Phosphorylated α-synuclein in skin nerve fibres differentiates Parkinson’s disease from multiple system atrophy

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Deposition of phosphorylated SNCA (also known as α-synuclein) in cutaneous nerve fibres has been shown pre- and post-mortem in Parkinson’s disease. Thus far, no pre-mortem studies investigating the presence of phosphorylated SNCA in skin sympathetic nerve fibres of multiple system atrophy, another synucleinopathy, have been conducted. In this in vivo study, skin from the ventral forearm of 10 patients with multiple system atrophy and 10 with Parkinson’s disease, together with six control subjects with essential tremor, were examined by immunohistochemistry. Phosphorylated SNCA deposits in skin sympathetic nerve fibres and dermal nerve fibre density were assessed. All patients with Parkinson’s disease expressed phosphorylated SNCA in sympathetic skin nerve fibres, correlating with an age-independent denervation of autonomic skin elements. In contrast, no phosphorylated SNCA was found in autonomic skin nerve fibres of patients with multiple system atrophy and essential tremor control subjects. These findings support that phosphorylated SNCA deposition is causative for nerve fibre degeneration in Parkinson’s disease. Moreover, pre-mortem investigation of phosphorylated SNCA in cutaneous nerve fibres may prove a relevant and easily conductible diagnostic procedure to differentiate Parkinson’s disease from multiple system atrophy.

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Abbreviations: ART = arterial blood vessels; CASS = Composite Autonomic Scoring Scale; H/M-ratio = heart to mediastinum ratio; MAP = arrector pili muscles; MIBG = metaiodobenzylguanidine; pSNCA = phosphorylated SNCA (α-synuclein); SGD = sweat glands dermal duct; SGS = sweat gland secretory portion; SRcontra = specific binding ratio contralateral to clinically affected side; UMSARS = Unified Multiple System Atrophy rating scale; UPDRS = Unified Parkinson’s Disease Rating Scale

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Introduction

Synucleinopathies such as idiopathic Parkinson’s disease and multiple system atrophy are sporadic, adult-onset neurodegenerative disorders, characterized by accumulation of modified SNCA that present clinically with a ‘Parkinson’ phenotype (Jellinger, 2003). Movement disorders (rigidity, hypokinesia) are the clinical hallmark of Parkinson’s disease and multiple system atrophy, whereas non-motor symptoms, foremost autonomic dysfunction as orthostatic hypotension, bladder dysfunction and constipation are more severe in multiple system atrophy (Barone et al., 2009). Thus, clinical differentiation between Parkinson’s disease and multiple system atrophy is challenging, especially in the early stage of the disease. In a clinicopathological study of 100 patients with Parkinson’s disease, sensitivity of clinical diagnostic criteria for Parkinson’s disease was limited to 90–91.1%, whereas the positive predictive value ranged between 92–98.6% (Hughes et al., 2001, 2002).

Serine 129 phosphorylated SNCA (pSNCA) is a major component of neuronal Lewy bodies in Lewy body disorders such as Parkinson’s disease and of glial cytoplasmic inclusions in multiple system atrophy (Fujiwara et al., 2002). In Parkinson’s disease, neuronal populations most vulnerable to Lewy body-related pathology include the substantia nigra, locus coeruleus, raphe nuclei, pedunculopontine nucleus, basal nucleus of Meynert and dorsal motor nucleus of the vagus (Jellinger, 2003). In addition, neurodegeneration in Parkinson’s disease leads to pathological SNCA deposits within peripheral autonomic nerve fibres innervating the heart, abdominopelvic organs, salivary glands, sympathetic ganglia as well as skin, which may contribute to autonomic dysfunction in Parkinson’s disease (Iwanaga et al., 1999; Minguez-Castellanos et al., 2007; Orimo et al., 2007, 2008; Beach et al., 2010, 2013; Del Tredici et al., 2010; Lebouvier et al., 2010; Cersosimo et al., 2011; Pouclet et al., 2012a; Folgoas et al., 2013; Hilton et al., 2014). In contrast, neurodegeneration in multiple system atrophy is largely limited to the CNS including autonomic centres of the brainstem (catecholaminergic neurons of the rostral ventrolateral medulla, nucleus of the solitary tract) causing predominant preganglionic autonomic failure (Sung et al., 1979; Wenning et al., 1996; Benarroch et al., 1998). Thus, detection of pSNCA in sympathetic postganglionic nerve fibres seems promising to differentiate Parkinson’s disease from multiple system atrophy. Recently, Donadio et al. (2014) demonstrated pSNCA in skin sympathetic nerve fibres of patients with clinically diagnosed Parkinson’s disease, but not in other tau-related parkinsonian syndromes such as progressive supranuclear palsy and corticobasal degeneration. To date, no comparative pre-mortem study assessed the differential involvement of skin nerve fibres with respect to pSNCA pathology in synucleinopathies such as Parkinson’s disease and multiple system atrophy. In the present study we tested the hypothesis that pSNCA can be detected in skin sympathetic nerve fibres in Parkinson’s disease but not multiple system atrophy. We performed skin punch biopsies and assessed the appearance of intra-axonal pSNCA in patients diagnosed as Parkinson’s disease and multiple system atrophy based on clinical consensus criteria, autonomic involvement and nuclear imaging.

Materials and methods

Patients

The study cohort was composed of 10 patients with Parkinson’s disease (three females; age 69.6 ± 8.77 years; disease duration 5.8 ± 4.34 years) and 10 patients with multiple system atrophy (three females, age 60.5 ± 10.96 years; disease duration 4.1 ± 2.10 years). Six subjects without a neurodegenerative disorder but essential tremor served as controls (two females, age 62.0 ± 10.96 years; disease duration 14.7 ± 11.8 years). Recruitment was limited to mild and moderate cases [Hoehn and Yahr stage 1–3, mean ± standard deviation (SD): 1.95 ± 0.9 points]. Patients were excluded, if they had a history of heart disease, dementia (Mini-Mental State Examination Score < 24 points), polyneuropathy, diabetes mellitus or alcoholism. Patients were enrolled from the movement disorders outpatient clinic of our hospital, Charité – University Medicine Berlin. The study was approved by our local ethical review board (approval number EA 2/133/09) and all participants gave their written informed consent before entering the study.

Diagnostic procedure

To increase diagnostic certainty, the clinical diagnosis of multiple system atrophy and Parkinson’s disease was performed according to current diagnostic criteria [Parkinson’s disease: UK Parkinson’s Disease Society Brain Bank criteria (Gibb and Lees, 1988; Hughes et al., 1992, 2001); multiple system atrophy: Gilman criteria (Gilman et al., 2008)] and validated by a clinical follow-up. Clinical classification of multiple system atrophy and Parkinson’s disease were further supported by nuclear imaging and autonomic reflex screen.

Clinical testing

All participants underwent a detailed neurological examination by two movement disorder specialists (C.N., A.L.). Motor impairment was evaluated by disease specific scoring scales [Parkinson’s disease: Unified Parkinson’s Disease Rating Scale, part III (UPDRS); multiple system atrophy: Unified Multiple System Atrophy rating scale, part II (UMSARS)]. All patients (multiple system atrophy and Parkinson’s disease) exhibited parkinsonism, which was unresponsive or only minimally responsive to levodopa treatment in those cases with multiple system atrophy (ΔUMSARS < 20%; off-state versus response to 200 mg levodopa/benserazide). A clinical follow-up exam was performed in all patients to gain information on disease progression and to validate the phenotype of the underlying disease.
Autonomic reflex testing

Autonomic reflex screening was performed by continuous heart rate recording (3-lead ECG-monitoring) and non-invasive blood pressure measurement based on beat-to-beat finger photoplethysmography (Finometer® mid, FMS) verified on the contralateral arm by automatic sphygmomanometry (Dinamap Pro 100, GE Healthcare). Data were digitized by an Analog-to-Digital converter (DI 720 series and DI-205-C, DATAQ Instruments, Inc.) with a sample rate of 500 Hz and 14-bit resolution using data acquisition software (WINDAQ®, DATAQ Instruments, Inc.). All vasoactive medications including antihypertensive and dopaminergic agents were discontinued for at least five half-lives before testing. Patients also had to refrain from alcohol, caffeine and nicotine for 24 h before the study. Autonomic reflex screening was performed in a supine position in a temperature-controlled room at least 3 h after a slight meal. Parasympathetic (heart rate response to deep breathing and Valsalva manoeuvre) and sympathetic (blood-pressure response to passive head up tilt and Valsalva manoeuvre) functions were graded by an age- and gender-adjusted Composite Autonomic Scoring Scale (CASS), an objective scale ranging from 0 (no deficit) to 7 (maximal deficit; without assessment of sudomotor function). Subjects with a summed score of 6 ≤ 3 on the CASS have mild autonomic failure; those with a summed score of 3 ≤ 6 have severe autonomic failure.

Single-photon emission computed tomography

123I-fluoropropyl (FP-CIT) SPECT reflects nigrostriatal transmission by visualizing presynaptic dopamine transporters in the striatum. SPECT imaging and data analysis was performed as described before (Plotkin et al., 2005). Briefly, 4 h after administration of 200 MBq 123I-FP-CIT the cerebral distribution of the tracer was assessed by a triple head camera system (Multispect 3, Siemens Medical Systems). The ratio of the specific binding within the putamen contralateral to the clinically more affected side in terms of movement impairment (SRcontra) versus an occipital reference region was used for further analysis.

The integrity of postganglionic sympathetic innervation of the myocardium was assessed by 123I-metaiodobenzylguanidine (123I-MIBG) myocardial scintigraphy after thyroid blockade with sodium perchlorate. Planar images were recorded 4 h post-injection by a double-headed gamma camera with a low energy high resolution (LEHR) collimator (either Millenium VG5 Hawkeye with VPC-45K collimator, GE Medical Systems-EU; or Symbia TruePoint SPECT-CT, Siemens) at a photopeak on 159 keV of a 15% window. SPECT imaging was performed on a 128 x 128 matrix as control. The heart to mediastinum ratio (H/M-ratio) of the 123I-MIBG uptake at 4 h was analysed, a cut-off value of 1.7 was defined.

Skin biopsy and immunohistochemistry

Skin biopsy, specimen preparation and staining

All subjects underwent 3 mm punch biopsy from the uninjured skin of the volar forearm of the clinically more affected side under 2% lidocaine local anaesthesia using sterile technique according to current guidelines (Lauria et al., 2010). The obtained biopsy consisted of a 3-mm core of skin (an ‘excision’) including epidermis and subpapillary dermis, to a depth of ~3 mm. The resulting lesion was allowed to heal by granulation with re-epithelialization. Biopsy specimens were immediately immersion-fixed with formaldehyde (formaldehyde 4% buffered, Herbeta Arzneimittel) for 24 h to 2 weeks, embedded in paraffin and sectioned in 3-µm thick sections by a microtome (HM335, MICROM Internation). One section per patient was stained with haematoxylin and eosin for morphological orientation within the sample. For immunohistochemistry, serial sections were stained by an auto-immunostainer (Benchmark or Benchmark XT, Ventana Medical Systems) with primary antibodies as listed in Table 1. Primary antibodies were detected by application of the iVIEW® DAB detection Kit (Ventana) based on a labelled streptavidin biotin method. Diaminobenzidine (Ventana) served as chromogen of the peroxidase reaction and counterstaining was performed with Blueing-haematoxylin (Ventana). Primary antibodies were omitted as negative controls. To validate the intra-axonal localization of pSNCA deposits we performed double-labeling immunofluorescence using anti-pSNCA and a neuronal marker [anti-protein gene product 9.5 (PGP 9.5)] in selected individuals. As secondary antibodies Cy3 and Alexa Fluor® were used. The fluorescence-labelled sections were mounted aqueously. Photomicrographs were taken with the Zeiss Observer.Z1 Microscope (Immunofluorescence).

Morphological analysis and assessment

Sections were examined with an Olympus BX 50 Microscope, and photomicrographic images were taken with the digital camera DP25 (Olympus) and cell®D software (Olympus Soft Imaging Solutions). A representative sample of each antibody was independently evaluated in a non-blinded manner by two neuropathologists (W.S., L.Z.). Skin sympathetic nerve fibres around sweat glands (dermal duct, SGD; secretory portion, SGS), arrector pili muscles (MAP) and arterial blood vessels (ART) were analysed within at least two histological levels. Antibody staining was assessed both qualitatively and semi-quantitatively. Detection rate (qualitative measure) was defined as the percentage of antibody positive skin elements (SGD, SGS, MAP, ART) related to all detected skin elements. Total-antibody-staining based on an antibody specific-semi-quantitative scoring scale. Phosphorylated SNCA antibody staining was graded as: 0 = absence of staining; 0.5 = slightly reduced staining pattern; 1 = strong staining pattern. The innervation of autonomic structures visualized by UCHL1-antibody and anti-TH were scored: 0 = absence of staining; 1 = severely reduced staining; 2 = slightly reduced staining; 3 = strong staining pattern (Supplementary Fig. 1). Assessment of antibody staining was limited to those nerve fibres that surrounded autonomic skin structures (SGD, SGS, MAP, ART). Staining of isolated, potentially sensory nerve fibres was ignored. Total antibody-staining (in per cent) was defined as the ratio between the actual detected staining and the maximum possible staining of an antibody summarized overall detected skin elements. As such, total pSNCA in sympathetic skin nerve fibres was assessed as: 100% x ∑ Score pSNCA (SGD + SGS + MAP + ART) / (maximum pSNCA score [1] x number of detected skin elements). Total UCHL1
and total TH were calculated as: 100% \times \sum \text{Score (SGD + SGS + MAP + ART)} / (\text{maximum score [3]} \times \text{number of detected skin elements}).

### Statistical analysis

Because of small sample size, normal distribution for quantitative data was assumed if skewness was $-1 < 0 > 1$ (Miles and Shevlin, 2001). For parametric data (BMI, $\Delta$SBP, H/M-ratio; $SR_{\text{contra}}$), between group analysis was performed by one-way ANOVA and Tukey’s multiple comparison test for post hoc analysis. Non-parametric data (age, disease duration, CASSv (vagal subscore); CASSa (adrenergic subscore); immunohistochemical scores) were analysed by Kruskal-Wallis test, and post hoc analysis consisted of Dunn’s multiple comparisons. Mann-Whitney U-test was performed to compare non-parametric data between two groups (disease duration of Parkinson’s disease and multiple system atrophy). Wilcoxon rank-sum test compared data of two correlated samples (sweat gland innervations SGD and SGS within one patient). Fisher’s exact test tested the relationship between disease and presence of pSNCA in sympathetic skin nerve fibres. Spearman’s rho ($\rho$) was used to express the relation between anti-pSNCA and anti-UCHL1, UPDRS, H/M-ratio, $SR_{\text{contra}}$, $\Delta$SBP and CASSv. Regression analysis estimated the relation between age and pSNCA in patients with Parkinson’s disease. Quantitative data were expressed as mean $\pm$ SD. Semi-quantitative and ordinal data were expressed as median [interquartile range, (IQR)]. A $P$-value $\leq 0.05$ was considered statistically significant, $P$-value adjustment strategy (Bonferroni) for multiple comparisons were performed when appropriate. Data were analysed using IBM® SPSS® Statistics software, Version 21.00.0.0 (IBM Corporation) and Prism 5 software for Windows, Version 5.02 (GraphPad Software, Inc.).

### Results

#### Clinical data

The study cohort comprised 26 patients diagnosed as Parkinson’s disease ($n = 10$), multiple system atrophy ($n = 10$) and essential tremor ($n = 6$, controls). Clinical phenotypes of multiple system atrophy were cerebellar in three cases and parkinsonian in seven cases. Clinical characteristics and results of autonomic function tests and
nuclear imaging are summarized in Supplementary Table 1 and Table 2. The initial diagnostic classification was confirmed by a clinical follow-up in all cases (mean follow-up period: 26 ± 13.7 month) except for one patient with multiple system atrophy and three controls that were lost to follow-up. Autonomic impairment, foremost adrenergic CASS score and orthostatic hypotension, was significantly higher in multiple system atrophy (P = 0.001) compared to Parkinson’s disease and control subjects, indicating the more severe and widespread autonomic involvement in the former. However, orthostatic hypotension of <20 mmHg occurred in 6 of 10 patients with Parkinson’s disease. Twenty-four patients underwent 123I-FP-CIT SPECT. Striatal dopaminergic function was impaired in all cases (multiple system atrophy, Parkinson’s disease) but preserved in controls (essential tremor) as indicated by significant reduction of SR (P < 0.001) contralateral to the clinically more affected side. Sympathetic cardiac innervation was significantly affected in both Parkinson’s disease and multiple system atrophy patients (H/M-ratio multiple system atrophy: 1.65 ± 0.32; Parkinson’s disease: 1.54 ± 0.43) but normal in controls (H/M-ratio controls: 2.33 ± 0.45). However, myocardial 123I-MIBG SPECT was not able to differentiate between a pre- and postganglionic autonomic lesion (H/M-ration multiple system atrophy versus Parkinson’s disease: P > 0.5; Tukey’s post hoc test), and predictability of individual cases was less distinct (unexpected 123I-MIBG reduction: 1/6 controls, 4/10 multiple system atrophy; unexpected normal 123I-MIBG: 3/10 patients with Parkinson’s disease).

**Innervation of autonomic skin structures**

UCHL1 immunoreactivity appeared as dotted and filamentous staining pattern in dermal nerve fibres of all patients and controls (detection rate 100%, Fig. 3A–I). UCHL1 staining in general was strong or slightly reduced (grading 2–3) with a predominance in SGS compared to SGD that was significant in Parkinson’s disease only [Parkinson’s disease P = 0.006; Wilcoxon rank-sum test (adjusted significance level P < 0.008)]. Total UCHL1 staining was significantly reduced in Parkinson’s disease when compared to multiple system atrophy and controls [UCHL1 total score: Parkinson’s disease 43% (38–56%), multiple system atrophy 67% (48–77%), controls 72.5% (64.3–83.5%); P = 0.004, Kruskal-Wallis test (adjusted significance level P < 0.008)] (Fig. 4B). In Parkinson’s disease, loss of UCHL1-positive nerve fibres was pronounced around SGD [Parkinson’s disease 1.0 (interquartile range, IQR 1.0–1.0); P = 0.007] and MAP [Parkinson’s disease 0.5 (IQR 0.5–0.6); P = 0.003]. UCHL1 staining in multiple system atrophy was slightly reduced around SGS [3.0 (IQR 2.0–3.0)] but pronounced around SGD [2.0 (IQR 2.0–3.0)] when compared to controls [SGS: 3.0 (IQR 3.0–3.0); SGD: 1.0 (IQR 1.0–1.0)]. Mann-Whitney U-test revealed a significant reduction of pilomotor nerve fibres in multiple system atrophy [1.0 (1.0–2.75)] compared to those of Parkinson’s disease [0.5 (0.5–0.63); U-test = 2.5; P = 0.003 (adjusted significance level P < 0.008)]. Because MAP was detected in only one essential tremor sample, the control group was excluded from statistical analysis.

Anti-TH staining resulted in a variable, less distinct dotted and filamentous pattern of nerve fibres surrounding autonomic skin structures. TH immunoreactivity was least frequent in controls (detection rate: controls 53%, multiple system atrophy 78%, Parkinson’s disease 65%). However, total-TH-staining did not differ significantly between groups [multiple system atrophy 37.5%, Parkinson’s disease 31%, controls 20%; P = 0.11, Kruskal-Wallis test (adjusted significance level P < 0.008)].

### Phosphorylated SNCA in skin sympathetic nerve fibres

Neuropathological data are summarized in Supplementary Table 2. Phosphorylated SNCA is visualized as fine-granular staining pattern in small nerve fibres surrounding autonomic skin structures. In patients with Parkinson’s disease, positive staining for pSNCA was observed in 7 of 10 SGS [0.5 (IQR 0–1.0); Fig. 1A and B], 8 of 10 ART [0.75 (IQR 0.38–1.0); Fig. 2A] and in three of three samples with MAP [0.5 (IQR 0.5–n.a.); Fig. 2B]. Phosphorylated SNCA staining was not discernible in nerve fibres supplying SGD of patients with Parkinson’s disease. The co-localization of anti-pSNCA and the neuronal marker UCHL1 as shown in Fig. 6 proves the intra-axonal localization of pSNCA deposits and distinguishes from unspecific background staining. In contrast to Parkinson’s disease, dermal autonomic nerves fibres of multiple system atrophy patients (Fig. 1C, Fig. 2C and D) and control subjects (Fig. 1D, Fig. 2E and F) were devoid of pSNCA [Fisher’s exact test χ² = 27; P < 0.001 (adjusted significance P < 0.0125)].

### Correlation of phosphorylated SNCA deposition and autonomic denervation

The parameter ‘total antibody-staining’ is a normalized semiquantitative measure of skin autonomic innervation (see ‘Materials and methods’ section) that allows between-group comparison independent from the number of detected dermal autonomic structures. Total pSNCA in patients with Parkinson’s disease ranged between 17–75% [33% (31–67%)] and was absent in multiple system atrophy patients and controls (Fig. 4A). There was a negative correlation between total pSNCA and the degree of dermal denervation assessed by anti-UCHL1 staining (ρ = −0.57; P = 0.002).

In Parkinson’s disease, total pSNCA did not correlate with clinical or nuclear imaging measures: disease duration (ρ = −0.57; P = 0.086), UPDRS (ρ = −0.38; P = 0.82), Hoehn and Yahr (ρ = −0.25; P = 0.492), H/M-ratio (ρ = 0.29; P = 0.423), SR_contra (ρ = 0.18; P = 0.62), ΔSBP (ρ = 0.4; P = 0.25), CASSv (ρ = 0.2; P = 0.638). Innervation
of autonomic skin structures (total UCHL1 staining) was significantly reduced in Parkinson’s disease ($P = 0.004$, see above). This loss of UCHL1 immunoreactivity was independent from patients age ($r = -0.12$; $P = 0.73$; Fig. 5) and disease duration ($r = -0.35$; $P = 0.31$).

**Discussion**

Our study addressed the detection and distribution of pathological SNCA in dermal sympathetic nerve fibres in vivo in two clinically similar but neuropathologically distinct neurodegenerative conditions, multiple system atrophy and Parkinson’s disease. The main results of our study are as follows: (i) pSNCA deposits were detected in nerve fibres innervating skin autonomic structures (sweat gland, arrector pili muscle and arterial blood vessels) in SNCA (alpha-synuclein) dependent neurodegenerative disorders; (ii) pSNCA deposition was limited to Parkinson’s disease whereas dermal nerve fibres of multiple system atrophy patients were devoid of pSNCA; and (iii) pSNCA deposition correlated with dermal denervation (UCHL1 staining).

Phosphorylated SNCA is considered the pathognomonic protein underlying Parkinson’s disease and multiple system atrophy. The neuropathological diagnosis of multiple system atrophy and Parkinson’s disease is based on specific regional distribution of pSNCA in glial cells and neurons of the CNS, respectively. The involvement of peripheral postganglionic autonomic neurons in Parkinson’s disease only led to the particular diagnostic value of autonomic function tests [thermoregulatory sweat test and quantitative sudomotor axon reflex test (QSART)] to separate Parkinson’s disease and multiple system atrophy (Kimpinski et al., 2012; Orimo et al., 2012). Nevertheless, sensitivity and specificity of clinical diagnostic tools remain limited (Kimpinski et al., 2012; Orimo et al., 2012).

Wang et al. (2013) reported a higher incidence of SNCA in sympathetic skin nerve fibres in patients with Parkinson’s disease compared to controls, and thus proposed SNCA deposits in skin nerve fibres as a diagnostic marker of Parkinson’s disease (Wang et al., 2013). However, the used antibody stained physiological soluble SNCA, a ubiquitous predominately presynaptic protein, and was not specific for the underlying pathogenic protein—pSNCA. Our finding of peripheral pSNCA deposits within skin nerve fibres is supported by post-mortem studies in neuro-pathologically confirmed Parkinson’s disease, demonstrating pSNCA in skin wedge samples of the upper arm, abdomen and scalp (Ikemura et al., 2008; Beach et al., 2010). In a recent study, Donadio et al. (2014) reported pSNCA in sympathetic nerve fibres of intravital cervical skin samples in 21/21 patients with Parkinson’s disease. Phosphorylated SNCA staining was pronounced in dermal nerve bundles and nerves of arterioles (22%, respectively).
and thus differed slightly from our findings (MAP 100%, ART 80% and SGS 70%).

In our present study, all patients with Parkinson’s disease presented pSNCA depositions within at least one dermal nerve fibre innervating autonomic skin structures (sensitivity of 100%). In contrast, evidence of dermal pSNCA deposits in previous histopathological studies ranged from 70% in skin samples of the upper arm and abdomen (Ikemura et al., 2008) to even 0% immunoreactivity in scalp and abdominal skin samples (Beach et al., 2010). Pre-mortem studies of patients with clinically diagnosed Parkinson’s disease present with likewise heterogeneous results; pSNCA was detected in skin samples of the distal and proximal leg, back and finger in 16/35 (45.71%) patients (Doppler et al., 2014); of the chest wall in 2/20 (10%) patients (Miki et al., 2010) but was absent in biopsies of the ventral forearm (Kawada et al., 2009) and distal leg (Navarro-Otano et al., 2014). The variable detection of pSNCA deposits in Parkinson’s disease skin biopsies may be a result of different skin sample size, tissue fixation, antibody pretreatment and biopsy site. The selection of the biopsy site should consider the physiological distribution of SNCA. Physiological SNCA is predominately located in presynaptic nerve terminals and implicated in synaptic plasticity (Watson et al., 2009), learning (George et al., 1995), neurotransmitter release (Burre et al., 2010), and synaptic vesicle pool maintenance (Dikiy and Eliezer, 2012). Nerve terminals are located at sympathetic effector

Figure 2 Immunohistochemistry with phosphorylated SNCA in arterioles (ART) and arrector pilorum muscles (MAP) of patients with Parkinson’s disease, multiple system atrophy and controls (essential tremor). In Parkinson’s disease, pSNCA was present in vasomotor (A) and pilomotor (B) sympathetic nerve fibres. In multiple system atrophy (C and D) and essential tremor (E and F) no pSNCA immunoreactivity was detectable in sympathetic nerve fibres of arterioles (C and E) and arrector pili muscles (D and F). The arrowheads indicate pSNCA deposition. The arrow marks an unspecific staining, presumably a macrophage. Counterstain = haematoxylin. Scale bar = 50 μm.
organs, e.g. sweat glands. Sweat gland density is higher at the ventral forearm (159/cm²) compared to the proximal sites abdomen (81–141/cm²), back (106–132/cm²), neck (126/cm²), upper arm (94–102/cm²) and distal sites such as leg (31–132/cm²), except finger (126–350/cm²) and scalp (70–195/cm²) (Taylor and Machado-Moreira, 2013). Previous studies proposed a proximo-distal gradient of pSNCA appearance with the highest incidence cervically (Donadio et al., 2014), and decreasing levels at the back (Doppler et al., 2014), abdomen, leg, upper arm or finger (Ikemura et al., 2008). Variable sweat gland density may contribute to this effect. High variability of pSNCA expression at different localizations requires a consensus with respect to biopsy location and sample preparation. Our data

Figure 3 UCHL1 Immunostaining of a dermal sweat gland duct (SGD) and secretory portion (SGS), arterioles (ART) and arrector pili muscles (MAP) in Parkinson’s disease (A–C), multiple system atrophy (multiple system atrophy, D–F) and controls (essential tremor, G–I). In controls, UCHL1 sympathetic skin nerve fibres were well preserved in sweat glands secretory portion (G), cutaneous arterioles (H) and arrector pili muscle (I). In contrast, patients with Parkinson’s disease had an obviously reduced innervation pattern in sweat gland secretory portion (A), and pilomotor nerve fibres (C) compared to controls (G–I). Sympathetic nerve fibres of sweat gland dermal duct and arrector pili muscle were more abundant in multiple system atrophy (D–F) than in Parkinson’s disease (G–I). Positive UCHL1 staining was verified by staining of nerve fascicles (N). T = sebaceous gland; Counterstain = haematoxylin. Scale bar = 50 µm.
promote the ventral forearm as preferred biopsy site for pSNCA detection in sympathetic skin nerve fibres.

The pathological hallmark of multiple system atrophy are cytoplasmatic pSNCA deposits in oligodendrocytes (glial cytoplasmatic inclusions) and to a lesser extend in nuclei of oligodendrocytes as well as in the nuclei and cytoplasm of neurons (Ahmed et al., 2012). The pathological process affects central and brainstem autonomic centres as well as preganglionic sympathetic neurons in the intermediolateral cell column (Wenning et al., 2004). Our present study is the first investigating pSNCA in dermal nerve fibres of multiple system atrophy patients in vivo. We demonstrate that despite the severe autonomic impairment, nerve fibres surrounding autonomic skin structures in multiple system atrophy are devoid of pSNCA deposits. Our finding is consistent with the results of Ikemura et al. (2008) demonstrating absence of pSNCA staining in skin samples of abdomen and upper arm of three patients with neuropathologically confirmed multiple system atrophy. In contrast, autopsy studies on peripheral sympathetic ganglia in multiple system atrophy patients revealed neuronal SNCA inclusions in 25% (Nishie et al., 2004) and Lewy bodies in late stage multiple system atrophy in 42.3% (mean disease duration 12.8 ± 5.3 years) (Sone et al., 2005). Furthermore, pSNCA has rarely been shown in the enteric and myocardial nervous system in multiple system atrophy (Orimo et al., 2008; Pouclet et al., 2012b). These findings might be limited to advanced stages of the disease as they were detected in post-mortem studies only, or in cases of concurrent Lewy body disorders. Our present study does not support the notion of an involvement of postganglionic sympathetic skin nerve fibres in multiple system atrophy with a clinical duration up to 7 years. Thus, testing sympathetic skin nerve fibres for pSNCA deposits appears worthy to differentiate Parkinson’s disease from multiple system atrophy. Additional studies with patients at different stages of the diseases are necessary to verify the involvement of skin sympathetic nerve fibres in multiple system atrophy.

Loss of dermal nerve fibres and reduced epidermal nerve fibre density in Parkinson’s disease has been repeatedly demonstrated (Dabby et al., 2006; Nolano et al., 2008; Novak et al., 2009; Navarro-Otano et al., 2014). However, quantification of autonomic dermal nerve fibres is complex due to insufficiency of sectional images depicting sterical organization of 3D sympathetic nerve fibre plexus. Accurate quantification requires immunofluorescence staining, confocal microscopy and is time consuming (Donadio et al., 2014). Our present study was not intended to access dermal nerve fibre density but detection of dermal

**Figure 4** Comparison of total pSNCA and total UCHL1 in sympathetic skin nerve fibres between controls (essential tremor), multiple system atrophy and Parkinson’s disease. Total antibody is the ratio of the summed scores of the present innervation for each detected skin element to the sum of the greatest possible score of detected skin elements. PSNCA was expressed and quantified in Parkinson’s disease (PD), but absent in essential tremor (ET) and multiple system atrophy (MSA) (A). Total UCHL1 was significantly reduced in Parkinson’s disease compared to multiple system atrophy and essential tremor (B). The data are presented as median ± IQR. *Tukey post hoc test P < 0.05.

**Figure 5** Linear regression of total UCHL1 versus age. Patients with Parkinson’s disease presented a loss of UCHL1 positive nerve fibres independent of age [Parkinson’s disease (PD) r² = 0.01; essential tremor (ET) r² = 0.00; multiple system atrophy (MSA) r² = 0.23]. Regression line is plotted for patients with Parkinson’s disease.
intra-axonal pSNCA depositions, which is why we deviated from the EFNS guidelines on the use of skin biopsy (Lauria et al., 2010). Nevertheless, our data support the finding of dermal denervation in Parkinson’s disease by a significant reduction of total-UCHL1 staining that occurred independent from patient’s age and correlated with the extent of intra-axonal pSNCA deposition. This is in line with recent studies proposing a length-independent loss of intraepidermal nerve fibres associated with appearance of pSNCA at proximal sites (Doppler et al., 2014) and sweat gland denervation correlating with pSNCA distribution (Donadio et al., 2014). To avoid any confounding effect of potential small-fibre neuropathy, we excluded patients suffering from diabetes mellitus or alcoholism.

The present study has several limitations. First, diagnostic classification of patients relied on clinical criteria and putative neuropathological abnormalities are not definite (no neuropathological confirmation available). To increase diagnostic reliability, additional diagnostic procedures (nuclear imaging, autonomic testing) and clinical follow-up supported the clinical diagnosis. Second, specificity of nuclear imaging, especially of $^{123}$I-MIBG-SPECT is limited as indicated by 8 of 26 conflicting $^{123}$I-MIBG results in our study. Overlapping results of myocardial $^{123}$I-MIBG SPECT in multiple system atrophy and Parkinson’s disease have been reported before (Kimpinski et al., 2012; Orimo et al., 2012). Although we excluded patients suffering from diabetes mellitus and alcoholism, inapparent peripheral neuropathy is highly prevalent in the age of Parkinson’s disease and potentially decreases nerve fibre density (total-UCHL1 staining). Anti-TH staining, applied to distinguish sympathetic from sensory dermal nerve fibres, resulted in highly variable patterns of immunoreactivity unsuitable for double-immunofluorescence staining (anti-UCHL1 and anti-TH). We therefore limited assessment of antibody staining to dermal nerve fibres surrounding autonomic skin structures (SGD, SGS, MAP, ART) ignoring isolated, potentially sensory nerve fibres. Whether those dermal nerve fibres are affected as well by pSNCA depositions is uncertain. Finally, semiquantitative scoring of staining patterns underlie subjectivity and thus reduce reliability.

In summary, we present the first in vivo comparison of pSNCA deposits in dermal sympathetic nerve fibres in Parkinson’s disease, multiple system atrophy and controls of similar age and gender. We were able to separate multiple system atrophy from Parkinson’s disease on the basis of peripheral pSNCA pathology that was limited to the latter. pSNCA deposits may contribute to cutaneous nerve fibre loss and be related to autonomic symptoms in Parkinson’s disease. Immunohistochemical analysis of pSNCA in skin biopsies appears sensitive, feasible, safe and inexpensive when compared to current diagnostic methods such as $^{123}$FP-CIT-SPECT and myocardial $^{123}$MIBG. Further studies with larger sample sizes and at

Figure 6 Evidence of intraaxonal localization of anti-pSNCA depositions. Double-labelling immunofluorescence of anti-pSNCA (A) and anti-protein gene product (B), UCHL1. Phosphorylated SNCA is strongly co-staining with UCHL1 within axonal structures surrounding sweat glands in a patient with idiopathic Parkinson’s disease indicating intra-axonal localization of pSNCA deposition (C). Scale bar = 50 μm.
different disease stages are needed to prove whether detection of peripheral pSNCA pathology in skin biopsies will increase diagnostic reliability of synucleinopathies.

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Supplementary material

Supplementary material is available at Brain online.

References


