 to 53% (Hughes *et al.* 2000). Recently, in a prospective study with 8 years follow-up, 78.2% of patients eventually developed dementia (Aarsland *et al.* 2003). In another prospective study the incidence of developing dementia in PD patients was up to six times higher compared to age-matched control subjects (Aarsland *et al.* 2001). In these prospective studies factors associated with the risk of dementia were found to be age (Levy *et al.* 2002), age at onset of PD (Mahieux *et al.* 1998), disease severity (Aarsland *et al.* 2001;Hughes *et al.* 2000), age at entry in the study (Hughes *et al.* 2000), confusional state (Stern *et al.* 1993), early hallucinations and the mixed tremor/akinetic form of PD (Aarsland *et al.* 2003).

The clinical profile of dementia in PD encompasses qualitatively the same deficits as in non-demented PD subjects but the impairments are more extensive and severe. A wide variety of cognitive deficits have been described, the most prominent of which is a deficit in executive function. Executive function is a broad term used to describe a range

of cognitive functions involved in the realisations of goal-directed, adaptive behaviour in response to new, challenging, environmental situations, including attention, inhibition, task management, planning, monitoring and coding (Smith and Jonides 1999). Impairment of executive functions such as impairment in concept formation, rule finding, problem solving, set elaboration and planning, set shifting and set maintenance have all been described in PD patients (Pillon *et al.* 1986;Litvan *et al.* 1991). The finding that patients have difficulties of internally cued behaviour and they substantially benefit from external cues to the fact that executive dysfunction in PD differs from that due to frontal cortical involvement (Owen *et al.* 1993).

Besides executive dysfunction, there is considerable evidence of deficits in visuospatial function even when tests contain few motor components (Boller *et al.* 1984). Tasks that require visuospatial analysis and orientation are the most affected, which suggests that visual perception is the most impaired function (Girotti *et al.* 1988). Nevertheless, with the exception of orientation, most authors believe that visuospatial dysfunction to be the result of high cognitive demand that is usually required by such tasks. Indeed, it would appear that visuospatial dysfunction in PD can be readily explained by the demand of visuospatial tasks on executive functions such as planning and shifting of attention (Brown and Marsden 1988).

Memory deficits including working memory, long-term memory, visuospatial memory and procedural memory have been frequently reported in demented PD patients but the impairment differs from the amnesia seen in patients with AD (Pillon *et al.* 1991). Deficits in learning of new information have been consistently described, although these deficits are less severe than that seen in AD (Pillon *et al.* 1991;Helkala *et al.* 1989). Several studies have shown that demented PD patients have impaired free recall similar to that seen in AD patients but PD patients benefit substantially from semantic cueing or probing which implies that new information are stored but not easily/readily accessed (Helkala *et al.* 1988;Pillon *et al.* 1993). Therefore, the problem in PD patients with dementia seems to be of retrieval and not of encoding. Indeed, recognition memory is often well preserved in PD patients with dementia. In addition, unlike AD aphasia, apraxia or agnosia are not very common in demented PD patients (Dubois and Pillon 1997;Huber *et al.* 1989). Quite analogous to visuospatial dysfunction, most authors

suggest that memory dysfunction in PD would for the most part be secondarily impaired due to the reliance of its manifestation on executive functionality.

Attention, as measured by parameters such as cognitive reaction and vigilance has been found to be impaired in PD patients with dementia (Litvan *et al.* 1991) and there is also evidence of fluctuations in attention (Ballard *et al.* 2002). These findings are very similar to those found in patients with dementia with Lewy bodies (DLB) (McKeith *et al.* 1996). Indeed, PD patients with dementia and DLB share many clinical features (Ballard *et al.* 2002) and are often difficult to distinguish other than by the temporal onset of dementia and psychosis in relation to parkinsonism (McKeith *et al.* 1996;McKeith *et al.* 2005). Therefore, according to clinical diagnostic criteria if extrapyramidal symptoms precede dementia by more than 12 months the patient is classified as having PD with dementia. If on the other hand, dementia and extrapyramidal symptoms develop together within a 12 month period or dementia precedes extrapyramidal symptoms (by approximately 1 year) then the patient is classified as having DLB (McKeith *et al.* 1996;McKeith *et al.* 2005).

Dementia in PD is a major risk factor for nursing home placement and the risk of death in patients with PD and dementia is substantially higher than those without dementia: the survival rate of patients with dementia was found to be significantly shorter compared to non-demented patients (Mindham *et al.* 1982;Boller *et al.* 1980).

1.2.4 Treatment

Treatment of PD is complex due to the progressive nature of the disease and the array of motor and non-motor features combined with early and late side effects associated with therapeutic interventions. A growing number of medical and surgical treatment options are available. Table 1.3 and 1.4 provide a general overview of the current treatments for PD motor, neuropsychiatric and autonomic symptoms. These tables were adopted from Rascol et al., (Rascol *et al.* 2002a) and modified based on information from the following references (Rascol *et al.* 2003;Rascol *et al.* 2002b;Rascol *et al.* 2001;Senard *et al.* 2001;Weintraub and Stern 2007;Bonuccelli *et al.* 2003).

Table 1.3 Therapeutic Interventions for Symptoms of PD

(Following page) *Abbreviations:* MAO-B: mono-amineoxidase-B

Drugs	Promote Dopamine Synthesis	Activate Specific Receptors	Prolong Dopamine Availability
Dopaminergic	Levodopa+ dopa-decarboxylase inhibitor	Dopamine agonists	MAO-B inhibitors
		Apomorphine Bromocriptine Tolcapone Cabergoline Dihydroergocriptine Lisuride Pergolide Piribedil Pramipexole Ropinirole	Selegiline
Antiglutamatergic		Amantadine	
Anticholinergic		Benztropine Biperiden Orphenadrine Procyclidine Trihexyphenidyl	

Surgery	Lesion	Deep Brain Stimulation	Transplantation
	Thalamotomy Pallidotomy Subthalamic nucleotomy	Thalamus Pallidum Subthalamic nucleus	Fetal mesencephalic cells

Rehabilitation	Rehabilitation procedures
	Physical therapy Occupational therapy Speech Therapy

Table 1.4 Therapeutic Interventions for Non-Motor Symptoms of PD

(Following page)

Mental Dysfunction	Medication
Dementia	Rivastigmine, donepezil, tacrine, galantamine
Psychosis/Drug-induced psychosis	Quetiapine, olanzapine, risperidone, zotepin, odensatron, rivastigmine, donepezil, tacrine, galantamine, clozapine
Depression	Tricyclic antidepressants, MAO-inhibitors, serotonin-reuptake-inhibitors, electroconvulsive therapy
Autonomic Dysfunction	
Orthostatic hypotension	Midodrine, fludrocortisone, dihydroergotamine, etilefrine, indomethacine, yohimbine
Urinary dysfunction	Oxybutinine, tolterodin, flavoxate, Propiverine, Prazosin
Gastrointestinal Dysfunction Constipation	Cisapride
Sexual dysfunction	Yohimbine, papaverine, sildenafil, vardenafil

1.3 Neuropathology of PD

1.3.1 Pre Alpha-Synuclein Era

1.3.1.1 Historical Observations

PD is considered to be a consequence of degeneration of the dopaminergic nigrostriatal system with associated changes in the neuronal cytoskeleton that result in the formation of Lewy bodies (LBs) (Forno 1996).

LBs were first described by the German pathologist Friedrich H. Lewy in 1912 in patients with PD (then known as paralysis agitans) (Lewy FH 1912). Lewy discovered these eosinophilic, round cytoplasmic inclusions in the dorsal motor nucleus of the vagus nerve (DMV), nucleus basalis of Meynert (NBM), paraventricular nucleus and lateral thalamic nucleus (Gibb and Poewe 1986). The involvement of LBs in the substantia nigra however, was first described by Tretiakoff in 1919, who coined the term "corps de Lewy" (Gibb and Poewe 1986) and also speculated that degeneration of the substantia nigra relates to the motor symptoms seen in the disease. Later, Hassler confirmed these findings and showed the focal distribution of pathology: the most pronounced nerve cell loss was found in the caudal and ventrolateral parts of the substantia nigra (Hassler R 1938). These findings on the neuronal depletion of nigral neurons were corroborated by later studies that also showed that the substantia nigra zona compacta (SNc) suffers a 60-85% loss of melanised, tyrosine hydroxylase containing neurons with a regional selectivity which is greatest in the lateral ventral tier (approx. 91%) followed by the medial ventral tier (approx. 71%) and the dorsal tier (approx 56%) (Fearnley and Lees 1991). With respect to LBs many later authors confirmed the presence of LBs in the substantia nigra as well as in other pigmented neurons of the brainstem most notably the locus coeruleus (LC) (Lipkin L.E 1959;Eadie M.J 1963;Greenfield J.G and Bosanquet 1953). As LBs were detected where most of the cell loss occurs in the PD brain, LBs were considered to be responsible for the neuronal degeneration. Thus, LBs became the *sine qua non* of PD and thus secured their position as pathological hallmarks of the disease along with the severe nerve depletion in the SNc. In fact, degeneration of the SNc and the occurrence of LBs in the remaining neurons constitute the anatomico-pathological hallmarks of the disease. However, LBs have also been observed and described in regions outside the substantia

nigra including the amygdala, hypothalamus, hippocampus, thalamus, pedunculopontine nucleus (PPN), olfactory bulb and the cerebral cortex (Braak and Braak 2000). Of all neuronal populations prone to develop LBs, the nerve cell loss is frequently observed in the DMV, SN and NBM however, it still remains to be elucidated why some neuronal populations are selectively vulnerable in PD, while others remain resistant.

1.3.1.2 Neuropathology of the Ageing Brain –Its Relation to PD

Studies on normal ageing have demonstrated that degeneration in the SNc is not seen in the normal ageing brain although cell loss occurs at approximately 4.7% per decade (Fearnley and Lees 1991). In fact, the regional pattern of cell loss is opposite to that seen in PD. It is generally agreed that neuronal loss in normal ageing is not sufficient to cause PD. Nevertheless, age is the main risk factor for PD (Esiri 2007). Interestingly 50% of those over 80 show some clinical features of parkinsonism (e.g. slow movement).

There have been a few studies to estimate the prevalence of LBs in normal elderly individuals (Lipkin L.E 1959;Woodard J.S 1962;Forno 1969). These have found a prevalence of incidential LBs of 5-10% in cases without parkinsonian manifestations. However, these studies they were heterogeneous as were derived from veterans and chronic disease hospitals or mental institutions and thus were biased towards psychiatric illnesses, age or male gender. The first study that could be reliably cited to have examined the prevalence of LBs in normal ageing is by Perry et al., (Perry *et al.* 1990). This study found that only 2% of subjects without any neurological or psychiatric condition had LBs. Therefore, LBs were concluded not to be an age-related feature but strongly associated to neurodegeneration. Interestingly, the prevalence of incidential LBs in the normal elderly population has been examined in a few subsequent studies (directly or indirectly) which have reported that rises between the 6^{th} and 9^{th} decades (Jellinger 2004;Saito *et al.* 2004). The LBs in all these studies occurred in the same brain regions as in PD. This condition of incidental LBs in cases without any neurologic or psychiatric illness is termed Incidental Lewy Body Disease (ILBD). Incidental Lewy bodies are thought to represent a pre-symptomatic phase of PD i.e. if the person had lived longer, parkinsonian clinical features were likely to have developed.

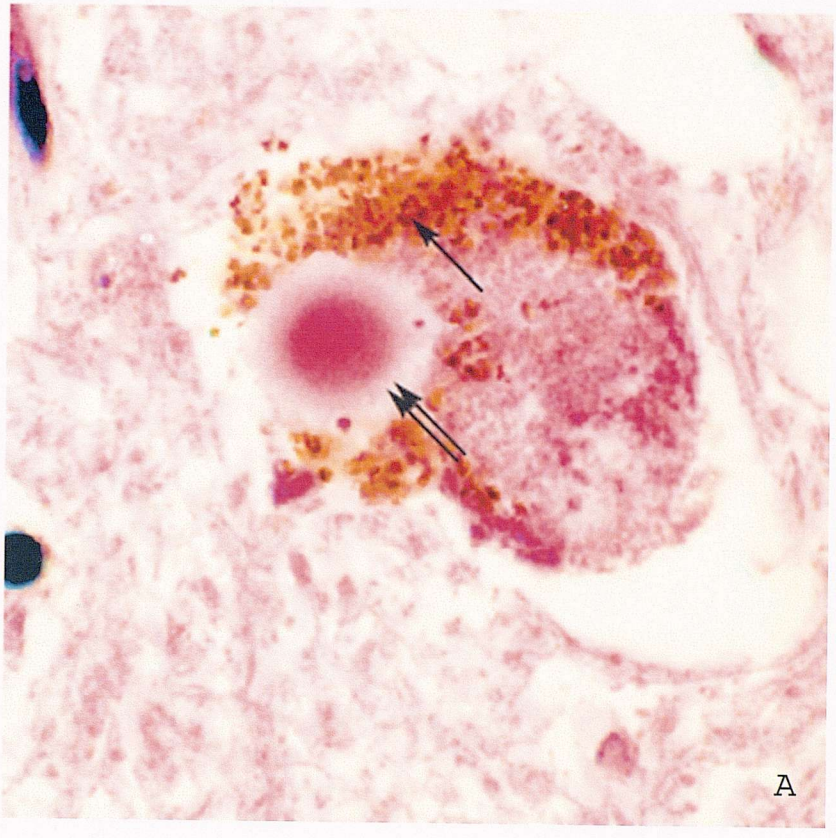
1.3.1.3 Detection of Lewy Bodies

LBs were initially identified with the use of conventional staining techniques such as haematoxylin and eosin (H&E) (Figure 1.1). The shape and size of LBs depends on the neuronal perikaryon it occupies but usually LBs have a spherical configuration around 5-25μm in diameter. In H&E staining LBs in the brainstem are considered to have a classical morphology consisting of a dark eosinophilic centre (body) surrounded by a paler halo. LBs in the cortex on the other hand, have a less conspicuous morphology and therefore are not readily evident on H&E stained sections (Lennox *et al.* 1989). Ultrastructurarly, LBs in the brainstem are formed of straight filaments of around 8-10nm in diameter that are arranged in a radial orientation around an electron dense core (Duffy PE and Tennyson VM 1965;Pollanen *et al.* 1993). The concentric lamination of LBs can consequently be explained by greater packing density of filaments in the core. LBs can extend into nerve cell processes or lie in the neuropil.

In the late 1980's specific detection methods involving immunohistochemistry (IHC) were introduced. The introduction of IHC identified a variety of different antigenic components within LBs that could be divided as the main structural elements (proteins, enzymes and passively entrapped proteins) (Pollanen *et al.* 1993;Galvin *et al.* 1999). The most consistently recognised proteins were the cytoskeletal proteins such as neurofilaments (NFs) and ubiquitin (Ub), a protein that is involved in the non-lysosomal degradation of cellular products (Goldman *et al.* 1983;Kuzuhara *et al.* 1988;Lowe *et al.* 1988). Comparison between antibodies raised against NFs and Ub showed that Ub IHC was a more reliable and sensitive method for the detection of LBs as antibodies against NFs only immunolabeled around half of the H&E stained LBs (Dale *et al.* 1992) (Figure 1.1). The use of Ub-IHC was even recommended by the first consortium guidelines (McKeith *et al.* 1996). In particular this antibody was especially helpful for the detection of cortical LBs that possessed a less stereotypical morphology in comparison to their brainstem counterparts (Kosaka 1978). Nevertheless, Ub immunoreactivity was not unique to LBs but was also present in other pathological inclusions such as neurofibrillary tangles (NFTs) that were difficult to distinguish from LBs (Lowe *et al.* 1988). For this reason, double immunostaining with both Ub and tau antibodies (tau: the main constituent of NFTs) was recommended especially in DLB where both NFTs and LBs co-exist (Dickson *et al.* 1989).

Figure 1.1 H&E and Ubiquitin stained sections from a PD case

(Following page) A) Lewy body in the substantia nigra neuron using hematoxylin-eosin staining is characterized by a red core and a surrounding white halo. Arrow - pigment melanin, a feature of substantia nigra neurons; double arrow - Lewy body on hematoxylin-eosin staining is characterized by red core and white halo. B) Lewy body characteristically stained by anti-ubiquitin antibody used in a routine neuropathological diagnostic procedure. The characteristic round shape with immunopositive core and pale ring around. Arrow - Lewy body. Magnification 20x (Image taken from Bogdanovic (Bogdanovic 2001).


A


B

1.3.2 Alpha-Synuclein (αSN)

1.3.2.1 Structure and Function

α-synuclein (αSN) is a member of the human synuclein protein family along with β-synuclein (βSN) and γ-synuclein (γSN). These proteins are small (19-20KDa) with a similar amino acid sequence but are encoded by different genes (Goedert 2001). The first synuclein to be discovered was αSN. αSN was first isolated in 1988 by Maroteaux and colleagues by expression screening from the electric organ of the fish *Torpedo californica* using an anti-serum against purified cholinergic synaptic vesicles (Maroteaux *et al.* 1988). The name synuclein was given to this protein as it was shown to localise in presynaptic nerve terminals and portions of the nuclear envelop (Goedert 2001). However, subsequent studies although confirming the presence of αSN in nerve terminals have failed to confirm its nuclear localisation, but the original nomenclature survived. αSN is abundantly found in the brain mainly as an isoform of 140 amino acids but a shorter alternatively spliced isoform of αSN 112 has also been identified although this isoform is predominately expressed in the heart, skeletal muscle and pancreas (Ueda *et al.* 1994). In fact αSN is the most studied member of the human synuclein protein family as it is the only one to be implicated directly in human neurological diseases (see following section). In 1990, Nakajo purified a presynaptic phosphoprotein (PNP14) from bovine brain that was very similar to αSN, which is now known as βSN. βSN like αSN is expressed predominately in the brain where is concentrated in the presynaptic nerve terminals. It consists of 134 amino acids and seems to be regulated by phosphorylation (Nakajo *et al.* 1990;Nakajo *et al.* 1993) (Figure 1.2). The third member of the human synuclein family, γSN was cloned from an EST library and identified as the breast cancer specific gene (BCSG1) whose expression was shown previously in invasive types of breast cancer (Ji *et al.* 1997). γSN consists of 127 amino acids and is predominantly expressed in peripheral sympathetic neurons (Akopian and Wood 1995) (Figure 1.2).

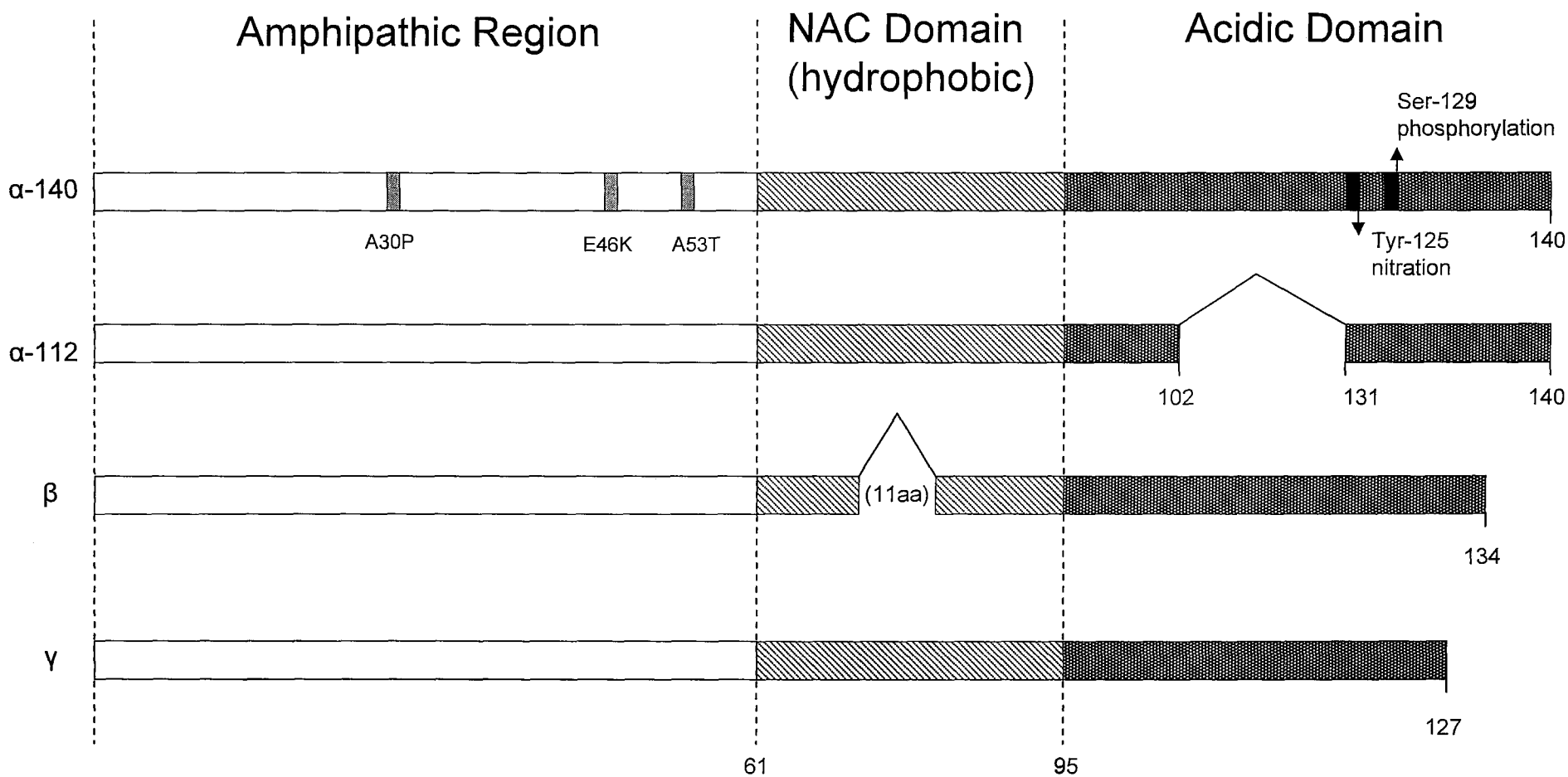
Structurally, αSN is composed of three main domains (Figure 1.2). The N terminal amphipathic domain is composed of imperfect 11 amino acid repeats with a consensus sequence (KTKEGV). This domain appears to be responsible for its lipid binding ability. The three known mutations (A30P, A53T and E46K) are also found in this domain.

Although, αSN has been shown in vitro to bind to monolayer phospholipid membranes acquiring an α-helical secondary structure (Davidson *et al.* 1998), not all cellular αSN seems to be linked to membranes, as it can also be purified from the cytosol (George *et al.* 1995;McLean *et al.* 2000). The middle hydrophobic domain, the NAC (non-amyloid component) domain is extremely amyloidogenic and is responsible for the ability of the protein to undergo a conformational change from a random coil to the β-sheet conformation required for its aggregation (Iwai *et al.* 1995). Interestingly, βSN has a deletion in the NAC domain which makes it non-amyloidogenic. The acidic C-terminal domain remains free and unfolded and does not associate with membranes (Eliezer *et al.* 2001).

Figure 1.2 The human synuclein family

(Following page) The different synucleins are shown and represented as a bar. The N-terminal amphipathic region, the hydrophobic region and the acidic tail are separated by vertical dashed lines and are differently hashed. The α-synuclein 112 splice variant as well as the β- and γ-synucleins are shown. The NAC region of βSN lacks 11 amino acids (residues 73-83). The phosphorylation and nitration sites of αSN are also indicated with black boxes. Image taken and modified from Lucking and Brice 2000 (Lucking and Brice 2000).

The Human Synuclein Family



The physiological function of αSN is largely unknown. Immunoelectron microscopic studies have demonstrated it to be closely localised and associated with synaptic vesicular membranes suggesting that αSN may play a role in function of synaptic vesicles (Jensen *et al.* 1998). Immunohistochemical analyses using laser scanning confocal microscope showed that αSN immunoreactivity was predominantly located in presynaptic regions. Due to this presynaptic localisation, αSN has been postulated to have a role in the regulation of synaptic plasticity (George *et al.* 1995), in neuronal differentiation (Bayer *et al.* 1999a), or in neurotransmitter release (Abeliovich *et al.* 2000). As in other proteins prone to aggregate, αSN is natively unfolded (Weinreb *et al.* 1996). On binding to phospholipid membranes it takes on an α-helical structure that may stabilise the vesicle membrane and inhibit their lysis (Davidson *et al.* 1998). It has also been demosnstrated *in vitro* and *in vivo* that αSN is an inhibitor of the enzyme phospholipase D2 which hydrolyses phosphatidylcholine to phosphatidic acid and may be involved in vesicle trafficking in the secretory pathway (Jenco *et al.* 1998). In developmental studies, expression of αSN was first detectable at embryonic days 12 to 15, followed by a dramatic decrease at later time points (Hsu *et al.* 1998). Its expression was detected earlier than another major synaptic vesicle protein, synaptophysin. These findings may suggest that αSN plays a role in synaptogenesis.

1.3.2.2 Pathological Significance of αSN

The role of αSN in the neurodegenerative diseases was demonstrated when Ueda and co-workers in 1993 isolated a 35 amino acid peptide (known as NAC, non amyloid component) from amyloid plaques of AD brain tissue (Ueda *et al.* 1993). cDNA of a larger NAC precursor (NACP) was cloned and afterwards this protein was identified as the human αSN, this is why αSN is still sometimes referred as the NACP. Nevertheless, subsequent studies failed to verify the presence of NAC in amyloid plaques (Bayer *et al.* 1999b;Culvenor *et al.* 1999). The finding that directly implicated αSN in neurodegenerative diseases was the discovery that αSN gene mutations can result in PD (Polymeropoulos *et al.* 1997;Kruger *et al.* 1998;Zarranz *et al.* 2004) (see also section 1.3.2.1). As in other neurodegenerative diseases, it became evident that the gene that was mutated in the inherited cases of the disease, also encoded for the protein that formed the main component of hallmark lesions of the disorder (Spillantini *et al.* 1997). Spillantini et

al., first reported that LBs and dystrophic neurites were robustly immunoreactive for αSN (Spillantini *et al.* 1997). Soon it was noticed that αSN was the main component of these structures, as the immunoelectron microscopic studies detected isolated filaments of 5-10nm in diameter from purified LBs to be intensely decorated with αSN (Spillantini *et al.* 1998;Spillantini *et al.* 1997;Wakabayashi *et al.* 1997). Further to this, Iwatsubo et al., prepared monoclonal antibodies using highly purified LBs from DLB patients and hence biochemically corroborated that αSN was accumulated in LBs (Iwatsubo *et al.* 1996). In fact αSN immunohistochemistry is now the gold standard for the detection and visualisation of LBs and its application has also demonstrated the presence of abnormal deposition of αSN in neuritic processes known as Lewy neurites (LNs). Western blot analysis and immunohistochemistry have shown that both LBs and LNs contain insoluble aggregates of full-length and truncated protein (Baba *et al.* 1998;Culvenor *et al.* 1999). All these genetic, biochemical and pathological studies have contributed to establishing the central role that αSN holds in the pathogenesis of PD and DLB.

1.3.2.3 αSN Aggregation and Putative Pathogenetic Mechanisms

αSN is one of several proteins associated with neurodegenerative diseases that have a high propensity to aggregate. In fact one of the most intriguing issues of medical pathology, in the post-genomic era, represented by 'Conformational diseases' whose pathogenesis is rooted in abnormalities of protein's three-dimensional structure (Soto 2003). In these diseases, one of which is PD, an important role is played by protein aggregation. Protein aggregation is a negative event in nature that represents a failure in the accomplishment of the correct spatial arrangement (folded state) necessary to a functional protein.

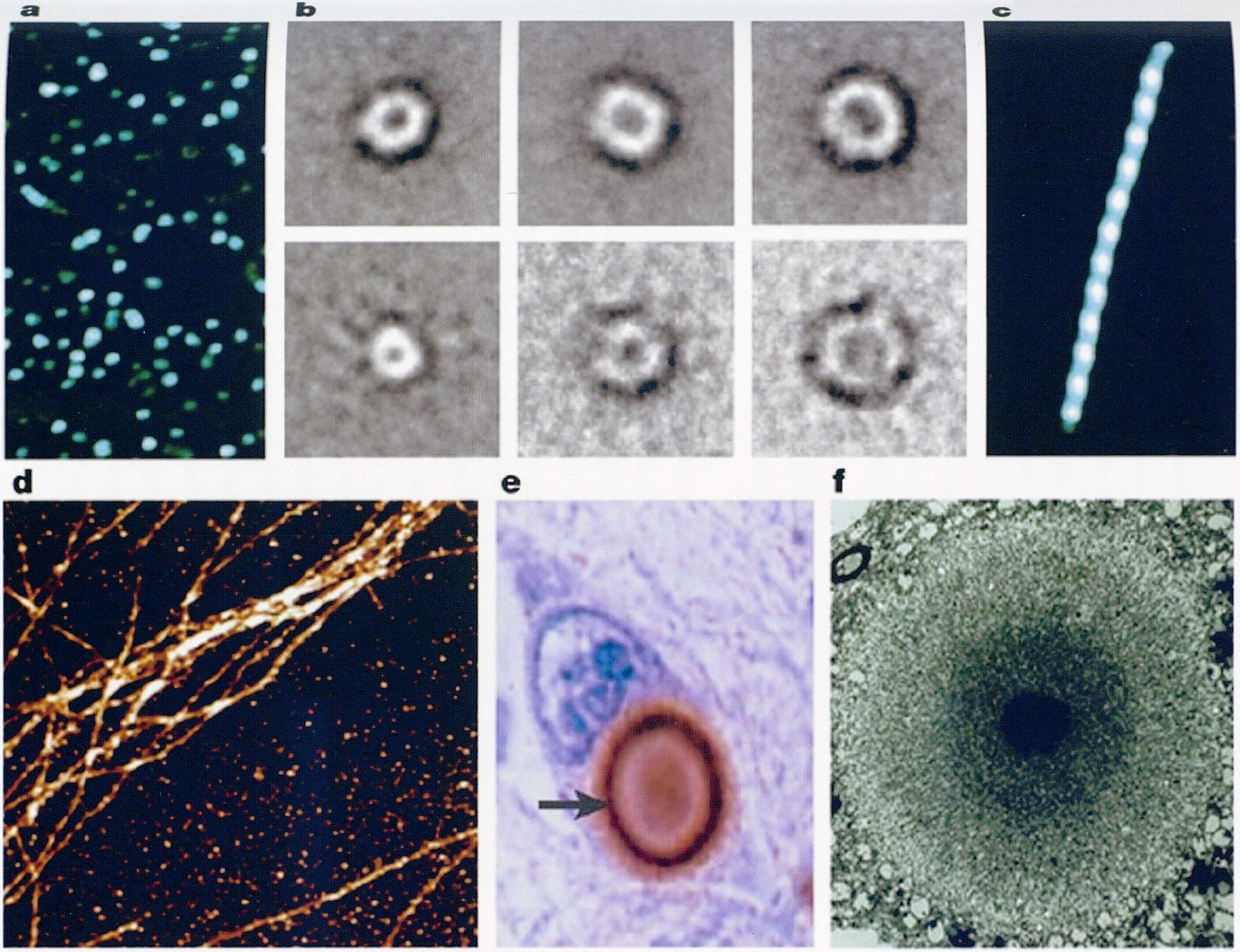
αSN is a natively unfolded protein that 15% is bound to cell membranes with a stable α-helical structure and the rest (85%) is free in the cytosol that is essentially disordered (McLean *et al.* 2000). The end result of αSN aggregation is the formation of insoluble polymers of protein known as fibrils. In this form β-pleated sheet-like bonding stabilises the aggregated form. It is thought that fibrillar αSN is the building block of LBs. The most direct evidence for this is immunogold labelling showing that αSN is present at

sites along the fibrils isolated from LBs (Crowther *et al.* 2000). LBs contain many proteins such as NFs and other cytoskeletal proteins suggesting that co-precipitants may be important for aggregation. However, fibrils can be formed *in vitro* from αSN alone, suggesting that this protein is sufficient to form inclusions (Uversky 2003). The fact that αSN is the most sensitive marker for LBs strengthens this argument that αSN is necessary for LB formation.

Further insights into αSN fibrillogenesis come from studies on the known mutations of the αSN gene. All known mutations (A53T, A30P and E46K) promote the formation of oligomeric species and not fibrils. Oligomeric species also known as protofibrils are intermediate species resembling an elongated filament that is formed in the fibrillisation from monomer to fibril (Lotharius and Brundin 2002). Protofibrils can form annular structures that have pore-like properties and may damage the membranes for instance by leakage of small molecules such as calcium and dopamine into the cell cytoplasm with toxic consequences. In this way αSN protofibrils could potentially disrupt ionic and metabolic homeostasis leading to toxicity by numerous downstream mechanisms (e.g. oxidative stress) (Lashuel *et al.* 2002). Recently, protofibrils of non-disease-associated proteins were shown to be cytotoxic, indicating that perhaps αSN protofibrils rather than the fibrils commonly found in the LBs and LNs are the deleterious species (Bucciantini *et al.* 2002). One hypothesis of the mechanism for LB formation is shown in Figure 1.3.

Figure 1.3 αSN fibrillogenesis

(Following page) One hypothesis for the formation of LBs is that αSN monomers join to generate oligomers (protofibrils) (a and b). These protofibrils have the potential to form elliptical or circular pores that can punctuate cell membranes, resulting in the release of cellular content and cell death. This supports the "protofibrilar hypothesis" outlined above. αSN protofibrils coalesce into fibrils (c and d) which have the β-pleated conformation of amyloid. The fibrils can aggregate into LB inclusions (e and f). f demonstrates the architecture of LBs that is characterised by an electron-dense core and a lighter surrounding halo. (Image taken from Couzin 2001 (Couzin 2001)).


a
b
c
d
e
f

A key unresolved issue is how the pathological aggregation of αSN as an inclusion is mechanistically linked to neuronal dysfunction or cell death that accounts for the manifestation of the clinical symptoms. One possible pathological mechanism would be that the aggregation of αSN disrupts normal cellular function. Nonetheless, loss of function appears unlikely as elimination of the αSN gene in knockout mice does not produce a characteristic phenotype of PD i.e. LBs, neuronal loss and motor deficits (Abeliovich *et al.* 2000). Therefore, a more plausible mechanism would be that the aggregation of αSN as an inclusion may itself possess the toxic gain of function that compromises the cell physiology, leading to demise of neurons. Current research efforts are directed towards a better understanding of the fatal insults that provoke αSN to aggregate as an inclusion. The mechanisms that have been proposed include overexpression of αSN, post-translational modification including oxidation and impaired degradation.

Various methods of overexpressing native or mutant αSN have been utilised in order to gain a further insight into the pathogenic potential of this protein. Transgenic mice and fruit flies that overexpress wild-type or mutant human αSN have been shown to exhibit abnormal cellular accumulation of αSN, neuronal loss and behavioural deficits (Meredith *et al.* 2004). In line with these studies fibrillar aggregates of αSN in an *in vitro* model demonstrated that accumulation of αSN induced apoptotic cell death (El-Agnaf *et al.* 1998;Zhou *et al.* 2000). Interestingly, the triplication of the normal αSN gene causes familial PD, further suggesting that mutant αSN behaves differently from wild-type protein in a quantitative rather than a qualitative manner (Singleton *et al.* 2003). However, the evidence from both *in vivo* and *in vitro* studies supporting the notion the overexpression of αSN could lead to cell death, is not in line with human studies that have failed to detect elevation of αSN mRNA in PD or DLB (Wirdefeldt *et al.* 2001).

The post-translational mechanisms that predispose αSN to undergo pathogenetic transformation are not very well understood. In transfected cell lines αSN becomes phosphorylated at serine residues 87 and 129 (Okochi *et al.* 2000). Phosphorylation at serine 129 *in vitro* promotes fibril formation (Fujiwara *et al.* 2002). Nevertheless, hyperphosphorylation of αSN may be a secondary event and not a prerequisite for the inclusion formation. In addition, under oxidative stress the tyrosine residue 125 of αSN can undergo nitration (Giasson *et al.* 2000). Similarly to phosporylation at Ser- 129,

nitration does not appear to be a requirement for αSN deposition (Gomez-Tortosa *et al.* 2002).

Given the anatomical pattern of neuropathology in PD, it was proposed that oxidative stress and dopamine contribute to the abnormal build up of αSN (Lotharius and Brundin 2002). In fact, in vitro studies have demonstrated that iron and free radical generators stimulate the production of αSN aggregates (Paxinou *et al.* 2001;Ostrerova-Golts *et al.* 2000). Further to this, studies have shown that inclusion formation is blocked by pre-treatment with antioxidants and or metal chelators (Hashimoto *et al.* 1999;Hsu *et al.* 2000). Perhaps the most direct evidence to implicate oxidative stress and dopamine in the formation of αSN aggregates comes from post mortem studies which have reported an increased oxidation state of the substantia nigra, increased iron levels, mitochondrial defects and decreases in antioxidant protective systems (Jenner and Olanow 1998). In addition, nigral dopaminergic neurons are particularly vulnerable to oxidative stress because of the dopamine auto-oxidation. *In vitro* studies have shown that overexpression of αSN induces apoptosis in the human dopaminergic cells (Xu *et al.* 2002). Therefore, the selective loss of dopaminergic neurons may be due to complex interactions between dopamine and αSN initiating a chain of events that lead to oxidative stress and increased production of free radicals. However, pathological changes in PD by no means are confined to the substantia dopaminergic neurons with many other neuronal populations including cholinergic, serotonergic and noradrenergic also been affected both in terms of αSN aggregates as well as neuronal loss (Jellinger 1997;Jellinger 1999;Jellinger 2000).

Perhaps the most studied hypothesis for the formation of αSN positive inclusions is that of impaired degradation. The ubiquitin-proteasome pathway (UPP) is responsible for degrading misfolded or damaged proteins. By inference its dysfunction would result in accumulation/aggregation of unwanted proteins such as αSN. Interestingly, two mutations parkin (Kitada *et al.* 1998) and ubiquitin C-terminal hydrolase L1 (UCH-L1) (Leroy *et al.* 1998) that encode key enzymes of the UPP become mutated in familial PD (see also section 1.1.3.1). Therefore, inhibiting the function of these enzymes by mutations could lead to disruption of UPP, impaired protein clearance and accumulation of αSN (McNaught and Olanow 2003). In support of this hypothesis, enzymatic proteasomal function has been observed to be reduced in the substantia nigra of PD patients (McNaught and Jenner 2001).

1.3.2.4 Neuronal Involvement - Lewy bodies, Lewy neurites

The most common type of αSN positive inclusion is the LB. However, the introduction of αSN IHC has revealed a wide range of αSN positive structures including "punctuate or diffuse" cytoplasmic staining, "grain-like dots" or "threads" in the neuropil, LBs with irregular shape, partially compact LBs and fully compact LBs (Kuusisto *et al.* 2003;Saito *et al.* 2003). In fact this spectrum of αSN staining has been interpreted to reflect the morphogenesis of LBs (Kuusisto *et al.* 2003). According to this hypothesis, the earliest stage comprises a punctuate and diffuse αSN accumulation that lacks immunoreactivity for Ub or a Ub binding protein known as p62 (Kuusisto *et al.* 2003). In later stages pale bodies appear. Pale bodies are immunopositive for αSN, Ub and p62 and have been reported to be more numerous in PD cases with short disease duration than those with longer disease duration (Wakabayashi *et al.* 1998) and thus thought to represent the precursors of LBs. In latter stages fully compact LBs appear (Kuusisto *et al.* 2003). The second most common αSN positive structure to be detected in the PD brain is LNs. LNs were not easily detectable before αSN IHC if at all and thus pathological significance is still largely unknown. LN type pathological change has only recently attracted attention. LNs are mostly observed in the amygdala, CA2 sector of the hippocampus and striatum (Duda *et al.* 2002;Dickson *et al.* 1991;Dickson *et al.* 1994).

The aggregation of αSN has been proposed to be initiated either in the somal or in the axonal compartment. According to the first hypothesis, although LBs are seen in the perikaryon studies suggest that protein aggregation appears initially in the axonal terminal, subsequently in the cell body and finally in the dendrite (Marui *et al.* 2002). Hence, according to this hypothesis initial pathology is in the axons leading to a "dying back" process that may cause functional and physiological disconnection of neural circuits perhaps even well before cell bodies are affected. This view is supported by studies showing initially LN type pathology (irrespective of anatomical localisation) in the brain (Braak *et al.* 2003). According to the second hypothesis LBs and LNs occur simultaneously (Katsuse *et al.* 2003).

Irrespective of the initial spatial distribution of αSN aggregates within neurons others have attempted to localise the induction site and propagation of αSN pathology within the brain. Professor Braak and colleagues have recently devised a staging system whereby αSN pathology progresses in a systematic fashion through the PD brain in six

distinct stages (Braak *et al.* 2003). According to this hypothesis αSN pathology begins in the lower brainstem and from there follows an ascending course to more rostrally situated regions eventually extending to the cerebral cortex. More specifically, based on precise patho-architectonic analysis, the earliest pathology is observed in the DMV (stage 1) whence it is thought to proceed in an upward direction via the pons (stage 2) to the midbrain (stage 3) and thence to the basal forebrain and mesocortex (stage 4) and finally to the neocortex (stages 5-6). Indeed this study reports early changes in the DMV and not in the SN. Although initially this hypothesis was very intriguing subsequent studies have questioned its reliability/validity (Parkkinen *et al.* 2005a;Jellinger 2003a).

1.3.2.5 Glial Involvement- Glial Cytoplasmic Inclusions (GCIs)

αSN aggregates are by no means found exclusively in neurons. Multiple System Atrophy (MSA) is a disease that is clinically characterised by any combination of extrapyramidal, pyramidal, cerebellar and autonomic features and neuropathologically by αSN deposition in oligodendrocytes as glial cytoplasmic inclusions (GCIs) (Papp *et al.* 1989;Gilman *et al.* 1999). GCIs can be found through out the white matter but in abundance are found in the substantia nigra, LC, inferior olives, cerebellum and regions in the spinal cord (Gilman *et al.* 1999). GCIs are sickle or flame-shaped inclusions that are positive for Ub but negative for NFs and glial fibrillary acidic protein (GFAP) (Castellani 1998). As αSN is produced in neurons the origin of αSN in oligodendrocytes has been debated. However, MSA is not the only disease where αSN positive glial inclusions are found. αSN positive inclusions in the astrocytes and oligodendrocytes in PD and DLB brains have been reported (Piao *et al.* 2000;Wakabayashi *et al.* 2000) as well as in astroglia and Schwann cells in the spinal cord in Amyotrophic Lateral Sclerosis (ALS) (Mezey *et al.* 1998).

In fact the term synucleinopathy has been recently introduced to describe several distinct neurodegenerative diseases that share, among other properties, the presence of abnormal αSN aggregates in neurons and/or glial cells. Some of these diseases include PD, MSA, DLB, sporadic and familial AD, Down syndrome and neurodegeneration with brain iron accumulation type 1 (NBIA I) ALS and pure autonomic failure (Gorell *et al.* 1994;Papp and Lantos 1994;Arawaka *et al.* 1998;Gai *et al.* 1998;Bayer *et al.* 1999b;Dickson *et al.* 1999;Mezey *et al.* 1998;Arai *et al.* 2000;Hamilton 2000;Lippa *et al.*

1999). Nevertheless, αSN aggregates are considered to be a primary causal pathology in PD, DLB and MSA.

1.3.3 Concomitant Pathologies in the PD brain

1.3.3.1 Alzheimer's Disease Type Pathology

αSN pathology is commonly found in sporadic and familial AD as well as Down syndrome (patients with a triplicate of amyloid precursor protein (APP)) with estimates ranging from 50 -60% (Hamilton 2000;Lippa *et al.* 1998;Victoroff *et al.* 1995). Vice versa, AD-related pathology (amyloid plaques and NFTs) is often found in PD brains to a varying degree (Jellinger 1997;Jellinger and Paulus 1992;Jellinger 1999;Jellinger *et al.* 2002;Jellinger 2000;Jellinger 2006;Jellinger 1991).

NFTs are one of the neuropathological hallmarks of AD (Braak *et al.* 2006a;Braak *et al.* 1998). Histologically, NFTs are clearly visualised by silver impregnation or amyloid stains and are tau-immunopositive. In AD brains, NFTs appear as flame-shaped or globoid masses of fibrous intraneuronal material, the morphology depending on the neuronal type and the developmental stage of the tangle. NFTs are accompanied by abnormal neuronal processes, i.e., neuropil threads (NTs) and dystrophic neurites that are also tau-immunoreactive. Ultrastructurally, NFTs mainly consist of dense bundles of paired helical filaments (PHFs) 8–20 nm across (Lee *et al.* 2001). PHFs may be associated with straight filaments and more complex types of fibrillar structures (Itoh *et al.* 1997;Gomez-Ramos and Moran 1998). The principal constituent of PHFs is the tau protein in its hyperphosphorylated form (Goedert *et al.* 1995). Immunohistochemistry also reveals the variable incorporation of Ub (Bancher *et al.* 1989) and a range of other proteins into NFTs (Lee *et al.* 2001). In AD brains, NFTs occur in the cerebral cortex and in several subcortical regions (Braak and Braak 1991). Within cortical areas, the distribution of NFTs suggests a hierarchical progression that first involves the transentorhinal cortex, from where the pathology spreads via limbic areas to the isocortex (Braak and Braak 1991). A growing number of studies have demonstrated tau-immunoreactive neuronal or glial inclusions in a variety of conditions beyond AD

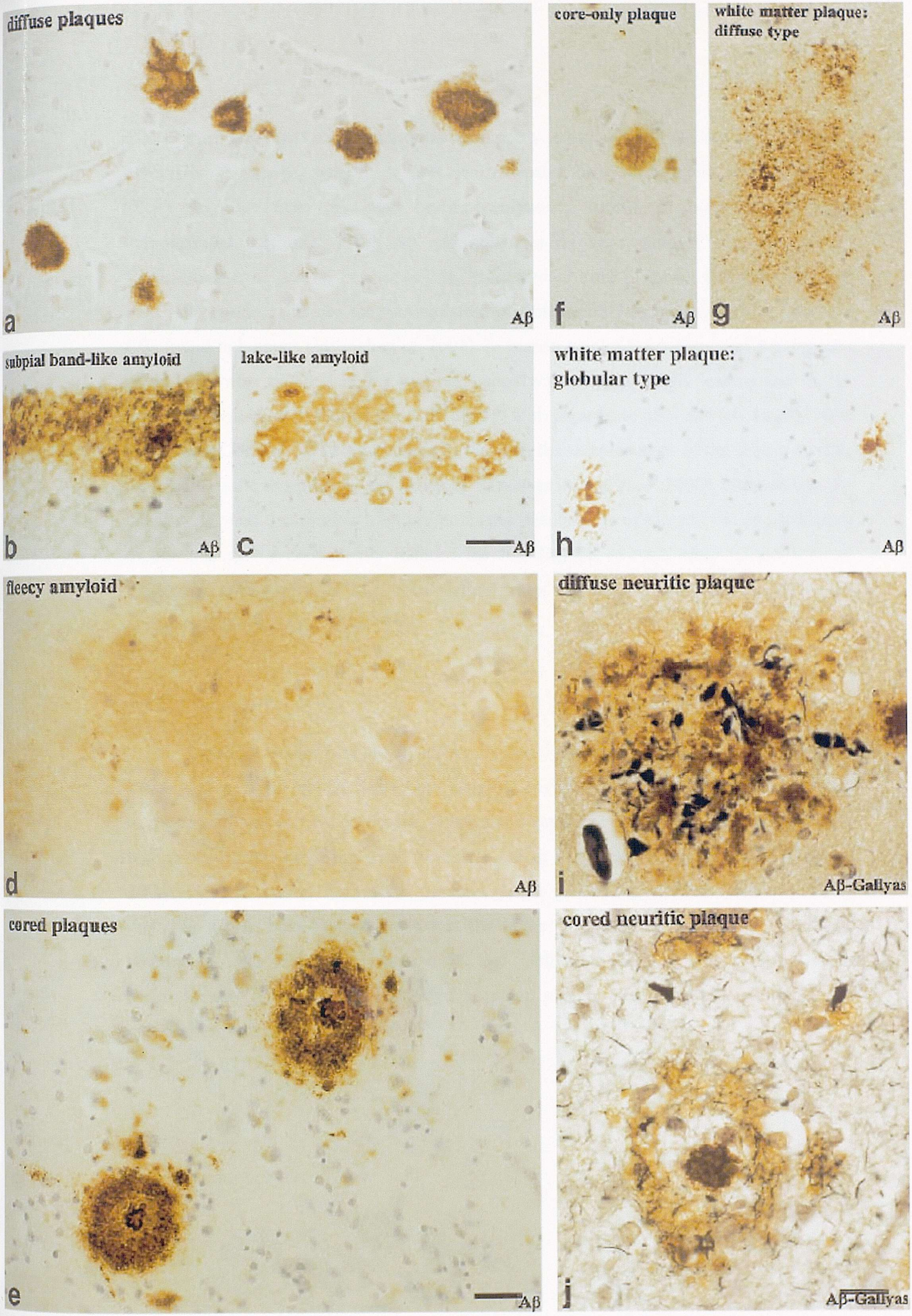
including progressive supranuclear palsy, Pick's disease, corticobasal degeneration and frontotemporal dementia with parkinsonism linked to chromosome 17 (Sorrentino and Bonavita 2007;Goedert 2004;Dickson 1998) collectively referred to as taupathies.

Amyloid plaques are another major histopathological hallmark found in the AD brain. The amyloid-β-peptide (Aβ) deposits extracellularly forming amyloid plaques in AD brain. There are two major types of amyloid plaques in AD brain: neuritic plaques and diffuse plaques. Neuritic plaques contain dense bundles of amyloid fibrils and are surrounded by dystrophic neurites, astrocytes, and microglia (Finder and Glockshuber 2007). Diffuse plaques contain nonstructured amyloid and are not surrounded by dystrophic neurites. There are studies suggesting that neuritic plaques may develop from diffuse plaques but this awaits verification. Amyloid plaques are formed of insoluble Aβ peptides that are cleaved from APP (Small and McLean 1999). Aβ peptides occur principally in two lengths, 40 or 42 amino acids, the latter being more susceptible to aggregate. In AD, the proportion of Aβ42 is increased, and the mutations causing familial forms of AD enhance the production of Aβ42. Interestingly, the levels of Aβ42 seem to be central to the pathogenesis of AD and correlate with the cognitive impairment in AD (Younkin 1995).

Although, plaques have been traditionally classified as either neuritic or diffuse their detection using IHC does not readily distinguish these plaque subtypes. Recently using Aβ IHC a wide range of morphologies of amyloid plaques has been described in AD but these different subtypes insofar have only been reported in the medial temporal lobe where NFTs are also found in abundance (Thal *et al.* 2000). On the basis of that amyloid plaques detected with Aβ IHC can be assigned to one of the following classes (see also Figure1.4): diffuse plaque, subpial band-like Aβ, lake-like amyloid, fleecy amyloid, cored plaque (classic/neuritic plaque), core-only plaque, white matter plaque.

Figure 1.4 Plaque type using Aβ IHC

(Following page) Diffuse plaques (a, i). a: diffuse non-neuritic plaque that is sharply delineated and evenly distributed, i: diffuse neuritic plaque that conatins Gallyas positive dystrophic neurites. Subpial band-like plaques (b). b: sharply-delineated, evenly distributed band-like Aβ, lake-like amyloid (c). c: sharply delineated, evenly distributed Aβ-deposits, fleecy amyloid (d). d: cloud like deposits, cored plaque (e, j). e: cored non-neuritic plaque; sharply delineated Aβ-deposits with an amyloid core in the centre surrounded by a corona of diffuse Aβ-deposits. Between core and corona is a small gap without Aβ. This non-neuritic plaque is free of dystrophic neurites containing neurofibrillary lesions as detectable with the Gallyas silver method. j: Cored neuritic plaque: cored plaque which contains Gallyas positive dystrophic neurites. Core-only plaque (f): f: pure, sharply delineated amyloid cores without associated diffuse Aβ-deposits, white matter plaque (g, h). g: diffuse type that is sharply delineated, evenly distributed Aβ-deposits within the white matter and h: globular type sharply delineated Aβ-deposits in the white matter which contain globular aggregates of Aβ. Calibration bars: a, b, e, f, g, h= 40 mm; c= 200 mm; d, i, j= 28 mm.(Image taken from (Thal *et al.* 2000)


diffuse plaques
a
Aβ
core-only plaque
f
Aβ
white matter plaque:
diffuse type
g
Aβ
subpial band-like amyloid
b
Aβ
lake-like amyloid
c
Aβ
white matter plaque:
globular type
h
Aβ
fleecy amyloid
d
Aβ
diffuse neuritic plaque
i
Aβ-Gallyas
cored plaques
e
Aβ
cored neuritic plaque
j
Aβ-Gallyas

The fact that αSN inclusions commonly co-exist with AD-related changes, especially in patients with more prevalent/extensive amyloid plaques and NFTs (i.e. DLB), suggests that they may be mechanistically linked. *In vitro* models have demonstrated that αSN binds Aβ and enhances its aggregation (Yoshimoto *et al.* 1995;Jensen *et al.* 1997). Further to this, in transgenic mice (a model that links AD and PD), Aβ peptide enhanced the accumulation of fibrillar αSN to inclusions that resemble LBs found in humans (Masliah *et al.* 2001). In human studies the presence of Aβ deposits in the neocortex of DLB brains was associated with increased αSN lesions and higher levels of insoluble Aβ (Pletnikova *et al.* 2005). With regards, to αSN and tau, studies have shown that LBs and NFTs are commonly found in the same neurons of the limbic system, especially in the amygdala in the brains of AD cases (Marui *et al.* 2000). Giasson and co-workers showed that *in vitro* αSN promotes the fibrillisation of tau and the co-incubation of tau and αSN synergistically induced the fibrillisation of each other (Giasson *et al.* 2003). In addition, αSN has been demonstrated to bind to tau and stimulate its phosphorylation (Jensen *et al.* 1999).

1.3.3.2 Vascular Pathology

Cerebrovascular pathology occurs frequently in the ageing brain and often co-exists with AD, PD and DLB. Cerebral amyloid angiopathy (CAA), the deposition of Aβ in meningeal and cortical arterioles, venules and capillaries, is a major histopathological feature in the AD brain but often is also found in PD (Zekry *et al.* 2002;Rektor *et al.* 2006). Several recent studies on the prevalence of cerebrovascular lesions (CVLs), including CAA, have demonstrated that the higher frequency is found in AD patients whereas in PD and DLB cases it is low (Jellinger 2003b). Therefore, although neurovascular dysfunction could have a major role in the pathogenesis of AD its contribution to PD is less likely clear.

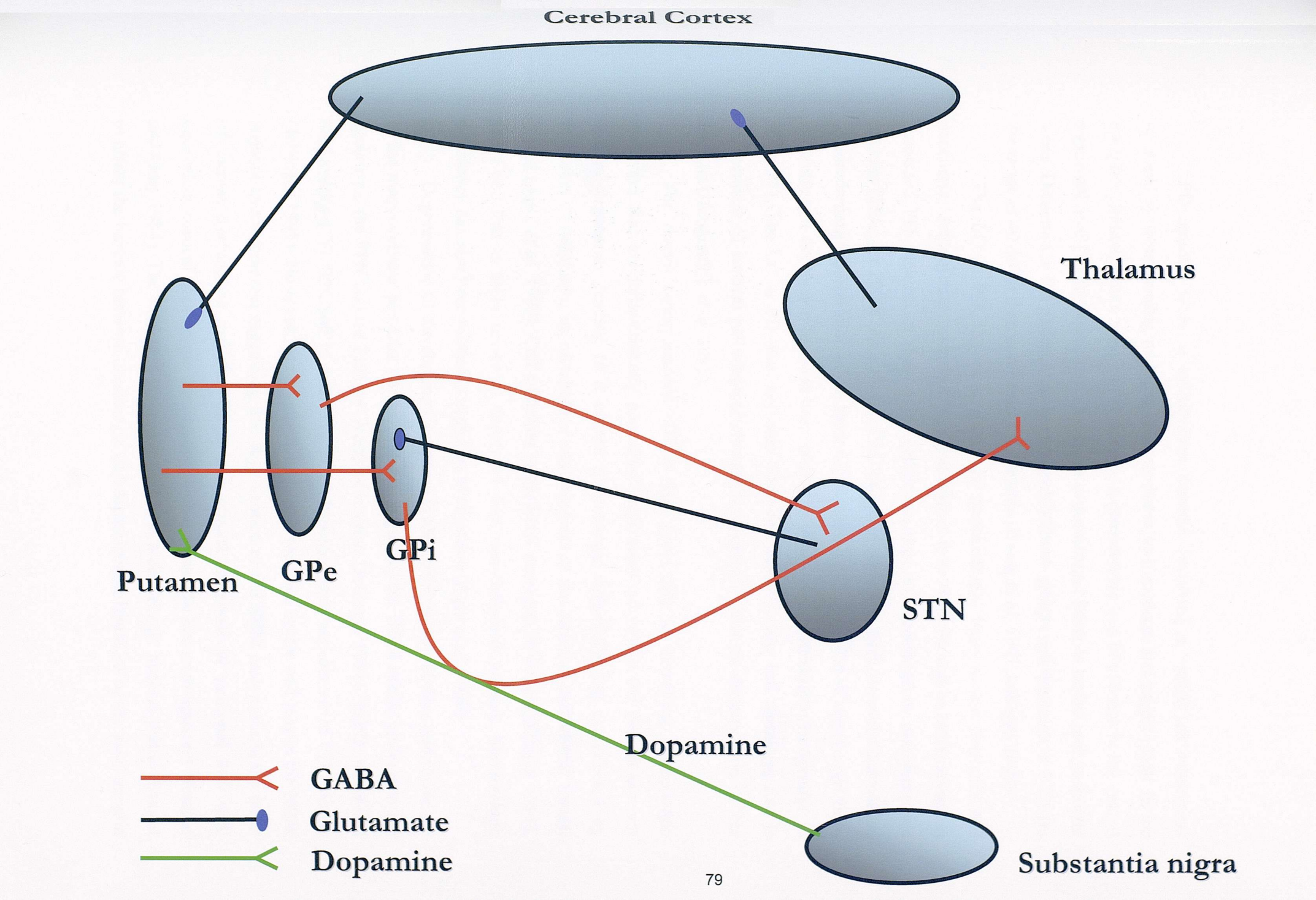
1.3.4 Neurochemical Deficits Associated with Motor and Neuropsychiatric Deficits in PD

The classical motor signs in PD are ascribed to dopaminergic cell loss in the SNc (Fearnley and Lees 1991). Progressive degeneration of the substantia nigra in PD leads to depletion of striatal dopamine with functional disturbances in the complex regulatory loops of the basal ganglia resulting in the clinical motor signs and symptoms (Figure 1.5). Neuronal damage in PD is not random (refer to section 1.3.1.1) and progresses slowly but unremittingly until a certain threshold is reached for the clinical manifestation of the disease. Degeneration of the nigro-striatal dopaminergic system has also been considered responsible for subtle cognitive changes such as impaired simultaneous test performance or inefficient reaction of retrieval process and other fronto-striatal deficits in PD resulting from disturbances in caudate outflow into the motor loop interconnecting the ventral striatum and frontal lobe (Pillon *et al.* 1994;Rolls 1994).

Figure 1.5 Circuitry of the basal ganglia

(Following page) Simplified schematic representation of the circuitry through the basal ganglia. Input arriving in the striatum is relayed to other basal ganglia nuclei through two pathways, known as the direct and indirect pathway. The direct pathway is the striatal projection to the internal segment of the globus pallidus. These medium spiny neurons express D1 receptors that are coupled via G_s proteins, to stimulation of adenyl cyclase. This population of neurons receive excitatory input from the SNc dopamine and glutamatergic cortical projections. Stimulation of the direct pathway leads to increased, inhibitory, GABA transmission in the internal segment of the globus pallidus. As a result of inhibiting these neurons, which exert an inhibitory (GABAergic) effect on the thalamus, activation of the direct pathway leads to disinhibition of the thalamus. The indirect pathway runs from the putamen to the internal globus pallidus initially via neurons projecting to the external segment of the globus pallidus. This population of striatal medium spiny neurons express dopamine D2 receptors, which are coupled via G_i proteins, to inhibition of adenylyl cyclase. Therefore, dopamine input from the SNc inhibits the activity of the indirect pathway. From the external globus pallidus a GABAergic projection runs to the subthalamic nucleus, which sends excitatory, glutamatergic input to the neurons of the internal globus pallidus. Activation of the GABA striatal projection to the external globus pallidus tends to suppress its inhibition of the subthalamic nucleus, increasing the excitatory drive to the internal globus pallidus and hence increasing inhibition of the thalamus. The pathophysiology in PD arises from an overactivity of the indirect pathway whereas the opposite is true for hyperkinetic disorders.

Abbreviations: STN: subthalamic nucleus, GPe: external segment of the globus pallidus, GPi: internal segment of the globus pallidus.


Cerebral Cortex
Thalamus
Putamen
GPe
GPi
STN
Dopamine
GABA
Glutamate
Dopamine
Substantia nigra

PD appears to be a multisystem disorder involving a variety of subcortical neuronal systems causing multiple neuromediator dysfunctions that might result in the complex clinical picture seen. In PD, there is a considerable loss of neurons in the ventral tegmental area (VTA) which provides major dopaminergic input to limbic and prefrontal areas. Dementia in PD patients has been associated with greater cell depletion in VTA (in the range of 40-60%) than non-demented patients (Zweig *et al.* 1993;Jellinger 1991).

The LC in PD, the main source of noradrenergic input to the forebrain and neocortex, demonstrates neuronal loss that ranges from 40-50% and is more severe in demented PD subjects or those with depression than non-demented or non-depressed patients (Jellinger 2000). As a result of LC neuronal depletion there is severe deprivation of noradrenergic innervation of the forebrain and neocortex the effect of which apart from dementia and depression has also been related to autonomic dysfunction (Hoogendijk *et al.* 1995). The LC in PD does not only show cell loss but also cell shrinkage that is independent of cortical pathological changes suggesting a primary degeneration of this nucleus (Hoogendijk *et al.* 1995).

The dorsal raphe nucleus (DRN) that gives rise to ascending serotonergic pathways that are bidirectionally connected with many centres in the central nervous system degenerate leading to a central serotonergic deficiency that is reflected by reduction of serotonin, its metabolites and receptors in the striatum and medial frontal cortex (Agid *et al.* 1990). Cell depletion in the DRN averages 20-40% ((Jellinger 1991). DRN cell loss is more severe in depressed than non-depressed patients. Serotonergic deficiency has also been related to cognitive dysfunction (Agid *et al.* 1990).

Degeneration of the cholinergic system is characterised by cell loss and shrinkage in the magnocellular posterior parts of the Ch_4 region of the NBM which projects to the neocortex, the PPN and the Edinger-Westphal nucleus (Jellinger 1991). In PD, NBM cell loss averages 30-40% and is much higher in demented than non-demented PD patients (Jellinger 1991). However, some authors have also reported severe cell loss in the NBM without overt dementia suggesting that degeneration of the NBM may precede the onset of mental dysfunction and that there is a critical threshold of neuronal loss with equivalent cortical cholinergic denervation before dementia becomes apparent (Gaspar and Gray 1984). The PPN pars compacta (PPNr) is a cholinergic nucleus that is thought to affect the balance between cholinergic and dopaminergic functions of the basal ganglia

(Graybiel *et al.* 1990;Steriade M 1990). Cell loss in the PPNr occurs in PD which ranges from 36 to 57% however, the clinical significance of this neuronal depletion remains unclear (Zweig *et al.* 1989). The Edinger-Westphal nucleus suffers a 54% neuronal loss in PD (Hunter 1985). This nucleus gives rise to cholinergic fibres to the ciliary ganglion regulating pupilloconstriction (Bender 1980) which may explain the abnormally slow pupillary responses to light in PD (see also section 1.2.2.1.5).

1.3.5 Clinico-pathological Correlations in PD Patients with Neuropsychiatric Deficits

One approach to neuropathological research is that of clinico-pathological correlations. This process of correlation permits the optimum characterisation of the brain in a diseased state as it examines the relationship between two negative events that characterise a disease; that of pathological change and the occurrence of clinical symptoms.

One of the most studied neuropsychiatric deficits in PD is dementia. Although, several clinico-pathological studies have been published, the underlying pathological conditions responsible for dementia in PD have been a matter of controversy, both in terms of site and type. The presence of LBs in limbic and neocortical regions has been suggested as a cause for dementia but this does not account for the co-existent AD type changes that are often found alongside αSN pathology. In cases with mixed pathology it is indeed difficult to determine whether αSN lesions or AD-type pathology are responsible for dementia, particularly in a clinically heterogeneous PD cohort where dementia may be present along with other neuropsychiatric deficits and medications that may aggravate the clinical picture. In addition, it remains debatable whether VH represent a primary clinical feature in disease progression reflecting specific neuroanatomical loci of pathology or a secondary feature of dementia, cognitive impairment and or drug treatment. Indeed, there are very few clinico-pathological studies on VH in comparison to those on dementia. Furthermore, the contribution of vascular changes (e.g. CAA) to the development of dementia and VH in PD has not been closely studied. The uncertainty of the pathological and anatomical basis of dementia and VH in PD more likely stems from methodological differences (case selection etc), differences in pathological protocols (e.g. staining methods, quantification methods etc) and different diagnostic criteria (e.g. early studies may have included cases with dementia as the presenting symptom followed by

parkinsonism (i.e. DLB)) used in each study. Furthermore, overlapping behaviours in PD complicate the analysis and make it difficult to examine changes related to a single clinical feature in isolation. Indeed, most of the previous reports that have focused on a single behavioural change fail to take into account concomitant neuropsychiatric symptoms in their subjects. On the other hand, the patho-anatomical bases of anxiety in PD are largely unknown as thus far there has been no clinico-pathological study to examine the pathological nature of this neuropsychiatric symptom.

Although, PD patients often develop dementia and VH later in the disease course, these neuropsychiatric manifestations predominate the clinical picture of DLB cases (Ince *et al.* 1998;McKeith *et al.* 2005). Clinicopathological studies in DLB cohorts point to LB densities in limbic lobe as pathological correlates of dementia in these patients (Uchikado *et al.* 2002). However, LB burden in limbic structures has also been associated as the pathological substrate of dementia in PD case series (Kovari *et al.* 2003;Mattila *et al.* 2000;Hurtig *et al.* 2000). In addition, studies on VH in DLB, PD and PDD point to LB pathology in amygdala as an important correlate of this clinical feature (Harding *et al.* 2002a;Harding *et al.* 2002b). On the whole, these studies demonstrate that the pathological correlates of dementia and VH in DLB and PD seem to converge. In fact, the relationship of DLB to PDD is unresolved and the topic of current research efforts. Several studies have now shown that with most diagnostic measures, there are few significant differences between DLB and PDD (Aarsland *et al.* 2004;McKeith and Mosimann 2004). Studying the patho-anatomical correlates of VH and dementia in PD one will by inference add to a greater understanding of the association between these two diseases.

1.4 Aims of the Thesis

From the introduction presented above it is clear that non-motor complications are frequent in PD patients and that in some cases they may overshadow the motor aspects of the disease. Among the most prominent non-motor deficits are neuropsychiatric complications with dementia, VH and anxiety having a major impact on the clinical course and prognosis of PD. Given the on-going controversy concerning the pathological and anatomical nature of these deficits in PD the primary aim of this thesis was to examine possible associations between these deficits and pathology in specific anatomical areas as a means of providing a better insight into their aetiopathogenesis. Having examined αSN pathology in a relatively large number of regions in the PD brain the need arose to define whether this PD-related pathological change followed the proposed predicted caudo-rostral spread as suggested by Professor Braak and colleagues. Thus, quite apart from the pathological basis of neuropsychiatric features of PD, a secondary aim of this thesis was also to examine the topographical distribution of αSN lesions in the PD brain.

The specific aims of this thesis were as follows:

1) To determine the relative contribution of αSN, AD-type pathology and vascular pathology in neocortical and subcortical regions known to play a role in cognitive function to dementia and VH in PD subjects presenting with these symptoms in isolation or in combination (Chapter 3).
2) To examine the nature and extent of striatal pathology in PD brains and study its clinical relevance to dementia and VH (Chapter 4).
3) To study the pathological and anatomical bases of anxiety in PD (Chapter 5)
4) To examine the topographical distribution of αSN pathology in a large series of PD brains (Chapter 6).

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Human Brain Samples

All post-mortem human brain specimens used in this research were obtained from the UK Parkinson's Disease Society Tissue Bank at Imperial College, London (UKPDSTB). The cases included in this thesis are those that were available from the UKPDSTB up to 15th of July 2006. A summary of the cases used and the brain areas examined in each study/experimental chapter are shown in Table 2.1 and 2.2. According to the dissection protocol used in the UKPDSTB, the brains were weighed, grossly examined and hemisected. The half used in this study was immersed in 10% buffered formalin for at least 2 weeks. Formalin-fixed, paraffin-embedded tissue was provided either in blocks from which sections were subsequently cut (as described in 2.2.3) or in 8μm sections on SuperFrost Plus slides.

2.1.2 Antibodies

The antibodies used in this research, their supplier, dilution and pre-treatment are summarised in Table 2.3. The selection of the αSN Ab used in the present research was based on its consistency and strong immunoreactivity with a variety of αSN-containing structures shown in a comparative study of different αSN Abs performed by Croisier et al., (Croisier *et al.* 2006). The selection of the tau and tyrosine hydroxylase (TH) Abs was based on their high specificity and sensitivity on human tissue shown in a comparative study of different tau and TH Abs performed by the UKPDSTB during the process of optimisation of Abs for diagnostic purposes. In addition, the tau antibody (clone AT8) used in this work is routinely used across many different centres for diagnostic purposes (Alafuzoff *et al.* 2006) and it has also recently used as the Ab of choice for staging AD neurofibrillary pathology according to the Braak and Braak scheme (Braak *et al.* 2006a).

The 1E8 Ab was a gift from GlaxoSmithKline (GSK), via Dr. Stephen Gentleman and was chosen on the basis of its intense staining pattern that covered the full range of Aβ-related pathology; diffuse and cored plaques (for definition of plaque types see section 2.2.5) and deposits in the walls of cerebral blood vessels (Gentleman *et al.* 1997). Further details about the antibodies are provided in section 2.2.4.3.

Table 2.1 Summary of cases examined in each chapter

(Following page)

Case Number	Gender	Age at Death (years)	Chapter studied
1	f	87	(6)
2	m	84	(3,4,6)
3	m	77	(6)
4	m	68	(excluded: DLB/AD)
5	f	80	(3,4,6)
6	m	72	(6)
7	m	78	(3,4,5,6)
8	m	72	(6)
9	f	86	(6)
10	f	81	(excluded: selection criteria not met)
11	m	71	(3,4,6)
12	m	42	(excluded: MSA)
13	m	78	(3,4,6)
14	m	79	(3,4,6)
15	m	70	(3,4,6)
16	f	85	(3,4,6)
17	m	75	(6)
18	m	82	(3,4,6)
19	f	72	(5,6)
20	m	75	(3,4,6)
21	m	76	(3,4,6)
22	f	75	(3,4,5,6)
23	m	82	(3,4,5,6)
24	m	81	(3,4,6)
25	f	63	(excluded: MSA)
26	m	74	(excluded: DLB)
27	m	74	(6)
28	m	82	(6)
29	m	76	(3,4,5,6)
30	m	73	(3,4,6)
31	m	81	(excluded: PSP)
32	m	89	(3,4,5,6)
33	f	74	(6)
34	f	84	(3,4,6)
35	m	77	(excluded: PSP)
36	m	77	(3,4,5,6)
37	m	87	(5,6)
38	f	79	(excluded: essential tremor)
39	f	82	(5,6)
40	m	92	(excluded: aging)
41	m	77	(5,6)
42	f	79	(3,4,6)
43	m	86	(excluded: MSA)
44	m	87	(3,4,6)
45	m	80	(3,4,6)
46	f	68	(excluded: aging)
47	m	78	(excluded: PSP)
48	m	71	(excluded: Possible Fragile-X ataxia)
49	m	75	(6)
50	f	82	(6)
51	m	80	(6)
52	f	71	(6)
53	m	76	(excluded: essential tremor)
54	m	75	(5,6)
55	f	76	(6)
56	m	54	(3,4,6)
57	m	83	(6)
58	f	58	(3,4,5,6)
59	m	81	(6)
60	m	84	(3,4,6)
61	m	78	(6)
62	m	73	(6)

Continued

63	f	80	(3,4,6)
64	m	88	(excluded: PSP)
65	f	84	(excluded: aging)
66	m	82	(6)
67	m	83	(6)
68	m	82	(6)
69	m	82	(3,4,6)
70	m	75	(6)
71	m	81	(excluded: aging)
72	m	62	(3,4,6)
73	m	73	(excluded: Frontotemporal lobar degeneration & MND)
74	m	85	(3,4,5,6)
75	m	66	(excluded: PSP)
76	m	61	(excluded: DLB)
77	m	58	(excluded: selection criteria not met)
78	m	79	(6)
79	f	78	(6)
80	m	71	(excluded: MSA)
81	m	73	(6)
82	m	82	(excluded: DLB)
83	f	77	(excluded: MSA)
84	m	86	(6)
85	m	78	(excluded: selection criteria not met)
86	f	87	(6)
87	f	80	(excluded: PSP)
88	f	81	(6)
89	m	70	(excluded: DLB)
90	f	83	(6)
91	m	50	(6)
92	m	83	(6)
93	f	81	(6)
94	m	75	(5,6)
95	m	74	(3,4,5,6)
96	m	88	(excluded: PSP)
97	f	88	(6)
98	m	75	(excluded: DLB)
99	m	83	(6)
100	m	81	(6)
101	m	69	(excluded: selection criteria not met)

Table 2.2 Summary of brain areas examined in each chapter

(Following page)

Brain Area	Chapter 3	Chapter 4	Chapter 5	Chapter 6
Superior Frontal gyrus	X	X		X
Cingulate gyrus	X		X	
Temporal Cortex	X	X	X	
Entorhinal Cortex	X	X	X	X
Hippocampus CA2 sector	X	X	X	X
Subiculum	X		X	
Amygdaloid Complex	X		X	X
Nucleus Basalis of Meynert	X		X	X
Caudate Nucleus		X		
Putamen		X		
Nucleus Accumbens		X		
Substantia Nigra				X
Locus Coeruleus			X	X
Raphe Nuclei			X	
Dorsal Motor Nucleus of Vagus				X
Spinal Cord				X
Total	8	7	9	9

Table 2.3 Description of antibodies used throughout this research

(Following page)

Name	Clone/Type	Epitope (Immunogen)	Pre-Treatment	Dilution	Source
αSN	42 (mouse monoclonal)	rat αSN_{15-123}	For dilution 1/1000 - 80% FA, 60min For dilution 1/300 - 100% FA, 5min	1/1000 for chapters 3, 4 and 5 and 1/300 for chapter 6	Becton-Dickinson
Tau	AT8 (mouse monoclonal)	tau phosphorylated at Ser-202/Thr-205	None	1/800	AutogenBioclear
Aβ	1E8 (mouse monoclonal)	residues 18-22 of the Aβ peptide	80% FA, 8min	1/1000	**
Tyrosine Hydroxylase	1B5 (mouse monoclonal)	carboxyl terminal end of the mouse tyrosine hydroxylase molecule	Ht EDTA	1/30	Vector

Abbreviations: αSN: alpha-synuclein, Aβ: beta-amyloid, FA: formic acid, EDTA: ethylenediaminetetraacetic acid, Ht: high temperature
**: gift from Dr. Stephen Gentleman

2.1.3 Chemicals

All chemical used in this research were pursued from VWR (www.VWR.com).

2.2 Methods

2.2.1 Neuropathological Assessment of Cases

Neuropathological assessment and diagnosis was performed by two clinical neuropathologists (Prof. Manuel B. Graeber and Dr. Federico Roncaroli) and one research neuropathologist (Dr. Stephen M. Gentleman), all from the Department of Neuropathology, Imperial College London. The diagnosis was based on slides provided by the UKPDSTB for αSN, tau and β-amyloid immunohistochemistry of the superior frontal gyrus, hippocampus, and midbrain. Screening of cases for confounding pathology was based on hematoxylin and eosin examination of a standard series of 18 tissue blocks following a standardized dissection procedure (Vonsattel *et al.* 1995). Neuropathological diagnosis was performed using international neuropathological consensus criteria for a definite diagnosis of PD (http://www.ICDNS.org). The extent of AD (both β-amyloid and tau) isocortical and/or entorhinal pathology was assessed using the grading system posted at http://www.ICDNS.org as well as the Braak and Braak staging to stage tangle pathology using AT8 immunohistochemistry (Braak *et al.* 2006a). A representative neuropathological report of a PD case is provided in appendix I.

2.2.2 Clinical Assessment of Cases

Clinical profiles of cases were compiled retrospectively from clinical records which included in all cases specialist secondary care physicians: consultant neurologist, geriatrician and psychiatrist notes by a movement disorder neurologist (Dr. Ronald K.B. Pearce). This clinical documentation was complemented by nurse specialist and therapist letters. Only subjects who had been evaluated by a clinician within 2 years prior to death

and had complete clinical histories were included in this work in order to reduce the possibility of incomplete ascertainment. The clinical diagnosis of PD followed established criteria whereby PD was considered to be present if the patient had at least 2 of the 4 cardinal symptoms (rigidity, hypokinesia, resting tremor and postural instability) and exhibited a positive response to levodopa (Daniel and Lees 1993) with a typical progressive course. The diagnosis of DLB was made if dementia preceded extrapyramidal symptoms by 2 years or they developed together within a 12-month period (McKeith *et al.* 1996). Patients were classified with probable DLB when cognitive impairment was accompanied with at least 2 of the 3 core features (extrapyramidal symptoms, fluctuating cognition and visual hallucinations) and with possible DLB when only 1 of these core features was present (McKeith *et al.* 1996). Patients with PD who developed late dementia (> 2 years after motor symptoms) were classified as PD with dementia (PDD) (Hurtig *et al.* 2000). The diagnosis of dementia was established on the basis of a recorded clinical diagnosis by the treating physician and satisfied the DSM-IV (American Psychiatric Association 1994) and ICD-10 clinical criteria for dementia. The diagnoses of visual hallucinations, anxiety, depression, delusions and paranoia were made by the treating physician and were clearly documented in the medical files. A representative clinical summary of a PD case is provided in appendix I.

2.2.2.1 Clinical Assessment of Signs and Symptoms

Demographic data, family history, gender, age at disease onset, age at death, duration of disease, laterality and treatment were abstracted and reviewed systematically with assistance of Dr. Ronald K.B. Pearce. In addition to these standard clinical parameters, particular attention was paid to the assessment of the clinical features reported. These included presenting symptoms (within 2 years after diagnosis: tremor, drooling, gait-balance abnormalities, akinetic-rigid, stiffness, cognitive dysfunction and autonomic deficits), motor deficits (>2 years after diagnosis: tremor, drooling, gait-balance abnormalities, akinetic-rigid, stiffness, laterality of hemi-parkinsonian signs and falls), neuropsychiatric complications (dementia, visual hallucinations, anxiety, depression, delusions and paranoia) and autonomic deficits (such as diarrhoea,

constipation, orthostatic hypotension, bladder dysfunction, sexual dysfunction, skin rash, speech problems, swallowing problems, drooling and sleep problems). Of the 101 cases included in this work, the clinical phenomenology of 81 cases was thoroughly examined. The incidence of the abovementioned deficits in those 81 cases is shown in figures 2.1, 2.2, 2.3 and 2.4. As this work was based on retrospective data, the clinical severity of presenting symptoms (within 2 years of diagnosis), motor deficits (>2 years of diagnosis) and neuropsychiatric complications was measured on a semi-quantitative global impression scale (0: absent, 1: mild, 2: moderate and 3: severe) with assistance of Dr. Ronald K.B. Pearce. A mild score indicates no significant disability or interference with every day living while a moderate score implies more troubling symptoms with some impact on quality of life while a severe score would entail significant disability/ impairment with profound disruption of activities of daily living and loss of independence. Judgments qualifying severity were very often present in the original clinical correspondence which, in all cases, provided a detailed narrative history of each individual's illness as reflected in GP, nurse specialist, neurologist/physician, geriatrician, psychiatrist and therapist letters. For dementia specifically the criteria used for the different grades are as follow: 0= No cognitive impairment recorded, 1= Mild dementia with minor impairment of activities of daily living (ADL) but with largely retained independence, 2= Moderate dementia with some loss of independence and ability to perform ADL with some supervision required and 3= Severe dementia with constant supervision/placement in institution/ inability to perform ADL. All data were entered on a database for subsequent analysis. Retrospective chart analysis from movement disorders specialists is a well accepted method of case ascertainment and has been frequently used in clinico-pathological studies involving both dementia and parkinsonism (Papapetropoulos *et al.* 2005b;Papapetropoulos *et al.* 2005a;Litvan *et al.* 1998).

Figure 2.1 Mode of presentation (presenting symptoms within 2 years of diagnosis; n=81)

(Following page)

Mode of Presentation

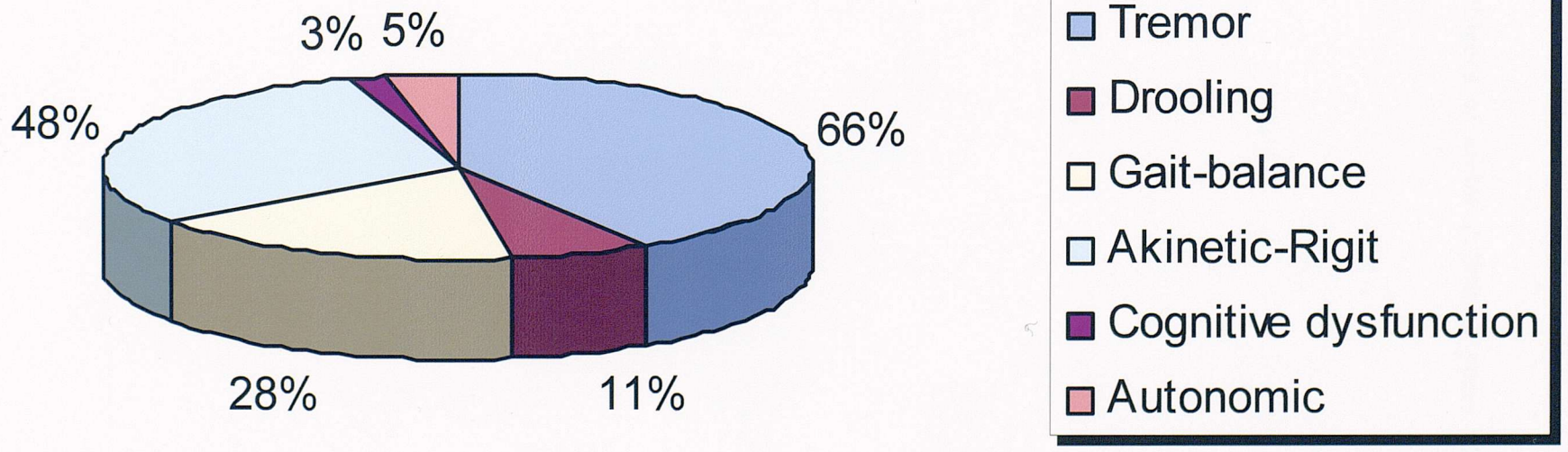
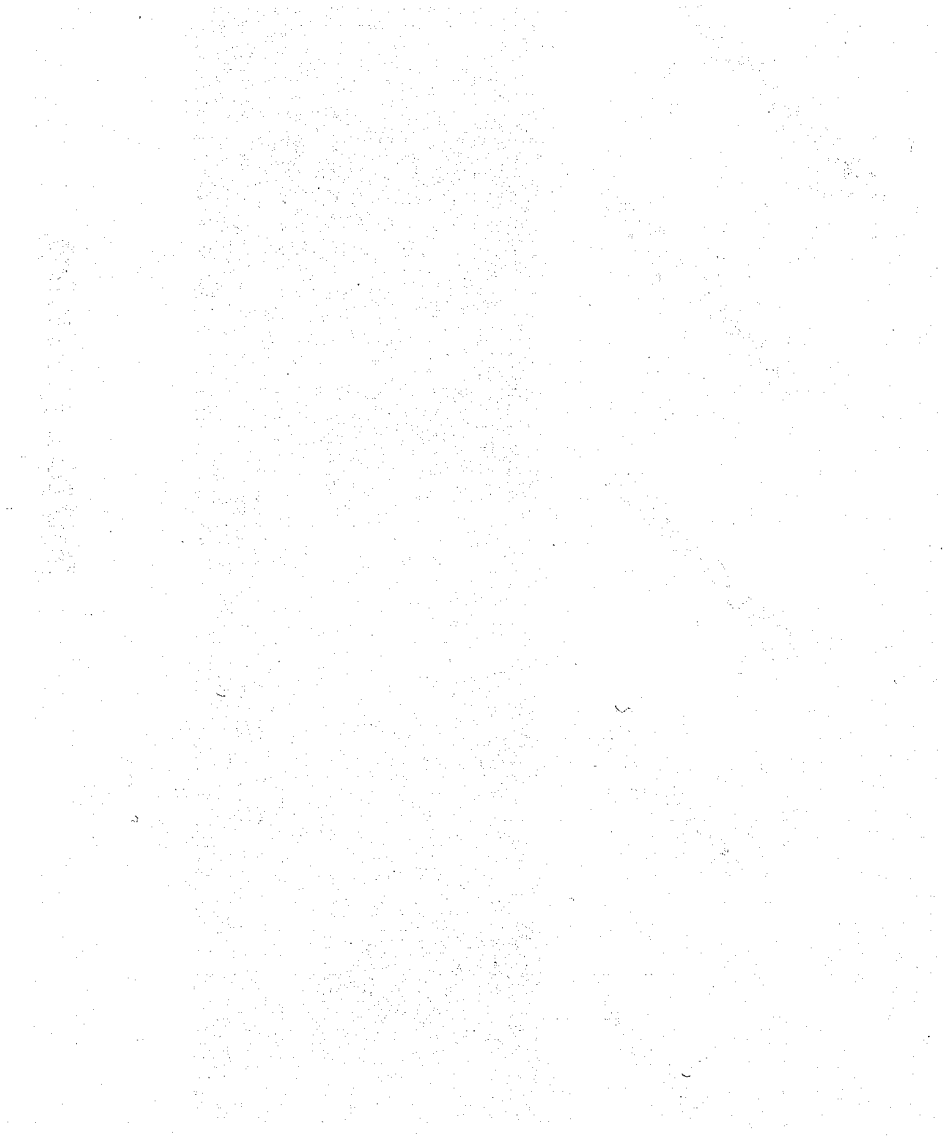


Figure 2.2 Cumulative incidence of motor deficits 2 years after clinical diagnosis (n=81).

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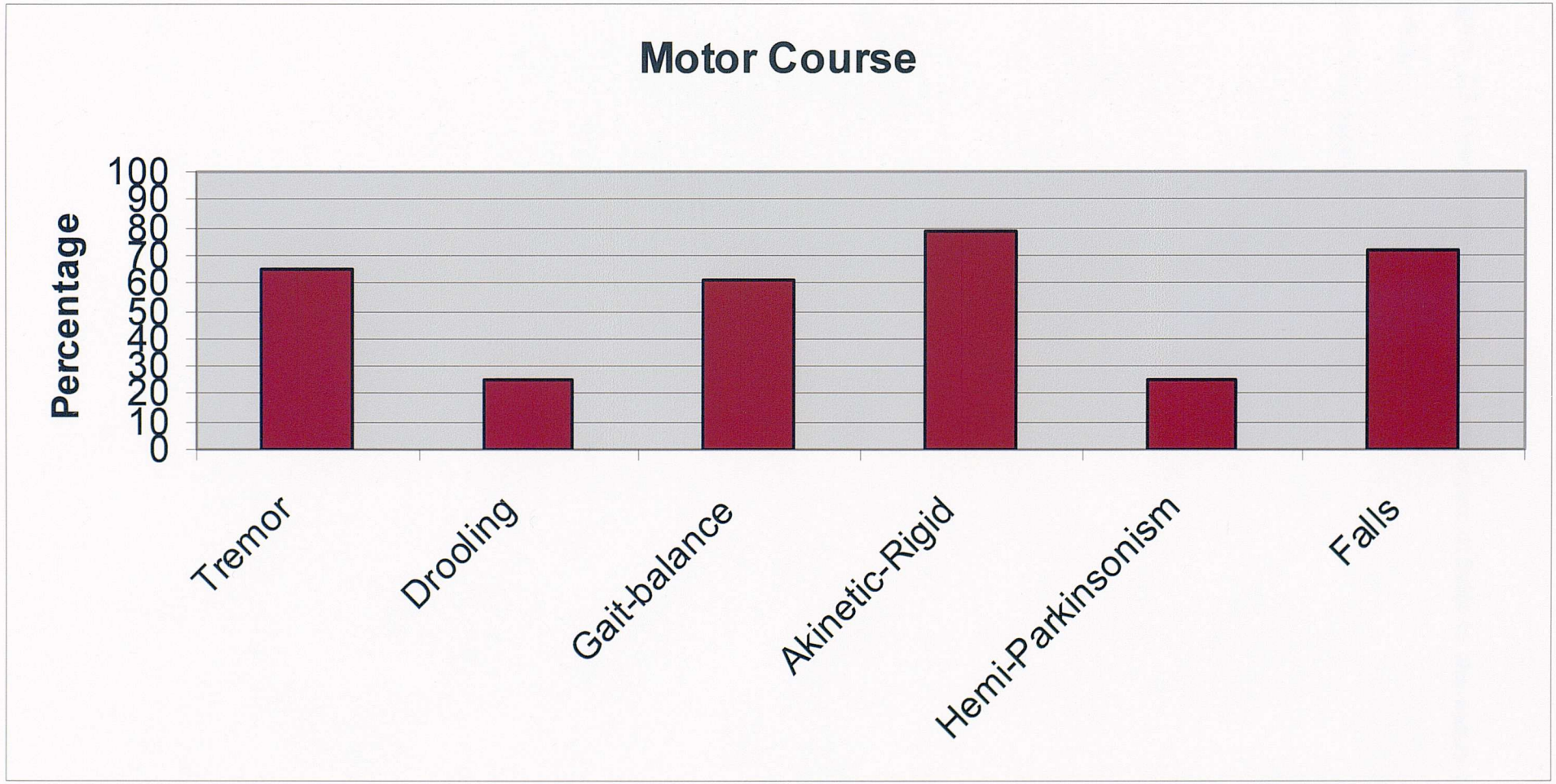

Motor Course
Percentage
100
90
80
70
60
50
40
30
20
10
0
Tremor
Drooling
Gait-balance
Akinetic-Rigid
Hemi-Parkinsonism
Falls

Figure 2.3 Cumulative incidence of autonomic deficits in the entire clinical course (n=81).

(Following page)

Autonomic Dysfunction

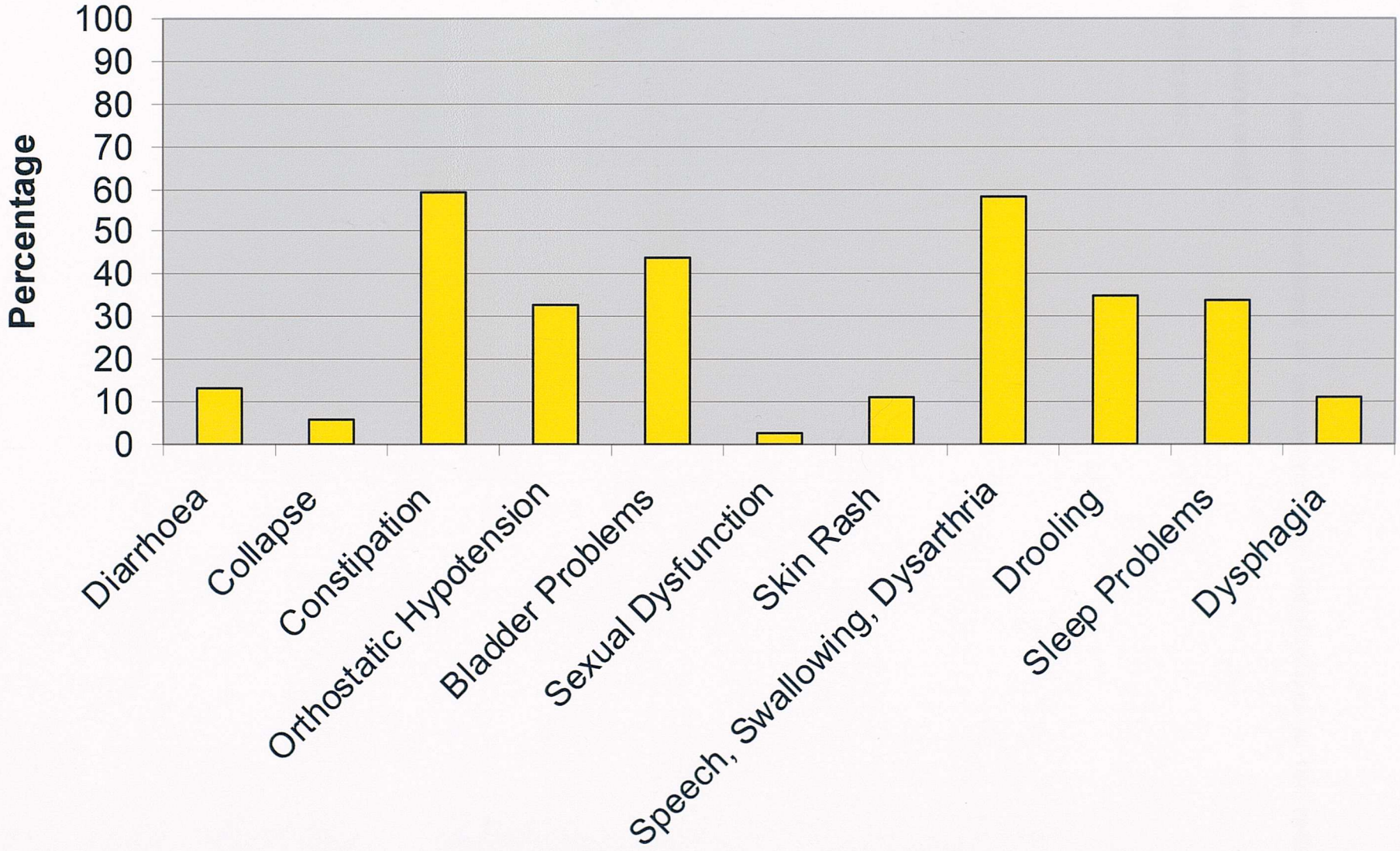
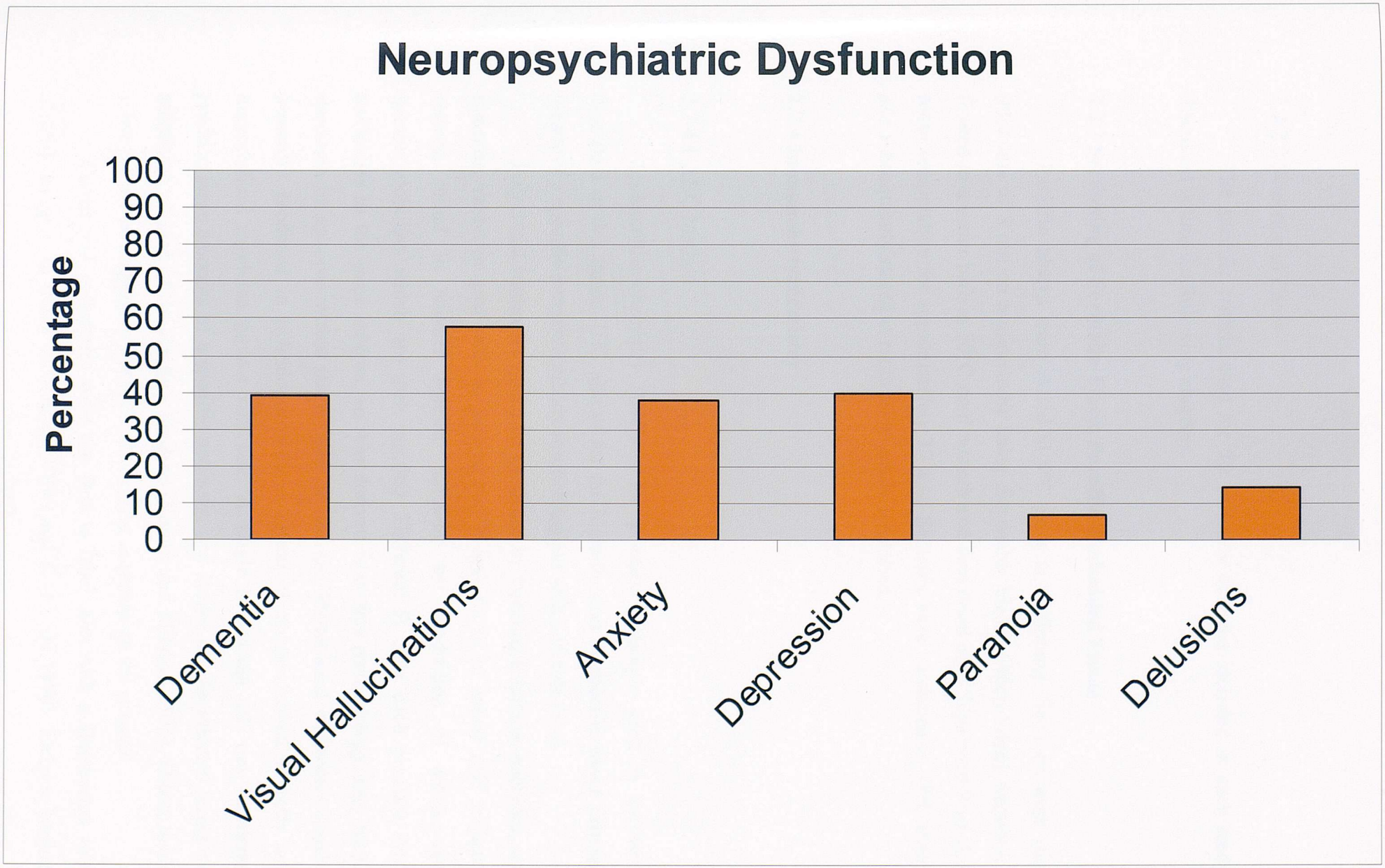


Figure 2.4 Cumulative incidence of neuropsychiatric complications in the entire clinical course (n=81).

(Following page)


Neuropsychiatric Dysfunction
Percentage
100
90
80
70
60
50
40
30
20
10
0
Dementia
Visual Hallucinations
Anxiety
Depression
Paranoia
Delusions

2.2.2.2 Selection of Cases

The specific criteria used for the selection of cases included in each study are discussed within the following chapters.

2.2.3 Sectioning of Formalin-Fixed, Paraffin-Embedded Tissue

Paraffin blocks were stored at 4°C prior to sectioning. Sections were cut at a thickness of 8 μm on a microtome using disposable blades (Accu-Edge). Sections were floated in a water bath at 50°C until smooth and then dried onto Superfrost Plus slides at room temperature for approximately 30 min. Sections were incubated at 37°C overnight and subsequently stored at room temperature until used.

2.2.4 Immunohistochemistry

2.2.4.1 ABC Method

Immunohistochemistry (IHC) is the primary technique used in the research described in this thesis. IHC allows for the identification of specific tissue proteins by means of a specific antigen/antibody reaction tagged with a visible label.

Polyclonal antibodies (pAb) are produced by injecting a suitable mammal, such as a mouse, rabbit or goat with an antigen that is specific to an amino acid sequence of interest found in another species, inducing the production of antigen-specific immunoglobulins which are then purified. Different B cells each produce different antibodies to the same antigen, so Abs derived from this methodology may bind to a spectrum of epitopes within the antigen of interest. Monoclonal antibodies (mAb) are generally produced in hybridoma cells, a fusion of mouse-derived B cells and an immortalised myeloma tumour cell line. A single hybridoma cell can be cloned to produce large amounts of a specific antibody, and clones can be selected based on the affinity to antigen of the Ab they produce (Kohler and Milstein 1975). Often, mAb can subsequently be mapped to a specific amino acid sequence on the antigen.

Coons and colleagues were the first to label Abs with a fluorescent label to identify antigens in tissue sections (COONS and KAPLAN 1950). Enzyme-conjugated

Abs allow for the visualisation of the antigen-Ab complex by a reaction between the enzyme and a substrate compound (Nakane and Pierce, Jr. 1967;Moriarty *et al.* 1973). This method is permanent and visible with a standard light microscope. The specificity and intensity of the immunohistochemical reaction can be increased through layering, using the primary Ab as an antigen for a subsequent, secondary Ab.

The avidin-biotinylated enzyme complex (ABC) was developed by Hsu et al. and relies on the very high affinity between avidin, a molecule found in egg white, for the vitamin biotin, which can also be conjugated to peroxidase or alkaline-phosphatase enzymes as well as Abs (Hsu *et al.* 1981a;Hsu *et al.* 1981b). Avidin, with four binding sites to biotin, forms a very high-affinity bridge between a biotinylated Ab and biotinylated enzyme, which can be visualised in the presence of the enzyme substrate.

2.2.4.2 Immunohistochemistry of Formalin-Fixed, Paraffin-Embedded Tissue

A detailed protocol can be found in appendix II. In the fume hood, sections are first dewaxed in xylene. They then pass through a series of four 5 min. alcohol washes (100%, 100%, 90% and 70%) in industrial methylated spirits (IMS) and are finally placed in distilled water. To block endogenous peroxidase activity, sections were placed in 0.3% hydrogen peroxide in methanol for 30mins. If a pre-treatment for antigen unmasking is required (see Section 2.1.2), it is carried out at this point. Sections are then rinsed in 1x PBS. The sections were then individually removed from the 1x PBS, wiped around and at the back, before the pap pen was applied around the tissue and subsequently placed in moist incubation chamber. Approximately 200µl per section of primary Ab (made up in 1st diluent to appropriate dilution, see appendix II) was applied on the tissue. Sections were incubated overnight at 4° C, then rinsed in 3 x 5 min washes of 1x PBS. Following the washes, the sections were incubated with a horse-anti-mouse secondary Ab at room temperature for 1 hour (made up in 2nd diluent to appropriate dilution, see Appendix II). After rinsing in 3 x 5 min washes in 1x PBS, the avidin-biotin tertiary complex (Vector ABC kit) was applied for 1 hour at room temperature (made up also in 2nd dilutent to appropriate dilution). After this period, a further 3 x 5 min washes in PBS were performed. Visualization of the reaction was achieved with the use of 3,3'-diaminobenzidine (DAB) chromogen (Vector kit, for recipe see Appendix II). Sections were incubated with DAB for 3 mins and rinsed in running water. Subsequently, slides

were lightly counterstained in haematoxylin and then rinsed in running water until the water became clear. Following this procedure, sections were dehydrated by moving them in the following solutions for 5mins each 1) 70% alcohol, 2) 90% alcohol, 3) 100% alcohol, 4) 100% alcohol, and then placed in xylene for complete dehydration. Using the xylene-based mountant DPX sections were then cover-slipped.

To ascertain the specificity of primary Ab staining a negative control slide was included in most instances (due to tissue availability), which was treated identically to the other slides with the exception that no primary Ab was applied to it. These slides were expected not to demonstrate any immunopositivity provided that no non-specific binding to tissue occurred in the rest of the reaction.

2.2.4.3 Antigen Unmasking

Formalin fixation and wax embedding leads to cross-linking of protein that may result in the "masking" of antigenic determinants and thus reducing the number of antigenic sites available for demonstration. Antigen unmasking is a process which makes tissue more permissive to Ab labelling (MacIntyre 2001). The structural biochemistry of antigen masking and unmasking is unclear. It has been reported that a need for antigen unmasking after formalin fixation is associated with the presence of a tyrosine residue in or near the Ab binding site and an arginine residue elsewhere on the antigen, suggesting that the Mannich reaction, in which formaldehyde and arginine can form a reactive intermediate that binds to tyrosine, may be involved (Sompuram *et al.* 2004).

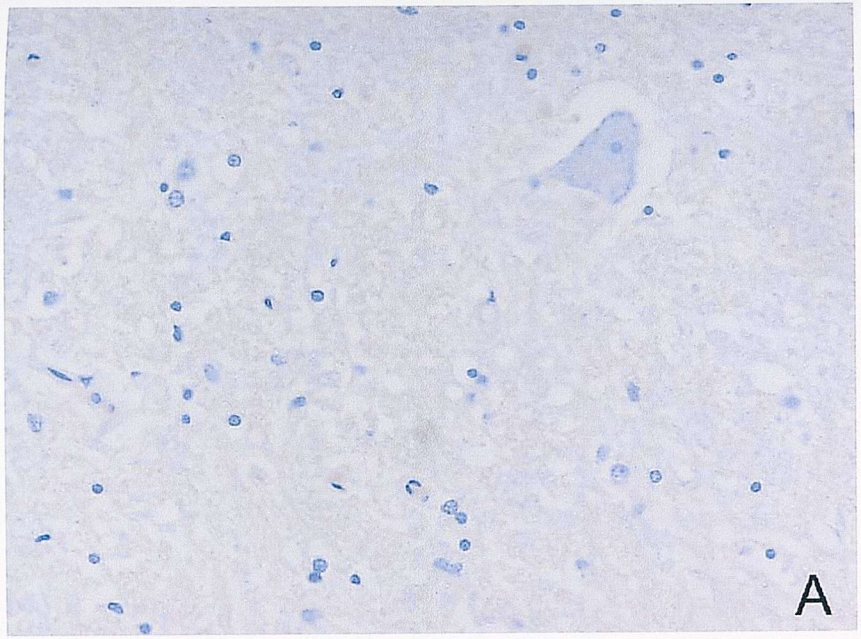
Two antigen unmasking techniques were used in this series of experiments. For the αSN Ab (described in Table 2.3), sections are incubated in 100% formic acid (FA) for 5 min or 80% FA for 60 min. For one Ab, anti-Aβ clone 1E8, a similar antigen unmasking pre-treatment consisting of an 8 min. incubation in 80% FA was used. Although, the selection and optimisation of the αSN Ab used in this research was performed elsewhere (Croisier *et al.* 2006) following recommendation of BrainNet Europe a new protocol was tested whereby sections are incubated for 60 min in 80% FA and with a dilution of the primary Ab 1/1000. Immunohistochemistry with this protocol revealed that αSN lesions were more clearly visible due to a less intensely stained background (Figure 2.5). In order to test the specificity of this protocol and to ensure that no normal αSN is picked up due to prolonged FA pre-treatment, immunohistochemistry

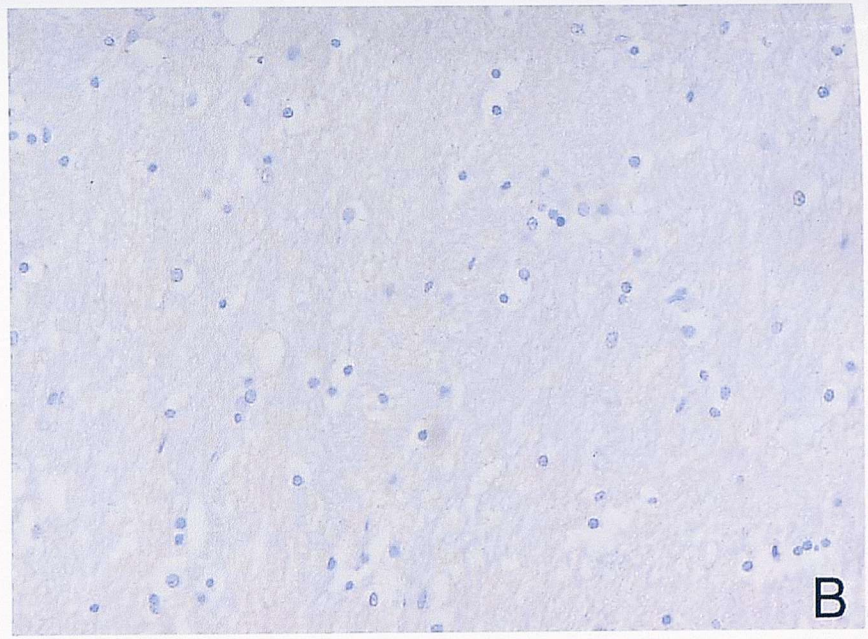
for αSN was performed in Alzheimer's disease (frontal cortex), Multiple Sclerosis (spinal cord) and Control (caudate-putamen) tissue (Figure 2.5). Immunostaining was absent in all three cases thus confirming specificity for pathological αSN inclusions. As a consequence, this protocol was adopted for chapters 3, 4 and 5 whereas for chapter 6 sections were incubated in 100% FA for 5 min and with a dilution of the primary Ab 1/300. Antigen retrieval methods have been shown to be particularly important in detection of pathologic forms of αSN. This is related to the fact that αSN is normally abundant in synaptic termini. Pre-treatments abolish much or all of the normal αSN and permit selective visualisation of pathologic αSN. For the anti-Aβ clone 1E8 Ab, optimisation was performed elsewhere and because it was non-commercial and only a limited supply was available, the established protocol was used (Table 2.3).

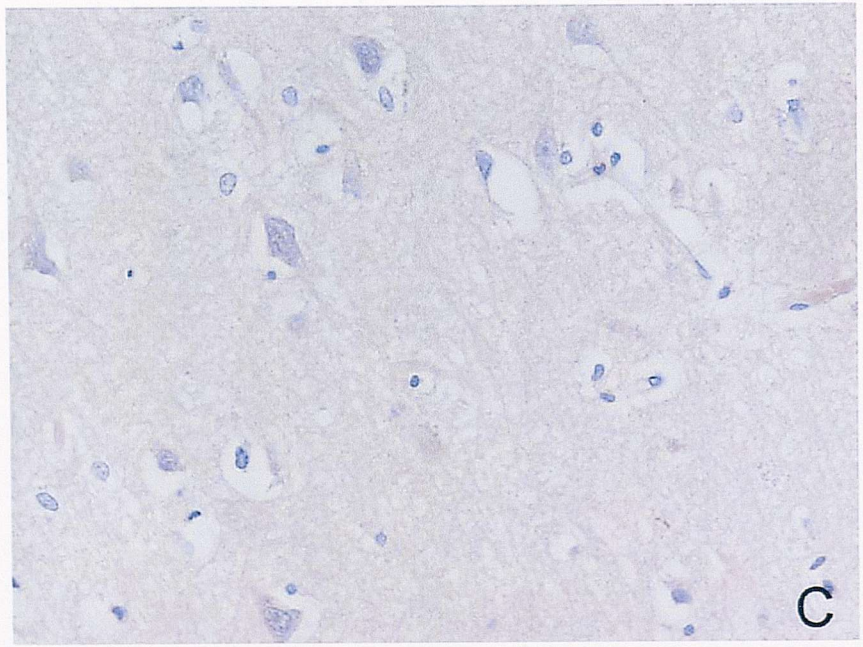
The other primary method of antigen unmasking, high temperature antigen unmasking (Ht), involves microwaving sections in a solution of 0.001M EDTA in distilled water, pH 8. Sections are placed in approximately 400 mL of EDTA and microwaved at 350W for 20 min (see appendix II).

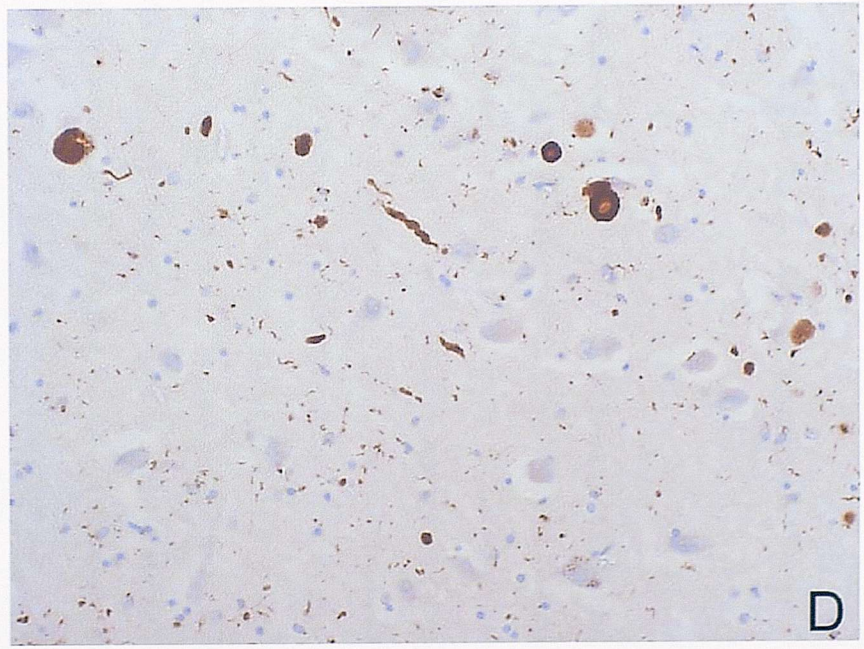
Figure 2.5 Specificity of α-synuclein immunohistochemistry with 1 hour FA pre-treatment.

(Following page). Immunoreactivity of αSN in A: MS tissue, B: Control and C: AD. The absence of staining confirms that 1 hour FA pre-treatment does not pick up normal αSN and thus verifies the specificity of the protocol. D: Representative αSN stained section (with 1 hour FA pre-treatment) from the hypothalamus of a PD case. Abbreviations: MS: Multiple Sclerosis, αSN: α-synuclein. Primary magnification for A-C: x40 and for D: x20.


A


B


C


D

2.2.5 Definition of αSN, and AD-type Pathology

The operational definitions of the neuropathological changes investigated in the present research are described below.

αSN pathology: The major histopathological hallmark of PD is the presence of LBs and LNs in the substantia nigra as well as various extranigral structures. The detection of LBs and LNs pre-αSN era is described elsewhere (see section 1.3.1.3). Following the recognition that Abs to αSN specifically label LBs and LNs as well as a wide range of αSN positive structures, IHC for αSN has become the preferred method in neuropathological research (see section 1.3.1.3). Given the superior nature of αSN Ab-based staining methods, in the present work αSN inclusions were visualised with the use of αSN IHC. αSN-containing inclusions displayed a variety of morphologies that are described in Table 2.4. Figure 2.6 illustrates the major morphologies of αSN inclusions observed in the present thesis but is by no means comprehensive. In the perikaryon of neurons, deposits were seen as compact spherical inclusions (i.e. LBs) (Figure 2.6 A, B and C), moderately immunoreactive deposits with irregular shape (Figure 2.6 D) or small particulate deposits (Figure 2.6 E). Less frequently, αSN aggregated also in glial cells (Figure 2.6 F). Combinations of the abovementioned morphologies were also observed, especially in the form of co-existing LBs with fine particulate deposits in the remaining cytoplasm (Figure 2.6 B). Multiple LBs in one neuron were principally found in the SN (Figure 2.6 C). αSN inclusions resembling the morphology and size of fully compact LBs were also seen extracellularly with no obvious association to a neuronal cell (Figure 2.6 A). These probably correspond to neuronal intracytoplasmic inclusions that were left behind when neurons died. Pathological involvement in neuronal processes (i.e. LNs) was predominantly seen in the CA2 sector of the hippocampus, NBM, amygdala and lower brainstem structures. LNs also displayed a range of morphologies varying from sausage-like, segmental serpentine to continuous serpentine and pearl-like arrangements (Figure 2.6 G-J)

Table 2.4 Diverse morphology of αSN-positive structures as detected with αSN IHC in the perikaryon of neurons and neuronal processes

(Following page)

Abbreviations: IHC: immunohistochemistry, LNs: Lewy neurites, LB: Lewy body

Morphology of αSN deposits in the perikaryon of neurons using αSN ICH

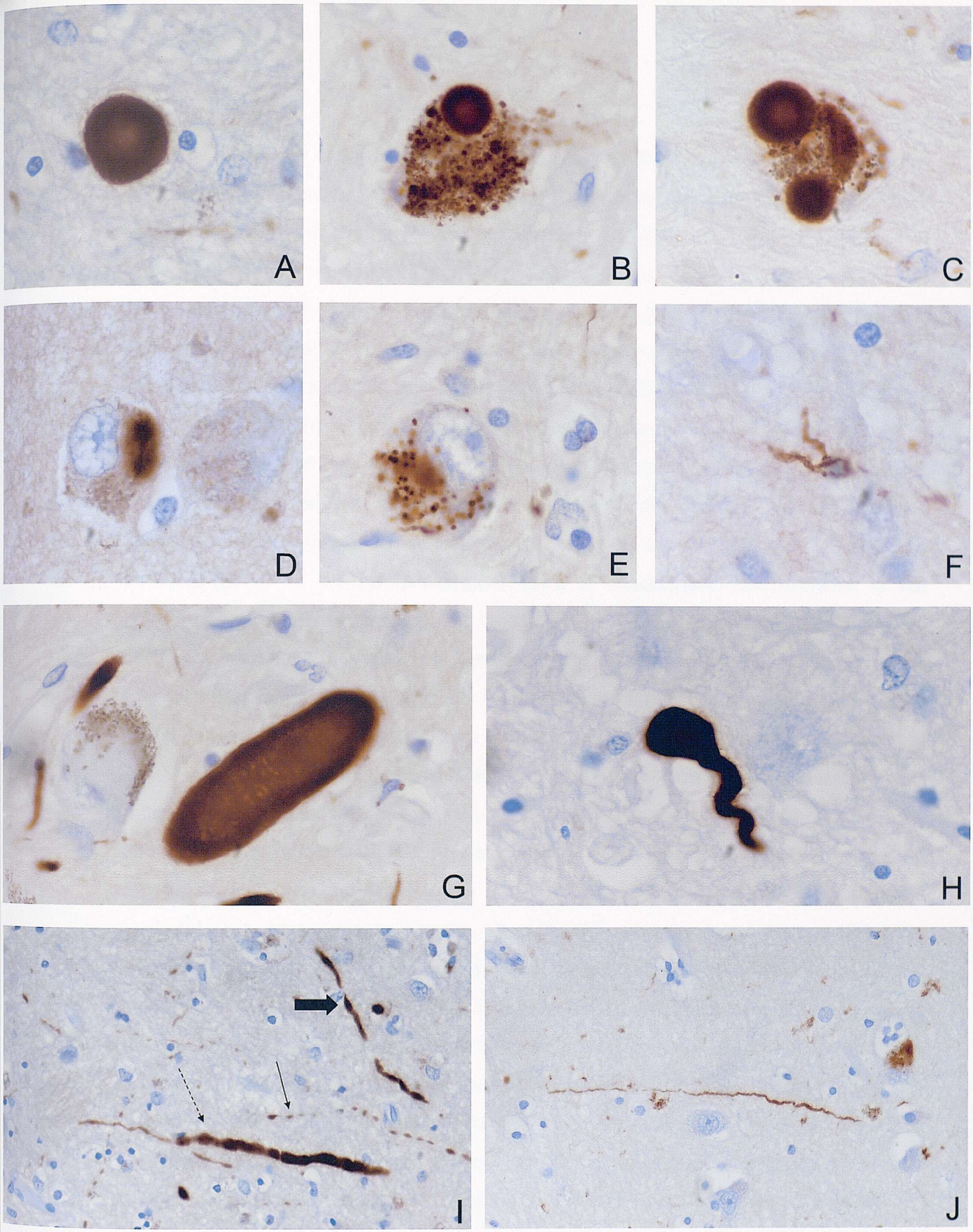
1) Small particulate deposits (dust-like particles)
2) Lightly immunoreactive with irregular shape
3) Lightly immunoreactive with sperical shape
4) Moderately immunoreactivity with irregular shape
5) Partly compact and partly loose
6)Compact structure and spherical with intense immunoreactivity with some fine dust-like particles in the remaining cytoplasm
7) Fully compact LB occupying the whole cytoplasm
8) Free-lying fully compact LB

Morphology of αSN deposits in neuronal processes (LNs) using αSN ICH

1) Sausage-like LNs
2) Segmental serpentine- like LNs
3) Continuous serpentine-like LNs
4) Pearl-like LNs

Figure 2.6 Heterogeneity αSN-positive inclusions

(Following page) A) Fully compact LB in the NBM with no obvious association to a neuron. B) Fully compact LB in the SN with small particulate deposits in the remaining of the cytoplasm. C) Several LBs in a pigmented neuron in the SN. D) Moderately immunoreactive deposit with irregular shape in the entorhinal cortex. E) Small particulate deposits in the SN. F) αSN in glial cell processes (possibly astrocyte) in the NBM. G) Large LN in the SN. H) LN terminating with a varicose in the DMV. I) LN exhibiting a pearl-like arrangement (arrow), segmental serpentine with dilatations (arrowhead), continuous serpentine-like with dilatations (dashed arrow) in the NBM. J) Long LN faintly stained in the AC. Magnification A-H: 100x oil objective, I and J: 40x.

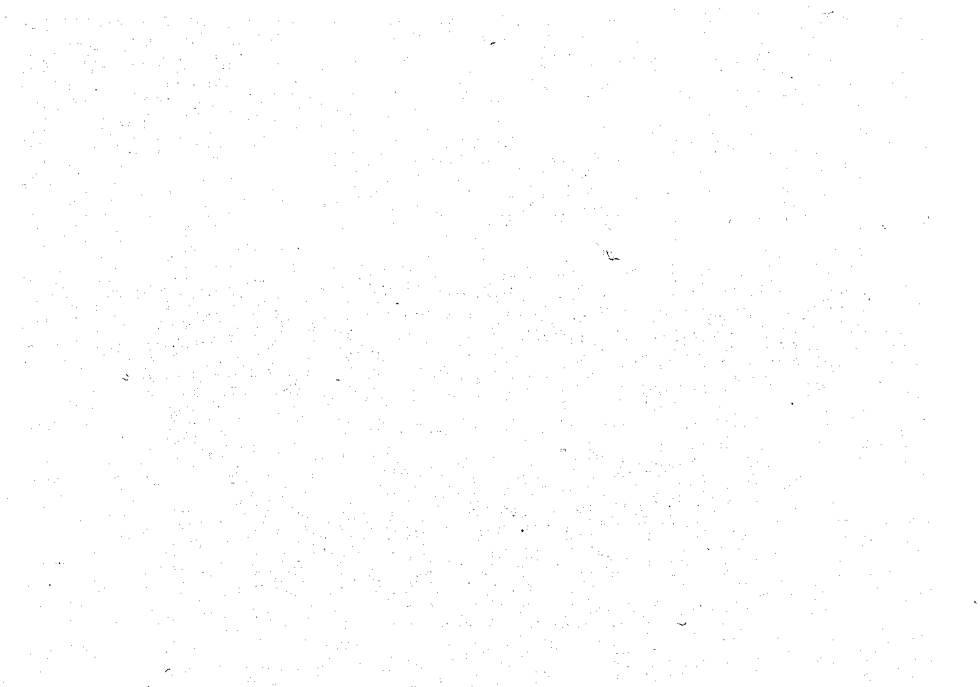

A
B
C
D
E
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G
H
I
J

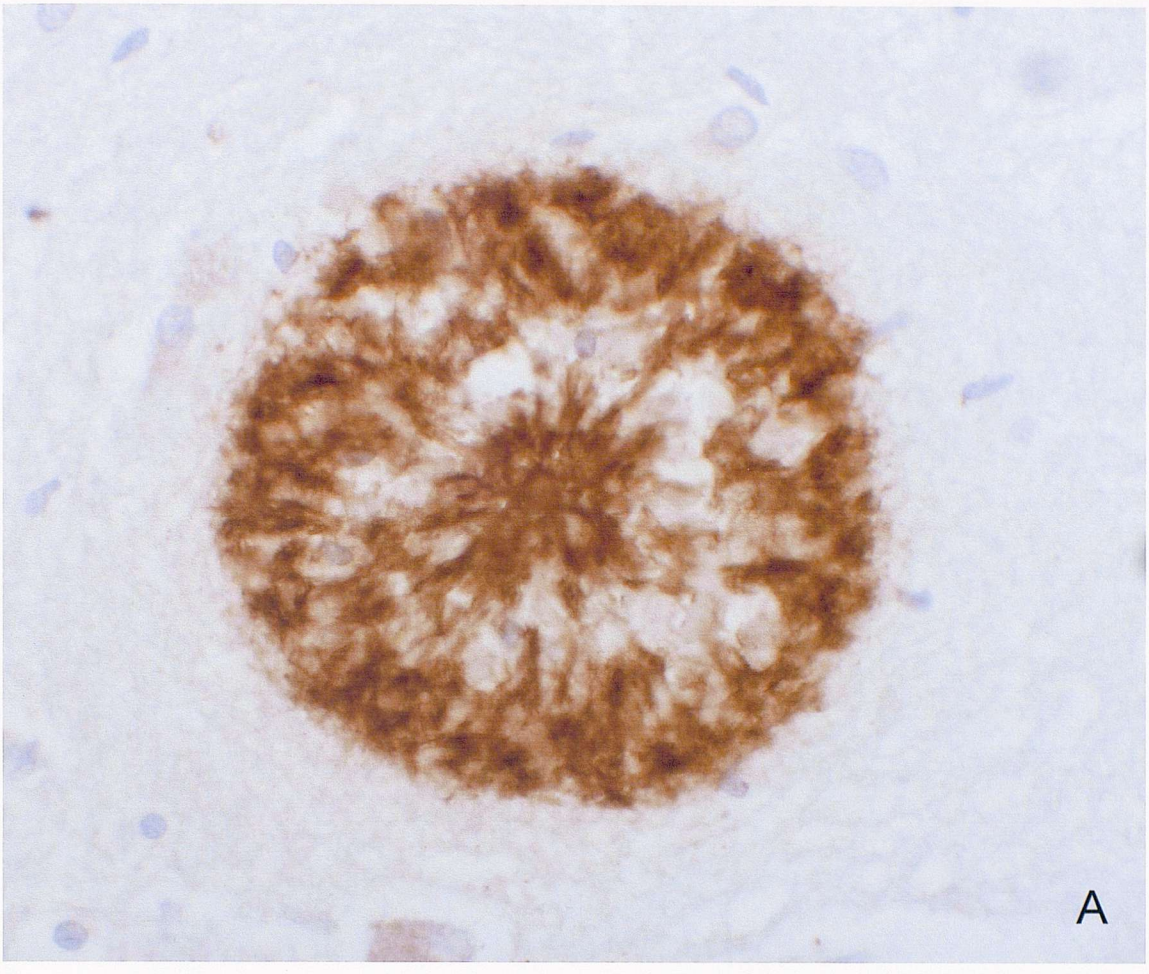
AD-type pathology (i.e. plaques and NFTs): As described in section 1.3.3.1 AD-type pathology is characterised by two major histopathological features- plaques and NFTs. Traditional histologic stains for the visualisation of AD pathology were developed empirically without understanding of their biological basis. Early descriptions of the AD histopathologic features were based on silver stains, amyloid stains and modifications thereof such as thioflavin-S fluorescence, Bielschowsky silver stain, Campbell-Switzer silver stain, Bodian stain and the Gallyas silver stain (Dickson 2005). Although, some of these stains (e.g. thioflavin-S fluorescence) are useful for the study of AD, they are not selective for a particular AD neuropathological feature. For instance, thioflavin-S fluorescence and Bielschowsky silver stain detect both plaques and NFTs while Campbell-Switzer silver stain detects plaques, NFTs as well as LBs (Braak and Braak 1999;McKee *et al.* 1991). In addition, there is considerable heterogeneity in the appearance of AD-type pathology of those early detection stains depending upon the particular laboratory in which it is performed and local modifications to the protocol. Immunohistochemical detection of AD-type pathology has proved superior to those early stains as it is specific and sensitive to a particular pathological feature (i.e. plaques or NFTs) and provides a biological meaningful stain that is derived from molecular and biochemical characteristics of the pathological changes under study. On the basis of the above argument in this work IHC was employed to detect AD-type changes- plaques and NFTs. Aβ IHC using the 1E8 Ab was used for the visualisation of amyloid plaques as well as CAA (described in chapter 3). Although, plaques have been traditionally classified as either neuritic or diffuse their detection using IHC does not readily distinguish these plaque subtypes (see also section 1.3.3.1). Using Aβ IHC a wide range of morphologies of amyloid plaques have been described in AD but these different subtypes insofar have only been reported in the medial temporal lobe (Thal *et al.* 2000) (see also section 1.3.3.1 Figure 1.4). In this work two major types of amyloid plaques predominated: cored and diffuse plaques. Plaques were classified as cored when they contained a central core composed of Aβ fibrils and surrounded by a corona of diffuse Aβ deposits. Between the core and the corona is a small gap without Aβ. On the other hand, diffuse plaques contained non-structured amyloid (Figure 2.7). Aβ IHC was also used for the visualisation of CAA changes (a description of CAA changes is provided in chapter 3 section 3.2.5). Tau IHC was employed for the visualisation of NFTs using the AT8 Ab.

NFTs appeared as flame-shaped or globoid masses of fibrous intraneuronal material often accompanied by abnormal neuronal processes, i.e., neuropil threads (NTs) and dystrophic neurites that were also tau-immunoreactive (Figure 2.8).

Figure 2.7 Visualisation of amyloid plaques.

(Following page). Different types of amyloid plaques using Aβ IHC. A: Cored plaque in the temporal cortex of a PD case. B: Diffuse plaque in the temporal cortex of the same PD case. Primary magnification x40.




A

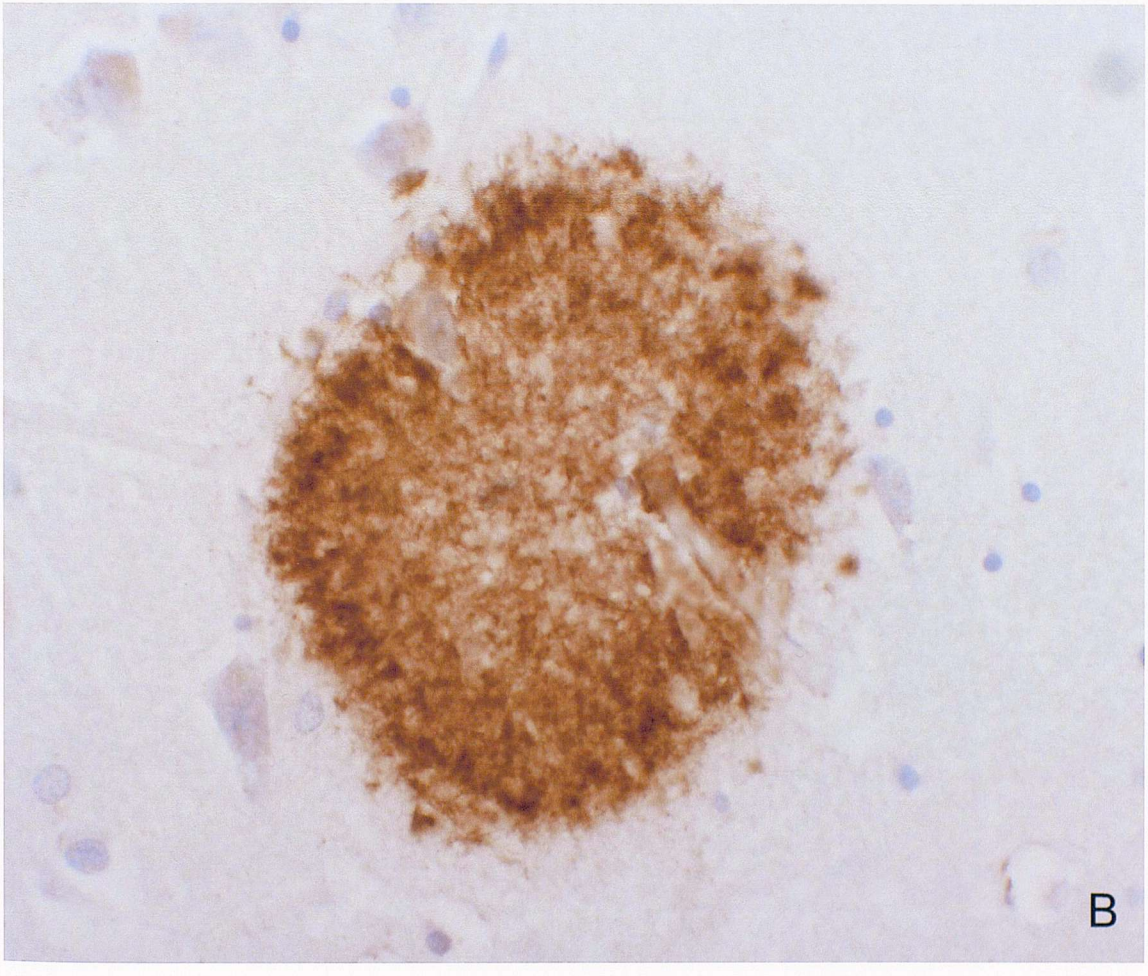
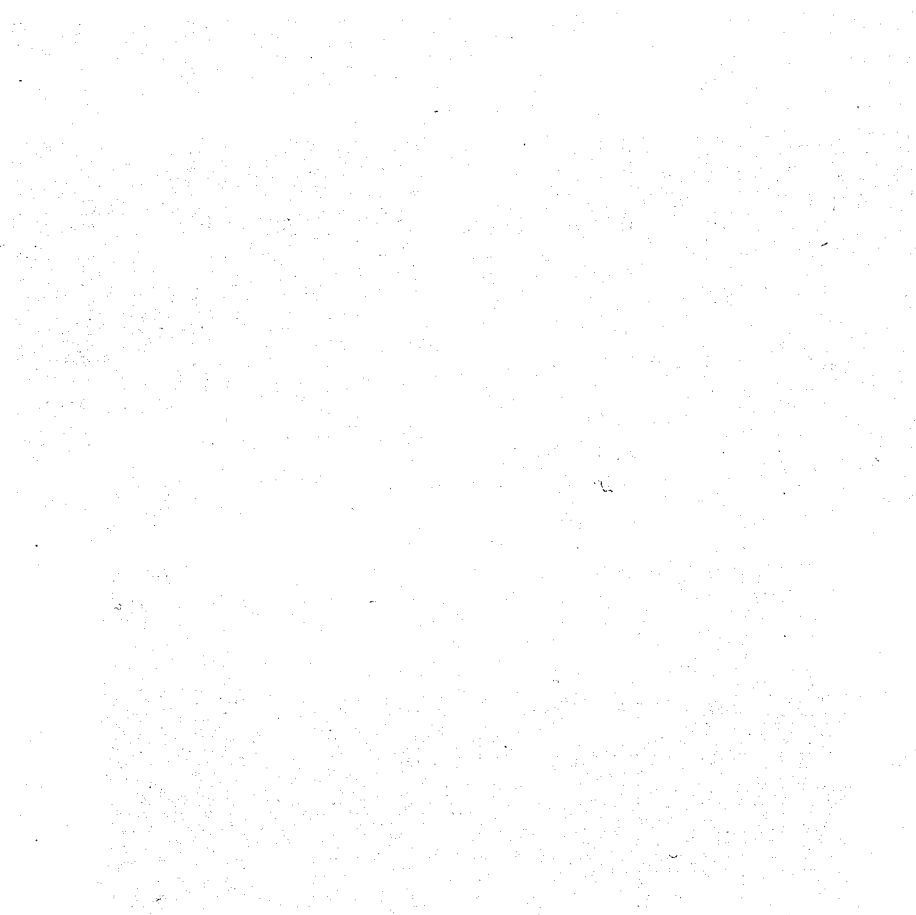
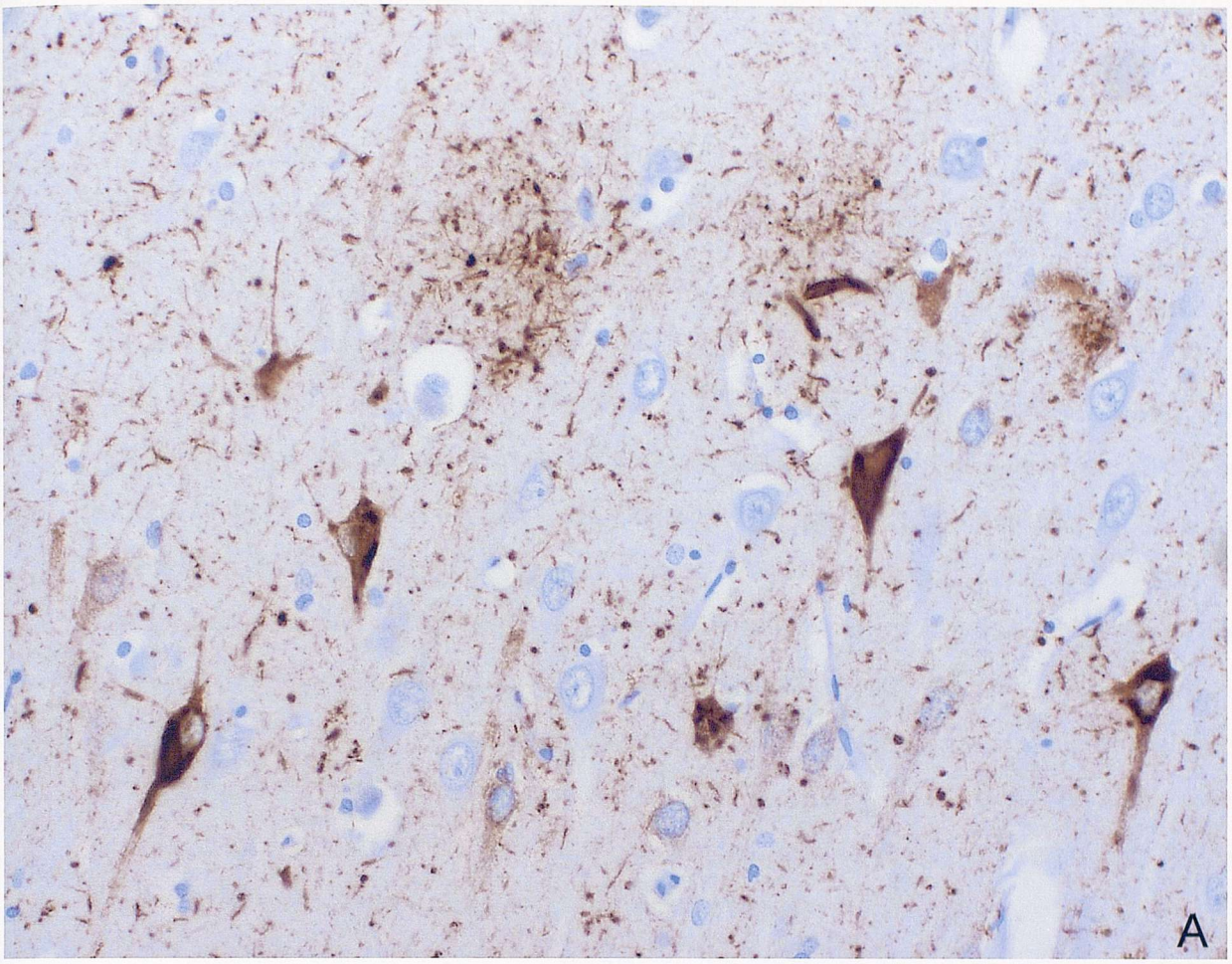
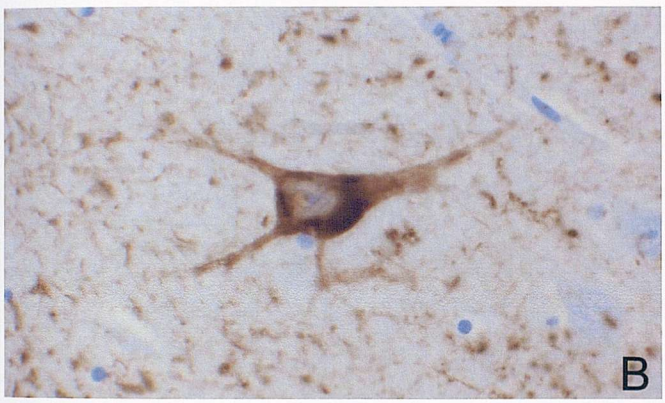

B

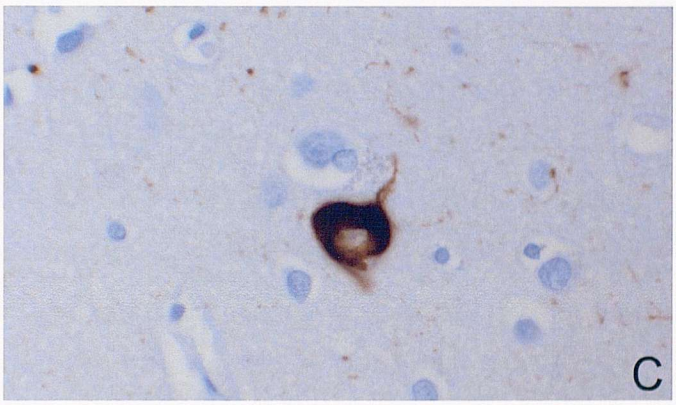
Figure 2.8 Visualisation of tau-positive inclusions.

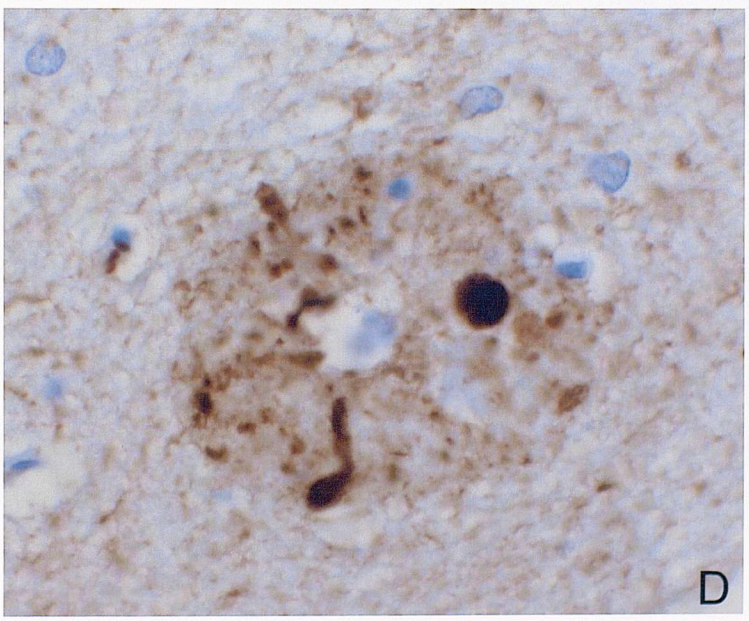
(Following page). Examples of tau inclusions using IHC. A: Overview of tau-positive inclusions in the CA1 sector of the hippocampus. NFTs as well as NTs and dystrophic neurites are clearly visible. B-C: NFTs appeared either as globoid or flame-shaped and often accompanied by NTs (entorhinal cortex). D: Dystrophic neurites in the entorhinal cortex forming a halo around a dense white core (β-amyloid). Primary magnification for A: x10 and for B-D: x40.




A


B


C


D

2.2.6 Analysis of Immunohistochemical Tissue Staining

The use of quantitative methods has become an increasingly important aspect in the study of neurodegenerative disorders. Various methodologies have been developed each of which has its own advantages and disadvantages. Image analysis systems have enabled images of histological sections to be captured and enhanced on a computer screen so that brain lesions can be quantified. However, this approach is vulnerable to numerous variables. Limiting a count to a particular area of the section, delineated anatomically or by the presence of another protein, precludes any differences in other areas from being detected. Automated cell counts do not distinguish cell types and measurements of the density of immunoreactivity across an area are prone to inaccuracies, as software tends to pick up haematoxylin-counterstained nuclei and, depending on the antibody used, cross-reactive blood vessels or lipofuscins. There is also little evidence of a linear relationship between the intensity of immunostaining and the amount of protein present.

Another relatively recent quantitative methodology is the so called unbiased or assumption-free stereology to distinguish it from the older assumption-based quantitative methods. Stereological methods have proved to be a valuable tool for gaining accurate quantitative information from histological sections. However, there is now an increasing consensus that 'unbiased' is a misnomer for stereological analysis because of ample evidence for potential biases - what could be unbiased theoretically, can well be biased when applied to real-world tissue sections (Benes and Lange 2001;Guillery and Herrup 1997;Guillery 2002). Indeed, there are some significant practical difficulties that in some studies preclude the use of stereology. One problem of using stereology in quantifying pathological lesions stems principally from the incomplete penetrance of the immunohistochemical stains as most of the Abs used in neuropathological research cannot penetrate the embedding mediums necessary for the preparation of thick tissue sections for stereological analysis (e.g. plastic techniques using glycol methacrylate) (Dorph-Petersen *et al.* 2001). Such incomplete stains not only will give false measurements of section thickness, but also incomplete counts. This problem could perhaps be overturned if fixed frozen tissue is used in conjunction with tissue permeabilisation (e.g. using triton). Although, this would increase the Ab penetration considerably, possible limitation would be the access to suitable sectioning device (e.g. freezing microtome or vibratome) and tissue shrinkage especially differential tissue

deformation on the z-axis. Another practical drawback is the necessity of using the entire brain region for stereological analysis (i.e. tissue availability). This in practice is not always possible especially in studies using human tissue.

Despite the advantages and disadvantages of different methods for quantifying brain lesions in neurodegenerative diseases the quantitative methodology to be adopted in any study should depend on the problem to be solved (i.e. should be relevant to the objective of the study). In this thesis the semi-quantitative methodology was used. Semi-quantitative severity ratings could be problematic in that it is not necessarily possible to replicate the methodology of a study without also replicating to some degree the training of the observer. It is also highly dependent on experience and familiarity with the stain under study. However, semi-quantitative methods allow for the assessment of large numbers of cases and are more robust to flaws in data acquisition (Armstrong 2003). Once the problem of inter-rater reliability is overcome, semi-quantitative methods have been shown to provide sufficiently accurate data (Armstrong 2003).

More specifically, in this work the immunoreactivity of αSN and AD type lesions was assessed using a CERAD-based (Mirra *et al.* 1991) visual impression of the numerical density of lesions under study ranging from 0-3 (absent-severe). Slides were assessed using an Olympus BX50 light microscope. The specific criteria and methodology applied are described thoroughly in the chapters in which it is assessed. Slides were randomised, their case and block number obscured with tape and marked with a number or letter prior to assignment of semi-quantitative ratings in order to minimise any bias held by the assessors. The reliability of semi-quantitative ratings was ensured as ratings were assigned by two independent observers. Cohen's kappa statistic was then used to test inter-rater reliability between the observers.

Chapter 3

Cortical and Limbic Pathology in Parkinson's Disease: Relation to Dementia and Visual Hallucinations

3.1 Introduction

Traditionally, PD has been characterised as a hypokinetic movement disorder with the main clinical features of resting tremor, bradykinesia, rigidity and abnormalities of gait, balance and posture (Lee *et al.* 1994;Lee *et al.* 1994). Experimental and clinico-pathological studies have shown that the primary anatomic substrate of these motor complications is the degeneration of the pigmented dopamine-producing neurons in the substantia nigra pars compacta (SNc) (see also section 1.3.4) (Forno 1996;Fearnley and Lees 1991). However, the complexity and variety of clinical symptoms experienced in PD (described in section 1.2) reflect an anatomically wide-ranging disease, a concept supported by animal studies, which have demonstrated that lesions restricted to the substantia nigra or dopamine pathways do not accurately model PD. Indeed, PD appears to be a multisystem disorder involving a variety of cortical and subcortical systems causing multiple neuromediator dysfunctions (Jellinger 1999). The present chapter will try to elucidate further the specific contribution of cortical and subcortical lesions to mental impairment in PD, with a focus on dementia and visual hallucinations (VH).

3.1.1 Clinico-pathological Studies of Dementia in PD

Contrary to initial assumptions that cognitive dysfunction is not an essential feature of PD, it has become increasingly apparent that patients with PD can have impairment of certain cognitive functions (described in section 1.2.3) and go on to develop dementia. Despite intense research efforts the underlying pathology and anatomical origin of dementia associated with PD remain a matter of controversy. Studies of clinico-pathological association in demented PD patients can be broadly categorised

into three groups by the suggested cause of dementia: subcortical pathology, limbic or cortical Lewy-body type pathology and those suggesting coincident AD-type pathology.

Degeneration of nigral neurons is the classical pathological feature in PD. The initial hypothesis therefore was that neuronal loss in the SNc also causes cognitive dysfunction although patients without cognitive impairment can have severe motor deficits. The first evidence to support this hypothesis was provided by Rinne and colleagues (Rinne *et al.* 1989), where they reported that cell loss in the medial substantia nigra was associated with dementia even when accounting for confounding Aβ burden. Jellinger and Paulus also reported a relationship between cell loss in the medial substantia nigra in demented PD patients although, this was associated with more severe AD-type pathology in the hippocampus and isocortex (Jellinger and Paulus 1992). Involvement of other subcortical nuclei such as the LC and the NBM have also been shown to contribute to dementia (Gaspar and Gray 1984).

LBs in the cortex and limbic structures are commonly found in PD brains to a varying degree. A number of clinico-pathological studies have reported an increased cortical LB burden in PD patients with dementia (Mattila *et al.* 2000;Mattila *et al.* 1998;Kovari *et al.* 2003;Hurtig *et al.* 2000). Mattila and colleagues have demonstrated a strong association between LBs in the frontal cortex and dementia, independently of concomitant AD pathology (Mattila *et al.* 2000). Similar results have been reported by Hurtig et al., where cortical LBs were found to be a more sensitive and specific correlate of dementia than AD-type pathology in 22 demented as compared with 20 non-demented PD subjects (Hurtig *et al.* 2000). In another study dementia correlated with LB densities in the entorhinal cortex and anterior cingulate gyrus (Kovari *et al.* 2003). Others have suggested LB and LN-type pathology in limbic structures (e.g. amygdala, hippocampus) to relate to cognitive decline and dementia in PD (Bertrand *et al.* 2003;Bertrand *et al.* 2004;Churchyard and Lees 1997). Overall, these studies point to LB-type degeneration in cortical and limbic regions as a main correlate of dementia in PD, independently of concomitant AD-type pathology.

A third group of studies suggest coincident AD-type pathology might cause dementia in PD. Boller et al., found AD-type pathology in all severely demented PD patients and only in a small proportion of non-demented (Boller *et al.* 1980). In another study amyloid deposits were found in 100% of cases with dementia but only 50% of those

who were non-demented (Mann and Jones 1990). In a large study of 610 patients with parkinsonism, 30% of those with PD were demented and dementia was associated with AD-related changes (Jellinger 1997). Finally, in 200 PD cases, 94% of those with dementia had cortical AD-type changes however, the relation to LB pathology was not investigated in this study (Jellinger *et al.* 2002).

Although studies have focused on PD-related and AD-type abnormal protein deposition as the possible substrate of dementia in PD, less attention has been given to vascular changes. CVLs, including CAA, the deposition of β-amyloid protein in meningeal and cortical (parenchymal) arteries, arterioles, veins and capillaries (Jellinger *et al.* 2002;Zekry *et al.* 2002;Preston *et al.* 2003) are frequently observed in the ageing brain and often co-exist with AD and PD. They may also play a significant synergistic role in the development of cognitive deficits and parkinsonism (Zekry *et al.* 2002;Rektor *et al.* 2006). In fact, CAA is a frequent, almost invariant component of the AD process. However, the contributory effect of CVLs and especially CAA in dementia in PD has gathered little attention. A recent autopsy study comparing 617 cases of PD with 535 age-matched controls revealed a 44.7% increase of CVLs which was slightly lower than in AD cases (Jellinger 2003b). In addition, the frequency of CVLs in demented PD cases was higher. In another study the occurrence of vascular β-amyloid in PD cases with dementia was greater than in age-matched controls, however due to inadequate clinical information a direct correlation with dementia was not possible (Mastaglia *et al.* 2003). Although, the association between CAA and AD has been extensively studied, the contributory factor of CAA in dementia in PD remains unclear.

3.1.2 Clinico-pathological Studies of Visual Hallucinations in PD and DLB

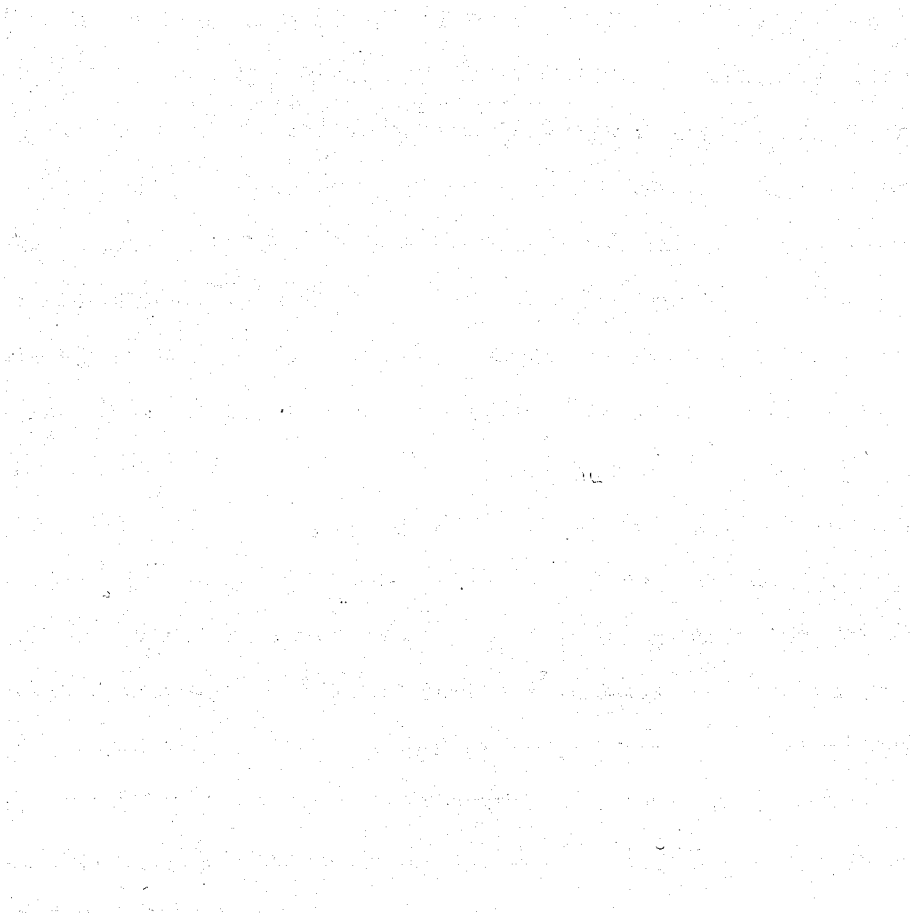
Although the most studied mental deficit in PD is dementia, VH are also a common symptom that often co-exist with dementia in PD patients with a recent literature survey demonstrating a prevalence in the range of 6-60% (Cummings 1991;Diederich *et al.* 2005) (for further information about VH in PD refer to section 1.2.3.2). Despite a high incidence there are very few neuropathologic studies of PD patients with VH and therefore the anatomico-pathological basis remains undefined although several studies point to a temporal lobe origin of this symptom. Harding et al., have demonstrated an increased burden of LBs in the inferior temporal cortex, parahippocampal gyrus and

amygdala in PDD and DLB cases with VH (Harding *et al.* 2002a). In a study where PD subjects were analysed without concomitant dementia an increased LB burden in the basolateral nucleus of the amygdala was associated with VH (Harding *et al.* 2002b). Recently, Papapetropoulos and colleagues have also reported an increased LB burden in the amygdala as well as frontal, temporal and parietal cortices in PD patients with VH (Papapetropoulos *et al.* 2006). On the other hand, imaging studies point to hypoperfusion in the inferior temporal gyrus, inferior parietal lobule, precuneus gyrus and occipital cortex in PD cases with VH as compared to those without (Matsui *et al.* 2006), while others point to the superior frontal gyrus and right fusiform gyrus as responsible for this complication (Stebbins *et al.* 2004;Oishi *et al.* 2005).

Many studies suggest that common factors associated with VH are higher age, duration of disease, cognitive impairment, medication, depression and sleep disturbances (Barnes and David 2001;Fenelon *et al.* 2000;Graham *et al.* 1997;Sanchez-Ramos *et al.* 1996). Although, higher doses of levodopa and other antiparkinsonian agents, such as dopamine agonists and anticholinergics have been associated with VH, a direct cause-effect relationship between a specific drug and hallucinations remains unclear (see also section intro 1.2.3.2).

3.1.3 Aims

In this study, the aim was to elucidate further the specific contribution of cortical and subcortical pathological lesions to dementia and VH in a relatively large PD cohort with minimal concomitant neuropsychiatric features. The pathology was assessed in eight brain regions that are affected in PD and which are known to play a role in cognitive function. The antiparkinsonian medication of the PD cases included in this work was also reviewed in order to gain a better understanding of any potential relationship with the clinical features under study.



3.2 Materials and Methods

3.2.1 Neuropathological Assessment

Neuropathological assessment and diagnosis were performed as described in section 2.2.1.

3.2.2 Selection of Cases

A total of 81 cases were included in this study. The cases were screened and the clinical severity of dementia, VH, anxiety, depression, delusions and paranoia were graded on a semi-quantitative global impression scale (0-3+, absent to severe) as described in section 2.2.2.1. For the purpose of the present study, cases were stratified into four groups according to clinical characteristics. 17 cases were identified with no dementia and no VH, 15 cases with moderate to severe scores for dementia and with VH (range 2-3), 7 cases with moderate to severe scores for VH alone without concomitant dementia (range 2-3) and 5 cases with moderate to severe scores for dementia alone without concomitant VH (range 2-3). Cases with severe co-existing neuropsychiatric features such as paranoia, delusions, depression and anxiety were excluded (n=26).

The clinical assessment of cases followed established criteria (see section 2.2.2). Upon disclosure of the clinical and neuropathological diagnoses, 9/17 cases with no dementia and no VH (with the exception of one case with mild VH) were diagnosed with PD (PD(H-) group), 1 case with Multiple System Atrophy (MSA), 1 case with Progressive Supranuclear Palsy (PSP), 3 cases with ageing-related changes and 3 cases with essential tremor. Out of the 15 cases with moderate to severe scores for dementia and VH 12 cases were diagnosed with PD (PDD(H+) group), 1 case with PD and AD and 2 cases with DLB. For cases with moderate to severe scores for VH alone without concomitant dementia 5 cases were diagnosed with PD (PD(H+) group) and 2 cases with MSA. For cases with moderate to severe scores for dementia without concomitant VH 4 cases were diagnosed with PD (PDD(H-) group) and 1 case with possible Fragile-X ataxia. All cases with a clinico-pathological diagnosis other than PD were excluded from the study. Thus, the cohort included a total of 30 PD cases of which 9 cases in the PD(H-)

group, 12 cases in the PDD(H+) group, 5 cases in the PD(H+) group and 4 cases in the PDD(H-) group (Table 3.1). For these cases gender, age at PD onset, age at death, duration of disease, as well as the clinical severity of the individual neuropsychiatric symptoms assessed are presented in Table 3.1. All apart from possibly one case were L-Dopa responsive (Table 3.1). Details of drug treatment including potential aggravating medication and cognitive enhancing agents are presented in Table 3.1. The mini mental state examination scores available for some of PDD cases are provided in Appendix III.

Table 3.1 Clinical data for the PD groups.

(Following page) *Abbreviations:* PDD(H-): PD cases with high clinical scores for dementia and no VH, PD(H+): PD cases with high clinical scores for VH and no dementia, PDD(H+): PD cases with high clinical scores for both dementia and VH, PD(H-): cognitively intact PD cases, D: Dementia, VH: Visual Hallucinations, A: Anxiety, De: Depression, Pr: Paranoia, Dl: Delusions PD: Parkinson's disease, m: male, f: female, semi-quantitative clinical severity scores 0: absent, 1: mild, 2: moderate, 3: severe, +: yes, -: no, ±: possibly

PD(H-) Group (n=9)	Case Number	Gender	Age at Onset (years)	Age at Death (years)	Duration of Disease (years)	Neuropathological Diagnosis (www.ICDNS.org)	ICDNS AD GRADE (www.ICDNS.org)	Braak AD-Stages	Clinical Severity Scores of Neuropsychiatric Features D	VH	A	De	Pr	DI	L-Dopa Responsiveness	Anti-Parkinsonian Medication	Other Medication*
	58	f	52	58	6	PD	isocortical 0 - entorhinal I	I-II	0	0	1-2	0	0	0	±	Madopar	-
	74	m	76	85	9	PD	isocortical II - entorhinal 0	I-II	0	0	1	0	0	0	+	Sinemet, Madopar, Pergolide	-
	95	m	47	74	27	PD	Isocortical I - entorhinal 0	I-II	0	0	0	1	0	0	+	Sinemet, Pergolide	-
	23	m	75	82	7	PD	isocortical I - entorhinal II	I-II	0	0	0	1	0	0	+	Sinemet	-
	7	m	68	78	10	PD	na	I-II	0	0	0	0	0	0	+	Amantadine	-
	22	f	65	75	10	PD	isocortical 0 - entorhinal 0	0	0	0	0	0	0	0	+	Madopar	-
	29	m	70	77	7	PD	isocortical 0 - entorhinal I	I-II	0	1	0	0	0	0	+	Madopar	-
	32	m	86	89	3	PD	isocortical 0 - entorhinal I	I-II	0	0	0	0	0	0	+	Madopar	-
	36	m	67	77	10	PD	isocortical 0 - entorhinal I	I-II	0	0	0	0	0	0	+	Madopar	-
	Mean		**67**	**77**	**9.8**												
PDD(H+) Group (n=12)																	
	5	f	57	80	23	PD	isocortical II - entorhinal II	I-II	2	3	0	0	0	0	+	Sinemet, Amantadine	-
	18	m	77	82	5	PD	isocortical I - entorhinal II	0	2-3	3	1	1	0	0	+	Madopar	Donepezil
	15	m	56	70	14	PD	isocortical IV - entorhinal III	III	2-3	2	1	0	0	1	+	Sinemet, Madopar	Olanzapine
	24	m	72	81	9	PD	isocortical 0 - entorhinal I	I-II	2-3	2	1-2	0	0	0	+	Sinemet, Madopar	-
	30	m	57	73	16	PD	isocortical I - entorhinal II	I-II	2	2	0	0	0	0	+	Benzhexol, Sinemet, Madopar	Olanzapine
	69	m	71	82	11	PD	isocortical I - entorhinal II	I-II	2	2-3	0	1	0	1-2	+	Ropinirole, Sinemet	Rivastigmine
	2	m	75	84	9	PD	isocortical 0 - entorhinal I	0	2	2	3	2	0	0	+	Pergolide, Sinemet	Donepezil, Risperidone
	16	f	67	85	18	PD	isocortical II - entorhinal I	I-II	2-3	2-3	1	1	0	0	+	Bromocriptine, Madopar, Sinemet	-
	44	m	74	87	13	PD	isocortical I - entorhinal II	I-II	2	3	0	0	0	0	+	Pergolide, Madopar	-
	13	m	54	78	24	PD	isocortical 0 - entorhinal I	I-II	2-3	3	2	0	0	0	+	Sinemet, Amantadine, Cabergoline	Olanzapine, Melleril
	34	f	73	84	11	PD	isocortical IV - entorhinal III	III	3	2	3	0	0	0	+	Sinemet	Diazepam, Olanzapine
	60	m	78	84	6	PD	isocortical II -entorhinal II	I-II	2	2	0	0	0	0	+	Pergolide, Sinemet, Ropinirole	Donepezil, Olanzapine
	Mean		**67**	**80**	**13.2**												
PDD(H-) Group (n=4)																	
	11	m	62	71	9	PD	isocortical III - entorhinal II	I-II	2	0	0	0	0	0	+	Sinemet	-
	45	m	61	80	19	PD	isocortical 0 - entorhinal III	III	2-3	0	2	2	0	1	+	Sinemet	-
	56	m	35	54	19	PD	isocortical 0 - entorhinal I	0	2	1-2	0	0	0	0	+	Bromocriptine, Sinemet, Pergolide, Madopar	-
	14	m	67	79	12	PD	isocortical IV - entorhinal III	I-II	3	1	0	0	0	0	+	Madopar	-
	Mean		**56**	**71**	**14.7**												
PD(H+) Group (n=5)																	
	63	f	67	80	13	PD	isocortical 0 - entorhinal I	I-II	0	3	0	0	0	0	+	Sinemet	-
	42	f	59	79	20	PD	isocortical 0 - entorhinal I	I-II	0	2	1-2	1	0	0	+	Sinemet, Amantadine, Cabergoline	Risperidone
	72	m	42	62	20	PD	isocortical 0 - entorhinal 0	0	0	2-3	0	0	0	0	+	Pergolide, Ropinirole, Pramipexole	-
	20	m	41	75	34	PD	isocortical I - entorhinal II	I-II	0	2	0	1	0	0	+	Pergolide, Sinemet, Artane	Quatiapine
	21	m	48	76	28	PD	isocortical 0 - entorhinal I	I-II	1	2-3	1	0	0	0	+	Amantadine, Pergolide, Sinemet, Madopar	-
	Mean		**51**	**74**	**23**												

3.2.3 Immunohistochemistry

Immunohistochemical reactions were performed as described in section 2.2.4. All 30 cases were stained for αSN for the identification of αSN lesions (Becton-Dickinson at a dilution of 1:1000), tau for visualization of neurofibrillary changes (Autogen Bioclear PHF-Tau (clone AT8) at a dilution of 1:800) and 1E8 for visualization of Aβ plaques (Aβ) (at a dilution of 1:1000).

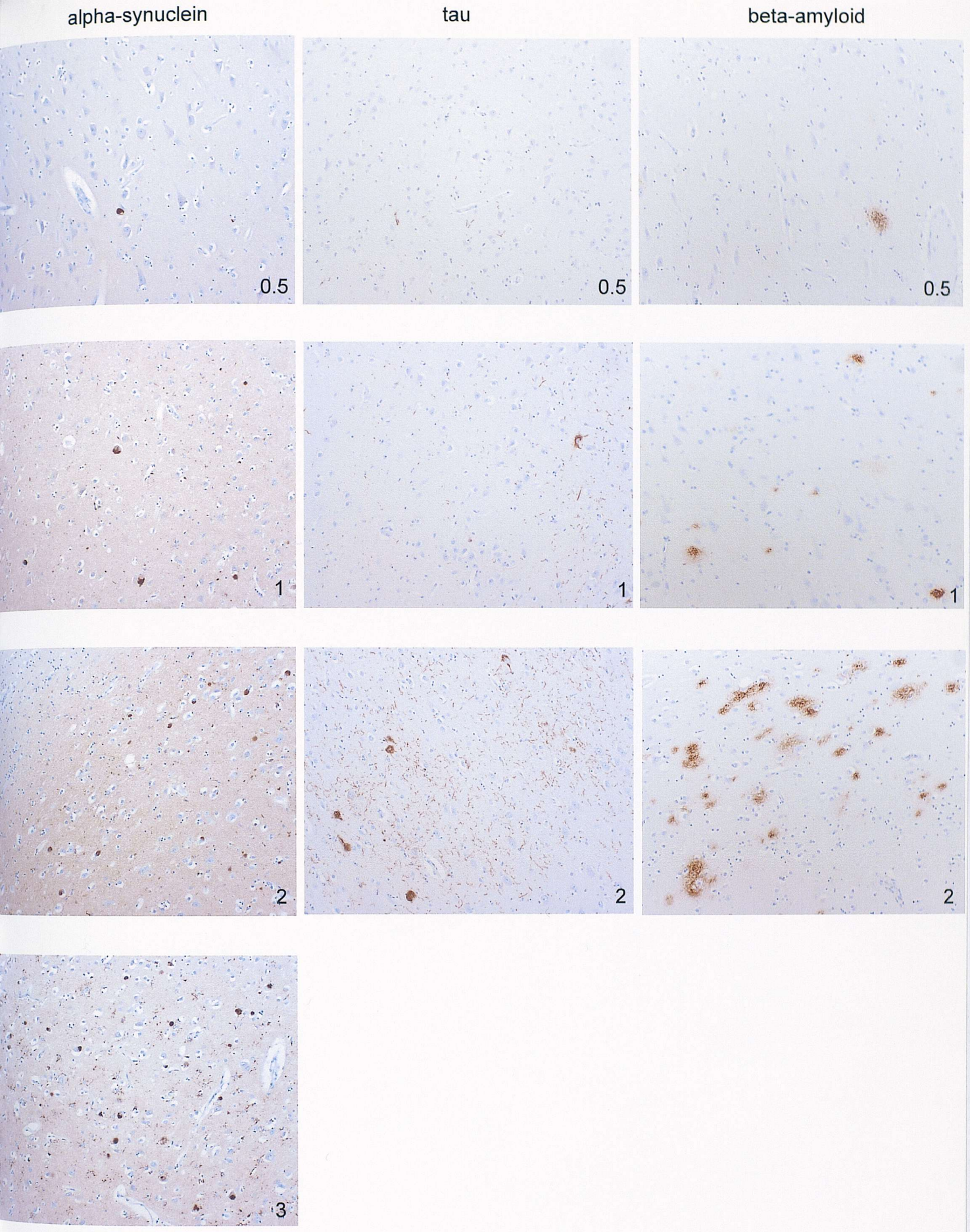
3.2.4 Semi-quantitative Assessment of αSN and AD Lesions

For each case, three representative sections from the superior frontal gyrus, cingulate gyrus, temporal cortex (middle temporal gyrus), entorhinal cortex, hippocampus CA2 sector, subiculum, amygdaloid complex and nucleus basalis of Meynert (NBM) were assessed for the occurrence of αSN, Aβ and tau pathological changes. The pathology was examined semi-quantitatively based on a scale ranging from 0-3 (absent to frequent; a pictorial representation of the different ratings of pathology is shown in Figure 3.1). Sections were screened in their entirety at 10x primary magnification for overall deposit burden. The occipital lobe was also examined in the same manner for the PD(H-) and PD(H+) groups only. Sections were graded by two investigators (Mr. Michail E. Kalaitzakis and Ms Lynne Christian). Cohen's kappa statistics revealed an inter-rater reliability of 0.8 for αSN, 0.9 for tau and 0.86 for Aβ (i.e. kappa statistic has a range of 0-1, with larger values indicating better reliability; generally a kappa of >0.70 is considered satisfactory). Intra-rater repeatability was also assessed by measuring the cingulate gyrus of five to six randomly selected subjects on five occasions at least a week apart. On each occasion, all operator-dependent processes (i.e. region of interest semi-quantitative assessment) were performed without reference to previous values. High intra-rater reliability was observed. The raw data for αSN, tau and Aβ pathology can be found in appendix III.

Figure 3.1 Semi-quantitative assessment of αSN, tau and Aβ deposition in a representative cortical region (i.e. entorhinal cortex).

(Following page) Semi-quantitative ratings of pathology. Assessment of αSN, tau and Aβ pathology in the regions under study was carried out using a CERAD-based (Mirra *et al.* 1991) visual impression of the numerical density of lesions ranging from 1-3 corresponding to sparse, moderate and frequent. A value of 0.5 was assigned to those fields where only a single lesion was present. In a few cases, where the density did not clearly fit into one of the main categories, intermediate values were assigned. No cases had a high enough density in cortical and subcortical areas to warrant a rating of 3 for tau and Aβ pathology. Magnification x10.




alpha-synuclein
tau
beta-amyloid
0.5
0.5
0.5
1
1
1
2
2
2
3

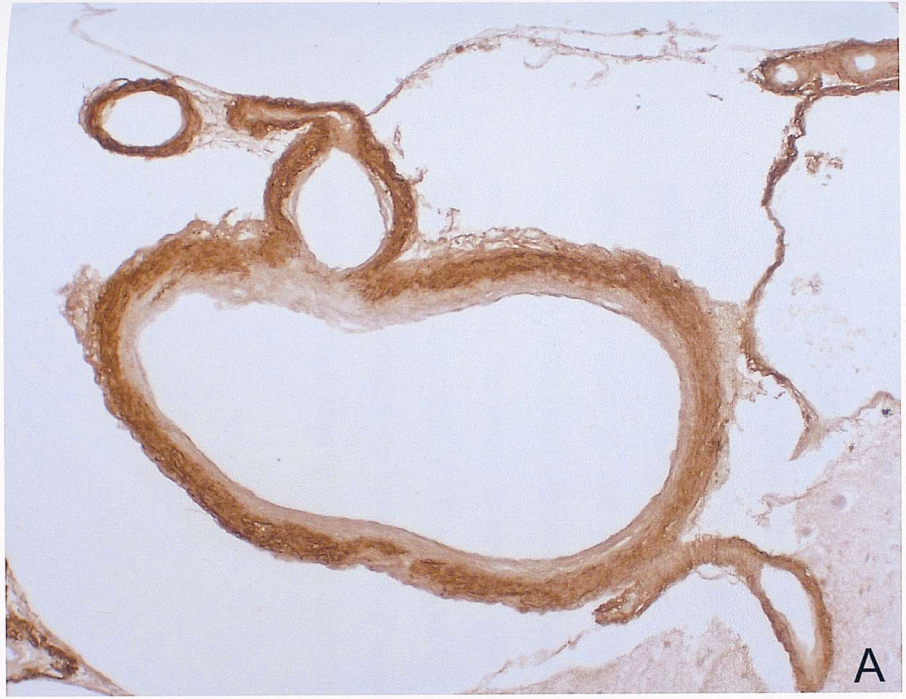
3.2.5 Assessment of Vascular β-Amyloid Deposits

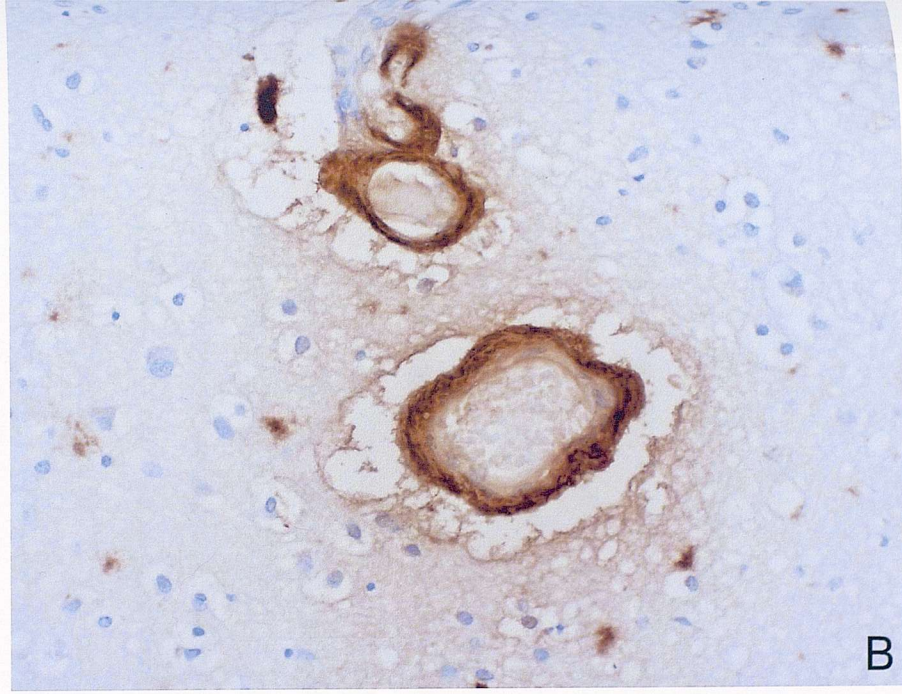
Assessment of β-amyloid deposition in large and small leptomeningeal vessels and medium sized and small parenchymal vessels was determined in superior frontal gyrus, cingulate gyrus and temporal cortex and occipital cortex (Figure 3.2). The presence of β-amyloid deposition in leptomeningeal and parenchymal vessel was noted as thickening of the basal membrane, stenosis of the vessel lumen or fragmentation of the internal elastic lamina. Given the difficulties of assessing the severity of these pathologies semi-quantitatively, the presence or absence was recorded.

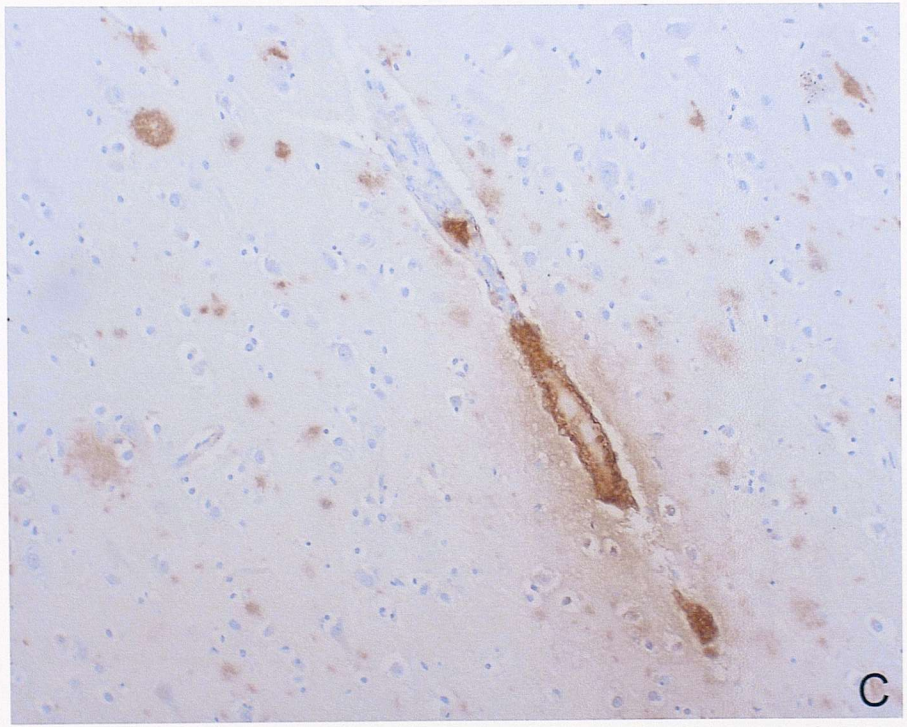
Figure 3.2 CAA changes in cortical regions

(Following page). A: Leptomeningeal Aβ deposition in the temporal cortex of a PD case. B: Parenchymal deposition of Aβ in medium sized vessels in the cingulate gyrus. C: Parenchymal penetrating vessel filled with Aβ in the cingulate gyrus. D: Parenchymal vessel with Aβ deposition in the superior frontal gyrus of a PD case. Magnification: 10x for A and C, 20x for B and D




A


B


C


D

3.2.6 Statistical Analysis

The association of αSN, tau and Aβ with dementia and VH plus the interaction between these two factors was tested using a two-way ANOVA in each anatomical region. Although these measures are on an ordinal scale, the use of a parametric ANOVA is appropriate (as opposed to a non-parametric ordinal regression) because the number of items in the scale is large. The X^2 test was used to detect possible significant differences in CAA changes in the populations of the different PD groups sampled. The extent of αSN, tau and Aβ burden in the occipital lobe between the PD(H-) and PD(H+) cases was examined with the use of the non-parametric Mann-Whitney *U*-test. The interaction between the clinical variables of age at death and duration of disease with the neuropathological variables (αSN, tau and Aβ) was assessed using Spearman's two-tailed correlation analysis (non-parametric). Ordinal regression was applied to detect relationships between age at disease onset, age at death and duration of disease with dementia and VH. Cohen's kappa statistic was used to test inter-rater reliability for the αSN, tau and Aβ semi-quantitative assessment between the two investigators. Statistical analysis was performed using the SPSS programme version 15.0 for Windows XP. P values <0.05 were considered significant.

The diagnostic performance of αSN and Aβ deposition in predicting dementia (regardless of the presence of VH) was calculated with measures of sensitivity, positive predictive value (PPV), specificity and negative predictive value (NPV). The methodology was adopted from Harding et al., (Harding and Halliday 2001). Briefly, sensitivity refers to the probability of having moderate to severe αSN and/or Aβ deposition with high clinical scores for dementia. Specificity refers to the probability of not reaching moderate to severe αSN and/or Aβ burden in cases not having high clinical scores for dementia. PPV refers to the probability of having high scores for dementia on reaching moderate to severe αSN and/or Aβ deposition. NPV refers to the probability of not having high scores for dementia in the absence of moderate to severe αSN and/or Aβ burden.

3.3 Results

3.3.1 Clinical Data

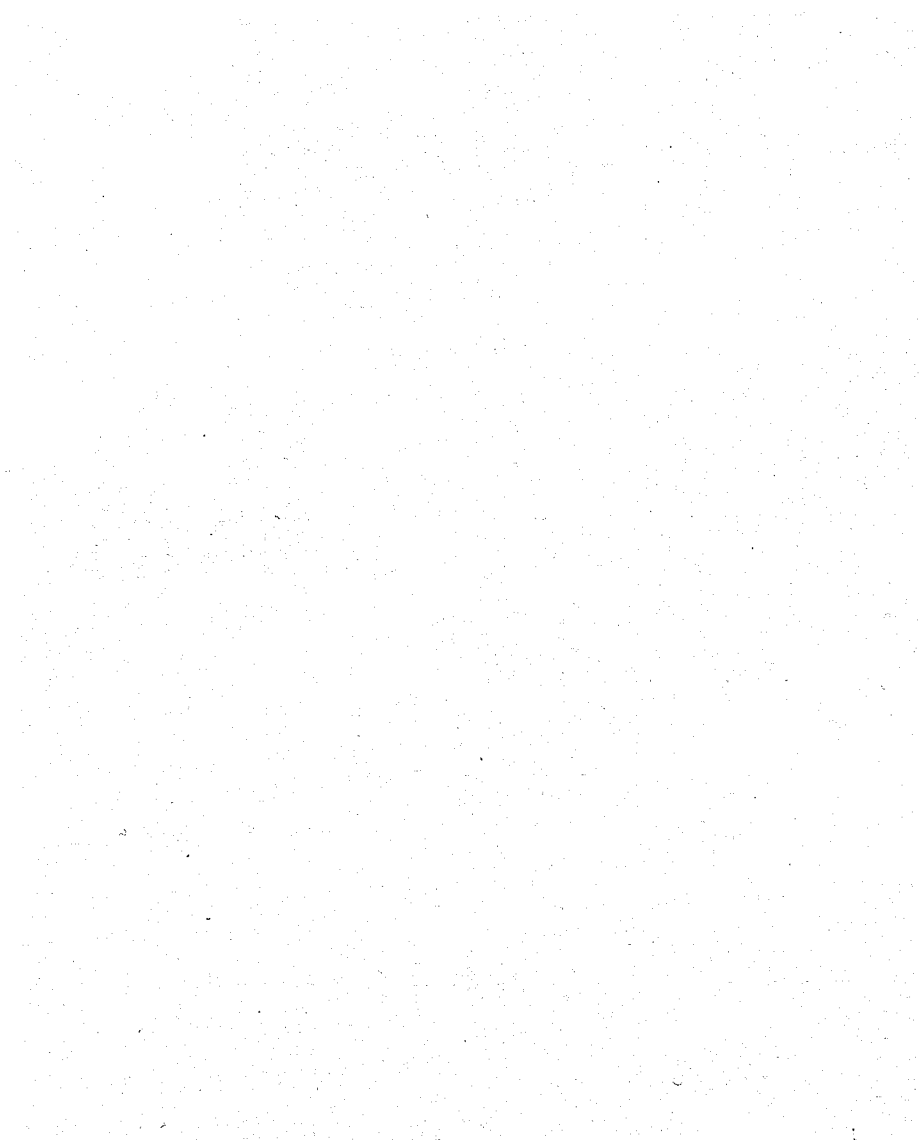
A total of 30 PD cases were included in this study (Table 3.1). Age at disease onset and age at death did not differ between groups, however PD(H+) cases had a statistically significant longer disease duration ($p=0.03$). All PD cases had received medication with the potential to exacerbate hallucinations (Table 3.1).

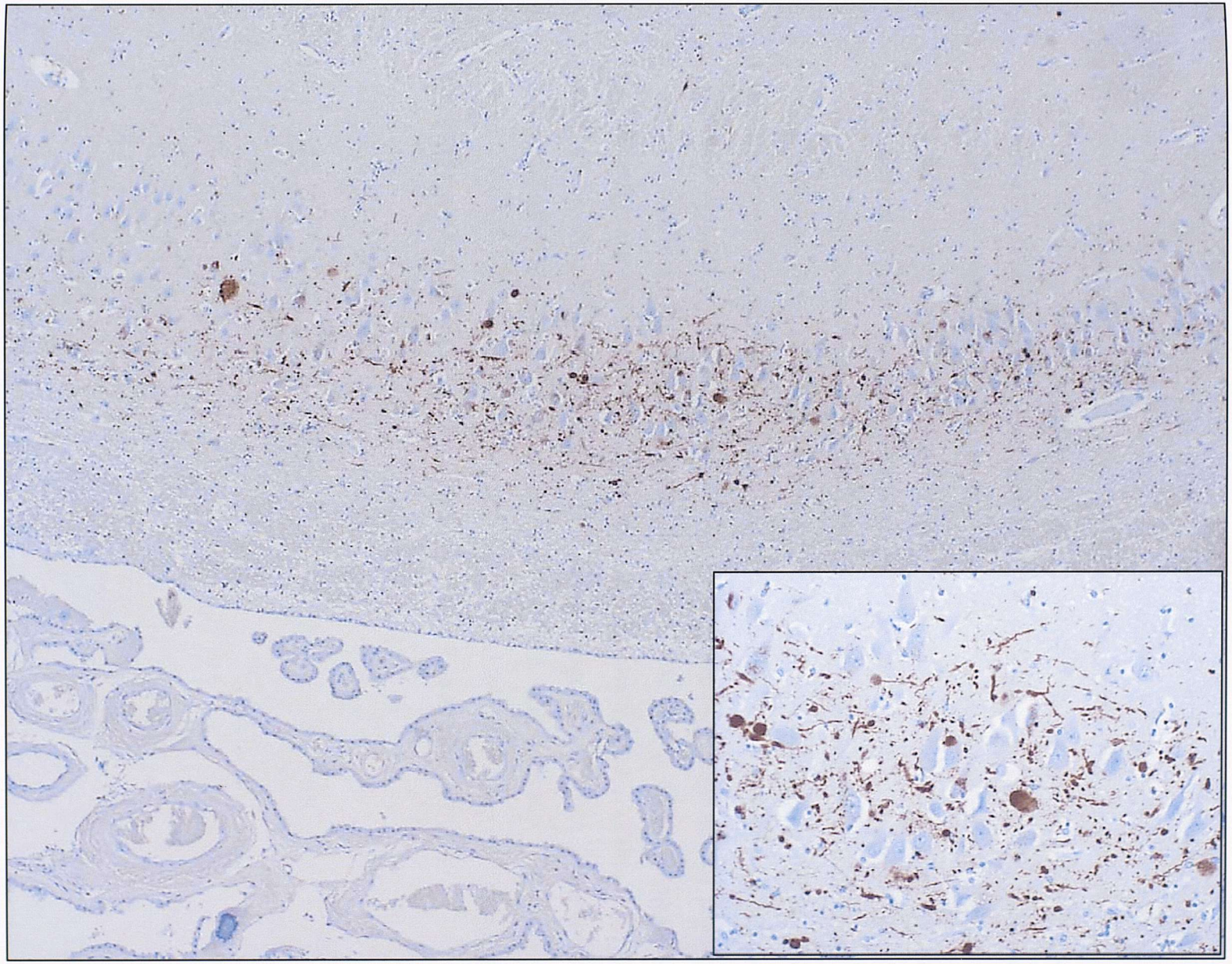
3.3.2 Histopathological Findings in Cortical and Subcortical Regions in the PD Brain

In cortical regions αSN lesions were predominantly found in the lower layers V and VI. LBs represented the primary type of αSN lesions in the cortex while LNs were rarely observed. Aβ and tau deposits were distributed evenly throughout the cortex with no clustering or patchy appearance of pathological lesions. Among subcortical areas a preferential susceptibility to LN-type pathology was seen in the CA2 sector of the hippocampus exhibiting a thick web of LNs (Figure 3.3) while neighbouring sectors remained relatively spared. In the amygdaloid complex and NBM both LB and LN-type pathology was observed with LBs as the principal pathological change.

Figure 3.3 LNs in the CA2 sector of the hippocampus

(Following page). Selective vulnerability of the CA2 sector of the hippocampus to LN-type pathology. Insert: higher magnification showing the web of LNs. Primary magnification 4x and for insert 10x.





3.3.3 Association of αSN, tau and Aβ Pathology with Dementia and VH

Statistical analysis revealed a significant association between dementia in PD and αSN burden in the cingulate gyrus (p=0.01), superior frontal gyrus (p=0.02), temporal cortex (p=0.02), entorhinal cortex (p=0.002), amygdaloid complex (p=0.007) and CA2 sector of the hippocampus (p=0.01) (Table 3.2). Although αSN burden did not associate with VH when present in isolation (PD(H+) group), a strong association was found between αSN burden in the amygdaloid complex (p=0.01) and VH in PD cases with concomitant dementia (PDD(H+)) (Figure 3.4, Table 3.2). Of note is that αSN load in the NBM did not associate with dementia. Interestingly, however αSN pathology in the NBM was severe in all groups studied independent of clinical phenotype.

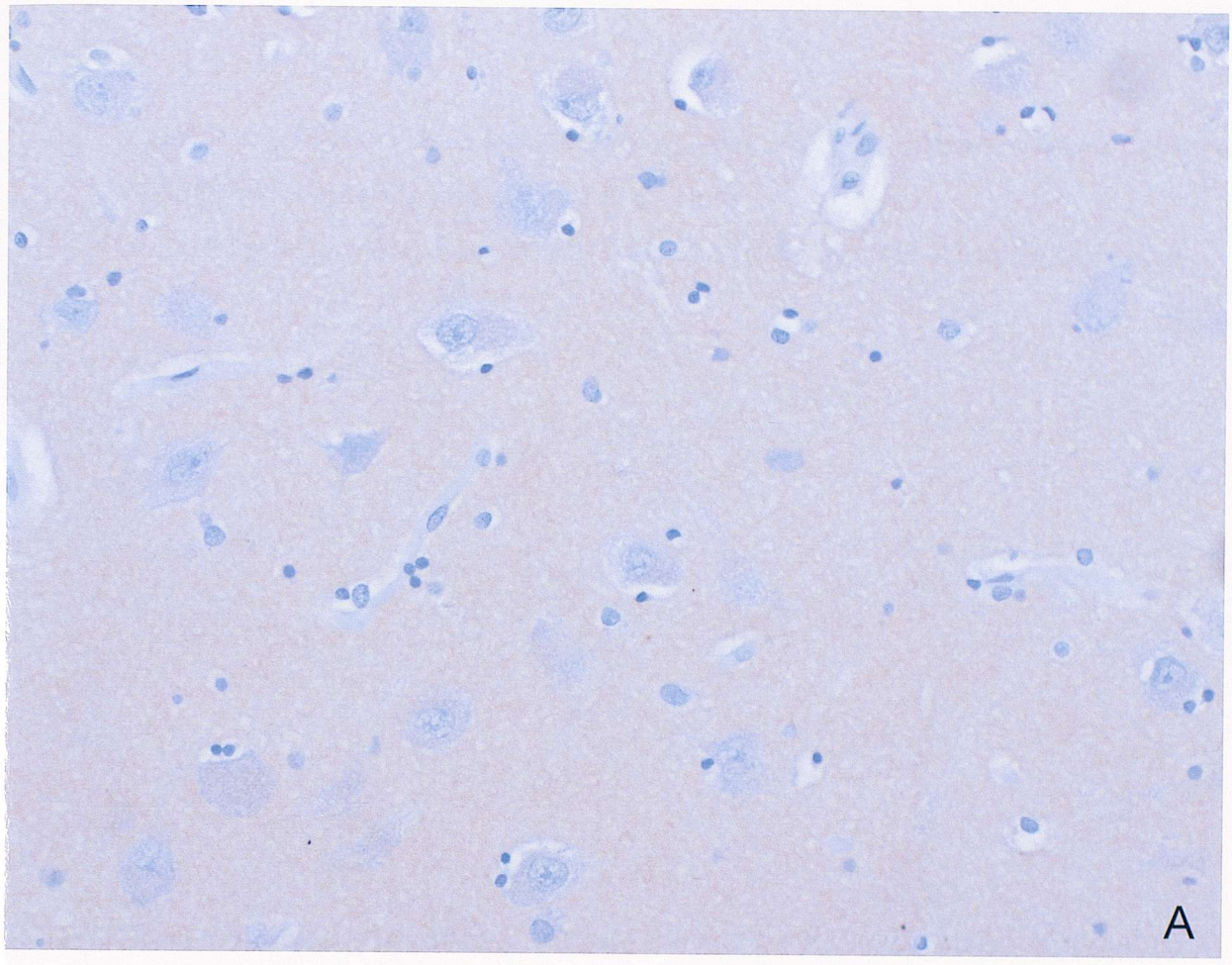
A strong association was also found between dementia and Aβ load in the cingulate gyrus (p=0.01), entorhinal cortex (p=0.002), amygdaloid complex (p=0.004) and NBM (p=0.02) whereas no association was detected between Aβ burden in PD cases with VH in isolation or with associated dementia (Table 3.2). Although tau pathology occurred mildly in all groups, tau burden in the CA2 sector of the hippocampus significantly associated with the presence of dementia (p=0.01) (Table 3.2). The associations observed herein cannot be attributed to an age related effect, as neither αSN nor tau or Aβ burden correlated with age at death or duration of disease.

Neither αSN nor tau was detected in the occipital lobe of PD(H-) or PD(H+) cases while the occipital Aβ burden was mild in both groups (Figure 3.5).

Figure 3.4 αSN burden in the amygdaloid complex

(Following page). Immunostaining for αSN in the amygdala of a PD case without VH (A) and a case with VH associated with dementia (B). Magnification x20

Abbreviations: PD: Parkinson's disease, VH: visual hallucinations, αSN: α-synuclein


A

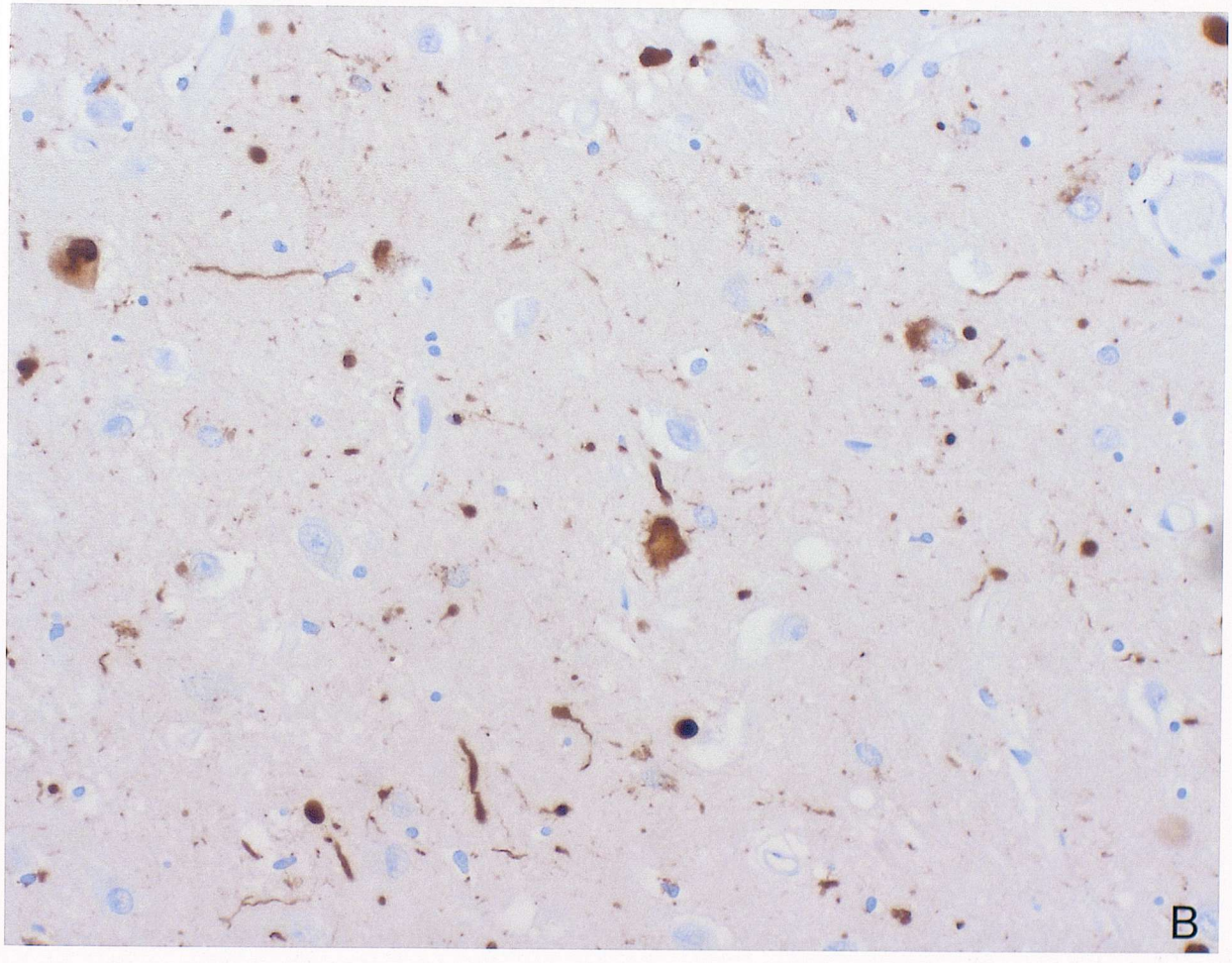

B

Table 3.2 Statistical associations between pathology in neocortical and subcortical regions with dementia and VH in PD patients

(Following page) *Abbreviations:* PD: Parkinson's disease, VH: visual hallucinations

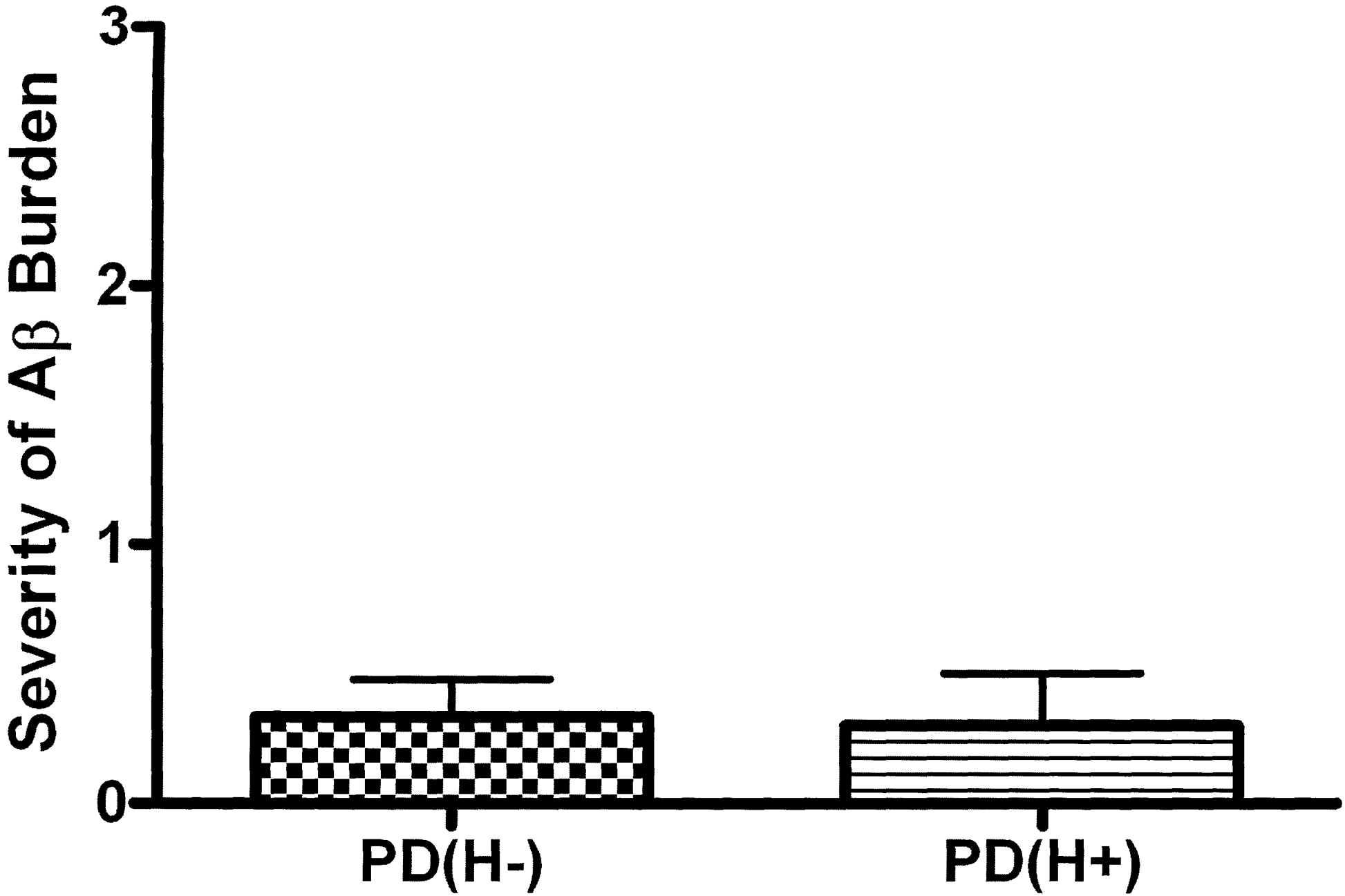
Brain Area	Dementia	Visual Hallucinations
αSN		
Superior Frontal gyrus	**0.02**	0.8
Cingulate gyrus	**0.01**	0.6
Temporal Cortex	**0.02**	0.4
Entorhinal Cortex	**0.002**	0.9
Hippocampus CA2 sector	**0.01**	0.2
Subiculum	0.08	0.6
Amygdaloid Complex	**0.007***	0.9
Nucleus Basalis of Meynert	0.4	0.5
Aβ		
Superior Frontal gyrus	0.08	0.5
Cingulate gyrus	**0.01**	0.3
Temporal Cortex	0.07	0.4
Entorhinal Cortex	**0.002**	0.9
Hippocampus CA2 sector	0.1	0.4
Subiculum	0.07	0.7
Amygdaloid Complex	**0.004**	0.7
Nucleus Basalis of Meynert	0.06	0.9
Tau		
Superior Frontal gyrus	0.2	0.8
Cingulate gyrus	0.5	0.8
Temporal Cortex	0.7	0.6
Entorhinal Cortex	0.4	0.5
Hippocampus CA2 sector	**0.01**	0.9
Subiculum	0.3	0.5
Amygdaloid Complex	0.1	0.6
Nucleus Basalis of Meynert	0.07	0.3

* In PD cases with dementia and concomitant visual hallucination p=0.01

Figure 3.5 Aβ burden in the occipital lobe

(Following page) The severity of Aβ deposition was similar between non-demented PD cases with or without VH.

Abbreviations: PD(H-): non-demented Parkinson's disease patients without visual hallucinations PD(H+): non-demented Parkinson's disease patients with visual hallucinations


Severity of Aβ Burden
3
2
1
0
PD(H-)
PD(H+)

As dementia associated with both αSN and AD-type pathology in various regions, in order to identify an anatomico-pathological marker that would differentiate PD cases with or without dementia (irrespective of the presence of VH), the sensitivity, PPV, specificity and NPV for all regions examined was calculated (i.e. PDD(H+) and PDD(H-) vs. PD(H-) and PD(H+) groups). The sensitivity and PPV for moderate to severe αSN burden in cases with dementia (PDD(H+) and PDD(H-) group) was highest in the range of 75-85% in the cingulate gyrus (Table 3.3). The sensitivity and PPV for Aβ deposition was also the highest in the cingulate gyrus and was 25% and 80% respectively. These values indicate that αSN in the cingulate gyrus is a better pathological marker for detecting cases with dementia with or without VH (Figure 3.6). Furthermore, the specificity and NPV for cases without dementia (i.e. PD(H-) and PD(H+) groups) was high in the range of 75-85%.

Table 3.3 Two by two contingency table for calculation of sensitivity, PPV, specificity and NPV for αSN burden in the cingulate gyrus in demented PD subjects regardless of the presence of VH

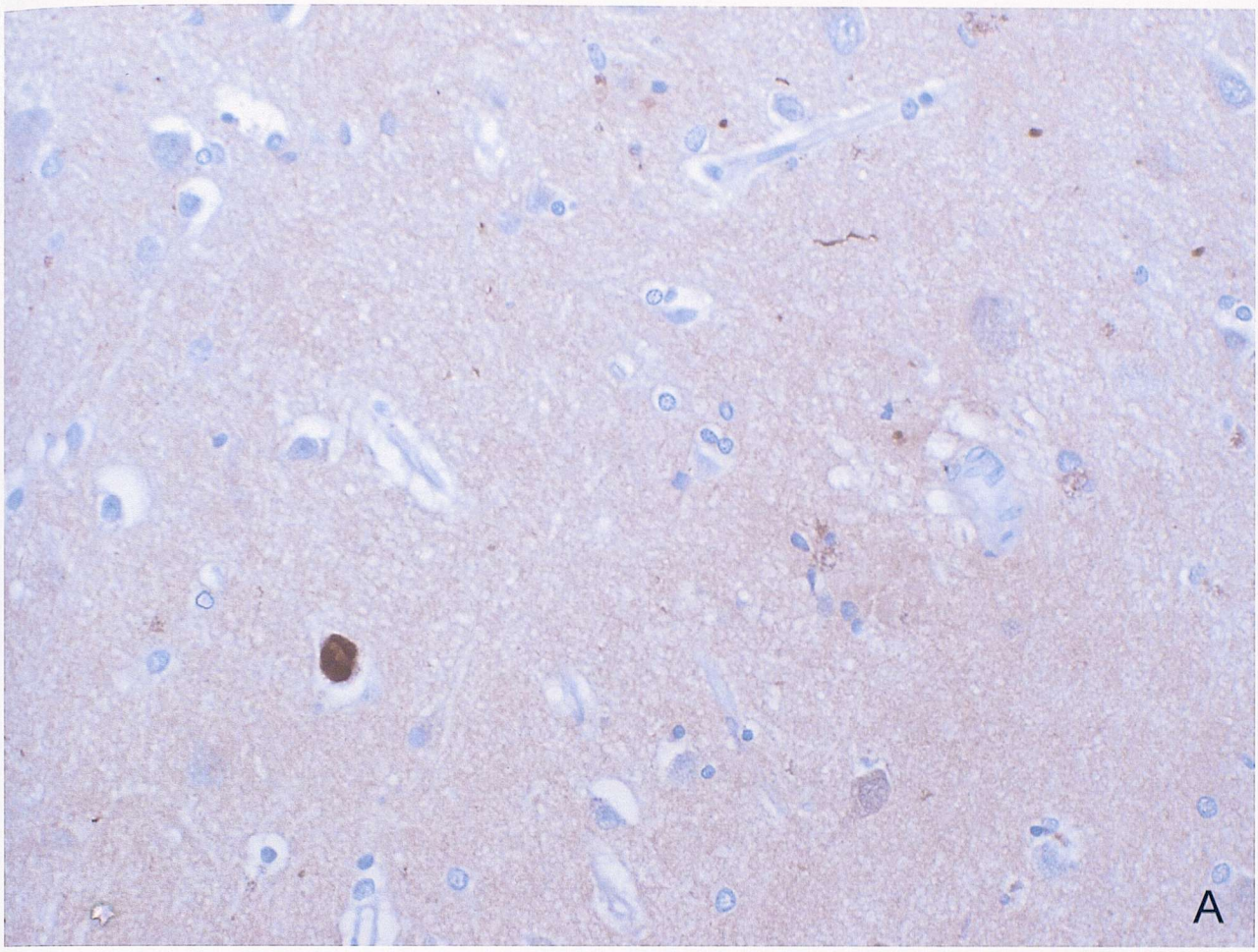
Sensitivity for demented PD cases (12/16X100)= 75%, PPV for demented PD cases (12/14X100)= 85%. Specificity for cases without dementia (12/14X100)= 85%, NPV for cases without dementia (12/16X100)= 75%

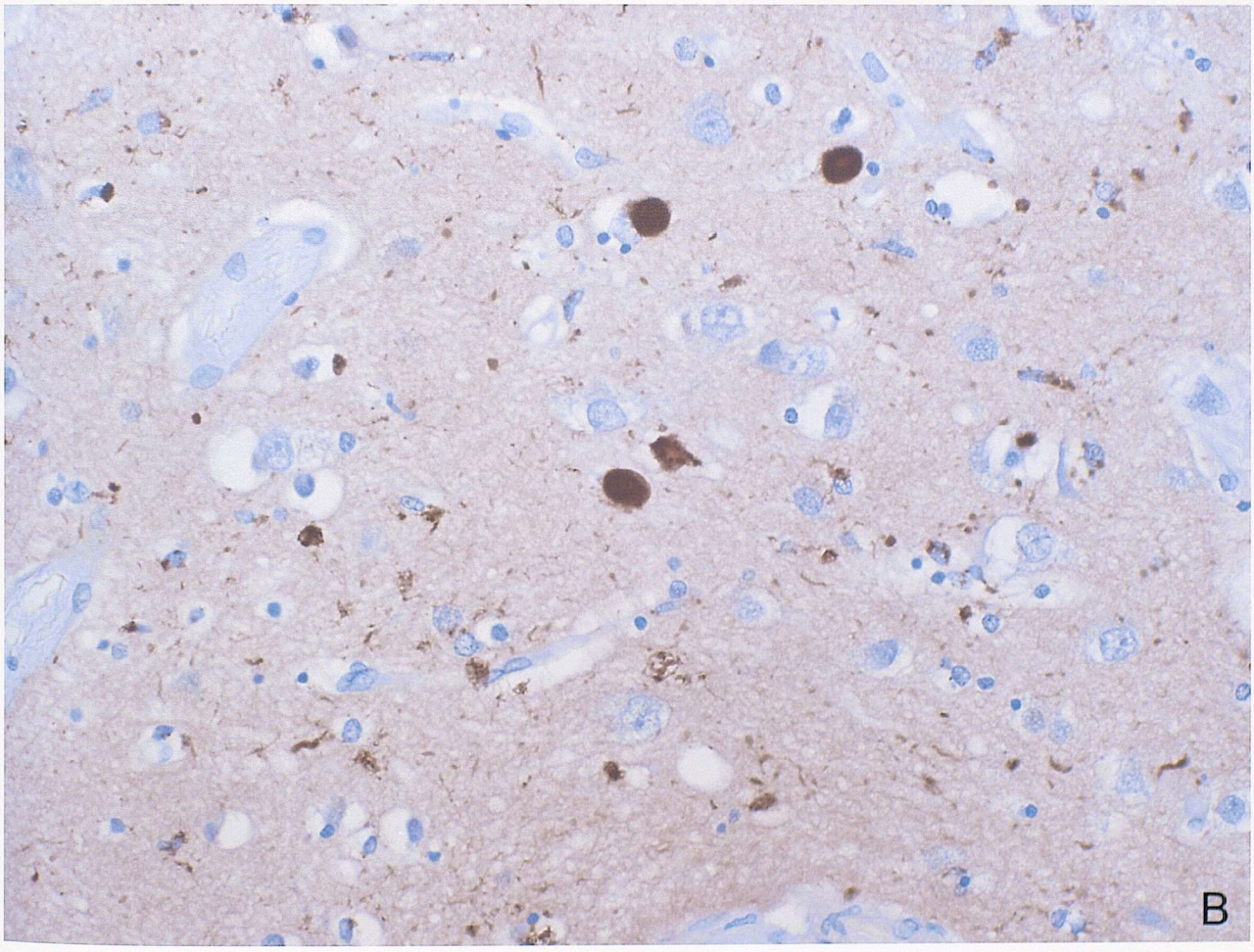
Cingulate Gyrus αSN burden	Clinically demented		Total number of cases
	yes	no	
2-3	12	2	14
0-1	4	12	16
Total Cases	16	14	30

Abbreviations: αSN: α-synuclein, PDD(H+): cases with high clinical scores for both dementia and visual hallucinations, PDD(H-): cases with high clinical scores for dementia but no visual hallucinations, PPV: positive predictive value, NPV: negative predictive value

Figure 3.6 αSN burden in the cingulate gyrus

(Following page) Immunostaining for αSN in the cingulate gyrus of a PD case without dementia or VH (A) in contrast to a case exhibiting both dementia and VH (B).
Abbreviations: PD: Parkinson's disease, VH: visual hallucinations, αSN: α-synuclein


A


B

3.3.4 Vascular Changes

CAA was observed in both leptomeningeal and cortical (parenchymal) vessels (Figure 3.2, Table 3.4). Leptomeningeal CAA changes in the cingulate gyrus, superior frontal gyrus and temporal cortex of demented PD subjects (i.e. PDD(H+) and PDD(H-)) were observed in 8/16, 7/16 and 10/16 cases respectively as opposed to 0/14, 3/14 and 1/14 among non-demented PD cases (PD(H-) and PD(H+)). Parenchymal CAA in the cingulate gyrus, superior frontal gyrus and temporal cortex of demented PD subjects was observed in 6/16, 7/16 and 4/16 cases respectively as opposed to 0/14, 0/14 and 1/14 non-demented PD subjects. Among PD cases with VH (irrespective of the presence of dementia) leptomeningeal CAA in the cingulate gyrus, superior frontal gyrus and temporal cortex was observed in 6/17, 7/17 and 8/17 cases respectively as opposed to 2/13, 3/13 and 3/13 PD cases without VH. Parenchymal CAA in the cingulate gyrus, superior frontal gyrus and temporal cortex of hallucinating PD cases was observed in 5/17, 6/17 and 4/17 cases respectively as opposed to 1/13, 1/13 and 1/13 of non-hallucinating PD cases. X^2 statistical analysis between demented and non-demented PD cases (i.e. PDD(H+) and PDD(H-) vs. PD(H-) and PD(H+)) revealed a strong association between dementia and CAA changes in leptomeningeal vessels in the cingulate gyrus ($p=0.001$) and temporal cortex ($p=0.0001$) and parenchymal Aβ deposition in the cingulate gyrus ($p=0.001$) and superior frontal gyrus ($p=0.001$) whereas no statistical significant association was observed between CAA and VH. Further to this, comparison of the occipital cortex of non-demented PD cases with and without VH did not demonstrate any differences with regard to vascular Aβ deposition. The CAA changes in the cingulate gyrus, superior frontal gyrus, temporal cortex and occipital cortex are presented in Table 3.4.

Table 3.4 Presence or absence of CAA changes in this cohort among the different PD groups.

(Following page). *Abbreviations:* PDD(H-): PD cases with high clinical scores for dementia and no VH, PD(H+): PD cases with high clinical scores for VH and no dementia, PDD(H+): PD cases with high clinical scores for both dementia and VH, PD(H-): cognitively intact PD cases, Lepto: leptomeningeal vessels, Parench: parenchymal vessels

	PD case	Cingulate Gyrus		PD case	Superior Frontal Gyrus		PD case	Temporal Cortex		PD case	Occipital Cortex	
		Lepto	Parench		Lepto	Parench		Lepto	Parench		Lepto	Parench
PD(H-)	58	0	0	58	0	0	58	0	0	58	0	0
	74	0	0	74	1	0	74	0	0	74	1	0
	95	0	0	95	1	0	95	1	1	95	0	1
	23	0	0	23	1	0	23	0	0	23	0	0
	7	0	0	7	0	0	7	0	0	7	0	0
	22	0	0	22	0	0	22	0	0	22	0	0
	29	0	0	29	0	0	29	0	0	29	0	0
	32	0	0	32	0	0	32	0	0	32	0	0
	36	0	0	36	0	0	36	0	0	36	0	0
PDD(H+)	5	1	1	5	1	1	5	1	0	5	-	-
	18	0	0	18	0	0	18	1	0	18	-	-
	15	0	0	15	0	0	15	0	0	15	-	-
	24	0	0	24	0	0	24	1	0	24	-	-
	30	1	0	30	1	1	30	1	0	30	-	-
	69	0	0	69	1	0	69	0	0	69	-	-
	2	1	0	2	1	0	69	0	0	69	-	-
	16	0	0	16	0	0	16	0	0	16	-	-
	44	0	1	44	1	1	44	1	1	44	-	-
	13	1	1	13	NA	1	13	1	1	13	-	-
	34	1	1	34	1	1	34	1	1	34	-	-
	60	1	1	60	1	1	60	1	1	60	-	-
PDD(H-)	11	0	0	11	0	0	11	0	0	11	-	-
	45	1	1	45	0	1	45	1	0	45	-	-
	56	0	0	56	0	0	56	0	0	56	-	-
	14	1	0	14	0	0	14	1	0	14	-	-
PD(H+)	63	0	0	63	0	0	63	0	0	63	0	0
	42	0	0	42	0	0	42	0	0	42	0	0
	72	0	0	72	0	0	72	0	0	72	0	0
	20	0	0	20	0	0	20	0	0	20	0	0
	21	0	0	21	0	0	21	0	0	21	1	1

-: not studied, NA: not available

3.4 Discussion

Dementia is a common complication in PD but its anatomical correlates remain a matter of controversy. For the cases examined in this study dementia was associated with significant degenerative changes in the limbic system. While the number of cases with dementia was relatively small (n=16), pathological changes in the amygdaloid complex, entorhinal cortex, cingulate gyrus and CA2 sector of the hippocampus are more frequently found with dementia in PD. Recent published findings demonstrate a relationship between LB densities in the parahippocampal gyrus and amygdala (Apaydin *et al.* 2002;Harding and Halliday 2001;Kovari *et al.* 2003;Mattila *et al.* 1999) and LNs in the CA2 sector of the hippocampus, with dementia in PD (Harding and Halliday 2001;Churchyard and Lees 1997). The data in this work are in agreement with these studies, pointing to the presence of limbic dysfunction as a central basis of dementia in PD. It is also of interest that the extent of αSN pathology in the NBM was high in both demented and cognitively intact cases highlighting a universal cholinergic deficit in PD, with or without clinical cognitive decline.

Some studies have demonstrated that increased numbers of cortical LBs correlate with cognitive decline independently of or in addition to AD type pathology (Mattila *et al.* 2000;Mattila *et al.* 1998;Hurtig *et al.* 2000). The results presented in this study suggest αSN pathology as a central correlate of dementia in PD. This is in agreement with a recent prospective community-based study indicating that LB pathology and not AD histopathological changes drive the progression of cognitive impairment in PD (Aarsland *et al.* 2005). However, AD-type pathology was also associated with dementia in this cohort. This finding, demonstrates that AD-type pathology may have a permissive effect in precipitating dementia in PD. In fact it has been recently shown with the use of both immunohistochemical and biochemical techniques that LBs in the cerebral cortex in PD patients are significantly increased in brains with a high β-amyloid load pointing to an interaction between these two pathologies at the molecular level (Pletnikova *et al.* 2005).

The pathological and clinical significance of LNs in PD is still uncertain. Although studies have focused on cortical and limbic LB-type pathology as the basis of dementia, in this work a strong association was also observed between αSN burden (i.e. LNs) in the CA2 sector of the hippocampus and dementia. LNs were not initially seen in PD (Dickson *et al.* 1991). However, several subsequent studies have observed such

pathology (Pollanen *et al.* 1993;Imamura *et al.* 2003;Imamura *et al.* 2005) and have seen a correlation with the presence of dementia in PD (Kim *et al.* 1995;Churchyard and Lees 1997). More specifically, Churchyard and Lees extended their analysis to find a correlation between LN burden and severity of dementia, although this correlation has remained controversial (de Vos *et al.* 1996;Mattila *et al.* 1999;Churchyard and Lees 1997) perhaps as ubiquitin immunohistochemistry was used which is a less sensitive method for the visualisation of LBs and LNs (see also section 1.3.1.3). The current work with the use of αSN immunohistochemistry corroborates the existence of numerous LNs in the CA2 sector of the hippocampus as well as its vulnerability to this type of pathology and consolidates previous findings of an association between LNs in the CA2 sector of the hippocampus with dementia in PD. Tau burden in the CA2 sector was also significantly associated with dementia in PD further supporting a primary role of this anatomical region for the presence of dementia in PD. The CA2 sector receives input from the hippocampal dentate gyrus, septal nuclei, entorhinal cortex, and supramammillary region of the hypothalamus and then projects to CA1 field which in turn projects to the subiculum (Giap *et al.* 2000). Pathological changes in CA2 will disrupt normal hippocampal function by interruption of its connections leading to impaired processing and transmission of information.

Previous studies have shown that high LB densities in the cingulate cortex are observed in other conditions such as DLB and Alzheimer's disease (Braak *et al.* 1996;Lippa *et al.* 1999;Harding and Halliday 2001;Rezaie *et al.* 1996). However, the results of this work indicate that αSN burden in the cingulate gyrus could differentiate cases with dementia from those without with high sensitivity and specificity. The positive predictive value (i.e. the probability of having high scores for dementia on reaching moderate to severe αSN burden in the cingulate gyrus) was 85%, further supporting a primary role of αSN in the pathological processes of cognitive dysfunction in PD as well as pointing to the cingulate gyrus as an important focus of damage in PD.

Recent surveys have revealed that anywhere from 6% to 60% of PD patients experience VH (Cummings 1991;Diederich *et al.* 2005). Risk factors for VH include higher age, duration of disease, cognitive impairment, medication, depression and sleep disturbances. In the group of patients characterized by VH but no dementia (PD(H+)) an association was observed with longer duration of disease which is in agreement with

previous reports (Barnes and David 2001;Fenelon *et al.* 2000). The most frequently incriminated drugs for the occurrence of VH in PD patients are dopaminergics (Moskovitz *et al.* 1978;Serby *et al.* 1978;Feigenson *et al.* 1976) anticholinergics (Goetz *et al.* 1982;de *et al.* 1982) and amantadine (Harper and Knothe 1973). Interestingly however, all of the cases in this study (with and without VH) received drugs with the potential to exacerbate hallucinations (it has to be noted though that the dosages were not available). Data from a community based-study in Norway involving 245 PD patients demonstrated an association of hallucinations with advanced age, cognitive decline and depression but not with antiparkinsonian medication (Aarsland *et al.* 1999a). In another study involving 102 PD patients, VH were associated with lower cognitive scores, lower visual acuity, higher Global Deterioration Scale scores and higher Unified Parkinson Disease Rating scores (Holroyd *et al.* 2001). However, there was no association found with the use of dopaminergic drugs, other medications, or L-dopa dosage or duration of treatment (Holroyd *et al.* 2001). Therefore the finding in this work supports previous reports (Aarsland *et al.* 1999a;Williams and Lees 2005;Holroyd *et al.* 2001) of no simple direct cause-effect connection between VH and medication. Thus, it appears that medication *per se* does not clearly account for the presence of VH. This underscores that a pathological substrate has a primary role in development of VH with a permissive effect of drug administration.

In this study the majority of cases with VH had concomitant dementia. In fact, many studies have found a significant strong association of cognitive impairment and VH (Poewe 2003;Klein *et al.* 1997). Excluding potential confounders such as medication, duration of disease and depression, at least three mechanisms have been proposed for this association. First, cognitive impairment may be a marker of more diffuse brain disease. Second, taking into account that VH occur in many neuropsychiatric diseases, they may represent a final common pathway reflecting of a general degradation in information processing. Third hallucinations, may relate to a specific impairment of complex visuo-spatial function in PD, with abnormalities in visual cognition, source monitoring or reality testing (Barnes *et al.* 2003). Interestingly a hypothesis derived from observations from AD relates the association between VH and dementia to disturbed cortical muscarinic receptor activities (Perry and Perry 1995).

It is now well established that the amygdala is a preferential site for LB formation in a variety of conditions including PD (Lippa *et al.* 1999;Lippa *et al.* 1998;Gelb *et al.* 1999;Yamazaki *et al.* 2000), although our understanding of the importance of this lesion in the pathogenesis of PD remains unclear. In this study a significant association was found between αSN burden in the amygdaloid complex with VH in demented PD cases. Interestingly, VH in non-demented PD subjects (PD(H+)) did not associate with αSN burden in the amygdaloid complex. Previous studies by Harding et al., have shown that LB burden in the amygdala positively correlate with VH in both demented and non-demented PD patients (Harding *et al.* 2002a;Harding *et al.* 2002b). Recently, Papapetropoulos and colleagues have also reported an increased LB burden in the amygdala, frontal, temporal and parietal cortices in PD subjects with VH (Papapetropoulos *et al.* 2006). The findings of the present study are in accord with clinico-pathological studies pointing to αSN in the amygdala as an important pathological substrate of VH in demented PD patients. The absence of association between αSN deposition in the amygdala and VH in non-demented PD patients may demonstrate that an increased burden of αSN in amygdala is not related to the presence of VH *per se* but rather that an underlying dementia is a significant determinant. However, the number of PD cases without dementia but with VH in this study was small which may account for the discrepancy between this report and with Harding's et al.,.

The extent and contributing effect of CAA changes on dementia and VH in PD has not been closely studied. The results of the present work indicate that CAA changes is a relatively common finding in PD brains with a higher incidence in demented PD cases as opposed to non-demented subjects. Furthermore, this report demonstrates that the presence of CAA significantly associates with dementia. CAA is a common pathological finding in the elderly and AD patients. Estimates of the prevalence of CAA from autopsy series are on the order of 10% to 40% in the general elderly population and approximately 80% in brains with AD (Jellinger 2002;Xu *et al.* 2003). Whereas autopsy studies may be biased by patient selection, a similar high prevalence of CAA has been observed in 21% of elderly brains in the population-based Medical Research Council (MRC) clinical-pathological series (Neuropathological Group.Medical Research Council Cognitive Function and Aging Study 2001). The occurrence of CAA changes has been shown to relate to dementia. Among subjects in the MRC study, CAA was identified in 34 of 93

with dementia versus only 7 of 99 without dementia, yielding an elevated odd ratio for dementia of 7.7 (Neuropathological Group.Medical Research Council Cognitive Function and Aging Study 2001). The relationship between CAA and dementia could easily be confounded by potential covariates such as age or presence of AD pathology. However, the effect of CAA remained independent when controlling for potential confounders such as age, brain weight, neuritic and diffuse plaques, neocortical and hippocampal NFTs, LBs and cerebrovascular disease. In another population-based study, the Honolulu-Asia Ageing Study, the presence of CAA in AD brains associated significantly with worse cognitive performance during life (Pfeifer *et al.* 2002). Like the finding in the MRC study, the effect of CAA was independent of potential confounders such as NFTs, age, plaque counts, infarctions and apolipoprotein E genotype. Further to this, CAA alone has been shown to be sufficient to cause dementia in some cases of hereditary cerebral hemorrhage with amyloidosis Dutch type (HCHWA-D) independently of AD type pathology (van Duinen *et al.* 1987). In addition, a recent autopsy study comparing 617 cases of PD with 535 age-matched controls revealed a 44.7% increase in CVLs, including CAA, which was slightly lower than AD cases and dementia was more frequent in the PD group with CVLs (Jellinger 2003b). In another study the prevalence of vascular β-amyloid in PD cases with dementia was higher than in age-matched controls (Mastaglia *et al.* 2003). Thus, the data of this work are in accord to those previous studies pointing to an important contribution of CAA to dementia not only in elderly and AD subjects but also in PD patients. Interestingly, VH did not associate with CAA although a slightly higher incidence was observed among PD cases with VH. More studies with larger number of PD cases are needed to unravel the influence CAA on dementia and VH in the PD brain.

3.5 Conclusions

This work demonstrates a widespread αSN pathology in the PD brain and provides evidence of a clinical relevance of αSN lesions in the limbic system with respect to the occurrence of dementia and VH in PD. Of particular interest is the finding that the NBM was equally affected in both demented and non-demented PD subjects indicating that the incidence of pathological changes in anatomical structures other than the NBM are obligatory for the presence of dementia in PD. The selective vulnerability of the CA2 sector of the hippocampus, with respect to neighbouring sectors and the finding that CA2 αSN deposition (i.e. LNs) associates with dementia supports a primary role of this anatomical region in the pathological processes of cognitive dysfunction in PD. In this study, although AD-related pathology associated with dementia in PD, statistical analysis demonstrated that αSN in the cingulate gyrus could differentiate cases with dementia from those without with high sensitivity and specificity and thus placing αSN pathology as a more sensitive indicator of dementia in PD than AD histopathological change which is in agreement with prospective studies (Aarsland *et al.* 2005). The significance of these relationships reported in this study will require further elucidation in larger cohorts.

VH in PD is a complex disturbance and its anatomical and pathological basis difficult to unravel, which is perhaps reflected in the few neuropathological studies of PD patients with VH. However, the present report indicates that αSN burden in the amygdala strongly associates with VH in demented PD subjects. Medication did not seem (at least directly) to contribute to the presence of VH in PD further supporting a substrate of brain pathology for this disabling symptom. However, further studies are warranted to elucidate the cause(s) of VH in demented and non-demented PD patients.

Finally, this report provides evidence that CAA changes can occur in the PD brain alongside PD and AD-related pathological lesions. Interestingly, the current work demonstrated that the presence of CAA relates to dementia in PD. However, further studies on the effect of CAA in PD clinical symptomatology in larger cohorts are needed.

Chapter 4

Striatal Pathology in Parkinson's Disease: Relation to Dementia and Visual Hallucinations

4.1 Introduction

Although most studies suggest that αSN-type lesions in neocortical and limbic structures are strongly associated with dementia and VH in PD (Mattila *et al.* 2000;Mattila *et al.* 1998;Kovari *et al.* 2003;Harding *et al.* 2002a;Harding and Halliday 2001;Hurtig *et al.* 2000) the exact morphological and pathophysiological substrate(s) of these deficits are still debatable. One reason for this debate is that dementia in the setting of PD more likely represents the confluence of additive or synergistic effects of several pathologies. Pathologically PD is classically characterised by loss of dopaminergic neurons in the SNc (Fearnley and Lees 1991) leading to striatal dopamine depletion. Although the motor manifestations of PD are related to this dopaminergic denervation of the striatum, the striatal nuclear complex has also been shown to play a major role in cognition (Nakano *et al.* 2000). Nevertheless, there are very few clinico-pathological studies that have investigated the clinical relevance of striatal pathology in PD (Liang T *et al.* 2006). The present chapter will therefore try to explore the relationship between striatal pathology and cognitive dysfunction in PD with a focus on dementia and VH.

4.1.1 Functional Anatomy of the Striatum

Today the striatum more often is divided into three functional territories. The sensorimotor territory (the putamen) receives input from motor cortices (primary motor cortex, supplementary motor area, premotor cortex) and projects back to them; the associative territory (dorsal caudate nucleus) which receives input from the prefrontal cortex and projects back to it and the limbic territory (ventral striatum; nucleus accumbens) which receives allocortical-mesocortical input and projects to anterior cingulate cortex and medial orbitofrontal cortices (Parent 1990;Parent and Hazrati 1995).

Thus, these territories process motor, cognitive and emotional or motivational information, respectively.

In the striatum 90-95% of the neurons are medium spiny neurons, which give rise to axons which leave the striatum and collaterals that remain within the striatum (Grofova 1975;Kitai and Kocsis 1979;Kawaguchi *et al.* 1990). These neurons are GABAergic and utilise enkephalin or substance P as co-transmitters (Penny *et al.* 1986;Gerfen 1984). The remaining striatal neurons are aspiny neurons thought to act as interneurons (Graybiel 1983). Some of these are also known to utilise acetylcholine as a neurotransmitter (Phelps *et al.* 1985).

Cortical input to the striatum comes mainly from layer 5, 3 and 6 (McGeorge and Faull 1989;Gerfen 1989;Gerfen 1989;Yelnik 2002) and primarily terminates on the medium spiny projection neurons (Dube *et al.* 1988;Sesack and Pickel 1990). The cortical input to the striatum is excitatory and utilises glutamate as its principal neurotransmitter (Fonnum *et al.* 1981). A topographic organization of these cortical inputs has been proposed so that the sensorimotor cortex projects to putamen and caudate nucleus and mesocortex and allocortex to ventral striatum (nucleus accumbens).

The striatum also receives input from the midline and intralaminar, ventral lateral posterior and posterior nuclei of the thalamus (Veening *et al.* 1980) which are excitatory (i.e. glutamatergic) (Kitai *et al.* 1976) (but do not appear to terminate on the same spiny neuron as a corticostriatal afferent (Dube *et al.* 1988). A dopaminergic input to the striatum originates from the retrorubral area, substantia nigra pars compacta, and ventral tegmental area (VTA) (Fallon and Moore 1978). Additionally, serotonergic input to the striatum comes from the dorsal raphe nucleus (Veening *et al.* 1980) and a modest projection from the mesopontine reticular formation also innervates the striatum (Jackson and Crossman 1983).

4.1.2 Pathology of the Striatum

An increased awareness of the complex circuitry of the basal ganglia and the advent of more sensitive methods of detecting pathological changes in the brain prompted recognition of degeneration of the striatum, to which the major efferents of the substantia nigra pars compacta project. There is evidence from imaging studies of volume loss of the left caudate nucleus and putamen in AD, DLB as well as other neurodegenerative

disorders associated with parkinsonism, such as MSA (Barber *et al.* 2002;Baron *et al.* 2001;Konagaya *et al.* 1994;Wakai *et al.* 1994;Schulz *et al.* 1994). PET and SPECT studies of the nigrostriatal dopaminergic system have demonstrated significant abnormalities in the striatum in several parkinsonian disorders including PD and DLB (Brooks 1993;Donnemiller *et al.* 1997). Bochelmann et al., and Bugiani et al., (Bockelmann *et al.* 1994;Bugiani *et al.* 1980) have reported reduced cell number in the striatum of PD brains whereas McNeill and colleagues have demonstrated loss of dendrites in medium spiny neurons, the major recipient of nigrostriatal input to the striatum (McNeill *et al.* 1988). Further to this, Lach et al., has shown abundance of dystrophic neurites in the caudate nucleus (Lach *et al.* 1992). Although numerous recent reports draw attention to the presence of Lewy neurites in patients with PD and DLB in the striatum, other studies have suggested that there is no striatal pathology in these disorders (Forno and Norville 1979;Forno 1996;Gomez-Tortosa *et al.* 2000;Irizarry *et al.* 1998). These conflicting results have prompted a debate over the definite pathological changes in the striatum in PD and DLB.

It was not until recently however, that Duda and colleagues showed with the use of novel synuclein monoclonal antibodies that there is indeed extensive αSN pathology in the striatum in LB disorders (Duda *et al.* 2002). The greatest density was found in patients with a combination of AD and DLB followed by cases with DLB alone. PD patients showed mild to moderate changes. Although this study described a previously underestimated pathology in the striatum in Lewy body diseases no correlation to the clinical deficits was performed thus the clinical relevance of αSN deposition in this region remains unclear.

Following Duba's findings, Liang and colleagues in a small group of patients with LB disorders reported a much higher burden of both Aβ and αSN pathologies in the striatum (particularly Aβ) of cases with DLB in comparison to those with the PDD phenotype who commonly had a paucity of both (Liang T *et al.* 2006). Jellinger and Attems have confirmed these findings in a larger cohort (n=17), pointing to a morphological distinction between DLB and PDD on the basis of differing striatal pathology (Jellinger and Attems 2006). More importantly, however, it is not clear whether striatal pathology could differentiate PD patients who develop dementia from those who remain cognitively intact or have mild or circumscribed cognitive impairments.

In addition, examination of striatal pathology in these previous studies was limited to the caudate nucleus and putamen omitting the ventral striatum (i.e. nucleus accumbens), a region strongly involved in cognition (Nakano *et al.* 2000).

4.1.3 Aims

Given a lack of information with regards to the clinical correlates and significance of striatal pathology in the PD brain, this study aimed to investigate the relationship between striatal pathology and dementia and/or VH in PD. To do this the burden of striatal αSN and AD type changes (both Aβ and tau) was determined in patients at the extremes of the spectrum of PD neuropsychiatric involvement in groups of PD patients with and without dementia and VH.

4.2 Materials and Methods

4.2.1 Neuropathological Assessment

Neuropathological assessment and diagnosis were performed as described in section 2.2.1.

4.2.2 Selection of Cases

The cohort examined in this work is the same as for chapter 3. Therefore, PD cases were stratified into four dementia status groups according to clinical characteristics as follows: 9 PD cases without dementia and without VH (with the exception of one case with mild VH) and with no or mild co-morbid neuropsychiatric features (PD(H-) group), 12 PD cases with severe dementia and with VH (range 2-3; PDD(H+) group), 4 PD cases with moderate to severe dementia alone without VH (range 2-3; PDD(H-) group) and 5 PD cases with severe VH alone without dementia (range 2-3; PD(H+) group) (Table 4.1). For selection of cases refer to section 3.2.2. In addition to neuropsychiatric deficits, the clinical severity of motor features was also recorded and rated. The assessment was performed on a semi-quantitative global impression scale (0-3+, absent to severe) with the assistance of Dr. Ronald K.B. Pearce. The clinical data of the cases are presented in Table 4.1.

Table 4.1 Clinical data for the PD groups.

(Following page) *Abbreviations:* D: Dementia, VH: Visual Hallucinations, A: Anxiety, De: Depression, Pr: Paranoia, Dl: Delusions, T: Tremor, G-B: Gait-Balance problems, A-R, S: Akinesia-Rigidity and Stiffness, H-P: Hemi-Parkinsonism, PD: Parkinson's disease, PDD(H-): PD cases with high clinical scores for dementia and no VH, PD(H+): PD cases with high clinical scores for VH and no dementia, PDD(H+): PD cases with high clinical scores for both dementia and VH, PD(H-): PD cases without dementia and without VH, m: male, f: female, semi-quantitative clinical severity scores 0: absent, 1: mild, 2: moderate, 3: severe, +: yes, -: no, ±: possibly

PD(H-) Group n=9	Case Number	Gender	Age at Onset (years)	Age at Death (years)	Duration of Disease (years)	Neuropathological Diagnosis (www.ICDNS.org)	ICDNS AD GRADE (www.ICDNS.org)	Braak AD-Stages	Clinical Severity Scores of Neuropsychiatric Features D	VH	A	De	Pr	DI	Clinical Severity Scores of Presenting Motor Deficits (within first 2 years) T	G-B	A-R,S	H-P	Clinical Severity Scores of Motor deficits (after 2 years) T	G-B	A-R,S	H-P	L-Dopa Responsiveness
	58	f	52	58	6	PD	isocortical 0 - entorhinal I	I-II	0	0	1-2	0	0	0	1-2	0	0	0	2	0	1-2	0	±
	74	m	76	85	9	PD	isocortical II - entorhinal 0	I-II	0	0	1	0	0	0	1-2	0	0	1-2	1	1	1	0	+
	95	m	47	74	27	PD	Isocortical I - entorhinal 0	I-II	0	0	0	1	0	0	1	0	1	1-2	1	1	2-3	1-2	+
	23	m	75	82	7	PD	isocortical I - entorhinal II	I-II	0	0	0	1	0	0	0	1-2	0	0	0	1	2-3	0	+
	7	m	68	78	10	PD	na	I-II	0	0	0	0	0	0	0	1	1	0	1	0	1-2	0	+
	22	f	65	75	10	PD	isocortical 0 - entorhinal 0	0	0	0	0	0	0	0	0	0	2	1-2	1	0	1	1	+
	29	m	70	77	7	PD	isocortical 0 - entorhinal I	I-II	0	1	0	0	0	0	1	0	1-2	1	1-2	2	1	1	+
	32	m	86	89	3	PD	isocortical 0 - entorhinal I	I-II	0	0	0	0	0	0	1	0	1	0	1-2	1-2	1	0	+
	36	m	67	77	10	PD	isocortical 0 - entorhinal I	I-II	0	0	0	0	0	0	0	0	0	0	0	1	1	0	+
	Mean		**67**	**77**	**9.8**																		
PDD(H+) Group n=12																							
	5	f	57	80	23	PD	isocortical II - entorhinal III	I-II	2	3	0	0	0	0	1	0	0	1	1	0	1-2	1	+
	18	m	77	82	5	PD	isocortical I - entorhinal II	0	2-3	3	1	1	0	0	0	1	1	0	0	1	0	0	+
	15	m	56	70	14	PD	isocortical IV - entorhinal III	III	2-3	2	1	0	0	1	0	1	1-2	0	0	0	1	0	+
	24	m	72	81	9	PD	isocortical 0 - entorhinal I	I-II	2-3	2	1-2	0	0	0	1-2	1-2	0	0	0	1-2	2	0	+
	30	m	57	73	16	PD	isocortical I - entorhinal II	I-II	2	2	0	0	0	0	1	0	0	1	2	1	1	1-2	+
	69	m	71	82	11	PD	isocortical I - entorhinal II	I-II	2	2-3	0	1	0	1-2	1	1	1-2	1	1	1-2	1-2	1-2	+
	2	m	75	84	9	PD	isocortical 0 - entorhinal I	0	2	2	3	2	0	0	0	0	1	1-2	2	1-2	1-2	1-2	+
	16	f	67	85	18	PD	isocortical II - entorhinal I	I-II	2-3	2-3	1	1	0	0	1-2	0	1	1	2	0	0	0	+
	44	m	74	87	13	PD	isocortical I - entorhinal II	I-II	2	3	0	0	0	0	1	0	0	1	1	1	1	1	+
	13	m	54	78	24	PD	isocortical 0 - entorhinal I	I-II	2-3	3	2	0	0	0	0	0	1	0	1	2	1	0	+
	34	f	73	84	11	PD	isocortical IV - entorhinal III	III	3	2	3	0	0	0	1-2	0	0	1-2	1	1-2	0	1-2	+
	60	m	78	84	6	PD	isocortical II -entorhinal II	I-II	2	2	0	0	0	0	0	0	1-2	1-2	0	0	2	0	+
	Mean		**67**	**80**	**13.2**																		
PDD(H-) Group n=4																							
	11	m	62	71	9	PD	isocortical III - entorhinal III	I-II	2	0	0	0	0	0	1-2	0	0	1	2	0	1-2	1	+
	45	m	61	80	19	PD	isocortical 0 - entorhinal III	III	2-3	0	2	2	0	1	0	0	1	1	0	0	1-2	0	+
	56	m	35	54	19	PD	isocortical 0 - entorhinal I	0	2	1-2	0	0	0	0	1	1-2	1-2	1-2	1	1	1	1	+
	14	m	67	79	12	PD	isocortical IV - entorhinal III	I-II	3	1	0	0	0	0	2-3	0	0	0	1	0	1	0	+
	Mean		**56**	**71**	**14.7**																		
PD(H+) Group n=5																							
	63	f	67	80	13	PD	isocortical 0 - entorhinal I	I-II	0	3	0	0	0	0	2	1	0	0	1	1	0	0	+
	42	f	59	79	20	PD	isocortical 0 - entorhinal I	I-II	0	2	1-2	1	0	0	0	1	0	0	0	1-2	3	0	+
	72	m	42	62	20	PD	isocortical 0 - entorhinal 0	0	0	2-3	0	0	0	0	1	0	0	1	1-2	1	0	1-2	+
	20	m	41	75	34	PD	isocortical I - entorhinal II	I-II	0	2	0	1	0	0	2	0	2	1-2	0	0	1	1	+
	21	m	48	76	28	PD	isocortical 0 - entorhinal I	I-II	1	2-3	1	0	0	0	2	0	2	2	0	2	0	0	+
	Mean		**51**	**74**	**23**																		

4.2.3 Immunohistochemistry

Immunohistochemical reactions were performed as described in section 2.2.4. All 30 cases were stained for αSN for the identification of αSN inclusions (Becton-Dickinson at a dilution of 1:1000), tau for visualization of neurofibrillary changes (Autogen Bioclear PHF-Tau (clone AT8) at a dilution of 1:800), 1E8 for visualization of Aβ plaques (Aβ) (at a dilution of 1:1000) and tyrosine hydroxylase (TH) for examination of nigrostriatal fibers (Vector at a dilution of 1:30)

4.2.4 Semi-quantitative Assessment of αSN and AD Lesions

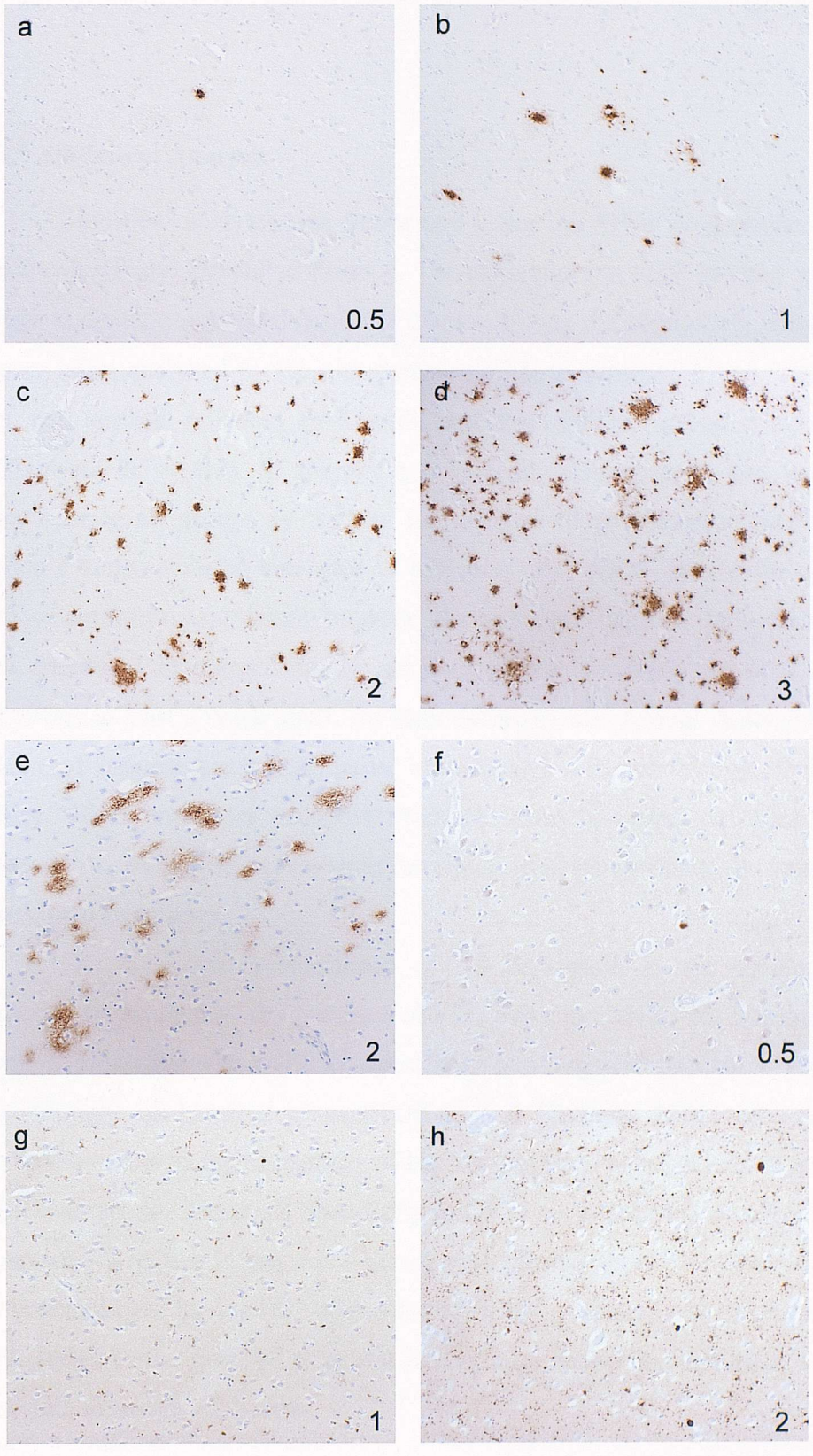
For each case, three representative sections from the caudate nucleus (CN), putamen (Put) and nucleus accumbens (NAcc) were assessed for the occurrence of αSN, Aβ and tau immunoreactivity respectively. The nucleus accumbens was defined as the roughly triangular portion of the striatum at the ventral edge of the internal capsule (Brilliant *et al.* 1997). The pathology was examined semi-quantitatively based on a scale ranging from 0-3 (absent to frequent; Figure 4.1). Sections were screened in their entirety at 10x primary magnification for overall deposit burden. Examination of αSN and AD pathology was also conducted in the temporal and entorhinal cortices, CA2 sector of the hippocampus and superior frontal gyrus and assessed semi-quantitatively from 0-3 (absent to frequent; for semi-quantitative ratings of pathology also refer to section 3.2.4). Sections were graded by two investigators (Mr. Michail E. Kalaitzakis and Dr. Stephen M Gentleman). Cohen's kappa statistics revealed an inter-rater reliability of 0.75 for αSN, 0.8 for tau and 0.84 for Aβ lesions (as mentioned in previous chapters, kappa statistic has a range of 0-1, with larger values indicating better reliability; generally a kappa of >0.70 is considered satisfactory). Intra-rater reliability was also examined (see section 2.2.6). The raw data for αSN, tau and Aβ pathology are given in Appendix IV.

Figure 4.1 Semi-quantitative assessment of αSN and Aβ deposition in the striatum from representative PD cases.

(Following page) Semi-quantitative ratings of pathology. Assessment of Aβ pathology in the striatum was carried out using a CERAD-based (Mirra *et al.* 1991) visual impression of the numerical density of plaques ranging from 1-3 (b, c, d) corresponding to sparse, moderate and frequent. A value of 0.5 was assigned to those fields where only a single lesion was present (a). In a few cases, where the density did not clearly fit into one of the main categories, intermediate values were assigned. Similar ratings were carried out on the Aβ pathology in the neocortex (e) and on the extent of αSN pathology in the striatum (f, g, h). Primary magnification x10

Abbreviations: αSN: α-synuclein, Aβ: amyloid β deposits




a
0.5
b
1
c
2
d
3
e
2
f
0.5
g
1
h
2

4.2.5 Statistical Analysis

Statistical analysis was performed using the SPSS programme version 15.0 for Windows XP and GraphPad Prism 4. The differences in αSN, tau and Aβ burden as well as age at death, onset and duration of disease among the cognitively impaired and PD(H-) groups were analyzed by the non-parametric Mann-Whitney *U*-test. The Kruskal-Wallis test was applied followed by Dunn's post hoc multiple group comparisons to detect differences in severity of tremor, gait-balance, akinetic-rigid and hemi-parkinsonism signs among all groups as well as for analysis of pathological data between groups. Cohen's kappa statistic was used to test inter-rater reliability for the αSN, tau and Aβ semi-quantitative assessment between the two investigators. Intra-rater reliability was also examined by measuring the striatum of five randomly selected subjects on five occasions at least a week apart. On each occasion, all operator-dependent processes (i.e. region of interest semi-quantitative assessment) were performed blinded to previous values. High intra-rater reliability was observed. Correlations were calculated with Spearman's two-tailed correlation analysis (non-parametric). P values <0.05 were considered significant.

The diagnostic performance of Aβ deposition in the striatum in predicting dementia (regardless of the presence of VH) was calculated with measures of sensitivity, positive predictive value (PPV), specificity and negative predictive value (NPV). The methodology of Harding et al.,(Harding and Halliday 2001) was adopted. Briefly, sensitivity refers to the probability of having moderate to severe Aβ deposition with high clinical scores for dementia. Specificity refers to the probability of not reaching moderate to severe Aβ burden in cases not having high clinical scores for dementia. PPV refers to the probability of having high scores for dementia with moderate to severe Aβ deposition. NPV refers to the probability of not having high scores for dementia in the absence of moderate to severe Aβ burden.

4.3 Results

4.3.1 Assessment of Clinical Data

A total of 30 cases were included in this study. Statistical analysis (Kruskal-Wallis) detected no differences in clinical severity of tremor, gait-balance, akinetic-rigid and hemi-parkinsonian signs among the different PD groups.

4.3.2 TH Striatal Pathology

TH immunohistochemistry was performed in all cases included in this study in order to examine the extent of dopamine denervation in the different regions of the striatum (i.e. putamen, caudate nucleus and nucleus accumbens). The overall pattern of cellular staining was noted in the CN, Put and NAcc. All cases showed severe dopaminergic terminal denervation of the striatum compared to a control.

4.3.3 αSN Striatal Pathology

In the PD(H-) group, 5/9 cases (55.5%) showed mild αSN pathology (LBs and LNs) in CN, Put and NAcc while in the remaining cases (44.5%) no αSN pathology was observed. No αSN staining was seen in the CN and Put in 40-50% of cognitively impaired cases while NAcc was negative in 14%. αSN pathology for the rest of the cognitively impaired cases was mild (with the exception of one case showing moderate αSN pathology) and when compared with the PD(H-) group no significant differences were detected (Figure 4.2). Total burden of αSN (expressed as the average load across all regions under examination) between the PD(H-) and all cognitively impaired groups did not differ significantly (Figure 4.3). When present, αSN lesions in the CN were mostly found laterally towards the internal capsule with the caudato-lenticular gray bridges also involved.

Figure 4.2 Severity of αSN, tau and Aβ pathology in the striatum of PD(H-), PDD(H-), PD(H+) and PDD(H+) patients. The columns represent mean values ± SEM.

(Following page) *Abbreviations:* PD: Parkinson's disease, PDD(H+): cases with high clinical scores for both dementia and VH, PDD(H-): PD cases with high clinical scores for dementia and no VH, PD(H+): PD cases with high clinical scores for VH and no dementia, PD(H-): PD cases without dementia and without VH, CN: Caudate Nucleus, Put: Putamen, NAcc: Nucleus Accumbens, αSN: α-synuclein, Aβ: amyloid β deposits. * denotes significant difference (Mann-Whitney *U*-test, $p<0.05$).

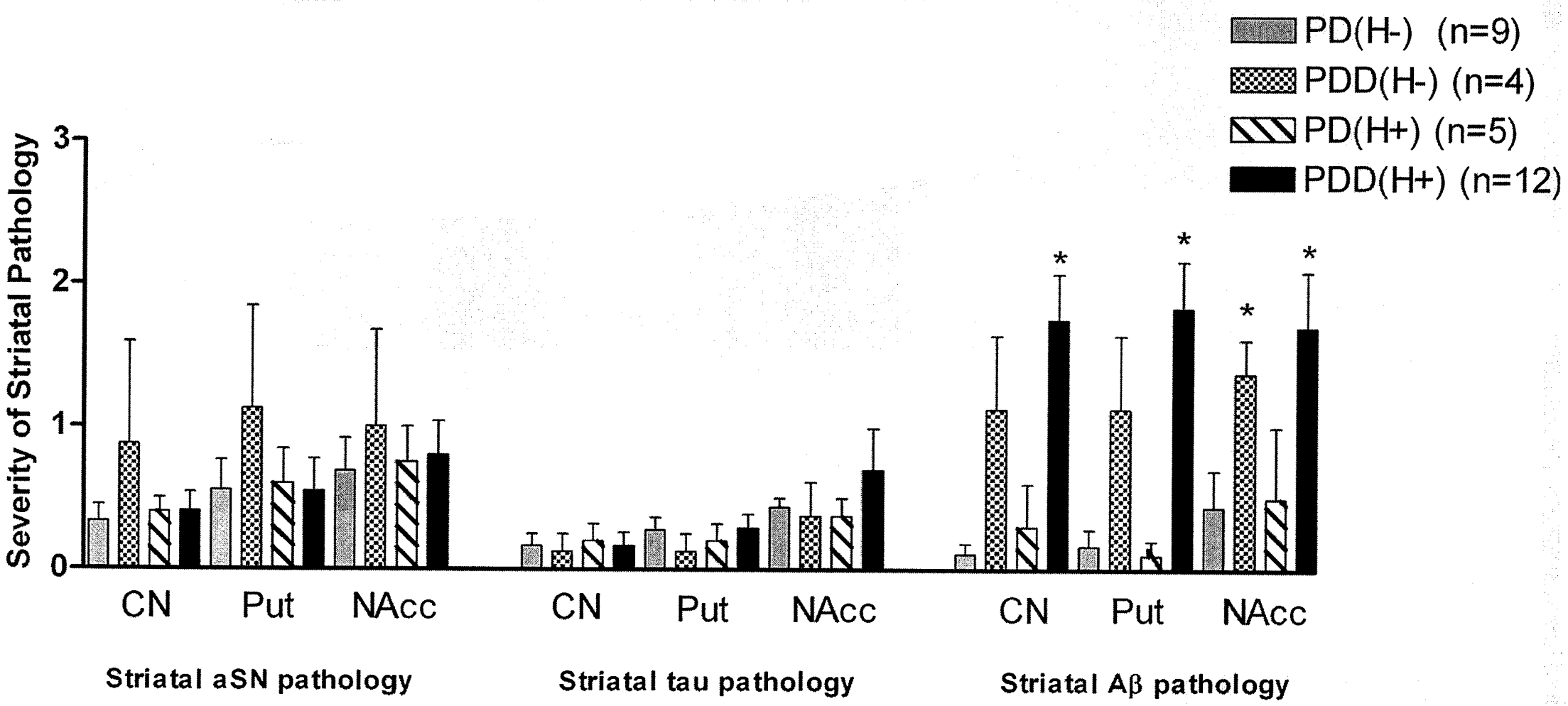
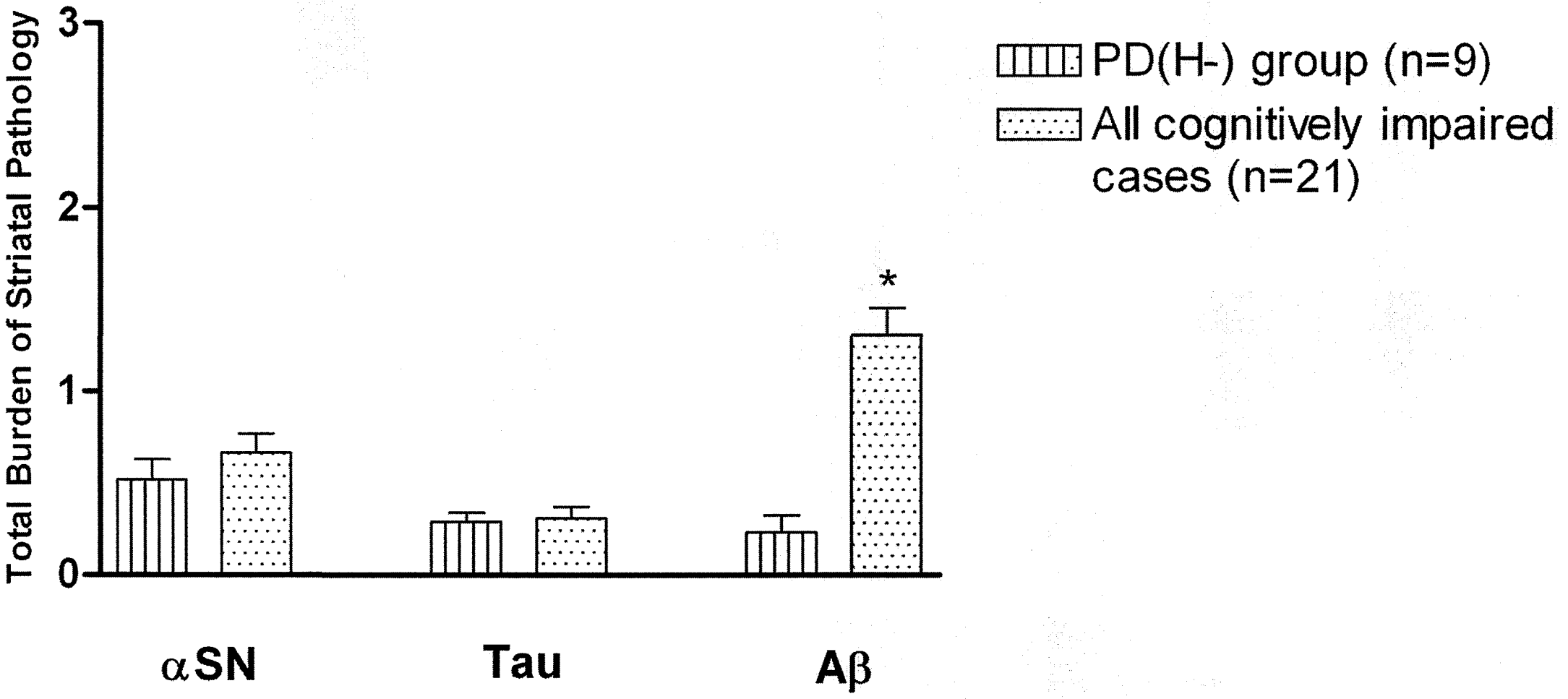

PD(H-) (n=9)
PDD(H-) (n=4)
PD(H+) (n=5)
PDD(H+) (n=12)
Severity of Striatal Pathology
0
1
2
3
CN
Put
NAcc
CN
Put
NAcc
CN
Put
NAcc
*
*
*
*
Striatal aSN pathology
Striatal tau pathology
Striatal Aβ pathology

Figure 4.3 Total burden of αSN, tau and Aβ pathology between PD(H-) and cognitively impaired (PD(H+), PDD(H-) and PDD(H+)) PD patients across all striatal regions examined. The columns represent mean values ± SEM.

(Following page) *Abbreviations:* PD: Parkinson's disease, PD(H-): PD cases without dementia and without VH, Cognitively Impaired (PDD(H+): cases with high clinical scores for both dementia and VH, PDD(H-): PD cases with high clinical scores for dementia and no VH, PD(H+): PD cases with high clinical scores for VH and no dementia), αSN: α-synuclein, Aβ: amyloid β deposits. * denotes significant difference (Mann-Whitney *U*-test, $p<0.05$).


Total Burden of Striatal Pathology
3
2
1
0
*
αSN
Tau
Aβ
PD(H-) group (n=9)
All cognitively impaired cases (n=21)

4.3.4 Tau Striatal Pathology

Tau pathology (neurofibrillary tangles and neuropil threads) in the PD(H-) group was mild in CN (3/9 cases; 33.3%), in Put (5/9 cases; 55.5%) and NAcc (7/9 cases; 77.7%), while the rest of the cases were negative. Tau pathology was also mild or absent in all cognitively impaired groups (PDD(H+), PD(H+), PDD(H-)) with the exception of one case demonstrating moderate tau pathological changes in NAcc. No statistical differences were detected between the PD(H-) and cognitively impaired groups with respect to tau burden in CN, Put and NAcc or the total tau striatal burden (Figure 4.2 and 4.3).

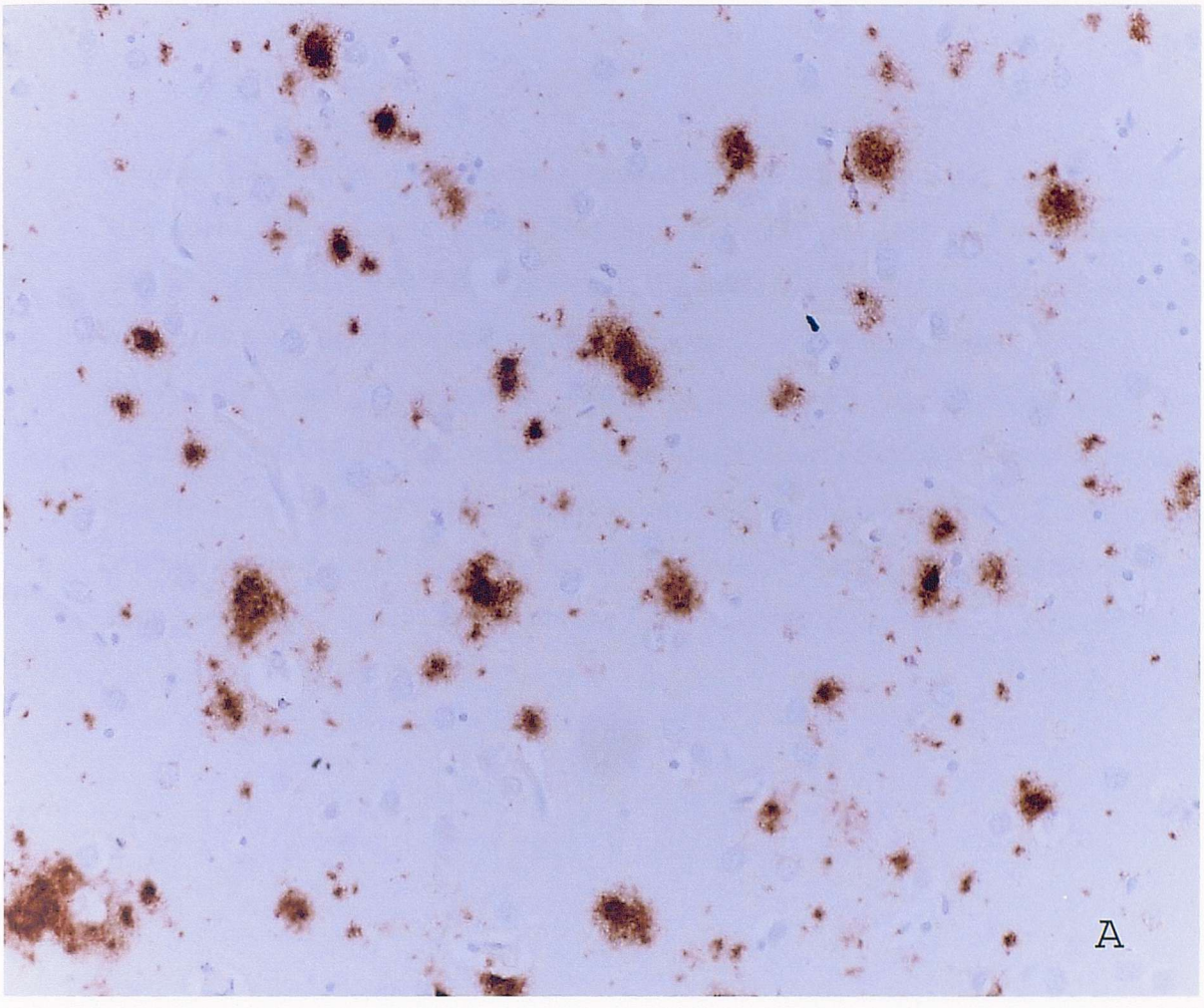
4.3.5 Aβ Striatal Pathology

The morphology of Aβ deposits were of four types in all striatal regions as follows: 1) large plaques that resemble diffuse cortical plaques, 2) smaller, more intensely stained deposits of a diffuse type that outnumbered the larger plaques by a factor of approximately 3:1, 3) small, presumably extracellular, deposits (size of a glial cell nucleus) and 4) dot-like aggregates (Figure 4.4). On the whole, Aβ lesions were more frequent and more severe than αSN and tau pathology in the demented [PDD(H-) and in PDD(H+)] groups. Aβ was absent in 7/9 of PD(H-) cases (77.7%) and only minimal in the remaining 2 cases (22.3%) in CN, Put and NAcc. Similarly, PD(H+) cases showed sparse Aβ deposition in all striatal regions, with the exception of one case showing moderate Aβ burden in NAcc. In contrast, PDD(H-) and especially PDD(H+) cases demonstrated moderate to severe Aβ pathology (Figure 4.2). When compared with the PD(H-) group, Aβ burden in the PDD(H-) cases was significantly greater in the NAcc ($p=0.04$) (Figure 4.2) whereas PDD(H+) cases showed a significantly higher burden of Aβ pathology in all striatal regions [CN ($p=0.002$), Put ($p=0.003$) and NAcc ($p=0.03$)] when compared with the PD(H-) group (Figure 4.2 and 4.4). Statistical analysis for group comparisons revealed a significant greater Aβ burden in the putamen of PDD(H+) cases in comparison to non-demented PD cases with VH (PD(H+)) (Kruskal-Wallis; $p=0.02$). These differences can not be attributed to age, as correlational analysis revealed that Aβ burden did not correlate with age at death. Comparison between non-demented and demented PD cases, irrespective of the presence of VH, revealed a significantly greater

Aβ burden in the latter group (p=0.001) (Figure 4.5). Furthermore, the severity of Aβ load in the striatum did not associate with the severity of Aβ deposits in cortical regions. Looking at the AD grading of isocortical and entorhinal pathology in demented PD cases (with and without VH) (Figure 4.6C), 60% of cases had abundant Aβ deposition in the striatum independently of cortical AD pathology. This was more apparent when looking at isocortical and entorhinal AD pathology independently (Figure 4.6B and C). Interestingly, demented cases (with and without VH) showed a significantly greater overall Aβ deposition in the striatum than in the cortex (Figure 4.7).

Figure 4.4 Immunostaining for β-amyloid deposits in the caudate nucleus.

(Following page) A) A large number of Aβ deposits in the caudate nucleus of a case with high clinical scores for both dementia and VH. B) Caudate nucleus without Aβ deposits from a PD case with no dementia and no VH. Magnification 20x.


A

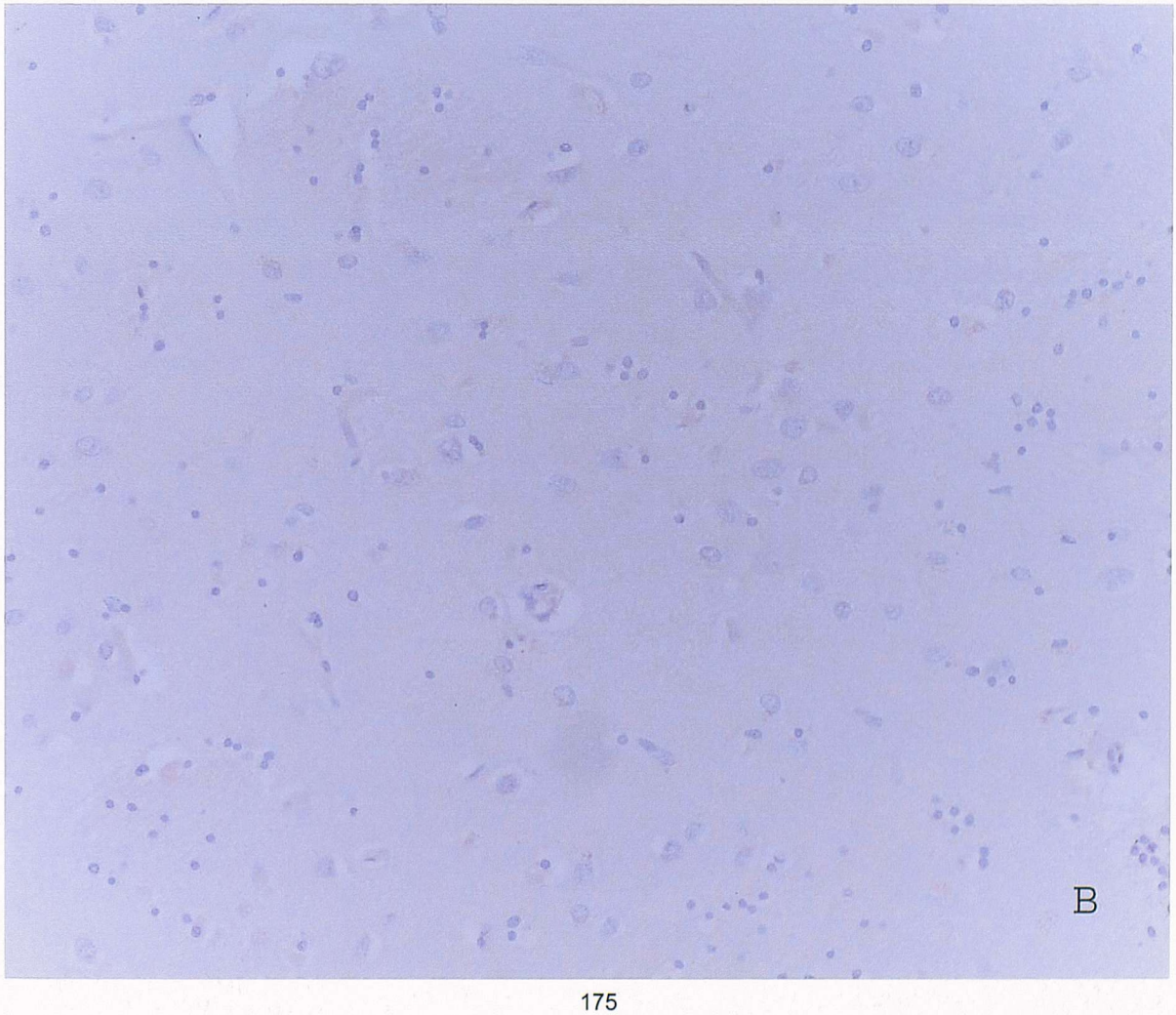

B

Figure 4.5 Scatter plot showing the extent of Aβ deposition in the striatum of demented PD subjects [(PDD(H+) and PDD(H-) (n=16)] and non-demented PD subjects [(PD(H-) and PD(H+) n=14)]. Each data point represents the averaged Aβ severity score for all striatal regions assessed (CN, Put and NAcc).

(Following page) *Abbreviations:* PDD(H+): cases with high clinical scores for both dementia and VH, PDD(H-): PD cases with high clinical scores for dementia and no VH, PD(H+): PD cases with high clinical scores for VH and no dementia, PD(H-): PD cases without dementia and without VH, PD: Parkinson's disease, CN: Caudate Nucleus, Put: Putamen, NAcc: Nucleus Accumbens, Aβ: amyloid β deposits, * denotes significant difference (Mann-Whitney *U*-test, $p<0.05$).

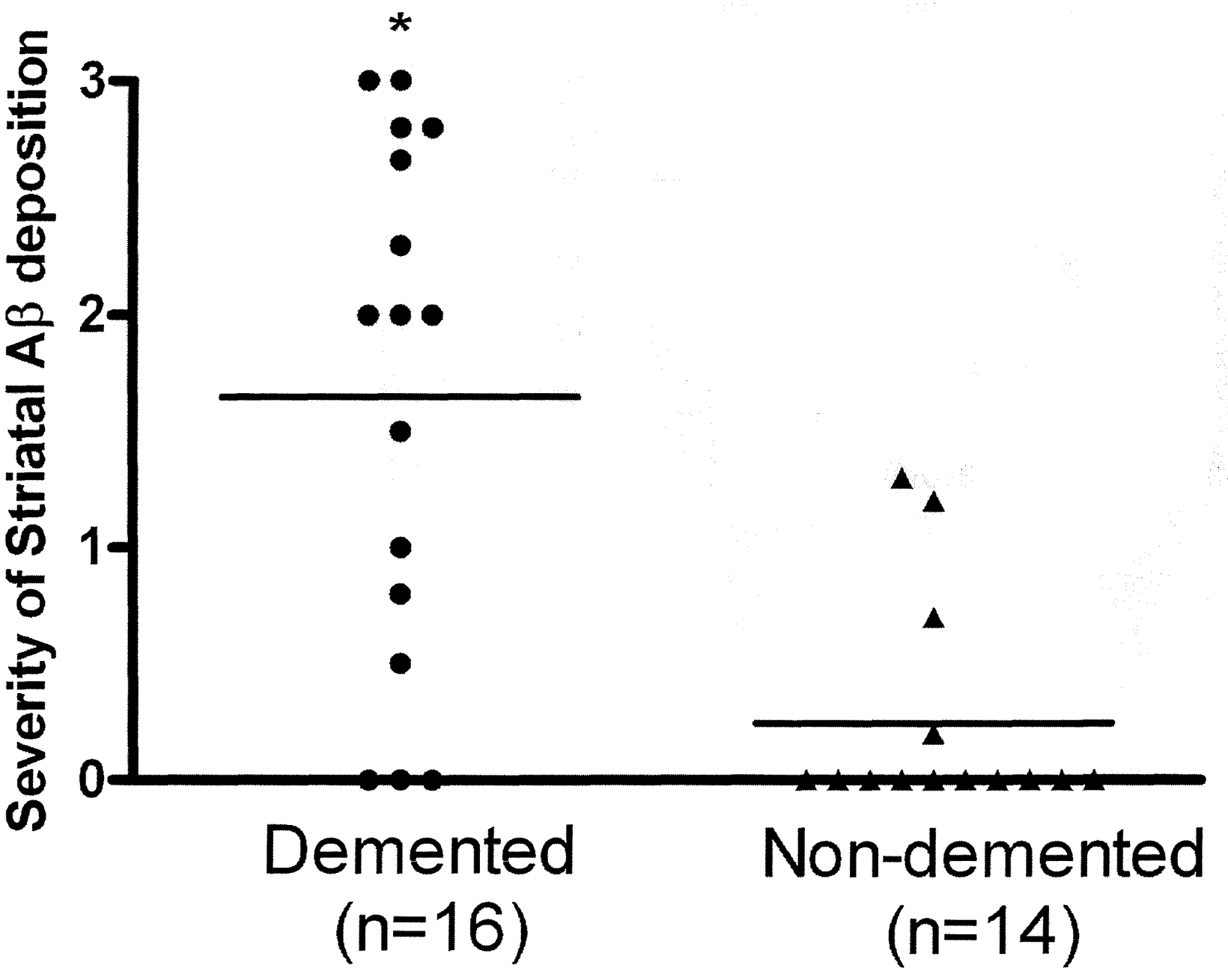

*
Severity of Striatal Aβ deposition
3
2
1
0
Demented
(n=16)
Non-demented
(n=14)

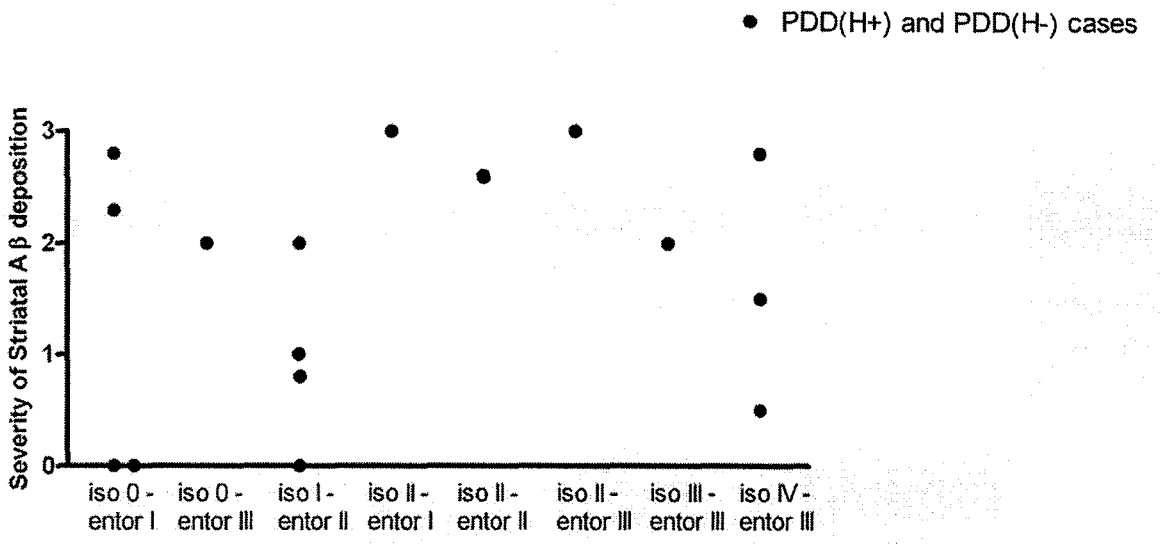
Figure 4.6 Scatter plots (A-C) showing the association between AD pathology in iso- and entorhinal cortices with the overall extent of Aβ deposition in the striatum of demented PD subjects (PDD(H+) and PDD(H-)) (n=16).

(Following page) A) Relation of iso- and entorhinal AD pathology with Aβ deposition severity in the striatum of demented PD subjects. B) Association between AD pathology in the entorhinal cortex with severity of striatal Aβ deposits. C) Relationship between AD pathology in isocortex and severity of striatal Aβ deposition. Striatal Aβ pathology in the striatum tends to dissociate from appreciable tandem AD co-morbidity in isocortex and entorhinal cortex.

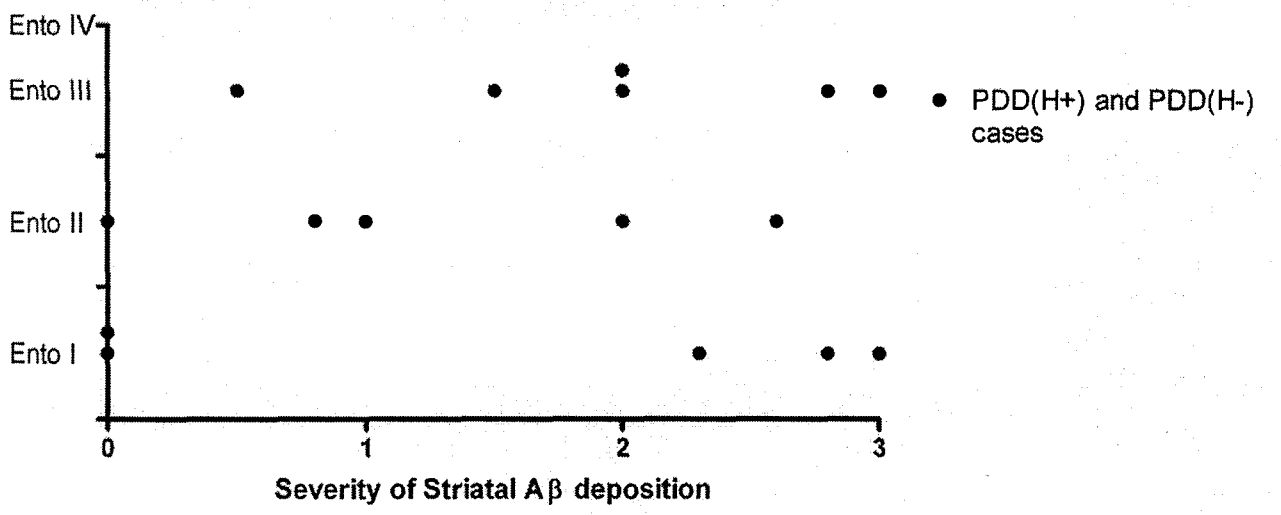
Each data point for the Aβ pathology in the striatum represents the averaged Aβ severity score across all striatal regions assessed (CN, Put and NAcc).

Abbreviations: PD: Parkinson's disease, AD: Alzheimer's disease, (PDD(H+): cases with high clinical scores for both dementia and VH, PDD(H-): PD cases with high clinical scores for dementia and no VH, Aβ: amyloid β deposits, CN: Caudate Nucleus, Put: Putamen, NAcc: Nucleus Accumbens

A


PDD(H+) and PDD(H-) cases
Severity of Striatal A β deposition
0
1
2
3
iso 0 - entor I
iso 0 - entor III
iso I - entor II
iso II - entor I
iso II - entor II
iso II - entor III
iso III - entor III
iso IV - entor III

B


Ento IV
Ento III
Ento II
Ento I
0
1
2
3
Severity of Striatal Aβ deposition
PDD(H+) and PDD(H-) cases

C

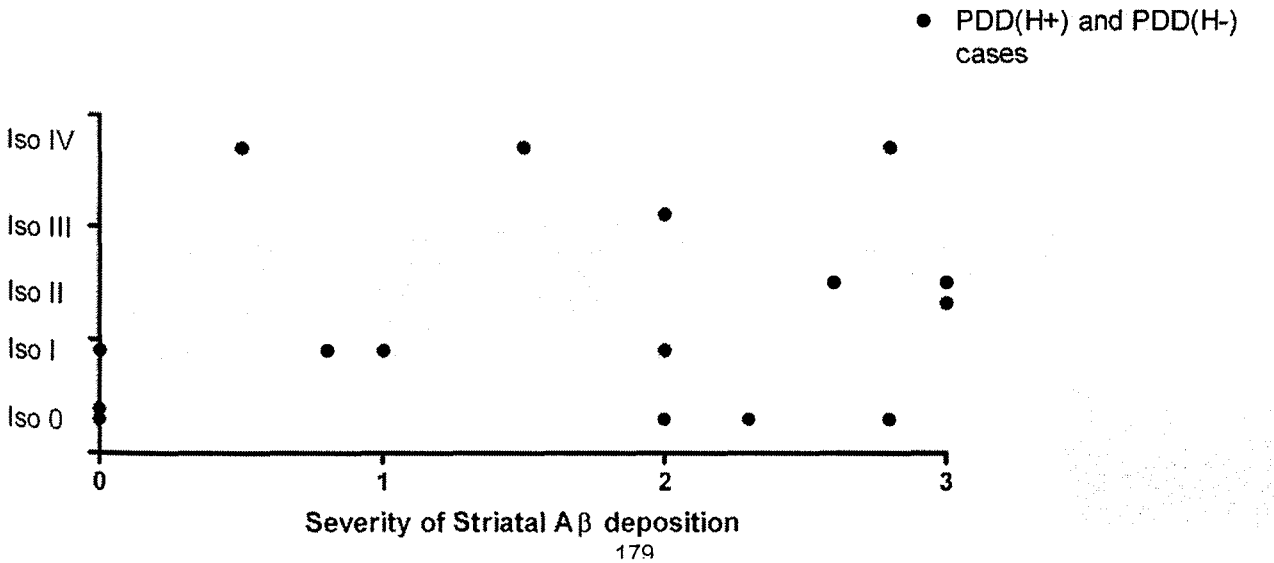
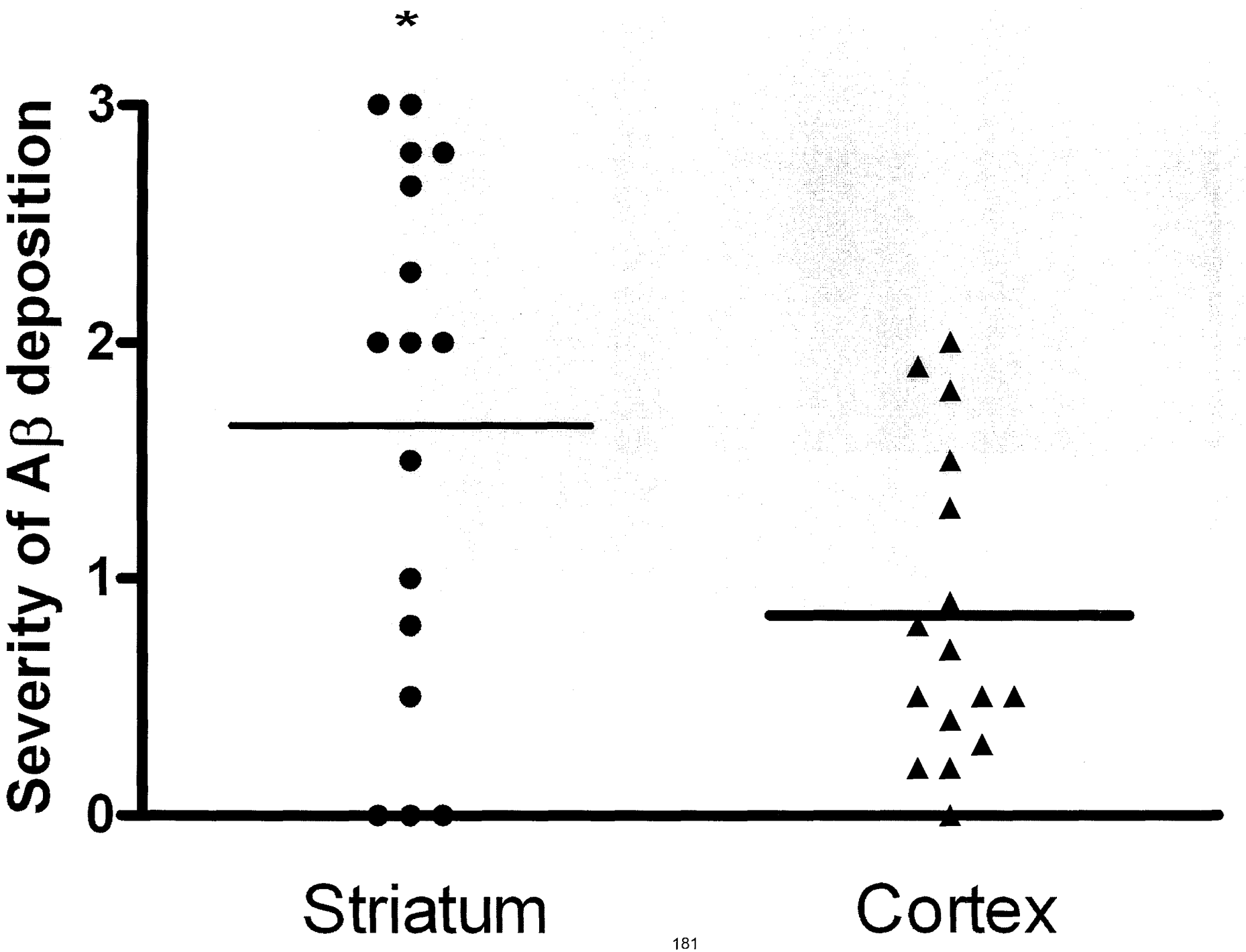

PDD(H+) and PDD(H-) cases
Iso IV
Iso III
Iso II
Iso I
Iso 0
0
1
2
3
Severity of Striatal Aβ deposition

Figure 4.7 Scatter plot showing the overall extent of Aβ deposition in the striatum and cortex of demented PD subjects (PDD(H+) and PDD(H-)) (n=16). A significantly greater deposition of Aβ burden is observed in the striatum than in the cortex (p=0.03).

(Following page) Each data point for the striatum represents the averaged Aβ severity score for all striatal regions assessed (CN, Put and NAcc) while each data point for the cortex corresponds to the semi-quantitative averaged Aβ severity score across the following cortical regions: temporal and entorhinal cortices, CA2 sector of the hippocampus and superior frontal gyrus.

Abbreviations: PDD(H+): cases with high clinical scores for both dementia and VH, PDD(H-): PD cases with high clinical scores for dementia and no VH, PD: Parkinson's disease, CN: Caudate Nucleus, Put: Putamen, NAcc: Nucleus Accumbens, Aβ: amyloid β deposits, * denotes significant difference (Mann-Whitney *U*-test, $p<0.05$).


*
Severity of Aβ deposition
3
2
1
0
Striatum
Cortex

In order to test the diagnostic performance of Aβ deposition in the striatum in predicting dementia (regardless of the presence of VH) the sensitivity, PPV, specificity and NPV in all striatal regions was calculated (Table 4.2). The sensitivity (i.e. the probability of having moderate to severe Aβ deposition with high clinical scores for dementia) was 56% whereas the PPV (the probability of having high clinical scores for dementia on reaching moderate to severe Aβ deposition) was 90%. Furthermore, the specificity and NPV for cases without dementia (i.e. PD(H-) and PD(H+) groups) was high in the range of 65-93% (Table 4.2). Further to this, total Aβ burden was significantly greater in the cognitively impaired cases than cognitively intact (p=0.0001) (Fig 3).

Table 4.2 Two by two contingency table for calculation of sensitivity, PPV, specificity and NPV for Aβ burden in the caudate nucleus, putamen and nucleus accumbens in clinically demented PD cases regardless of the presence of VH (i.e. PDD(H+) and PDD(H-) groups).

(Following page) Sensitivity of demented PD cases (9/16X100)= 56%, PPV for demented PD cases (9/10X100)= 90%. Specificity for cases without dementia (13/14X100)= 92.8%, NPV for cases without dementia (13/20X100)= 65%

Abbreviations: Aβ: amyloid β plaques, PPV: positive predictive value, NPV: negative predictive value

Two by two contingency table for calculation of sensitivity, PPV, specificity and NPV for Aβ burden in the CN, Put and NAcc in demented PD cases regardless of visual hallucinations [(PDD(H+) and PDD(H-) group]

Caudate Nucleus, Putamen and Nucleus Accumbens Aβ burden	Clinically Demented		Total number of cases
	yes	no	
2-3	9	1	10
0-1	7	13	20
Total Cases	16	14	30

4.4 Discussion

Recent pathological studies point to LB-type pathology in limbic and neocortical regions as a main cause of cognitive impairment/dementia and VH in PD (Mattila *et al.* 2000;Mattila *et al.* 1998;Hurtig *et al.* 2000;Harding and Halliday 2001;Apaydin *et al.* 2002;Aarsland *et al.* 2005;Kovari *et al.* 2003;Hurtig *et al.* 2000), while others have not found a clear relationship (Parkkinen *et al.* 2005a). The present literature is further confounded by the AD type changes that often co-exist with αSN pathology. Despite extensive anatomical surveys in the literature, striatal pathology has not been examined in detail. In this study striatal αSN deposition was mild in all striatal regions under investigation, with no statistical differences detected between different PD clinical phenotypes with or without dementia. Furthermore, all groups showed minimal or absent striatal tau pathology. These findings demonstrate that there is no evident association between αSN and tau pathology in the striatum with dementia or VH. This result by implication provides further evidence that LBs in neocortical and limbic structures are more likely responsible for the development of dementia and VH in PD.

While αSN and tau pathology did not differ between PD demented and non-demented and between hallucinating and non-hallucinating groups, Aβ deposition in the striatum distinguished PD patients with dementia from those without. More specifically, an increased Aβ in the NAcc of cases with dementia but without VH (PDD(H-)) was observed and when we looked at dementia with VH as an added complication (PDD(H+)), all striatal regions exhibited increased Aβ burden and this increase was independent of the extent of co-morbid cortical AD pathology. The observation that Aβ deposition was minimal in the PD(H+) group implies that increased striatal Aβ deposition does not relate to the presence of VH but rather to cognitive impairment. The results of the present work also indicate that Aβ burden in all striatal regions could differentiate cases with dementia from those without with high sensitivity and specificity, further supporting a primary role of striatal Aβ deposition in the pathological processes of cognitive dysfunction in PD as well as pointing to the striatum as an important focus of damage in PD. It is worth noting that the clinical severity of motor deficits was similar among all PD groups demonstrating that the pathology observed was not related to motor features. Altogether, the results demonstrate a greater Aβ striatal pathology in PD cases with cognitive impairment/dementia in the absence of prominent cortical Aβ deposition.

Early reports found no clear abnormality of the striatum in PD (Forno and Norville 1979). However, two recent studies have revealed an abundant Aβ burden in the striatum of DLB cases that was more pronounced than in PDD, pointing to a morphological distinction between those two diseases (Jellinger and Attems 2006;Liang T *et al.* 2006). Herein, the existence of cases with extensive striatal Aβ pathology is confirmed and also this work shows for the first time a clinical relevance of Aβ deposition in the striatum in PD patients.

The emerging view since the mid-1970s has been that dementia can be separated into cortical and subcortical types, a distinction that has been supported from both the clinical (Pillon *et al.* 1993) and aetiological perspectives (Darvesh and Freedman 1996). In cortical dementias with AD as the prototype of the most common dementing illness (dementia of the AD-type), neuropathological changes occur predominantly in cortical regions (e.g. temporal, frontal and parietal cortices). Senile plaques and NFTs populate the cortex and there is generalised cortical atrophy, especially in frontal and temporal lobes, with neuronal degeneration affecting particularly the outer three layers. The typical clinical findings include dyscalculia, aphasia, apraxia and agnosia that are said to be indicative of cortical dysfunction. On the other hand, in subcortical dementias the lesions occur predominantly in the basal ganglia, the brainstem and the cerebellum (Darvesh and Freedman 1996) and the clinical picture is correspondingly different with a profound slowness of thought and information processing (bradyphrenia), memory disturbances, frontal executive dysfunction and changes in personality and affect in the absence of aphasias, apraxias and agnosias (Cummings 1986). Although, subcortical dementia may result from numerous diseases, PD is one of the most common. The result of this work demonstrates the presence of prominent Aβ deposition within the striatum of demented patients with PD that dissociated from appreciable tandem AD co-morbidity and this offers a firm pathological substrate for features ascribed to subcortical dementia in these individuals giving rise to a distinctive clinical presentation.

Although AD and PD are largely understood as distinct disease entities, the frequent overlap of clinical and pathological features raises the possibility that these neurodegenerative diseases may involve common pathogenetic pathways (Kotzbauer *et al.* 2001). Many AD patients develop extrapyramidal symptoms whereas a high proportion of PD patients become demented (Galvin *et al.* 2001). Pathological studies

have shown that LBs are frequently found in AD with some reports estimating αSN positive inclusions in 50-60% of patients with AD or Down syndrome (Lippa *et al.* 1998;Hamilton 2000). The findings of the present work lead to further acknowledgement of a molecular link between PD and AD with respect to Aβ deposition, with the topography of involvement in subcortical regions (i.e. striatum) to be distinctive in those cases with concomitant dementia (i.e. PDD) independently of a formal second diagnosis of AD in these brains.

There is considerable diversity of opinion regarding the significance of plaque formation in the neocortex to the etiopathogenesis of both AD and PD (Arnold *et al.* 1991;Arriagada *et al.* 1992a;Terry 1996;Mattila *et al.* 2000;Kovari *et al.* 2003). For example there have been inconsistent reports on the correlation between Aβ burden and various measures of clinical deficit in both diseases (Vickers *et al.* 2000). Furthermore, in elderly subjects without overt dementia, plaques are found in the neocortex and viewed as part of normal ageing (Arriagada *et al.* 1992b). In contrast, Aβ deposition in the striatum is universally found in AD brains but is rarely observed in non-demented elderly individuals (Gearing *et al.* 1993;Suenaga *et al.* 1990). Further to this, according to the phases of β-amyloid deposition in the human brain proposed by Thal et al., Aβ deposition in the striatum occurs in phase 3 with associated clinical dementia and thus it appears to reflect a disease specific pathological change in clinical proven AD subjects (Thal *et al.* 2002). This evidence makes the striatum as an important anatomical region that is closely related to dementia in AD. This study confirms these previous reports and shows for the first time that striatal pathology in the PD brain strongly associates with clinical dementia.

In PD the striatum, a primary affected brain area, shows an heterogeneous pattern of dopamine denervation as reflected by tyrosine hydroxylase immunoreactivity (Graybiel *et al.* 1987). The dorsolateral caudate-putamen exhibits more severe dopamine denervation than the ventral striatum (nucleus accumbens) (Perez-Otano *et al.* 1994). It is thus possible that the pattern of dopamine terminal loss may influence the distribution of Aβ deposits. However, according to the present findings the distribution of plaques in different regions within the striatum did not appear to be affected by the diminution of tyrosine hydroxylase which was universal in the striatum irrespective of dementia status. These results suggest that progression to clinical dementia in PD is associated with an increased Aβ burden in the striatum that does not reflect tyrosine hydroxylase positive

terminal nigrostriatal denervation. Further studies are needed however, to unravel any possible links between the chemoarchitecture of the striatum and striatal Aβ distribution.

Previous studies have shown that the morphology of striatal Aβ plaques in Lewy body disorders is of the diffuse cortical type (Jellinger and Attems 2006) however, this study shows that striatal Aβ deposits portray a diversity of morphologies that by and large could be grouped into four categories. This finding demonstrates that the Aβ deposits in the striatum may not represent morphologically a mere extension of the cortical plaques, but may relate to the mosaic distribution of striatal projection systems. The striatum exhibits a mosaic pattern with markers for a variety of neurotransmitters and other substances (Gerfen 1984). While patches or striosomes are enriched in enkephalin and substance P immunolabel, the matrix is acetylcholinesterase-positive (Gerfen 1984). These two compartments of the striatum also exhibit different patterns of connectivity with other brain regions (Gerfen 1984;Kincaid and Wilson 1996). Although in this study Aβ deposition was not examined in relation to the striatal mosaic pattern, it is possible that the different morphologies of Aβ deposits observed in this study reflect striatum's complex yet well-established heterogeneous microenvironment. Further evidence that striatal plaques may not represent a mere extension of cortical plaques comes from morphological studies in AD brains which report that striatal diffuse plaques are smaller than their cortical counterparts and in the range of 10μm to 30μm in diameter while diffuse plaques in the neocortex reaching up to 1000μm in diameter (McKenzie *et al.* 1996). Although, both cortical and striatal diffuse plaques are comprised of β-amyloid terminating at residue 42 and contain little or no $A\beta_{40}$, striatal plaques do not label with antibodies to apolipoprotein E (Gearing *et al.* 1995;Kida *et al.* 1994). In contrast, cortical diffuse plaques are apolipoprotein E-positive (Kida *et al.* 1994;Gearing *et al.* 1995). These differences, in addition to the fact that the predominant plaque type in the striatum of AD brains, regardless of age and disease duration, is the diffuse plaque (Gearing *et al.* 1993;Braak and Braak 1990;Suenaga *et al.* 1990) supports a distinct Aβ pathological change in the striatum. This is also reflected by the diverse morphologies of Aβ deposits reported in this PD study and needs further elucidation.

A dopaminergic deficit is regarded as the main neurochemical impairment in PD. In this cohort, we found a universal dopaminergic denervation of the striatum. This finding demonstrates that loss of striatal monoaminergic terminals is severe in all PD

cases with or without cognitive impairment. The present results are in agreement with imaging studies showing that the rate of striatal dopaminergic loss does not seem to increase as dementia develops (Colloby *et al.* 2005). Furthermore, the daily clinical experience that dementia does not improve with levodopa treatment suggests that the dopaminergic deficit is not the main neurochemical impairment responsible for dementia in PD.

While part of the motor loop, the striatum also plays a major role in cognition and behavior (Nakano *et al.* 2000). The CN contributes to memory (Wise 1996), learning (Nakano 2000;Knowlton *et al.* 1996) and behavioral functions and lesions lead to apathy and decreased recent memory (Schultz *et al.* 1997). The NAcc receives input from prefrontal and limbic regions (Nakano *et al.* 2000), its efferents primarily pass to extrapyramidal centers but also to a number of limbic-related structures including the septum and the bed nucleus of the stria terminalis (Newman and Winans 1980). Abnormal deposition of Aβ plaques would disrupt striatal function by interruption of limbic connections and thus directly or indirectly contribute to dementia in PD. Nevertheless, the cellular mechanisms of this increased Aβ pathology in the striatum of PD brains are unclear. However, recent studies suggest an interaction between Aβ pathology and αSN lesions (Pletnikova *et al.* 2005). In DLB brains the presence of Aβ deposits in the neocortex was associated with increased αSN lesions and higher levels of insoluble Aβ (Pletnikova *et al.* 2005). These data suggest a synergistic reaction between Aβ and αSN as has been suggested also for tau and αSN (Galpern and Lang 2006;Iseki *et al.* 2003) although the specific molecular mechanisms of this synergistic interface between abnormally aggregated proteins awaits further clarification.

Predominant motor symptoms (bradykinesia, rigidity and tremor) have been differentially linked to severity of intellectual decline in PD. Research has demonstrated that bradykinesia and rigidity correlate with cognitive impairment, whereas tremor does not (Iwasaki *et al.* 1989;Huber *et al.* 1992;Huber SJ 1991;Portin RJ 1989). Portin and colleagues (Portin RJ 1989) found that patients with predominant bradykinesia and rigidity were more likely than patients with tremor to show mental status decline. Huber and colleagues (Huber SJ 1991) compared patients with predominant tremor to those with predominant bradykinesia and rigidity and found only the latter group of patients exhibited deficits in memory, visuospatial and executive skills. Iwasaki and colleagues

(Iwasaki *et al.* 1989) reported a negative correlation between bradykinesia and visuospatial tasks and rigidity and memory tasks in patients with PD. These findings, apart from suggesting that the pathophysiological mechanisms responsible for producing bradykinesia and rigidity may also underlie PD-related cognitive deficits also point to an interaction between motor symptoms and cognitive decline. Although the present study did not aim to examine the influence of motor disability on cognitive dysfunction, the finding that motor deficits were similar among the PD groups irrespective of dementia status argues against a straightforward association between cognition and motor deficits rather the relationship seems to be more complex as supported by previous reports (Katzen *et al.* 2006) that implicate laterality of motor disability as an important determinant for the development and severity of mental deficits. Further studies are needed to explain the relationship between predominant motor deficits in PD on cognition and behaviour.

4.5 Conclusions

The present work demonstrates an increased Aβ striatal burden in PD cases with dementia as opposed to non-demented PD subjects. This finding provides direct evidence of a clinical relevance of striatal pathology in PD. Of particular interest was the finding that the increased striatal Aβ pathology was observed in the absence of prominent cortical Aβ deposition which offers a firm pathological substrate for features ascribed to subcortical dementia in these individuals giving rise to a distinctive clinical presentation. Nevertheless, the neuropathology of demented PD individuals emphasise the interaction of both cortical and subcortical lesions (see also chapter 3). Also, the findings of this study provide further acknowledgement of a molecular link between PD and AD with respect to Aβ deposition, as well as highlighting a primary role of the striatum in the pathological processes of cognitive dysfunction in PD.

The present work also shows that striatal Aβ deposits portray a diversity of morphologies that may not represent a mere extension of cortical plaques, but may relate to the intrinsic mosaic distribution of striatal projection systems. Further studies are needed to unravel the possible links between the chemoarchitecture of the striatum and the distribution of striatal Aβ plaques in the brain of both PD and AD subjects.

Chapter 5

Anxiety in Parkinson's Disease: Possible Anatomical and Pathological Correlates of this Disabling Symptom

5.1 Introduction

Dementia and VH have been recognised as two major non-motor complications typically associated with PD. However, PD is accompanied by a spectrum of psychiatric symptoms with more than 60% of PD patients having one or more neuropsychiatric symptoms (Aarsland and Karlsen 1999;Aarsland *et al.* 1999c) including depression, apathy, agitation, delusions, euphoria, paranoia, irritability and anxiety. The most studied of these features is depression (Tandberg *et al.* 1996), while much less attention has been given to anxiety despite an up to 40% incidence among PD sufferers (Lauterbach and Duvoisin 1991;Starkstein SE *et al.* 1993) and an increased risk for anxiety in PD patients compared to non-PD age-matched controls (Richard *et al.* 1996;Menza and Mark 1994) (see also section 1.2.3.1). Nevertheless, there is no study to date investigating whether PD individuals with anxiety can be differentiated from those without at a neuropathological level. The present chapter will therefore try to investigate possible pathological correlates of anxiety in PD.

5.1.1 Anxiety in PD

Anxiety is a state characterized by a vague and unpleasant sense of apprehension, often accompanied by autonomic symptoms such as palpitations and dry mouth. Anxiety can be beneficial when warning of impending danger, but it may also be pathologic if symptoms are prolonged or excessive or if they occur inappropriately. An anxiety disorder connotes the presence of a significant disturbance interfering with normal well being due to anxiety. There are several different types of anxiety disorders which are distinguished from one another by their clinical context symptom constellation, response to medication, and in some cases presumed etiologic mechanism. While in many studies

the type of anxiety disorder is not specified by the authors others have reported a wide range of anxiety disorders in patients with PD. In a study by Stein and colleagues of the nine PD patients with significant anxiety disorder, one had generalised anxiety disorder, two had panic disorder, one had long standing panic disorder with depression, one had panic disorder with social phobia, three had social phobia alone, and one had anxiety disorder not otherwise specified (Stein *et al.* 1990). Vazquez et al., described only panic disorder, whereas Schiffer et al., reported the presence of panic disorder and generalised anxiety disorder (Vazquez *et al.* 1993;Schiffer *et al.* 1988). In familial parkinsonism the following rates have been reported: simple phobia; 34.27%, agoraphobia; 15.8%, obsessive-compulsive disorder; 13.2%, panic disorder; 7.9%, generalised anxiety disorder; 5.3% and social phobia; 5.3% (Lauterbach and Duvoisin 1991). Thus the diagnoses of anxiety disorders in patients with PD appear to be clustered in the panic disorder, phobic disorder and generalised anxiety disorder (Menza *et al.* 1993;Lauterbach and Duvoisin 1991;Stein *et al.* 1990;Schiffer *et al.* 1988). Nevertheless, further studies are needed in larger patient populations and standard psychiatric interviews to clarify the relative frequencies of the various anxiety disorder types in PD.

5.1.2 Pathophysiology of Anxiety in PD

Despite a high incidence of anxiety in PD it is still unclear why PD patients are especially prone to anxiety. Anxiety may relate to the psychological and social difficulties of the disease. Patients with PD experience higher stress than people without the disease (Ellgring *et al.* 1990). Patients with PD may suffer from social anxiety: patients are afraid of being negatively evaluated in public and thus anxiety and social withdrawal may then result. However, Schiffer et al., found a higher frequency of anxiety in depressed parkinsonian patients than in depressed multiple sclerosis patients (Schiffer *et al.* 1988). Therefore anxiety in PD may not simply be the result of psychological and social difficulties of the disease but may reflect specific neurobiological mechanisms.

Anxiety in PD may relate to the neurochemical changes of the disease itself. There is evidence to suggest that dopamine may be involved in the development of anxiety directly (Stein *et al.* 1990) or via its interaction with the variable deficits of noradrenalin and serotonin that are also known to occur in PD (Lauterbach and Duvoisin 1991;Iruela *et al.* 1992). The ventral tegmental area and the locus coeruleus which give rise to

mesolimbic and noradrenergic pathways, demonstrate significant neuronal loss in PD (Forno 1996). Dopamine inhibits the rate of firing of the locus coeruleus and a loss of dopamine inhibition could explain the high prevalence of anxiety in PD. More specifically, there is strong evidence to suggest that noradrenergic dysfunction, particularly of α_2-adrenergic receptors, and perhaps in the locus coeruleus itself, in the development of primary anxiety disorders especially panic disorder (Nutt and Lawson 1992;Heninger and Charney 1988). Interestingly, several abnormalities in the noradrenergic system have been also discovered in PD (Agid *et al.* 1989). Bernal and co-workers suggest that untreated PD is associated with a significant reduction in α_2-adrenergic sensitivity (Berlan *et al.* 1989). It is thus possible that PD patients are more vulnerable to panic attacks because they have an alteration of the α_2-adrenergic receptor.

Anxiety may be caused by abnormalities of serotonin. A functional polymorphism in the promoter region of the transporter gene has been recently linked to anxiety. Menza and colleagues have reported that PD patients who carry the short allele of the serotonin transporter score significantly higher than non-carriers on anxiety scales (Menza *et al.* 1999). These findings suggest that a genetic defect may play role in susceptibility to anxiety in PD.

The potential role of GABA in the genesis of anxiety is suggested by the efficacy of benzodiazepines for the treatment of panic disorder and generalised anxiety disorder; these anxiolytic drugs produce their effects by activating GABA receptors in the brain (Charney *et al.* 1990). In most of the PD brain examined so far, an increased concentration of GABA in putamen and pallidum and a decreased concentration in cortical areas have been observed (Agid *et al.* 1989).

5.1.3 Aims

Aside from the neurotransmitter systems and their possible link to anxiety in PD there is no up-to-date neuropathological study to examine pathologic burden in relation to anxiety in the PD brain. Therefore, this study aimed to investigate the relationship between αSN and AD-type pathology (both tau and Aβ) in cortical, limbic and brainstem nuclei in PD patients with and without anxiety as a means of identifying possible pathological correlates of anxiety in PD and thus provide a better understanding of the pathophysiological mechanisms responsible for this disabling symptom.

5.2 Materials and Methods

5.2.1 Neuropathological Assessment

Neuropathological assessment and diagnosis were performed as described in section 2.2.1.

5.2.2 Selection of Cases

A total of 81 cases were included in this study. The cases were screened and the clinical severity of dementia, VH, anxiety, depression, delusions and paranoia were graded on a semi-quantitative global impression scale (0-3+, absent to severe) by two investigators as described in section 2.2.2.1. Out of the 81 cases initially screened 57 received a clinical and neuropathological diagnosis of PD. Of these cases 44% experienced anxiety alone or in combination with other neuropsychiatric signs. Anxiety co-existed with dementia and VH in 29% of cases while anxiety in combination with depression occurred in 22%. Interestingly, 21% of cases had anxiety without depression. Among non-demented PD subjects 14% had anxiety. The degree of co-morbidity between anxiety, delusions and paranoia was less pronounced (12.2% and 7% respectively). For the purpose of the present work, PD cases were categorised according to anxiety status as follows: PD cases without anxiety and with no co-morbid neuropsychiatric features (n=9) (PD (A-) group), and PD cases with moderate to severe clinical scores for anxiety (n=6) (range 2-3; PD (A+)) (Table 5.1).

In addition to neuropsychiatric deficits the clinical severity of motor features was also recorded and rated in this study. The assessment was performed on a semi-quantitative global impression scale (0-3+, absent to severe) by two investigators. The clinical data of the cases are presented in Table 5.1.

Table 5.1 Clinical data for the PD groups.

(Following page) *Abbreviations:* PD(A+): PD cases with high clinical scores for anxiety, PD(A-): cognitively intact PD cases, D: Dementia, VH: Visual Hallucinations, A: Anxiety, De: Depression, Pr: Paranoia, Dl: Delusions T: Tremor, G-B: Gait-Balance problems, A-R, S: Akinesia-Rigidity and Stiffness, H-P: Hemi-Parkinsonism, PD: Parkinson's disease, m: male, f: female, semi-quantitative clinical severity scores 0: absent, 1: mild, 2: moderate, 3: severe, +: yes, -: no, ±: possibly

Table 1 Clinical data for the PD groups

PD(A-)	Case Number	Gender	Age at Onset (years)	Age at Death (years)	Duration of Disease (years)	Neuropathological Diagnosis (www.ICDNS.org)	ICDNS AD GRADE (www.ICDNS.org)	Braak AD-Stages	Clinical Severity Scores of Neuropsychiatric Features D	VH	A	De	Pr	DI	Clinical Severity Scores of Presenting Motor Defic (within first 2 years) T	G-B	A-R,S	H-P	Clinical Severity Scores of Motor deficits (after 2 years) T	G-B	A-R,S	H-P	L-Dopa Responsiveness
	58	f	52	58	6	PD	isocortical 0 - entorhinal I	I-II	0	0	1-2	0	0	0	1-2	0	0	0	2	0	1-2	0	±
	74	m	76	85	9	PD	isocortical II - entorhinal 0	I-II	0	0	1	0	0	0	1-2	0	0	1-2	1	1	1	0	+
	95	m	47	74	27	PD	Isocortical I - entorhinal 0	I-II	0	0	0	1	0	0	1	0	1	1-2	1	1	2-3	1-2	+
	23	m	75	82	7	PD	isocortical I - entorhinal II	I-II	0	0	0	1	0	0	0	1-2	0	0	0	1	2-3	0	+
	7	m	68	78	10	PD	na	I-II	0	0	0	0	0	0	0	1	1	0	1	0	1-2	0	+
	22	f	65	75	10	PD	isocortical 0 - entorhinal 0	0	0	0	0	0	0	0	0	0	2	1-2	1	0	1	1	+
	29	m	70	77	7	PD	isocortical 0 - entorhinal I	I-II	0	1	0	0	0	0	1	0	1-2	1	1-2	2	1	1	+
	32	m	86	89	3	PD	isocortical 0 - entorhinal I	I-II	0	0	0	0	0	0	1	0	1	0	1-2	1-2	1	0	+
	36	m	67	77	10	PD	isocortical 0 - entorhinal I	I-II	0	0	0	0	0	0	0	0	0	0	0	1	1	0	+
	Mean		**67**	**77**	**9.8**																		
PD(A+)																							
	19	f	54	72	18	PD	isocortical I - entorhinal 0	I-II	0-1	0	2	1	0	1	3	0	0	3	3	0	2	0	+
	37	m	85	87	2	PD	isocortical 0 - entorhinal I	I-II	0	0	2	0	0	0	1-2	1-2	2	0	1-2	1	1	0	+
	39	f	69	82	13	PD	isocortical I - entorhinal I	I-II	0	1	3	3	0	0	1-2	0	0	1	1	1-2	1-2	0	+
	41	m	67	77	10	PD	isocortical I - entorhinal II	I-II	1	0	3	3	0	0	1-2	0	0	1-2	1-2	0	0	1-2	+
	54	m	64	75	11	PD	isocortical III - entothinal III	I-II	0-1	0	2	0	0	0	1	0	0	1-2	1-2	1	0	1	+
	94	m	61	75	14	PD	isocortical 0 - entorhinal II	I-II	0	2	2	0	0	0	1-2	0	1	1	1-2	0	1	1-2	+
	Mean		**66.6**	**78**	**11.3**																		

5.2.3 Immunohistochemistry

Immunohistochemical reactions were performed as described in section 2.2.4. All 15 cases were stained for αSN for the identification of αSN inclusions (Becton-Dickinson at a dilution of 1:1000), tau for visualization of neurofibrillary changes (Autogen Bioclear PHF-Tau (clone AT8) at a dilution of 1:800) and 1E8 for visualization of Aβ plaques (Aβ) (at a dilution of 1:1000).

5.2.4 Semi-quantitative Assessment of αSN and AD Lesions

For each study case, three representative sections from the temporal, cingulate and entorhinal cortices as well as the hippocampal CA2 sector, subiculum, amygdala, nucleus basalis of Meynert (NBM), locus coeruleus and raphe nuclei were assessed for the occurrence of αSN, Aβ and tau immunoreactivity. The semi-quantitative rating scheme was used based on a scale ranging from 0-3 (absent to severe) as described in previous chapters 3 and 4. Sections were graded by two investigators (Mr. Michail E. Kalaitzakis and Dr. Stephen M Gentleman). Cohen's kappa statistics revealed an inter-rater reliability of 0.75 for αSN, 0.8 for tau and 0.8 for Aβ. For visual representation of the severity rating system please refer to image Figure 3.1. The raw data for αSN, tau and Aβ pathology are given in Appendix V.

5.2.5 Statistical Analysis

Statistical analysis was performed using the SPSS programme version 15.0 for Windows XP and GraphPad Prism 4. The differences in αSN, tau and Aβ burden as well as age at death, onset and duration of disease among PD cases with anxiety and those without (the PD(A-) group) were analyzed by the non-parametric Mann-Whitney *U*-test. The Kruskal-Wallis test was applied followed by Dunn's post hoc multiple group comparisons to detect differences in severity of tremor, gait-balance, akinetic-rigid and hemi-parkinsonism signs among the groups. Cohen's kappa statistic was used to test inter-rater reliability for the αSN, tau and Aβ semi-quantitative assessment between the two investigators. P values <0.05 were considered significant.

5.3 Results

5.3.1 Assessment of Clinical Data

Gender, age at PD onset, age at death, duration of disease, as well as the clinical severity of the individual neuropsychiatric and motor symptoms assessed are presented in Table 5.1. All apart from possibly one case were L-Dopa responsive (Table 5.1). Age at death, age at disease onset and duration of disease did not differ between the PD(A+) and PD(A-) groups. PD(A+) cases had a significantly greater degree of tremor severity ($p=0.01$) within the first 2 years of the disease course. No differences were observed with respect to the other motor signs assessed.

5.3.2 Pathological Data

The PD(A-) group comprised 9 PD cases without anxiety and with no or only mild concomitant neuropsychiatric signs. Among this group LBs were sparse in the neocortical, limbic and brainstem regions. Of note, however, is the extent of αSN pathology in the NBM among the PD(A-) group which was clearly more pronounced than the other regions assessed. Most of the PD(A-) cases displayed a mild degree of AD type pathological change.

In the PD(A+) group there was a trend towards increased αSN burden in all regions under investigation (Figure 5.1). However, when compared with the PD(A-) group this was only significantly greater for the amygdaloid complex ($p=0.004$), CA2 sector of the hippocampus ($p=0.008$) and locus coeruleus ($p=0.04$) (Figure 5.1 and Figure 5.2). Among the PD(A+) group however, there were three cases with concomitant depression. Exclusion of these cases from the analysis resulted in a loss of statistical significance for the locus coeruleus although an increased αSN burden in the amygdala and CA2 sector of the hippocampus was still apparent ($p=0.01$ and 0.04, respectively). Neither tau nor Aβ burden in any region showed any relationship to anxiety.

Figure 5.1 αSN burden in the PD(A+) and PD(A-) groups

(Following page) Semi-quantitative assessment of αSN burden in cases with anxiety reveals a significant difference when compared with the PD(A-) group in the CA2 sector of the hippocampus, amygdaloid complex and locus coeruleus. The columns represent mean values ± SEM.

Abbreviations: PD(A+): PD cases with high clinical scores for anxiety, PD(A-): cognitively intact PD cases, C: Cingulate gyrus, T: Temporal Cortex, EC: Entorhinal Cortex, AC: Amygdaloid Complex, NBM: Nucleus Basalis of Meynert, CA2: CA2 sector of the Hippocampus, SUB: Subiculum, LC: Locus Coeruleus, RN: Raphe Nuclei * denotes significant difference (Mann-Whitney *U*-test, $p<0.05$).



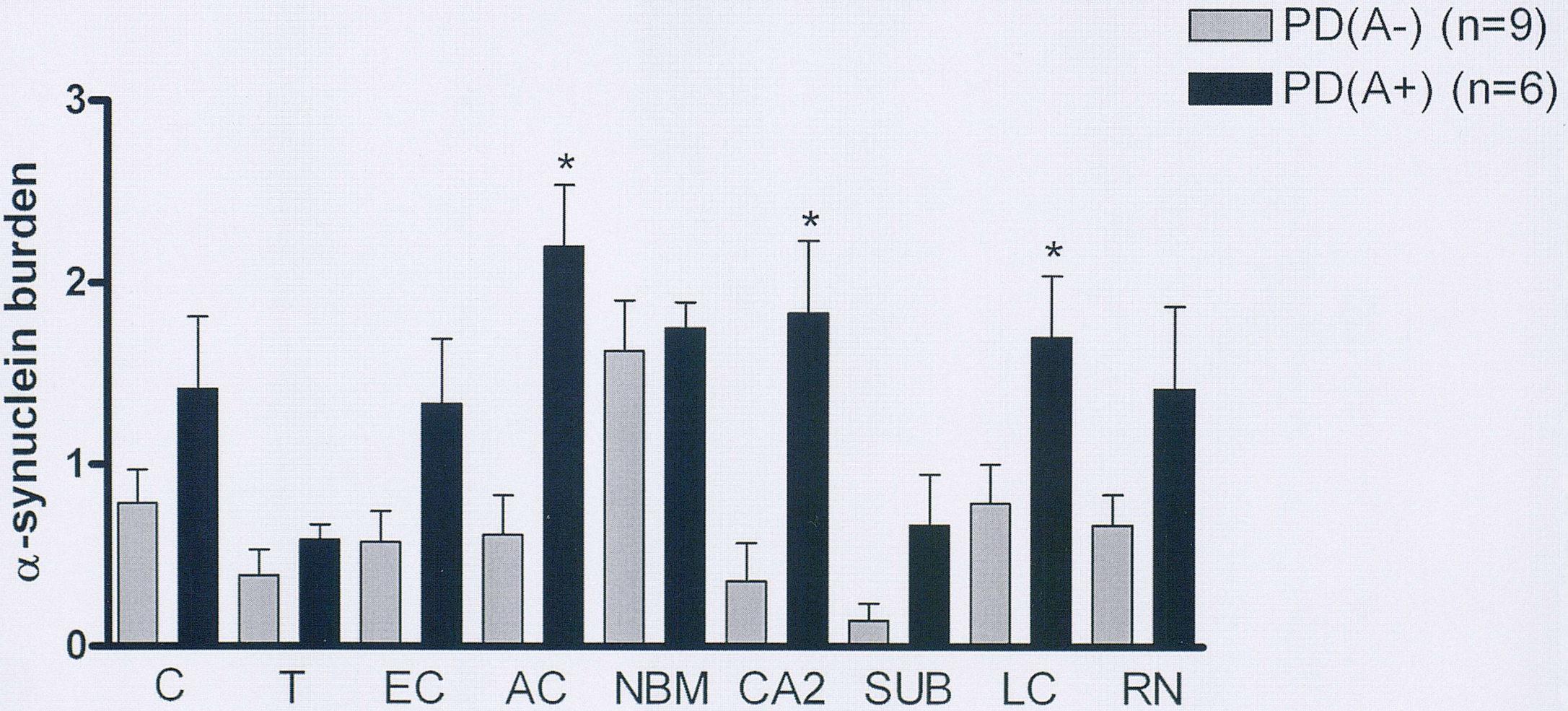
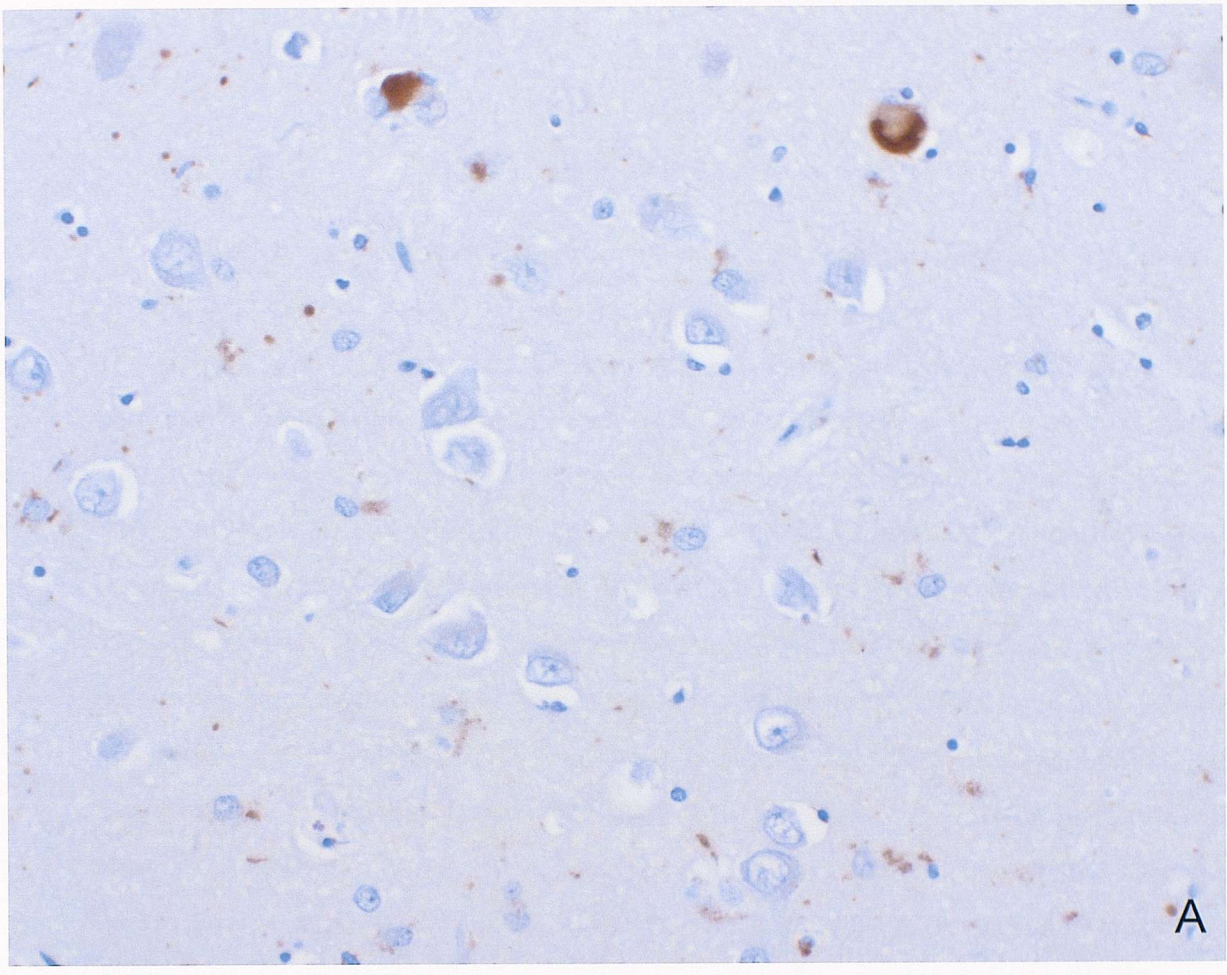
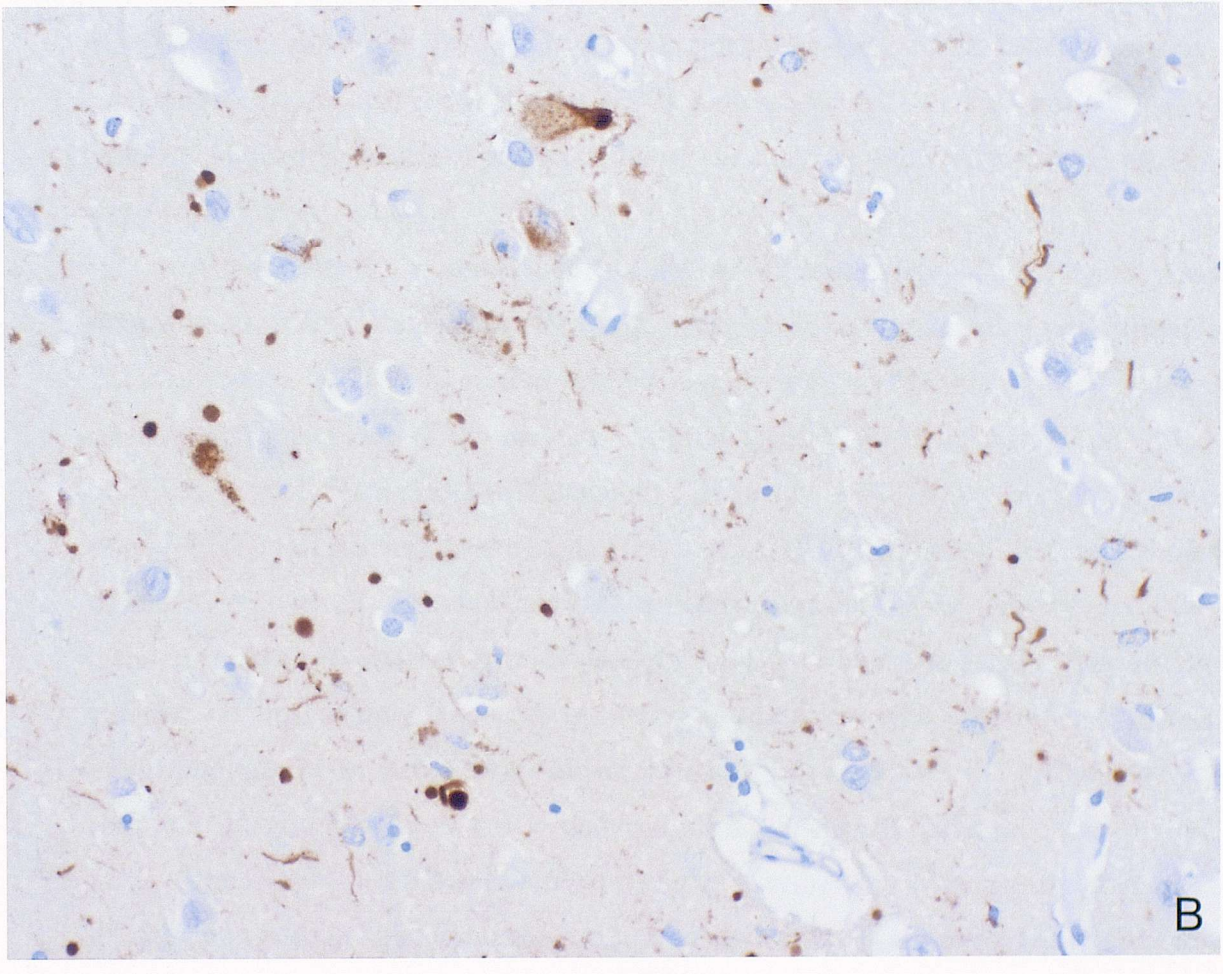

PD(A-) (n=9)
PD(A+) (n=6)
α-synuclein burden
0
1
2
3
*
*
*
C
T
EC
AC
NBM
CA2
SUB
LC
RN

Figure 5.2 Photomicrographs of the amygdala of a PD case with anxiety and a PD case without

(Following page) αSN immunostaining of a PD case without anxiety (A) and a PD case with anxiety (B). Primary magnification 20x


A


B

5.4 Discussion

Anxiety is a common complication of PD (Cummings 1992). In this study the overall frequency of anxiety of 44% is consistent with previous reports (Richard *et al.* 1996;Walsh and Bennett 2001). Anxiety and other neuropsychiatric disturbances, particularly depression, commonly co-exist in PD. Patients with the disease have significantly higher levels of depression and anxiety than normal controls (Hillen and Sage 1996) and in fact anxiety in combination with depression may represent a specific depressive subtype in PD. Henderson and colleagues have found a prevalence of 38% of PD patients with depression and anxiety compared with 8% of healthy spouse controls: depression and anxiety were highly correlated in PD patients (Henderson *et al.* 1992). In this cohort the prevalence of 22% of what is slightly less than previous studies, more likely reflects the smaller sample size studied (n=57). Although, studies have reported a close link between anxiety and depression, anxiety can clearly occur in the absence of depression. A study by Liu et al., revealed that, although the severities of anxiety and depression via standardised rating scales were generally correlated, 14 of 58 PD patients (24%) determined to be free of depressive symptoms fulfilled DSM-III-R criteria for generalised anxiety disorder (Liu *et al.* 1997). In this cohort, 21% of the cases had anxiety without depression confirms previous reports that anxiety can occur without associated depressive symptomatology.

Although there is some evidence for an increased rate of anxiety in primary dementia disorders (Wands *et al.* 1990) the relationship between anxiety and dementia in PD is uncertain. Most of the studies that examined anxiety in PD either failed to comment on the cognitive status of the patients (Schiffer *et al.* 1988;Henderson *et al.* 1992) or deliberately excluded patients with dementia (Stein *et al.* 1990;Menza *et al.* 1993;Siemers *et al.* 1993). Nevertheless, Iruela et al., (Iruela *et al.* 1992) has found that the prevalence of anxiety is lower in demented than non-demented patients. On the contrary, in this study 29% of demented patients had co-existent anxiety while among the non-demented patients 14% had anxiety. Thus, our results are in agreement with Lauterbach (Lauterbach 1993) pointing to an increased incidence of anxiety in demented PD patients, although studies in a larger population are needed to confirm the present findings.

The relationship between anxiety and motor symptoms is controversial. A number of studies have found that the severity of motor state in PD is not related to anxiety (Stein

et al. 1990;Menza *et al.* 1993). However, some authors have reported higher degrees of anxiety in patients who experience 'on-off' motor fluctuations (Menza *et al.* 1993;Henderson *et al.* 1992). One could conjecture that the unpredictable nature of the on-off states might result in anxiety behaviour similar to that which occurs in laboratory animals after they are exposed to unpredictable aversive stimuli (Seligman 1968). Most authors have noted that in patients who do experience the on-off phenomenon, anxiety tends to occur most often during the 'off' phase (Stein *et al.* 1990;Siemers *et al.* 1993;Henderson *et al.* 1992). Further to this, one study revealed that in most patients mood or anxiety improved significantly from 'off' to 'on', but then worsened again in the 'on' state when dyskinesias appeared (Menza *et al.* 1990). In this PD(A+) group we noted a significantly greater degree of tremor severity, presenting within the first two years of the disease course, although no differences were detected with respect to severity of other motor signs assessed between the PD(A+) and PD(A-) groups. Thus the results of the present report support previous studies of a connection between the motor state and anxiety in PD and accentuate tremor as an important clinical feature relating to anxiety. However, it has to be noted that in the present study it was not possible examine the severity of anxiety during on-off fluctuations.

The findings of this report point to αSN pathology in the amygdala and hippocampus as possible candidate regions underlying the pathogenesis of anxiety in PD. Although these regions have been previously related to dementia and VH (Harding *et al.* 2002a;Samuel 1998) in PD the results of the present study show that they may also underlie anxiety as the PD(A+) cohort was relatively free of dementia and VH. In fact it is not difficult to rationalise an involvement of amygdala and hippocampus in the pathogenesis of anxiety in PD as these regions have also been implicated as dysfunctional in pure mood disorders (Drevets 1998). For example, in panic disorder amygdalohippocampal structures have a central role in the elaboration of panic attacks and related symptoms of arousal and fearful avoidance (Grove *et al.* 1997). The amygdala is a key structure for emotional processing in humans and mediates fear processing and anxiety (LeDoux 2000) while it is also highly interconnected with other regions of the limbic system (Swanson and Petrovich 1998). Electrical stimulation of the amygdala in humans evokes strong visceral and emotional fears responses while ablation studies in primates suggest loss of the fear response to previously threatening stimuli, as part of the

the Kluver-Bucy syndrome (Davis 1997;Deakin JFW and Graeff FG 1991). It is also of interest to note that the amygdala provides a widespread efferent network with projections to centres regulating endocrine and autonomic functions (Swanson and Petrovich 1998). It is not then surprising that PD patients with anxiety experience more complaints related to autonomic function (e.g. postural dizziness, urinary frequency, dry mouth) than controls, possibly as a result of amygdala pathology (Berrios *et al.* 1995).

Anxiety in PD has been mainly investigated in neuroimaging studies, both directly and indirectly. In a study by Remy et al., (Remy *et al.* 2005) anxiety severity negatively correlated with bilateral amygdala [^{11}C]RTI-32 binding in PD subjects. Furthermore, Benkelfat and colleagues(Benkelfat *et al.* 1995) found rCBF increases in amygdala associated with cholecystokinin$_4$-induced anxiety. Although no study so far has implicated hippocampal abnormalities in PD patients with anxiety, the hippocampus may facilitate the presence of anxiety by retrieval of fear-signifying memories and thus work in close proximity with the amygdala, with which it is reciprocally interconnected. The neuropathological correlates of anxiety in PD have not been previously studied. The findings reported herein are largely in agreement with previous functional imaging studies implicating the amygdala as a possible candidate for anxiety in PD but also point for the first time to hippocampal pathology as an important region for the development of anxiety in PD although the exact pathophysiological mechanisms remain to be elucidated.

The pathological basis of depression in PD is not yet fully understood. Although this study did not aim to describe an association between focal pathology and depression, we did find that αSN pathology in the locus coeruleus was increased in PD subjects with both depression and anxiety. The fact that αSN in the locus coeruleus did not differ significantly between the PD(A-) and PD(A+) group (when depressed cases were excluded) may suggest that αSN in the locus coeruleus plays an important role in the development of depression. Although depression in PD has been associated with noradrenaline deficiency in PD patients (Scatton *et al.* 1983), damage to the locus coeruleus occurs both with and without clinical manifestations of depression (Sandyk 1989) thus leaving the definitive contribution of noradrenaline to depression in PD unclear. To our knowledge this is the first study to observe an association between depression and αSN burden in locus coeruleus but does require further study.

5.5 Conclusions

The present work provides the first preliminary clinico-pathological investigation of anxiety in PD. The findings demonstrate an increased αSN burden in the amygdala and hippocampus CA2 sector in PD cases with anxiety. Nevertheless, it is important to note that the PD(A+) group had other neuropsychiatric deficits while the PD(A-) group was cognitively intact thus the pathological changes observed may be a surrogate marker of extranigral involvement of more severe disease. The present findings can be confirmed if two additional PD groups had been studied that would include PD cases without anxiety but with additional neuropsychiatric deficits and PD cases with anxiety without any concomitant neuropsychiatric symptoms. Even with these reservations this study provides evidence that anxiety in PD is not simply the result of psychological and social difficulties of the disease but rather possibly relates to specific neurobiological mechanisms. These findings (although preliminary) possibly also shed some light on common neurobiological mechanisms of anxiety in PD and mood disorders. Further studies with well matched PD groups for anxiety are needed to confirm the involvement of amygdala and CA2 sector of the hippocampus in the pathogenesis of anxiety in PD.

Chapter 6

The dorsal motor nucleus of the vagus is not an obligatory trigger site of Parkinson's disease: A critical analysis of α-synuclein staging

6.1 Introduction

The term "synucleinopathy" has been used broadly to designate a spectrum of progressive degenerative disorders of the human nervous system that share, among other properties, the presence of abnormal αSN immunoreactive inclusion bodies in neurons and/or macroglial cells (Takeda *et al.* 1998;Galvin *et al.* 2001). The term currently encompasses neurobiologically and neuropathologically diverse, but also, in part, clinically overlapping entities, such as multiple system atrophy (MSA), PD, dementia with Lewy bodies (DLB), the Lewy body variant of Alzheimer's disease (LBVAD), and neurodegeneration with brain iron accumulation type 1 (NBIA I) (Gorell *et al.* 1994;Papp and Lantos 1994;Arawaka *et al.* 1998;Gai *et al.* 1998;Bayer *et al.* 1999b;Dickson *et al.* 1999).

PD is the most frequently occurring synucleinopathy. Involvement of the substantia nigra and obliteration of dopaminergic neurons in the pars compacta are universally acknowledged hallmarks of PD (Mann and Yates 1983;Graybiel *et al.* 1990;Agid *et al.* 1993). However, as described in earlier chapters, PD is a multisystemic illness with pathology occurring in a variety of cortical and subcortical regions including the olfactory bulb and related areas, the dorsal motor nucleus of the vagal nerve and adjoining intermediate reticular zone, the tegmental pedunculopontine nucleus, nonthalamic nuclear nuclei with diffuse projections to cortical and subcortical regions, intralaminar and midline thalamic nuclei, the amygdala, NBM, trans- and entorhinal cortices, hippocampal formation, temporal and frontal cortices (Richard *et al.* 2002;Braak *et al.* 2003;Pahapill and Lozano 2000;Rub *et al.* 2002;Braak and Braak 2000;Braak *et al.* 1994;Braak *et al.* 1998;Rub *et al.* 2002;Jellinger 1991). The question had arisen as to whether these αSN lesions in the PD brain evolve simultaneously or whether regions show different susceptibility to develop this PD-related pathology. Recently, a few

studies have suggested that involvement of αSN pathological lesions in the PD brain is not random but in fact follows a predictable stereotypic pattern.

6.1.1 Staging of PD-related pathology

A prerequisite for the post-mortem diagnosis of PD is the assessment of the pathognomonic lesions (Gibb *et al.* 1991). As a result of a conformational change in the predominantly presynaptic protein αSN and the subsequent failure of intracellular proteasomal recycling systems to eliminate the misfolded proteins (see also section 1.3.2.3), affected neurons develop LNs in their cellular processes and LBs in their somata. In addition, abnormal neurofilaments, synphilin, and the stress protein ubiquitin also contribute to the formation of these nearly insoluble aggregates (Trojanowski and Lee 2000;Takeda *et al.* 1998;Gai *et al.* 1995;Spillantini *et al.* 1998;Baba *et al.* 1998;Trojanowski and Lee 1998;Golbe 1999).

On the basis of the occurrence of these PD-related histopathological hallmarks in the PD brain (LBs and LNs), Professor Braak and colleagues have proposed that PD progresses in six neuropathological stages (Braak *et al.* 2003). The proposed staging system rests in the following two assumptions. The first assumption is that incidental Lewy body pathology is the first step along a disease continuum. If one regards PD as a dynamic biological process then the so-called incidental lesions (presence of LBs and LNs at neuropathological examination in neurologically unimpaired individuals) represent presymptomatic correlates of a pathological condition ultimately capable of leading to the manifestation of clinical PD. The second prerequisite for staging PD is that the pathological process associated with PD (i.e. LBs and LNs) increases in extent and severity with disease progression. Based on these assumptions the design for the staging system included both clinically PD cases as well as cases with incidental LBs and LNs therefore presumably reflecting the entire spectrum of the PD pathological process.

Stage 1

In stage 1 αSN lesion in the brain are seen in the dorsal motor nucleus of the vagus nerve (DMV) and olfactory bulb and related portions of the anterior olfactory nucleus (Braak *et al.* 2003). LBs and LNs are limited to these 2 regions and cases in stage

1 do not demonstrate αSN pathology in any other brain region. Since the pathology does not advance further from olfactory structures into the neocortex, the DMV is viewed as the main start point.

Stage 2

αSN pathology in the DMV is more severe than in stage 1. In stage 2 lesions occur in the caudal raphe nuclei, gigantocellular reticular nucleus and locus coeruleus. In the first 2 stages of the disease the pathology is confined to the lower and upper brainstem. This means that the pathological process that ultimately leads to clinical PD does not begin in the substantia nigra (SN).

Stage 3

In stage 3 the pathology in caudal regions (regions in stages 1 and 2) increases progressively in severity. The brain structures affected in this stage are the amygdala, Nucleus Basalis of Meynert (NBM), pedunculopontine nucleus and most notably the substantia nigra pars compacta. At this stage the cerebral cortex is uninvolved.

Stage 4

In stage 4 cortical LBs and LNs appear in the temporal mesocortex (transentorhinal regions and the CA2 sector of the hippocampus). The neocortex is not affected. It is in this stage and in some cases in stage 3 that the presymtomatic phase gives rise to the clinically recognisable phase of the disease.

Stage 5

In stage 5 the density of lesions in the temporal mesocortex is more striking and the disease is present in the insular and cingulate mesocortex. The temporal neocortex becomes involved.

Stage 6

In stage 6 the key feature is the involvement of nearly the entire neocortex. The premotor areas, the primary motor field, the first order sensory association and primary sensory areas are affected but show mild changes.

6.1.2 Important considerations of the proposed PD-staging system

The criteria by which Professor Braak initially classified cases creates an inherent bias in experimental design. Cases with no clinical symptoms of PD are screened and categorised based on the presence of αSN deposition in the DMV prior to an examination of additional anatomical areas. In another study by the same research group (DelTredici *et al.* 2002), cases with αSN in the DMV were described as also showing inclusions in additional lower brainstem nuclei, particularly the locus coeruleus and in the olfactory bulbs. These are considered secondary induction sites on the basis that they are never the sole area in which inclusions are observed. However this is an experimental impossibility given that all cases were initially pre-selected on the basis of pathological inclusions in the DMV.

In the Braak et al. paper (2003) the clinical profiles of the 'incidental' pathology subjects are not described in a clear or concise fashion. There are cases with "mild alterations" and cases with "a few cases with severe pathology were misdiagnosed or did not receive a clinical diagnosis". This lack of clinical information is a serious failing in a paper that surports to define a definitive pathological staging system ranging from normal controls to full blown PD, for ultimately the integrity of the proposed system must rely on strict clinico-pathological correlation.

It is also very interesting that involvement of the SN was found in none of the 'incidental' Lewy body disease cases. Classical 'Incidental Lewy body Disease' (ILBD) is based on the finding of Lewy bodies in the susbtantia nigra in individuals without a history of any clinical sign of PD. It is reckoned that this pre-clinical phase of PD pathology is present in 10% of those in their seventies, 15% of those in their eighties, 20% of those in their nineties and in a third or more of those living over the age of 100. These results were obtained using older techniques less sensitive than current αSN immunohistochemical staining. Thus, there should have been about 3 cases with nigral involvement from the 'incidental' group described by the Braak et al., or even more than

this number, considering that all of these subjects were pre-selected on the basis of αSN pathology already present in the lower brainstem and were studied with highly sensitive αSN immunohistochemistry. We know that one must loose 80% of striatal dopamine and about 60% of substantia nigra neurons at least before the disease becomes clinically manifest (Bernheimer *et al.* 1973). The preclinical phase of the illness may last anywhere from 5-20 years. Thus, it is highly surprising and appears implausible that none of the 'incidental' cases described had any pathological involvement in the substantia nigra. Indeed, this implies that none of these individuals would have developed clinically apparent PD over the ensuing decade at least.

In addition, to the abovementioned important concerns about the Braak PD staging scheme although the system indicates an ascending path of αSN pathology, lower parts of the nervous system (i.e. spinal cord) were not examined and therefore the comparative incidence of αSN pathology in the spinal cord and DMV has not been studied. αSN lesions in the spinal cord are often found in both PD and neurologically unimpaired subjects (Bloch *et al.* 2006;Wakabayashi and Takahashi 1997b;Wakabayashi and Takahashi 1997a).

6.1.3 Aims

Given these important issues with regards to the experimental design used by the Braak research group, the aim of this work was based upon the following view: It would be necessary initially to screen clinically and neuropathologically well substantiated PD cases for pathology in multiple vulnerable regions (e.g. the locus coeruleus, substantia nigra, hippocampus and meso- and neocortex) and confirm that they all show additional inclusions in the DMV before it can be considered a probable candidate for the site in which αSN inclusions are first observed in clinically unaffected cases. This study therefore aimed to determine the incidence and topographic distribution of αSN lesions in a large unselected PD cohort collected by the UKPDSTB at Imperial College, London. The pathology was examined in eight brain regions along the rostro-caudal axis in addition to the spinal cord.

6.2 Materials and Methods

6.2.1 Neuropathological Assessment

Neuropathological assessment and diagnosis were performed as described in section 2.2.1. All cases with a clinico-pathological diagnosis other than PD were excluded from this study.

6.2.2 Clinical Assessment and Selection of Cases

The clinical assessment of cases followed established criteria (see section 2.2.2). A total of 101 cases were examined. These cases represent the first 101 cases accrued by the UKPDSTB. From this sample 30 cases were diagnosed clinically or neuropathologically with a condition other than PD (e.g. 6 MSA, 8 PSP, 3 ET, 7 DLB, 5 with age-related changes and 1 with possible fragile-X ataxia) and thus excluded from further analysis. Therefore a total of 71 cases well substantiated with a clinical and neuropathological diagnosis of PD were included in this study (50 males, 21 females, age range between 50 and 89, mean age at death 78 years; Table 6.1).

6.2.3 Immunohistochemistry

Immunohistochemical reactions were performed as described in section 2.2.4. All cases were stained for αSN for the identification of αSN inclusions. In this study for antigen unmasking, 5 minutes formic acid (100%) pre-treatment was performed with a dilution of 1:300 of the primary antibody (Becton-Dickinson).

6.2.4 Semi-quantitative assessment of αSN lesions

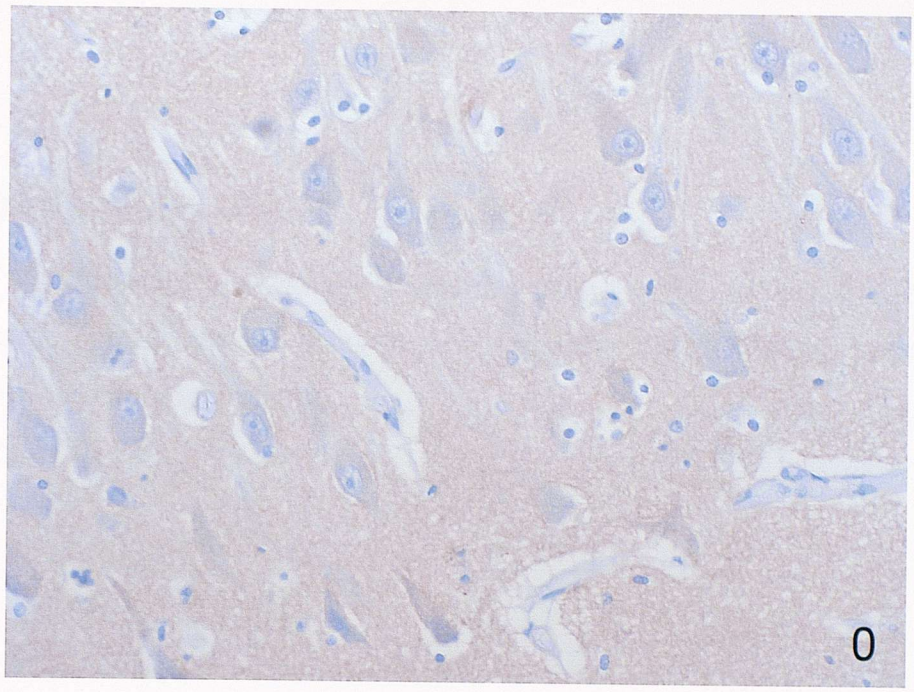
Representative sections from the dorsal motor nucleus of vagus (DMV), locus coeruleus (LC), substantia nigra (SN), Nucleus Basalis of Meynert (NBM), amygdaloid complex (AC), CA2 sector of the hippocampus (CA2), entorhinal cortex (EC; mesocortex) and superior frontal gyrus (SFG; neocortex) were obtained and the burden of αSN was assessed semi-quantitatively as follows: -= absent, += mild or moderate and

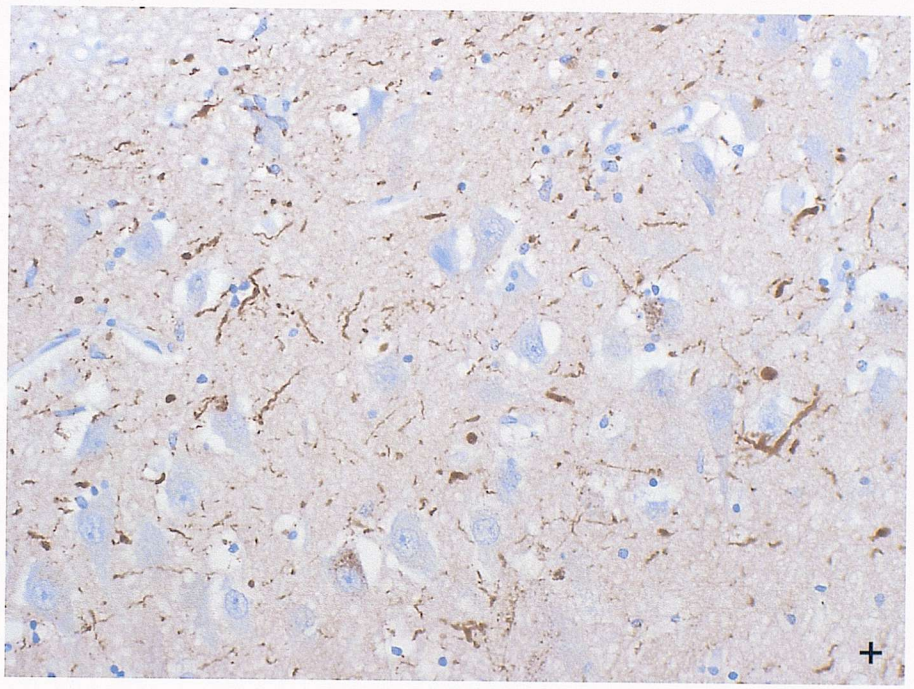
++= severe (Figure 6.1). The spinal cord (SC) of 24 PD cases was also examined. αSN positive lesions in the SC were examined at the cervical, thoracic and lumbar levels and the most severely affected structure was taken for rating using the same semi-quantitative rating scale used previously (-= absent, += mild or moderate and ++= severe). The absence of αSN positivity in the DMV and SC was verified in each case through examination of three additional consecutive sections. DMV sections were carefully inspected for integrity of preservation of vagal neurons in addition to assessment of αSN burden.

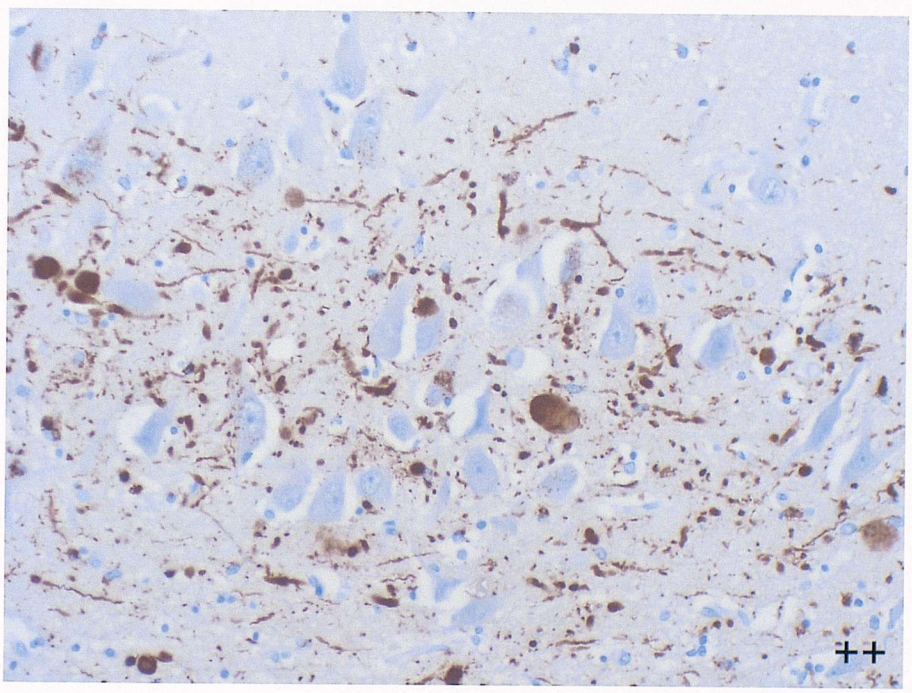
Figure 6.1 Semi-quantitative assessment of αSN positive lesions

(Following page). The pathology was either absent (0) or present in scant to moderate severity (+) or severely and significant present (++). Representative section from the CA2 sector of the hippocampus.




0


+


++

6.2.5 Statistical Analysis

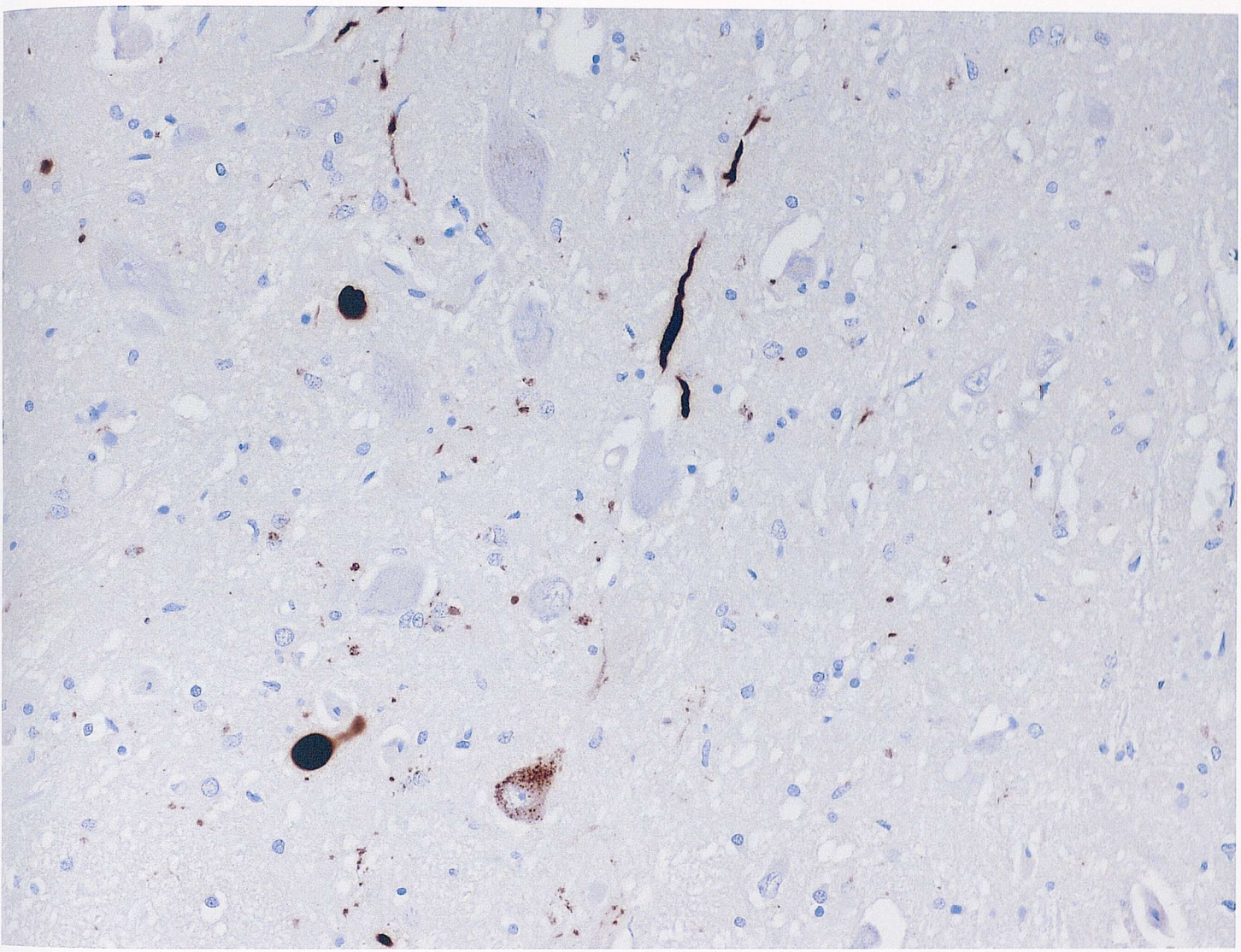
Statistical analysis was performed using GraphPad Prism 4. Differences between groups were determined using the non-parametric Mann-Whitney *U*-test. P values <0.05 were considered significant.

6.3 Results

A total of 71 PD cases (30 PDD and 41 PD) were included in this study, none of which had received a diagnosis of AD or DLB. In no case was αSN pathology confined solely to the SN. αSN immunoreactive deposits displayed a variety of morphologies. A photographic representation of the heterogeneity of LB and LN-type lesions are given in Figure 2.6. LB and LN-type lesions in the spinal cord are shown in Figure 6.2.

Figure 6.2 LBs and LNs in the spinal cord

(Following page) Lewy bodies and Lewy neurites in the intermediate lateral column of the thoracic spinal cord (case 17). Magnification 20x



6.3.1 Incidence of αSN pathology in brainstem and telencephalic regions

In our cohort, the SN was affected in 100% of cases. The NBM was affected in 98.5% of the cases, making it the second most vulnerable nucleus. The melanised neurons of the LC were αSN immunopositive in 97% and the DMV in 92.9% of cases (Figure 6.3). The AC (90.1%) and EC (90.1%) were equally affected. The CA2 sector of the hippocampus was affected in 85.9% of the cases and the least affected region was the SFG (80.2%) (Table 6.1).

Figure 6.3 Microphotograph of a coronal section of the medulla from a PD case

(Following page) αSN positive inclusions (LBs and LNs) are seen in the DMV (dotted box) of PD case 65. αSN positive LNs could be traced from the DMV area to the peripheral portion of the vagus nerve (arrows). Primary magnification 4x

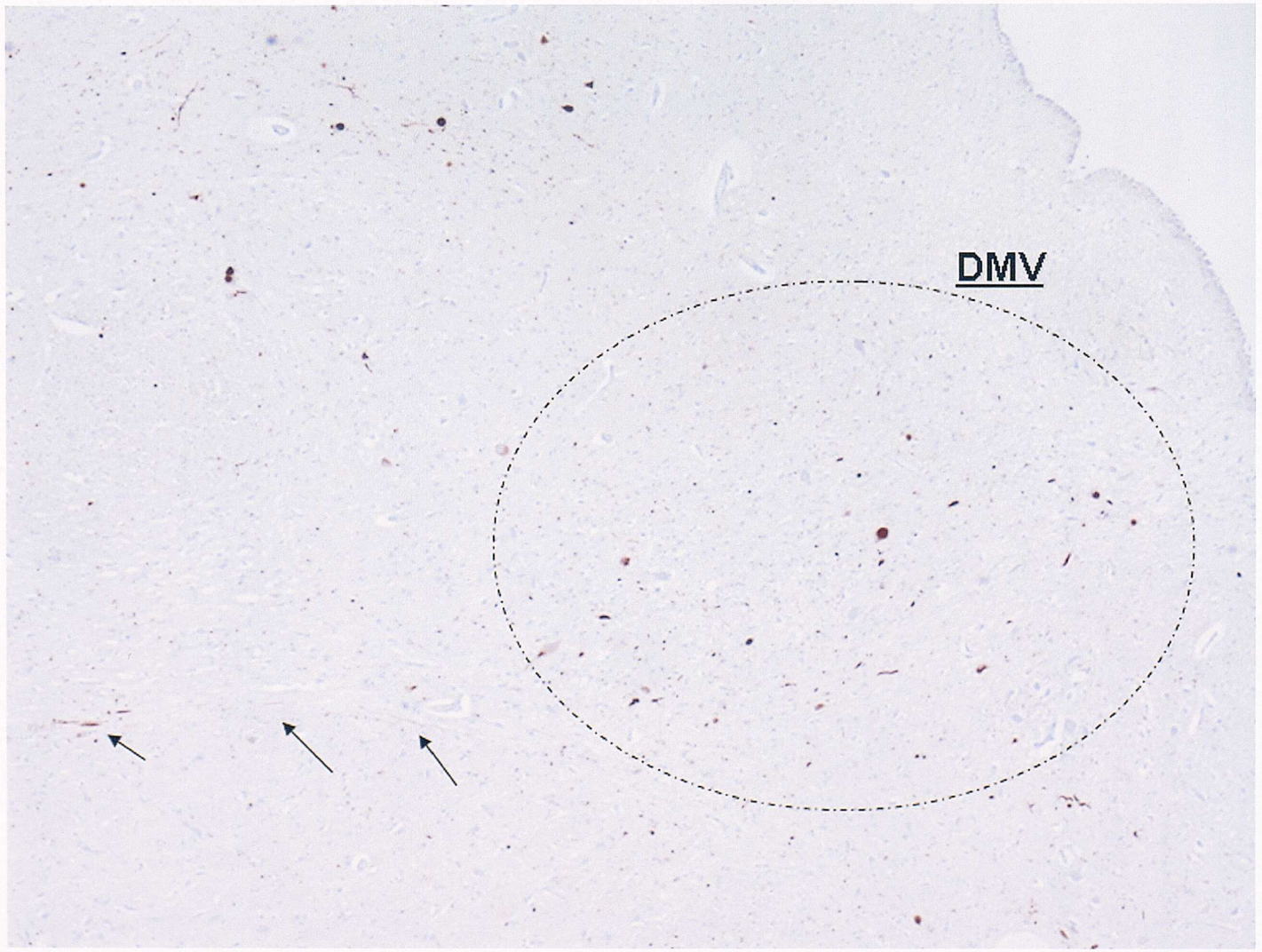

DMV

Table 6.1 Clinical characteristics and topographic distribution of αSN lesions in the PD brain.

(Following page) The occurrence of αSN is indicated as -: absent, +: mild and moderate, and ++: severe.

Abbreviations: PD: Parkinson's disease, PDD: Parkinson's disease with dementia, DMV: dorsal motor nucleus of vagus, LC: locus coeruleus, SN: substantia nigra, NBM: Nucleus Basalis of Meynert, AC: amygdaloid complex, CA2: CA2 sector of the hippocampus, EC: entorhinal cortex and SFG: superior frontal gyrus.

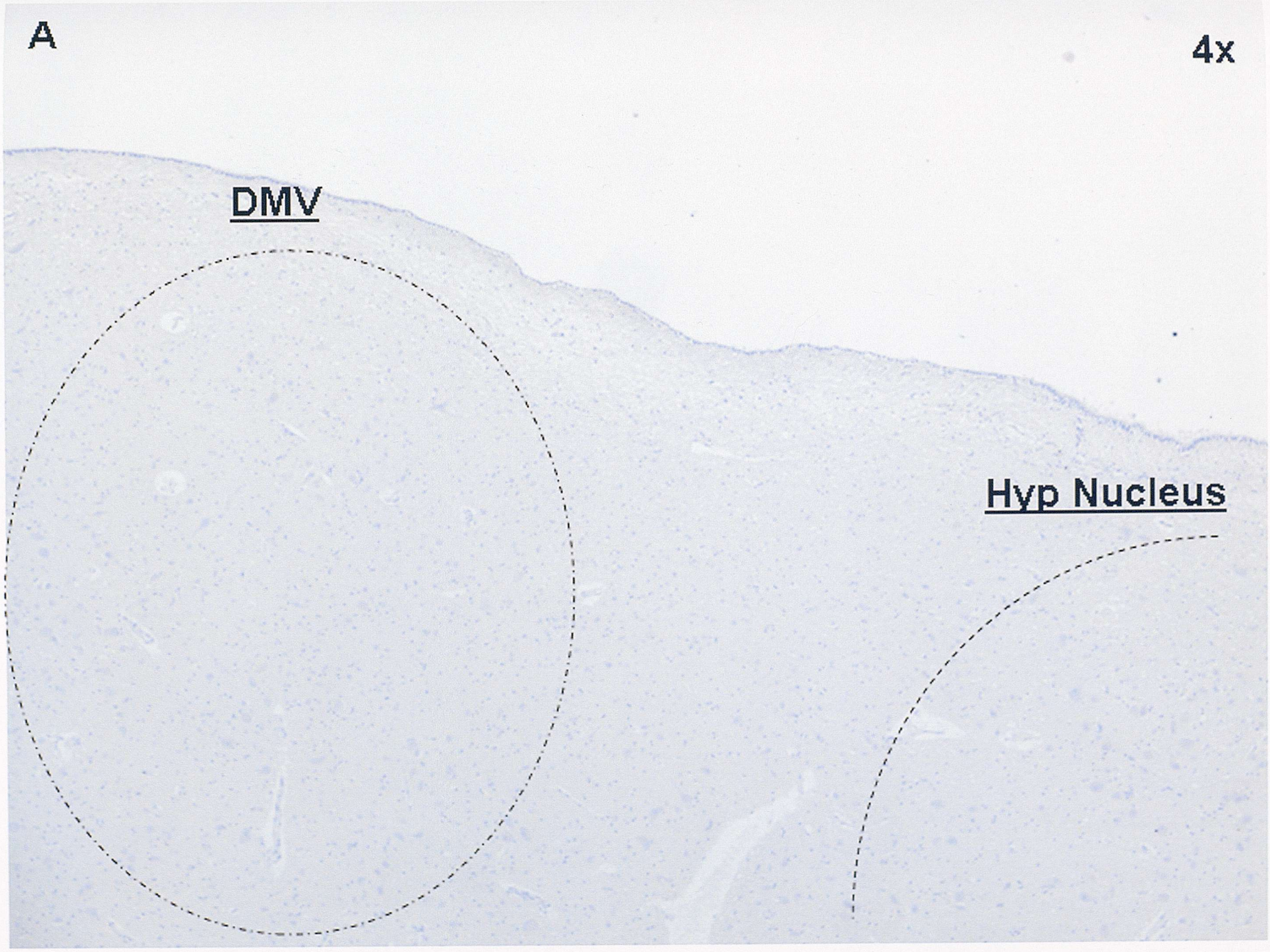
Actual Number	PD case	Age at onset (yrs)	Age at death (yrs)	Duration	sex	Clinical Diagnosis	Neuropathological Diagnosis (http://www.ICDNS.org)	ICDNS AD GRADE (http://www.ICDNS.org)	Braak AD-Stages	Distribution of aSN inclusion							
										DMV	LC	SN	NBM	AC	CA2	EC	SFG
PD01	1	75	87	12	f	PDD	PD	isocortical 0 - entorhinal I	I-II	+	+	+	+	+	+	+	+
PD05	2	57	80	23	f	PDD	PD	isocortical II - entorhinal III	I-II	+	+	+	+	++	++	+	+
PD07	3	68	78	10	m	PD	PD	na	na	+	+	+	+	-	+	+	+
PD08	4	62	72	10	m	PD	PD	isocortical I - entorhinal I	I-II	+	+	+	+	-	-	+	+
PD13	5	54	78	24	m	PDD	PD	isocortical 0 - entorhinal I	I-II	+	+	+	+	+	++	+	+
PD17	6	57	75	18	m	PDD	PD	isocortical I - entorhinal II	I-II	+	+	+	++	+	+	+	+
PD18	7	77	82	5	m	PDD	PD	isocortical I - entorhinal II	0	+	+	+	+	+	+	+	+
PD21	8	49	76	27	m	PD	PD	isocortical 0 - entorhinal II	I-II	+	++	+	++	+	++	+	+
PD22	9	65	75	10	f	PDD	PD	isocortical 0 - entorhinal 0	0	+	-	+	+	+	+	+	-
PD23	10	75	82	7	m	PD	PD	isocortical I - entorhinal II	I-II	+	+	+	+	+	+	-	+
PD24	11	72	81	9	m	PDD	PD	isocortical 0 - entorhinal I	I-II	+	+	+	++	+	+	+	+
PD27	12	60	74	14	m	PD	PD	isocortical 0 - entorhinal I	I-II	++	++	++	++	++	+	+	-
PD28	13	64	82	18	m	PDD	PD	isocortical I - entorhinal II	I-II	+	+	+	+	+	+	+	+
PD29	14	69	76	7	m	PD	PD	isocortical 0 - entorhinal I	I-II	+	+	+	+	+	+	+	+
PD39	15	69	82	13	f	PD	PD	isocortical I - entorhinal III	I-II	+	+	+	+	++	+	+	-
PD41	16	67	77	10	m	PDD	PD	isocortical I - entorhinal I	I-II	+	+	+	-	+	+	+	+
PD44	17	74	87	13	m	PDD	PD	isocortical I - entorhinal II	I-II	+	+	+	+	+	+	+	+
PD45	18	61	80	19	m	PDD	PD	isocortical 0 - entorhina III	III	++	++	++	++	++	++	+	++
PD49	19	67	75	8	m	PD	PD	isocortical I - entorhinal I	I-II	+	+	+	+	+	+	+	-
PD51	20	75	80	5	m	PD	PD	isocortical 0 - entorhinal 0	0	+	+	+	+	+	-	-	-
PD52	21	48	71	23	f	PDD	PD	isocortical I - entorhinal II	0	+	+	+	+	+	+	+	-
PD54	22	64	75	11	m	PD	PD	isocortical III - entothinal III	I-II	+	+	+	+	+	-	+	+
PD61	23	74	78	4	m	PD	PD	isocortical IV - entorhinal III	III-IV	+	+	+	++	+	+	+	+
PD62	24	51	73	22	m	PD	PD	isocortical 0 - entorhinal I	I-II	+	+	+	+	+	+	+	+
PD67	25	74	83	9	m	PDD	PD	isocortical 0 - entorhinal I	I-II	+	+	+	++	+	++	+	+
PD68	26	53	82	29	m	PD	PD	isocortical II - entorhinal III	I-II	+	+	+	+	+	+	+	+
PD78	27	64	79	15	m	PD	PD	isocortical I - entorhinal II	I-II	+	+	+	+	+	-	+	+
PD79	28	59	78	19	f	PDD	PD	isocortical 0 - entorhinal I	I-II	++	+	+	+	++	++	+	+
PD80	29	76	83	7	f	PDD	PD	isocortical IV - entorhinal III	III	+	+	+	++	++	+	+	+
PD91	30	49	50	1	m	PD	PD	isocortical I - entorhinal I	I-II	+	+	+	+	+	-	-	-
PD92	31	70	83	13	m	PDD	PD	isocortical I - entorhinal II	I-II	+	+	+	++	+	+	+	+
PD93	32	67	81	14	f	PD	PD	isocortical I - entorhinal II	I-II	+	+	+	+	+	+	+	+
PD94	33	61	75	14	m	PD	PD	isocortical 0 - entorhinal II	I-II	+	+	+	+	+	+	+	+
PD95	34	37	74	37	m	PD	PD	isocortical I - entorhinal 0	I-II	+	+	+	++	+	+	+	+
PD97	35	77	88	11	f	PDD	PD	isocortical I - entorhinal II	I-II	+	+	+	+	-	-	-	+
PD99	36	72	83	11	m	PDD	PD	isocortical I - entorhinal II	I-II	+	+	+	+	+	+	+	+
PD70	37	57	75	18	m	PD	PD	isocortical 0 - entorhinal I	I-II	++	++	++	++	++	+	+	+
PD84	38	78	86	8	m	PD	PD	isocortical 0 - entorhinal I	I-II	++	+	++	+	++	+	+	+
PD03	39	74	77	3	m	PD	PD	isocortical I - entorhinal I	I-II	+	+	++	++	+	+	+	++
PD19	40	54	72	18	f	PD	PD	isocortical I - entorhinal 0	I-II	+	++	++	+	+	+	+	+
PD20	41	41	75	34	m	PD	PD	isocortical I - entorhinal II	I-II	+	-	++	+	++	++	++	+
PD30	42	57	73	16	m	PDD	PD	isocortical I - entorhinal II	I-II	+	+	++	++	+	+	+	+
PD34	43	73	84	11	f	PDD	PD	isocortical IV - entorhinal III	III-IV	+	++	++	++	+	+	+	+
PD36	44	67	77	10	m	PD	PD	isocortical 0 - entorhinal I	I-II	+	+	++	++	-	-	+	+
PD50	45	68	82	14	f	PD	PD	isocortical 0 - entorhinal 0	0	+	++	++	++	+	+	+	+
PD56	46	35	54	19	m	PDD	PD	isocortical 0 - entorhinal I	0	+	++	++	++	++	++	+	+
PD60	47	78	84	6	m	PDD	PD	isocortical II -entorhinal II	I-II	+	++	++	++	++	++	+	+
PD63	48	67	80	13	f	PD	PD	isocortical 0 - entorhinal I	0	+	++	++	++	+	-	-	+
PD66	49	73	82	9	m	PDD	PD	isocortical 0 - entorhinal I	I-II	+	++	++	++	+	+	+	-
PD69	50	71	82	11	m	PDD	PD	isocortical I - entorhinal II	I-II	+	+	++	++	+	++	+	+
PD72	51	42	62	20	m	PD	PD	isocortical 0 - entorhinal 0	0	+	+	++	+	+	+	+	+
PD74	52	76	85	9	m	PD	PD	isocortical II - entorhinal 0	I-II	+	+	++	+	+	+	+	+
PD81	53	64	73	9	m	PD	PD	isocortical I - entorhinal II	I-II	+	+	++	++	++	+	+	+
PD88	54	67	81	14	f	PDD	PD	isocortical I - entorhinal III	I-II	+	++	++	++	++	+	+	+
PD100	55	77	81	4	m	PD	PD	isocortical I - entorhinal III	I-II	+	++	++	++	++	+	+	+
PD02	56	75	84	9	m	PDD	PD	isocortical 0 - entorhinal I	0	++	+	++	+	-	++	+	-
PD33	57	59	74	15	f	PD	PD	isocortical 0 - entorhinal I	I-II	++	+	+	+	+	+	+	-
PD42	58	60	79	19	f	PD	PD	isocortical 0 - entorhinal I	I-II	++	+	+	+	+	+	+	+
PD57	59	69	83	14	m	PD	PD	isocortical 0 - entorhinal I	I-II	++	++	++	++	++	+	+	-
PD86	60	78	87	9	f	PD	PD	isocortical 0 - entorhinal I	I-II	++	+	+	++	+	+	+	-
PD11	61	62	71	9	m	PDD	PD	isocortical III - entorhinal III	I-II	+	+	+	+	+	+	++	++
PD15	62	56	70	14	m	PDD	PD	isocortical IV - entorhinal III	III	+	++	+	+	++	+	++	+
PD16	63	67	85	18	f	PDD	PD	isocortical II - entorhinal I	I-II	+	+	+	++	+	++	++	+
PD32	64	86	89	3	m	PD	PD	isocortical 0 - entorhinal I	I-II	+	+	+	+	-	-	-	+
PD37	65	85	87	2	m	PD	PD	Isocortical 0 - entorhinal I	I-II	+	+	+	+	++	++	++	+
PD55	66	58	76	18	f	PD	PD	isocortical III - entothinal III	I-II	+	+	+	+	++	+	++	+
PD06	67	62	72	10	m	PD	PD	isocortical 0 - entorhinal I	0	-	+	+	+	+	+	+	-
PD09	68	71	86	15	f	PD	PD	isocortical I - entorhinal II	I-II	-	+	+	+	+	na	+	+
PD14	69	67	79	12	m	PDD	PD	Isocortical IV - entorhinal III	I-II	-	+	+	+	+	+	na	+
PD58	70	52	58	6	f	PD	PD	isocortical 0 - entorhinal I	I-II	-	+	++	+	-	-	-	+
PD59	71	66	81	15	m	PD	PD	isocortical 0 - entorhinal 0	I-II	-	+	+	+	+	+	+	-

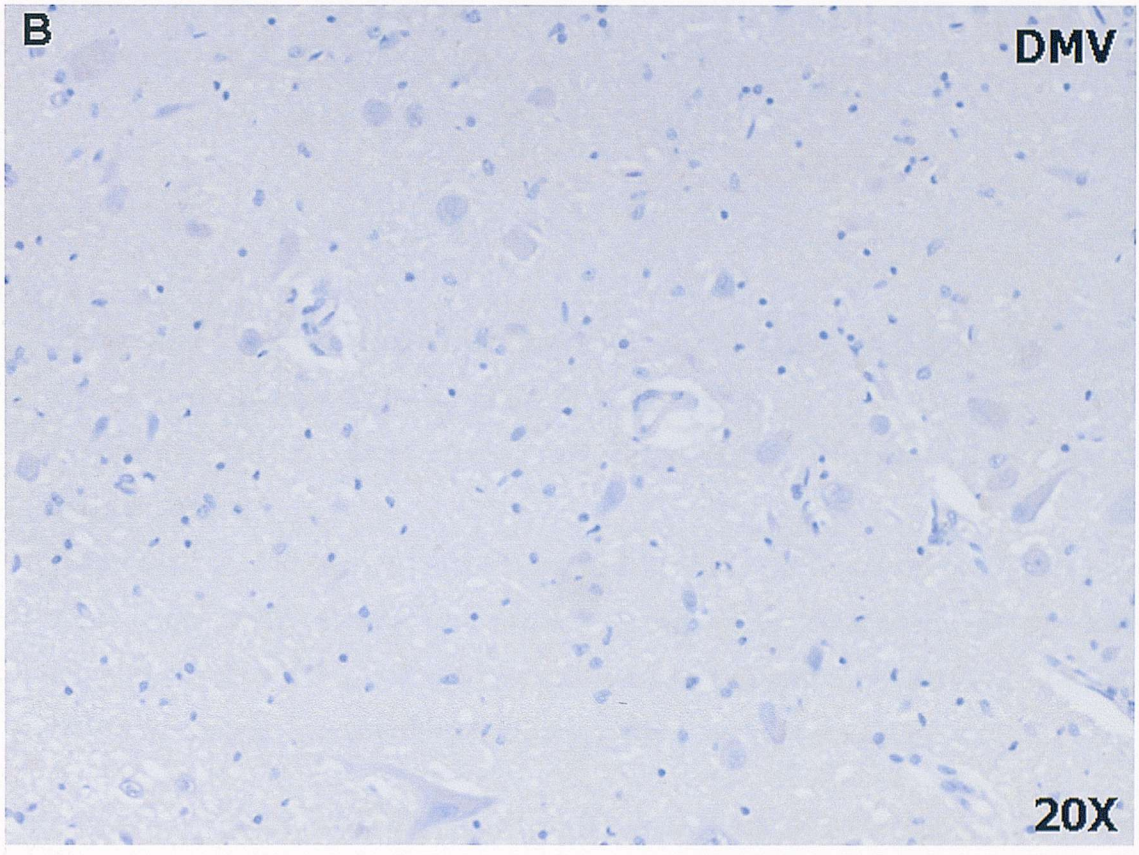
6.3.2 Topography of αSN lesions

The topographical distribution of αSN lesions is shown in table 6.1. In 38 cases (1-38) αSN inclusions were seen in nearly all regions under investigation with little or no αSN interregional variability. Among these cases the most frequently severely affected region was the NBM (11/38). Seventeen cases (39-55) showed a severe load of αSN in the SN with mild to moderate involvement of the DMV. These cases did not demonstrate neuronal loss in the DMV sufficient to explain the insignificant deposition of αSN. Cases with high densities of αSN in the SN also exhibited severe lesions in the LC and NBM. In 5 cases (56-60) the DMV was severely affected in the absence of prominent involvement of more rostral meso- and neocortical regions. Among these cases, 4 (56, 57, 59 and 60) showed no αSN pathology in the frontal cortex despite severe pathology in the DMV. In cases 61-66 the meso-cortex was severely affected together with pathology in the basal forebrain nuclei NBM and AC with lesser affection of lower brainstem structures including the DMV despite preservation of neurons in this nucleus. In 5 cases (67-71) no αSN lesions were seen in the DMV in spite of the presence of αSN inclusions in more rostrally situated regions (Figure 6.4). It is of interest to note that case 70 showed severe involvement of the SN in the absence of αSN pathology in the DMV. All of the PD cases devoid of αSN in the DMV demonstrated well preserved neurons in this nucleus.

Figure 6.4 Absence of αSN pathology in the DMV of a PD case

(Following 2 pages) A) Low magnification of a medullary section from PD case 71 showing absence of αSN positivity. B) Higher magnification of DMV from PD case 71 showing preservation of neurons but absence of αSN immunopositivity. Primary magnification for A) 4x and for B) 20x.


A
4x
DMV
Hyp Nucleus


B
DMV
20X

6.3.3 αSN pathology in the Spinal Cord and its relation to DMV lesions

In a subset of cases (n=24) αSN pathology at all levels of the SC (cervical, thoracic and lumbar) was also examined. We assessed all cases (n=5) that were negative for αSN in DMV, as well as 19 others that were randomly selected from the initial 71 cases of PD/PDD (Table 6.2). In cases with DMV affection, involvement of the SC was observed in 89.4% (17/19) of cases while only 2 cases (54 and 34) had no αSN pathology in the SC. One case (16) showed higher density of αSN lesions in the SC than in the DMV. In cases with no αSN lesions in the DMV (67-71), 60% (3/5) showed αSN inclusions in the SC.

Table 6.2 Clinical characteristics and topographic distribution of αSN lesions in the spinal cord and supraspinal regions in the PD brain.

(Following page) The occurrence of αSN is indicated as -: absent, +: mild and moderate, and ++: severe.

Abbreviations: PD: Parkinson's disease, PDD: Parkinson's disease with dementia, SC: spinal cord, DMV: dorsal motor nucleus of vagus, LC: locus coeruleus, SN: substantia nigra, NBM: Nucleus Basalis of Meynert, AC: amygdaloid complex, CA2: CA2 sector of the hippocampus, EC: entorhinal cortex and SFG: superior frontal gyrus.

Actual Number	PD case	Age at onset (yrs)	Age at death (yrs)	Duration	sex	Clinical Diagnosis	Neuropathological Diagnosis (http://www.ICDNS.org)	ICDNS AD GRADE (http://www.ICDNS.org)	Braak AD-Stages	SC	Distribution of aSN inclusion							
											DMV	LC	SN	NBM	AC	CA2	EC	SFG
PD07	3	68	78	10	m	PD	PD	na	na	+	+	+	+	+	-	+	+	+
PD17	6	57	75	18	m	PDD	PD	isocortical I - entorhinal II	I-II	+	+	+	+	++	+	+	+	+
PD18	7	77	82	5	m	PDD	PD	isocortical I - entorhinal II	0	+	+	+	+	+	+	+	+	+
PD21	8	49	76	27	m	PD	PD	isocortical 0 - entorhinal II	I-II	+	+	++	+	++	+	++	+	+
PD22	9	65	75	10	f	PD	PD	isocortical 0 - entorhinal 0	0	+	+	-	+	+	+	+	+	-
PD23	10	75	82	7	m	PD	PD	isocortical I - entorhinal II	I-II	+	+	+	+	+	+	+	+	+
PD29	14	69	76	7	m	PD	PD	isocortical 0 - entorhinal I	I-II	+	+	+	+	+	+	+	+	+
PD41	16	67	77	10	m	PD	PD	isocortical I - entorhinal I	I-II	++	+	+	+	-	+	+	+	+
PD44	17	74	87	13	m	PDD	PD	isocortical I - entorhinal II	I-II	+	+	+	+	+	+	+	+	+
PD94	33	61	75	14	m	PD	PD	isocortical 0 - entorhinal II	I-II	+	+	+	+	+	+	+	+	+
PD95	34	37	74	37	m	PD	PD	isocortical I - entorhinal 0	I-II	-	+	+	+	++	+	+	+	+
PD30	42	57	73	16	m	PDD	PD	isocortical I - entorhinal II	I-II	+	+	+	++	++	+	+	+	+
PD34	43	73	84	11	f	PDD	PD	isocortical IV - entorhinal III	III-IV	+	+	++	++	++	+	+	+	+
PD60	47	78	84	6	m	PDD	PD	isocortical II -entorhinal II	I-II	+	+	++	++	++	++	++	+	+
PD74	52	76	85	9	m	PD	PD	isocortical II - entorhinal 0	I-II	+	+	+	++	+	+	+	+	+
PD88	54	67	81	14	f	PDD	PD	isocortical I - entorhinal III	I-II	-	+	++	++	++	++	+	+	+
PD02	56	75	84	9	m	PDD	PD	isocortical 0 - entorhinal I	0	+	++	+	++	+	-	++	+	-
PD15	62	56	70	14	m	PD	PD	isocortical IV - entorhinal III	III	+	+	+	++	+	+	++	+	++	+
PD32	64	86	89	3	m	PD	PD	isocortical 0 - entorhinal I	I-II	+	+	+	+	+	+	-	-	-	+
PD06	67	62	72	10	m	PD	PD	isocortical 0 - entorhinal I	0	-	-	+	+	+	+	+	+	+	-
PD09	68	71	86	15	f	PD	PD	isocortical I - entorhinal II	I-II	+	-	+	+	+	+	na	+	+
PD14	69	67	79	12	m	PDD	PD	isocortical IV - entorhinal III	I-II	+	-	+	+	+	+	+	na	+
PD58	70	52	58	6	f	PD	PD	isocortical 0 - entorhinal I	I-II	-	-	+	++	+	-	-	-	+
PD59	71	66	81	15	m	PD	PD	isocortical 0 - entorhinal 0	I-II	+	-	+	+	+	+	+	+	-

6.3.4 Clinical assessment of PD cases without DMV affection

The mode of presentation as well as neuropsychiatric and autonomic disturbances experienced by cases without DMV affection are presented in Table 6.3. Age at disease onset, age at death and duration of disease did not differ between cases with and without DMV involvement. PD cases with no αSN lesions in the DMV demonstrated a diverse clinical picture and were all L-Dopa responsive with the exception of possibly one (Table 6.3). None of these cases was classified as atypical.

αSN pathology in relation to dementia

Dementia was present in 42% of the entire cohort examined. In comparison to non-demented PD cases, individuals with dementia demonstrated more severe αSN deposition in the CA2 sector of the hippocampus (p=0.006). The NBM was severely involved in 47% of cases with dementia and 32% of cases without, while the SN was severely affected in 30% and 36%, respectively.

Table 6.3 Clinical characteristics of PD cases without DMV involvement.

(Following page)

Abbreviations: +: yes, -: no, ±: possibly, R: right,

Actual Number	PD Case	Mode of Presentation	Neuropsychiatric Complications	Autonomic Complications	L-Dopa Responsiveness
PD06	67	Asymmetric (R) Akinetic-Rigid Syndrome	Dementia, Confusion	Bladder Symptoms	±
PD09	68	Tremor, Poor Balance	Depression, Apathy	Constipation. Bladder Frequency	+
PD14	69	Tremor	Confusion, Hallucinations, Memory Loss	Sialorrhoea, Constipation	+
PD58	70	Tremor	Confusion, Anxiety	Sialorrhoea	+
PD59	71	Tremor	Hallucinations	Dysphagia, Dysarthria, Nocturia, Diarrhoea, Constipation	+

6.4 Discussion

Intense research has been directed at elucidating the molecular pathophysiology of αSN in PD (Baba *et al.* 1998;Lotharius and Brundin 2002) with effort also focused on exploring the distribution of αSN in the PD brain to obtain a better understanding of the disease process (DelTredici *et al.* 2002;Braak *et al.* 2004;Braak *et al.* 2003). Some studies using αSN immunohistochemistry have found αSN pathology in the DMV but not in SN (DelTredici *et al.* 2002;Braak *et al.* 2003) and have interpreted this as indicate of an early pathological stage of αSN deposition. Based on observations of post-mortem tissue from diagnosed cases with PD as well as cases without PD, a staging system has been proposed (Braak *et al.* 2004;Braak *et al.* 2003). According to this system, early stages show pathological inclusions in the lower brainstem and no clear clinical manifestations. In later stages the disease advances to the upper brainstem including the substantia nigra (SN) and then moves from mesocortex to neocortex, with the pathology in caudal regions increasing progressively in severity (Braak *et al.* 2003). In the present study, 53% of cases showed a distribution pattern of αSN which is compatible with this view of a caudo-rostral spread of αSN through the PD brain. We have not seen cases that would suggest weak alpha-synuclein labelling of the DMV as a result of neuronal cell loss. If we accept the assumption that the extent and severity of αSN lesions increase as the disease progresses (i.e. this is a prerequisite according to the proposed staging scheme), a significant number of cases did not follow this predicted pattern of spread, with 24% showing severe involvement of the SN in the absence of prominent DMV pathology. In 9%, the mesocortex was severely affected together with basal forebrain nuclei, NBM and AC, with less pronounced pathology in lower brainstem structures including the DMV. Interestingly, in 7% of cases with typical clinical features of PD, the DMV was not affected, but αSN inclusions were found in the SN, NBM, AC, CA2 sector of the hippocampus, EC and SFG. Jellinger and Parkkinen et al have also reported multiple αSN inclusions with preservation of vagal medullary nuclei (Jellinger 2003a;Parkkinen *et al.* 2005a). Our results highlight the involvement of multiple neuronal populations in the PD brain and contest the theory that the DMV is an obligatory trigger site for αSN pathology. Although the design of our study does not allow us to pinpoint trigger site(s) or how αSN evolves in the PD brain in early disease our data clearly demonstrate that the DMV is not always affected in a significant number of otherwise typical and clinico-pathologically

confirmed PD cases. Our results also underscore the pathological regional heterogeneity of disease affliction in the PD brain which is in line with molecular findings (Moran *et al.* 2006).

An important consideration for the acceptance of the proposed PD staging system is that of the criteria used in case selection by Braak and colleagues. Cases with no clinical symptoms of PD were selected based on the presence of αSN deposition in the DMV prior to an examination of additional anatomical areas. Thus, the pre-selection of brains showing involvement of the DMV represents a serious bias and confounding factor in the interpretation of their data. Interestingly, in a recent study by this research group a number of cases (19/301) diverged from their staging scheme (Braak *et al.* 2006b). In these cases olfactory structures and amygdala were predominantly involved in the absence of brainstem pathology (Braak *et al.* 2006b). This is in line with our own results.

The search for an induction site of PD-related pathology has relied solely on the presence or absence of αSN immunostaining. However, one needs to ask whether the presence of αSN inclusions *per se* is indicative of PD at all. Aside from the obvious problem that there is no way pathologically to differentiate 'sub-clinical' PD from any other synucleinopathy in its 'sub-clinical' phase, a fundamentally more important concern is that perhaps not enough is known about αSN inclusions for them to be regarded with such assurance as pathological indicators and thus incorporated as the sole pathology in a disease staging scheme. Parkkinen et al., (Parkkinen *et al.* 2005b) have recently reported widespread and abundant αSN pathology in a neurologically unimpaired subject. While αSN inclusions may be indicative of an underlying pathological process they are not necessarily pathogenic in that process. By restricting our observations to αSN inclusions alone, we may be excluding brain regions that are affected differently. For example, the pre-supplementary motor area shows a significant loss of neurons in PD, but no αSN deposition is observed there (Macdonald and Halliday 2002). It could be that different pathological processes occur in different brain regions in PD. Thus, looking at other indicators of disease, such as nerve cell number and glial activation (Croisier and Graeber 2006), is mandatory. These other indicators must also be addressed in the brain of ageing individuals without signs of Parkinsonism as well as in PD cases, if we wish to better and more fully document disease progression in PD.

One must loose 80% of striatal dopamine and about 60% of SN neurons before PD becomes clinically manifest and the preclinical/premotor phase of the illness may last anywhere from 5-20 years (Hawkes C.H and Deeb J 2006). It is generally known that olfaction, autonomic function and sleep are perhaps the best substantiated symptoms in this preclinical period. It may be argued that the predictive power of these clinical signs would identify subjects at risk for the future development of PD and provide a window of opportunity for early intervention. However, a variety of premorbid indicators of PD exist that challenge the clinical significance of such prodromal features. For instance, the Honolulu-Asia Aging study suggested the mid-life obesity and slow reaction time as the most significant premorbid indicators of PD (Ross *et al.* 2006;Abbott *et al.* 2002). In addition, depression, anxiety, phobia and impaired color discrimination have all been shown as important premotor features (Lauterbach *et al.* 2004;Nilsson *et al.* 2001;Postuma *et al.* 2006). These features implicate widespread brain involvement and do not support a medullary primary focus of pathology. Furthermore, the clinical relevance of the proposed staging scheme has been recently challenged by Parkkinen and colleagues (Parkkinen *et al.* 2005a).

Until now few studies have investigated the presence of αSN immunoreactive lesions in the spinal cord of aged neurologically unimpaired as well as PD cases (Bloch *et al.* 2006;Wakabayashi and Takahashi 1997b;Wakabayashi and Takahashi 1997a). In PD, LBs have been documented in the intermediolateral nucleus of the thoracic spinal cord (Wakabayashi and Takahashi 1997a;Wakabayashi and Takahashi 1997b;Oppenheimer 1980) and in various grey areas of the spinal cord including the posterior, lateral and anterior horns (Hishikawa *et al.* 2003). More importantly, however, the comparative incidence of αSN pathology in the spinal cord and DMV has not been closely studied. In this cohort 89.4% of cases with DMV pathology showed αSN inclusions in the spinal cord highlighting a near universal occurrence of αSN positive lesions in the spinal cord of PD patients. In a recent study by Bloch et al., (Bloch *et al.* 2006) a high incidence of αSN lesions in the spinal cord was also reported in neurologically unimpaired individuals underscoring pathological involvement of the spinal cord at an early stage probably far below disease threshold for motor symptoms of PD. Nonetheless, Bloch et al., found no cases with isolated spinal or medullary pathology and thus have proposed that αSN spread could follow both an ascending course to more rostrally situated regions as well as

descending towards the spinal cord. However, in this cohort 60% of cases with no αSN in the DMV showed αSN lesions in the spinal cord. This result questions both an ascending as well as a descending course of αSN pathology spread in the PD brain and further challenges the existence of a medullary induction site of αSN pathology in the PD brain.

Although it becomes increasingly evident that αSN pathology in the SC is a common histopathological feature of PD (Wakabayashi and Takahashi 1997b;Wakabayashi and Takahashi 1997a;Braak *et al.* 2007) the clinical relevance of such pathology remains unclear. Pathological changes in the intermediate lateral column of the thoracic spinal cord including neuronal loss and LB inclusions have been implicated in autonomic dysfunction in PD (e.g. orthostatic hypotension)(Wakabayashi and Takahashi 1997a). In addition, the parasympathetic autonomic nuclei of the sacral cord give rise to sacral nerves that supply the descending and distal colon. Pathological changes in this region may contribute to colonic dysmotility leading to constipation(Oyanagi *et al.* 1990). Indeed, bowel problems in PD are common and it has been recently shown that constipation is a risk factor for PD decades before typical Parkinsonian motor symptoms appear (Abbott *et al.* 2001;Abbott *et al.* 2007). The findings of the present study of a high incidence of αSN pathology in the SC support previous reports that the central and peripheral autonomic nervous system is heavily involved. Systematic studies are warranted to examine the clinical correlates of αSN pathology in the SC.

In this study αSN was found in the NBM in 99% of the cases thus demonstrating that this nucleus is particularly vulnerable to αSN pathology in PD. In addition it was the most severely affected region in both demented and non-demented PD cases. The NBM was one of the first nuclei in which LBs were first observed in PD by Lewy in 1913. Degeneration of the NBM is characterized by neuronal loss and shrinkage more noticeably in the Ch4 region which projects to the neocortex (Nieuwenhuys R *et al.* 1988;Steriade M and Biesold D 1990). Studies have shown that in PD, the NBM cell depletion ranges from 0-77% and more severe involvement of the intermediate and posterior parts of Ch4, with comparative preservation of the Ch2 and Ch3 portions, where significant neuronal loss occurs in AD (Etienne *et al.* 1986b;Etienne *et al.* 1986a). The extent of NBM cell loss shows no correlation to age or duration of illness (Tagliavini *et al.* 1984). Irrespective of cortical pathology, NBM neuronal loss has been reported to be

much higher in demented PD subjects (50-77%), in whom it approaches the values in AD, than in non-demented PD patients (0-40%), who show values slightly higher than those aged-matched controls. In other studies, however, considerable NBM cell loss (about 50%) has also been reported in non-demented PD subjects (Gaspar and Gray 1984) with no relationship between cell counts and mental status (Tagliavini *et al.* 1984;Paulus and Jellinger 1991). The finding of the present work of a predominant involvement of αSN deposition in the NBM in PD subjects confirms previous reports of a primary degenerative process of the cholinergic forebrain system in PD as well as corroborates the results presented in chapter 3 where the extent of αSN inclusions in the NBM was high in both demented and non-demented PD patients highlighting a universal cholinergic deficit in PD, with or without clinical cognitive decline. Given the heterogeneity of cell loss in the different subdivisions of the NBM as well as its primary degeneration in PD both in terms of neuronal depletion and αSN deposition, further studies are needed to unravel the basic mechanisms by which this nucleus is affected in the PD brain.

6.5 Conclusions

The results of the present study demonstrate a predominant involvement of the NBM pointing to a universal cholinergic deficit in the PD brain which is in agreement with previous findings. In addition, the distribution of αSN pathology in this PD cohort followed the recent Braak staging scheme in 53% of the cases analysed, however, a significant number of cases did not seem to fit this predicted caudo-rostral spread. At variance to the proposed staging scheme, a number of subjects demonstrated severe involvement of the SN with less affection of the DMV, while others showed severe DMV αSN deposition with no affection of the meso- or neocortex. Other PD subjects exhibited severe αSN pathology in the NBM and AC with less affection of the DMV and a number of cases demonstrated no αSN pathology in the DMV despite αSN lesions in more rostrally situated regions. Of particulate interest is the finding that αSN lesions in the SC was a common pathological finding in PD subjects with or without DMV involvement which further challenges the theory that the DMV is the trigger site for αSN pathology in the PD brain.

Additional studies are needed to confirm Braak's findings as well as further analysis of αSN distribution in the ageing brain is required to redress Braak's confounding systematic bias in deciding to exclude cases without DMV inclusions. While, the clinical and pathological significance of αSN deposition at the molecular level has yet to be determined, studies looking at other indications of disease, such as decreased cell number and glial activation, suggest that degeneration is not confined solely to inclusion-rich areas. These other indicators must also be addressed in the ageing brain of individuals without signs of Parkinsonism as well as in PD cases with proper experimental design, if we wish to fully document disease progression in PD.

Chapter 7

General Discussion

The aim of this thesis was to examine possible associations between the most common neuropsychiatric complications associated with PD (dementia, VH and anxiety) and pathology in specific anatomical areas as a means of providing a better insight into their aetiopathogenesis and thus provide a bridge between clinical phenomenology and regional pathological involvement in the PD brain. The results presented herein demonstrate that PD as well as AD-related pathology in limbic and striatal regions play a principal role in the development of cognitive and behavioural dysfunction in PD. In addition, the present thesis demonstrated that the dorsal motor nucleus of the vagus is not an obligatory trigger site of Parkinson's disease.

7.1 Methodological Considerations

The number of cases studied in chapters 3 and 5 may be viewed as relatively small but they were carefully drawn from a larger cohort with stringent selection criteria applied. While for some variables correlations and associations may be more evident in larger samples, the careful case selection employed in these chapters greatly strengthens the positive clinico-pathological associations for the neuropsychiatric features assessed. The sample size presented in chapter 3 is similar to Churchyard and Lees (1997) and other previous clinico-pathological studies such as Mattila et al., 2000, Harding et al., 2002a,b, Hurtig et al., 2000, Bertrand et al., 2004 ((Mattila *et al.* 2000;Harding *et al.* 2002a;Harding *et al.* 2002b;Hurtig *et al.* 2000;Bertrand *et al.* 2004) that have investigated the pathological correlates of dementia and VH in PD. Furthermore, the studies presented in chapters 3 and 4 have the advantage that PD subjects presented with the two clinical signs under study (dementia and VH) in different combinations and with minimal to absent concomitant neuropsychiatric disturbances (e.g. anxiety, depression) thus enabling a greater understanding of the anatomical and pathological basis of dementia and VH. This is very important as previous reports failed to take into account concomitant

neuropsychiatric deficits in the subjects analysed. The sample size for Chapter 4 is the largest studied so far while for chapter 6 it is similar to previous studies (Braak *et al.* 2003;Parkkinen *et al.* 2005a).

For all cases the clinical diagnosis was based on established criteria (section 2.2.2). However, our report has limitations germane to all retrospective clinico-pathological studies. Although clinical details were available for all cases studied, incomplete and inaccurate observations may have introduced an ascertainment bias for some of our clinical severity scores. For example, it may be that some patients in the PD(H-) group had cognitive impairment, dementia or VH that was not recorded in the patient notes and therefore may have been underestimated by our assessment. A selection bias that typically operates in brain-bank samples (e.g. patients with more severe or rapidly progressive disease) cannot be excluded, although every attempt was made to limit any potential bias. Further to this, PD patients often present with several types of behavioural change. Thus, overlapping behaviours complicate the analysis and make it difficult to examine changes related to a single behaviour in isolation. However, in all studies presented in this thesis every effort was made to isolate dementia, VH and anxiety and assess their anatomical and pathological correlates.

Data obtained from postmortem tissues may be influenced by variations in tissue processing. All immunohistochemical reactions were performed with the same reagents and by the same investigator (Mr Michail E. Kalaitzakis) to reduce variability. In addition, the specificity of immunodetection was ensured by employing well-characterized and/or widely used antibodies (e.g., AT8 and αSN (clone 42) see section 2.1.2) and negative and positive controls for every immunohistochemical run.

Neuropathological studies do not allow the elucidation of the molecular mechanisms underlying disease state. However, while descriptive in character, when combined with clinical information this permits the optimum characterisation of the brain in a diseased state and consequently the data provided can be instrumental for the testing of new hypotheses and for the delineation of novel concepts.

7.2 General Conclusions

In Chapter 3, the relationship between pathological protein deposition (both PD- and AD-related) and the presence of dementia and VH alone or in combination was investigated by assessing eight regions of the brain that have been shown to play a role in cognitive function. The results of this work demonstrate that αSN in limbic regions strongly relates to the occurrence of dementia in PD while AD-type pathological change may have a permissive and or synergistic effect in precipitating dementia in PD. An important finding of this work was also that αSN pathology in the NBM was high in both demented and cognitively intact cases highlighting a universal cholinergic deficit in PD, with or without clinical cognitive decline. This finding demonstrates that degeneration of the NBM alone is not sufficient to cause dementia in PD thus the contribution of additional anatomical regions (i.e. limbic structures, striatum) are necessary for the development of dementia. Furthermore, the findings of this study also show that αSN in the amygdala closely relates to the presence of VH but only in those cases with concomitant dementia. Overall, the data presented in chapter 3 apart from providing further evidence of a clinical relevance of αSN deposition in the PD brain also point to pathological changes in the limbic system as an important determinant for progression to clinical dementia and VH in PD.

In Chapter 4 the relationship between striatal pathology and dementia and VH in PD was investigated as a means of examining further the link between neuropathology and cognitive dysfunction. The reason striatum was chosen as a possible candidate for exploration was the central role of these nuclei in PD pathophysiology and their putative role in cognitive function. The results of this work demonstrate an increased Aβ striatal burden in PD cases with cognitive impairment/dementia in the absence of prominent cortical Aβ deposition. In fact this is the first report to demonstrate a clinical relevance of striatal pathology with respect to cognitive dysfunction in PD. Further to this, the findings of this work also underscore a molecular link between PD and AD with respect to Aβ deposition. This is of particular importance as it is still unclear whether PD and AD represent separate and distinct disease entities or extremes of a common neurodegeneration. The results of chapter 4 clearly demonstrate a link between PD and AD and thus challenge the notion that NFT, Aβ plaques and αSN inclusions are specific markers for AD and PD, respectively. Indeed the view that these abnormal protein

deposits represent morphologic features that are inherent to these diseases and that they provide a valid means by which each disease can be morphologically identified and defined is no longer tenable. However, further research is needed to explore at the molecular level the interrelationship between PD and AD. Also, in this study a link between the chemoarchitecture of the striatum and the distribution of striatal Aβ plaques was considered, as the morphology of striatal Aβ plaques did not seem to represent a mere extension of their cortical counterparts. Taken as a whole, chapters 3 and 4 demonstrate that dementia in the setting of PD more likely represents the confluence of additive and/or synergistic effects of pathologic involvement of limbic and striatal regions while VH seem to associate with pathological changes in limbic structures (i.e. amygdala). However, it has to be noted that the selection of brain regions examined may have introduced a bias and thus the findings do not exclude the contribution of additional anatomical areas for the development of dementia or VH in PD.

In Chapter 5 a common but understudied neuropsychiatric complication associated with PD symptomatology was investigated. The aim of this chapter was to examine the possible anatomico-pathological correlates of anxiety in PD and thus further our understanding of the possible substrate(s) of this rather neglected psychiatric abnormality. The findings point to amygdala and the CA2 sector of the hippocampus as key regions for the development of this disabling symptom. In fact this is the first study to explore the neuropathological basis of anxiety in the PD brain and although preliminary possibly sheds some light on common neurobiological mechanisms of anxiety in PD and mood disorders. A link between anxiety and tremor was also found pointing to an influence of motor disability on behaviour in PD. Overall the results presented in this research may provide the backbone for future studies into anxiety in PD.

An improved understanding of the anatomical and pathological basis of cognitive and psychiatric symptoms in PD may ultimately contribute towards the evolution of better treatment strategies for these aspects of the illness and by studying the relationship between centres in the brain that control behaviour in PD disease state one will by inference add to a greater understanding of brain function in normal individuals.

Having looked at αSN pathology in a relatively large number of regions in the PD brain the need arose as to whether this PD-related pathological change indeed follows the proposed predicted caudo-rostral spread suggested by Professor Braak and colleagues.

Thus, quite apart from the pathological basis of neuropsychiatric features of PD studied in the previous chapters, chapter 6 dealt with the examination of the topographic distribution of αSN in the PD brain. The findings of this study showed that a significant number of cases did not fit the proposed predicted caudo-rostral spread of αSN pathology with some cases (n=5) showing no αSN pathology in the DMV despite αSN lesions in more rostrally situated regions including the SN and cortex. In addition, this investigation demonstrated that αSN in the SC is a common pathological finding in PD subjects with or without DMV affection. Taking together these results, this chapter contests the theory that the DMV is an obligatory trigger site for αSN pathology. These data can be instrumental for future studies by highlighting the need to study additional PD-related pathological changes (i.e. decreased cell number, glial activation) if we wish to fully document disease progression in PD.

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Appendix I

Representative Neuropathological Report of a PD case (PD69)

Clinico-pathological Autopsy Report

CLINICAL SUMMARY

Name: **Date of Birth**: **Age at death:** 82
Sex: Male **GP:**

Clinical cause of death: Ischaemic & degenerative heart disease, carcinoma of ? liver.
Quality of notes: Good
Age at Onset: 71 (1993, Dx 1995)
Disease Duration: 11 years
Presentation: Akinetic-rigid syndrome (shuffling, festinating gait, poor fine motor skills, flexed posture, drooling, micrographia, cogwheeling L wrist, then later R arm signs).
Disease Details: Two years of symptoms prior to formal diagnosis. Akinetic-rigid syndrome with L wrist rigidity.
Drugs: Selegiline, Sinemet 110, Sinemet Plus & CR, Ropinirole, Entacapone Quetiapine, Risperidone, Fludrocortisone, Amitriptyline, Clonazepam, Rivastigmine, Cipramil, Sertraline.
Complications: Falls. Restless legs. Nocturnal jerking of legs (?REM sleep disorder). Wearing-off episodes/ Freezing/ On-off fluctuations. Dyskinesia when on.
Autonomic: Drooling. Swallowing problems. Soft monotonous voice/ gruff Somnolence/ Disturbed sleep. Postural hypotension; Bradycardia. Sexual dysfunction/ Impotence. Nocturia. Seborrhoeic rash.
(Reduced smell & taste on Selegiline)
Psychiatric: Depression. Memory loss. Dementia (visuospatial problems/ dressing apraxia). Hallucinations (upsetting, worse with medication). Delusions. Agitation.
Premorbid & Risks: Non-smoker. Golfer.

(Detailed Clinical History available)

MACROSCOPIC NEUROPATHOLOGICAL FINDINGS:
(Photographic documentation available)

Brain weight: 1,204 grams

The meninges overlying the convexity are translucent. The gyration appears to be normal with the exception of the right occipital pole where there is a small arachnoid cyst which measures about 1 x 0.8 cm in diameter. The uncus hippocampi is indented on both sides, more so on the left. The blood vessels at the base of the brain are not preserved but the middle cerebral artery bilaterally does not show any atherosclerotic deposits. The nigra is pale especially in its lateral parts. Inspection of the coronal sections does not reveal significant abnormalities with the exception of a small accumulation of what appears to be blood in the base of the anterior horn of the left lateral ventricle. This may just be a preparation artefact but histology is taken. The ventricular system is somewhat compressed. There are no other abnormalities.

Brain regions sampled: 1) Superior frontal gyrus, 2) cingulate gyrus, 3) nucleus accumbens, 4) putamen and caudate, 5a) nucleus basalis of Meynert, 5b) globus pallidus, 6) hypothalamus with mamillary body, 7) amygdala, 8) thalamus, 9) hippocampus, 10) temporal cortex, 11) precentral gyrus, 12) visual cortex, 13) parietal lobe, 14) cerebellum and dentate nucleus, 15) substantia nigra, 16) pons at the height of the locus coeruleus, 17) medulla with inferior olive, 18) spinal cord at three levels.

Immunohistochemical labellings performed: anti-alpha synuclein (monoclonal, BD), anti-A-beta peptide (residues 8-17) (monoclonal, NCL), anti-tau (monoclonal AT8, NBS Biological) and anti-ubiquitin (monoclonal, Dako).

MICROSCOPIC FINDINGS

The haematoxylin-eosin stained sections of the superior frontal gyrus, cingulate, precentral gyrus, parietal lobule, temporal neocortex, primary visual cortex, nucleus accumbens, nucleus pallidus, hypothalamus, thalamus, amygdala, medulla and spinal cord look unremarkable. Putamen shows artefacts due to poor fixation and embedding. The section containing nucleus basalis of Meynert is characterised by reactive astrocytosis and deposition of haemosiderin. A few nerve cells show large, eosinophilic cytoplasm. The section from hippocampus demonstrates loss of pyramidal nerve cells in the CA1 sector of the hippocampus and the subiculum. The cerebellum shows a mild reaction of Bergmann's glia and reactive astrocytosis of the dentate. There is severe loss of pigmented nerve cells from the substantia nigra associated with reactive gliosis, an increase in the number of microglial cells and free-lying neuromelanin pigment. Some pigmented nerve cells contain Lewy bodies. Severe loss of pigmented neurones is also found in the locus coeruleus. The section from the block labelled „lesion" shows periventricular white matter with no significant pathological changes.

Alpha synclein: Immunostains for alpha-synuclein demonstrate diffuse, finely granular immunolabelling in the grey matter of the superior frontal gyrus, and a few neurons containing Lewy bodies. Accumulation of alpha-synuclein is seen in the nucleus basalis and the amygdala, more prominent in the cortical nucleus, hippocampus, substantia nigra and pons. The hippocampus shows alpha-

synuclein positive Lewy body-like structures in a few nerve cells, and prominent alpha-synuclein accumulation is seen in dendrites and axons of the CA2 sector. Lewy body-like alpha-synuclein immunoreactive inclusions are also seen in the subicular and entorhinal cortex. The substantia nigra shows widespread accumulation of alpha-synuclein in nerve cells, the neuropil and oligodendroglia. Alpha-synuclein positive inclusions are also present in the locus coeruleus, neurons of the raphe nucleus, the pontine reticular formation and the hypoglossal nucleus

Tau and A-beta peptide: There are A-beta positive, tau-negative diffuse and focal deposits in the superior frontal gyrus and the entorhinal cortex. Immunostaining for phosphorylated tau demonstrates neurofibrillary tangles in some nerve cells and axons of the hippocampus and entorhinal cortex. These features are consistent with isocortical grade 1 and entorhinal grade 2 Alzheimer-type pathology (www.ICDNS.org). There is no tau accumulation in the substantia nigra.

CLINICAL DIAGNOSIS: Akinetic-rigid syndrome with typical progression (cognitive & autonomic involvement) complicated by dyskinesia.

NEUROPATHOLOGICAL DIAGNOSIS: Alpha-synucleinopathy, brain stem-predominant, consistent with clinical Parkinson's disease (www.ICDNS.org)

Yours sincerely,

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09/09/2005 Department of Neuropathology

Representative Clinical Summary of a PD case (PD69)

PD69 31/05/1922 M
Quality of Notes: Good
Presentation: Akinetic-rigid syndrome (shuffling, festinating gait, poor fine motor skills, flexed posture, drooling, micrographia, cogwheeling L wrist, then later R arm signs)
Age of onset: 71 (1993, Dx 1995) **Disease Duration: 11 years** Two years of symptoms prior to formal diagnosis. Akinetic-rigid syndrome with L wrist rigidity.
Drugs: Selegiline, Sinemet 110, Sinemet Plus & CR, Ropinirole, Entacapone Quetiapine, Risperidone, Fludrocortisone, Amitriptyline, Clonazepam, Rivastigmine, Cipramil, Sertraline
Complications: Falls Restless legs Nocturnal jerking of legs (?REM sleep disorder) Wearing-off episodes/ Freezing/ On-off fluctuations Dyskinesia when on
Autonomic: Drooling Swallowing problems Soft monotonous voice/ gruff Somnolence/ Disturbed sleep Postural hypotension; Bradycardia Sexual dysfunction/ Impotence Nocturia Seborrhoeic rash (Reduced smell & taste on Selegiline)
Psychiatric: Depression Memory loss Dementia (visuospatial problems/ dressing apraxia) Hallucinations (upsetting, worse with medication) Delusions Agitation

Premorbid & Risks:
Non-smoker. Golfer.

Neuropathological Diagnoses:
Primary:

Secondary:

Remarks:

PD69 31/05/1922 M

Medical Practitioner
(Medical Advisor to Foreign Office)

Cause of death (09/09/2004): "Ischaemic & degenerative heart disease, CA of liver"(?correct?)

GP: Dr

CLINICAL HISTORY:
1995
Two year history of "Deteriorating golf swing & unusual fatigue on exercise. Restless aching legs when resting". Golf handicap falling off. Festinating gait, tendency to drool, poor fine motor skills & micrographia. Rigidity at L wrist. Slower, stiffer, flexed with a shuffling gait, poverty of arm swing & impassive face. Speech soft & monotonous. Marked improvement on Sinemet 110 & significant improvement adding Selegiline to Sinemet 110 QD. Mild extrapyramidal features.
1996
Mild bradykinesia, little tremor. BP 130/80 lying & 105/70 standing. Poor smell. Mobility reduced off Selegiline. Stable on Sinemet Plus QD. Sexual dysfunction.
1997
More of a struggle to keep mobile, especially playing golf. Falls asleep in day. Insomnia/ restless legs & L leg tremor at night. More akinetic-rigid than tremulous. BP drops from 140/70 to 120/70. To increase Sinemet.
1998
Depression, poor memory (vs. pseudo-dementia). More flexed & slow in thought and movement.
1999
Improved mobility, alertness & sleep on low dose Ropinirole.
2000
Dyskinetic fidgets.
2001
Muscle spasms & twitching evenings/ nights. To increase Ropinirole. Off episodes reduced on increased Ropinirole. CT scan: some generalised atrophy. Some help with dressing & meals. Difficulty in bed turning over. Minimal bradykinesia in R

hand & reduced arm swing on R. Difficulty with spatial awareness & short-term memory (great trouble on escalators).
2002
On/ off & freezing. Dressing apraxia & perseveration. Good stride but reduced arm swing on R. R arm bradykinesia & arm dyskinesia. Tone normal. Word-finding problems, speech worsening, losing track, worried about dementia or Alzheimer's. Walked with upright posture, good arm swing, minimal rigidity & no tremor.
2003
Neuropsychological assessment: impaired (esp. visual function) not to drive. Entacapone effective in reducing wearing-off episodes but increased hallucinations & disturbed thoughts. On Ropinirole 16 mg/ day. Confused & muddled. Speech gruff, word-finding difficulties, swallowing problems.
2004
Confused ("rapidly dementing"), hallucinosis (upsetting), visuospatial problems & dressing apraxia. Falls. Risperidone for agitation. Jerking ("myoclonic") spasms of legs. Better on Rivastigmine & decreased hallucinations. To decrease Ropinirole. Generally bradykinetic.

OTHER MEDICAL:
2004
Seborrhoeic excema. CA liver.
2003
Senile dementia.
2002
Bradycardia & Hypotension (170/70 dropping to 120/60). Cardiology opinion.
2000-2001
Sensorineural hearing loss consistent with age. Basal Cell Carcinoma
1999
Age-related macular degeneration
1998
L inguinal hernia repair. "Neurotic depression reactive type".
1993
Fungal infection hand & groin. Persistent excema.
1988
Seborrhoeic keratosis
1954

FAMILY HISTORY:
None

MEDICATION:
Selegiline, Sinemet 110, Sinemet Plus, Sinemet CR, Ropinirole, Sertraline, Quetiapine, Risperidone, Entacapone, Fludrocortisone, Quinine, Amitriptyline, Clonazepam, Rivastigmine, Cipramil.

Appendix II

Standard Operating Procedure

De-waxing, dehydrating and clearing of paraffin embedded human sections

De-waxing sections

Slides are placed in the appropriate racks, and then moved through the following solutions:

1. Xylene	5 mins
2. Xylene	5 mins
3. 100% alcohol	5 mins
4. 100% alcohol	5 mins
5. 90% alcohol	5 mins
6. 70% alcohol	5 mins
7. Distilled water	5 mins

Dehydrate and clearing sections

1. Distilled water	5 mins
2. 70% alcohol	5 mins
3. 90% alcohol	5 mins
4. 100% alcohol	5 mins
5. 100% alcohol	5 mins

6. Xylene 5 mins
7. Xylene 5 mins

Application: Indirect immunoperoxidase technique using monoclonal/polyclonal antibodies. On Human Tissue (Immunocytochemistry)

1. Sections to Water (refer to Appendix II on de-waxing)
2. Block in Hydrogen peroxide/methanol 30 mins
3. Rinse in PBS 3 X 5 mins
4. Carry out any pre-treatments (see next page)
5. Rinse in water 5 mins
6. Rinse in PBS 3 x 5 mins
7. Individually remove sections from the PBS, wipe around sections and the back of the slide apply pap pen around the sections and place in moist incubation chamber.
8. Add 1st layer antibody at appropriate concentration and incubate overnight at 4°C
   The antibody was diluted at correct concentration and that the sections DO NOT dry out.
9. Rinse in PBS 3 x 5 mins
10. Secondary antibody added at a concentration of 1:100 room temp 1hour
    For Rabbit polyclonal 1°antibody a goat anti-rabbit 2° layer is used

    Four Mouse monoclonal 1° antibody a horse anti-mouse 2° layer is used
11. Rinse in PBS 3 X 5 mins
12. Third layer complex incubate sections in 3rd layer 1 hour
    Vectastain elite regents A & B should be combined to a final concentration of 1:200 with respect to both components (e.g. 5µl A + 5µl B : 990µl Diluent) This layer was made up at least 30 minutes in advance.
13. Rinse in PBS 3 x 5 mins

14. Visualize: 5mins

Incubate the slides with 1 aliquot of DAB into 400mls of PBS 16 drops of Hydrogen peroxide.

15. Remove the slides and rinse in running water

    Waste DAB to be put in appropriate container for disposal

16. Lightly counter stain in Haematoxylin

17. DCM (refer to Appendix II on de-hydration of sections)

Formic acid pre-treatment of paraffin wax sections sections.

- After de-waxing and blocking of endogenous peroxidases in sections, sections were placed into a coplin jar of 80% or 100% Formic acid for 8 or 60 minutes.
- Slides were then removed and place into running water.

Microwave pre-treatment of tissue sections.

- Sections placed in plastic rack
- The rack was placed in a suitable microwave container, filled with an adequate amount of EDTA.
- Sections were microwaved on medium (40%) for 10mins and then a further 10mins on medium high (40%)
- The dish was then removed from the microwave and placed in a sink of cold water for at least 20 minutes in order to cool the solution and slides down.

Buffers and Solutions

10x Phosphate Buffered Saline (PBS)

27g Sodium dihydrogen orthophosphate dehydrate
193g di-sodium hydrogen orthophosphate 12 hydrate
20g Potassium chloride
800g Sodium chloride
Made up to 10l with dH_2O, pH 7.4

1x PBS

1l of 10x PBS
9l dH_2O
pH 7.4

3,3'-diaminobenzidine (DAB)

5g DAB powder
200ml dH_2O
(4ml aliquots of this solution was stored in a freezer and used)

Primary diluents

490ml PBS
1.5ml triton X-100
1.5g sodium azide
10ml of normal horse serum

Second and third diluents

500ml PBS
1.5ml triton X-100

EDTA Microwave solution

- Weigh out 0.3722g of EDTA
- Add the EDTA to 1 liter of distilled water
- MIX well
- Adjust the pH to 8 using either HCL or sodium hydroxide.
- Solution stored until ready to use.

Publications arising from this work

1. M.E. Kalaitzakis, M.B. Graeber, S.M. Gentleman, R.K.B. Pearce (2007). The dorsal motor nucleus of the vagus is not an obligatory trigger site of Parkinson's disease: A critical analysis of α-synuclein staging *Neuropath. Appl. Neuro.* (in press)

2. M.E. Kalaitzakis, M.B. Graeber, S.M. Gentleman, R.K.B. Pearce (2008). Striatal β-Amyloid Deposition in Parkinson's Disease with Dementia. *J. Neuropath. Exp Neurol* (in press)

Appendix III

αSN

	Cingulate Gyrus	Superior Frontal Gyrus	Temporal Cortex	Entorhinal Cortex	Amygdaloid Complex	NBM	CA2	Subiculum
PD(H-) Group	0.5	0.5	0	0	0	2	0	0
	1.5	1	0.5	1	1.5	2	0.5	0.5
	1	1	1	1	1	2	1.5	0.5
	1	0	1	1	0.5	0.5	0.5	0
	0.5	0.5	0	0.5	0	1.5	0.5	0
	0.5	0	0	1	1.5	1	0.5	0
	0	0.5	0.5	0.5	1	1	0	0
	0	0.5	0	0	0	3	0	0
	1	0.5	0.5	0.5	0	1	0	0
PDD(H+) Group	1	1	1	3	3	2	2	0
	2	0.5	0.5	1	1	2	1	0.5
	1.5	1	1.5	2.5	3	2.5	1.5	0.5
	2	0.5	1	1	1.5	3	3	0
	1.5	1	0.5	1	0.5	2.5	0.5	0
	1.5	1	1	2	2	2	2.5	0
	0.5	0.5	1.5	1	1.5	1	0.5	2
	1	1.5	1	1	1	2	3	2
	2.5	0	0.5	3	0	1	2	1
	1.5	1	1.5	2	2	2	2.5	0.5
	2.5	1.5	1.5	1.5	1.5	2.5	0.5	1.5
	2	1	1	2	2.5	2	3	1
PDD(H-) Group	2	1.5	2.5	3	2	2.5	2	0
	2.5	2	1.5	2	3	2.5	1.5	1
	2	1	1	1.5	2.5	2	1	1
	0.5	0.5	0	2	2	2	2	0
PD(H+) Group	1	0.5	0.5	0	1	3	0	0
	1	0.5	1	1	2	2	0.5	0
	1	1	1	0.5	1.5	2	0.5	0
	1.5	1	1	2.5	2.5	2	3	0.5
	1.5	1	1	1	0.5	2	1.5	1

Aβ

	Cingulate Gyrus	Superior Frontal Gyrus	Temporal Cortex	Entorhinal Cortex	Amygdaloid Complex	NBM	CA2	Subiculum
PD(H-) Group	0	0	0.5	0	0	0	0	0
	0.5	0.5	0.5	0.5	0.5	0.5	0	0.5
	0.5	0.5	0.5	0.5	0.5	0	0	0.5
	0	0.5	0.5	0.5	0.5	0	0	0
	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
	0.5	0.5	0.5	0.5	0.5	0	0	0
	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0
PDD(H+) Group	1.5	2	2	1.5	0.5	0.5	0.5	0.5
	0.5	0.5	0.5	0.5	0.5	0	0	0
	1.5	1	2	1.5	0.5	1	0.5	1
	0.5	0	0.5	0	0.5	0	0	0
	1.5	1	1	1	0.5	2	0	0.5
	0.5	0	0.5	0.5	0.5	0.5	0	0.5
	0.5	0.5	0.5	1	0.5	0	0	0.5
	1.5	0.5	0.5	0.5	1	0	0.5	0.5
	0	0	0.5	0.5	0	0	0	0
	2	2	1.5	2	1	2	1	0
	2	2	2	1.5	1	1.5	0.5	0.5
	1	0.5	0.5	0.5	0.5	0	0	0.5
PDD(H-) Group	1.5	2	2	1.5	1	1	0	0
	2	0.5	1.5	1	1	2	0.5	1
	0	0	0	0	0	0	0	0
	0.5	0.5	0.5	1	0.5	1	0	0
PD(H+) Group	0.5	0	0	0	0	0	0	0
	0	0	0	0	0	0	0.5	0
	0	0	0	0	0	0	0	0
	1.5	1.5	2	0.5	0.5	0.5	0	0
	0.5	0.5	1.5	0.5	0.5	0.5	0	0

Tau

	Cingulate Gyrus	Superior Frontal Gyrus	Temporal Cortex	Entorhinal Cortex	Amygdaloid Complex	NBM	CA2	Subiculum
PD(H-) Group	0.5	0	0	1	0.5	0.5	0	0
	0.5	0	0	1	0	0.5	0.5	0
	0	0	1	1	1.5	0	1	0
	0	0	0	0.5	0.5	0	0	0.5
	0.5	0	0.5	1	0.5	0.5	0	0.5
	0.5	0.5	0.5	1	1	1	0.5	0.5
	0	0	0.5	1.5	0.5	1	0.5	0.5
	0	0.5	0.5	0.5	0.5	1	0.5	0
	0	0.5	0.5	1	0.5	0.5	0	0.5
PDD(H+) Group	1	0	1	1	1.5	0	1	1
	0.5	1	0	0	0	0	0.5	0.5
	1	1	2	1	2	1	1	2
	0	0	0	0	0.5	0	1.5	0
	0	0	0	1	0	2	0	0
	0.5	0	0	1	0.5	0.5	0.5	0.5
	0	0	0	1.5	1	1	0.5	0
	0	0	0	1	1	0	1.5	2
	0	1	0	1	0	0.5	0.5	0
	0.5	0.5	0	1	0.5	1.5	0.5	0.5
	0.5	1	1	1	1.5	2	1	1
	0	0	0	1	1	0.5	1.5	0.5
PDD(H-) Group	0.5	1	1	1.5	1.5	0	0	0
	0.5	0	0.5	1	1.5	2	1.5	1
	0	0	0	0	0	1	1	0
	0	0	0	1	0.5	0.5	1	0
PD(H+) Group	0	0	0	0	0.5	0.5	0.5	0
	0	0	0	0.5	0.5	0	0	0.5
	0	0	0	0.5	0	0.5	0.5	0.5
	0.5	0.5	0.5	1.5	0.5	0.5	1	0
	0.5	0	0.5	1.5	0.5	1	0.5	1

	Recorded MMSE
PDD(H+)	
	-
	-
	19/30
	-
	20/30
	-
	4/10
	-
	-
	-
	16/30
	11/28
PDD(H-)	
	20/30
	20/30
	-
	20/30

Appendix IV

αSN

	Caudate Nucleus	Putamen	Nucleus Accumbens
PD(H-) Group	0.5	0.5	0
	0.5	0.5	1
	1	1	1
	0.5	1	1
	0	0	1
	0	0	0
	0.5	0.5	0.5
	0	0	0
	0	0	0
PDD(H+) Group	0	0	0.5
	0	0	0.5
	0.5	0.5	1
	0.5	0.5	2
	1	1.5	0.5
	0.5	0.5	1
	1	2	0.5
	0	0	0
	0	0	0
	0.5	0	0.5
	0	0	1.5
	1	1.5	0.5
PDD(H-) Group	0	0	2
	2	2	0.5
	0.5	1.5	0
	0	0	0.5
PD(H+) Group	0.5	1	0.5
	0.5	1	1.5
	0.5	1	0.5
	0.5	0	0.5
	0	0	0.5

Aβ

	Caudate Nucleus	Putamen	Nucleus Accumbens
PD(H-) Group	0	0	0
	0.5	0.5	0
	0.5	1	2
	0	0	0.5
	0	0	0
	0	0	0
	0	0	0
	0	0	0
	0	0	0
PDD(H+) Group	3	3	3
	1	1	0.5
	3	3	2.5
	0	0	0
	2	2	2
	1	1	1
	2	2	3
	3	3	3
	0	0	0
	2.5	3	3
	1	1.5	2
	2.5	2.5	3
PDD(H-) Group	2	2	2
	2	2	2
	0	0	0
	0.5	0.5	0.5
PD(H+) Group	0	0	0
	0	0	0
	0	0	0
	1.5	0.5	2
	0	0	0

Tau

	Caudate Nucleus	Putamen	Nucleus Accumbens
PD(H-) Group	0	0.5	0.5
	0	0.5	0.5
	0	0	0.5
	0	0	0.5
	0.5	0	0
	0.5	0.5	0.5
	0	0.5	0
	0.5	0.5	0.5
	0	0	0.5
PDD(H+) Group	0	0	0.5
	0	0.5	1
	1	1	0
	0	0	0.5
	0.5	0.5	0
	0	0	0.5
	0	0.5	0.5
	0	0	0
	0	0	0.5
	0	0.5	2
	0.5	0.5	0.5
	0	0	0
PDD(H-) Group	0	0	1
	0.5	0.5	0
	0	0	0.5
	0	0	0
PD(H+) Group	0.5	0	0.5
	0	0.5	0.5
	0	0.5	0
	0.5	0	0.5
	0	0	0

Appendix V

αSN

	Cingulate Gyrus	Temporal Cortex	Entorhinal Cortex	Amygdaloid Complex	NBM	CA2	Subiculum	Locus Coeruleus	Raphe Nuclei
PD(A-) Group	0.5	0	0	0	2	0	0	0.5	0.5
	1.5	0.5	1	1.5	2	0.5	0.5	0.5	1.5
	1	1	1	1	2	1.5	0.5	1.5	1
	1	1	1	0.5	0.5	0.5	0	1.5	1
	0.5	0	0.5	0	1.5	0.5	0	0.5	0.5
	0.5	0	1	1.5	1	0.5	0	0	0
	0	0.5	0.5	1	1	0	0	0.5	1
	0	0	0	0	3	0	0	1	0.5
	1	0.5	0.5	0	1	0	0	0.5	0
PD(A+) Group	1	0.5	1.5	1.5	1.5	1.5	0.5	2.5	2
	2.5	1	3	3	1.5	3	2	2	2
	0	0.5	1	3	2	3	0.5	2	1
	1.5	0.5	0.5	2	2	1.5	0	2	3
	2.5	0.5	1	2	2	0.5	0.5	0.5	0
	1	0.5	1	1.5	1.5	1.5	0.5	1.5	0.5

Aβ

	Cingulate Gyrus	Temporal Cortex	Entorhinal Cortex	Amygdaloid Complex	NBM	CA2	Subiculum	Locus Coeruleus	Raphe Nuclei
PD(A-) Group	0	0.5	0	0	0	0	0	0	0
	0.5	0.5	0.5	0.5	0.5	0	0.5	0	0
	0.5	0.5	0.5	0.5	0	0	0.5	0	0
	0	0.5	0.5	0.5	0	0	0	0	0
	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0
	0.5	0.5	0.5	0.5	0	0	0	0	0
	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0
PD(A+) Group	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0
	1	1.5	1	2.5	0	0	1	0	0
	0.5	1	0	0.5	na	0	0	0	0
	2	1.5	2	2	0	0.5	1	0	0
	0.5	0.5	0.5	1	0	0	0	0	0

Tau

	Cingulate Gyrus	Temporal Cortex	Entorhinal Cortex	Amygdaloid Complex	NBM	CA2	Subiculum	Locus Coeruleus	Raphe Nuclei
PD(A-) Group	0.5	0	1	0.5	0.5	0	0	0.5	0
	0.5	0	1	0	0.5	0.5	0	0.5	0.5
	0	1	1	1.5	0	1	0	0.5	0.5
	0	0	0.5	0.5	0	0	0.5	0.5	0.5
	0.5	0.5	1	0.5	0.5	0	0.5	0.5	0.5
	0.5	0.5	1	1	1	0.5	0.5	0.5	0.5
	0	0.5	1.5	0.5	1	0.5	0.5	na	na
	0	0.5	0.5	0.5	1	0.5	0	0.5	0
	0	0.5	1	0.5	0.5	0	0.5	0.5	0
PD(A+) Group	0	0	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	0.5	0.5	1	2.5	0.5	1	1	0.5	0.5
	0	0.5	1	2	0.5	0.5	0.5	0.5	1
	0	0.5	1	na	0.5	0.5	1.5	na	na
	0	0.5	0.5	1	0.5	1	0.5	0.5	0
	0	0	1	0	0.5	0.5	0.5	0	0