Supplemental Methods

Skin biopsy

Three mm punch biopsies were taken from proximal and distal hairy skin sites. The proximal site included the cervical C8 paravertebral area whereas distal sites were located in the thigh (15 cm above the patella) and distal leg (10 cm above the lateral malleolus). A second skin biopsy was taken 3-4 centimetres away from the first sample to assess the pattern of intraneural α-synuclein deposits.\textsuperscript{1,2} We were unable to take skin samples from a thigh (in 1 DLB patient), a leg (in 1 different DLB patient) and thigh and leg (a third DLB patient) because of patient’s refusal. According to previously published procedures\textsuperscript{3} skin samples were immediately fixed in cold Zamboni’s fixative and kept at 4°C overnight.

Phosphorylated α-synuclein deposits

Ten μm sections were obtained using a freezing sliding microtome (HM550, Thermo Scientific, Walthan, MA, USA) to evaluate α-synuclein deposits.\textsuperscript{1,2,4} They were double-immunostained overnight with a panel of primary antibodies including rabbit monoclonal phosphorylated α-synuclein at Ser 129 (p-syn; 1:500, abcam, Cambridge, UK, cat. num. ab-51253) or mouse p-syn (1:4000, BioLegend, San Diego, CA, USA, cat. num. 825701), mouse pan-neuronal marker protein gene product 9.5 (1:750; Abcam, Cambridge, UK, cat. num. ab72911), rabbit tyrosine-hydroxylase (TH, 1:1000, Novus Biologicals, Littleton, CO, USA, cat. num. NB300-109), to identify the noradrenergic fibers and rabbit vasoactive intestinal peptide (VIP, 1:1000, Incstar, Stillwater, MN, USA, cat. num. NB300-109) co-localized in the sudomotor cholinergic fibers.\textsuperscript{3} Sections were then washed and secondary antibodies were added for an incubation of 1 hour. As secondary antibodies, an anti-mouse Alexa Fluor(R) 488 (1:400; Jackson ImmunoResearch, West Grove, PA, USA, cat. num. 715-545-150) or rabbit cyanine dye fluorophores 3.18 (1:200 when double-stained with p-syn or 1:800 double-stained with VIP and TH, Jackson ImmunoResearch, West Grove, PA, USA; cat. num. 711-165-152) were used. The microscope analysis and criteria followed to define a p-syn positivity were previously described.\textsuperscript{1,2,4} Shortly, sections were initially viewed and analyzed under a Zeiss fluorescent microscope. The correspondence between rabbit p-syn and mouse PGP staining helped to verify the intraneuronal deposits excluding possible non-specific staining arising from the background. The analysis was made in a blinded fashion by two authors with expertise in immunoflorescent analysis (DV and IA). P-syn staining was rated in each skin site as the percentage of autonomic structures or nerve bundles showing a positive staining at high magnification (x40). For a 3D co-localization analysis of mouse p-syn with specific autonomic
markers (i.e. rabbit TH or VIP) digital images were also acquired using a laser-scanning confocal microscope (Leica DMIRE 2, TCS SL, Leika Microsystems, Heidelberg, Germany). Each image was collected in successive frames of 1-2 μm increments on a Z-stack plan at the appropriate wavelengths for the fluorophores coupled to secondary antibodies with a x20 or x40 plan apochromat objective and subsequently projected to obtain a double-stained 3D digital image by a computerized system (LCS lite, Leica Microsystems, Heidelberg, Germany).

Skin innervation
Additional 50 μm sections from the same skin sample were obtained during the freezing sliding microtome session. Twelve free-floating sections were incubated overnight with a panel of primary antibodies, including the rabbit pan-neuronal marker protein gene product 9.5 (1:1000; AbD Serotec, Raleigh, NC, USA, cat. num. 7863-0504) and mouse collagen IV (ColIV, 1:800, Chemicon, Temecula, CA, USA, cat. num. MAB1910). Sections were then washed and secondary antibodies, labeled with mouse Alexa Fluor(R) 488 (see above) and rabbit cyanine dye fluorophores 3.18 (1:800 see above) were added for an overnight incubation. Sections were initially viewed under a Zeiss fluorescent microscope (model Axioskop 40; Jena, Germany). Autonomic innervation density was quantified using the previously described automated method showing a high interobserver and intraobserver reliability.1-4 Briefly, this method is based on a technique known as the ‘unsharp mask filter’ which creates a composite image by subtracting the background color in the out of focus image from the base image expressing the autonomic innervation staining (Image Pro Plus, Media Cybernetics). Target autonomic structures included arrector pilum muscle and sweat gland. The corresponding innervations were quantified as PGP percent area providing a stronger staining easier to quantify than the specific autonomic markers but which was correlated with the innervation quantified by the specific autonomic markers.3 Autonomic innervation score in each skin site usually represented the mean of three different target structures identified by ColIV staining. Epidermal nerve fiber density was calculated by considering a single epidermal nerve fiber marked by PGP 9.5 crossings of the dermal–epidermal junction. A 3D study of the innervation pattern was made using a laser-scanning confocal microscope as specified above.

Statistical analysis
Statistical analyses were performed using SPSS 15.0 for Windows. One-way analysis of variance (ANOVA) followed by a post hoc LSD test was performed for comparison of the age at examination. The difference in sex distribution among groups was evaluated with the χ2 test. Parametric tests were used as Kolmogorov–Smirnov testing showed that the variables were normally distributed. Differences in skin innervation scores among groups were assessed by
ANOVA followed by Bonferroni post hoc test for pair wise comparisons. Unpaired Student’s t test was used to compare clinical parameters between DLB and NSD and abnormal p-syn deposits and skin innervation scores between DLB patient subgroups (autonomic vs non autonomic symptom; dementia vs parkinsonism onset symptom; with vs without L-DOPA treatment). Linear correlation using Pearson test was used to correlate the percentage of p-syn deposits in analysed skin samples with clinical parameters and the innervation scores. The chosen level of statistical significance was p<0.05 for all tests.

References