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Impact of α-Synuclein Pathology on Aging

Doctoral dissertation

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ABSTRACT

A new direction to research on pathogenesis of Parkinsons's disease (PD) and related disorders was initiated after two breakthrough discoveries that brought α-synuclein (αS) to the fore; mutations in the αS gene were found to be a cause of familial PD and the fibrillar αS was detected as a major component of the hallmark inclusions. Since these intracytoplasmic αS-positive inclusions are often accompanied by neuronal loss, they were believed to compromise the cellular functions leading to the subsequent presentation of clinical symptoms. However, this now appears to be an oversimplified theory as there are many factors that oppose the pathognomonic nature of the αS-positive inclusions.

In the present series of studies, we aimed to clarify the impact of various αS-positive inclusions on the aging process by examining their incidence and topographical distribution throughout the brain in a large elderly population (n=904). The αS-positive inclusions were observed in around 15% of these aged individuals, by screening the most vulnerable nuclei; substantia nigra (study **I**), basal forebrain nuclei (study **II**) and dorsal motor nucleus of vagus (study **III**). Case selection was shown to contribute substantially to this rate (Study **I**). Also, the prevalence of αS-positive inclusions increased with age but was not influenced by gender.

The prevalence of αS-positive inclusions has been considered to be influenced by concomitant Alzheimer's disease (AD) pathology. In our material (study **II**), we did not detect αS-positive inclusions in AD patients as frequently as reported before. Since the amygdala had been reported to be particularly sensitive to the buildup of αS in AD, we concentrated on the examination of regional distribution of αS pathology in subjects with or without AD pathology.

In study **III**, the clinical relevance of αS-positive inclusions was assessed with a unique design whereby the selection of material was entirely based on the presence of αS pathology regardless of the clinical phenotype. The retrospective clinical assessment revealed that only 30% of all αS-positive cases (n=106) had been diagnosed with a neurodegenerative disorder. In particular, extrapyramidal symptoms were identified only in a quarter of all cases with brainstem αS-positive inclusions, and cognitive impairment in 35% of cases with cortical inclusions. Thus, assessment of distribution or load of αS pathology did not permit a reliable post mortem diagnosis of either of these symptoms.

Since some neurologically unimpaired subjects showed a reasonable burden of αS pathology, we decided to compare the immunohistochemical profile of one of these asymptomatic individuals with a patient with clinical syndrome of dementia with Lewy bodies (study **IV**). We found no differences in the extent of vascular pathologies, gliosis, or in apoptosis. The only discrepancies were the striatal beta-amyloid aggregates found in the symptomatic patient alone but the clinical relevance of this finding needs to be clarified.

In study **V,** we examined the topographical distribution of αS-positive glial inclusions and their clinical relevance in the cases (n=5) that had been selected from our large study population because of their predominant glial αS pathology in the midbrain.

Taken together (**IV** and **V**), our findings suggest that fibrillar αS-positive inclusions observed at autopsy are not specific hallmarks related to any particular clinical symptomatology, and it may well be that earlier events of fibrillogenesis or some additional factors are required to trigger the neuronal dysfunction.

National Library of Medicine Classification: WL 359, WT 155, QW 504.5, WT 104
Medical Subject Headings: alpha-synuclein; Alzheimer disease; aging; humans; immunohistochemistry; inclusion bodies; Lewy bodies; neurodegenerative diseases/pathology; Parkinson disease

"The conventional view serves to protect us from the painful job of thinking."

-John Kenneth Galbraith-

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Kuopio, May 2005

Laura Parkkinen

ABBREVIATIONS

αS	α-synuclein
Aβ	beta-amyloid
AC	amygdaloid complex
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
Apo E	apolipoprotein E
BFB	basal forebrain
CAA	cerebral amyloid angiopathy
CA1-4	CA 1-4 subfields of the hippocampus
CBD	corticobasal degeneration
CERAD	Consortium to Establish a Registry for Alzheimer's Disease
CG	cingulate gyrus
CNS	central nervous system
CT	computer tomography
CVD	cardio/cerebrovascular disease
DSM	Diagnostic and Statistical Manual of Mental Disorders
DLB	dementia with Lewy bodies
DMV	dorsal motor nucleus of vagus
EEG	electroencephalography
EP	epilepsy
EPSs	extrapyramidal symptoms
FCx	frontal cortex
FTD	frontotemporal dementia
GCI	glial cytoplasmic inclusions
GFAP	glial fibrillary acidic protein
HLA	human leukocyte antigen
H&E	haematoxylin and eosin
HP-τ	hyperphosphorylated tau
IHC	immunohistochemistry
LB	Lewy body
LC	locus coeruleus

MCI	mild cognitive impairment
MHC	major histocompatibility complex
MMSE	Mini-Mental state examination
MSA	multiple system atrophy
MRI	magnetic resonance imaging
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association
NF	neurofilament
NFT	neurofibrillary tangle
NP	neuritic plaque
NPH	normal pressure hydrocephalus
NBM	nucleus basalis of Meynert
PCx	parietal cortex
PHG	parahippocampal gyrus
PD	Parkinson's disease
PDD	Parkinson's disease with dementia
PUT	putamen
SN	substantia nigra
SND	striatonigral degeneration
SPSS	Statistical Package for Social Sciences
TCx	temporal cortex
TIA	transient ischemic attack
Ub	ubiquitin
VaD	vascular dementia
WMD	white matter degeneration

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by the Roman numerals **I-V**.

I Parkkinen L, Soininen H, Laakso M, Alafuzoff I (2001). α-Synuclein pathology is highly dependent on the case selection. *Neuropathology and Applied Neurobiology* 27(4), 314-325.

II Parkkinen L, Soininen H, Alafuzoff I (2003). Regional distribution of α-synuclein pathology in unimpaired aging and Alzheimer's disease. *Journal of Neuropathology and Experimental Neurology* 62(4), 363-367.

III a Parkkinen L, Kauppinen T, Pirttilä T, Autere J, Alafuzoff I. (2005). α-Synuclein pathology does not predict extrapyramidal symptoms or dementia. *Annals of Neurology* 57 (1), 82-91.

III b Parkkinen L, Kauppinen T, Pirttilä T, Autere JM, Alafuzoff I. (2005) In reply to "alpha-synuclein aggregation and its relation to neurodegenerative diseases" by Papapetropoulos S and Mash DC. *Annals of Neurology* 57 (4), 605-606.

IV Parkkinen L, Pirttilä T, Tervahauta M, Alafuzoff I. (2005). Widespread and abundant α-synuclein pathology in a neurologically unimpaired subject. *Neuropathology* (in press).

V Parkkinen L, Hartikainen P, Alafuzoff I. (2005). Significance of glial α-synuclein-positive inclusions with respect to the clinical symptoms. Submitted for publication.

CONTENTS

1. INTRODUCTION

The age-associated neurodegenerative disorders are an expanding social dilemma with the increasing longevity of the population. In addition, especially Europe, United States and Japan will start to resemble a global old-age home as the proportion of the elderly population increases and this will be accompanied by an elevated incidence of neurodegenerative disorders. Thus, longer life expectancy and the reversed pyramid of age will both contribute to this enormous public health burden. The most common clinical symptoms related to the neurodegenerative processes are cognitive and movement impairments. The therapeutics currently available are merely palliative *i.e.* they are able to alleviate symptoms but not to prevent the disease process. The successful treatment of neurodegenerative diseases will require an elucidation of the mechanisms of neuronal death in molecular level which is a major challenge of current research.

Many neurodegenerative disorders are characterized by dysfunctional metabolism of certain proteins required for the normal cellular function. These proteins become aberrantly aggregated into fibrillar inclusions that can be detected in post mortem analysis, and their presence is required for the definite and accurate diagnosis of a particular neurodegenerative disorder. Since these proteinaceous inclusions are often detected in the same brain regions as the neuronal loss, they have been considered to disrupt the cellular functions leading to demise of affected neurons and this then accounts for the clinical phenotype. One of these proteinaceous inclusions is a Lewy body (LB) the presence of which in the substantia nigra (SN) and other pigmented brainstem nuclei is considered to be a diagnostic hallmark for Parkinson's disease (PD), whereas their occurrence in the cortical neurons is pathognomonic for dementia with Lewy bodies (DLB). The genetic linkage of α-synuclein (αS) protein to the familial PD and its presence in the LBs and related pathological inclusions have been the trigger for researchers to gather a vast amount of data to support the role of αS protein in neurodegeneration (Polymeropoulos et al. 1997, Spillantini et al. 1997). However, the pathogenetic mechanisms by which αS aggregation could disable and eventually kill the neuron remain unknown. It needs to be resolved what causes these proteinaceous inclusions to form and whether αS-positive inclusions are an epiphenomenon or the actual cause of cell death. Many studies with methods ranging from cell-free systems to animal models have tried to delineate the factors that are

responsible for the fibrillogenesis and aggregation αS into inclusions (Lotharius and Brundin 2002, Caughey and Lansbury 2003, Dev et al. 2003). On the other hand, recent biophysical studies have suggested that the aggregation of αS fibrils into compact inclusion may actually be neuroprotective as these structures may represent one way to sequester the toxic early products of fibrillogenesis (Goldberg and Lansbury 2000, Caughey and Lansbury 2003, Olanow et al. 2004). Taken together, the bridge between the αS-positive inclusions and the origin of clinical symptoms is still wide and a matter of considerable debate.

Even with the avalanche of knowledge on posttranslational modifications and numerous factors that promote the aggregation process of αS, it is still essential to examine the impact of αS pathology in human studies with as large study populations as possible containing both symptomatic and asymptomatic subjects. Many previous autopsy studies have focused their examination only on patients displaying specific clinical signs, and moreover, to a limited number of normal controls. Findings of positive correlations between pathological markers and clinical symptoms have then further promoted the "inclusion theory". However, the significance of any pathologic change in relation to symptoms can only be evaluated by studying a larger pathologically derived sample. The pathologic nature can be strengthened by ruling out the presence of a particular change in the asymptomatic subjects. In view of the above considerations, the present series of studies was initiated to clarify the role of αS pathology in aging and in neurodegenerative disorders by examining its incidence, topographical distribution and clinical relevance. In the following review of the literature, the evolution of knowledge regarding the inclusions considered at present to be pathognomonic for PD, DLB and other synucleinopathies is discussed in a chronological order.

2. REVIEW OF THE LITERATURE

2.1 Pre-α-synuclein era

2.1.1 Lewy body pathology in parkinsonism

Parkinson's disease is the most common movement disorder, estimated today to affect ~1% of the general population at the age of 65, increasing up to 5% by the age of 85 (Forman et al. 2004). PD is considered to be the consequence of changes in the neuronal cytoskeleton that result in the formation of LBs in certain specific neuronal populations (Gibb and Lees 1988). Thus, the neuronal damage in PD does not occur at random and the locus of the most crucial pathology involves the degeneration of SN inputs to the striatum (Braak and Braak 2000). The underlying pathological processes progress slowly but unremittingly until they reach a certain threshold for the clinical manifestation of the disease. Clinically, the syndrome is characterized by cogwheel rigidity, bradykinesia (generalized slowness of motor activity), resting tremor and postural instability (Daniel and Lees 1993).

Lewy bodies have already almost a 100-year history as they were first described by a German pathologist Friedrich H. Lewy in 1912 in patients with paralysis agitans, later named idiopathic Parkinsonism or PD (Lewy 1912). Lewy first discovered the eosinophilic cytoplasmic inclusions in the neurons of dorsal motor nucleus of vagus (DMV), nucleus basalis of Meynert (NBM), paraventricular nucleus and lateral thalamic nucleus (Gibb and Poewe 1986, Drach et al. 1997). The involvement of LBs in SN, however, was first described by Tŕetiakoff in 1919, who coined the term "corps de Lewy" (Tretiakoff 1919, Gibb and Poewe 1986). He also described the degeneration of SN in PD and speculated that there might be a connection between this phenomenon and extrapyramidal symptoms (EPSs). Later, many authors have confirmed the presence of LBs in all pigmented neurons of the brainstem including locus coeruleus (LC) (Greenfield and Bosanquet 1953, Lipkin 1959, Bethlem and Den Hartog Jager 1960, Eadie 1963). They were invariably detected within the same nuclei where most of the cell loss was documented in PD, and consequently the LBs were considered to be responsible for the neuronal degeneration. Thus, LBs secured their position as pathological hallmarks, the *sine qua non* of PD (Forno 1996). However, LBs have been described also in many other regions of the nervous system such as amygdala, hippocampal region, hypothalamus, and less consistently in the cerebral cortex, thalamus and autonomic ganglia (Braak and Braak 2000). Of all neuronal

types prone to develop LBs, the nerve cell loss is frequently observed in the DMV, SN and NBM. Thus, the neurodegeneration and presence of hallmark inclusions do not unequivocally coincide. It still remains to be elucidated why some neuronal types are selectively vulnerable in PD, whereas others remain resistant.

2.1.2 Lewy body pathology in dementia

In 1961, Okazaki and Lipkin described widely disseminated LBs throughout the cortex in two patients with a progressive dementia associated with flexion of all extremities but without parkinsonian stigmata (Okazaki et al. 1961). Until then, it had been thought that LBs were confined to the deep structures of the brain, and the only clinical implications were the EPSs in PD. This initial observation was soon confirmed by Japanese reports (Kosaka et al. 1976, Kosaka et al. 1978), and together these unique observations gave rise to the assumption that a diffuse cortical spread of LB pathology could also be responsible for the cognitive impairment. Cortical LBs were especially evident in the small to medium-sized pyramidal neurons of the deep layers of the frontal, temporal, insular and cingulate cortices (Kosaka et al. 1984), whereas they appeared fairly seldom in the parietal and occipital cortices (Harding and Halliday 1998).

Much of the early work on the association of LBs to dementia originated from Japan, where the term diffuse LB disease (DLBD) was coined (Yoshimura 1983). A few years later, the first patients with DLBD were reported in Europe and United States (Dickson et al. 1987, Gibb et al. 1987). Shortly after, Perry *et al.* drew attention to a group of elderly patients who were demented with a clinical picture atypical of Alzheimer's disease (AD) *i.e.* a more rapid deterioration, early and prominent hallucinatory and behavioural disturbances, and associated mild parkinsonian features (Perry et al. 1989). The neuropathological evaluation of these subjects revealed an intermediate amount of cortical LBs, more abundant than that normally found in PD but less than that reported in DLBD, and yet another classification, the senile dementia of LB type (SDLT), emerged (Perry et al. 1990a). Moreover, often the patients with DLBD also had neuritic plaques (NPs) equivalent to the density found in patients with AD, and thus these patients were termed as having Lewy body variant AD (LBVAD) (Hansen et al. 1990).

Indeed, the terminology that had been used in the literature to describe the patients with cortical LBs was rather incoherent, depending on the variable emphasis on clinical versus pathological

findings. In 1996, the first International Workshop met to set common consensus guidelines for the clinical and pathological diagnosis of DLB in an effort to reconcile the differences in the nomenclature (McKeith et al. 1996). The division of DLB into three types; brainstem, limbic and neocortical was based on the earlier work by Kosaka *et al.* (Kosaka et al. 1984), and this also laid the foundation for the concept that LB disorders (PD and DLB) form a spectrum wherein the clinical manifestation depends on the anatomical distribution and severity of the LB pathology (Kosaka et al. 1984, Perry et al. 1990a, McKeith et al. 1996, Ince et al. 1998, Hishikawa et al. 2003).

2.1.3 Lewy body pathology in aging

Estimations have been made of the prevalence of LB pathology in the normal elderly population (Lipkin 1959, Woodard 1962, Forno 1969). These studies investigated very heterogeneous patient populations derived from the veterans' or chronic disease hospitals or mental institutions, and subsequently were biased towards psychiatric illness, age and/or male gender. The incidental finding of LBs in cases without any parkinsonian manifestations in these studies varied from 5-10% with an age-related rise (Gibb and Lees 1988). However, the first study that can be cited correctly to represent “normal” aged population found LBs in only 2% of subjects without any neurological and/or psychiatric disorders (Perry et al. 1990b). Therefore, LBs were concluded not to be a common age-associated feature but to be specifically related to neurodegenerative diseases. The incidental LBs reported by several autopsy studies, normally occurred in the same locations as in PD, only varying in degree, and for that reason they were thought to represent a presymptomatic phase of PD. Given that the person had lived longer, parkinsonian clinical features were supposed to have developed.

2.1.4 Detection of Lewy bodies

Initially, LBs were identified with conventional staining techniques such as haematoxylin and eosin (H&E) until more specific detection methods involving immunohistochemistry (IHC) were introduced in the late 1980's. The shape and size of the LBs depends on the configuration of the neuronal perikaryon it occupies, but often they possess a conspicuously spherical configuration around 5-25μm in diameter. In H&E staining, the brainstem LBs are considered to have a classical structure with a darker eosinophilic centre (body) surrounded by a paler halo. The cortical LBs tend to have a less conspicuous morphology and therefore, are not as readily identified in H&E stained sections (Lennox et al. 1989). Ultrastructurally, the brainstem LBs

are formed of straight filaments of 8-10nm in diameter arranged in radial orientation around an electron dense core (Duffy and Tennyson 1965, Pollanen et al. 1993). The concentric lamination of LBs can consequently be explained by greater packing density of filaments in the core. Lewy bodies can extend into nerve cell processes or lie free in the neuropil (extracellular LBs).

Soon after the original representation of LB, a lighter-coloured inclusion, lacking the characteristic body/halo profile, was described (Redlich 1930). The presumption was that these two inclusions were related. The pale bodies were larger, up to 30μm in diameter, and more irregular in shape with a rather homogeneous granular structure normally surrounded by a rim of melanin granules. Pale bodies were non-eosinophilic (Pappolla et al. 1988), and ultrastructurally, they were comprised of disorganized and sparsely arranged fibrils intermixed with granular matter and vacuoles (Gibb et al. 1991, Takahashi et al. 1994a). These structures have been mainly reported in the SN and LC (Gibb et al. 1991), but occasionally also in the cortex (Lennox et al. 1989). Pale bodies were suggested to represent a stage in the evolution of LBs as both forms share antigenic determinants (Lennox et al. 1989, Dale et al. 1992, Takahashi et al. 1994a). In addition, both inclusions are typically found simultaneously, and frequently even co-occur in the same neuron (Pappolla et al. 1988, Gibb et al. 1991). In contrast, LBs and pale bodies have been suggested to represent separate entities due to the lack of any apparent intermediate forms (Gibb et al. 1991).

Characterization of LB by using IHC identified a host of different antigenic components within this inclusion that could be divided as the main structural elements; proteins that reflect the cellular adaptive response to LB fibrils; enzymes; and passively entrapped proteins (Pollanen et al. 1993, Galvin et al. 1999a). The most consistently recognized protein constituents were cytoskeletal proteins such as neurofilaments (NFs) and ubiquitin (Ub), a protein involved in the nonlysosomal degradation of cellular products (Goldman et al. 1983, Kuzuhara et al. 1988, Lowe et al. 1988). The comparison of antibodies against NFs and Ub showed that whereas most LBs were Ub-positive, the antibodies against NFs only immunolabeled around half of the H&E stained LBs (Dale et al. 1992). Thus, Ub-IHC became the most reliable and sensitive method for identification of LBs and its use was also recommended by the first consortium guidelines (McKeith et al. 1996). In particular, the antibody was found especially helpful for the detection of cortical LBs that possessed a less stereotyped morphology (Kosaka et al. 1978). Thus, although LBs were first described solely in a movement disorder, their immunoreactive

unveiling largely contributed to their possible role also in dementia by improving their visualization in the cortex. Ubiquitin immunoreactivity, however, was not unique to the LBs but presented also 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 was recommended, especially in DLB, where both NFTs and LBs often co-exist (Dickson et al. 1989).

2.2 Pathological significance of α-synuclein

Synuclein was described in 1988 when it was cloned from the electric organ of the fish *Torpedo californica* by screening an expression library with an antiserum raised against purified cholinergic synaptic vesicles (Maroteaux et al. 1988). The protein was named synuclein because it was initially found within presynaptic nerve terminals and portions of the nuclear envelope (Giasson et al. 2004). Later, this nuclear localization was not confirmed, but the original nomenclature survived. The role of αS in the neurodegeneration was proposed five years later after the isolation of the αS fragment, the non-amyloid component (NAC), from the amyloid plaques in AD (Ueda et al. 1993). This is why αS is still occasionally referred to as the precursor protein of the non-amyloid component of the neuritic plaques (NACP). However, subsequent work failed to confirm the presence of NAC in the amyloid plaques (Bayer et al. 1999b, Culvenor et al. 1999). The breakthrough that directly implicated αS in neurodegenerative diseases came from genetics. Polymeropoulos *et al*. reported an autosomal dominant mutation in the αS gene that resulted in parkinsonism in a large Italian-American kindred and in three unrelated Greek families (Polymeropoulos et al. 1997). Two additional point mutations in the αS gene have since been identified (Kruger et al. 1998, Zarranz et al. 2004). As in some 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 inclusions of that disorder. Spillantini *et al.* first reported that LBs and dystrophic neurites were robustly immunoreactive for αS (Spillantini et al. 1997). Soon, it was noticed that αS was actually 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 αS antibodies (Spillantini et al. 1997, Wakabayashi et al. 1997, Baba et al. 1998, Spillantini et al. 1998b). In addition, Iwatsubo *et al.* prepared monoclonal antibodies using highly purified LBs from the brains of patients with DLB, and hence, biochemically corroborated that αS was accumulated in LBs (Iwatsubo et al. 1996). All these early genetical,

biochemical and pathological studies together contributed to the substantial role of αS that it presently holds in the pathogenesis of PD and DLB.

2.2.1 Structure and normal function

The human synuclein protein family consists of four (α-, β-, γ-synucleins and synoretin) small proteins (19-20kDa) with a relatively similar amino acid sequence but encoded by different genes (Goedert 2001). α-Synuclein is abundantly expressed in the brain mainly as an isoform of 140 amino acids, although a shorter alternatively sliced isoform αS 112 has been identified (Ueda et al. 1994, Beyer et al. 2004). α-Synuclein has been studied most extensively as it alone is directly implicated in human neurological disorders. Although β-and γ-immunoreactive axonal pathology was reported in a study that restricted its analysis to the hippocampus in PD and DLB (Galvin et al. 1999b), the cytoplasmic LB-like inclusions have been reported to be only immunoreactive for αS (Spillantini et al. 1997, Spillantini et al. 1998b). Furthermore, β- and γ-synucleins have failed to assemble into filaments *in vitro* (Biere et al. 2000). In addition, there is no genetic evidence linking β-and γ-synuclein genes to any neurological disease. β-Synuclein, which has the greatest homology to αS, is also concentrated in the presynaptic nerve terminals, and therefore, may have a similar physiological function with αS (Nakajo et al. 1990, Jakes et al. 1994). γ-Synuclein instead is often found in the malignant breast and ovarian tumours (Ji et al. 1997, Lavedan et al. 1998, Bruening et al. 2000), whereas its close homologue synoretin is predominantly expressed in the retina (Surguchov et al. 1999).

Structurally, αS is composed of three modular domains (Figure 1). The N-terminal amphipathic region of αS (residues 1-60), that harbours imperfect 11 amino acid repeats with consensus sequence (XKTKEGVXXXX) similarly to the apolipoproteins, appears to be responsible for the lipid-binding ability. The three known mutations are placed in this area as well. However, not all cellular αS is membrane-bound and at least 50% can be found free in the cytosol (Lotharius and Brundin 2002). The middle hydrophobic NAC (residues 61-95) comprises the highly amyloidogenic domain that is responsible for the ability of protein to undergo a conformational change from a random coil to the β-sheet conformation required for its aggregation (Iwai et al. 1995b). The acidic C-terminal tail (residues 96-140) remains free and unfolded, and does not associate with membranes (Eliezer et al. 2001). In conclusion, the structure of αS appears to be highly dynamic and context dependent (Clayton and George 1999).

THE HUMAN SYNUCLEIN FAMILY

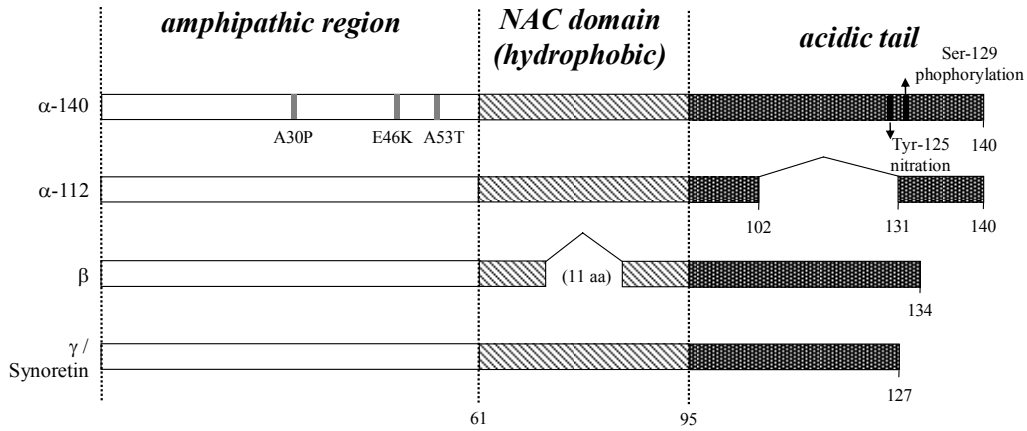

Figure 1. Structure of the synuclein family. The different domains are separated by vertical dashed lines and shaded area. The α-112 splice variant lacks 28 amino acids (103-130) within the acidic tail, whereas the NAC region in β-synuclein lacks 11 central amino acids (73-83). Three αS mutations are indicated with grey boxes, whereas the black boxes roughly show the location of post-translational modifications. (Modified from Lucking and Brice 2000, Giasson et al. 2004)

The physiological function of αS is not established, but the electron microscopic studies and biochemical analyses have shown that αS is closely localized and associated with synaptic vesicular membranes (George et al. 1995, Iwai et al. 1995a, Jensen et al. 1998). Due to its presynaptic localization, αS 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). Similarly to other proteins prone to aggregate, αS is natively unfolded (Weinreb et al. 1996). On binding to phospholipids membranes, it takes on an α-helical structure that may stabilize the vesicle membranes and inhibit their lysis (Davidson et al. 1998). Another mechanism for the inhibition of neurotransmitter release e.g. dopamine by αS could occur via inhibition of phospholipase D2 which is required for the vesicle budding (Jenco et al. 1998).

2.2.2 Pathogenetic mechanisms

The key unresolved issue is how the pathological aggregation of αS as an inclusion is mechanistically linked to neuronal dysfunction or death that accounts for the presentation of clinical symptoms. One possible pathogenetic mechanism could be that the aggregation of αS disrupts its normal cellular function and this leads to underlying neuropathology. However, loss of function appears unlikely since elimination of the αS gene in knockout mice did not produce a characteristic phenotype of PD *i.e.* LBs, neuronal loss and motor deficits (Abeliovich et al. 2000). Hence, the aggregation of αS as an inclusion may itself possess the toxic gain of function that compromises the cell physiology, leading to demise of neurons. Thus, current efforts of research ranging from cell-free systems to animal model studies have focused on striving to understand what is/are the fatal insults that provoke αS to aggregate as an inclusion. The suggested mechanisms (Figure 2.) comprise of 1) over-expression; 2) post-translational modification including 3) oxidation; 4) impaired degradation of αS; and 5) toxicity of protofibrils.

2.2.3 Overexpression of α-synuclein

The discovery of mutations in αS raised an immediate question of how genetics and the underlying pathology of PD were linked together. Although the mutations appear to increase the rate of filament assembly (Conway et al. 1998, El-Agnaf et al. 1998b), *in vitro* studies have also shown the wild-type αS to aggregate into filamentous inclusions that ultrastructurally resemble the inclusions found in human disease (Giasson et al. 1999). The assembly into filaments is a concentration-dependent process, so the increased expression of αS (either wild- type or mutant) due to saturation effects appears to be the key pathogenetic event in the mechanism of αS aggregation (Cole and Murphy 2002). Moreover, the triplication of the normal αS gene causes familial PD, further suggesting that mutant αS behaves differently from wild-type protein in a quantitative rather than a qualitative manner (Singleton et al. 2003). Various methods of overexpressing native and mutant versions of αS have been utilized in order to gain insight into the pathogenetic potential of the protein.

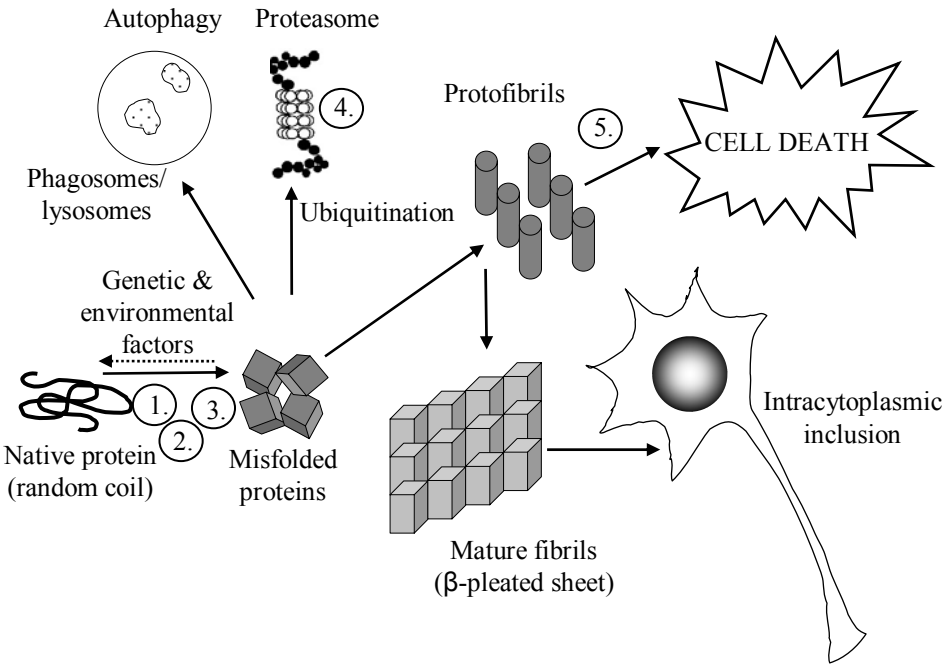

Figure 2. Model of protein misfolding and fibrillization that leads to deposition of aggregated protein in the cytoplasm of the neuron. 1) Mutations 2) post-translational modifications and 3) oxidation may all accelerate the formation of misfolded proteins that are can be eliminated by the autophagy or the ubiquitin-proteasome systems. Thus, 4) the dysfunction of proteasome may lead to increased accumulation of fibrils, whereas 5) the annular prorofibrils may disrupt the vesicle integrity and thereby trigger the cell death. (Modified from Forman et al. 2004)

The neurotoxic ability of fibrillar aggregates was further emphasized by *in vitro* studies that found accumulation of αS to induce apoptotic cell death (El-Agnaf et al. 1998a, Zhou et al. 2000). In line with these cellular studies, transgenic mice and fruit flies that overexpress wild-type or mutant human αS have been characterized as exhibiting abnormal cellular accumulation of αS, neuronal loss and behavioural deficits (Meredith et al. 2004). Although several recently developed animal models of PD appear to mimic many facets of the disease, there is a large diversity in the ultrastructural profile of αS-positive inclusions and in the phenotype of neuronal loss (Dev et al. 2003). Although promising, these transgenic mouse models lack a selective loss of dopaminergic neurons but do exhibit motor neuron degeneration in the spinal cord and this defect may be more likely responsible for the observed motor deficits (van der Putten et al. 2000, Giasson et al. 2002, Lee et al. 2002). At best, some loss of dopaminergic terminals has been observed in rodents (Masliah et al. 2000). In transgenic fruit flies, on the other hand, a selective dopaminergic cell loss has been shown to occur together with inclusions that

ultrastructurally better resemble those inclusions found in humans (Feany and Bender 2000). In addition to transgenesis, a more targeted and stable overexpression of αS obtained by viral vectors has produced better corresponding phenotypes (Betarbet et al. 2000, Kirik et al. 2002, Kirik et al. 2003). However, the evidence from both *in vitro* and *in vivo* studies supporting the notion that the overexpression of αS could lead to death of the affected neurons, is not in line with human studies that have failed to detect elevation of αS mRNA in PD or DLB patients (Neystat et al. 1999, Wirdefeldt et al. 2001). For this reason, the results obtained from these experimental animal studies should not be taken as unequivocal proof that the neurodegeneration in humans is caused simply by an increased dose of αS.

2.2.4 Post-translational modifications

Not much is known about specific post-translational modifications that may predispose αS to undergo this pathogenetic transformation. In transfected cell lines, αS becomes constitutively phosphorylated at serine residues 87 and 129 (Okochi et al. 2000). *In vitro* studies have also shown that the phosphorylation of αS at Ser-129 promotes fibril formation (Fujiwara et al. 2002). Furthermore, an antibody that specifically recognizes the phosphorylated Ser-129 generates a specific and intense immunolabelling of the filaments present in LBs or neurites (Fujiwara et al. 2002). It is possible, however, that hyperphosphorylation of αS is a secondary phenomenon resulting from fibrillogenesis, and not an actual prerequisite for the inclusion formation. Under oxidative stress, the tyrosine-residue 125 of αS can undergo nitration (Giasson et al. 2000). Using antibodies to specific nitrated tyrosine residues of αS, an intensive labelling of signature inclusions of synucleinopathies has been demonstrated (Duda et al. 2000). Nonetheless, similarly to phosphorylation, nitration does not appear to be a requirement for αS deposition (Gomez-Tortosa et al. 2002).

2.2.5 Oxidative stress and dopamine

In recognition of the anatomical pattern of neurodegeneration in PD, it is reasonable to propose that oxidative stress and dopamine contributes to the abnormal buildup of αS (Lotharius and Brundin 2002). Some *in vitro* studies have shown that iron and free radical generators as well as nitrative insults stimulate the production of αS-positive aggregates (Ostrerova-Golts et al. 2000, Paxinou et al. 2001). Further support for this hypothesis has been provided by studies where inclusion formation was blocked by pretreatment with antioxidants and /or metal chelators

(Hashimoto et al. 1999, Hsu et al. 2000). Yet, the most elegant support for oxidative stress has been provided by numerous animal models induced by mitochondrial toxins that inhibit the complex I such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and pesticides (rotenone) that appear to mimic many pathological and phenotypic facets of PD (Betarbet et al. 2000, Kowall et al. 2000).

Post mortem studies of PD have clearly indicated that the SN of these patients has existed in a state of oxidation with increased levels of iron, mitochondrial defects and decreases in antioxidant protective systems (Jenner and Olanow 1998). In addition, nigral dopaminergic cells are particularly vulnerable to oxidative stress because of dopamine auto-oxidation. Thus, the selective loss of nigral dopaminergic neurons in PD may be due to complex interaction of αS with intracellular dopamine, initiating a chain of events that leads to oxidative stress and increased production of free radicals. However, it should be noted that not all dopaminergic neurons have a tendency to develop cytoskeletal abnormalities and it appears that the melanized dopaminergic neurons are differentially susceptible in PD (Hirsch 1992). *In vitro* studies have shown that the overexpression of αS induces apoptosis only in the human dopaminergic cells, whereas in the cortical neurons it may be neuroprotective (Xu et al. 2002). This also implies that the neurotoxicity of αS would somehow be dopamine-or catecholamine-dependent. However, pathological changes in PD by no means involve exclusively catecholaminergic cells in PD as the formation of αS-positive inclusions and degeneration can also be detected in several cholinergic, serotonergic, glutamatergic and GABAergic neurons (Braak and Braak 2000).

2.2.6 Impaired degradation of α-synuclein

It is generally thought that the formation of αS-positive inclusions results merely from the increased expression of the protein but the possibility of impaired degradation provides an alternative hypothesis. The ubiquitin-proteasome pathway (UPP) is a cellular system that is responsible for degrading damaged or misfolded proteins. By inference, the failure of this mechanism would lead to accumulation and aggregation of unwanted proteins, such as αS in the cytosolic space. Interest in this concept was stimulated by the fact that two genes that become mutated in familial PD, parkin (Kitada et al. 1998) and ubiquitin C-terminal hydrolase L1 (UCH-L1) (Leroy et al. 1998), encode for the proteins that are key enzymes and components of the UPP. Parkin is an ubiquitin ligase which is required for tagging a chain of Ub molecules

to the unwanted protein as a sign for proteasomal destruction. The UCH-L1 enzyme instead is involved in breaking down polyubiquitin to the monoubiquitin needed by parkin to label proteins for their degradation. Hence, inhibiting the function of either enzyme by mutations could lead to disruption of UPP, impaired protein clearance and accumulation of αS (McNaught and Olanow 2003). Moreover, UCH-L1, parkin, ubiquitin and proteasomal elements are all components of LBs, further supporting the involvement of UPP (Lowe et al. 1990, Ii et al. 1997, Schlossmacher et al. 2002).

In support of the theory of proteolytic stress, enzymatic proteosomal function was observed to be reduced in the SN of PD patients (McNaught and Jenner 2001). This proteosomal impairment has been studied in experimental models by using the proteasome inhibitor lactacystin. *In vitro*, exposure to this compound has resulted in relatively selective dopaminergic cell death together with αS-positive inclusion formation (Rideout et al. 2001, McNaught et al. 2002b). Also, rats treated with stereotaxic injection of lactacystin became progressively bradykinetic, adopted a stooped posture and displayed head tilting, and moreover, showed dopaminergic degeneration with αS-positive inclusions bodies (McNaught et al. 2002a). Tofaris *et al.* have shown that proteosomal inhibition leads to an increase of non-ubiquitylated αS, suggesting that αS can be degraded by proteasome in Ub-independent manner (Tofaris et al. 2001). This may possibly explain how αS could accumulate in those inclusions that are Ub-negative.

Although, it is indeed intriguing and unlikely fortuitous that mutations causing familial PD converge to encode for the UPP, these mutations could be responsible for the impaired protein clearance in only a very small portion of patients. The cause of proteosomal inhibition in idiopathic PD is at present unknown, although accumulating oxidative stress has been implicated (McNaught and Olanow 2003). In addition, even if the dysfunctional UPP would cause the proteins to accumulate as inclusions, it remains enigmatic how this instigates the demise of neurons.

2.2.7 α-Synuclein protofibrils

Accumulating evidence from biophysical studies has recently indicated that the αS fibrils themselves may not be the pathogenetic species (Goldberg and Lansbury 2000, Volles and Lansbury 2003). According to the "protofibril hypothesis", it is the transient small units of β-

sheet containing oligomers of αS, *i.e.* protofibrils that are more toxic than the insoluble fibrillar form. The development of cell-free systems has demonstrated that these protofibrils are hollow cylinders capable of permeabilizing synthetic vesicles (Volles et al. 2001, Lashuel et al. 2002). The fibrillar αS shows no such permeabilization activity. Insertion of annular pores into the membrane by protofibrils could result in the leakage of small molecules such as calcium and dopamine into the cell cytoplasm with toxic consequences. In this way, the αS protofibrils could potentially disrupt ionic and metabolic homeostasis leading to toxicity by numerous downstream mechanisms (e.g. oxidative stress). Two known αS mutations have been shown to enhance the rate of protofibril formation *in vitro* (Conway et al. 2000), but it would be interesting to determine whether *in vivo* animal models would produce consistent results.

2.3 The spectrum of synucleinopathies

After the breakthrough discovery of filamentous αS-positive inclusions in the neuronal cytoplasm of PD and DLB, numerous studies have subsequently depicted a variety of αS-positive configurations seen in selectively vulnerable neurons. Surprisingly, a supposedly neuronal αS protein was soon found to accumulate also in the oligodendrocytes as glial cytoplasmic inclusions (GCIs) in patients with multiple system atrophy (MSA) (Spillantini et al. 1998a, Tu et al. 1998, Wakabayashi et al. 1998a, Wakabayashi et al. 1998b). Further investigation has revealed αS-immunopositive aggregation in several other neurodegenerative disorders such as sporadic and familial AD (Lippa et al. 1998, Hamilton 2000); Down syndrome (Lippa et al. 1999a); amyotrophic lateral sclerosis (ALS) (Mezey et al. 1998); the parkinsonism-dementia complex of Guam (Yamazaki et al. 2000) and pure autonomic failure (Arai et al. 2000). Also, the αS-positive axonal swellings, known as spheroids hallmark neurodegeneration with brain iron accumulation type 1 (NBIA1), previously known as Hallevorden-Spatz disease (Wakabayashi et al. 1999, Neumann et al. 2000), and have been reported to occur also after traumatic brain injury (Newell et al. 1999). Hence, the aggregation of αS protein appears to be a common denominator for several distinct neurodegenerative disorders that are presently named with an umbrella term “synucleinopathies” (Jellinger 2003b). However, the αS-positive inclusions are considered to be a primary causal pathology generally only in PD, DLB and MSA.

2.3.1 Neuronal involvement

The most common type of αS-positive inclusion is the compact LB, but αS-IHC has enabled the detection of less conspicuous types of immunoreactivity, described as "punctate or diffuse" staining in the cytoplasm and "grain-like dots" or "threads" in the neuropil (Wakabayashi et al. 1998a, Kuusisto et al. 2003, Saito et al. 2003). The extracellular inclusions that appear to lie freely in the neuropil most likely represent a cross-section of larger elongated neurites, in the same way as grains or dots represent the side view of smaller neurites. The neurites are most frequently observed in the accessory cortical and central nuclei of amygdala (Iseki et al. 1995), CA2 region of the hippocampus (Dickson et al. 1991) and striatum (Duda et al. 2002).

The aggregation of αS has been proposed to be initiated either in the somal or in the axonal compartment. Although LBs are usually seen in the soma, protein aggregation has been suggested to begin in the axon, and only then to continue through the cell soma into the dendrites (Marui et al. 2002). According to this "neuritic dystrophy hypothesis" (Trojanowski et al. 1998, Duda 2004), the axonal transport blockage leads eventually to the supersaturation of αS together with other anterogradely transported proteins in the somatic compartment as LBs. However, the initial pathology is in the axons leading to a "dying back" process that may cause functional and physical disconnection of neuronal circuits even though the cell bodies of neurons may somehow survive with inclusions for some time. This view has been supported by studies that have noted the preliminary pathology despite the anatomic location to be the αS-positive neurites (Del Tredici et al. 2002, Braak et al. 2003).

According to a second hypothesis, the appearance of neurites occurs together with more well-defined inclusions after the initial perikaryal accumulation of αS (Katsuse et al. 2003). Some studies have attempted to determine the antigenic profile of LBs at different stages in the process of their formation (Wakabayashi et al. 1998a, Gomez-Tortosa et al. 2000b, Kuusisto et al. 2003). The spectrum of αS staining varying from punctate and diffuse cytoplasmic staining to variform compact inclusions has been interpreted to reflect the morphogenesis of LBs (Kuusisto et al. 2003). The earliest stage of punctate and diffuse αS accumulation lacks immunoreactivity for Ub or Ub-binding protein p62, whereas these proteins appear to be invariably present in the pale bodies (Kuusisto et al. 2003). Pale bodies are however strongly αS-immunoreactive (Irizarry et al. 1998, Gomez-Tortosa et al. 2000b, Kuusisto et al. 2003), and this must have contributed to the fact that higher numbers of αS-positive structures have been reported in comparison to the numbers detected with Ub-IHC (Gomez-Tortosa et al. 2000b,

Goedert 2001). Ubiquitination appears to be a late event and not required for the inclusion formation itself (Tofaris et al. 2001, Sampathu et al. 2003). The sequential incorporation of αS>p62>Ub suggests that punctate/diffuse cytoplasmic staining, pale bodies and LBs originate as successive stages of a complex aggregation process (Kuusisto et al. 2003), but how this relates to cell physiology remains to be elucidated.

Recently, a few studies have also attempted to localize the induction site and the manner of progression for the αS pathology to obtain a deeper insight into the pathogenesis of PD and DLB. The topographical distribution of LBs in PD and DLB does not appear to follow a consistent hierarchical spread as is seen with NFTs in AD (Braak and Braak 1991). However, a few years ago, Braak *et al.* devised a staging system whereby αS pathology progresses in a systematic fashion through the brain (Braak et al. 2003). Based on the precise architectonic analysis, the proposed sequence was suggested to begin from the medulla (stage 1), where it proceeds with an upward progression via pons (stage 2) to the midbrain (stage 3), and then to the basal prosencephalon and mesocortex (stage 4) and finally to the neocortex (stages 5-6). Notably, these studies observed very early αS-immunoreactive structures in DMV and not in the SN in contrast to the previous assumptions (Del Tredici et al. 2002). However, some deviations from the stereotypic topographic distribution have been reported (Jellinger 2003a), and understanding of what these outliers really mean is crucial.

2.3.2 Glial involvement

MSA is a unique synucleinopathy in the sense that αS aggregation is predominantly detected in the glial compartment. It is an adult-onset, progressive neurodegenerative disorder clinically characterized by any combination of extrapyramidal, pyramidal, cerebellar and autonomic features. MSA is mainly sporadic although one family has recently been described with autosomal dominant inheritance (Wullner et al. 2004). The concept of MSA was first proposed by Graham and Oppenheimer in 1969 with the intention to merge three separate conditions with overlapping clinical features: olivopontocerebellar atrophy, striatonigral degeneration and Shy-Drager syndrome (Graham and Oppenheimer 1969). The clinicopathological entity of MSA was further consolidated twenty years later when glial cytoplasmic inclusions (GCIs) were identified in all three conditions (Papp et al. 1989). Thus, neuropathologically, MSA is characterized by multisystem neuronal degeneration with unique oligodendroglial inclusion pathology. In

addition to selective neuronal loss, iron pigment accumulation, causing a discolouration of the striatum is often encountered (Dickson et al. 1999).

Today, the αS-positive GCIs are the defining neuropathological feature required for the definite diagnosis of MSA (Papp et al. 1989, Gilman et al. 1999). GCIs can be found throughout the white matter but their greatest abundance occurs in striatum, SN, LC, pontine nuclei, inferior olives, cerebellum and regions of the spinal cord. They are sickle or flame-shaped inclusions that can be readily detected also with Gallyas silver staining and antibodies to Ub but are negative for neurofilaments and glial fibrillary acidic protein (GFAP) (Castellani 1998). The immunohistochemical markers and ultrastructural characteristics have confirmed the oligodendroglial origin of GCIs (Burn and Jaros 2001). Cells that contain GCIs show a positive staining for several oligodendroglial markers such as carbonic anhydrase isoenzyme II, Leu-7 and transferrin (Lantos and Quinn 2003). Ultrastructurally, CGIs are composed of a meshwork of randomly arranged, loosely packed filaments of 5-18nm in diameter that contain αS as a major component (Spillantini et al. 1998a). These filaments are heavily coated with amorphous material and arrange in parallel bundles, and this together with less soluble αS in GCIs has been reported to be the main differences between the GCIs and LBs (Campbell et al. 2001). To a lesser extent, some αS aggregation in MSA can also be found in the nucleus of oligodendrocytes, in the cytoplasm and nucleus of some nerve cells and in their neuritic processes (Lin et al. 2004). Since the αS is produced almost exclusively by neurons, the origin of αS inside the oligodendrocytes has been debated. One plausible explanation may be its uptake from dying neurons or alternatively there may be overexpression of αS for some unknown reason specifically in glia. Whatever the mechanism, this defect may eventually compromise nerve cell viability and function via the oligodendrocyte-myelin-neuronal axon pathway (Jellinger 2003).

However, it should be kept in mind, that MSA is not the only disease characterized by αS-positive glial inclusions. The αS-positive inclusions have been reported in both astrocytes and oligodendrocytes also in PD and DLB (Piao et al. 2000, Wakabayashi et al. 2000). Astroglia and Schwann cells in the spinal cord have been found to be αS-positive in ALS (Mezey et al. 1998).

2.3.3 Concomitant pathologies

α-Synuclein-positive inclusions have been reported to be a regular finding, estimates ranging from 50-60%, in familial and sporadic AD patients, as well as in Down syndrome patients with a triplicate APP (Lippa et al. 1998, Lippa et al. 1999a, Hamilton 2000, Arai et al. 2001). Vice versa; AD-related pathology is also often discovered in patients with a clinicopathological diagnosis of DLB (Gomez-Tortosa et al. 2000a, Londos et al. 2001, Jellinger 2003b). The fact that αS pathology commonly co-exists with AD-related pathology *i.e.* is more prevalent in those patients with NPs/NFTs than in the individuals without, suggests that these two types of pathologies might somehow be mechanistically linked (Giasson et al. 2003b). Although there still is no agreement on the presence of NAC domain of αS in the NPs (Ueda et al. 1993, Masliah et al. 1996, Bayer et al. 1999b, Culvenor et al. 1999, Hashimoto et al. 2000, Iwai 2000), at least in *in vitro* models, αS has been found to bind to Aβ and to enhance its aggregation (Yoshimoto et al. 1995, Jensen et al. 1997). Furthermore, in the transgenic mouse model linking AD and PD, Aβ peptide enhances the accumulation of fibrillar αS to inclusions that better resemble the LBs found in humans (Masliah et al. 2001). The double mutant mice in comparison to single αS transgenics also show a more severe cholinergic degeneration in NBM and development of earlier motor impairment, thus supporting the role of Aβ as the principle culprit in the pathogenesis of synucleinopathies as well. Furthermore, LBs and NFTs have been reported to frequently coexist in the same neurons of the limbic areas, especially in the amygdala in the brains of patients with AD (Marui et al. 2000), DLB (Iseki et al. 1999) and Parkinsonism-Dementia complex of Guam (Forman et al. 2002). Giasson *et al.* demonstrated *in vitro* that αS promotes the fibrillization of tau, whereas the coincubation of αS and tau synergistically induced the fibrillization of each other (Giasson et al. 2003a). These authors claimed that even in those cases where αS aggregates are not detectable a limited amount of amyloidogenic αS fibrils could still serve as seeds for the tau fibrillization. α-Synuclein has also been shown to bind to tau and to stimulate its phosphorylation (Jensen et al. 1999). Recent double immunostaining with tau and αS revealed tau-positive LBs in most examined DLB cases (Ishizawa et al. 2003). The tau-immoreactivity was mainly detected in the periphery of the αS-positive LBs, whereas in other neurons, the double immunoreactivity represented merely the overlay of LBs and NFTs. However, tau deposition appears unlikely to be a primary pathogenetic mechanism for the aggregation of αS because only a minority of LBs were found to be tau-immunopositive, and thus the significance of interactions between tau and αS remains to be determined.

The frequency of concomitant severe cerebrovascular changes in pathologically confirmed DLB patients is low, and thus cognitive impairment in DLB appears to be independent from the coexistent vascular changes (Jellinger 2003c). Origin of dementia in DLB remains disputable but appears mainly attributable to either cortical LBs or AD-related pathology or mixture of both.

2.4 Clinico-pathological correlations

2.4.1 Parkinson's disease

Clinical manifestation of idiopathic PD is considered to require the deterioration of SN. Lewy bodies are found in clinically diagnosed PD patients with estimates ranging from 75% to 85% according to clinico-pathological studies (Hughes et al. 1992, Hughes et al. 2002). The prospectively evaluated PD patients without LBs usually have AD-related or vascular changes. Thus, it has been suggested that PD should be regarded as a disease caused by many different pathologies, and its pathological diagnosis should require only the degeneration of SN and not the presence of LBs (Forno 1996).

2.4.2 Dementia with Lewy bodies

Dementia is a variable but common manifestation occurring in 10-40% of patients during late progression of PD (Apaydin et al. 2002). Dementia associated with cortical LBs is usually differentiated into patients with primary PD that develop dementia and into patients with DLB. In the former, the initial clinical symptoms are the EPSs, whereas in the latter cognitive impairment appears simultaneously (McKeith et al. 1996). Neuropathologically, these entities cannot be distinguished (Harding and Halliday 2001, Jellinger 2003a).

Pure DLB is rare (Uchikado et al. 2002), but the bulk of studies has still emphasized the role of cortical LB pathology in the generation of cognitive impairment in both PD and DLB (Haroutunian et al. 2000, Hurtig et al. 2000, Mattila et al. 2000, Harding and Halliday 2001, Apaydin et al. 2002, Kovari et al. 2003). Dementia has also been attributed to the density of dystrophic neurites in the CA2 region of the hippocampus (Dickson et al. 1991) but recent studies have failed to confirm this hypothesis (Hurtig et al. 2000). A few studies have reported correlations between the severity of dementia and cortical LB density (Haroutunian et al. 2000, Mattila et al. 2000, Kovari et al. 2003). Studies on diagnostic accuracy of DLB have gained

variable results. Whereas Hohl *et al.* estimated diagnostic accuracy to be 50% (Hohl et al. 2000), Londos *et al.* reported only 38% of prospectively examined clinically diagnosed DLB cases to have αS-positive LBs (Londos et al. 2001). Most of the misdiagnosed DLB cases had pure AD-related pathology. On the other hand, Mc Keith *et al.* have reported as high as 95% specificity in the prospective validation of Consensus criteria for the diagnosis of DLB (McKeith et al. 2000). It appears that patients clinically diagnosed with DLB often show cortical LBs (high specificity), however, there are many subjects with cortical involvement that do not fulfil these clinical criteria (low sensitivity). In the retrospective study of consecutive clinical autopsy cases, Lindboe and Hansen found all the cases with cortical LBs to be free of dementia (Lindboe and Hansen 1998). One recent prospective clinicopathological study strengthened this notion by reporting only approximately one third of subjects with cortical LBs to fulfil the clinical diagnosis of DLB (Lopez et al. 2002).

Several autopsy series have examined the proportion of DLB in demented *i.e.* have attempted to estimate how much of dementia overall can be explained by cortical LBs (Perry et al. 1989, Perry et al. 1990a, Galasko et al. 1994, Kalra et al. 1996, Drach et al. 1997, Bowler et al. 1998, Lim et al. 1999, Akatsu et al. 2002, Barker et al. 2002). This is a difficult task as classical AD-related changes, NPs and NFTs, often co-exist with the cortical LB pathology as mentioned above. These cases with mixed pathologies present an additional complication in attempts to unravel the causative pathologies involved in cognitive impairment.

2.4.3 Multiple system atrophy

Few studies have tried to evaluate the relationship between the αS-positive glial inclusions and the clinical features of MSA. Neuronal loss appears to be clinically most relevant (Papp and Lantos 1994), and no direct association between load of inclusions and clinical features has been detected. However, some studies have shown a significant correlation between the density αS-positive glial inclusions and the severity of neuronal loss (Ozawa et al. 2004). It is not known whether the development of glial inclusions is a synchronous process with neuronal degeneration, a phenomenon preceding it or simply an epiphenomenon or response to some other primary mechanism. One suggestion has been that the glial cells are dysfunctioning and in this way evoke the secondary neuronal death. Even if this is true, a straightforward relationship between these two pathologic changes, such that a certain threshold density of αS-positive glial inclusions would trigger the neuronal loss, is unlikely to be achieved.

3. AIMS OF THE STUDY

Since αS-positive inclusions appear to be a common denominator for a group of neurodegenerative disorders in which they have a general tendency to accumulate in the degenerating brain regions, these proteinaceous inclusions have been considered to be causative for neuronal dysfunction. However, recently accumulating evidence has challenged this pathognomonic role for the αS-positive inclusion. Thus, the primary aim of this study was to clarify the impact of αS pathology in the aging process in general.

The specific aims were as follows:

1. To estimate the prevalence of αS pathology in an unselected aged population, and to examine the influence of sampling strategies, age and gender (**Study I**)

2. To estimate the prevalence of αS pathology in sporadic AD patients, and furthermore, to examine the regional distribution of αS pathology in relation to concomitant AD-related pathology and cognitive impairment (**Study II**).

3. To identify region(s) of the brain where the αS pathology begins (incipient synucleinopathy) and how it progresses through the brain (topographical distribution) (**Study III**).

4. To assess the clinical relevance of αS-positive inclusions in a large post mortem study where the selection of material is entirely based on the presence of αS pathology regardless of the clinical phenotype (**Study III**)

5. To compare the immunohistochemical profile between a neurologically unimpaired subject and a patient with DLB both of whom exhibited a similar extent of αS pathology. (**Study IV**)

6. To examine the topographical distribution of αS-positive glial cytoplasmic inclusions and their clinical relevance in cases selected from a large post mortem material on the basis of the presence of αS-positive glial inclusions in the midbrain. (**Study V**).

4. SUBJECTS AND METHODS

4.1 Selection of subjects

The study population consisted of a maximum of 904 elderly individuals who underwent an autopsy including an examination of the brain during the years 1996-2000 in the Department of Pathology of the Kuopio University Hospital. The total population within the area of registry of eastern Finland is estimated to be 250 000 with an annual rate of death of 2000 individuals. The autopsy rate is estimated to be 40%, and therefore, in any given year around 800 individuals are subjects of a postmortem examination. Participants of this study represent around 10% of all who died and roughly 25% of all the autopsied individuals over the 5 years that the survey encompassed.

Subjects were members of five distinct cohorts each subordinated to different sampling strategies. Patients of the first group (DEMENTIA) were derived from a longitudinal follow-up study of patients with dementia of Alzheimer's type from the geriatric department of Harjula Hospital in Kuopio. The members of second group (VANHUS) were individuals from an ongoing prospective study of aging, and results of their cognitive performance screening have been published (Koivisto et al. 1995). All patients in the above groups had undergone a neurological examination prior to death. The rest of the subjects were derived from a cohort of consecutive clinical autopsy cases collected for one year (CLINICAL), a cohort of forensic autopsy cases collected for six months (FORENSIC), and a cohort of neurological or neurosurgical referral cases from Eastern Finland that were not part any of above studies (REFERRAL).

4.2 Clinical assessment

Clinical data was compiled retrospectively from the hospital records and re-evaluated by a standardized manner for each patient by neurologists blinded to the neuropathological findings. We only included subjects that had been evaluated by a clinician within approximately one year in order to reduce the possibility of incomplete ascertainment. The diagnosis of dementia was based on the criteria of the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) (McKhann et al. 1984) and the Diagnostic and Statistical Manual of Mental Disorders, Revised Third Edition, 1987. The diagnosis of PD followed the criteria established by the United

Kingdom Parkinson's Disease Society Brain Bank whereby PD was considered to be present if the patient had at least 2 of the 4 cardinal symptoms (resting tremor, rigidity, hypokinesia and postural instability) and exhibited a positive response to levodopa (Daniel and Lees 1993). The diagnosis of DLB was based on the consensus guidelines whereby patients were classified with probable DLB when the cognitive impairment was accompanied with at least 2 of the 3 core features (EPSs, fluctuating cognition and visual hallucinations), and with possible DLB when only 1 core feature was present (McKeith et al. 1996). Those patients with PD who developed late dementia (>2 years after EPSs) were classified as PD with dementia (PDD) (Hurtig et al. 2000). The diagnosis of MSA was based on the consensus guidelines (Gilman et al. 1999) whereby patients were classified as probable when at least two of the three core features were present (autonomic/urinary dysfunction plus poorly levodopa responsive parkinsonism or cerebellar ataxia).

4.3 Neuropathological assessment

According to the dissection protocol used in Kuopio University Hospital, the brains were weighed, evaluated for grossly detectable lesions and vessel abnormalities, perfused with and immersed in 10% buffered formalin for at least one week and cut in coronal slices of 1 cm thickness. Brain specimens were taken from 15 standard regions, embedded in paraffin and cut into the 7μm-thick sections that were stained routinely applying H&E.

For study **I and IV**, AD-related pathology, i.e. NPs, were quantified in three cortical areas stained with Bielschowsky silver impregnation. As described by Mölsä *et al.* (Molsa et al. 1987), the above inclusions were counted in each cortical region within the microscopic field magnified at ×100 and semiquantitatively scored from 1 to 10. The total score (0-30) was the sum of scores in frontal, temporal and parietal cortices. In study **II**, all subjects were classified into neuropathological groups as recommended by the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (Mirra et al. 1991). In this classification, the NP score (some, moderate and numerous) is related to the age of the subjects and then to the clinical signs of dementia giving different categories of likelihood of AD. In study **III** and **IV,** subjects were classified into six neuropathological stages (I-VI) as recommended by the guidelines established by Braak *et al.* (Braak and Braak 1991), and for this both the modified Bielschowsky silver impregnation and hyperphosphorylated tau (HP-τ) IHC were used. This classification is based on the topographical distribution of NFTs rather than their quantification.

In study **III**, the extent of vascular lesions was also estimated on the H&E stained slides and graded on a 3-step scale as 0 = no macro/microscopic lesions found, 1 = only microscopic lesions found and 2 = both macro/microscopic lesions found.

4.4 Immunohistochemistry

The expression of αS was visualized with IHC in the brain areas summarized in Table 1 for the each study (I-V). In studies **I** and **IV**, the expression of β-amyloid (Aβ) was estimated in frontal, temporal and parietal cortices and the extent given as stained area fraction (Alafuzoff et al. 1999). In study **IV**, each brain area examined were additionally immunostained with antibodies to Ub, p62, GFAP and human major histocompatibility complex (MHC) class II glycoprotein. Sections were deparaffinized, rehydrated, subjected to epitope unmasking treatments and immunostained with specific antibodies. The antibodies, dilutions and pretreatments for IHC are described in Table 2. For detection, the Histostain SAP kit (Zymed, San Francisco, CA) was used together with Vector-Red chromogen (Vector Laboratories, Burlingame, CA) (I-III). With this method, the reddish αS-positive inclusions were easily identified especially in the subcortical nuclei filled with brown neuromelanin. Additionally, the Histostain SP kit (Zymed) with Romulin AEC chromogen (Biocare Medical, Walnut Creek, CA) was used that more precisely revealed the morphology (III-V).

Table 1. Summary of brain areas examined in each study.

Brain area	*Study I*	*Study II*	*Study III*	*Study IV*	*Study V*
Frontal cortex			x	x	x
Temporal cortex			x	x	x
Parietal cortex			x	x	x
Precentral cortex				x	x
Occipital cortex				x	x
Cingulate gyrus	**x**		x	x	x
Parahippocampal gyrus including hippocampus	**x**		x	x	x
Basal forebrain including nucleus basalis of Meynert and amygdala		**x**	**x**	x	x
Striatum			x	x	x
Thalamus				x	x
Midbrain including substantia nigra	**x**	**x**	**x**	x	x
Pons including locus coeruleus		x	x	x	x
Medulla including vagus		x	**x**	x	x
Vermis				x	x
Cerebellum				x	x
Total	3	4	10	15	15

Table 2. Description of the antibodies.

Name, type	*Epitope*	*Pretreatment*	*Dilution*	*Source*
Synuclein-1, mouse mAb	rat αS $_{15-123}$, human αS $_{91-99}$	80% FA, 60 min	1:1000	Transduction Laboratories (Lexington, KY, USA)
NCL-ASYN, mouse mAb	human αS$_{1-140}$	autoclave+ 80% FA 5 min	1:1000	Novocastra (Newcastle upon Tyne, UK)
AT8, mouse mAb	HP-τ	-	1:500	Innogenetics (Ghent, Belgium)
M0872, mouse mAb	Aβ	80% FA 6 h	1:100	DakoCytomation (Glostrup, Denmark)
Z0458, rabbit pAb	Ub	-	1:500	DakoCytomation
p62 lck ligand, mouse mAb	human p62 $_{257-437}$	autoclave	1:1000	Transduction Laboratories
Z0334, rabbit pAb	GFAP	0.03% Protease XXIV 10 min	1:4000	DakoCytomation
MHC class II glycoprotein, mouse mAb	HLA-DR/DP/DQ β chain	autoclave	1:100	DakoCytomation

Key: Aβ= beta-amyloid; αS = alpha-synuclein; FA = formic acid; GFAP = glial fibrillary acidic protein; HLA = human leukocyte antigen; HP-τ = hyperphosphorylated tau; mAb = monoclonal antibody; pAb = polyclonal antibody; Ub = ubiquitin.

4.5 Quantification of α-synuclein-positive inclusions

The number of intensely stained αS-positive inclusions was counted within the microscopic field magnified at ×200 (diameter of 1mm) and assessed semiquantitatively in all brain areas examined. The synuclein index, used in study **I,** was based on the number of αS-positive inclusions together with assessment of dystrophic neurites (few, some and several). On the subsequent quantification in study **III**, the inclusions and neurites were reported separately. The

αS-positive inclusions in the cortical areas (parahippocampal and cingulate gyri, temporal, frontal, parietal) and amygdala were assessed according to the established pathological guidelines (McKeith et al. 1996). In the NBM and subcortical regions, αS-positive inclusions were counted within entire nuclei and assessed following an arbitrary grading system from 0 to +++. The elongated αS-positive neurites were semiquantitatively assessed in the putamen and in the CA2 region of the hippocampus from 0 to ++. A more precise assessment, that is, a comparison of two different antibodies against αS and their morphological spectrum was carried out in study **IV** that only included two subjects. In study **V**, the GCIs and neuronal inclusions were assessed from 0 to +++.

4.6 Microscopy and photomicrography

The sections were analyzed using a Nikon Optishot-2 microscope equipped with a Nikon Coolpix 990 digital camera. Digital images in studies IV and V were taken using a Leica DM4000 B microscope equipped with a Leica DFC 320 digital camera.

4.7 Statistical analysis

Statistical analyses were conducted with SPSS program for Windows (SPSS Inc., Chicago, IL). In study **I**, Student's t-test and one-way analysis of variance (ANOVA) were used to compare means between the groups. Also, non-parametric Mann-Whitney U and Kruskal-Wallis tests were applied to detect differences in the synuclein index that were not normally distributed. The correlations were tested using Pearson two-tailed correlation test, whereas the comparison of two correlation coefficients was performed by Fischer's Z transformation test. In study **III**, the Pearson χ^2 analysis was used. The level of significance was $p<0.05$ in all analyses.

5. RESULTS

5.1 Influence of case selection on the prevalence of α-synuclein pathology (I)

The αS-positive LBs and dystrophic neurites in the SN were present in 109 (14%) of 774 subjects over 40 years of age examined in study **I**. Dementia was present in 27% (209/565) of the total population. The prevalence of αS pathology varied extensively between the five study groups, ranging from 8% to 27% (Table 3). It was highest in the DEMENTIA group that had the oldest mean age of death (84 years), and naturally contained the highest proportion of demented patients (85%) and subjects with AD pathology (68%). Conversely, the lowest prevalence was found in the FORENSIC group with 20 years younger mean age of death, and a low occurrence of dementia (15%) and AD pathology (5%). The FORENSIC group had a male preponderance (2.5:1), whereas the DEMENTIA group contained many more females (4.7:1). The synuclein index differed significantly between the five study groups (Kruskall-Wallis, p=0.027), being highest in the DEMENTIA group and lowest among the consecutive CLINICAL autopsy cases.

Table 3. Demographics and pathological findings in study groups.

Study group	*N*	*Gender (F/M)*	*Age*	*Dementia*	*AD-related pathology*	*αS pathology*
DEMENTIA	103	85/18	84	85%	68%	27%
AGEING	69	45/24	81	29%	17%	20%
CLINICAL	262	116/146	71	12%	4%	13%
FORENSIC	121	35/86	62	15%	5%	8%
REFERRAL	234	114/120	71	26%	15%	11%
ALL	774	384/390	72	27%	17%	14%

Our total material included 209 patients that were clinically diagnosed with cognitive impairment, and αS pathology was detected in 49 (23%) of these demented patients. In around half (n=24, 11% of all demented), αS-positive inclusions were distributed throughout the limbic cortex including parahippocampal or cingulate gyri. In the 565 non-demented subjects, 60

(11%) had αS pathology; in most cases (n=51) this was restricted to the area of SN. The remaining nine non-demented subjects displayed a remarkable αS pathology throughout their cortices. When all those cases that had any psychiatric or neurological symptoms (n=112) were excluded, "incidental αS pathology" was found in 9% of the group. In most incidental cases, αS-positive inclusions were restricted to the brainstem area, but surprisingly, a few subjects also exhibited some cortical inclusions. The synuclein index was significantly greater in the demented than in the non-demented αS-positive cases (Mann-Whitney, p=0.001).

5.2 Influence of age and gender on the prevalence of α-synuclein pathology (I)

The mean age at death in the total study population (n=774) was 72±13 (age range from 40 to 102 years), whereas it was slightly higher among the 109 αS-positive cases (76.5±11 years). Table 4 shows the prevalence of αS pathology in increments of ten years. A clear age-related rise was detected in the prevalence of αS-positive inclusions between the 4th and 9th decades, increasing from 3.7% to 18.6% (altogether 15% increase). There was a decrease back to 0% after the age of 100, but the number of centenarians (n=3) was far too small to draw any conclusions. There was no significant correlation between the synuclein index and age at death (Pearson, p=0.083). Distribution between genders was rather even in the total study population, and also among αS-positive cases (Table 5). Furthermore, there was no gender-related difference in the synuclein index (p=0.123).

Table 4. The influence of age on the αS pathology.

Decade specific prevalence rate	*40-49*	*50-59*	*60-69*	*70-79*	*80-89*	*90-99*	*>100*
All	54	99	146	232	197	43	3
αS-positive cases	2 **(3.7%)**	5 **(5.1%)**	17 **(11.6%)**	41 **(17.7%)**	36 **(18.3%)**	8 **(18.6%)**	0 **(0%)**

Table 5. The influence of gender on the αS pathology.

Gender specific prevalence rate	*Female*	*Male*
All	325	340
αS-positive cases	59 (**18.2%**)	50 (**14.7%**)

5.3 Relationship of α-synuclein pathology to concomitant AD pathology (I-II)

In study **I**, a large number of αS-positive cases showed concomitant AD pathology (59% NPs, 34% NFTs and 68% some Aβ expression), but no correlation was found between the synuclein index and the above variables. A statistical difference (Kruskal-Wallis, p=0.008) was however detected between the αS-positive cases categorized according to assessment of plaque density (none, sparse, moderate and frequent), such that the group without NPs had a much lower synuclein index than the groups with moderate or frequent NPs. In study **II**, that included more participants (n=904), definite or probable AD was assigned using CERAD criteria to 131 subjects (Table 6). Twenty-six of these patients (20%) had concomitant αS pathology. This study also included 105 cognitively unimpaired subjects who displayed mostly moderate to frequent NPs (CERAD class of possible AD b), and αS pathology was seen in 15% of these cases. Taken together, αS-positive inclusions were detected in 42 (18%) of 236 subjects with more pronounced AD pathology.

Table 6. The prevalence of αS pathology according to CERAD rating.

Cognitive impairment			*CERAD rating*	*All examined cases*	*αS-positive cases*
	All	αS+ cases		n	n (% of all)
Yes	209	43 (21%)	definite AD	89	14 (16%)
			probable AD	42	12 (29%)
			possible ADa	31	7 (23%)
			normal c	47	10 (21%)
No	695	78 (11%)	possible ADb	105	16 (15%)
			normal b	78	10(13%)
			normal a	512	52 (10%)
			Total	904	121 (13%)

norm a=no histological evidence of AD/no clinical history of dementia; norm b=histological evidence of AD is uncertain/no clinical history of dementia; possADb=histologic evidence is suggestive or indicative of AD/no clinical history of dementia; norm c=no histologic evidence of AD/clinical history of dementia; possADa=histologic evidence of AD is uncertain/clinical history of dementia; prAD=histologic evidence is suggestive of AD/clinical history of dementia; def AD=histologic evidence is indicative of AD/clinical history of dementia.

5.4 Incipient α-synuclein pathology and topographical distribution (II-III)

In study **II**, the αS-positive inclusions were found in 121 (13%) out of the total 904 subjects in either BFB nuclei (n=13), midbrain (n=23) or in both of these areas (n=85). When lower brainstem nuclei were included into the analysis, 2 of 13 cases with BFB inclusions and 17 of

23 cases with SN inclusion were revealed to have additional αS-positive inclusions in the LC and/ or DMV. Taken together, 11 subjects were identified with αS pathology restricted to the BFB nuclei and 6 to the SN.

In study **III**, additional screening of DMV of 904 subjects was carried out. This yielded 149 subjects (16%) that displayed αS-positive inclusions in one of the most vulnerable areas reported 1) DMV; 2) SN; and/or 3) BFB nuclei. Out of the 149 subjects with αS pathology, 43 had to be excluded because of inadequate clinical documentation, and ultimately, study III comprised 106 αS-positive cases. The topographical distribution of αS-positive inclusions enabled the division of subjects into ten distinct stages (Table 7). These included 24 subjects with αS-positive inclusions in the lower brainstem but not in the SN (stage 1-2). We also isolated three cases with only minor affection of SN (stage 3) and nine cases (stage 5) with inclusions in both SN and BFB nuclei, but in all the lower brainstem nuclei were unaffected. In a further three cases, BFB nuclei were severely affected together with some cortical αS-positive inclusions, but the whole brainstem (including SN) remained preserved (stage 6).

Table 7. Topographical distribution of αS-positive inclusions in relation to clinical symptoms.

Stage	*Area(s) affected*	*Braak LB stage*	*N*	*EPSs n (%)*	*Cognitive impairment n (%)*
1	DMV only	1	6	0 (0%)	0 (0%)
2	DMV+LC+ putamen	2	18	2 (11%)	1 (6%)
3	SN only	?	3	0 (0%)	0 (0%)
4	Entire brainstem (DMV+LC+SN)	3	5	1 (20%)	1 (20%)
5	SN+BFB nuclei (no lower brainstem)	?	9	1 (11%)	3 (33%)
6	BFB nuclei only	?	3	1 (33%)	3 (100%)
7	BFB nuclei+entire brainstem	4	18	1 (6%)	3 (17%)
8	PHG or CG or both gyri + all above	5-6	17	4 (24%)	3 (18%)
9	TCx or FCx or both cortices+ all above	5-6	14	5 (36%)	5 (36%)
10	All areas examined	5-6	13	12 (92%)	6 (46%)

5.5 Retrospective assessment of clinical data (III)

The retrospective clinical assessment revealed that only 32 (30%) of the 106 αS-positive cases had been diagnosed with a neurodegenerative disorder; 20 of these with either PD or DLB. Other neurodegenerative diagnoses included seven with AD, two with frontotemporal dementia, one with striatonigral degeneration, one with corticobasal degeneration and one with radiation-induced white matter degeneration. Forty-two (40% of αS-positives) patients had exhibited other neurological disorders, primarily cardio/cerebrovascular diseases such as vascular dementia (n=4), stroke (n=15) or transient ischemic attack (n=9). Two patients were diagnosed with normal pressure hydrocephalus and four with epilepsy. Eight patients categorized under other neurological/psychiatric diseases included three with cephalalgia, three with vertigo, one with depression, and one with essential tremor. The remaining 30% of these αS-positive cases were neurologically unimpaired.

5.6 Load of α-synuclein pathology in relation to EPS and cognitive impairment (III)

In particular, the EPSs were identified in 25% of all the cases with αS-positive inclusions in the brainstem neurons (n=103), and cognitive impairment in 35% of the cases with cortical inclusions (n=46). Chi-square analysis of the inclusion score by region with respect to EPSs gave the following results: SN p=0.034, LC p=0.051, DMV p=0.038, and putamen p=0.008. According to this assessment, the αS-positive inclusions correlated with EPSs in all areas except LC. However, an extensive load of αS-positive inclusions (+++) was also seen in the brainstem nuclei in cases without any EPSs; in SN (n=5), in LC (n=6) and in DMV (n=9).

Similarly, χ^2 analysis in relation to cognitive impairment gave the following results: PHG p=0.002, CG p=0.001, TCx p=0.001, FCx= 0.017, PCx p=0.068, AC p=0.000, NBM p=0.625, and CA2 p=0.020. Thus, the αS-positive inclusion scores in a number of regions did correlate with dementia (except parietal cortex and NBM). Correspondingly to those subjects without EPSs, an extensive cortical αS pathology (>5 inclusions per microscopic field) was detected in a substantial portion of the non-demented subjects. Only the frontal and parietal cortices remained without severe αS pathology in the cognitively unimpaired subjects.

5.7 Comparison of a neurologically unimpaired subject to a patient with DLB (IV)

The topographical distribution in both subjects corresponded to Braak's PD stage 5-6 (Braak et al. 2003). The neurologically unimpaired subject was discovered to fulfil the Consensus pathological criteria for neocortical DLB with a LB score of 8 (McKeith et al. 1996). The frontal and parietal cortices showed <5 αS-positive inclusions (score 1) per field, whereas in the temporal cortex and parahippocampal and cingulate gyri, their number exceeded 5 (score 2). In the patient with the classical clinical syndrome of DLB, all cortical areas displayed <5 αS-positive inclusions (score 1) per field except for the parahippocampal gyrus (score 2) and thus, the patient fulfilled the Consensus pathological criteria for limbic DLB with a LB score of 6 (McKeith et al. 1996). We used a wide range of antigenic determinants and an in situ end-labeling technique but we detected no differences in vascular pathologies, in gliosis, or in apoptosis that would have explained the discrepant clinical endpoints. Some mild spongiform change was detected in the parahippocampal gyri of both subjects and in the amygdala of DLB patient. The only differences noted were the Aβ-positive aggregates in the putamen found exclusively in the symptomatic patient.

5.8 Topographical distribution and load of glial α-synuclein-positive inclusions with respect to the clinical symptoms (V)

The topographical distribution and load of αS-positive glial inclusions are assessed in Table 8. The αS-positive GCIs were found throughout the white matter, with the greatest abundance in cerebellum, basis pontis, putamen and globus pallidus. They were least frequent in case 1, whereas cases 4 and 5 were most affected. The neuronal loss (Table 9) was in general most prevalent in the basis pontis and in the lateral SN. The most severe neuronal loss was seen in cases 1, 3 and 5 that had variable densities of αS-positive GCIs.

According to retrospective clinical assessment, four of the five subjects with αS-positive GCIs had been diagnosed with MSA. In cases 1, 3 and 5, the predominant motor feature was cerebellar ataxia fulfilling the subtype MSA-C, whereas in case 4 it was parkinsonism (subtype MSA-P). Autonomic failure was present in all four cases. In case 2, the only registered neurological deficit was dizziness. This patient had an acute Puumala virus infection that was also the primary cause of death, verified by postmortem investigation.

Table 8. Topograhical distribution and load of αS-positive glial inclusions in the white matter.

Brain area	*Case 1*	*Case 2*	*Case 3*	*Case 4*	*Case 5*
Vermis and cerebellum	+++	++	+++	+++	++
Medulla					
periaxial structures	+	++	++	+++	na
pyramids	+	+	++	++	na
Pons					
periaxial structures	+	++	+	++	++
basis pontis	++	++	+++	+++	+++
Midbrain					
periaxial structures	+	++	++	++	++
crus cerebri	0	+	+	++	++
Basal forebrain/ Striatum					
fornix	0	na	+	+	+
capsula interna (anterior)	0	+	+	++	++
capsula interna (posterior)	0	+	+	++	+
capsula externa	0	0	0	++	++
capsula extrema	0	0	0	+	++
amygdala	0	+	+	+	++
caudatus	+	+	+	++	++
globus pallidus	+	+	++	+++	+++
putamen	+	+	+++	+++	+++
Cingulate gyrus	0	+	++	++	+++
Pre/post central gyrus	0	+	+	+	+
Parietal lobe	0	+	+	+	++
Temporal lobe	0	+	+	+	+
Frontal lobe	0	0	+	+	++
Occipital lobe	0	0	na	0	+

Table 9. Assesment of neuronal loss in the cerebellum and brainstem nuclei.

Brain area	*Case 1*	*Case 2*	*Case 3*	*Case 4*	*Case 5*
Cerebellum					
Dentate nucleus	+	0	+	0	0
Purkinje cell layer	+	0	++	+	0
Medulla					
DMV	+	+	+	0	na
Hypoglossal nucleus	0	0	0	0	na
Inferior olivary nucleus	++	0	++	0	na
Pons					
LC	++	0	++	0	++
Raphe nucleus	+	0	0	+	+
Basis pontis	+	0	+	+	++
Midbrain					
Oculomotor nucleus	0	0	0	0	0
SN (lateral)	++	+	++	+	++

6. DISCUSSION

The aim of the present series of studies was to examine the impact of αS-positive inclusions in aging and in various neurodegenerative disorders. We examined the incidence, topographical distribution and clinical relevance of αS pathology in a large post mortem study (n=904) including subjects from five distinct study cohorts each subordinated to different sampling strategies. Although our study was largely hospital-based and participants represented only around 10% of all deceased over the five years that the survey encompassed, we feel that it is fairly representative for the population at large because it includes both diseased and non-diseased subjects.

6.1 Prevalence of α-synuclein-positive inclusions

From our large autopsy cohort, we screened 149 (16%) subjects that exhibited αS-positive structures in one of the three nuclei reported to be most vulnerable; amygdala, SN and DMV. The screening process was observed to have only a slight effect (3% change) on the prevalence. In study **I,** where we screened only SN, we detected αS-positive inclusions in 14% (109/774); in study **II,** adding BFB nuclei to the analysis found 13% (121/904); and in study **III,** adding DMV detected 16% (149/904). In study **I**, the case selection showed a more profound effect on the prevalence of αS pathology ranging from 8% to 27% according to sampling strategies. It was most prevalent in the group that had the highest mean age at death, female preponderance, the greatest proportion of demented and subjects with concomitant AD-related pathology. When one variable was investigated at a time, prevalence of αS pathology was shown to increase with age but was not influenced by gender. The age-related rise was detected from 5.1% to 18.6% between the sixth and ninth decades. A similar age-specific increase has been reported by several other groups (Gibb and Lees 1988, Perry et al. 1990b, Forno and Langston 1993, Wakisaka et al. 2003) Furthermore, similar to the recent report of Saito *et al.*, we found the average age of death to be greater in subjects with αS-positive inclusions compared to those without these inclusions, implying that they are an age-associated change similar to NPs and NFTs (Saito et al. 2004). We detected no gender differences among the αS-positive cases in line with most other large autopsy studies (Esiri et al. 2001, Saito et al. 2004), although both a female (Wakisaka et al. 2003) and male (Rosenberg et al. 2001) preponderance have been reported.

Table 9. Main autopsy studies estimating prevalence of αS/LB pathology in the aged population.

Study	*Study population (autopsy rate)*	*n*	*Dementia*	*F/M*	*Age*	*IHC*	*LB pathology of all*	*LB pathology of all demented*	*LB pathology of all non-demented*
Forno *et al.* (1993)	Consecutive autopsy cases (45-50%)	1199	na	na	na	-	8%	na	na
Ince *et al.* (1996)	Random sample of elderly (41%)	92	75%	67/25	85	Ub	20%	20%	17%
Lindboe *et al.* (1998)	Consecutive autopsy cases (31%)	284	na	107/177	72	Ub	8%	na	na
Esiri *et al.* (2001)	Prospectively followed elderly population	209	48%	119/90	85	Ub	11%	12%	9%
Parkkinen *et al.* (2001)	Consecutive autopsy cases and prospectively followed elderly population	774	27%	384/390	72	αS	14%	23%	11%
Hishikawa *et al.*(2003)	Consecutive autopsy cases (70.5%)	102	28%	51/51	80	αS	23%	41%	15%
Saito *et al.* (2004)	Consecutive autopsy cases	1241	43%	578/663	81	αS	21%	na	na
Mikolaenko *et al.* (2005)	Prospectively followed elderly population	117	43%	32/85	87	αS	25%	na	na

In study **I**, we detected αS-positive inclusions more frequently in the demented subjects with an average of 23% in compared to 11% of non-demented. The load of αS pathology was also found significantly greater among the demented individuals at variance to study by Wakisaka *et al.* who found no change in LB score between demented and non-demented subjects (Wakisaka et al. 2003). Our results are summarized together with the main autopsy studies estimating the prevalence of αS/LB pathology in Table 9. The overall range from 8% to 25% of these studies may be attributable to the source of recruitment of the subjects (e.g. nursing home, community brain bank, clinical research centre or specialized hospital). Our results are rather similar to the findings of the British community-based neuropathology study of the elderly in relation to prospectively evaluated dementia status (Esiri et al. 2001). At variance with our study, they found no age-related differences in the LB pathology which was probably due to the rather small size of their LB-positive cases as the detection of age-related changes requires a wider distribution of ages. Also, the prevalence among the demented (12%) was lower than we detected (23%) in our study, despite the fact that the overall rate of demented in the study of Esiri *et al.* was almost twice as high as we found, this being probably attributable to their higher mean age of death. Interestingly, although our DEMENTIA cohort resembled the elderly individuals described in another study by Ince *et al.* with a rather high proportion of demented patients with AD-related pathology, the prevalence of αS pathology was higher among our dementia cohort (27% v. 20%) (Ince et al. 1995). The prevalence of 8% reported by Lindboe and Hansen corresponded well with our results with the consecutively collected CLINICAL and FORENSIC autopsy cases (Lindboe and Hansen 1998).

Altogether, in study **I,** we found 11% of non-demented subjects with αS pathology. When we excluded all those cases that had any parkinsonian or other neurological symptoms, we observed an incidental finding of αS-positive inclusions in 9%, restricted mostly to the brainstem area, but in a few subjects also cortical inclusions were detected. These incidental LBs have been reported in 5 to 10% by many earlier studies already before the era of αS (Lipkin 1959, Woodard 1962, Forno 1969, Forno and Langston 1993, Lindboe and Hansen 1998), but although these studies have excluded patients with PD, the diagnosis of other neuropsychiatric symptoms has been poor even in the best of circumstances. Incidental LB pathology was reported in only 2% of subjects examined in a retrospective study that screened to exclude all patients with neurological and/or psychiatric disorders (Gibb and Lees 1988). However, a few years ago, a large prospective study reported that 9% of their controls exhibited subcortical Lewy bodies, but they did not extend their clinical assessment beyond the diagnosis

of dementia to other forms such as EPSs (Esiri et al. 2001). The autopsy studies of cognitively and neurologically intact elderly subjects who have been prospectively studied have almost invariably focused on the analysis of AD-related pathology. Nonetheless, the normal aging study of Davis *et al.* described 8% of subjects with LBs (Davis et al. 1999), and moreover, in a few subjects the distribution and extent of inclusions were compatible with the neocortical category of DLB as outlined by the consensus guidelines. This appears to be the first report of numerous cortical LBs in unimpaired subjects. Few years later, another clinopathological study reported a clinically normal subject with also neocortical distribution of LBs (Richard et al. 2002). However, it is very likely that some degenerative changes may have been overlooked because αS-IHC was not used, and the original screening process was limited to SN and LC. In another study, αS-positive LBs (in few subjects also found in cortical samples) were detected in 13% of normal elderly subjects (Knopman et al. 2003).

6.2 Impact of concomitant AD-related pathology on α-synuclein pathology

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 (Perl et al. 1998, Kotzbauer et al. 2001). Many AD patients develop EPSs, whereas a high proportion of PD patients become demented (Galvin et al. 2001). Thus, numerous studies from *in vitro* to clinical level have tried to determine the complex relationship between αS and AD-related pathologies. It has been recognized for some time that LBs are also frequently seen in AD and these cases have been assessed as suffering LBVAD (Hansen et al. 1990). Some reports have estimated αS-positive inclusions in the 50% to 60% of patients with familial or sporadic AD or Down syndrome (Lippa et al. 1998, Lippa et al. 1999a, Hamilton 2000, Arai et al. 2001). In study **II**, we found 18% of the subjects with pronounced AD-related pathology to exhibit αS-positive inclusions, which is much less than the previously reported incidences in general. However, our result is similar to that of De Lucia *et al.* who found αS-positive inclusions in only 16% of AD cases (De Lucia et al. 2002). Recently Mikolaenko *et al.* reported that 30% of AD cases exhibited αS pathology (Mikolaenko et al. 2005). However, when they examined the frequency according to those subjects fulfilling the CERAD criteria for definite AD, 39% were detected with αS-positive inclusions, a number much higher than we found (16%). The proportion of αS pathology in AD is likely to depend on the criteria used for the pathological diagnosis of AD because the LBs have been reported to be primarily found in plaque-predominant AD (Hansen et al. 1993). When AD cases are

selected according to CERAD criteria that take into consideration not only the deposition of NPs but also the age and clinical signs of dementia, a higher percentage of subjects with LBs will usually be encountered. In comparison, in Braak staging, where AD is assigned only according to progression of NFTs (Ince et al. 1998), neither the age or the influence of dementia will alter the incidence of LB pathology.

Since αS pathology was not seen in the majority of AD patients, our study did not confirm the concept that the aggregation process is a primarily result of some common interrelated pathogenesis. The recognition of the fact that these two entities often co-exist does not necessarily indicate that one is caused by the other and this proposal is also supported by the fact that both αS and AD-related pathologies can develop independently from each other. In line with this, in study **I**, we found no correlation between the density of LBs and the extent of AD-related changes. Nonetheless, like others, we found αS-positive inclusions to be more frequent in the brains of patients with AD-related changes than in those without (20%v 10%, respectively), suggesting that when NPs and NFTs are present in the brain they may in some way facilitate the formation of αS-positive inclusions. Alternatively, it appears possible that αS-positive inclusions can promote the formation of AD-related pathology. There is compelling evidence suggesting that all three amyloid proteins, Aβ, tau and αS may act in synergy, by enhancing the aggregation of each other when they are simultaneously present (Masliah et al. 2001, Giasson et al. 2003a).

6.3 Topographical distribution of α-synuclein pathology

Already in the beginning of the 1980's, Kosaka *et al.* proposed a subdivision of subjects with LB pathology into three groups, namely brainstem, limbic and neocortical subtypes and the presently operative clinicopathological Consensus criteria are based on this concept (Kosaka et al. 1984, McKeith et al. 1996). In study **III**, where the subjects were selected disregarding the clinical symptoms, the topographical distribution of αS-positive inclusions enabled the division of our subjects into ten groups. This distribution largely followed the recent Braak staging of αS pathology with some deviations (Del Tredici et al. 2002, Braak et al. 2003). At variance, we identified a number of subjects where DMV was not affected but αS-positive inclusions were found in SN and/or BFB nuclei. Similarly, Jellinger has recently reported subjects with multiple αS-positive inclusions but preservation of medullary nuclei (Jellinger 2003a). One possible explanation for the varying results might stem from how the subjects were selected. In the

studies of Del Tredici and Braak *et al.*, as in ours, the subjects were screened from a larger autopsy series (Del Tredici et al. 2002, Braak et al. 2003). The notable difference was however, that their selection was based on the presence of αS pathology in the DMV alone, whereas our screening process also included three other nuclei reported to be vulnerable: SN, NBM and amygdala. Therefore, their initial screening process must have inevitably eliminated those with αS pathology restricted to these areas. The fact that αS-positive inclusions can appear without the involvement of the DMV, however, calls into question the theory that this region is the trigger site for αS pathology. The amygdala has been reported to be severely affected with LB pathology both in PD and in DLB (Braak et al. 1994, Rezaie et al. 1996). Indeed, this region appears to be the most preferable site for the αS to aggregate also in patients with sporadic (Hamilton 2000, Arai et al. 2001) or familial AD (Lippa et al. 1998), Down syndrome (Lippa et al. 1999a) and even in patients with Parkinsonism-Dementia Complex of Guam (Yamazaki et al. 2000). In agreement with these studies, in study **II**, we found the amygdala to be particularly vulnerable to αS pathology, and sometimes this was even the sole area of affection in subjects with severe concomitant AD-related changes. This suggests that an alternative induction site and route of progression for αS pathology might exist when coexistent AD-related pathology or other risk factors are present.

6.4 Clinico-pathological correlations with α-synuclein pathology

Study **III**, had a unique design in the sense that the selection of the material was entirely based on the presence of αS pathology irrespective of clinical phenotype. This enabled us to assess the valid clinical relevance of αS pathology. Most studies seeking a connection between a particular pathologic change and a clinical symptom have focused their examination on a limited number of patients displaying specific clinical signs and have contained very few controls. However, in our opinion, the true significance of any pathological change in relation to the clinical symptoms can only be evaluated by examining a large unselected sample. Now, if the strength of study **III** lies in above, the retrospective clinical assessment can be with justification considered to be one of its main weaknesses. Admittedly, some subtle extrapyramidal signs or mild cognitive impairment may have gone unnoticed, especially in subjects that were not neurologically evaluated during life. However, we included only cases with high-quality and recent (a maximum of 1 year before death) clinical documentation which ultimately led to the exclusion of 30% of our original αS-positive cohort. All neurologically unimpaired subjects had visited a general physician just prior to death or had been under a

continuous clinical follow-up because of some chronic terminal illness such as neoplasia. Thus, we feel confident that no full blown parkinsonian syndrome or dementia would have remained undetected.

Our retrospective clinical assessment revealed that only 30% of 106 αS-positive cases were overall diagnosed with a neurodegenerative disorder. In particular, the EPSs have been identified in only one quarter of cases with αS-positive inclusions in the brainstem neurons, and cognitive impairment in 35% of cases with cortical inclusions. Although, we identified a positive correlation between the load of αS-positive inclusions in many brainstem areas and putamen with respect to EPSs, we also found an extensive burden of brainstem αS pathology in some subjects without any EPSs. As a result, we conclude that EPSs cannot not be predicted based on the load of αS pathology in any of the brainstem areas. In addition, our findings do not support the concept whereby the difference between incidental LB disease and PD is attributable to the degree of αS pathology in the brainstem (Del Tredici et al. 2002). The load of αS-positive LBs has been reported to show a "∩"-shaped distribution over time where the number of inclusions increases with the progression of the disease until the neurons start to die (Duda 2004). This reciprocal relationship between number of inclusions and neuronal loss has been used to rationalize why in the end stage of the disease, the load of αS-positive lesions can be rather low. However, although we did not extend our analysis to the quantification of neuronal loss in SN in study **III,** it was evident that no normal curve could be drawn as far too many outliers e.g. those without neuronal loss and many αS-positive inclusions existed.

Numerous studies have focused on the role of cortical αS-positive LBs in the generation of dementia in DLB and PD (Hurtig et al. 2000, Mattila et al. 2000, Harding and Halliday 2001, Apaydin et al. 2002, Kovari et al. 2003). Many of these studies have included subjects with concomitant AD-related pathology influencing the outcome. At the end of the disease, it is difficult to discern the underlying cause for the dementia in the patients with "mixed-pathologies". Furthermore, the temporal sequences for the development of multiple pathologies would be very difficult to study. Similarly to the above studies, we found a correlation between several cortical areas and dementia even though our retrospective and semiquantitative analysis of αS-positive subjects was not designed to assess this correlation. However, we also identified some subjects with a relatively high burden of cortical αS pathology but who were cognitively unimpaired. Thus, it appears that the assumption by which the absolute number of cortical LBs alone would be responsible for the development of cognitive decline in PD or DLB is over-

simplistic. This has been also proposed by Colosimo *et al.* who reported that around half of examined PD patients with cortical LBs did not present any history of cognitive impairment (Colosimo et al. 2003).

Surprisingly, in study III, we identified subjects with a reasonably high burden of αS pathology in both brainstem and cortical areas without any neurological or psychiatric symptoms. This was mainly due to our unselective screening process that included all subjects with αS pathology. Thus, it appears that there are too many cases that do not fit into the classic diagnostic compartments. This is an important finding as hitherto the incidental αS/LB pathology has been considered to merely represent a presymptomatic phase of the disease (Davis et al. 1999). Thus, our findings raise the important question of why some individuals can tolerate αS pathology without exhibiting any symptoms. Our knowledge of the biology of αS and its aggregation is insufficient to understand how it affects the cell viability, and is likely that some unrecognized factors of decisive importance mediate the neuronal dysfunction. Thus, until we solve these issues it does not seem feasible or even possible to achieve optimal clinico-pathological correlations in the diagnosis of PD or DLB (McKeith et al. 2004).

6.5 Evidence against pathognomonic nature of α-synuclein-positive inclusions

Similarly to our study **IV**, Van Duinen and coworkers described widespread and numerous cytoplasmic LB-like inclusions in a patient without any apparent clinical signs of parkinsonism or dementia. The inclusions they described lacked immunoreactivity for αS, which they concluded to explain their harmless nature (van Duinen et al. 1999). This inference was contradicted by our study where inclusions in the neurologically unimpaired subject were readily labelled with αS antibodies, suggesting that the incorporation of αS into the inclusions is not detrimental to the viability of the affected neurons. Both these case reports have, however, demonstrated that the presence of numerous cytoplasmic inclusions, both subcortical and cortical, is not exclusively restricted to symptomatic individuals.

The same main conclusion was derived from study **V**, where the retrospective clinical assessment revealed that one individual showing abundant αS-positive glial inclusions was neurologically unimpaired. Similarly to the design of the study **III**, here we also selected all subjects from a large post mortem material with predominant αS glial pathology regardless of their clinical phenotype. In this way, we were able to assess the actual clinical relevance of αS-

positive glial inclusions as well. The neuronal loss in our study appeared to be clinically highly relevant, and not due to the presence of αS-positive glial inclusions. Although, Ozawa *et al.* did recently show a significant correlation between the density αS-positive glial inclusions and the severity of neuronal loss (Ozawa et al. 2004), we found no such apparent relationship in our cases. Severe neuronal loss was seen in subjects with variable densities of glial inclusions. The fact that our neurologically unimpaired subject displayed numerous αS-positive GCIs without any apparent neuronal loss suggests that glial dysfunction precedes neuronal degeneration and death. However, it may equally well mean that neuronal loss and formation of glial inclusions may be unrelated phenomena, which is also suggested by the partially non-overlapping distribution of these pathologic changes (Papp and Lantos 1994).

Although there is a large amount of data supporting the pathogenetic causal role of αS-positive inclusions in clinical symptomatology, there are also some critical shortcomings to this hypothesis. First, the neuronal loss in PD is disproportionately high in comparison to the small number of inclusions that can be observed in post mortem analysis (Terry 2000). Indeed, it seems unlikely that every dying neuron would go through a stage of formation of compact αS-positive inclusions. One could argue that the inclusions might be degraded rapidly as the cells die but this seems unlikely due to the highly insoluble nature of LBs (Pollanen et al. 1993). Some studies have shown a different compartmentalization for the neuronal loss and LBs within the nuclei (Gibb and Lees 1991), whereas others have detected LBs in nuclei in which the neurons are intact (Kremer and Bots 1993), suggesting that the inclusion formation is not a general indication of neuronal dropout at all. Furthermore, there is no substantial neuronal loss in the DLB patients with widespread cortical αS-positive inclusions (Lippa et al. 1994, Gomez-Isla et al. 1999). Thus, it appears that the cortical distribution of LBs is not linked with the cell loss in a similar way as occurs in the brainstem structures. Notably, the only repeatedly reported neuronal loss in DLB occurs in NBM (Lippa et al. 1999b), and this could explain the enhanced efficacy of acetylcholinesterase inhibitors in DLB (Simard and van Reekum 2004).

The disparity between the number of αS-positive inclusions and the magnitude of neuronal loss is supported by studies that have shown that the inclusion-containing neurons can remain viable for many years (Hatanpaa et al. 1996, Morsch et al. 1999). Moreover, some reports have shown that the LB-containing neurons appear morphologically healthier (cell and nucleolar size) than the adjacent non-LB-containing neurons (Gertz et al. 1994). Also, it appears that those neurons that undergo an apoptotic-like cell death are those without somal LBs (Tompkins and Hill

1997). Some *in vitro* studies have clearly shown that overexpression of wild-type αS has a protective effect against the apoptotic stimuli (da Costa et al. 2000, Lee et al. 2001). Furthermore, synucleins have been reported to possess a chaperone-like activity *in vitro* in that they can suppress the aggregation of other proteins (Souza et al. 2000). However, it remains to be determined whether they retain this activity *in vivo*. There is also accumulating evidence from the animal models (Auluck et al. 2002, Lo Bianco et al. 2002, Gispert et al. 2003) indicating that the formation of αS-positive inclusions and neuronal loss may not be linked in a causal chain. This may also apply to the αS-positive glial inclusions and multisystem neurodegeneration in MSA as the recently developed transgenic mouse model of the disease that overexpresses αS in the oligodendrocytes does not show any motor impairment (Kahle et al. 2002). Furthermore, PD can develop without αS-positive LB pathology at all as is seen in autosomal recessive juvenile parkinsonism with the parkin mutation (Takahashi et al. 1994b, van de Warrenburg et al. 2001).

One could even speculate that the presence of inclusions may represent a protective mechanism on the part of surviving cells, i.e. those cells unable to form inclusions may be at most risk of dying without leaving any tombstones behind (Colosimo et al. 2003, Tanaka et al. 2004). Some *in vitro* work has lead to the speculation that abnormal αS metabolism leads to neurodegeneration prior to the formation of characteristic inclusion bodies (Saha et al. 2000). This view is supported by many biophysical studies that have suggested that it is a protofibrillar form of αS rather than the "mature" fibrils which is responsible for the cell death (Goldberg and Lansbury 2000, Volles et al. 2001, Caughey and Lansbury 2003). The aggregation process of αS may still be the main event in the pathogenesis of synucleinopathies, but the actual "death signal" may be emitted during a much earlier stages along the fibrillogenesis. The aggregation of αS into insoluble filamentous structures that is detected in the post mortem examination would reduce the concentration of the "toxic form" of αS and protect cells from αS-induced cell death (Olanow et al. 2004, Tanaka et al. 2004). This proposal could have serious ramifications for the design of potential therapeutics because drugs that prevent fibrillization (protofibril-to-fibril formation) but allow oligomerization might accelerate the disease progression by causing accumulation of toxic species (Lansbury 1999, Conway et al. 2000). On the other hand, characterization of early stages of the aggregation process might provide the key to developing effective novel therapies that target the precise pathogenic process (Conway et al. 2001, Sulzer 2001). In conclusion, αS-positive inclusions could be the last resort and a desperate attempt by a neuron to save itself by sequestering toxic protein intermediates. If this is

true, inclusion formation should be viewed in a completely new light; not as an enemy that needs to be eliminated but as a last loyal warrior on the battlefield of neuronal survival.

CONCLUSIONS

This series of studies was carried out in order to clarify the role of αS pathology in aging and in neurodegenerative disorders by examining its incidence, topographical distribution and clinical relevance. The main strength of our study lies in the unbiased selection of material as all the cases with αS pathology were included regardless of their clinical phenotype.

The following conclusions can be made:

1. Some αS-positive inclusions can be found in every sixth elderly individual (prevalence ~15%), and the case selection highly influences the prevalence of αS pathology. Age, dementia and co-existent AD pathology all have some bearing on the prevalence of αS pathology, whereas gender appears not to have any effect.

2. The majority (80%) of AD patients did not have concomitant αS pathology. However, when these two pathologies do co-exist, αS pathology may induce the AD-related pathology, and vice versa.

3. Alternative routes must exist for the sequence whereby αS pathology commences from the medullary nuclei.

4. The distribution or load of αS-positive inclusions in either neurons or glia cannot be used to predict with certainty the EPSs and cognitive impairment or clinical features of multiple system atrophy. Thus, it appears that αS-positive inclusions should not be considered as specific hallmarks related to a particular clinical symptomatology.

5. The identification of subjects with a high burden of αS pathology (both neuronal and glial) who exhibit no clinical symptoms suggests that there must be some unrecognized factors of decisive importance involved in mediating neuronal and glial dysfunction.

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APPENDIX: ORIGINAL PUBLICATIONS (I-V)

I

α-Synuclein pathology is highly dependent on the case selection

Parkkinen L, Soininen H, Laakso M, Alafuzoff I

Neuropathology and Applied Neurobiology 2001; 27(4): 314-325.

II

Regional distribution of α-synuclein pathology in unimpaired aging and Alzheimer's disease

Parkkinen L, Soininen H, Alafuzoff I

Journal of Neuropathology and Experimental Neurology 2003; 62(4): 363-367.

IIIa

α-Synuclein pathology does not predict extrapyramidal symptoms or dementia

Parkkinen L, Kauppinen T, Pirttilä T, Autere J, Alafuzoff I.

Annals of Neurology 2005; 57 (1): 82-91.

IIIb

In reply to "alpha-synuclein aggregation and its relation to neurodegenerative diseases" by Papapetropoulos S and Mash DC

Parkkinen L, Kauppinen T, Pirttilä T, Autere JM, Alafuzoff I

Annals of Neurology 2005; 57 (4): 605-606.

IV

Widespread and abundant α-synuclein pathology in a neurologically unimpaired subject

Parkkinen L, Pirttilä T, Tervahauta M, Alafuzoff I

Neuropathology (in press)

V

Significance of glial α-synuclein-positive inclusions with respect to the clinical symptoms

Parkkinen L, Hartikainen P, Alafuzoff I

Submitted for publication

PUBLICATIONS
SERIES OF REPORTS, DEPARTMENT OF NEUROLOGY

1. **Juhani Partanen (1978):** Time-locked phenomena of human motor unit potentials. An electromyographic study of satellites and doubles.
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