Title: Small fiber neuropathy and phosphorylated alpha-synuclein in the skin of E46K-SNCA mutation carriers

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ABSTRACT

Background and objective: In 2004 we described the E46K mutation in alpha-synuclein gene (E46K-SNCA), a rare point mutation causing an aggressive Lewy body disease with early prominent non-motor features and small fiber denervation of myocardium. Considering the potential interest of the skin as a target for the development of biomarkers in Parkinson's Disease (PD), in this work we aimed to evaluate structural and functional integrity of small autonomic nerve fibers and phosphorylated alpha-synuclein (p-synuclein) deposition in the skin of E46K-SNCA carriers as compared to those observed in parkin gene mutation (PARK2) carriers and healthy controls. ***Patients and methods:*** We studied 7 E46K-SNCA carriers (3 dementia with Lewy bodies, 2 pure autonomic failure, 1 PD and 1 asymptomatic), 2 PARK2 carriers and 2 healthy controls to quantify intraepidermal nerve fiber density and p-synuclein deposition with cervical skin punch biopsies (immunohistochemistry against anti PGP9.5/UCHL-1, TH and p-synuclein) and sudomotor function with electrochemical skin conductance (ESC) (SudoScan). ***Results:*** All E46K-SNCA carriers had moderate to severe p-synuclein deposits and small fiber neurodegeneration in different epidermal and dermal structures including nerve fascicles and glands, especially in carriers with Pure Autonomic Failure, while p-synuclein aggregates where absent in healthy controls and in one of two PARK2 carriers. The severity of the latter skin abnormalities in E46K-SNCA were correlated with sudomotor dysfunction (lower ESC) in hands (p=0.035). ***Interpretation:*** These results together with our previous findings support the relevance of E46K-SNCA mutation as a suitable model to study small fiber neuropathy in Lewy body diseases.

INTRODUCTION

In the last years, different authors have reported that alpha-synuclein aggregates, the main component of Lewy bodies, can be detected within cutaneous autonomic small fibers of patients with Parkinson's Disease (PD) using the skin punch biopsy [1,2]. It has been described that accumulation of synuclein-immunoreactive deposits is most prominent in sympathetic adrenergic nerve fibers innervating the erector pili muscles but is also present in sudomotor nerve fibers, i.e. sympathetic cholinergic fibers [3]. Nonetheless, other studies have reported more prominent deposits in nerve fibers of skin vessels [4]. Growing evidence supports the detection of phospho-synuclein (p-synuclein) aggregates in cutaneous nerves as a promising approach to improve the identification of patients with synucleinopathies, including PD [1,4], pure autonomic failure (PAF) [5], REM sleep behavior disorder (RBD) [6] or dementia with Lewy Bodies (DLB) [7]. In addition to morphological abnormalities, several studies have quantified non-invasively sudomotor function abnormalities in synucleinopathies with different technological approaches such as quantitative sudomotor axon reflex testing [8,9] or skin electroconductance (ESC) measurement tools [10,11].

Considering the great heterogeneity of idiopathic PD (iPD), the study of clinically and pathophysiologically homogeneous genetic PD variants becomes of the utmost importance for the development of biomarkers in iPD. In 2004 we described for the first time in the literature a family of the Basque Country (Spain) with the E46K mutation, one of the three known missense point-mutations in the alpha-synuclein gene (SNCA)[12]. SNCA-linked mutations are considered a rare condition as they are

limited to specific families and series around the world [13]. Experimental studies with E46K-SNCA mutation have shown its strong tendency towards fibril formation and its outstanding pathogenicity [14,15]. Moreover, our clinical and post-mortem follow-up observations on E46K-SNCA carriers support that this mutation is a prototypical model for pure and aggressive Lewy body diseases. The clinical phenotype of E46K-SNCA mutation is characterized by an early onset parkinsonism with prominent non-motor features including dementia, autonomic dysfunction and sleep disturbances [16–18]. Neuropathological examination of the index case revealed extensive Lewy bodies and neurites in cortical and subcortical structures of the brain, meeting the pathological criteria for DLB [16]. Interestingly, in addition to CNS involvement, the postmortem examination of the myocardium in two symptomatic carriers [16] together with in vivo studies using autonomic functional tests and myocardial metaiodobenzylguanidine (MIBG) scintigraphy [19,20] support that E46K-SNCA mutation induces a prominent autonomic neuropathy that affects small noradrenergic sympathetic fibers. On the opposite side, Parkin gene mutation (PARK2) is associated to a disease in which autonomic abnormalities and myocardial sympathetic denervation are subtle [20,21] and Lewy bodies are virtually absent, especially in homozygous PARK2 variants [22].

Except for a recent letter reporting pathological skin findings in two PARK2 carriers [23], as far as we are concerned no studies have been published describing structural or functional correlates of small nerve fiber neuropathy in the skin of genetic carriers of PD. In this work, we aimed to evaluate and describe the integrity of small autonomic nerve fibers and p-synuclein deposition in the skin of E46K-SNCA carriers, with different phenotypes and their correlation with sudomotor function. The study fo

E46K-SNCA mutation carriers constitutes the ideal scenario to analyze the presence of p-synuclein aggregates in the peripheral nervous system of patients with PD, since this mutation induces a genetically defined aggressive Lewy body disease with prominent autonomic failure, cognitive and motor symptoms.

MATERIAL AND METHODS

We performed a cross-sectional study of 7 E46K-SNCA carriers from the same single family, 6 symptomatic (3 dementia with Lewy bodies, 2 pure autonomic failure (PAF) and 1 PD) and 1 asymptomatic, 2 carriers of heterozygous PARK2 mutation and 2 healthy controls (HC). See table 1 for further details on demographical and disease-related information of individual participants. Subjects were recruited in the Department of Neurology of Cruces University Hospital. The study procedures were approved by the regional Basque Clinical Research Ethics Committee. All participants gave written informed consent prior to their participation in the study, in accordance to tenets of Declaration of Helsinki.

Skin Biopsies

We obtained 4-mm diameter skin punch biopsies from the cervical C7 region in all subjects. Biopsies were performed under aseptic and anesthetic conditions, following recommendations by Donadio et al. [24] to improve phosphorylated synuclein detection. Samples were collected at Cruces University Hospital, fixed in 4% formaldehyde for 24 hours, embedded in paraffin and serially cut into 5 µm sections for immunohistochemistry.

Immunohistochemistry studies

Slides were oven-heated at 60ºC for 30 minutes, immersed in Xylene (5 minutes, three times) and then rehydrated in decreasing ethanol concentrations (100% - 95%, 70% - 50%) and in distilled and tap water (5 minutes each). Endogenous peroxidase activity was inhibited incubating sections in tap water with 0.02 % hydrogen peroxide (H_2O_2) for 15 minutes. For antigen retrieval, sections were incubated in 0.01 M citrate buffer, pH 6.0, for 20 min at 96 ºC, in a PT module TS (Thermo Fisher Scientific). Then, tissue sections were rinsed in 0.1 M PBS (5 min, three times) and incubated overnight in DAKO antibody diluents (S2022) containing the corresponding primary antibody: polyclonal antibody against protein gene product (anti-PGP 9.5; 1/1000; AB1761: Millipore Billerica, MA, USA) as a neuronal marker for skin innervation; monoclonal mouse antibody against tyrosine hydroxylase (anti-TH monoclonal mouse; 1:1000; MAB5280: Millipore, Billerica, MA, USA) for noradrenergic fibre staining; monoclonal mouse antibody against phospho-synuclein (anti-p-synuclein S129 monoclonal mouse; pSyn#64, 1:2000, WAKO, Japan) as a marker of PD pathology. Samples were rinsed three times for 5 min in PBS and incubated for 30 min at room temperature with biotinylated goat anti-rabbit IgG (E0432, Dako, Denmark) or biotinylated goat anti-mouse IgG (E0433, Dako, Denmark) diluted 1:200 in PBS. Sections were developed using 3, 3´-diaminobenzidine tetrahydrochloride with H_2O_2 (DAB Kit, Vector Laboratories) and counterstained with Nissl. The sections were mounted on gelatinized slides, coverslipped with DPX mounting medium (BDH) and examined by light microscopy. Each staining also included a negative control in which the primary

antibody was omitted. In order to better characterize the deposits, we performed double-labeling of aggregates with a combination of antibodies against p-synuclein and Thiflavin S. Detection fluorescence was performed with secondary antibodies coupled to fluorescent markers. These sections were coverslipped with PBS-Glicerol (1:1) and examined under a confocal microscope (LSM 510 meta, zeiss).

Quantification of the skin nerve fiber density and synuclein deposits

Samples were viewed and digitalized with an Olympus BX-51 microscope equipped with an Olympus DP-70 digital camera at 60x using CAST grid software (Olympus, Denmark). The dermis was included for quantifying PGP immunoreaction. Non-consecutive sections were stained to avoid overquantification. For the analysis, we included thirty images taken from each sample, using a lens of 60x magnification. For every case, we included 30 sections of the sample that were randomly digitalized and analyzed. We used a computer-assisted image analysis with a macro of instructions to be executed in the software ImageJ (Wayne Rasband, NH, USA). The same threshold limits were defined for immunohistochemistry images of PGP-9.5. Intraepidermal nerves were counted when crossing or originating at dermal-epidermal junction, branching in the dermis out of the virtual line at the dermal-epidermal junction was excluded from quantitation. We measured the immunoreactivity of the PGP-ir axons by using the ImageJ software, with a specific image-processing tool. Blinded and randomized quantification of all samples was performed by the same examiner (MCA) using the same microscope. Intra-observer agreement was evaluated by counting the number of nerve fibers per area twice, one month apart. There was good

intraobserver reliability of PGP-ir measures. The results for epidermal nerve fiber density were expressed as number of fibers/area.

Lastly, we evaluated in all participants the degree of p-synuclein deposits within nerve fascicles with a semi-quantitative visual score: 'absent' (score 0); '+', slight (score 1); '++', moderate (score 2); '+++', severe (score: 3).

Sudomotor testing

Overall sudomotor function was evaluated with SudoScan device (Impeto Medical, Paris France), which acquires non-invasively through reverse iontophoresis ESC measures in hands and feet using two sets of large-area stainless steel plate electrodes. ESC is expressed in micro-Siemens (μS) and constitutes a surrogate measure of postganglionic sympathetic (cholinergic) function. During testing, subjects were required to place their hands and feet on electrode plates for approximately 2 minutes. We obtained separate average ESC measures for each limb (right palm, left palm, right foot sole and left foot sole) as well as the bilateral ESC averages for palms and foot soles. For this study, we only used bilateral ESC averages for hands and feet as surrogate markers of overall sudomotor function. SudoScan has been used to test sudomotor function, without any commercial interest. Further details on SudoScan technology are detailed elsewhere [25]. This technique was not conducted to analyze sympathetic cholinergic function, as SudoScan is not appropriate for the latter purpose. It is important to stress here that ESC measurements from SudoScan are not specific enough to ascertain the participation of specific pathophysiological mechanisms, including loss of sudomotor fibers, sweat gland atrophy, reduced number

of sweat glands, or glandular dysfunction caused by toxic, metabolic, or other disorders.

Statistics

We performed descriptive and correlation analyses for E46K-SNCA carriers using the statistical software package SPSS 13 for Windows (SPSS, Chicago, IL). Given the limited sample size, the correlations of nerve fiber density with age, years of disease duration and p-synuclein deposition scores were performed with the nonparametric Spearman´s rho test.

RESULTS

The demographical and clinical features of study participants and the results of the analysis of nerve fiber density, degree of p-synuclein deposits and electrochemical conductance of the skin are displayed in Table 1. All E46K-SNCA carriers had p-synuclein inclusions in cutaneous sympathetic fibers at different degrees of severity (Table 1, Figure 1 and Figure 2). All the samples had TH-immunoreactive nerve fibers, revealing the presence of noradrenergic nerve fibers in skin samples. Nerve fibers were identified surrounding sweat grands, hair follicles and erector pili muscles. Interestingly, for those E46K-SNCA carriers with clinical phenotypes suggesting a diffuse CNS involvement (e.g. DLB and PD phenotypes) (A03, A05 and A07) the severity of p-synuclein deposits was rather heterogeneous, from mild to moderate, while for those E46K-SNCA with manifestations restricted to the autonomic nervous system (PAF) (A04 and A06) the degree of p-synuclein was consistently high. Contrarily, healthy controls and one of the two PARK2 carriers (P02) did not show p-synuclein

inclusions in nerve fibers. Figure 1 illustrates the distribution of p-synuclein deposition in epidermis, glands and nerve fascicles between E46K-SNCA cases (A02, A06 and A07) and two reference cases with practically absent p-synuclein pathology (P02 and H01), and the presence of p-synuclein deposits within nerve fascicles. Regarding the quantification of skin innervation, even though the number of studied healthy controls was not enough to perform group comparisons, following the descriptive purpose of the analysis, when we analyzed the relation between nerve fiber density and the degree of p-synuclein aggregates we observed a statistically significant negative correlation between both measures (r = -0.889, p = 0.002) (mean fiber density for severe p-synuclein aggregates: 9.02 fibers/area; for moderate p-synuclein: 12.24 fibers/area; for mild p-synuclein: 15.42 fibers/area; for absent p-synuclein: 17,23 fibers/area). It is worth mentioning that the density of nerve fibers was low and p-synuclein deposition moderate to severe in the asymptomatic E46K-SNCA mutation carrier with normal physical and ancillary examinations (A02) (8.83 fibers/area and moderate degree of p-synuclein deposits) and in the PARK2 mutation carrier (P03) who had an exceptionally long disease duration (30 years) (9.62 fibers/area and severe p-synuclein). However, in the overall analysis the correlation with nerve fiber density was not significant for age (r = -0.072, p = 0.844) or disease duration (r = -0.235, p = 0.513) (Figure 3).

In terms of the evaluation of sudomotor function with SudoScan, we found that compared to the healthy control the decrease of ESC in patients was more pronounced in hands than in feet (average difference of 11.89 µS in hands and 2.66 µS in feet). In general, patients with low fiber density were those who had lower ESC values,

especially in hands (Table 01). In fact, there was a significant positive correlation between ESC values in the upper extremities and nerve fiber density (r = 0.669, p = 0.035) or the degree of p-synuclein inclusions (r = -0.889, p = 0.002) (Figure 3). However, the correlation between ESC in feet and nerve fiber density was not significant (r = 0.185, p = 0.608). Of note, for the asymptomatic E46K-SNCA carrier (A02) and for the PARK2 patient (P02), despite the number of epidermal nerve fibers (below 9 fibers/area), the ESC remained in normal ranges both in hands and in feet (above 70 µS), which may suggest the existence of possible mechanisms of compensation for sudomotor function in both cases.

DISCUSSION

In this study, we provide for the first time, evidences of the existence of small fiber neuropathy in the skin of E46K-SNCA carriers, one of the best known in vivo genetic models for PD and Lewy body disorders. This small nerve fiber degeneration was accompanied in all E46K-SNCA carriers by moderate to severe aggregates of p-synuclein in skin biopsies, especially in patients with predominant autonomic features. In opposition, p-synuclein aggregates were absent in healthy controls and in one out of two PARK2 mutation carriers. Moreover, the severity of fiber density and p-synuclein deposition in the skin of E46K-SNCA carriers were correlated with the deterioration of sudomotor function as measured by electroskin conductance, which provides a correspondence between structural and functional injury to our results (Figure 3). All these findings, together with our previously published evidences on autonomic myocardial denervation [16,19], support the relevance of E46K-SNCA mutation as a

suitable model of small fiber neuropathy related to p-synuclein pathology, in Lewy body diseases.

It is remarkable that we identified aggregates of p-synuclein and low density of small nerve fibers in the skin not only in symptomatic but also in young asymptomatic E46K-SNCA carriers. More specifically, we observed higher loads of skin p-synuclein in those E46K-SNCA carriers with prominent dysautonomia, which is in line with a recent study by Donadio et al. demonstrating wider p-synuclein deposits in autonomic skin nerves of iPD patients with orthostatic hypotension versus those without orthostatic hypotension [26]. Interestingly, in our study, the degree of p-synuclein deposits in the skin of E46K-SNCA carriers with PAF (A04 and A06) was consistently severe and accompanied by low fiber density. Whereas for those patients with diffuse CNS involvement (DLB and PD phenotypes) (A03, A05 and A07) the severity p-synuclein aggregates and skin denervation was heterogeneous from one patient to the other. This may imply that the progression of the pathogenic process is different between members of the same family, Suggesting the existence of different phenotypes within the same genotype, which may be linked to epigenetic factors. Nevertheless, these findings suggest that the degree of small fiber neuropathy and p-synuclein pathology in the skin of Lewy body diseases might progress together with neurovascular dysautonomia, which is also linked small fiber neuropathy of vessels. Recently, the first results of a prospective registry on PAF were published [27] estimating a risk of phenoconversion to PD or DLB of 34% after 4 years of follow-up. For our study participants, both E46K-SNCA carriers with PAF phenotype had significant noradrenergic sympathetic denervation in cardiac MIBG scintigraphy

[16,19], orthostatic hypotension and abundant p-synuclein aggregates in skin nerve fibers. When clinical evaluations where performed they did not have motor nor cognitive abnormalities, although their chronological age (55 and 58 years) was in the usual age range for the clinical onset of CNS symptoms in diffuse Lewy body diseases. One year later, one of them (A06) developed smell loss and RBD.

Regarding the asymptomatic E46K-SNCA carrier (A02), p-synuclein deposits were found in skin samples and they were accompanied by low nerve fiber count per area, although the patient did not present any autonomic or neurological sign or symptom. We hypothesize that the deposition of synuclein may occur prior to the development of any symptom, highlighting that the loss of fibers and the presence of synuclein aggregates might be detected early in disease progression (Figure 1).

We also observed that small nerve fiber count and the degree of p-synuclein deposition in the skin of E46K-SNCA carriers were positively correlated with sudomotor dysfunction (ESC reduction) in hands, but not in feet (Figure 3). In fact, differences in ESC between patients and healthy controls were more pronounced in hands than in feet. SudoScan derived ESC measures have good correlation with skin nerve fiber density [28] and display an optimal performance for discriminating controls from patients with small fiber neuropathy due to different conditions [29]. Since SudoScan measures ESC in hands and feet simultaneously, it potentially allows performing inferences regarding topographical progression of small fiber neuropathies. Several studies have been performed so far with SudoScan comparing sudomotor function in controls and diabetic patients and, although with some conflicting results, most of them demonstrated in diabetic patients stronger sudomotor dysfunction (lower ESC

values) in feet than in hands [29], which is in opposition to our findings in E46K-SNCA carriers. Although different studies support that cutaneous small fiber neuropathy in iPD is proportional to disease duration and follows left-right progression and is related to severity of motor manifestations [30], few have analyzed the precise topographical evolution of skin denervation and its structural-functional correlates. Donadio et al. demonstrated p-synuclein deposits in a proximal-distal gradient with the highest rate of positivity in the cervical site (i.e., close to the spinal ganglia) and lowest rate in the leg [2], which potentially suggests the existence of a spreading phenomenon of p-synuclein from spinal ganglia along the peripheral nervous system in iPD. Thus, we hypothesize that the preferential dysfunction of small fibers in hands of E46K-SNCA carriers may be related to the anatomical vicinity between cervical spinal nerve roots and brainstem autonomic nuclei, where, according to Braak staging, CNS Lewy body pathology is suspected to begin in synucleinopathies.

One of the main limitations of the present study is related to the small sample size, which may limit the generalizability of results. However, considering that E46K-SNCA is an extremely rare genetic abnormality and its uniqueness as one of the best in vivo models for Lewy body diseases, we believe that this study might provide relevant insights for the understanding of small fiber neuropathy in iPD and its associated symptoms and may support the validation of skin biomarkers for synucleinopathies.

Although p-synuclein skin deposits were absent in one out of two PARK2 carriers, we observed severe p-synuclein aggregates in the skin of the other PARK2 participant (P03). Interestingly, an immunohistochemical study of the skin in a single exon 4 deletion heterozygous PARK2 case showed p-synuclein deposits in small dermal nerve

fiber bundles, that were not present in a second PARK2 case with a compound heterozygous PARK2. While homozygous PARK2 variants have been almost unambiguously associated to absent alpha-synuclein deposits and Lewy body pathology, brain autopsy findings in heterozygous PARK2 carriers have been conflicting [22]. In addition, the existence of "incidental Lewy body disease" must be taken into consideration, since it is present up to 25% of healthy subjects and at mild degree in certain homozygous PARK2 cases. Moreover, since p-synuclein deposits tend to increase with age and disease duration in PD, these factors might also explain the severe skin p-synuclein deposits observed in the second PARK2 case (P03), who was relatively old and had a markedly long disease duration. Regarding the methodology, although the most commonly used technique calculates the linear density of intraepidermal nerve fibers per millimeter of skin [31], others have used alternative methods in order to allow an easier evaluation of dermal nerve fibers [32]. Moreover, immunohistochemistry analyses were performed using anti-phospho-synuclein antibodies, following standardized neuropathological assessment, in a blind way. Non-consecutive samples were used for immunohistochemical purposes, to prevent over-quantification. Thus, the presence of synuclein aggregates was analyzed avoiding overlooking the same area and ensuring penetrance of the antibody. On the other hand, it should be also considered that the antibodies against synuclein and phospho-synuclein could identify different forms of synuclein aggregates, such as fibers and dots. In terms of p-synuclein peripheral deposits, we performed Thioflavin-immunofluorescence staining to better characterize the deposits (Figure 2), showing the colocalization between p-synuclein deposits and Thiflavin. In terms of the selection of the anatomical location for skin punch-biopsies, we chose the cervical region since it

has been described as the most sensitive in terms of synuclein deposits [2]. Lastly, considering the statements of recent studies supporting the lack of validity of SudoScan as a measurement tool for autonomic function [33,34] it is important to mention that in the present study we use the ESC measurements exclusively as surrogates to sudomotor function, not as a precise measure of autonomic function.

In summary, with this study we identified for the first time moderate to severe small nerve fiber differences between patients, and p-synuclein deposits in the cervical skin of all studied E46K-SNCA carriers, regardless of being symptomatic or asymptomatic. Moreover, the nerve fiber density and p-synuclein aggregates in the cervical skin were singular in this group of patients with severe autonomic manifestations and they significantly correlated with sudomotor dysfunction in hands. For future studies on E46K-SNCA carriers, it is key to understand why patients with the same mutation have different outcomes. Studies of alpha-synuclein expression in cell cultures using neurons derived from reprogrammed induced pluripotent stem cells carrying this mutation will help to understand these differences, opening an opportunity window to unravel PD pathophysiology and to develop future therapies.

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FIGURE LEGENDS

Figure 1. Anti-tyrosine hydroxylase (Anti-TH) staining of epidermis demonstrating the integrity of noradrenergic nerve fibres and anti-p-synuclein immunohistochemical staining of epidermis-dermis, skin glands and nerve fascicles demonstrating the degree and distribution of p-synuclein skin aggregates (arrows) for E46K-SNCA carriers (A02, A06 and A07) with p-synuclein aggregates versus two cases with low or absent p-synuclein aggregates, one symptomatic PARK2 carrier (P02) and healthy control (H01). Magnification: black reference lines within each image correspond to 50 µm.

Figure 2. Double immunofluorescence staining showing phospho-synuclein positive aggregates (a, d), Thioflavin S-ir aggregates (b, e). Merge images showing colocalization (c, f), in a SNCA-E46K carrier. Scale bar (a, b, c), magnification 100x (Image crop) (d, e, f).

Figure 3. Scatter plot illustrating the **correlation** between electroconductance in hands and density of fibers, p-synuclein deposits and density of fibers and p-synuclein deposits and electroconductance in hands.

Contributors: MC, IG and JCG designed the study; JG performed skin biopsies; MC, RSP, MRL processed biospecimens; MC performed immunohistochemistry and counting of intraepidermal nerve fiber density; IG, MA and AM performed electrochemical skin conductance studies; MC and JCG performed statistical analysis and created the figures and tables; MC, IG and JCG interpreted the results of the analysis with subsequent substantial contributions from all the co-authors. MC, IG and

JCG drafted the manuscript, to which all the authors contributed with revisions and approved the final version.

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Title: Small fiber neuropathy and phosphorylated alpha-synuclein in the skin of E46K-SNCA mutation carriers

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ABSTRACT

Background and objective: In 2004 we described the E46K mutation in alpha-synuclein gene (E46K-SNCA), a rare point mutation causing an aggressive Lewy body disease with early prominent non-motor features and small fiber denervation of myocardium. Considering the potential interest of the skin as a target for the development of biomarkers in Parkinson's Disease (PD), in this work we aimed to evaluate structural and functional integrity of small autonomic nerve fibers and phosphorylated alpha-synuclein (p-synuclein) deposition in the skin of E46K-SNCA carriers as compared to those observed in parkin gene mutation (PARK2) carriers and healthy controls. ***Patients and methods:*** We studied 7 E46K-SNCA carriers (3 dementia with Lewy bodies, 2 pure autonomic failure, 1 PD and 1 asymptomatic), 2 PARK2 carriers and 2 healthy controls to quantify intraepidermal nerve fiber density and p-synuclein deposition with cervical skin punch biopsies (immunohistochemistry against anti PGP9.5/UCHL-1, TH and p-synuclein) and sudomotor function with electrochemical skin conductance (ESC) (SudoScan). ***Results:*** All E46K-SNCA carriers had moderate to severe p-synuclein deposits and small fiber neurodegeneration in different epidermal and dermal structures including nerve fascicles and glands, especially in carriers with Pure Autonomic Failure, while p-synuclein aggregates where absent in healthy controls and in one of two PARK2 carriers. The severity of the latter skin abnormalities in E46K-SNCA were correlated with sudomotor dysfunction (lower ESC) in hands (p=0.035). ***Interpretation:*** These results together with our previous findings support the relevance of E46K-SNCA mutation as a suitable model to study small fiber neuropathy in Lewy body diseases.

INTRODUCTION

In the last years, different authors have reported that alpha-synuclein aggregates, the main component of Lewy bodies, can be detected within cutaneous autonomic small fibers of patients with Parkinson's Disease (PD) [1,2]. It has been described that accumulation of synuclein-immunoreactive deposits is most prominent in sympathetic adrenergic nerve fibers innervating the erector pili muscles but is also present in sudomotor nerve fibers, i.e. sympathetic cholinergic fibers [3]. Nonetheless, other studies have reported more prominent deposits in nerve fibers of skin vessels [4]. Growing evidence supports the detection of phospho-synuclein (p-synuclein) aggregates in cutaneous nerve as a promising approach to improve the identification of patients with synucleinopathies, including PD [1,4], pure autonomic failure (PAF) [5], REM sleep behavior disorder (RBD) [6] or dementia with Lewy Bodies (DLB) [7]. Several studies have quantified non-invasively sudomotor function abnormalities in synucleinopathies with different technological approaches such as quantitative sudomotor axon reflex testing [8,9] or skin electroconductance (ESC) measurement tools [10,11].

Considering the great heterogeneity of idiopathic PD (iPD), the study of pathophysiologically homogeneous genetic PD variants becomes of the utmost importance for the development of biomarkers in iPD. In 2004 we described for the first time in the literature a family of the Basque Country (Spain) with the E46K mutation, one of the three known missense point-mutations in the alpha-synuclein gene (SNCA)[12], limited to specific families [13]. Experimental studies with E46K-SNCA

mutation have shown its strong tendency towards fibril formation and its outstanding pathogenicity [14,15]. The clinical phenotype of E46K-SNCA mutation is characterized by an early onset parkinsonism with prominent non-motor features including dementia, autonomic dysfunction and sleep disturbances [16–18]. Neuropathological examination of the index case revealed extensive Lewy bodies and neurites in cortical and subcortical structures of the brain, meeting the pathological criteria for DLB [16]. In addition to CNS involvement, the postmortem examination of the myocardium in two symptomatic carriers [16] together with in vivo studies using autonomic functional tests and myocardial metaiodobenzylguanidine (MIBG) scintigraphy [19,20] support that E46K-SNCA mutation induces a prominent autonomic neuropathy that affects small noradrenergic sympathetic fibers. On the opposite side, Parkin gene mutation (PARK2) is associated to a disease in which autonomic abnormalities and myocardial sympathetic denervation are subtle [20,21] and Lewy bodies are virtually absent, especially in homozygous PARK2 variants [22].

Except for a recent letter reporting pathological skin findings in two PARK2 carriers [23], no studies have been published describing structural or functional correlates of small nerve fiber neuropathy in the skin of genetic carriers of PD. In this work, we aimed to evaluate and describe the integrity of small autonomic nerve fibers and p-synuclein deposition in the skin of E46K-SNCA carriers, with different phenotypes and their correlation with sudomotor function. The study of E46K-SNCA mutation carriers constitutes the ideal scenario to analyze the presence of p-synuclein aggregates in the peripheral nervous system of patients with PD, since this mutation

induces a genetically defined aggressive Lewy body disease with prominent autonomic failure, cognitive and motor symptoms.

MATERIAL AND METHODS

We performed a cross-sectional study of 7 E46K-SNCA carriers from the same single family, 6 symptomatic (3 dementia with Lewy bodies, 2 pure autonomic failure (PAF) and 1 PD) and 1 asymptomatic, 2 carriers of heterozygous PARK2 mutation and 2 healthy controls (HC). See table 1 for further details of individual participants. Participants were recruited in the Department of Neurology of Cruces University Hospital. The study procedures were approved by the regional Basque Clinical Research Ethics Committee. All participants gave written informed consent prior to their participation in the study, in accordance to tenets of Declaration of Helsinki.

Skin Biopsies

We obtained 4-mm diameter skin punch biopsies from the cervical C7 region, under aseptic and anesthetic conditions, following recommendations by Donadio et al. [24]. Samples were fixed in 4% formaldehyde for 24 hours, embedded in paraffin and serially cut into 5 µm sections for immunohistochemistry.

Immunohistochemistry studies

Slides were oven-heated at 60ºC for 30 minutes, immersed in Xylene (5 minutes, three times) and then rehydrated in decreasing ethanol concentrations (100% - 95%, 70% - 50%) and in distilled and tap water (5 minutes each). Endogenous peroxidase activity was inhibited incubating sections in tap water with 0.02 % hydrogen peroxide (H_2O_2)

for 15 minutes. For antigen retrieval, sections were incubated in 0.01 M citrate buffer, pH 6.0, for 20 min at 96 ºC, in a PT module TS (Thermo Fisher Scientific). Then, tissue sections were rinsed in 0.1 M PBS (5 min, three times) and incubated overnight in DAKO antibody diluents (S2022) containing the corresponding primary antibody: polyclonal antibody against protein gene product (anti-PGP 9.5; 1/1000; AB1761: Millipore Billerica, MA, USA) as a neuronal marker for skin innervation; monoclonal mouse antibody against tyrosine hydroxylase (anti-TH monoclonal mouse; 1:1000; MAB5280: Millipore, Billerica, MA, USA) for noradrenergic fibre staining; monoclonal mouse antibody against phospho-synuclein (anti-p-synuclein S129 monoclonal mouse; pSyn#64, 1:2000, WAKO, Japan) as a marker of PD pathology. Samples were rinsed three times for 5 min in PBS and incubated for 30 min at room temperature with biotinylated goat anti-rabbit IgG (E0432, Dako, Denmark) or biotinylated goat anti-mouse IgG (E0433, Dako, Denmark) diluted 1:200 in PBS. Sections were developed using 3, 3´-diaminobenzidine tetrahydrochloride with H_2O_2 (DAB Kit, Vector Laboratories) and counterstained with Nissl. The sections were mounted on gelatinized slides, coverslipped with DPX mounting medium (BDH). Each staining also included a negative control in which the primary antibody was omitted.

Quantification of the skin nerve fiber density and synuclein deposits

Samples were viewed and digitalized with an Olympus BX-51 microscope equipped with an Olympus DP-70 digital camera at 60x (Olympus, Denmark). For every case, we included 30 sections of the sample, using a lens of 60x magnification, that were randomly digitalized and analyzed. We used a computer-assisted image analysis with a macro of instructions to be executed in the software ImageJ (Wayne Rasband, NH,

USA). Intraepidermal nerves were counted when crossing or originating at dermal-epidermal junction, branching in the dermis out of the virtual line at the dermal-epidermal junction was excluded from quantitation. Blinded and randomized quantification of all samples was performed by the same examiner (MCA) using the same microscope. Intra-observer agreement was evaluated by counting the number of nerve fibers per area twice, one month apart. There was good intraobserver reliability of PGP-ir measures. The results for epidermal nerve fiber density were expressed as number of fibers/area. The degree of p-synuclein deposits in nerve fascicles was quantified using a semi-quantitative visual score: 'absent' (score 0); '+', slight (score 1); '++', moderate (score 2); '+++', severe (score: 3).

Sudomotor testing

Overall sudomotor function was evaluated with SudoScan device (Impeto Medical, Paris France), which acquires non-invasively through reverse iontophoresis ESC measures in hands and feet using two sets of large-area stainless steel plate electrodes. ESC is expressed in micro-Siemens (μS) and constitutes a surrogate measure of postganglionic sympathetic (cholinergic) function. During testing, subjects were required to place their hands and feet on electrode plates for approximately 2 minutes. We obtained separate average ESC measures for each limb (right palm, left palm, right foot sole and left foot sole) as well as the bilateral ESC averages for palms and foot soles. For this study, we only used bilateral ESC averages for hands and feet as surrogate markers of overall sudomotor function. SudoScan has been used to test sudomotor function, without any commercial interest. Further details on SudoScan technology are detailed elsewhere [25]. This technique was not conducted to analyze

sympathetic cholinergic function, as it is not appropriate for that and a more solid functional test should be used for that purpose. It is important to stress here that ESC measurements from SudoScan are not specific enough to ascertain the participation of specific pathophysiological mechanisms, including loss of sudomotor fibers, sweat gland atrophy, reduced number of sweat glands, or glandular dysfunction caused by toxic, metabolic, or other disorders.

Statistics

We performed descriptive and correlation analyses for E46K-SNCA carriers using the statistical software package SPSS 13 for Windows (SPSS, Chicago, IL). Given the limited sample size, the correlations of nerve fiber density with age, years of disease duration and p-synuclein deposition scores were performed with the nonparametric Spearman´s rho test.

RESULTS

The demographical and clinical features of study participants and the results of the analysis of nerve fiber density, degree of p-synuclein deposits and electrochemical conductance of the skin are displayed in Table 1. All E46K-SNCA carriers had p-synuclein inclusions in cutaneous sympathetic fibers at different degrees of severity (Table 1, Figure 1 and Figure 2). All the samples had TH-immunoreactive nerve fibers, revealing the presence of noradrenergic nerve fibers in skin samples. Nerve fibers were identified surrounding sweat grands, hair follicles and erector pili muscles. Interestingly, for those E46K-SNCA carriers with clinical phenotypes suggesting a diffuse CNS involvement (e.g. DLB and PD phenotypes) (A03, A05 and A07) the severity

of p-synuclein deposits was rather heterogeneous, from mild to moderate, while for those E46K-SNCA with manifestations restricted to the autonomic nervous system (PAF) (A04 and A06) the degree of p-synuclein was consistently high. Contrarily, healthy controls and one of the two PARK2 carriers (P02) did not show p-synuclein inclusions in nerve fibers. Figure 1 illustrates representative images of anti-p-synuclein immunohistochemical staining of epidermal nerve fascicles in patients with moderate to severe degree of p-synuclein deposition (A07, A02 and A06) and in participants without deposits (P02 and H01). Regarding the quantification of skin innervation, the number of healthy controls is not enough to perform statistical comparisons between groups. Nevertheless, following the descriptive purpose of the analysis, we found a significant inverse correlation between nerve fiber density and the degree of p-synuclein aggregates in the epidermis (r = -0.889, p = 0.002) (mean fiber density for severe p-synuclein aggregates: 9.02 fibers/area; moderate p-synuclein: 12.24 fibers/area; mild p-synuclein: 15.42 fibers/area; absent p-synuclein: 17,23 fibers/area). It is worth mentioning that the density of nerve fibers was low and p-synuclein deposition moderate to severe in the asymptomatic E46K-SNCA mutation carrier (A02) (8.83 fibers/area and moderate degree of p-synuclein deposits) and in the PARK2 mutation carrier (P03) who had an exceptionally long disease duration (30 years) (9.62 fibers/area and severe p-synuclein). However, in the overall analysis the correlation with nerve fiber density was not significant for age (r = -0.072, p = 0.844) or disease duration (r = -0.235, p = 0.513).

In terms of the evaluation of sudomotor function with SudoScan, we found that compared to the healthy control the decrease of ESC in patients was more pronounced

in hands than in feet (average difference of 11.89 µS in hands and 2.66 µS in feet). In general, patients with low fiber density were those who had lower ESC values, especially in hands (Table 01). In fact, there was a significant positive correlation between ESC values in the upper extremities and nerve fiber density ($r = 0.669$, $p = 0.035$) or the degree of p-synuclein inclusions ($r = -0.889$, $p = 0.002$). Of note, for the asymptomatic E46K-SNCA carrier (A02) and for the PARK2 patient (P02), despite the number of epidermal nerve fibers (below 9 fibers/area), the ESC remained in normal ranges both in hands and in feet (above 70 µS), which may suggest the existence of possible mechanisms of compensation for sudomotor function in both cases.

DISCUSSION

In this study, we provide for the first time, evidences of the existence of small fiber neuropathy in the skin of E46K-SNCA carriers, one of the best known in vivo genetic models for PD and Lewy body disorders. This small nerve fiber degeneration was accompanied in all E46K-SNCA carriers by moderate to severe aggregates of p-synuclein in skin, especially in patients with predominant autonomic features. In opposition, p-synuclein aggregates were absent in healthy controls and in one out of two PARK2 mutation carriers. Moreover, the severity of fiber density and p-synuclein deposition in the skin of E46K-SNCA carriers were correlated with the deterioration of sudomotor function as measured by electroskin conductance, providing correspondence to our results. All these findings, together with our previously published evidences on autonomic myocardial denervation [16,19], support the relevance of E46K-SNCA mutation as a suitable model of small fiber neuropathy related to p-synuclein pathology.

It is remarkable that we identified aggregates of p-synuclein and low density of small nerve fibers in the skin also in young asymptomatic E46K-SNCA carrier. We observed higher loads of skin p-synuclein in those E46K-SNCA carriers with prominent dysautonomia, which is in line with a recent study by Donadio et al. demonstrating wider p-synuclein deposits in autonomic skin nerves of iPD patients with orthostatic hypotension [26]. Interestingly, in our study, the degree of p-synuclein deposits in the skin of E46K-SNCA carriers with PAF (A04 and A06) was consistently severe and accompanied by low fiber density. Whereas for those patients with diffuse CNS involvement (DLB and PD phenotypes) (A03, A05 and A07) the severity of p-synuclein aggregates and skin denervation was heterogeneous. This may imply that the progression of the pathogenic process is different between members of the same family, leading to different phenotypes within the same genotype, attending possibly to epigenetic factors. Nevertheless, these findings suggest that the degree of small fiber neuropathy and p-synuclein pathology in the skin of Lewy body diseases might progress together with neurovascular dysautonomia. Recently, the first results of a prospective registry on PAF were published [27] estimating risk of phenoconversion to PD or DLB. For our study participants, both E46K-SNCA carriers with PAF phenotype had significant noradrenergic sympathetic denervation in cardiac MIBG scintigraphy [16,19], orthostatic hypotension and abundant p-synuclein aggregates in skin nerve fibers without motor or cognitive abnormalities. One year later, one of them (A06) developed smell loss and RBD.

Regarding the asymptomatic E46K-SNCA carrier (A02), p-synuclein deposits were found in skin samples and they were accompanied by low nerve fiber count per area.

We hypothesize that the deposition of synuclein may occur prior to the development of any symptom, highlighting that the loss of fibers and the presence of synuclein aggregates might be detected early in disease progression.

We also observed that small nerve fiber count and the degree of p-synuclein deposition in the skin of E46K-SNCA carriers were positively correlated with sudomotor dysfunction (ESC reduction) in hands. In fact, differences in ESC between patients and healthy controls were more pronounced in hands than in feet. SudoScan derived ESC measures have good correlation with skin nerve fiber density [28] and display an optimal performance for discriminating controls from patients with small fiber neuropathy due to different conditions [29]. Several studies have been performed so far with SudoScan comparing sudomotor function in controls and diabetic patients and, although with some conflicting results, most of them demonstrated in diabetic patients stronger sudomotor dysfunction (lower ESC values) in feet than in hands [29], which is in opposition to our findings in E46K-SNCA carriers. Although different studies support that cutaneous small fiber neuropathy in iPD is proportional to disease duration, follows left-right progression and is related to severity of motor manifestations [30], few have analyzed the precise topographical evolution of skin denervation and its structural-functional correlates. Donadio et al. demonstrated p-synuclein deposits in a proximal-distal gradient with the highest rate of positivity in the cervical site [2], which potentially suggests the existence of a spreading phenomenon of p-synuclein. Thus, we hypothesize that the preferential dysfunction of small fibers in hands of E46K-SNCA carriers may be related to the anatomical vicinity between cervical spinal nerve roots and brainstem autonomic nuclei, according to Braak staging.

One of the main limitations of the present study is related to the small sample size. However, considering E46K-SNCA is unique and as one of the best in vivo models for Lewy body diseases, we believe that this study might provide relevant insights for the understanding of small fiber neuropathy in iPD and may support the validation of skin biomarkers for synucleinopathies.

Although p-synuclein skin deposits were absent in one out of two PARK2 carriers, we observed severe p-synuclein aggregates in the skin of the other PARK2 participant (P03). While homozygous PARK2 variants have been almost unambiguously associated to absent alpha-synuclein deposits and Lewy body pathology, brain autopsy findings in heterozygous PARK2 carriers have been conflicting [22]. In addition, the existence of incidental Lewy body disease must be taken into consideration, since it is present up to 25% of healthy subjects and at mild degree in certain homozygous PARK2 cases. Moreover, since p-synuclein deposits tend to increase with age and disease duration in PD, these factors might also explain the severe skin p-synuclein deposits observed in the second PARK2 case (P03), who was relatively old and had a markedly long disease duration. Regarding the methodology, although the most commonly used technique calculates the linear density of intraepidermal nerve fibers per millimeter of skin [31], others have used alternative methods in order to allow an easier evaluation of dermal nerve fibers [32]. Moreover, immunohistochemistry analyses were performed using anti-phospho-synuclein antibodies, following standardized neuropathological assessment, in a blind way. Non-consecutive samples were used for immunohistochemical purposes, to prevent over-quantification. In terms of p-synuclein peripheral deposits, we performed Thioflavin-immunofluorescence staining

(Figure 2) showing colocalization with p-synuclein deposits. In terms of the location of skin punch-biopsies, the cervical site has been described as the most sensitive [2]. Recent studies [33,34] show contradictory results regarding its validity as a measure of autonomic function. Here we use the ESC measurements as surrogates of sudomotor function, we do not use this method as an autonomic function measure.

In summary, with this study we identified for the first time moderate to severe small nerve fiber differences between patients, and p-synuclein deposits in the cervical skin of all studied E46K-SNCA carriers, regardless of being symptomatic or asymptomatic. Moreover, the nerve fiber density and p-synuclein aggregates in the cervical skin significantly correlated with sudomotor dysfunction in hands. Future studies on E46K-SNCA carriers to understand why patients with the same mutation have different outcomes will open a window of opportunity to unravel PD pathophysiology and to develop future therapies.

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FIGURE LEGENDS

Figure 1. Anti-p-synuclein immunohistochemical staining of epidermal nerve fascicles in cases with moderate-severe degree (A07, A02 and A06) and with absence (P02 and H01) of p-synuclein deposition. Magnification: 50X; black reference lines within each image correspond to 50 µm.

Figure 2. Anti-tyrosine hydroxylase (Anti-TH) staining of epidermis demonstrating the integrity of noradrenergic nerve fibres (a,b,c) and anti-p-synuclein immunohistochemical staining of epidermis-dermis (d,f,g), skin glands (h,I,j) and nerve fascicles (k,l,m) demonstrating the degree and distribution of p-synuclein skin aggregates (arrows) for an E46K-SNCA symptomatic carrier (A07) with marked p-synuclein aggregates (g,j,m) versus two cases with low or absent p-synuclein aggregates, one symptomatic PARK2 carrier (P02) (f,I,j) and healthy control (H01). Magnification: black reference lines within each image correspond to 50 µm.

Figure 3. Double immunofluorescence staining of skin nerve fascicles showing phosho-synuclein positive aggregates (a, d), Thioflavin S-ir aggregates (b, e). Merge images showing colocalization (c, f). Scale bar (a, b, c), magnification (d, e, f)

Contributors: MC, IG and JCG designed the study; JG performed skin biopsies; MC, RSP, MRL processed biospecimens; MC performed immunohistochemistry and counting of intraepidermal nerve fiber density; IG, MA and AM performed electrochemical skin conductance studies; MC and JCG performed statistical analysis and created the figures and tables; MC, IG and JCG interpreted the results of the analysis with subsequent substantial contributions from all the co-authors. MC, IG and

JCG drafted the manuscript, to which all the authors contributed with revisions and approved the final version.

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Table(s)

ID	Group	Phenotype	Age (years)	Gender	Disease duration (years)	Summary of clinical manifestations	Degree of p-syn deposits	Nerve fiber density (fibers / area)	ESC Hands (µS)	ESC Feet (µS)	LEDD	SCOPA-AUT
A03	E46K-SNCA	DLB	51	Female	8	Moderate-severe motor & cognitive symptoms, visual hallucinations, sleep disturbances, dysautonomia with OH	+	15.42	78	77	1480	14
A05	E46K-SNCA	DLB	56	Male	14	Moderate-severe motor & cognitive symptoms, visual hallucinations, vivid nightmares, OH, hyposmia	++	14.7	69	54	430	17
A01	E46K-SNCA	PD	31	Male	2	Mild motor symptoms, vivid nightmares and POTS	++	13.18	69	66	380	2
A06	E46K-SNCA	PAF	58	Male	2	Cough syncopes. OH (Tilt Table Test). Myocardial denervation on myocardial MIBG scintigraphy	+++	12.96	67	85	-	10
P03	PARK2	PD	73	Male	30	Moderate motor symptoms, dystonia	+++	9.62	73	83	200	
A02	E46K-SNCA	Asymptomatic carrier	34	Male	---	Asymptomatic. Normal physical & ancillary examinations*	++	8.83	81	83	-	2
A04	E46K-SNCA	PAF	55	Female	1	OH during Tilt Table Test. Myocardial denervation on myocardial MIBG scintigraphy.	+++	8.38	63	55	-	2
A07	E46K-SNCA	DLB	63	Male	12	Severe motor & cognitive symptoms, OH	+++	5.11	52	54	1300	23
P02	PARK2	PD	71	Female	5	Mild motor symptoms	-	21.45	78	70	240	17

H01	HC	Control	34	Male	---	---	-	19.38	83	73	-	0
H02	HC	Control	49	Female	---	---	-	10,87			-	0

Table 1. Demographical and clinical features, including L-Dopa doses and SCOPA-Aut scores, degree of p-syn deposits, nerve fiber density of the skin and electrochemical conductance values in participants.

The degree of phosphorylated alpha-synuclein deposition is displayed in the following way: - none, + slight, ++ moderate, +++ severe. PD: Parkinson's disease; * Absence of PD motor signs, normal orthostatic stress test, brain *DaTscan* and myocardial MIBG scintigraphy. HC: healthy controls; E46K-SNCA: carriers of E46K mutation in alpha-synuclein gene; PARK2: carriers of Parkin gene mutation; DLB: Dementia with Lewy Bodies; PAF: Pure Autonomic Failure; p-syn: phosphorylated alpha-synuclein; ESC: electrochemical skin conductance; µS: micro Siemens; OH: orthostatic hypotension; POTS: Postural Orthostatic Tachycardia Syndrome. MIBG: metaiodobenzylguanidine.

Figure(s)

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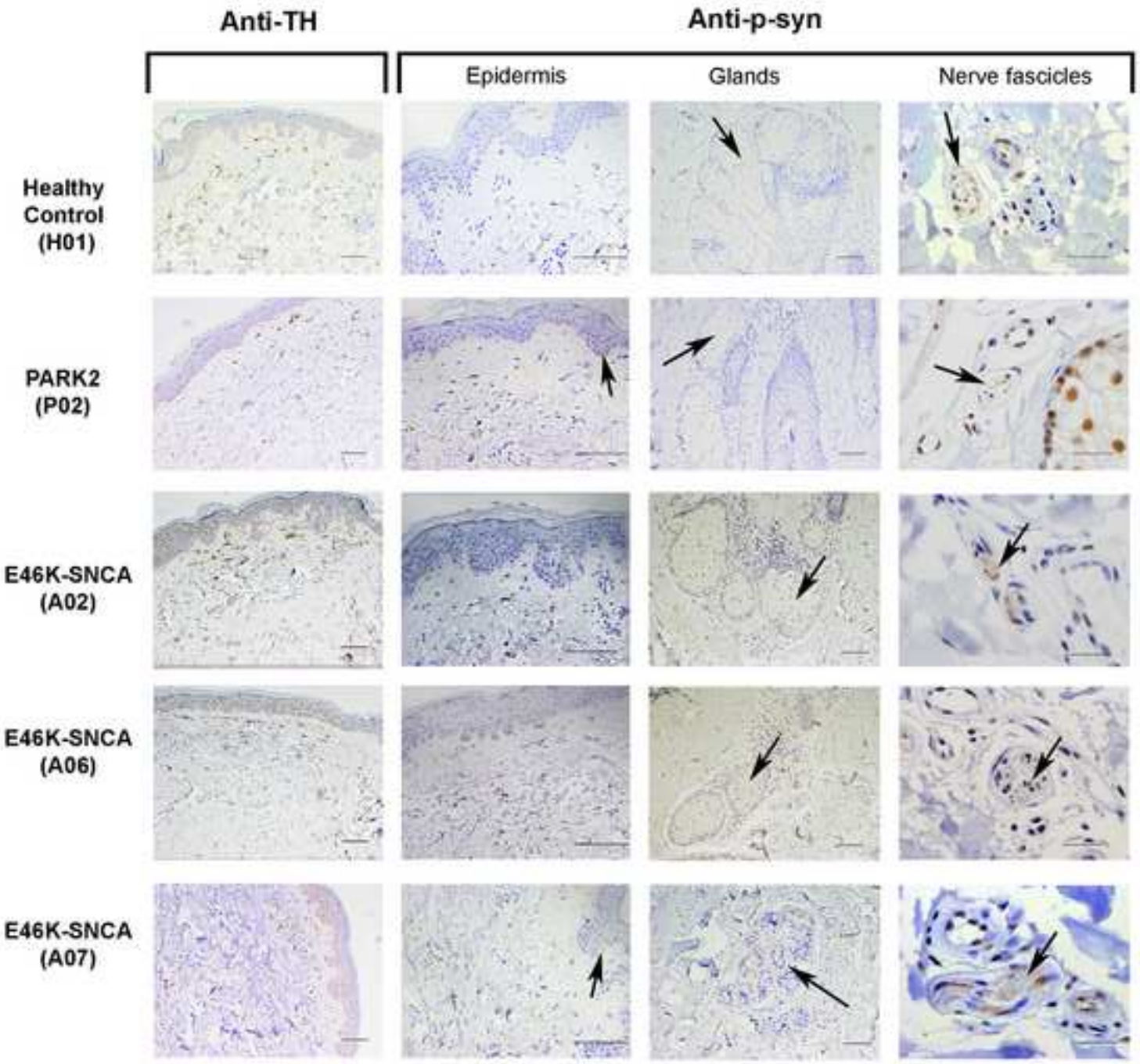

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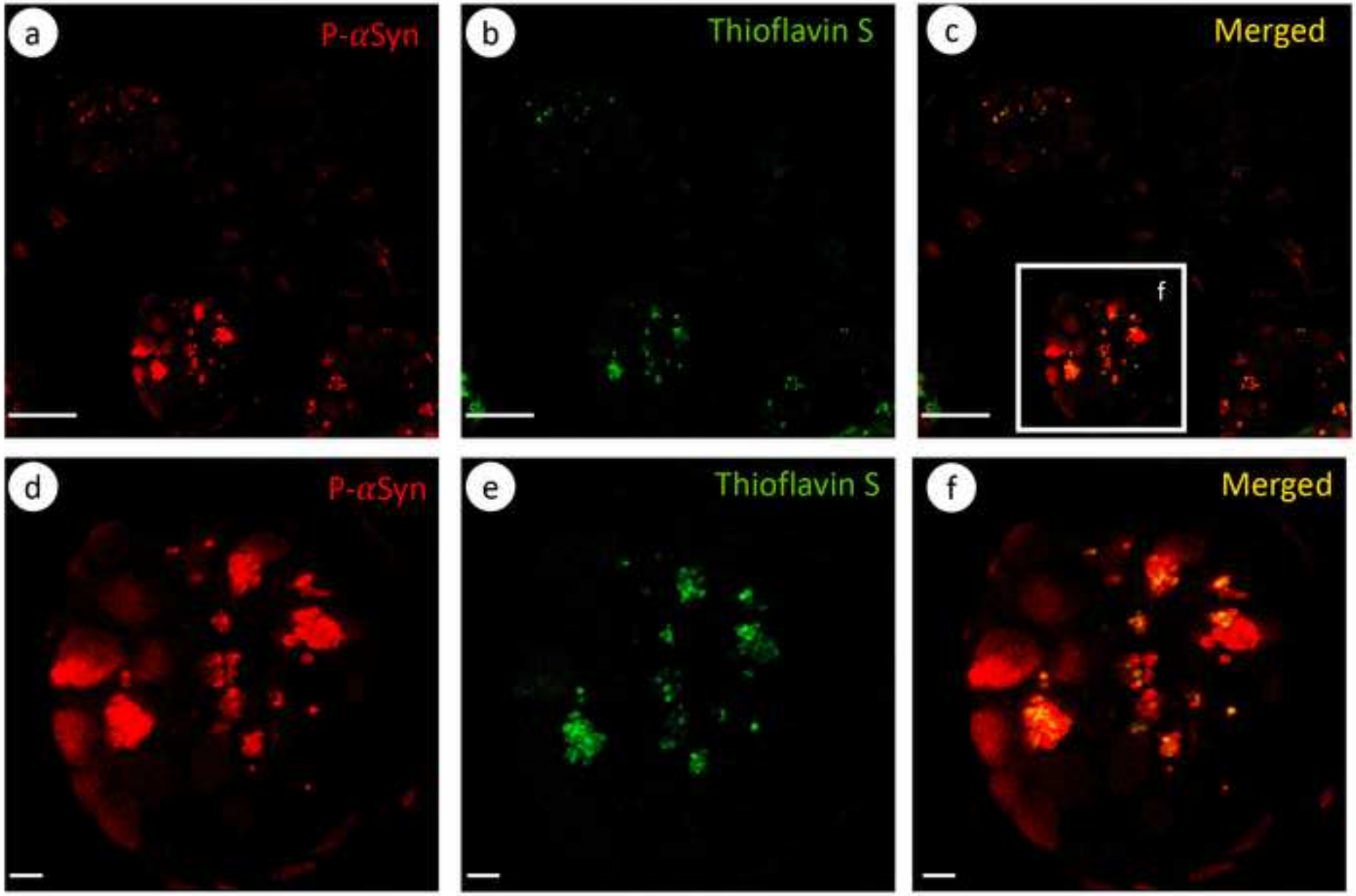

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