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# Brain α-synuclein accumulation in multiple system atrophy, Parkinson's disease and progressive supranuclear palsy: a comparative investigation

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α-Synuclein is a major component of Lewy bodies and glial cytoplasmic inclusions, pathological hallmarks of idiopathic Parkinson's disease and multiple system atrophy, and it is assumed to be aetiologically involved in these conditions. However, the quantitative status of brain α-synuclein in different Parkinsonian disorders is still unresolved and it is uncertain whether α-synuclein accumulation is restricted to regions of pathology. We compared membrane-associated, sodium dodecyl sulfate-soluble α-synuclein, both the full-length 17 kDa and high molecular weight species, by western blotting in autopsied brain of patients with Parkinson's disease (brainstem-predominant Lewy body disease: $n = 9$), multiple system atrophy ($n = 11$), progressive supranuclear palsy ($n = 16$), and of normal controls ($n = 13$). Brain of a patient with familial Parkinsonism-dementia due to α-synuclein locus triplication (as positive control) showed increased membrane-associated, sodium dodecyl sulfate-soluble α-synuclein levels with abundant high molecular weight immunoreactivity. In multiple system atrophy, a massive increase in 17 kDa membrane-associated, sodium dodecyl sulfate-soluble α-synuclein was observed in highly pathologically affected regions, including putamen (+1760%, range +625–2900%), substantia nigra [+1000% (+356–1850%)], and white matter of internal capsule [+2210% (+430–6830%)] together with numerous high molecular weight species. Levels of 17 kDa membrane-associated, sodium dodecyl sulfate-soluble α-synuclein were only modestly increased in less affected areas (cerebellar cortex, +95%; caudate, +30%; with both also showing numerous high molecular weight species) and were generally normal in cerebral cortices. In both Parkinson's disease and progressive supranuclear palsy, membrane-associated, sodium dodecyl sulfate-soluble α-synuclein levels were normal in putamen and frontal cortex whereas a trend was observed for variably

increased 17 kDa membrane-associated, sodium dodecyl sulfate-soluble α-synuclein concentrations [+184% (−60% to +618%)] with additional high molecular weight species in Parkinson's disease substantia nigra. No obvious correlation was observed between nigral membrane-associated, sodium dodecyl sulfate-soluble α-synuclein accumulation and Lewy body density in Parkinson's disease. Two progressive supranuclear palsy cases had membrane-associated, sodium dodecyl sulfate-soluble α-synuclein accumulation in substantia nigra similar to multiple system atrophy. Several Parkinson's disease patients had very modest high molecular weight membrane-associated, sodium dodecyl sulfate-soluble α-synuclein accumulation in putamen. Levels of 17-kDa membrane-associated, sodium dodecyl sulfate-soluble α-synuclein were generally positively correlated with those of high molecular weight membrane-associated, sodium dodecyl sulfate-soluble α-synuclein and there was a trend for a positive correlation between striatal dopamine loss and 17-kDa membrane-associated, sodium dodecyl sulfate-soluble α-synuclein concentrations in multiple system atrophy. Brain membrane-associated, sodium dodecyl sulfate-soluble α-synuclein accumulations in Parkinson's disease and multiple system atrophy are regionally specific, suggesting that these sporadic α-synucleinopathies, unlike familial Parkinsonism-dementia, are not associated with a simple global over-expression of the protein. Despite a similar extent of dopamine depletion, the magnitude of brain membrane-associated, sodium dodecyl sulfate-soluble α-synuclein changes is disease specific, with multiple system atrophy clearly having the most severe accumulation. Literature discrepancies on α-synuclein status in 'Parkinson's disease' might be explained by inclusion of cases not having classic brainstem-predominant Lewy body disease and by variable α-synuclein accumulation within this diagnostic classification.

# Introduction

The discoveries of α-synuclein gene mutations (Polymeropoulos *et al.*, 1997; Singleton *et al.*, 2003) in autosomal-dominant hereditary Parkinsonism and α-synuclein protein as the major component of Lewy bodies (Spillantini *et al.*, 1997, 1998*b*; Wakabayashi *et al.*, 1997) and glial cytoplasmic inclusions (GCIs) (Spillantini *et al.*, 1998*a*; Wakabayashi *et al.*, 1998), the pathological hallmarks of idiopathic Parkinson's disease (Forno, 1996) and multiple system atrophy (Papp *et al.*, 1989; Papp and Lantos, 1994), respectively, have provided clues to the pathogenesis of Parkinson's disease and associated illnesses. Collectively, these illnesses, including Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy, are now described as 'α-synucleinopathies' (Goedert and Spillantini, 1998).

α-Synuclein is a small (140 amino acids), naturally unfolded, cytosolic protein that associates occasionally with membranes (Maroteaux and Scheller, 1991; Ueda *et al.*, 1993; Jakes *et al.*, 1994; Irizarry *et al.*, 1996; Weinreb *et al.*, 1996); however, under certain pathological conditions, it can aggregate and become insoluble, accumulating in various neuronal or glial inclusion bodies including Lewy bodies and GCIs. A number of studies have examined α-synuclein accumulation in post-mortem brain of patients with different Parkinsonian conditions and revealed the presence of membrane associated, sodium dodecyl sulfate (SDS)-soluble α-synuclein [α-synuclein$_M$: full-length and non-reducible, high molecular weight (HMW) oligomers/polymers] and, to a lesser extent, SDS-insoluble and non-reducible (tough) large α-synuclein aggregates that can be extracted by urea or formic acid (Table 1). However, as elaborated in the 'Discussion' section, many conflicting findings have been reported regarding, for example, the question of abnormal levels of α-synuclein in the substantia nigra in

Parkinson's disease (unchanged, Fuchs *et al.*, 2008; slight increase, Xu *et al.*, 2002; massive increase, Devi *et al.*, 2008; Shehadeh *et al.*, 2009). Part of the uncertainty in the literature can be explained by the selection in some investigations of only a small number of subjects and/or brain regions for examination. The neurochemical studies have also generally focused on qualitative similarity in α-synuclein accumulation among the α-synucleinopathies, with possible quantitative differences not addressed. Indeed, the limited information on quantitation of brain α-synuclein in Parkinsonian conditions is surprising and might be related to the laborious effort required for quantitative western blotting analysis in subfractionated regional tissue samples, uncertainty in selecting an appropriate procedure for tissue extraction (e.g. a mild detergent is unlikely to solubilize HMW species), and possibly by the assumption that the status of α-synuclein in the disorders has already been established. Further, findings in dementia with Lewy bodies have often been generalized, possibly inappropriately, to classical 'uncomplicated' idiopathic Parkinson's disease (sporadic Parkinson's disease with brainstem-predominant type of Lewy bodies; Li *et al.*, 2005; Anderson *et al.*, 2006; Kramer and Schulz-Schaeffer, 2007). In this regard, it continues to be debated whether the two conditions represent a continuum within the spectrum of Lewy body disease or, despite some overlap, are clinically and pathologically distinct entities (see Jellinger, 2008 for a review). This confusion regarding the status of α-synuclein in Parkinson's disease may be partly related to recent histochemical reports of wide-spread α-synuclein immunoreactive pathology (Lewy bodies, Lewy neurites, glial inclusions) in brain of some patients with Parkinson's disease (Hishikawa *et al.*, 2001; Duda *et al.*, 2002; Braak *et al.*, 2003, 2007; Mori *et al.*, 2008), raising the issue whether α-synuclein accumulation in Parkinson's disease is regionally specific or as diffuse as in dementia with Lewy bodies.

**Table 1** α-Synuclein in multiple system atrophy and Parkinson's disease

| References | Regions | n | Buffer-soluble | | Buffer-insoluble | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | SDS-soluble | | SDS-insoluble[a] | |
| | | | 17 kDa | HMW | 17 kDa | HMW | 17 kDa | HMW |
| **Multiple system atrophy** | | | | | | | | |
| Tu et al., 1998 | Frontal GM | 2 | ↔ | NE | ↔ | NE | NE | NE |
| | Frontal WM | 2 | ↔ | NE | ↑800% | NE | NE | NE |
| Dickson et al., 1999 | Putamen | 7 | ↔[b] | –[b] | ↑2700% | + | ↔ | + |
| | 8 other GM | 1 | ↔[b] | –[b] | ↑ | + | ↑ | ± |
| Duda et al., 2000 | Cerebellar WM | 3 | ↓ | – | ↑ | + | ↑ | + |
| Campbell et al., 2001 | Pons | 4 | ↔ | – | ↑500% | + | ↑ | + |
| | Frontal WM | 4 | ↑ | – | ↑700% | + | ↔ | – |
| | Cerebellar WM | 4 | ↔ | – | ↑470% | + | ↔ | – |
| Kahle et al., 2002 | Cerebellar WM | 2 | ↑ | + | ↑ | + | ↑ | + |
| Pawlyk et al., 2003 | Cerebellar WM | 3 | ↔ | NE | ↑ | NE | NE | NE |
| Meulener et al., 2005 | Cerebellar WM | 2 | ↔ | – | ↑[c] | +[c] | NE | NE |
| Uryu et al., 2006 | Pons | 10 | ↔ | – | NE | NE | ↑ | + |
| **Parkinson's disease** | | | | | | | | |
| Langston et al., 1998 | Caudate | 14 | ↔ | – | NE | NE | NE | NE |
| | Frontal cortex | 14 | ↔ | – | NE | NE | NE | NE |
| Culvenor et al., 1999 | Cingulate cortex | 4 | ↔ | – | ↑↔ | ± | ↑↔ | ± |
| Duda et al., 2000 | Cerebellar WM | 1 | ↔ | – | ↔ | – | ↔ | – |
| Campbell et al., 2000 | Temporal cortex | 4 | ↔ | – | ↔ | – | ↑↔ | ± |
| Campbell et al., 2001 | SN | 3 | ↔ | – | ↔ | + | ↑ | + |
| Kahle et al., 2001 | Parietal cortex | 1 | NE | NE | NE | NE | ↑ | + |
| Xu et al., 2002 | SN | 6 | ↑64%[b] | +[b] | ↑ | + | NE | NE |
| | VTA | 6 | ↔[b] | –[b] | ↔ | – | NE | NE |
| Sharon et al., 2003 | Frontal cortex | 5 | NE | + | NE | NE | NE | NE |
| Tofaris et al., 2003 | SN, cingulate and frontal cortices | 3 | ↔[b] | –[b] | ↑[f] | +[f] | | |
| Pletnikova et al., 2005 | Cingulate cortex | 4[d] | ↔[b] | –[b] | ↑↔ | ± | NE | NE |
| | | 6[e] | ↔[b] | –[b] | ↑ | + | NE | NE |
| Liu et al., 2005 | SN | 1 | ↔ | – | ↔ | + | ↑ | + |
| Deramecourt et al., 2006 | Temporal cortex | 2 | ↔ | – | ↔ | – | NE | NE |
| Uryu et al., 2006 | Cingulate cortex | 9 | ↔ | – | NE | NE | ↑ | + |
| Devi et al., 2008 | SN | 11 | ↑ 10-fold (17 kDa) in mitochondria. HMW, NE. | | | | | |
| | Striatum | 5 | ↑ 6-fold (17 kDa) in mitochondria. HMW, NE. (ELISA and WB) | | | | | |
| Fuchs et al., 2008 | SN,MO,Cerebellum, Cingulate cortex | 8–17 | ↔ Total α-synuclein protein in any brain regions examined (ELISA, guanidine HCl extracts). | | | | | |
| Shehadeh et al., 2009 | SN | 5 | ↑ 11-fold in total α-synuclein (TRIzol extracts). HMW, NE. | | | | | |

Levels of full-length 17 kDa α-synuclein were increased (↑), decreased (↓), or normal (↔). HMW species were positive (+) or undetected (–). The method employed was western blotting unless otherwise indicated. SDS = sodium dodecyl sulfate; HMW = high molecular species of α-synuclein; GM = gray matter; WM = white matter; SN = substantia nigra; VTA = ventral tegmental area; MO = medulla oblongata; NE = not examined; ELISA = enzyme-linked immunosorbent assay; WB = western blotting.
a The SDS-insoluble fraction was extracted with either urea or formic acid.
b Include both buffer soluble and Triton X-100 or Nonidet P-40 soluble fractions.
c Sarkosyl instead of SDS was used.
d Parkinson's disease cases with dementia.
e Parkinson's disease cases with dementia plus Aβ deposits.
f The mild detergent-insoluble fraction was extracted with both SDS and urea.

Similarly, in the case of multiple system atrophy it has been assumed that α-synuclein accumulates throughout the brain (Trojanowski and Revesz, 2007) although neurochemical evidence employing an attempt at quantitation (e.g. by western blots) appears to be based on data from only a single case study (Dickson et al., 1999). Most studies of multiple system atrophy have been restricted to only a few brain areas with a key degenerative area, the substantia nigra, yet to be examined (Table 1).

Our study was designed to address the literature deficiencies by systematic examination of brain α-synuclein and comparison of protein levels in multiple brain areas (pathological and non-pathological), in representative numbers of patients with idiopathic Parkinson's disease (brainstem-predominant type of Lewy body disease) and multiple system atrophy. We selected a western blotting procedure for α-synuclein measurement to confirm absolutely whether the protein measured was the full-length protein or HMW species. As a positive control, a single case of familial Parkinsonism-dementia with triplicated α-synuclein locus was also included. In addition, we examined patients with progressive supranuclear palsy as a presumed negative control as this atypical

Parkinsonian condition is classified as a tauopathy (Albers and Augood, 2001) rather than synucleinopathy, although it shares nigrostriatal dopamine loss with Parkinson's disease and multiple system atrophy.

# Materials and methods

## Patients

Autopsied brains, collected at the Centre for Addiction and Mental Health in Toronto through collaboration with neurologists, were obtained from a total of 11 patients with multiple system atrophy, 9 patients with Parkinson's disease, 16 patients with progressive supranuclear palsy, and 13 normal subjects. No significant difference (one-way ANOVA) was found in post-mortem interval (in hours) among the four groups (control: $13.6 \pm 1.7$; multiple system atrophy: $13.1 \pm 1.6$; Parkinson's disease: $13.2 \pm 1.9$; progressive supranuclear palsy: $11.4 \pm 1.3$; mean $\pm$ SEM), or in age between control ($70.8 \pm 2.4$ years) and patients with multiple system atrophy ($65.4 \pm 2.7$ years), Parkinson's disease ($76.7 \pm 3.1$ years), or progressive supranuclear palsy ($73.4 \pm 2.3$ years) although the Parkinson's disease patients had significantly higher age than those with multiple system atrophy ($P < 0.05$). One half-brain was used for neuropathological examination, whereas the other half was frozen for neurochemical analyses. The characteristics of the patients are summarized in Supplementary Table 1. No detailed information on the neuropsychological or mental function of the patients was available. The causes of death for the control subjects were cardiovascular illnesses (9), bronchopneumonia (1), cancer (2), and natural death (1).

## Neuropathological assessment

Neuropathological findings of neuronal loss and gliosis and assessment of the pathological hallmarks including GCIs and Lewy bodies in brains of patients with multiple system atrophy and Parkinson's disease are summarized in Supplementary Table 2. Lewy bodies (by routine haematoxylin-eosin stain) were confirmed in all of the Parkinson's disease patients but were absent in multiple system atrophy. The presence of GCIs (by Bielschowsky silver impregnation) was confirmed in five multiple system atrophy patients whereas the analysis of the remaining patients was conducted prior to the consensus statement on the diagnosis of multiple system atrophy in 1999 (Gilman et al., 1999), which incorporated assessment of the neuropathology of GCIs. For all of the progressive supranuclear palsy cases, pathological examination confirmed the absence of Lewy bodies and the presence of neuronal loss and gliosis in substantia nigra, globus pallidus, subthalamic nucleus, brainstem, and cerebellar dentate nucleus together with tau-positive neurofibrillary tangles (by Bielschowsky silver impregnation). α-Synuclein immunohistochemistry was not performed on any of the subjects and it is possible that some cortical Lewy bodies might have been undetected by our conventional methods.

## A familial Parkinsonism-dementia case with triplicated α-synuclein locus

An affected case (Muenter et al., 1998: Patient IV-7) in a large familial Parkinsonism-dementia pedigree (Iowa kindred associated with α-synuclein locus triplication; Waters and Miller, 1994; Muenter et al., 1998; Gwinn-Hardy et al., 2000; Singleton et al., 2003) was also examined. Neuropathological examination disclosed severe degeneration of the substantia nigra, remarkable neuronal loss and vacuolation (or spongiosis) in the temporal cortex, and widespread subcortical and cortical Lewy bodies in patients with familial Parkinsonism-dementia, including our case, from the Iowa kindred. Using α-synuclein immunostaining, neuronal and glial inclusions resembling those reported by Gwinn-Hardy et al. (2000) were observed in the degenerated putamen of our patient (unpublished data, L.S. Forno). Quantitative genomic analysis of α-synuclein exons 2 and 6 (Farrer et al., 2004) showed a doubling of genomic copy number in our patient with familial Parkinsonism-dementia, confirming α-synuclein locus triplication (Singleton et al., 2003).

## Tissue sample preparation for SDS-polyacrylamide gel electrophoresis

Brain dissection followed published procedures (Kish et al., 1988). Whole homogenate was prepared by sonicating brain samples for 15 s at $4^{\circ}$C in 25 mM Tris-HCl (pH 7.4) buffer containing protease inhibitors. An aliquot of homogenate was centrifuged at 18,000$g$ for 10 min and the supernatant was saved as a buffer-soluble fraction (Langston et al., 1998; Mash et al., 2003). The membrane fraction was prepared by centrifugation at 35,000$g$ for 15 min as described previously (Wilson et al., 1996) and the pellet was re-suspended in 10% SDS. We confirmed that the neuron specific enolase (control protein for cytosolic fraction) and the synaptosome-associated protein 25 (SNAP-25: control protein in the membrane fraction), were recovered by 89% and 84%, respectively, suggesting high efficiency of recovery. In each of the three brain fractions (homogenate, cytosolic, membrane), total protein content was determined by Bio-Rad assay.

## Western blotting

Using quantitative blot immunolabeling (Furukawa et al., 2002a, b), levels of the full length, 17 kDa α-synuclein were measured with a monoclonal antibody (Syn-1, clone 42, epitope at amino acids 91–96, Anderson et al., 2006; or amino acids 91–99, Perrin et al., 2003) from Transduction Laboratories (Lexington, KY, USA). The specificity was confirmed by using purified recombinant α-synuclein (Alpha Diagnostic International, San Antonio, TX, USA) and by three other antibodies against different epitopes of human α-synuclein, including polyclonal antibodies from Synaptic Systems (SS, raised in rabbit, against amino acids 2–25) and Oncogene (raised in guinea pig, against amino acids 123–140) and by another monoclonal antibody from Zymed (clone LB509, epitope at amino acids 115–122, Jakes et al., 1999) (Fig. 1). Five concentrations of tissue standard (whole homogenate protein, 10–50 μg/lane; soluble fraction protein, 10–50 μg/lane; membrane fraction protein, 5–40 μg/lane), consisting of a pool of human frontal cortex, were loaded onto each gel to construct a standard curve. Immunoreactivities were visualized with chemiluminescence reagents and quantified by a computer-based imaging device (MCID, Imaging Research Inc., St. Catharines, ON, Canada). The amount of 17 kDa α-synuclein immunoreactivity (for simplicity referred to as 'protein') was interpolated from the tissue standard curve, which had been calibrated by using purified recombinant α-synuclein, and expressed in ng/μg protein. At the same time, levels of the HMW α-synuclein-immunoreactive species in the membrane fraction were determined by taking the optical density of the whole smear bands above α-synuclein, normalized against the same tissue standard curve for the 17 kDa α-synuclein on the same blot, and expressed in arbitrary units (AU) equivalent to μg tissue standard protein (17 kDa)/μg tissue sample protein. In addition,
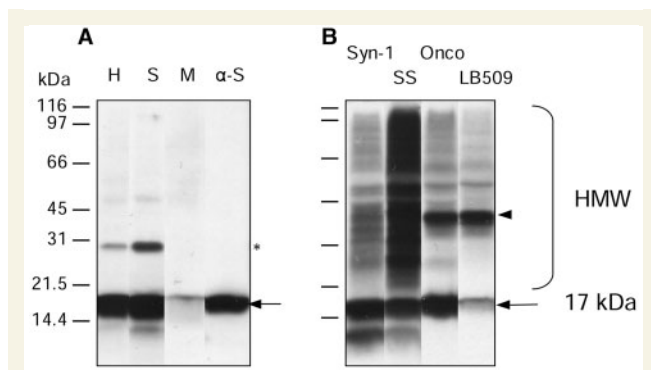
**Figure 1** Western blot of α-synuclein in human brain. (**A**) α-synuclein in homogenate (H), cytosolic (S), and membrane (M) fractions of pooled human frontal cortex (30 μg protein each) detected by the monoclonal antibody from Transduction Laboratory (Syn-1, clone 42). Purified recombinant human α-synuclein (α-S, 40 ng) was used as the positive control. (**B**) α-synuclein immunoreactivity in putamen of a patient with multiple system atrophy (MSA Case 4) as detected by different antibodies, including the monoclonal Syn-1 (epitope at amino acids 91–96), a rabbit polyclonal antibody from Synaptic Systems (SS, epitope 2–25), a guinea pig polyclonal antibody from Oncogene (Onco, epitope 123–140), and the monoclonal LB509 from Zymed (epitope 115–122). The arrow identifies the full-length 17 kDa α-synuclein(-s) and the bracket identifies HMW α-synuclein immunoreactive species. *Identifies a non-specific band at ∼31 kDa recognized by the second antibody alone upon longer exposure. Note in (**B**) the protein band at ∼40 kDa (arrowhead) preferentially recognized by both C-terminal antibodies (Onco and LB509) and the relatively higher reactivity of the N terminal antibody (SS) to the HMW species versus the 17 kDa band.

levels of neuron specific enolase, SNAP-25, and αB-crystallin, as another component of GCIs and a 23 kDa membrane protein (Pountney *et al.*, 2005), were also determined by the same quantitative immunoblotting technique.

## Measurement of tissue dopamine levels

Dopamine levels in putamen and caudate were measured by high-performance liquid chromatography-electrochemical detection (Wilson *et al.*, 1994), with those for patients with Parkinson's disease (caudate and putamen), multiple system atrophy (putamen), and progressive supranuclear palsy (putamen, 10 of the 16 subjects) previously published (Tong *et al.*, 2004; Kish *et al.*, 2008).

## Statistical analyses

Statistical analyses on differences in the outcome measures among the control and the Parkinsonian conditions were carried out using non-parametric Kruskal–Wallis ANOVA followed by Dunn's multiple comparison tests or Mann–Whitney U-tests. Correlations were examined by Pearson product-moment or Spearman rank order correlation analyses as indicated in the text. The criterion of statistical significance was $P < 0.05$.

# Results

## Immunoblotting assay of α-synuclein in human brain

As expected, the full-length 17 kDa α-synuclein protein was highly enriched (87% on average) in the cytosolic fraction in normal human brain (Fig. 1A). The low molecular weight protein bands ($\leqslant 12$ kDa) were detected by both Syn-1 and SS antibodies but not by those from Oncogene or LB509 (Fig. 1B) and therefore were presumably C-terminal truncated α-synuclein fragments. The HMW α-synuclein immunoreactive species, best observed in putamen of multiple system atrophy patients (Fig. 1B, see also below in Fig. 3), were detected by all four antibodies although of different relative sensitivity and preference. The C-terminal antibodies (Oncogene, LB509) preferentially recognized an α-synuclein immunoreactive protein band at approximately 40 kDa which presumably represents an α-synuclein dimer (Duda *et al.*, 2000; Sharon *et al.*, 2003) or ubiquitin modified α-synuclein (Sampathu *et al.*, 2003; Anderson *et al.*, 2006). The N-terminal antibody (SS) differed from the others by relatively stronger reactivity with the smear HMW species than with the 17 kDa band, which is consistent with similar findings with other N-terminal antibodies (Waxman *et al.*, 2008) and might suggest that C-terminal truncated α-synuclein fragments are major components of many of the HMW species, corresponding to its increased hydrophobicity and propensity to aggregate (Li *et al.*, 2005; Liu *et al.*, 2005). In the following neurochemical studies, quantitative or semi-quantitative measurements of the full-length 17 kDa α-synuclein and the HMW species were carried out by using the Syn-1 antibody. The between-gels coefficients of variance of replicate samples from a single extraction were 13%, 15% and 15% for membrane and cytosol fractions and homogenate, respectively.

## α-Synuclein in normal human brain

As expected, 17 kDa α-synuclein$_M$ brain immunoreactivity was enriched in grey matter regions in normal human brain, with the white matter sample from the rostral limb of the internal capsule containing a very low level of α-synuclein$_M$ (Table 2). Mean levels of 17 kDa α-synuclein$_M$ in the substantia nigra pars compacta were lower than those in other grey matter brain regions. This was also true for α-synuclein in tissue homogenate and in the cytosolic fraction: substantia nigra (homogenate: $1.81 \pm 0.18$ ng/μg protein; supernatant: $5.10 \pm 0.55$ ng/μg protein); putamen (homogenate: $7.04 \pm 0.50$ ng/μg protein; supernatant: $10.5 \pm 0.63$ ng/μg protein); and frontal cortex (homogenate: $8.75 \pm 0.66$ ng/μg protein; supernatant: $11.9 \pm 1.1$ ng/μg protein).

We did not observe markedly above-normal accumulation of HMW α-synuclein$_M$ in normal brains except in two cases (Table 2, see Figs 2B, 3B and 5B). Control 10 had high levels of α-synuclein$_M$ accumulation in frontal (HMW, 5.10 AU; 17 kDa, 5.75 ng/μg protein) and occipital (HMW, 1.24 AU; 17 kDa, 3.54 ng/μg protein) cortices, and some HMW α-synuclein$_M$ in the caudate (0.15 AU; normal 17 kDa, 2.43 ng/μg protein), with

**Table 2 Levels of α-synuclein in the brain membrane fraction (α-Synuclein$_M$) of patients with multiple system atrophy, Parkinson's disease, progressive supranuclear palsy, hereditary Parkinsonism-dementia and of normal controls**

| | SNpc | Putamen | Caudate | Cerebellar cortex | WM | A10 | A22 | A18 |
|---|---|---|---|---|---|---|---|---|
| **17 kDa α-synuclein (ng/μg protein)** | | | | | | | | |
| Controls (n = 10 or 13)[a] | 0.26±0.08 (0.03–0.87) | 0.94±0.08 (0.63–1.74) | 2.14±0.18 (1.35–3.03) | 1.09±0.09 (0.64–1.47) | 0.06±0.01 (0.02–0.12) | 1.73±0.36 (0.75–5.75) | 1.87±0.16 (0.79–2.70) | 1.76±0.22 (0.89–3.54) |
| Hereditary Parkinsonism-dementia (n = 1) | 2.81 | 4.51 | 5.06 | NE | NE | 3.88 | 6.05 | 1.96 |
| Progressive supranuclear palsy (n = 16 or 8)[b] | 0.70±0.27 (0.12–3.83) | 0.82±0.06 (0.54–1.06) | NE | NE | NE | 1.28±0.21 (0.48–2.39) | NE | NE |
| Multiple system atrophy (n = 8–11)[c] | 2.86±0.37** (1.18–5.07) | 17.5±2.77** (6.82–28.3) | 2.78±0.38 (1.29–4.82) | 2.13±0.35 (0.59–3.64) | 1.42±0.45** (0.32–4.24) | 1.91±0.26 (1.03–4.42) | 2.70±0.40 (0.76–4.64) | 1.69±0.21 (0.86–3.16) |
| Parkinson's disease (n = 9) | 0.74±0.19 (0.10–1.86) | 1.14±0.13 (0.77–1.85) | NE | NE | NE | 1.43±0.10 (1.06–1.84) | NE | NE |
| **HMW α-synuclein (arbitrary unit)** | | | | | | | | |
| Controls (n = 10 or 13)[a] | ND | ND | 0.15 ND in others | ND | ND | 5.10, 0.60 ND in others | ND | 1.24 ND in others |
| Hereditary Parkinsonism-dementia (n = 1) | 5.41 | 4.73 | 5.04 | NE | NE | 4.26 | 10.8 | 0.36 |
| Progressive supranuclear palsy (n = 16 or 8)[b] | 0.52, 2.54, ND in others | ND | NE | NE | NE | 2.72, ND in others | NE | NE |
| Multiple system atrophy (n = 8–11)[c] | 4.02±0.95 (0.34–8.55) | 11.1±1.9 (3.27–20.6) | 2.26±0.88 (0.08–8.82) | 1.28±0.52 (0.16–5.67) | 0.55±0.22 (0.13–1.94) | 1.37, 0.93 ND in others | 1.45, 0.80 ND in others | 0.66 (ND in others) |
| Parkinson's disease (n = 9) | 0.48±0.14† (0.09–1.50) | 0.66, 0.51, 0.28, ND in others | NE | NE | NE | ND | NE | NE |

Data represent mean ± SEM (range) or individual values of exceptional cases in some brain regions. SNpc = substantia nigra pars compacta; WM = white matter at rostral internal capsule; A10, A22, A18 = Brodmann cerebral cortical (grey matter) areas 10 (frontal), 22 (temporal), and 18 (occipital), respectively. HMW = high molecular weight; ND = not detectable; NE = not examined.

a n = 13 except for n = 10 for SNpc, caudate, cerebellar cortex, and white matter of control subjects.

b n = 16 for SNpc and n = 8 for putamen and frontal cortex of progressive supranuclear palsy cases.

c n = 11 except for n = 10 for putamen and cerebellar cortex or n = 8 for WM of multiple system atrophy cases.

** $P < 0.001$ for the difference from the controls in 17 kDa α-Synuclein$_M$ (Kruskal–Wallis ANOVA followed by Dunn's multiple comparison tests or Mann–Whitney U-test); †$P < 0.002$, Parkinson's disease versus multiple system atrophy in HMW α-Synuclein$_M$ (Mann–Whitney U-test).
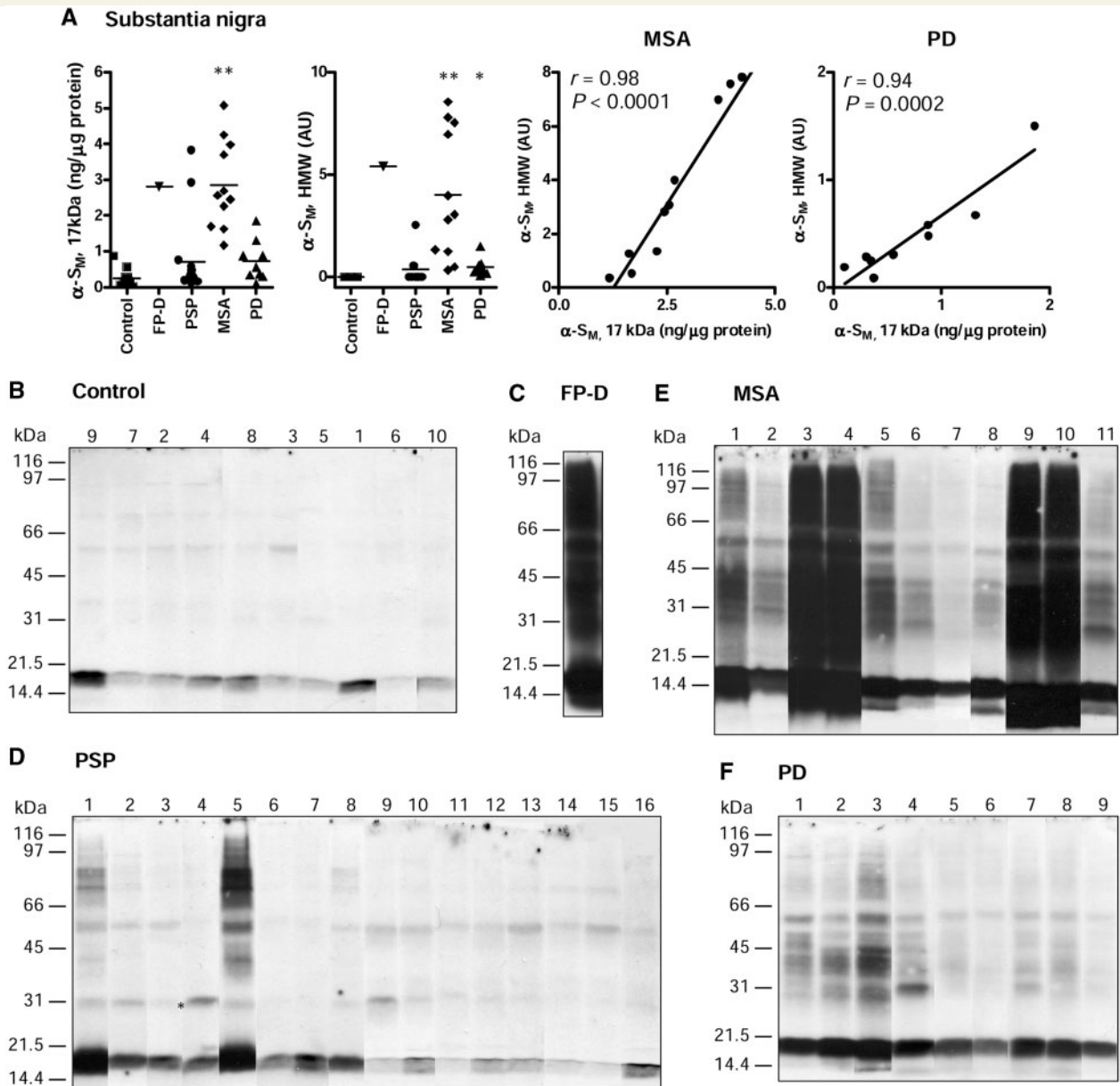
Figure 2 $\alpha$-Synuclein in membrane fraction ($\alpha$-$S_M$) of substantia nigra in patients with multiple system atrophy (MSA), Parkinson's disease (PD), progressive supranuclear palsy (PSP), familiar Parkinsonism-dementia with triplicated $\alpha$-synuclein locus (FP-D), and control subjects. (**A**) Scatter plots of levels of the 17 kDa and HMW $\alpha$-synuclein$_M$ and the correlations (Pearson). **$P<0.001$, multiple system atrophy versus control; *$P<0.05$, Parkinson's disease versus control (Kruskal–Wallis ANOVA followed by Dunn's multiple comparison tests). (**B**)–(**F**) Individual western blots (over-exposure) showing variability among subjects in HMW $\alpha$-synuclein$_M$ species. * Identifies a non-specific band at ~31 kDa recognized by the second antibody alone. Note two exceptional progressive supranuclear palsy cases (Cases 1 and 5) with increased 17 kDa and HMW $\alpha$-synuclein$_M$.

other brain regions examined normal. Control 11 showed mild HMW $\alpha$-synuclein$_M$ accumulation only in the frontal cortex (0.60 AU; normal 17 kDa, 0.99 ng/µg protein).

## $\alpha$-Synuclein in familial Parkinsonism-dementia

The patient with familial Parkinsonism-dementia showed marked $\alpha$-synuclein$_M$ accumulation throughout the brain (Table 2). For the

17 kDa $\alpha$-synuclein$_M$, the magnitude of increase was most marked in the substantia nigra (10-fold), intermediate in temporal and frontal cortices and striatum (2.2- to 4.8-fold), and least in the occipital cortex (+12%). All brain regions showed numerous HMW $\alpha$-synuclein$_M$ species (see Figs 2C, 3C and 5C), which were most prominent in the temporal cortex and least so in the occipital cortex. Across brain regions, there was a significant positive correlation (Pearson) between levels of HMW and 17 kDa $\alpha$-synuclein$_M$ ($r=0.83$, $P=0.04$). Levels of SNAP-25, a control
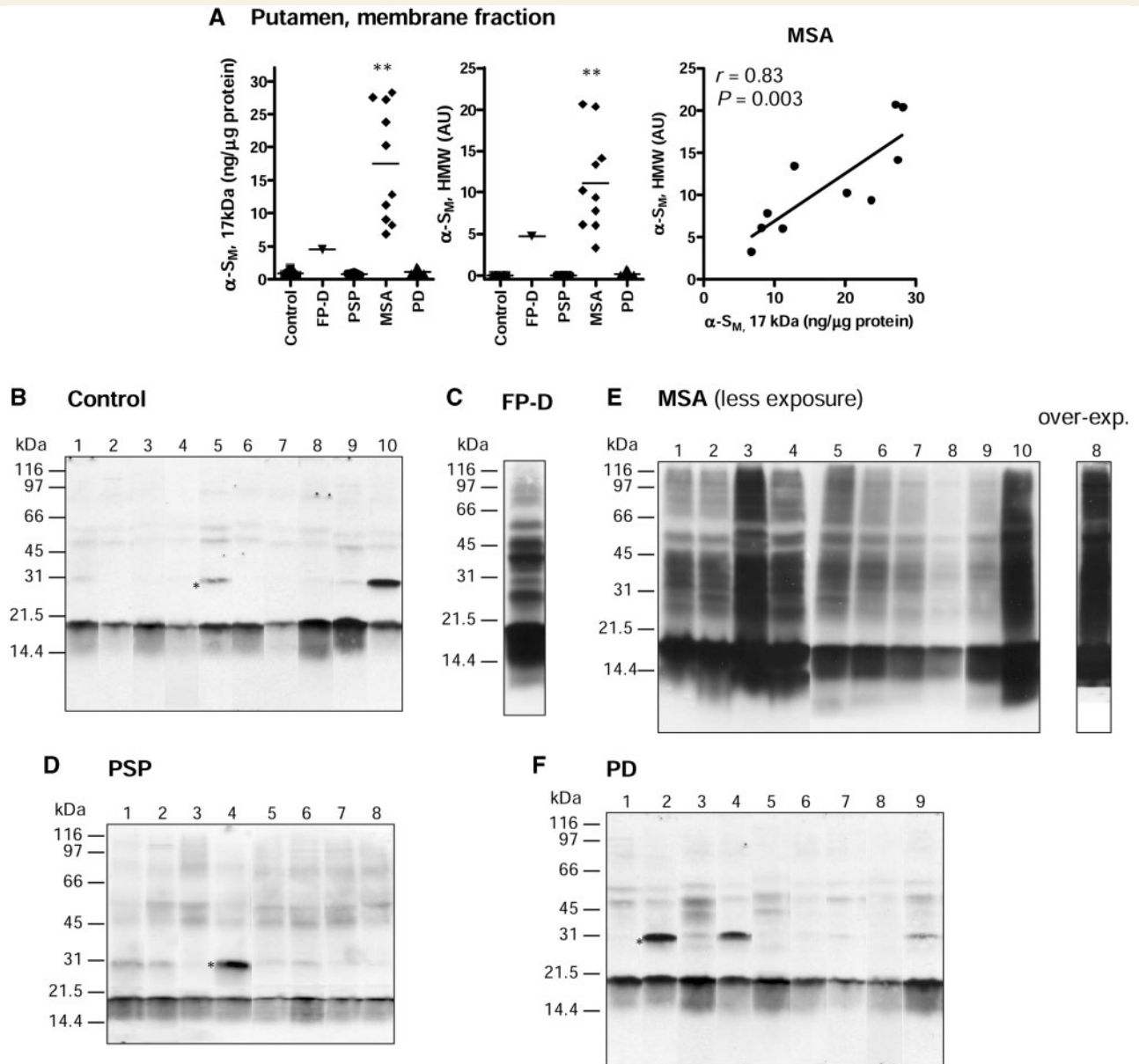
**Figure 3** α-Synuclein in membrane fraction (α-S$_M$) of putamen in patients with multiple system atrophy (MSA), Parkinson's disease (PD), progressive supranuclear palsy (PSP), familiar Parkinsonism-dementia with triplicated α-synuclein locus (FP-D), and control subjects. (**A**) Scatter plots of levels of 17 kDa and HMW α-synuclein$_M$ and the correlation (Pearson). **P < 0.001, multiple system atrophy versus control (Kruskal–Wallis ANOVA followed by Dunn's multiple comparison tests). (**B**)–(**F**) Individual western blots [over-exposure except that indicated in (**E**)] showing variability among subjects in HMW α-synuclein$_M$ species. * Identifies a non-specific band at ∼31 kDa recognized by the second antibody alone. Note modestly increased HMW species in three cases of Parkinson's disease (Cases 3, 5 and 9).

protein, were decreased by 27% in substantia nigra but were normal in putamen (Supplementary Figure).

In the cytosolic fraction, only 17 kDa α-synuclein could be detected and levels were normal in putamen (Fig. 4A) and increased (+115%) in the frontal cortex (25.5 versus 11.9 ± 1.1 ng/µg protein in controls). In contrast, levels of the control protein neuron specific enolase were normal in these brain areas (data not shown).

## α-Synuclein in progressive supranuclear palsy

In progressive supranuclear palsy, 17 kDa and HMW α-synuclein$_M$ species were generally normal (Table 2; see Figs 2D–5D). The increase (+171%) in 17 kDa α-synuclein$_M$ observed in the substantia nigra in progressive supranuclear palsy was explained by two progressive supranuclear palsy cases who had high levels of
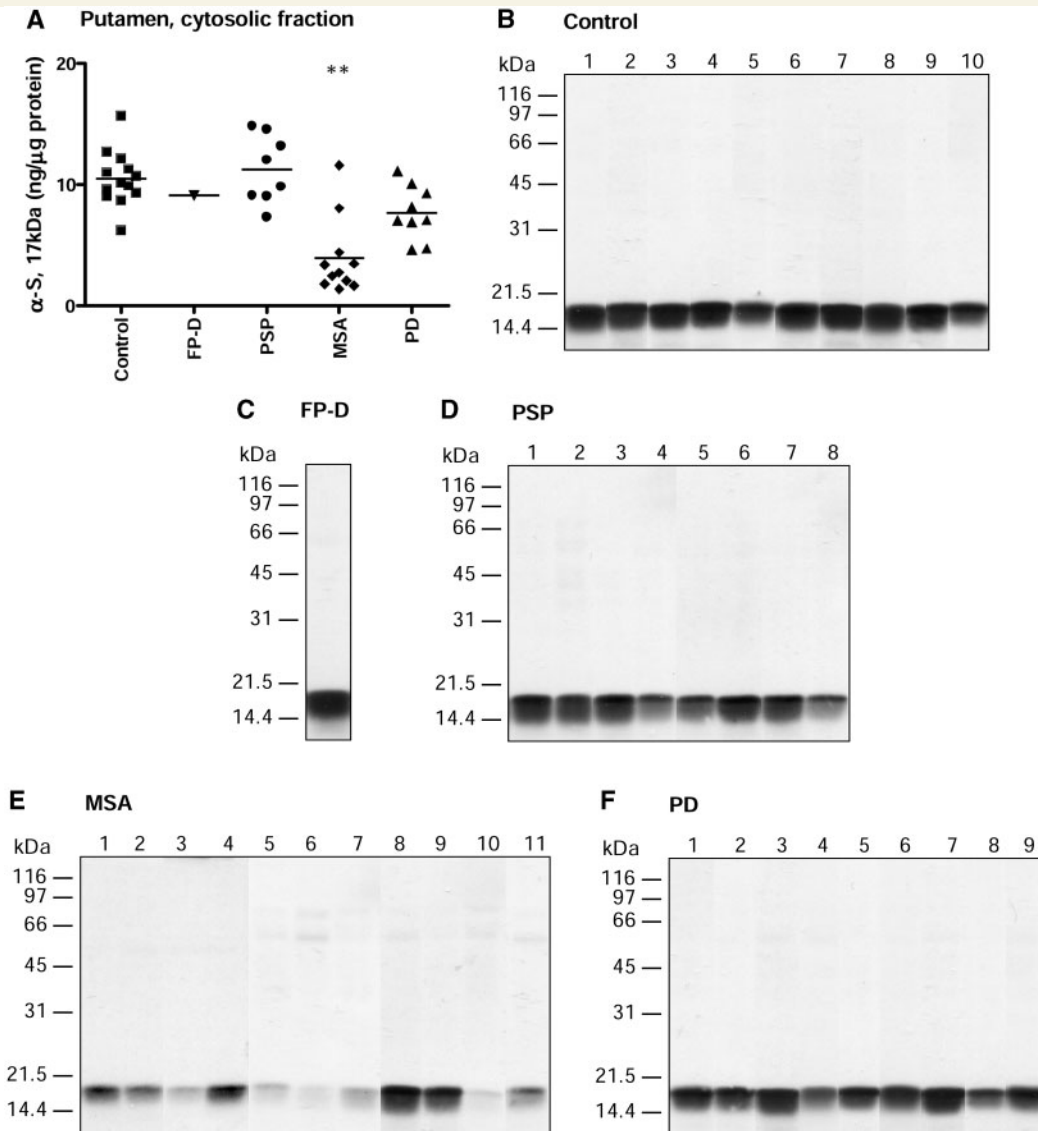
**Figure 4** $\alpha$-Synuclein in cytosolic fraction of putamen in patients with multiple system atrophy (MSA), Parkinson's disease (PD), progressive supranuclear palsy (PSP), familiar Parkinsonism-dementia with triplicated $\alpha$-synuclein locus (FP-D), and control subjects. (**A**) Scatter plots of levels of the full-length 17 kDa $\alpha$-synuclein. **$P < 0.001$, multiple system atrophy versus control (Kruskal–Wallis ANOVA followed by Dunn's multiple comparison tests). (**B**)–(**F**) Individual western blots (over-exposure) showing variability among subjects.

17 kDa $\alpha$-synuclein$_M$ (Cases 1 and 5, 2.92 and 3.83 ng/$\mu$g protein, respectively) similar to that in familial Parkinsonism-dementia (Fig. 2A) and moderate accumulation of HMW $\alpha$-synuclein$_M$ (0.52 and 2.54 AU). In the frontal cortex (Fig. 5D), another patient with progressive supranuclear palsy (Case 3) had moderate accumulation of HMW $\alpha$-synuclein$_M$ (2.72 AU) despite a normal level of 17 kDa $\alpha$-synuclein$_M$ (1.54 ng/$\mu$g protein). Levels of SNAP-25 were significantly decreased in the substantia nigra (−28%) but were normal in the putamen (Supplementary Figure).

## $\alpha$-Synuclein in multiple system atrophy

In multiple system atrophy, we observed heterogeneous $\alpha$-synuclein$_M$ accumulation both among brain regions (Table 2)

and individual subjects (Figs 2E–5E). The putamen had an 18-fold increase (range from +625% to +2900%) in 17 kDa $\alpha$-synuclein$_M$ and the highest levels of HMW species observed in our investigation (Fig. 3A). The white matter had a 23-fold increase (+430% to +6830%) in 17 kDa $\alpha$-synuclein$_M$; however, due to its low $\alpha$-synuclein content, levels of the HMW species were correspondingly much lower. The substantia nigra of multiple system atrophy showed a magnitude of $\alpha$-synuclein$_M$ accumulation similar to that of the familial Parkinsonism-dementia case, with a 10-fold increase in 17 kDa $\alpha$-synuclein$_M$ (+356% to +1850%) and a high level of HMW $\alpha$-synuclein$_M$ (Fig. 2A). In other brain regions, levels of 17 kDa $\alpha$-synuclein$_M$ were normal or only modestly increased (cerebral and cerebellar cortices, caudate: −4% to +95%, non-significant) but with caudate and
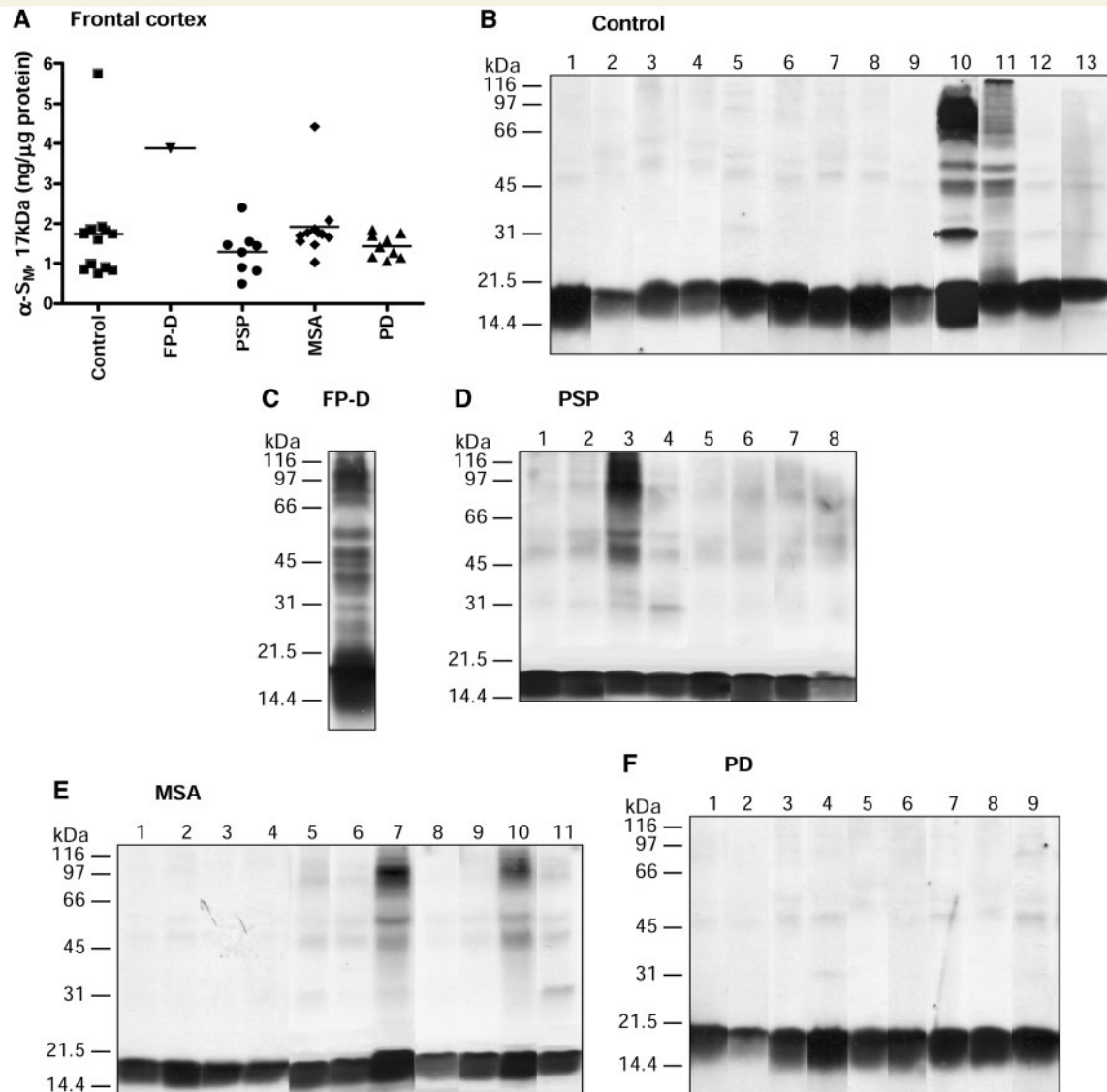
**Figure 5** α-Synuclein in membrane fraction (α-S$_M$) of frontal cortex in patients with multiple system atrophy (MSA), Parkinson's disease (PD), progressive supranuclear palsy (PSP), familiar Parkinsonism-dementia with triplicated α-synuclein locus (FP-D), and control subjects. (**A**) Scatter plots of levels of 17 kDa α-synuclein$_M$. (**B**)–(**F**) Individual western blots (over-exposure) showing variability among subjects. *Identifies a non-specific band at ~31 kDa recognized by the second antibody alone. Note increased HMW species in two control subjects 10 and 11, two multiple system atrophy Cases 7 and 10 and one progressive supranuclear palsy Case 3.

cerebellar cortex also showing mild to high levels of HMW species in all the cases (Table 2). Two cases (Cases 7 and 10, Fig. 5E) showed moderate accumulation of HMW α-synuclein$_M$ in the cerebral cortices (for occipital cortex, only in Case 7); however, for Case 7 the cerebral cortical α-synuclein$_M$ change was not correlated with that in subcortical regions (compare with Fig. 2E and 3E). The two multiple system atrophy cases differed from the subject with familial Parkinsonism-dementia in relative involvement of putamen (multiple system atrophy ≫ familial Parkinsonism-dementia) and cerebral cortices, in particular the temporal cortex (familial Parkinsonism-dementia ≫ multiple system atrophy).

Despite heterogeneity among individuals, there were significant positive correlations (Pearson) between levels of HMW and 17 kDa α-synuclein$_M$ in each brain region examined

($r = 0.67$–$0.98$, $P < 0.05$; see Figs. 2A and 3A for examples). Among the areas of pathology in multiple system atrophy, significant correlations (Pearson) in levels of the 17 kDa α-synuclein$_M$ were observed between putamen and substantia nigra ($r = 0.66$, $n = 10$, $P = 0.04$), caudate and white matter ($r = 0.78$, $n = 8$, $P = 0.02$), and caudate and cerebellar cortices ($r = 0.74$, $n = 10$, $P = 0.01$). In the striatum (putamen and caudate) but not in other brain regions, there were significant positive correlations (Spearman) between pathological ratings of neuronal loss/gliosis and levels of α-synuclein$_M$, both 17 kDa ($r = 0.84$, $n = 21$, $P < 0.0001$) and HMW species ($r = 0.75$, $n = 21$, $P < 0.0001$). Dopamine concentrations (see Supplementary Table 1) were severely reduced from control levels [-96% (−83% to −99.5%)] in putamen of all of the subjects whereas in the caudate, levels of
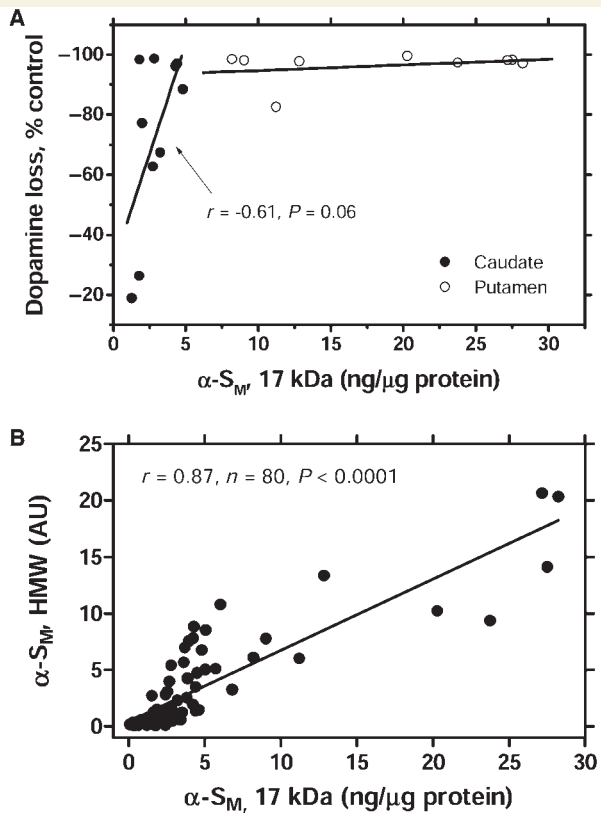
**Figure 6** (**A**) Correlations (Pearson) between levels of 17 kDa α-synuclein in membrane fraction (α-S$_M$) and dopamine loss in caudate and putamen of patients with multiple system atrophy (MSA). Dopamine levels in putamen of the multiple system atrophy patients were previously published (Tong *et al*., 2004). (**B**) Correlations (Pearson) between levels of 17 kDa α-synuclein$_M$ and HMW species. See Table 2 for number of cases for each brain regions included (*n* = 80 for total data points).

the neurotransmitter were variably decreased [−73% (−19% to −99%)]. In the caudate there was a trend for a positive correlation (Pearson) that just missed statistical significance (*P* = 0.06) between levels of 17 kDa α-synuclein$_M$ and dopamine loss (Fig. 6A).

In contrast to the α-synuclein levels, nigral concentrations of αB-crystallin in the same membrane fraction were not increased in all of the patients with multiple system atrophy [23.6 ± 2.2 (7.5–34.0) μg tissue standard/10 μg protein] compared with the normal control subjects [21.5 ± 2.7 (15.7–37.1)]. On the other hand, levels of SNAP-25 were significantly decreased in the membrane fraction of substantia nigra (−40%) and putamen (−42%) in multiple system atrophy (Supplementary Figure).

Examination of the cytosolic fraction of putamen and frontal cortex in the multiple system atrophy cases revealed a significant decrease in soluble 17 kDa α-synuclein levels in the putamen (by 63%; Fig. 4A and E) but not in the frontal cortex (10.3 ± 1.3 versus 11.9 ± 1.1 ng/μg protein in controls). Levels of neuron specific enolase in the cytosolic fraction of multiple system atrophy putamen were significantly decreased by 25%. After adjustment

for tissue and neuronal loss against neuron specific enolase, the multiple system atrophy putamen still had a significant 52% loss of cytosolic 17-kDa α-synuclein as compared with control values.

Comparing the subtypes of multiple system atrophy, there were trends (0.05 < *P* < 0.1) for higher levels of α-synuclein$_M$ accumulation in the striatonigral areas in multiple system atrophy with predominant Parkinsonian features than in multiple system atrophy with predominant cerebellar features (Supplementary Table 3).

## α-Synuclein in Parkinson's disease

Qualitatively, the substantia nigra of the patients with Parkinson's disease showed some generally 'modest' α-synuclein$_M$ accumulation (Fig. 2F), with levels of HMW and 17 kDa α-synuclein$_M$ significantly correlated with each other (Fig. 2A). Quantitatively, however, the magnitude of the increased 17 kDa α-synuclein$_M$ [+184%, (−60% to +618%)] was much less than that in multiple system atrophy and the increase was only statistically significant before correction for multiple comparison (*P* = 0.01, Mann–Whitney U-test). Examination of individual subject data revealed a wide scatter of values in which some values fell within the normal control range (Fig. 2A). Nigral levels of HMW α-synuclein$_M$ in Parkinson's disease were also significantly lower than in multiple system atrophy (Table 2). Levels of soluble 17 kDa α-synuclein in the cytosolic fraction of substantia nigra in Parkinson's disease were not significantly different from those of the controls (4.70 ± 0.44 versus 5.10 ± 0.55 ng/μg protein in controls). Nigral levels of SNAP-25 were normal in Parkinson's disease (Supplementary Figure).

In putamen of the patients with Parkinson's disease levels of the 17 kDa α-synuclein$_M$ (Table 2 and Fig. 3A) and SNAP-25 (Supplementary Figure) were normal. However, three Parkinson's disease patients (3, 5 and 9) had a modest accumulation of HMW α-synuclein$_M$ and also had the three highest levels of 17 kDa α-synuclein$_M$ in putamen of Parkinson's disease (Fig. 3F, see Table 2 and Supplementary Table 1 for individual values). In the cytosolic fraction, the putamen showed a slight, but not statistically significant reduction of soluble 17 kDa α-synuclein (by 27%) as compared with the controls (Fig. 4A and F).

The frontal cortex of Parkinson's disease was normal in 17 kDa α-synuclein immunoreactivity in both membrane (Table 2 and Fig. 5A) and cytosolic fractions (10.3 ± 1.4 versus 11.9 ± 1.1 ng/μg protein in controls) and was without any increased HMW species (Fig. 5F).

Employing all of the different brain regions examined with detectable HMW α-synuclein$_M$, there was a significant positive correlation (Pearson) between levels of HMW and 17 kDa α-synuclein$_M$ (Fig. 6B). No other statistically significant correlations (Spearman or Pearson) were observed between levels of α-synuclein$_M$ accumulation in any of the brain regions examined and retrospective data obtained from subject profiles including age, age of disease onset, post-mortem time, estimated disease duration, disease stage (Hoehn–Yahr for Parkinson's disease), striatal dopamine levels (for Parkinson's disease and progressive supranuclear palsy), and pathological ratings of neuronal

loss/gliosis and Lewy bodies (for Parkinson's disease nigra, see Supplementary Table 2).

# Discussion

The major finding of our study is that, in contrast to the global α-synuclein$_M$ (both 17 kDa and HMW species) accumulation in familial Parkinsonism-dementia with triplicated α-synuclein locus, the accumulation in Parkinson's disease, multiple system atrophy and progressive supranuclear palsy was, in general, specific to regions of pathology, with multiple system atrophy clearly having the most severe α-synuclein$_M$ accumulation. However, we also found high variability in protein levels amongst individual subjects, e.g. above-normal α-synuclein$_M$ in substantia nigra of some patients with progressive supranuclear palsy, in putamen of some Parkinson's disease cases, and in frontal cortex of some multiple system atrophy and progressive supranuclear palsy patients and even control subjects, which could relate to similar 'discrepancies' in the literature.

## Methodological considerations and limitations

We measured the full-length 17 kDa α-synuclein by quantitative immunoblotting whereas the HMW species could only be quantified in a crude semi-quantitative manner. Although it is reasonable to compare the HMW species, as a semi-quantitative estimate, between subjects, it is not possible to compare its absolute intensity to that of the 17 kDa band because of possibly different transfer efficiency and antibody reactivities. However, the good correlation between levels of the HMW species and 17 kDa α-synuclein$_M$ provides some justification for our semi-quantitative analyses and also suggests that an equilibrium might exist among the different pools of α-synuclein species and that analysis of the membrane-associated forms is relevant.

We focused primarily on a simple, crude membrane fraction preparation rather than complex sub-fractionations with multi-step centrifugations and extractions by different detergents, organic solvents and/or chaotropic reagents as used previously by many investigators (Table 1). Our rationale was to reduce as much as possible disturbance to endogenous α-synuclein because, given its natively unfolded structure, it can be vulnerable to the above mentioned reagents (Weinreb et al., 1996) and because our primary interest was the quantitative comparison of α-synuclein accumulation in different Parkinsonian conditions rather than a qualitative sub-fraction distribution. Recently, a sandwich enzyme-linked immunosorbent assays have been developed to measure in body fluids (cf. Mollenhauer et al., 2008) and in brain extracts levels of total α-synuclein and in particular of its soluble oligomers in the cytosolic fraction (Paleologou et al., 2009). Our assay did not include α-synuclein soluble oligomers, which, given its low abundance, might not be achieved by a quantitative western blotting procedure. In future, it would be of interest to establish whether levels of α-synuclein$_M$ are correlated with those of the soluble oligomers in the Parkinsonian brains. It should also be pointed out that compared with western blotting, enzyme-linked immunosorbent assay of total α-synuclein appeared to give lower values of concentration in blood (Barbour et al., 2008) and even more so in the brain (Paleologou et al., 2009 versus Iwai et al., 1995 and this study). The reason for the discrepancy is unknown but could be related to different reagents and procedures employed for brain α-synuclein extraction and the possible complex in vivo status of α-synuclein, e.g. interaction with other proteins including parkin (Choi et al., 2001), a proteasomal protein (Snyder et al., 2003), 14–3–3 protein (Xu et al., 2002), and DJ-1 (Meulener et al., 2005).

## α-Synuclein in normal human brain

Our observation in two (of 13) neurologically normal control subjects of α-synuclein$_M$ accumulation in cerebral cortices but not in subcortical areas including substantia nigra is somewhat surprising as it is generally assumed that 'incidental α-synuclein pathology' should propagate caudo-rostrally from the brainstem to the neocortex (Braak et al., 2003; McKeith et al., 2005; but see Jellinger, 2003; Attems and Jellinger, 2008; Kalaitzakis et al., 2008; Zaccai et al., 2008; see also Burke et al., 2008; Jellinger, 2008; Parkkinen et al., 2008 for discussions). In principle, the two cases might have incidental Lewy pathology in the cerebral cortices without the participation of midbrain, which could be related to normal aging without clinical consequence or a preclinical stage of, perhaps, a dementing illness.

## α-Synuclein accumulation in familial Parkinsonism-dementia

Our familial Parkinsonism-dementia case findings are consistent with western blot reports of the Iowa kindred (Gwinn-Hardy et al., 2000; Miller et al., 2004) and a Swedish-American family (Farrer et al., 2004). Interestingly, we demonstrated a massive accumulation of α-synuclein$_M$ in substantia nigra of the member of Iowa kindred whereas in another (clinically similar) member of the kindred the substantia nigra appeared to be much less affected by α-synuclein accumulation (see Fig. 7, Gwinn-Hardy et al., 2000). Pathological examination of both cases revealed severe neuronal loss and only a few Lewy bodies in substantia nigra. Possibly, shorter disease duration (9 versus 15 years) and/or older disease onset (46 versus 32 years old) in our case could explain the difference; although our study of multiple system atrophy and Parkinson's disease (see below) did not reveal a clear relationship between α-synuclein$_M$ accumulation and disease duration or age of onset. We also report here similar α-synuclein$_M$ accumulation in putamen and caudate of the familial Parkinsonism-dementia case (Table 2), which is consistent with the global nature of the disorder and similar extent of dopamine loss in the two striatal subdivisions of the subject (Muenter et al., 1998). Unexpectedly, our familial Parkinsonism-dementia patient with doubling in gene dosage showed a more than 2-fold increase in 17 kDa α-synuclein$_M$ in severely affected regions (e.g. 10-fold increase in substantia nigra). This suggests the presence of additional genetic and/or environmental factors that may modulate the steady-state level of α-synuclein$_M$ in this autosomal dominant disorder.

## α-Synuclein in progressive supranuclear palsy

We did not observe significant $\alpha$-synuclein$_M$ accumulation in most of the patients with progressive supranuclear palsy except in 2 (of 16) cases in substantia nigra and in a third subject in frontal cortex. These findings confirm histochemical observations of rare $\alpha$-synuclein pathology in progressive supranuclear palsy (Tsuboi *et al.*, 2001; Mori *et al.*, 2002). The exceptional cases might reflect incidental $\alpha$-synuclein pathology in normal aging (Tsuboi *et al.*, 2001), as we did observe occasional $\alpha$-synuclein$_M$ accumulation in two of our aged controls, or coexisting multiple system atrophy/Parkinson's disease-like $\alpha$-synuclein pathology (e.g. Lewy neurites) accompanying progressive supranuclear palsy (Mori *et al.*, 2002; Uchikado *et al.*, 2006) that was missed by our conventional methods.

## α-Synuclein accumulation in multiple system atrophy

To our knowledge, this is the first systematic and quantitative study of $\alpha$-synuclein accumulation in both white and grey matter (including especially substantia nigra) regions in a representative number of patients with multiple system atrophy. Our finding in putamen of a massive accumulation of $\alpha$-synuclein$_M$ is consistent with that of Dickson *et al.* (1999). The observation of mostly normal levels of $\alpha$-synuclein in the frontal cortex is similar to that of Tu *et al.* (1998). However, in a single case report, Dickson *et al.* (1999) demonstrated increased $\alpha$-synuclein (full-length and HMW) in the SDS-soluble fraction of nine grey matter regions including the frontal cortex, suggesting a global accumulation of $\alpha$-synuclein$_M$ in multiple system atrophy brain. In contrast, our findings, by examination of a more representative number of cases, suggest that $\alpha$-synuclein$_M$ accumulation, while showing marked individual heterogeneity, is generally regionally specific, with the putamen clearly having the most severe accumulation of $\alpha$-synuclein$_M$ (more in fact than in familial Parkinsonism-dementia) but with cerebral cortices generally spared. Nevertheless, we did find that in some multiple system atrophy cases (e.g. Cases 7 and 10), there was $\alpha$-synuclein$_M$ accumulation in cerebral cortices and, in this respect, are consistent with the single case observation of Dickson.

A limitation of our study is that GCIs were only examined in 5 of the 11 patients. Nevertheless, we did not observe any qualitative or quantitative differences in $\alpha$-synuclein$_M$ accumulation between the two sub-groups, consistent with our assessment of the multiple system atrophy cases. In addition, the extent of 17 kDa $\alpha$-synuclein$_M$ accumulation (expressed as %change) among the brain regions examined (white matter $\approx$ putamen $>$ substantia nigra $>$ cerebellar $>$ caudate $>$ cerebral cortices) correspond approximately to qualitative regional pathological findings (neuronal loss, gliosis and GCIs where available). However, the normal concentrations of $\alpha$B-crystallin in multiple system atrophy nigra suggest that the accumulation of monomeric $\alpha$-synuclein$_M$ is not merely caused by the formation of GCIs. Indeed, in a multiple system atrophy case with only occasional

brain GCIs (Case 9, Supplementary Table 2), the substantia nigra showed striking accumulation of $\alpha$-synuclein$_M$ (Fig. 2E).

In putamen, the brain area with the most severe $\alpha$-synuclein$_M$ accumulation and abundant GCIs, there was also a marked depletion of cytosolic $\alpha$-synuclein (Fig. 4A and E). These observations circumstantially support a possible role of increased $\alpha$-synuclein insolubility in the formation of GCIs in multiple system atrophy and are consistent with previous findings of Tu *et al.* (1998) and Campbell *et al.* (2001) (but see Dickson *et al.*, 1999).

## α-Synuclein accumulation in Parkinson's disease

Our data suggest that $\alpha$-synuclein$_M$ accumulation in Parkinson's disease is quite heterogeneous amongst individual subjects and is, on average, much less severe than in familial Parkinsonism-dementia and multiple system atrophy, with the substantia nigra bearing the brunt of the changes.

The literature has been contradictory on the question of nigral $\alpha$-synuclein accumulation in Parkinson's disease (Table 1), in which findings appear to be dependent on the methods used for tissue extraction. Similarly, $\alpha$-synuclein mRNA expression data in this key pathological brain region of Parkinson's disease are also not consistent (increased: Rockenstein *et al.*, 2001; Chiba-Falek *et al.*, 2006; Grundemann *et al.*, 2008; unchanged: Tan *et al.*, 2005; decreased: Kingsbury *et al.*, 2004; Papapetropoulos *et al.*, 2007; Simunovic *et al.*, 2008; Bossers *et al.*, 2009). However, our findings are consistent with western blot reports showing an increase in the membrane-associated $\alpha$-synuclein in Parkinson's disease substantia nigra (full-length plus HMW, not necessarily full-length alone; Campbell *et al.*, 2001; Xu *et al.*, 2002; Liu *et al.*, 2005; Devi *et al.*, 2008) but normal levels in the cytosolic fraction (Campbell *et al.*, 2001; Liu *et al.*, 2005). The heterogeneity we observed in nigral $\alpha$-synuclein$_M$ levels (Fig. 2A; see also below), with many values falling within the range of normal controls, is also consistent with the contradictory reports in the literature (Table 1). We did not observe any correlation between $\alpha$-synuclein$_M$ accumulation and nigral Lewy body intensity (Supplementary Table 2). This observation is in agreement with some findings in the cerebral cortex in patients with dementia with Lewy bodies (Klucken *et al.*, 2006; but see Li *et al.*, 2005), although we are cautious in this interpretation due to a lack of quantitative assessment of Lewy bodies and Lewy neurites. Further studies by $\alpha$-synuclein immunohistochemistry would help resolve this question in Parkinson's disease.

We did not observe regionally wide-spread, extra-nigral $\alpha$-synuclein$_M$ accumulation in Parkinson's disease as suggested by some recent immunohistochemical reports (Hishikawa *et al.*, 2001; Duda *et al.*, 2002; Braak *et al.*, 2003, 2007; Mori *et al.*, 2008) except for some mildly increased HMW $\alpha$-synuclein species in putamen in three subjects (Fig. 3F). Our observation is more in line with the classic brainstem-predominant Lewy body pathology in idiopathic Parkinson's disease, i.e. low incidence of Lewy bodies in the striatum and frontal cortex. There are several possible explanations for the apparent discrepancy between the neurochemistry and histochemistry findings. First, our cases had been screened by

pathological examination to exclude those with diffuse Lewy bodies and therefore we aimed to represent only the 'classical' brainstem-predominant type of Parkinson's disease, i.e. at Braak stage III/IV. Indeed, in a case of dementia with Lewy bodies (data not shown), much higher levels of α-synuclein$_M$ accumulation (both 17 KDa and HMW) were observed in the putamen in addition to those in cerebral cortices, consistent with immunohistochemical findings. In this respect, we suggest that some of the variability in the literature on α-synuclein status in Parkinson's disease might be explained by the failure to distinguish, in some studies, between classic brainstem-predominant Lewy body disease and dementia with Lewy bodies. The variance observed in our study could also involve inclusion of subjects with and without cognitive impairment. Second, there are differences between cortical/striatal Lewy bodies/Lewy neurites and brainstem Lewy bodies in Parkinson's disease, including size, density, staining pattern, and antibody reactivities, which might make α-synuclein-positive inclusions in cerebral cortex/striatum in Parkinson's disease less resistant to extraction and evade our neurochemical detection. However, cortical Lewy bodies as observed in dementia with Lewy bodies appear to be as 'tough' as those in the substantia nigra in Parkinson's disease (Campbell et al., 2001). Finally, the evidence that detection of wide-spread α-synuclein immunoreactive pathology, in particular in oligodendrocytes (Hishikawa et al., 2001) or astrocytes (Braak et al., 2007) in Parkinson's disease depends on the type of antibody employed or a special pretreatment (Duda et al., 2002; Braak et al., 2007) suggests that the α-synuclein pathology revealed by immunohistochemistry might not be caused by α-synuclein$_M$ accumulation but rather by conformational changes or changes in partnership status of α-synuclein. Surprisingly, the antibodies employed in the histochemical study did not recognize the massive α-synuclein$_M$ accumulation in putamen of multiple system atrophy (Duda et al., 2002) found in the present study, suggesting possible differences in neuronal (Lewy bodies) and glial (GCIs) α-synuclein conformation/accumulation (cf. Croisier et al., 2006).

## Multiple system atrophy versus Parkinson's disease

We now show in a single comparative investigation striking quantitative differences in α-synuclein$_M$ accumulation between Parkinson's disease and multiple system atrophy, in particular in substantia nigra (Fig. 2). This much more severe α-synuclein$_M$ accumulation, together with wider regional involvement, might in part underlie the more aggressive progress of disease in multiple system atrophy than in Parkinson's disease, although we did not observe a significant correlation between levels of α-synuclein$_M$ and estimated duration of either disease. On the other hand, the more prolonged disease duration in Parkinson's disease might have caused gradual loss of Lewy bodies and α-synuclein with the loss of dopamine neurons, resulting in less α-synuclein$_M$ accumulation at an advanced stage. This relationship between α-synuclein accumulation and disease progression between the disorders was also noted for dementia with Lewy bodies and Parkinson's disease with dementia, with the former having higher levels of α-synuclein pathology and shorter disease duration (Jellinger and Attems, 2006).

Our observation in multiple system atrophy suggests a positive association between striatal (caudate and putamen), but not substantia nigra, dopamine loss and α-synuclein$_M$ accumulation in the striatum. This is indicated by the much more marked increase in α-synuclein$_M$ levels in the putamen versus the caudate, in which the putamen was more affected by dopamine loss, and the positive correlation, that just missed statistical significance, between 17 kDa α-synuclein$_M$ levels and dopamine loss in caudate (the striatal subdivision in which there was some variability in dopamine concentrations). In addition, α-synuclein$_M$ accumulation was also correlated with neuronal loss/gliosis in the striatum. These findings suggest that there might be a causal relationship between striatal α-synuclein pathology and dopamine neuronal terminal damage, perhaps also suggesting a dying-back (striatum to nigra) degenerative mechanism in multiple system atrophy. We did not observe such correlations in Parkinson's disease. Indeed, there was a non-significant trend for a positive correlation (Pearson) between levels of the 17 kDa α-synuclein$_M$ in the substantia nigra and dopamine levels in the caudate in Parkinson's disease ($r = 0.57$, $P = 0.11$). In this respect our data are consistent with other findings pointing towards a lack of correlation between α-synuclein pathology and clinical symptoms in Lewy body disorders (Parkkinen et al., 2005, 2008; Burke et al., 2008; Jellinger, 2008). Indeed, the presence of α-synuclein immunoreactive Lewy inclusions could represent a neuroprotective process (Kramer and Schulz-Schaeffer, 2007; Burke et al., 2008). Although our finding might seem at variance with a recent observation by Kovacs et al. (2008) of an inverse correlation between nigral α-synuclein burden and concentration of a dopamine marker (dopamine transporter) in putamen, this investigation did not distinguish (as was our aim) between brainstem-predominant Lewy body disorders and dementia with Lewy bodies, thus allowing for the possibility of a lack of correlation between α-synuclein and brain pathology in classic Parkinson's disease (see also Jellinger, 2008). Further studies are needed to address the fundamental question whether it is dysregulated α-synuclein metabolism that does in fact drive the pathology of Parkinson's disease.

## Funding

## Supplementary material

Supplementary material is available at Brain online.

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