



Spectrum of abnormalities of sympathetic tyrosine hydroxylase and alpha-synuclein in chronic autonomic failure

Risa Isonaka¹ · Patti Sullivan¹ · Yunden Jinsmaa¹ · Abraham Corrales¹ · David S. Goldstein¹ 

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Abstract

Objective Lewy body forms of primary chronic autonomic failure (CAF) such as incidental Lewy body disease (ILBD), Parkinson's disease (PD), and pure autonomic failure evolving into dementia with Lewy bodies (PAF+DLB) feature cardiac sympathetic denervation, whereas multiple system atrophy (MSA) in most cases does not. What links Lewy bodies with cardiac sympathetic denervation in CAF? In familial PD, abnormalities of the alpha-synuclein (AS) gene cause CAF and cardiac sympathetic denervation; and in sporadic PD, brainstem Lewy bodies contain AS co-localized with tyrosine hydroxylase (TH), a marker of catecholaminergic neurons. Cytotoxicity from AS deposition within sympathetic neurons might explain noradrenergic denervation in Lewy body forms of CAF. We used immunofluorescence microscopy (IM) to explore this possibility in sympathetic ganglia obtained at autopsy from CAF patients.

Methods Immunoreactive AS and TH were imaged in sympathetic ganglion tissue from 6 control subjects (2 with ILBD), 5 PD patients (1 with concurrent PSP), and 3 patients with CAF (2 PAF + DLB, 1 MSA).

Results MSA involved normal ganglionic TH and no AS deposition. In ILBD TH was variably decreased, and TH and AS were co-localized in Lewy bodies. In PD TH was substantially decreased, and TH and AS were co-localized in Lewy bodies. In PAF + DLB TH was virtually absent, but AS was present in Lewy bodies. The PD + PSP patient had AS co-localized with tau but not TH.

Conclusions Sympathetic denervation and intraneuronal AS deposition are correlated across CAF syndromes, consistent with a pathogenic contribution of synucleinopathy to cardiac noradrenergic deficiency in Lewy body diseases.

Keywords Synuclein · Autonomic · Sympathetic · Immunofluorescence · Lewy

Abbreviations

AS	Alpha-synuclein
DA	Dopamine
DOPAL	3,4-Dihydroxyphenylacetaldehyde
ILBD	Incidental Lewy body disease
IM	Immunofluorescence microscopy
MSA	Multiple system atrophy
NE	Norepinephrine
OH	Orthostatic hypotension

PAF + DLB	Pure autonomic failure evolving into dementia with Lewy bodies
PD	Parkinson's disease
PSP	Progressive supranuclear palsy
TH	Tyrosine hydroxylase

Synopsis

Cardiac sympathetic denervation characterizes Lewy body diseases. We obtained evidence for a range of abnormalities in sympathetic ganglionic immunoreactive tyrosine hydroxylase (TH) and alpha-synuclein (AS) in chronic autonomic failure (CAF) syndromes. The finding of correlated abnormalities of TH and AS across different forms of CAF is consistent with a pathogenic contribution of synucleinopathy to cardiac noradrenergic deficiency in Lewy body diseases.

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✉ David S. Goldstein
goldsteind@ninds.nih.gov

¹ Clinical Neurocardiology Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health, 10 Center Drive MSC-1620, Building 10 Room 8N260, Bethesda, MD 20892-1620, USA

Introduction

Lewy body forms of primary chronic autonomic failure (CAF), such as incidental Lewy body disease (ILBD), Parkinson's disease (PD), and pure autonomic failure evolving into dementia with Lewy bodies (PAF + DLB), feature cardiac sympathetic denervation [11, 14, 34], whereas multiple system atrophy (MSA) in most cases does not [4, 29–31].

What links Lewy bodies with cardiac sympathetic denervation in CAF? In familial PD abnormalities of the alpha-synuclein (AS) gene cause not only parkinsonism but also CAF and cardiac sympathetic denervation [18, 37]. In sporadic PD, brainstem Lewy bodies contain AS [38] colocalized with immunoreactive tyrosine hydroxylase (TH) [9], a catecholamine neuronal marker in the brain [5, 6] and sympathetic nervous system [1, 32]. AS deposition in sympathetic neurons might therefore cause or contribute to noradrenergic denervation in Lewy body forms of CAF.

We used immunofluorescence microscopy (IM) for immunoreactive TH, AS, and other related proteins to address the possibility of AS deposition in sympathetic neurons in ganglia obtained at autopsy from CAF patients.

Since interference with vesicular sequestration, which is mediated by the type 2 vesicular monoamine transporter (VMAT2), enhances the susceptibility of catecholaminergic neurons [3, 15, 39, 40] and is impaired in PD [15, 28, 35], we also explored the possible association between PD and decreased VMAT2 as revealed by immunostaining for the two forms of the VMAT in sympathetic ganglion tissue from PD patients and controls.

Finally, we examined sympathetic ganglion tissue from a patient with PD and progressive supranuclear palsy (PD + PSP). PSP is thought to be a tauopathy [7], with no consistent evidence for CAF or cardiac sympathetic denervation [23, 25]; however, whether tau co-localizes with TH and AS in PD + PSP has been unknown.

Methods

Subjects

In a total of 14 subjects, post-mortem tissue harvesting of a thoracic sympathetic trunk for research purposes was done with the consent of the next of kin. There were 6 control subjects (2 with central neural ILBD), 5 PD patients (1 with concurrent PSP, denoted PD + PSP), 2 patients with PAF that had evolved into dementia with Lewy bodies and parkinsonism (PAF + DLB), and a patient with MSA.

Sympathetic ganglionic tissues from the control subjects and PD patients were obtained from the Banner Sun Health Research Institute (Sun City, AZ, USA). The two patients

with PAF + DLB were followed at the NIH Clinical Center; one was autopsied near the place of death and the other at the NIH Clinical Center. The MSA patient was autopsied near the place of death. The post-mortem interval was less than 24 h in all cases. The frozen specimens were delivered in dry ice and kept frozen at -80°C until processed for immunofluorescence microscopy.

Immunofluorescence microscopy (IM)

OCT-embedded frozen tissues on slides were obtained from the Banner Sun Health Research Institute. Frozen samples sent to our lab were embedded in OCT and sliced into 10- μm -thick sections (Histoserv, Germantown, MD, USA).

The tissues were treated with glycine and then with 1% bovine serum albumin, 0.3% Triton-X, and 10% normal donkey serum in phosphate-buffered saline. The samples were incubated with rabbit anti-TH antibody (Pel-Freez Biologicals, Rogers, AR, USA) and mouse monoclonal anti-AS (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Other primary antibodies were rabbit anti-VMAT1 (ImmunoStar, Hudson, WI, USA) and monoclonal mouse anti-VMAT2 (R&D Systems Inc., Minneapolis, MN, USA). After 24 h at 4°C , secondary antibodies were applied (Cy3-conjugated anti-rabbit secondary antibody from Jackson Immune Research Labs; Alexa 488-conjugated anti-mouse IgG secondary antibody from Thermo Scientific, Inc., Rockford, IL, USA; and Hoechst 33342 trihydrochloride trihydrate from Thermo Scientific, Inc.) for 1 h at room temperature. The immunostained tissues were mounted on slides with anti-fade reagent and examined via immunofluorescence confocal microscopy using a Zeiss LSM 880 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

Multiplex IM

For multiplex immunofluorescence staining, the sections were immunoreacted for 24 h at 4°C using a mixture of the following immunocompatible primary antibodies: rabbit anti-TH (Pel-Freez Biologicals), mouse IgG monoclonal anti-AS (Santa Cruz Biotechnology), sheep anti-choline acetyltransferase (ChAt, Abcam, Cambridge, MA, USA), chicken anti-Tau (Abcam). This was followed by incubation for 1 h at room temperature with Hoechst 33342 trihydrochloride trihydrate (Thermo Scientific, Inc.), Alexa 488-conjugated anti-mouse IgG secondary antibody (Thermo Scientific, Inc.), Alexa 546-conjugated anti-rabbit secondary antibody (Thermo Scientific, Inc.), Alexa 594-conjugated anti-chicken secondary antibody (Thermo Scientific, Inc.), and Alexa 790-conjugated anti-sheep secondary antibody (Jackson Immune Research Labs). The immunostained tissues were mounted on slides as described above with anti-fade reagent using Immu-Mount medium. Microscopic

imaging was done using a multichannel wide-field epifluorescence microscope—an Axio Imager.Z2 slide scanning fluorescence microscope (Zeiss) equipped with a 20×/0.8 Plan-Apochromat (Phase 2) nonimmersion objective (Zeiss), a high-resolution ORCA-Flash4.0 sCMOS digital camera (Hamamatsu), and a 200 W X-Cite 200DC broadband lamp source (Excelitas Technologies). The images were captured using the ZEN 2 image acquisition and analysis software program (Zeiss).

Myocardial tissue norepinephrine (NE)

Apical myocardial tissue contents of NE were assayed by batch alumina extraction followed by liquid chromatography with series electrochemical detection, as described previously [21].

Results

Postmortem sympathetic ganglion tissue in four control subjects without Lewy body disease (see Fig. 1a for example) and the MSA patient (Fig. 1b) showed abundant immunoreactive TH-containing fibers and no AS. Apical myocardial NE content in the MSA patient was normal at 4.0 pmol/mg wet weight (control 2.0 ± 0.3 , $N = 21$).

In the two controls with central neural ILBD, the sympathetic ganglion tissue showed co-localization of AS with TH in Lewy bodies and Lewy neurites (see Fig. 2a for example). Immunoreactive TH was not quantified but seemed decreased in one subject and normal in the other. Apical myocardial NE content was low at 0.06 pmol/mg in the first and normal at 2.9 pmol/mg in the other.

All five patients with PD had obviously decreased TH, AS deposition, and Lewy bodies containing co-localized

Fig. 1 Immunofluorescence microscopic images of postmortem sympathetic ganglion tissue in a control subject (a) and a patient with MSA (b). The panels show overlays of cell nuclei (blue), immunoreactive TH (red), and AS (green). The MSA patient has normal sympathetic noradrenergic innervation

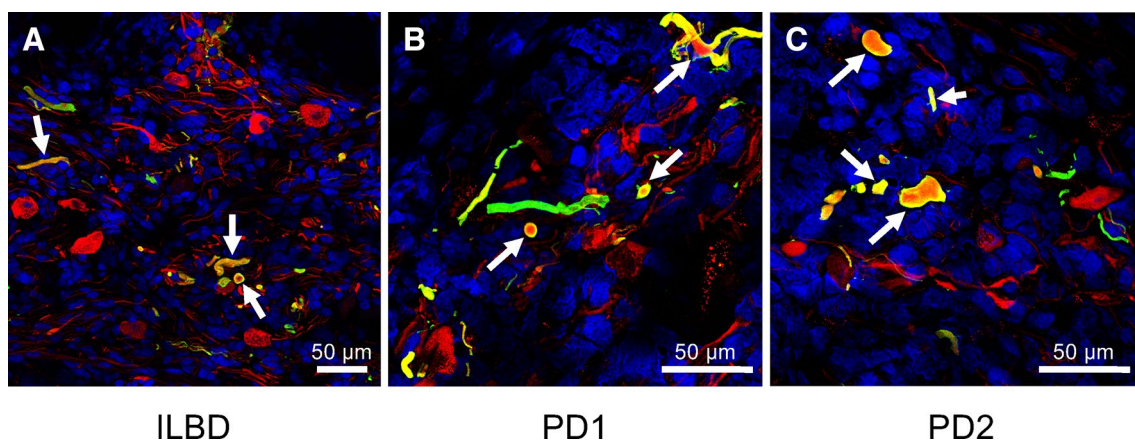
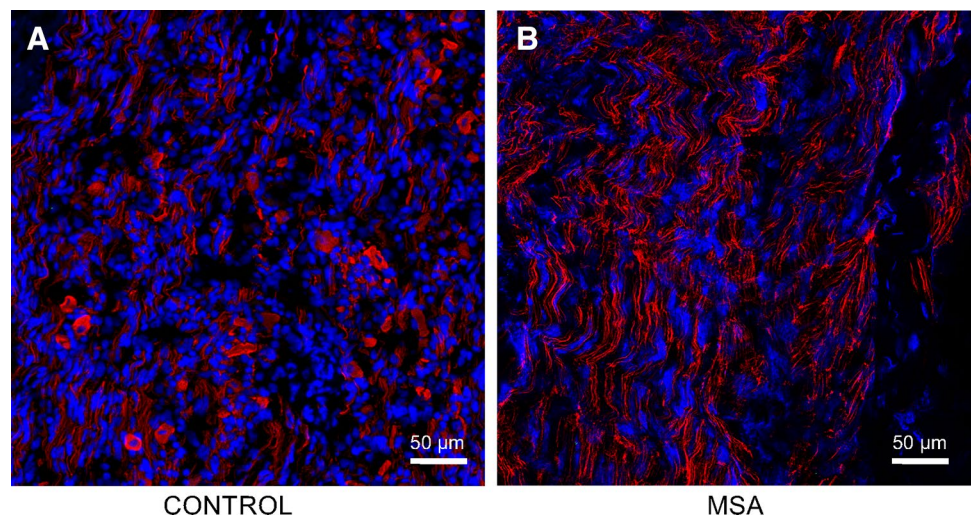


Fig. 2 Immunofluorescence microscopic images of postmortem sympathetic ganglion tissue in a patient with a ILBD and b, c two patients with PD. The panels show overlays of cell nuclei (blue),

immunoreactive TH (red), and AS (green). The ILBD patient has mildly decreased immunoreactive TH, with co-localized AS and in Lewy bodies and neurites (white arrows)

TH with AS (see Fig. 2b, c for examples). The Lewy bodies often had a “fried egg” appearance, with a red center, corresponding to TH without co-localized AS, and a yellow surround, corresponding to co-localized TH with AS. There were no examples of AS (green) surrounded by co-localized TH with AS. Apical myocardial NE content was variable, ranging from 0.01 to 1.6 pmol/mg (mean 0.40 ± 0.31 pmol/mg).

Both patients with PAF + DLB had remarkably decreased TH and abundant AS deposits (see Fig. 3a for example).

Lewy bodies contained AS with variable amounts of co-localized TH, including some Lewy bodies with little or no TH (white arrows in Fig. 3b). Apical myocardial NE contents were low at 0.02 and 0.05 pmol/mg.

Figure 4 shows multiplex fluorescence immunohistochemistry images from the same PAF + DLB patient as in Fig. 3. The tissue was stained for nuclei, TH, AS, tau, and ChAT simultaneously. There was scant TH, a large amount of AS, little ChAT, and hardly any tau. There were several

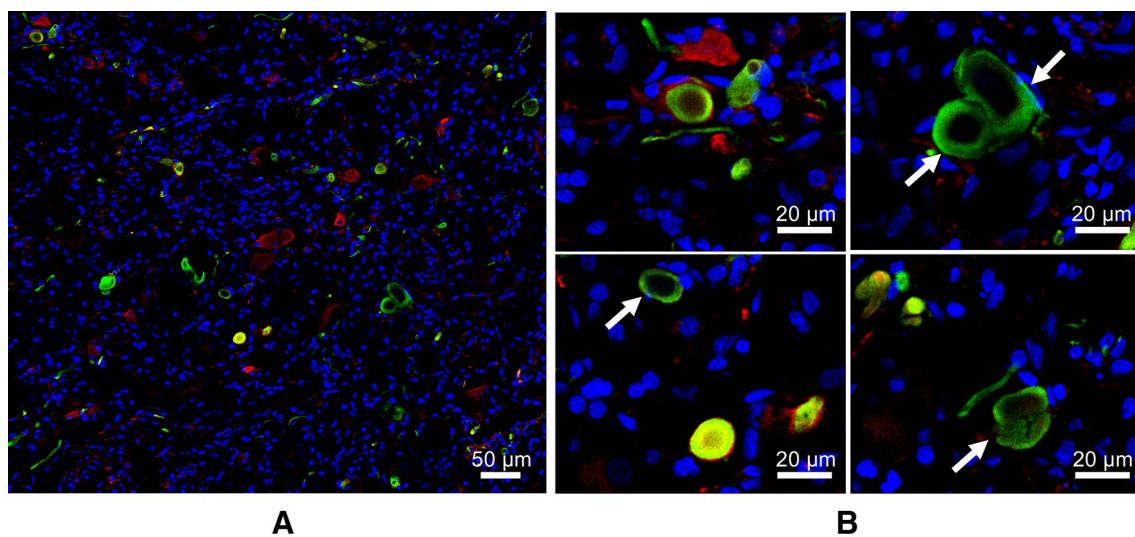


Fig. 3 Immunofluorescence microscopic images of postmortem sympathetic ganglion tissue from a patient with PAF + DLB. **a** Low magnification; **b** Lewy bodies. The Lewy bodies had relatively little or even no TH

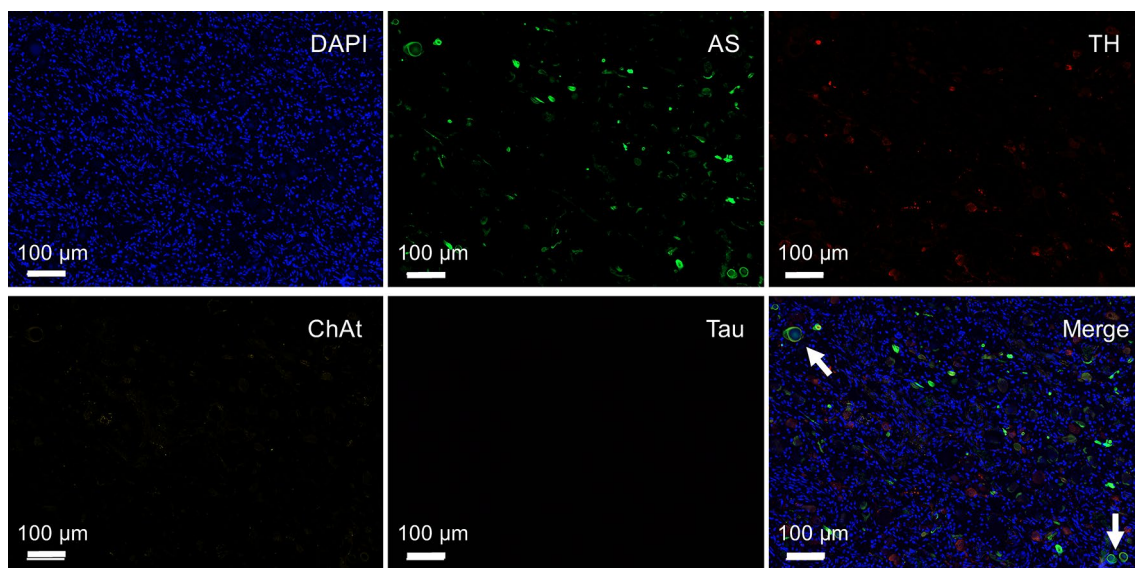
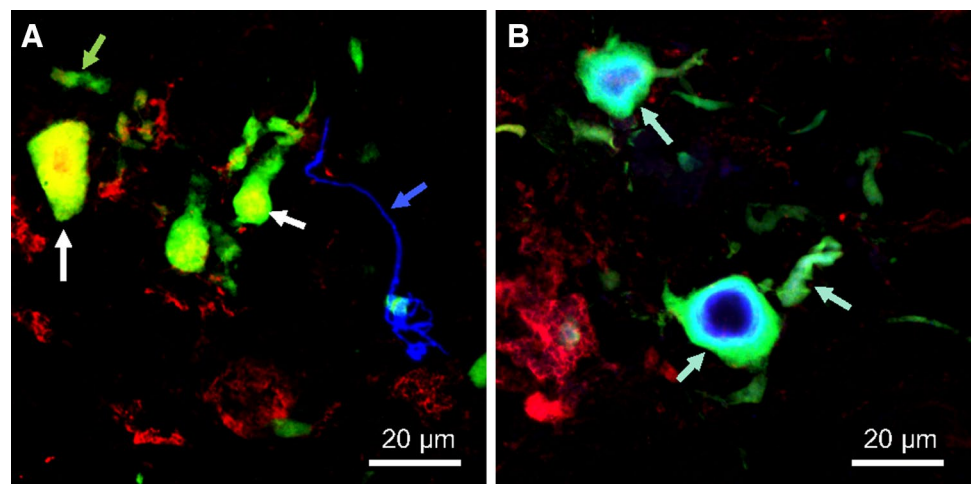


Fig. 4 Multiplex fluorescence immunohistochemistry images of postmortem sympathetic ganglion tissue from the same patient with PAF + DLB as in Fig. 3. The *panels* show cell nuclei, AS, TH, ChAT, and tau in the same slice from the same slide. There is abun-

dant AS, decreased TH, and hardly any ChAT or tau. Lewy bodies with AS and no TH are indicated by the *white arrows* in the merged image

Fig. 5 Immunofluorescence microscopic images of postmortem sympathetic ganglion tissue from a patient with PD + PSP. TH is in red, AS is in green, and tau is in blue. Note the co-localization of AS with TH in Lewy bodies (white arrows), AS-containing Lewy neurites (green arrow), and Lewy bodies or neurites with co-localized AS and tau (light blue arrows)



Lewy bodies with AS and no TH (white arrows in the merged image in Fig. 4).

The patient with PD + PSP had neurites that contained tau (blue arrow in Fig. 5a), AS (green arrows), or both (light blue arrow in Fig. 5b). TH seemed decreased, and AS was co-localized with TH in some Lewy bodies (white arrows in Fig. 5a) and co-localized with tau in others (light blue arrows in Fig. 5b). Apical myocardial NE content was decreased at 0.30 pmol/mg.

Control subjects without Lewy body disease had abundant VMAT1 and VMAT2 in sympathetic ganglionic cells (see Fig. 6a for example). In contrast, all four PD patients had virtually no VMAT2 (see Fig. 6b for example), while VMAT1 was still present.

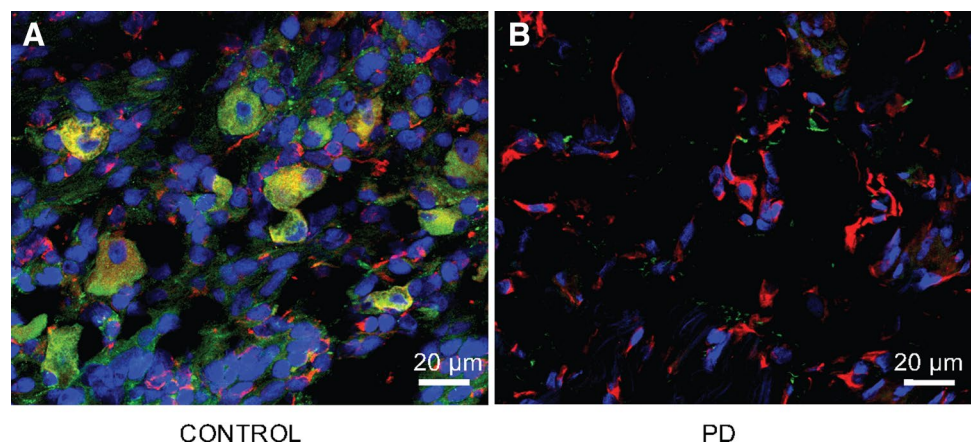
Discussion

This report describes particular patterns of TH loss and AS deposition in sympathetic ganglion tissue across different forms of synucleinopathy. We noted a spectrum of lesions, from no abnormalities in MSA to moderately decreased TH

in ILBD, severely decreased TH in PD, and virtually absent TH in PAF + DLB. In the Lewy body forms of synucleinopathy, AS and TH were co-localized in sympathetic neurons. AS was also co-localized with TH or tau in sympathetic neurons in a patient with PD + PSP. Moreover, we noted for the first time a close correspondence of IM results in sympathetic neurons to myocardial NE contents in the same patients. The findings provide important new support for the view that intraneuronal synucleinopathy contributes to sympathetic neuronal loss and cardiac noradrenergic deficiency in Lewy body diseases.

The demonstration of normal TH and no discernible AS in MSA confirms that cardiac sympathetic noradrenergic innervation is generally intact and that there is no ganglionic AS deposition in this disease [29, 30]. Normal myocardial NE content was documented in the same MSA patient [19]. In two control subjects with neuropathologically identified central neural ILBD, we noted co-localization of AS with TH in Lewy bodies and Lewy neurites. There seemed to be decreased immunoreactive TH in one subject, which would confirm previously published findings for this condition [8, 34]. The same subject had decreased myocardial NE. The

Fig. 6 Immunofluorescence microscopic images of postmortem sympathetic ganglion tissue from a control subject (a) and a patient with PD (b). The panels show overlays of cell nuclei (blue), VMAT1 (red), and VMAT2 (green). The PD patient has virtually no VMAT2, although VMAT1 is present



CONTROL

PD

other subject with central neural ILBD had Lewy bodies and Lewy neurites in sympathetic ganglion tissue but normal immunoreactive TH and normal myocardial NE. This pattern would be consistent with synucleinopathy preceding myocardial sympathetic neurodegeneration, as has been demonstrated in inherited synucleinopathies [18, 33, 37, 41]. In ILBD, AS was localized in a specific manner with respect to TH, in that a central core of immunoreactive TH was surrounded by co-localized AS and TH.

In all five PD patients there was a pattern of clearly decreased ganglionic TH associated with local AS deposition, in line with findings reported by others [34]. We noted co-localization of TH with AS in Lewy bodies and Lewy neurites. The distribution of TH with respect to AS within Lewy bodies was quite similar in the PD patients and the controls with ILBD. The spatial arrangement of TH and AS also closely resembled that in brainstem Lewy bodies in PD [9]. We found no instances of the converse organization (AS core surrounded by co-localized TH and AS).

A recently published study on the natural history of neurogenic OH noted that PAF can progress to DLB with parkinsonism [26]. We call this phenomenon PAF + DLB. In this study, both of the patients with PAF + DLB had virtually absent immunoreactive TH in sympathetic ganglion tissue. Instead of Lewy bodies containing co-localized TH and AS, the Lewy bodies often had a rim of AS alone and a core with hardly any immunoreactive TH in it. In one patient, the fibrils of ganglionic AS were so extensive we gained the impression that the AS was originally within TH-positive nerve fibers that had since disappeared, leaving AS fibrils as a kind of “skeletal” remains. Multiplex IM imaging in one of the PAF + DLB patients showed that the AS deposition and drastic loss of TH were unassociated with the concurrent deposition of tau or choline acetyltransferase. As reported elsewhere [19], both PAF + DLB patients had marked myocardial NE depletion, documenting extensive cardiac sympathetic denervation.

PSP is thought to reflect a form of tauopathy [7]. In a patient with PD + PSP we found decreased immunoreactive TH and co-localization of AS with tau and with TH in Lewy bodies and Lewy neurites. Decreased myocardial NE content confirmed that a cardiac sympathetic noradrenergic lesion was present in this patient.

If AS deposition in sympathetic neurons played a pathogenic role in the CAF attending Lewy body diseases, what would the neurotoxic mechanisms be? Accumulating evidence points to interference by synucleinopathy in intraneuronal vesicular storage [13]. Synucleinopathy permeabilizes vesicles [36], decreases VMAT2 expression [22], and decreases vesicle numbers [12]. Striatal vesicles isolated from PD patients exhibit decreased vesicular uptake associated with profoundly decreased VMAT2 protein [35]. Moreover, in both the PARK1 form of inherited PD due

to mutation of the AS gene and the PARK4 form due to triplication of the normal AS gene, there is neuroimaging evidence of decreased vesicular storage in the residual myocardial sympathetic nerves [13, 18, 37]. Finally, as reported here, PD patients have drastically decreased immunoreactive VMAT2 in sympathetic ganglion tissue.

A vesicular storage defect would be expected to impede NE synthesis in sympathetic nerves, as synthesis takes place within the vesicles. This would explain cardiac NE deficiency and clinical manifestations of sympathetic neurocirculatory failure but would not explain denervation. A shift in the fate of cytoplasmic catecholamines from vesicular sequestration to oxidative deamination in cardiac sympathetic nerves [21] would be expected to build up cytoplasmic levels of the autotoxic dopamine metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL) [20]. DOPAL potentially oligomerizes AS [2, 10, 24, 36], converting AS to its putatively toxic form [27, 36, 42]. Thus, DOPAL and AS together could participate in positive feedback loops that threaten the integrity of and eventually kill sympathetic noradrenergic neurons [16, 17].

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to disclose.

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