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Axonal α-synuclein aggregates herald centripetal degeneration of cardiac sympathetic nerve in Parkinson's disease

Satoshi Orimo,[1,2] Toshiki Uchihara,[2] Ayako Nakamura,[2] Fumiaki Mori,[3] Akiyoshi Kakita,[4] Koichi Wakabayashi[3] and Hitoshi Takahashi[4]

[1]Department of Neurology, Kanto Central Hospital, 6-25-1 Kami-Yoga, Setagaya-ku, 158-8531, [2]Department of Neurology, Tokyo Metropolitan Institute for Neuroscience, Tokyo, [3]Department of Neuropathology, Institute of Brain Science, Hirosaki University School of Medicine, Hirosaki and [4]Department of Pathology, Brain Research Institute, University of Niigata, Niigata, Japan

Correspondence to: Dr Satoshi Orimo, Department of Neurology, Kanto Central Hospital, 6-25-1 Kami-Yoga, Setagaya-ku, 158-8531 Tokyo, Japan.
E-mail: orimo@kanto-ctr-hsp.com

Degeneration of the cardiac sympathetic nerve occurs in both Parkinson's disease (PD) and dementia with Lewy bodies and begins early in the disease progression of PD, accounting for reduced cardiac uptake of *meta*-iodobenzylguanidine even in the early stages of Lewy body disease (LBD). We previously demonstrated that degeneration of the distal axons of the cardiac sympathetic nerve precedes loss of their mother neurons in the paravertebral sympathetic ganglia, suggesting distal dominant degeneration of the cardiac sympathetic nerve in PD. Because α-synuclein is one of the key molecules in the pathogenesis of this disease, we further investigated how α-synuclein aggregates are involved in this distal-dominant degeneration. Both cardiac tissues and paravertebral sympathetic ganglia were obtained for comparison from 20 patients with incidental Lewy body disease (ILBD), 10 with PD, 20 with multiple system atrophy (MSA) and 10 control subjects. Immunohistochemical analysis was performed using antibodies against tyrosine hydroxylase (TH) as a marker for sympathetic nerves, phosphorylated neurofilament as a marker for axons and phosphorylated α-synuclein for pathological deposits. We found that (i) α-synuclein aggregates in the epicardial nerve fascicles, namely the distal axons of the cardiac sympathetic nerve, were much more abundant in ILBD with preserved TH-ir axons than in this disease with decreased TH-ir axons and PD; (ii) α-synuclein aggregates in the epicardial nerve fascicles were closely related to the disappearance of TH-ir axons; (iii) in ILBD with preserved TH-ir axons, α-synuclein aggregates were consistently more abundant in the epicardial nerve fascicles than in the paravertebral sympathetic ganglia; (iv) this distal-dominant accumulation of α-synuclein aggregates was reversed in ILBD with decreased TH-ir axons and PD, which both showed fewer of these axons but more abundant α-synuclein aggregates in the paravertebral sympathetic ganglia and (v) MSA was completely different from ILBD and PD based on the preservation of TH-ir axons and the scarcity of α-synuclein aggregates in either the cardiac tissues or the paravertebral sympathetic ganglia. These findings indicate that accumulation of α-synuclein aggregates in the distal axons of the cardiac sympathetic nervous system precedes that of neuronal somata or neurites in the paravertebral sympathetic ganglia and that heralds centripetal degeneration of the cardiac sympathetic nerve in PD, which sharply contrasts with slight changes in MSA. This chronological and dynamic relationship between α-synuclein aggregates and distal-dominant degeneration of the cardiac sympathetic nervous system may represent the pathological mechanism underlying a common degenerative process in PD.

Keywords: α-synuclein; cardiac sympathetic nerve; paravertebral sympathetic ganglia; Parkinson's disease; multiple system atrophy

Abbreviations: ABC = avidin–biotin–peroxidase complex; ILBD = incidental Lewy body disease; ir = immunoreactive; MIBG = *meta*-iodobenzylguanidine; MSA = multiple system atrophy; NF = neurofilament; PD = Parkinson's disease; TH = tyrosine hydroxylase

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Introduction

The neuropathological hallmark of Parkinson's disease (PD) is the presence of neuronal intracytoplasmic inclusions known as Lewy bodies (LB) and Lewy neurites in various regions of the nervous system. α-Synuclein is a pre-synaptic protein that maintains synaptic integrity and functions together with regulation of dopamine synthesis (Abeliovich *et al*., 2000; Perez *et al*., 2002; Chandra *et al*., 2004) and is a major constituent of LB and Lewy neurites in PD and dementia with LB (Spillantini *et al*., 1997; Wakabayashi *et al*., 1997; Baba *et al*., 1998) as well as glial cytoplasmic inclusions in multiple system atrophy (MSA) (Gai *et al*., 1998). Therefore, PD, dementia with LB and MSA are collectively grouped as α-synucleinopathies (Galvin *et al*., 2001), while pathological mechanisms that may distinguish these entities remain to be clarified. Using α-synuclein immnohistochemistry, Braak and colleagues reported detailed pathological stages for the progression of PD. Early pathological changes in the brain begins in the lower part of the brainstem, especially in the dorsal vagal nucleus or the olfactory bulb (anterior olfactory nucleus) before nigral involvement occurs (Braak *et al*., 2003). Bloch and colleagues reported that the autonomic nuclei of the spinal cord and the peripheral autonomic nervous system are the earliest affected structures next to the lower brainstem and the olfactory bulb (Bloch *et al*., 2006).

Reduced cardiac uptake of *meta*-iodobenzylguanidine (MIBG), a physiological analogue of noradrenaline (Wieland *et al*., 1980), on [^{123}I] MIBG myocardial scintigraphy or that of fluorodopamine on 6-[^{18}F] fluorodopamine positron emission tomography have been reported in patients with PD, dementia with LB, pure autonomic failure and α-*synuclein*-associated PD (Goldstein *et al*., 1997; Yoshida *et al*., 1997; Yoshita, 1998; Braune *et al*.,1999; Orimo *et al*., 1999; Druschky *et al*., 2000; Taki *et al*., 2000; Watanabe *et al*., 2001; Yoshita *et al*., 2001; Singleton *et al*., 2004). We demonstrated that degeneration of the cardiac sympathetic nerve is profound in the presence of LB, as in PD and dementia with LB (Orimo et al., 2005*a*). Moreover, degeneration begins in early in the disease process of PD, accounting for the reduced cardiac MIBG uptake even in the early stages of Lewy body disease (LBD) (Orimo et al., 2007*b*). This is in sharp contrast with good preservation of both MIBG uptake and axons of the cardiac sympathetic nerve in the absence of LB as in most cases of MSA, progressive supranuclear palsy, Alzheimer's disease and *Parkin*-associated PD (Orimo *et al*., 2005*a*, *b*). Moreover, we demonstrated that degeneration of the cardiac sympathetic nerve precedes neuronal cell loss of the paravertebral sympathetic ganglia, suggesting distal-dominant degeneration in this pathological mechanism (Orimo et al., 2005*a*). Iwanaga and colleagues reported α-synuclein-immunoreactive (ir) LB and Lewy neurites in the cardiac plexus of PD and incidental Lewy body disease (ILBD) (Iwanaga *et al*., 1999). However, the significance of α-synuclein aggregates in the pathological mechanism underlying the degenerative process of the cardiac sympathetic nervous system remains to be clarified. Moreover, if α-synuclein aggregates accumulate in both cardiac tissues and the paravertebral sympathetic ganglia, in which location does α-synuclein accumulate earlier?

Slight reduction of cardiac uptake of MIBG has also been reported in some patients with MSA (Yoshita, 1998; Orimo *et al*., 1999; Braune *et al*, 1999; Druschky *et al*., 2000; Taki *et al*., 2000). We previously examined the cardiac tissues and paravertebral sympathetic ganglia from 15 patients with MSA immunohistochemically and reported that the mild degeneration of the cardiac sympathetic nerve can occur, accounting for the slight reduction of cardiac uptake of MIBG (Orimo *et al*., 2007*a*). However, whether α-synuclein aggregates are related to mild degeneration of the cardiac sympathetic nerve remains to be clarified. Although PD and MSA are collectively grouped as α-synucleinopathies, we hypothesized that these distinct patterns of degeneration of the cardiac sympathetic nervous system are related to different patterns of accumulation of α-synuclein aggregates in these two diseases.

In this study, in order to clarify the significance of α-synuclein aggregates in the pathological mechanism underlying the degenerative process of the cardiac sympathetic nervous system in PD and MSA, we immunohistochemically examined cardiac tissues and the paravertebral sympathetic ganglia from patients with ILBD, PD and MSA.

Materials and Methods

Subjects

Tissue samples were obtained from the Department of Pathology, Brain Research Institute, University of Niigata. Patients with ILBD were defined as having no history of Parkinsonian symptoms or signs despite the presence of LB in the substantia nigra and/or locus coeruleus on routine histopathology or α-synuclein immunohistochemistry. Each patient with ILBD was rated as stage 2 or 3 based on Braak staging (Braak *et al*., 2003). The pathological diagnosis of PD and that of MSA were confirmed by α-synuclein immunohistochemistry. Control subjects without neurodegenerative disorders, heart diseases or diabetes mellitus were also examined. The post-mortem interval was within 12 h in all patients and control subjects. The summary of the clinical characteristics in each subject is shown in Table 1. The mean ages of ILBD ($n = 20$, 72.6 ± 7.7 years) and MSA ($n = 20$, 67.6 ± 5.9 years) did not significantly differ from that of the controls (67.0 ± 10.4 years), whereas the mean age of PD ($n = 10$, 76.8 ± 9.0 years) was significantly higher than that of the controls ($P < 0.05$). Patients with ILBD and 15 of 20 patients with MSA were from the series we reported previously (Orimo *et al*., 2007*a*, *b*).

Immunohistochemistry

In each of the 60 subjects, blocks were taken from the anterior wall of the left ventricle and the paravertebral sympathetic ganglia (stellate or upper thoracic) as previously reported (Orimo *et al*., 2005*a*). Tissues were fixed with formalin for 3–4 weeks, embedded in paraffin, sectioned at a thickness of 4 μm, and stained with hematoxylin and eosin. Other sections were immunostained with

Table 1 Clinical characteristics and immnohistochemical findings

Patient	Diagnosis	Age	Sex	Heart					SG
				TH	NF	syn + F/F	%	Score	Score
1	ILBD(a)	70	m	3	3	34/34	100	3	2
2	ILBD(a)	58	f	3	3	29/30	96.7	2	1
3	ILBD(a)	87	f	3	3	15/18	83.3	2	1
4	ILBD(a)	66	m	2/3	3	0/40	0	0	0
5	ILBD(a)	62	f	2/3	3	11/13	84.6	2	1
6	ILBD(a)	80	m	2/3	2/3	14/14	100	3	0.5
7	ILBD(a)	72	m	2/3	3	20/20	100	2.5	2
8	ILBD(a)	79	m	2/3	3	12/18	66.7	2	0
9	ILBD(a)	71	m	2/3	2/3	15/15	100	2.5	1
10	ILBD(a)	76	m	2/3	2/3	30/30	100	2	1
11	ILBD(b)	61	m	2	2	1/7	14.3	1	1
12	ILBD(b)	74	m	2	2/3	0/16	0	0	0
13	ILBD(b)	77	m	2	2	0/1	0	0	0
14	ILBD(b)	75	m	1/2	2	4/38	10.5	1	2
15	ILBD(b)	75	m	0/2	1/2	18/21	85.7	1.5	1
16	ILBD(b)	76	f	0/1	2	2/10	20	1	0
17	ILBD(b)	62	f	0	2	6/12	50	1	1
18	ILBD(b)	69	m	0	2	2/10	20	1	2
19	ILBD(b)	81	f	0	1/2	0/4	0	0	1
20	ILBD(b)	79	f	0	0	1/4	25	1	3
21	PD	61	m	0	1/2	3/24	12.5	1	2
22	PD	74	m	0	1	12/29	24.2	1	3
23	PD	68	f	0	0/1	4/23	17.4	1	3
24	PD	84	f	0	0/1	4/36	11.1	1	3
25	PD	75	m	0	0	3/17	17.6	1	3
26	PD	80	f	0	2	3/12	25	1	n.e.
27	PD	70	m	0	1/2	0/8	0	0	n.e.
28	PD	83	f	0	1/2	0/13	0	0	n.e.
29	PD	92	f	0	1/2	0/20	0	0	n.e.
30	PD	81	f	0	0	0/28	0	0	n.e.
31	MSA-C	58	m	3	3	0/3	0	0	n.e
32	MSA-P	63	m	3	3	0/11	0	0	0
33	MSA-P	63	f	3	3	0/3	0	0	n.e
34	MSA-C	65	m	3	3	0/7	0	0	n.e
35	MSA-C	66	m	3	3	0/4	0	0	n.e
36	MSA-C	72	f	3	3	0/8	0	0	0
37	MSA-P	62	f	2/3	3	0/11	0	0	0
38	MSA-C	65	f	2/3	3	0/23	0	0	0
39	MSA-C	65	m	2/3	3	0/30	0	0	1
40	MSA-C	69	m	2/3	3	0/19	0	0	0
41	MSA-C	72	m	2/3	3	0/20	0	0	0
42	MSA-C	79	m	2/3	3	0/14	0	0	0
43	MSA-C	59	m	2	3	0/22	0	0	0
44	MSA-C	67	f	2	3	0/10	0	0	n.e
45	MSA-P	72	m	2	3	0/14	0	0	0
46	MSA-C	72	f	2	3	0/13	0	0	0
47	MSA-C	73	f	2	3	3/9	33.3	1	1
48	MSA-C	60	m	1/2	2/3	0/13	0	0	0
49	MSA-C	77	m	1/2	2/3	0/4	0	0	0
50	MSA-C	72	m	0/1	0/1	2/10	20	1	2
C1	C	55	f	3	3	0/13	0	0	0
C2	C	55	m	3	3	0/23	0	0	0
C3	C	62	m	3	3	0/3	0	0	0
C4	C	75	f	3	3	0/14	0	0	0
C5	C	77	m	3	3	0/13	0	0	0
C6	C	83	f	3	3	0/7	0	0	0
C7	C	60	m	2/3	3	0/10	0	0	0
C8	C	61	f	2/3	3	0/11	0	0	0
C9	C	63	m	2/3	2/3	0/13	0	0	0
C10	C	79	m	2/3	3	0/42	0	0	0

ILBD(a) = incidental Lewy body diseases with preserved tyrosine hydroxylase (TH)-immunoreactive (ir) axons; ILBD(b) = incidental Lewy body diseases with decreased TH-ir axons; PD = Parkinson's disease; NF = neurofilament; syn+F = the number of α-synuclein positive fascicles; F = the number of fascicles examined; SG = paravertebral sympathetic ganglia; n.e: Not examined; see text for detailed semi-quantification; MSA-C = multiple system atrophy cerebellar; MSA-P = multiple system atrophy parkinsonism; C = control.

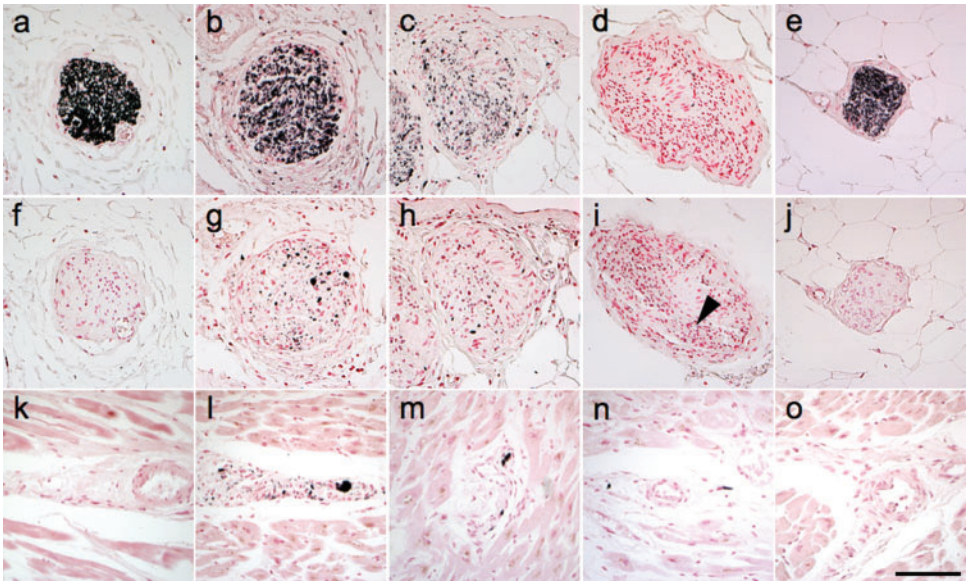

Fig. 1 α-Synuclein and tyrosine hydroxylase (TH) immunostaining in ILBD, PD, MSA and control subjects. In controls, TH-immunoreactive (ir) axons were abundant (a) but there were no α-synuclein aggregates observed in the epicardial nerve fascicles (f) or in the myocardium (k). In ILBD with preserved TH-ir axons (b), α-synuclein aggregates were more abundant (g, l) than in ILBD with decreased TH-ir axons (c) (h, m). In PD, TH-ir axons were absent to nearly absent (d) and α-synuclein aggregates were absent to sparse (i, n). The arrowhead indicates α-synuclein aggregates (i). In MSA, TH-ir axons were abundant (e) but α-synuclein aggregates were absent (j, o). a, f, k = control I; b, g, l = patient I; c, h, m = patient I4; d, j, n = patient 22; e, j, o = patient 32, a, b, c, d, e = TH; f, g, h, i, j = α-synuclein (epicardial nerve fascicle); k, l, m, n, o = α-synuclein (myocardium); ILBD = incidental Lewy body disease, PD = Parkinson's disease, MSA = multiple system atrophy, Bar indicates 100 μm.

monoclonal antibodies against tyrosine hydroxylase (TH) (TH16; Sigma, St Louis, MO, USA; 1: 3000), phosphorylated neurofilament (NF) (SMI-31; Sternberger Immunochemicals, Baltimore, MD, USA; 1: 10 000) or phosphorylated α-synuclein (#64; WAKO, Osaka, Japan; 1:5000)(Fujiwara *et al.*, 2002), using the avidin–biotin–peroxidase complex (ABC) method with a Vectastain ABC kit (Vector, Burlingame, CA, USA). Peroxidase labelling was visualized with diaminobenzidine with nickel as a chromogen, and then stained sections were lightly counterstained with nuclear fast red solution.

For double immunofluorolabelling, sections were incubated with a mixture of anti-phosphorylated α-synuclein mouse monoclonal antibody (1: 2000) and anti-TH rabbit polyclonal antibody (CA-101bTHrab, Protos Biotech Corp.; 1: 1000) at 4°C for 2 days. These antibodies were visualized with a mixture of anti-mouse IgG made in sheep conjugated with Alexa546 (Molecular Probe. Eugene, OR, USA; 1:200) and anti-rabbit IgG made in goat conjugated with Alexa488 (Molecular Probe; 1:200).

Semi-quantification

The numbers of TH- and NF-ir axons of epicardial nerve fascicles were assessed using a semi-quantitative rating scale: −, absent or nearly absent (0); +, sparse (1); ++, moderate (2); +++, numerous (3) as described elsewhere (Orimo *et al.*, 2007*b*). If fascicles showing 'rating scales 2 and 3' were almost evenly distributed in the subject, we described the rating scale of the subject as 2/3. The severity of α-synuclein aggregates in the epicardial nerve fascicles and in the paravertebral sympathetic ganglia was assessed semi-quantitatively as follows: −, absent or not discernible (score: 0); +, slight (score: 1); ++, moderate (score: 2); +++, severe (score: 3). If fascicles showing 'severity scores 2 and 3' were almost evenly distributed in the subject, we described the severity score of the subject as 2.5 to facilitate accurate statistical analysis.

Statistical analysis

The values for semi-quantification of α-synuclein aggregates and the frequency of epicardial nerve fascicle with α-synuclein aggregates relative to the number of total fascicles were expressed as means ± standard error (SEM). Significance of differences in the average in each group was analysed by the Mann–Whitney U-test. A P-value <0.05 was considered significant.

Results

Cardiac tissues

Summary of the semi-quantitative rating scale for TH- and NF-ir axons, and their relations to α-synuclein aggregates in the epicardial nerve fascicles in patients with ILBD, PD, MSA and control subjects are shown in Table 1.

In controls, numerous TH- (Fig. 1a) and NF-ir axons were seen both in the epicardial nerve fascicles and in the myocardium. However, there were no α-synuclein aggregates observed in either the epicardial nerve fascicles (Fig. 1f) or myocardium (Fig. 1k). In ILBD, the number of TH- and NF-ir axons varied among patients. In 10 ILBD patients [patients 1–10, ILBD(a)], the number of TH- (Fig. 1b) and NF-ir axons ranged from moderate to numerous as seen in the controls, and α-synuclein aggregates were observed in 9 of these 10 patients, both

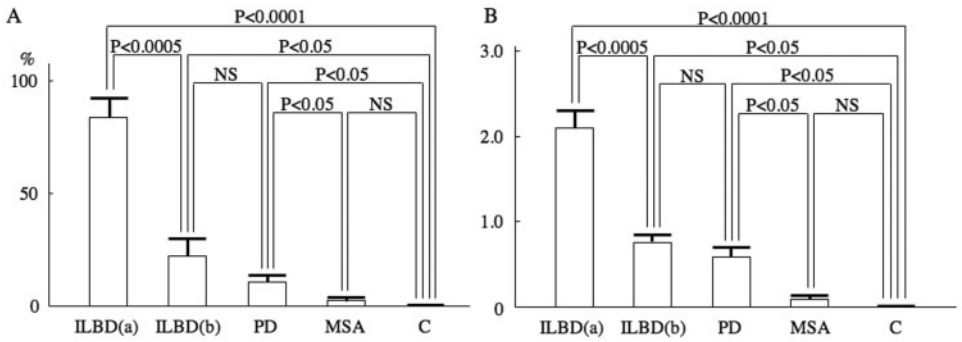

Fig. 2 Comparison of the relative frequencies of epicardial nerve fascicles with α-synuclein aggregates (**A**) and the severity score of α-synuclein aggregates (**B**). The relative frequency of epicardial nerve fascicles with α-synuclein aggregates and the severity score of α-synuclein aggregates in ILBD with preserved TH-ir axons were significantly higher than that in ILBD with decreased TH-ir axons ($P < 0.0005/P < 0.0005$), PD ($P < 0.0001/P < 0.0005$), MSA ($P < 0.0001/P < 0.0001$) or controls (C) ($P < 0.0001/P < 0.0001$), respectively.

in the epicardial nerve fascicles (Fig. 1g) and in the myocardium (Fig. 1l, patients 1–3, 5–10). The severity of α-synuclein aggregates was moderate to severe (score: 0–3; 2.1 ± 0.22), and the relative frequency of epicardial nerve fascicles with α-synuclein aggregates ranged from 0% to 100% ($83.1 \pm 14.5\%$). In the remaining 10 ILBD patients [patients 11–20, ILBD(b)], the number of TH-ir axons ranged from sparse to moderate (Fig. 1c) in four patients (patients 11–14), and absent or nearly absent to sparse in six patients (patients 15–20). α-Synuclein aggregates were observed in the epicardial nerve fascicles in 7 of 10 patients (Fig. 1h, patients 11, 14–18, 20) and in the myocardium in 7 of 10 patients (Fig. 1m, patients 13–19). The severity of α-synuclein aggregates was, at most, slight to moderate (score: 0–1.5; 0.75 ± 0.10), and the relative frequency of epicardial nerve fascicles with α-synuclein aggregates ranged from 0% to 85.7% ($22.6 \pm 24.2\%$), indicating that the loss of TH-ir axons is associated with less abundant and less frequent α-synuclein aggregates in ILBD.

In PD, TH-ir axons were absent to nearly absent (Fig. 1d). α-Synuclein aggregates were observed in 6 of the 10 patients in the epicardial nerve fascicles (Fig. 1i: arrowhead, patients 21–26) and in 4 of 10 patients in the myocardium (Fig. 1n, patients 21, 22, 24, 26). The severity of α-synuclein aggregates was slight (score: 0–1; 0.6 ± 0.11), and the relative frequency of epicardial nerve fascicles with α-synuclein aggregates was far less frequent, ranging from 0% to 25% ($10.8 \pm 10.2\%$).

In MSA, TH-ir axons were well preserved in 12 patients with MSA (Fig. 1e, patients 31–42), slightly to moderately decreased in seven (patients 43–49) and markedly decreased in one MSA patient associated with concurrent LB pathology in the paravertebral sympathetic ganglia and dorsal vagal nucleus (patient 50). In 17 of 20 patients with MSA (patients 31–34, 36–46, 48, 49), there were no α-synuclein aggregates observed in either the epicardial nerve fascicles (Fig. 1j) or myocardium (Fig. 1o). α-Synuclein aggregates were observed in the epicardial nerve fascicles in two patients (patients 47, 50), and one of these two showed concurrent LB pathology as described above (patient 50). α-Synuclein aggregates were also observed in the myocardium in one MSA patient (patient 35). In two pure MSA patients (patients 35, 47), one exhibited a slight decrease in the number of TH-ir axons with preserved NF-ir axons (patient 47), and the other exhibited good preservation of both TH- and NF-ir axons (patient 35). The severity of α-synuclein aggregates was very slight (score: 0–1; 0.1 ± 0.07), and the relative frequency of epicardial nerve fascicles with α-synuclein aggregates was very low, ranging from 0% to 33.3% ($3 \pm 2\%$).

The relative frequency of epicardial nerve fascicles with α-synuclein aggregates (Fig. 2A) and the severity score of α-synuclein aggregates (Fig. 2B) in ILBD with preserved TH-ir axons [ILBD(a)] were significantly higher than that in ILBD with decreased TH-ir axons [ILBD(b)], PD, MSA or controls.

With double immunofluorolabelling, we analysed the relationship between α-synuclein aggregates and degeneration of the cardiac sympathetic nerve. There were various patterns of the relationship between α-synuclein aggregates and TH-ir axons: (i) well preserved TH-ir axons and no α-synuclein aggregates; (ii) several small round or thread-like α-synuclein aggregates on TH-ir axons (Fig. 3a, e and i); (iii) enlarged α-synuclein aggregates along with TH-ir axons when TH immunoreactivity was still well preserved (Fig. 3b, f and j); (iv) increased number and quantity of α-synuclein aggregates with diminished TH-immunoreactivity, mainly from the centre of α-synuclein aggregates (Fig. 3c, g and k); and (5) decreased number of α-synuclein aggregates with regression of TH-immunoreactivity (Fig. 3d, h and l).

Sympathetic ganglia

In controls, neither neuronal cell loss nor α-synuclein aggregates were observed in the paravertebral sympathetic ganglia. In ILBD, there was no apparent neuronal cell loss. α-Synuclein aggregates were observed in 8 of 10 patients with preserved TH-ir axons [ILBD(a), patients 1–3,

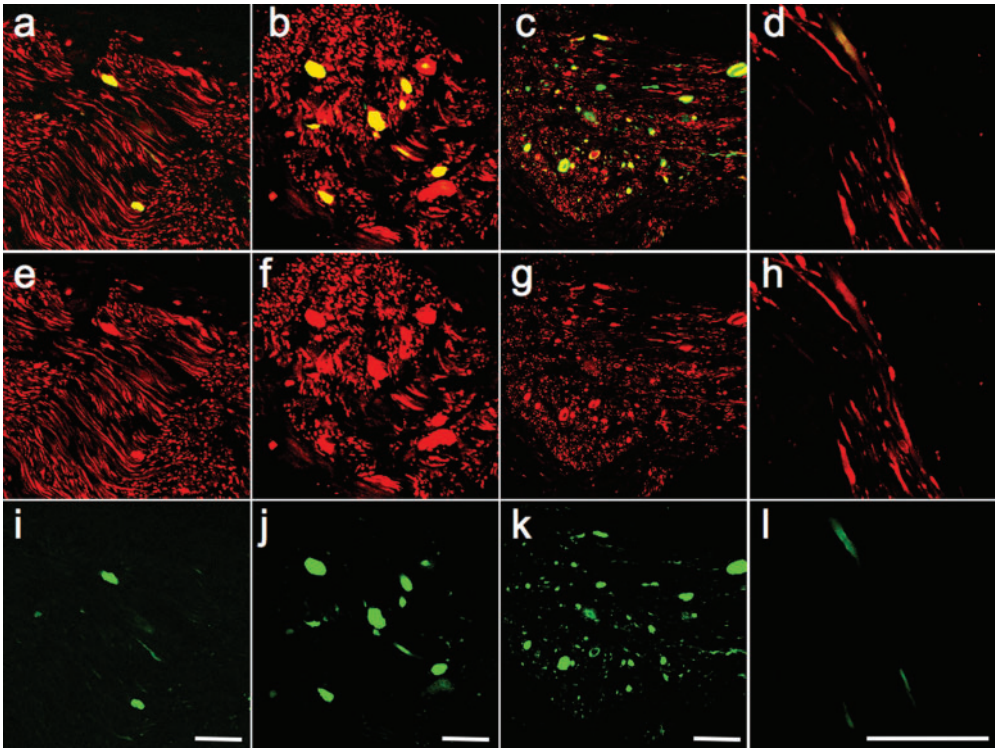

Fig. 3 α-Synuclein and TH double immunofluorolabelling of epicardial nerve fascicles in ILBD. There were various patterns of the relationship between α-synuclein aggregates and TH-ir axons: (i) Several small round or thread-like α-synuclein aggregates on TH-ir axons (a, e, i); (ii) enlarged α-synuclein aggregates along with the TH-ir axons when TH-immunoreactivity was still well preserved (b, f, j); (iii) increased number and quantity of α-synuclein aggregates with diminished TH-immunoreactivity, mainly from the center of α-synuclein aggregates (c, g, k); and (iv) decreased number of α-synuclein aggregates with regression of TH-immunoreactivity (d, h, l). Red: TH, green = α-synuclein, yellow = merge; a, e, i = patient 2; b, f, j = patient 2; c, g, k = patient 10; d, h, l = patient 15. Bar indicates 50 μm.

5–7, 9, 10] in the paravertebral sympathetic ganglia. α-Synuclein aggregates in the paravertebral sympathetic ganglia were consistently less abundant than those in the epicardial nerve fascicles. Moreover, the severity of α-synuclein aggregates was, at most, slight in the paravertebral sympathetic ganglia (Fig. 4a and b) even in patients with abundant α-synuclein aggregates in the epicardial nerve fascicles (Fig. 4e and f, patients 6, 9). Contrarily, in ILBD with decreased TH-ir axons [ILBD(b)], α-synuclein aggregates in the paravertebral sympathetic ganglia (Fig. 4c) were equal or more abundant than those in the epicardial nerve fascicles (Fig. 4g, patients 11–14, 17–20). In PD, α-synuclein aggregates in the paravertebral sympathetic ganglia were observed in all patients examined (patients 21–26). The severity of α-synuclein aggregates was moderate to severe in the paravertebral sympathetic ganglia (Fig. 4d), but was absent to slight in the epicardial nerve fascicles (Fig. 4h). In MSA, α-synuclein aggregates in the paravertebral sympathetic ganglia were observed in 3 of 15 patients examined (patients 39, 47, 50). The severity of α-synuclein aggregates was slight in two (patients 39, 47) and moderate in one patient with concurrent LB pathology as described earlier (patient 50).

Discussion

In the present study, on the cardiac sympathetic nervous system, phosphorylated α-synuclein aggregates were observed in 18 of 20 (90%) ILBD, in 6 of 10 (60%) PD, in 3 of 20 (15%) MSA and 0 of 10 (0%) control subjects on 4 μm-thick paraffin-embedded sections of the heart. Of the three MSA patients with α-synuclein aggregates, one was associated with ILBD (patient 50), and the other two (patient 35, 47) were pure MSA. α-Synuclein aggregates in the distal axons were much more abundant in ILBD than in PD, which is consistent with the previous report (Iwanaga *et al.*, 1999). Bloch reported that α-synuclein pathology was observed in the peripheral autonomic nervous system of ILBD; the oesophageal myenteric plexus (14 of 17; 82%), the paravertebral sympathetic chain (14 of 17; 82%) and the vagal nerve (12 of 16; 75%) on 4μm-thick paraffin-embedded sections fixed with 4% formaldehyde (Bloch *et al.*, 2006). Interestingly, Minguez-Castellanos reported that α-synuclein pathology was observed in autonomic plexuses of surgically resected specimens of abdominopelvic organs in 9% of subjects without known neurodegenerative disorders (Minguez-Castellanos *et al.*, 2007). Braak reported that Lewy neurites or LB were observed in the alimentary

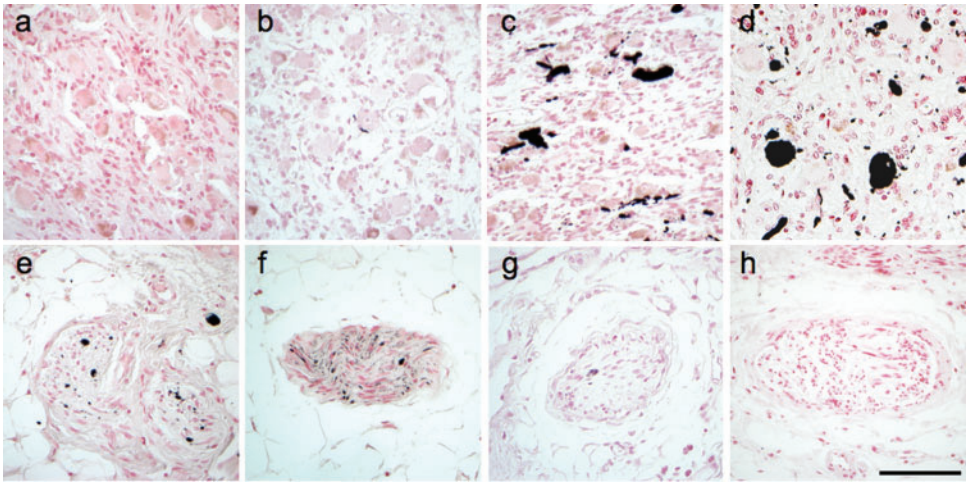

Fig. 4 α-Synuclein immunostaining of the epicardial nerve fascicles and the paravertebral sympathetic ganglia in ILBD and PD. In ILBD with preserved TH-ir axons, α-synuclein aggregates were absent or sparse in the paravertebral sympathetic ganglia (a, b) even when there were abundant α-synuclein aggregates in the epicardial nerve fascicles (e, f). Contrarily, in ILBD with decreased TH-ir axons and PD, α-synuclein aggregates in the paravertebral sympathetic ganglia (c, d) were much more abundant than those in the epicardial nerve fascicles (g, h). a, b, c, d = paravertebral sympathetic ganglia; e, f, g, h = epicardial nerve fascicle; a, e = patient 6; b, f = patient 9; c, g = patient 14; d, h = patient 25. Bar indicates 100 μm.

autonomic nervous system of the stomach or the distal oesophagus in two of two ILBD and three of three PD on 150 μm-thick cryostat sections (Braak *et al.*, 2006). Although they reported that the paraffin sections were of little use for demonstration of thread-like aggregates within the intramucosal and submucosal axons of the Meissner plexus (Braak *et al.*, 2006), we were successful in demonstrating α-synuclein aggregates in the distal axons of the cardiac sympathetic nerve, most evidently in ILBD. It is possible to conclude that α-synuclein aggregates of the peripheral autonomic nervous system including the alimentary tracts and the cardiac sympathetic nerve are consistently and earliest affected structures in ILBD, which indicates very early involvement of the peripheral autonomic nervous system in PD.

The relative frequency of fascicles containing α-synuclein aggregates and the mean severity score of α-synuclein aggregates were the highest in ILBD with preserved TH-ir axons [patients 1-10, ILBD(a)] (Fig. 2A and B). These findings suggest that in PD, axonal α-synuclein aggregates are an earlier and a more upstream lesion occurring prior to the decrease in TH-immunoreactivity, namely degeneration of the cardiac sympathetic nerve. The reason for sparse α-synuclein aggregates in the distal axons of the cardiac sympathetic nerve in PD is presumably explained by the profound depletion of axons of the cardiac sympathetic nerve, which differs from the observation that α-synuclein aggregates are more abundant in the PD brain than in the ILBD brain (Braak *et al.*, 2003). In MSA, the number of TH-ir, but not that of NF-ir axons, was slightly to moderately decreased in 6 of 15 patients with MSA, suggesting that mild involvement of the cardiac sympathetic nerve can occur in MSA (Orimo *et al.*, 2007a). However, α-synuclein aggregates were not detected except in two patients with pure MSA and in one patient associated with ILBD. The severity of α-synuclein aggregates was very limited, and the relative frequency of fascicles with α-synuclein aggregates was very low. In two pure MSA with α-synuclein aggregates, degeneration of the cardiac sympathetic nerve, if any, was not related to α-synuclein aggregates. We recently reported that the number of TH-ir neurons in the paravertebral sympathetic ganglia was slightly to moderately decreased in 6 of 15 MSA, of whom four showed mild degeneration of the cardiac sympathetic nerve, implying a possible relationship between involvement of the cardiac sympathetic nerve and neuronal cell damage of the paravertebral sympathetic ganglia in MSA (Orimo *et al.*, 2007*a*). Taken together, the pathological mechanism underlying the degenerative process of the cardiac sympathetic nervous system in MSA sharply contrasts with that in PD.

Double immunofluorolabelling for α-synuclein and TH confirmed the close relationship between α-synuclein aggregates and degeneration of the distal axons, and further demonstrated pathological mechanisms underlying degeneration of the cardiac sympathetic nerve (Fig. 3). We speculate on the degenerative process of the cardiac sympathetic nerve. Initially, small amounts of α-synuclein aggregates accumulate in TH-ir axons (Fig. 3a, e and i). These aggregates enlarge in association with TH-ir axons (Fig. 3b, f and j). Then, TH-immunoreactivity begins to decrease mainly from the center of α-synuclein aggregates (Fig. 3c, g and k). Subsequently, TH-ir axons begin to deplete, followed by disappearance of α-synuclein aggregates (Fig. 3d, h and l). These findings clearly indicate that α-synuclein aggregates progressively accumulate initially in

the distal TH-ir axons, followed by a decrease in TH-immunoreactivity of the distal axons in parallel with disappearance of α-synuclein aggregates. Mori and colleagues reported a close relationship between α-synuclein aggregates and decreased TH-immunoreactivity of pigmented neurons in the substantia nigra and the locus coeruleus in PD (Mori *et al.*, 2006). Although they speculated that the decreased TH-immunoreactivity might represent a cytoprotective mechanism in PD because this decreased TH-immunoreactivity indicates a reduction in dopamine synthesis, leading to a decrease in cytotoxic α-synuclein oligomer (Perez *et al.*, 2002), the chronological relationship between these events remains to be clarified. This study using a sufficient number of subjects with ILBD and PD demonstrated a chronological change in α-synuclein aggregation and degeneration of the cardiac sympathetic nerve, where one of the earliest changes specific to PD is clinically detectable by [^{123}I] MIBG myocardial scintigraphy. At present, it remains to be clarified why these aggregates accumulate initially in the axons of the cardiac sympathetic nerve. Recently, studies related to axonal transport and neurodegeneration have been reported (Roy *et al.*, 2005). One of these showed that there was significant age-related retardation in the normal axonal transport of α-synuclein (Li *et al.*, 2004), suggesting that age-related retardation of α-synuclein transport leads to the accumulation of α-synuclein over time (Roy *et al.*, 2005). We speculate that these aggregates may secondarily cause some disturbance in axonal flow, eventually resulting in degeneration of the cardiac sympathetic nerve.

In ILBD with preserved TH-ir axons [ILBD(a)], α-synuclein aggregates in the epicardial nerve fascicles were consistently much more abundant than those in the paravertebral sympathetic ganglia. Moreover, in two ILBD, α-synuclein aggregates were nearly absent in the paravertebral sympathetic ganglia even when there were abundant α-synuclein aggregates in the epicardial nerve fascicles. This finding suggests that pathological α-synuclein synthesized in the neuronal somata of the paravertebral sympathetic ganglia are transported to the distal axons of the cardiac sympathetic nerve to form α-synuclein aggregates at a very early stage [Fig. 5, ILBD(a)]. Then, α-synuclein aggregates accumulate in the neuronal somata or neurites in the paravertebral sympathetic ganglia with progression of the disease process [Fig. 5, ILBD(b), PD], supporting our previous conclusion that degeneration of the cardiac sympathetic nerve precedes neuronal cell loss in the paravertebral sympathetic ganglia in PD (Orimo *et al.*, 2005a). Indeed, the previous neuropathological studies showed that accumulation of α-synuclein aggregates in neurites, that is Lewy neurites, precedes that in neuronal somata, mainly LB (Del Tredici *et al.*, 2002; Braak *et al.*, 2003; Saito *et al.*, 2003). This chronological and dynamic relationship between α-synuclein aggregates and distal-dominant degeneration of the cardiac sympathetic nervous system sharply contrasts with the slight changes in MSA (Fig. 5, MSA) and may represent the pathological mechanism underlying a common degenerative process in PD.

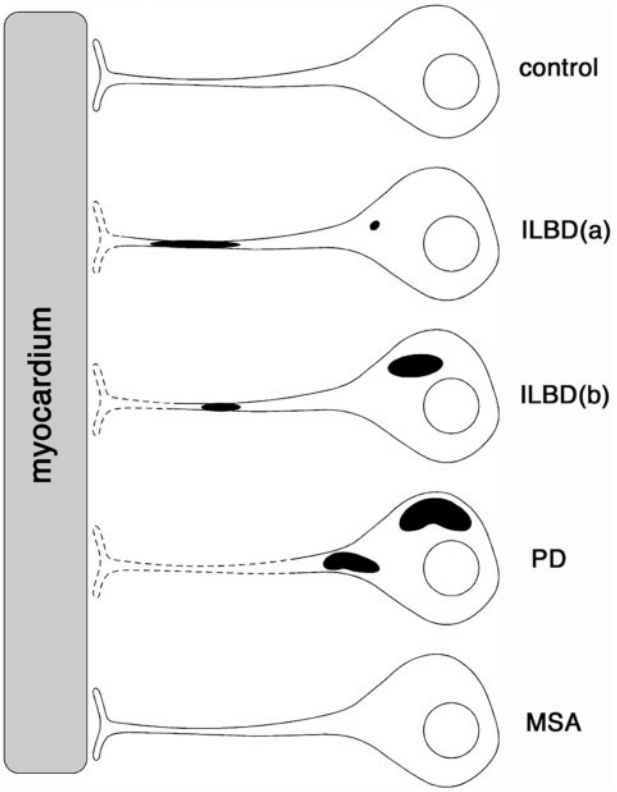

Fig. 5 Schematic illustration of the degenerative process of the cardiac sympathetic nervous system. In ILBD(a) α-synuclein aggregates abundantly accumulate in the distal axons in contrast to sparse α-synuclein aggregates in the paravertebral sympathetic ganglia. In ILBD(b), α-synuclein aggregates in the distal axons diminish, while they increase in number and quantity in the paravertebral sympathetic ganglia. In PD, α-synuclein aggregates in the distal axons disappear with regression of TH-ir axons, whereas α-synuclein aggregates accumulate much more abundantly in the paravertebral sympathetic ganglia. In MSA, α-synuclein aggregates are basically not observed as seen in controls, with a few exceptions. Black shading indicates α-synuclein aggregates. The line indicates the outline of TH-ir axons or their mother neurons. The dotted line indicates degeneration of TH-ir axons. ILBD(a) = ILBD with preserved TH-ir axons, ILBD(b) = ILBD with decreased TH-ir axons.

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