Journal of Neurochemistry

JNC

JOURNAL OF NEUROCHEMISTRY | 2013 | 126 | 673–684

doi: 10.1111/jnc.12251

ORIGINAL ARTICLE

Heat-mediated enrichment of α-synuclein from cells and tissue for assessing post-translational modifications

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Abstract

α-synuclein (α-syn) is the major component of Lewy bodies, a pathological hallmark of Parkinson's disease and other synucleinopathies. The characterization of α-syn post-translational modifications (PTMs), thought to interfere with its aggregation propensity and cellular signaling, has been limited by the availability of extraction methods of endogenous protein from cells and tissues, and by the availability of antibodies toward α-syn PTMs. Here, by taking advantage of α-syn thermostability, we applied a method to achieve high enrichment of soluble α-syn both from cultured cells and brain tissues followed by proteomics analysis. Using this approach, we obtained 98% α-syn sequence coverage in a variety of model systems, including a transgenic mouse model of PD, and validated the strategy by identifying previously described PTMs such as phosphorylation and N-terminal acetylation. Our findings demonstrate that this procedure overcomes existing technical limitations and can be used to facilitate the systematic study of α-syn PTMs, thereby enabling the clarification of their role under physiological and pathological conditions. Ultimately, this approach may enable the development of novel biomarkers and strategies for therapeutic intervention in synucleinopathies.

Keywords: Parkinson's disease, post-translational modifications, proteomics, α-synuclein.

J. Neurochem. (2013) **126**, 673–684.

Parkinson's disease (PD) is the most common progressive neurodegenerative movement disorder, with a prevalence of ~ 2% in people over 65 years of age. Pathologically, it is characterized by the loss of predominantly nigrostriatal dopaminergic neurons and the accumulation of proteinaceous cytoplasmic inclusions known as Lewy bodies (LBs). These are primarily composed of α-syn, a protein of unclear function (Gai *et al.* 2000; Braak *et al.* 2003). The majority of

Received February 27, 2013; revised manuscript received March 12, 2013; accepted March 25, 2013.

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Abbreviations used: α-syn, α-synuclein; IPTG, isopropyl β-D-1-thiogalactopyranoside; LBs, Lewy bodies; PTMs, post-translational modifications; TNM, tetranitromethane.

PD cases are sporadic, although several genes are associated with familial forms, namely mutations and multiplications in the gene encoding for α-syn, *SNCA* (Polymeropoulos *et al.* 1997; Kruger *et al.* 1998; Zarranz *et al.* 2004).

α-syn is the target of several PTMs including oxidation, phosphorylation, nitration, sumoylation, ubiquitylation, and N-terminal acetylation (Hirsch 1993; Castellani *et al.* 1996; Giasson *et al.* 2000; Fujiwara *et al.* 2002; Hasegawa *et al.* 2002; Anderson *et al.* 2006; Choi and Lim 2010). However, their contribution to disease progression and pathobiology is still unclear.

Thus far, the majority of PTMs were detected in α-syn extracted from LBs (Anderson *et al.* 2006). One of the major technical limitations for the characterization of α-syn PTMs is the complexity of its extraction. Alone or in combination, different techniques, such as density gradient centrifugation, ion-exchange, size-exclusion, immunoaffinity chromatography, and immunoprecipitation, can be used to enrich α-syn for subsequent analyses of PTMs (Przedborski *et al.* 2001; Iwatsubo 2003; Broersen *et al.* 2006). Moreover, although western blotting experiments using antibodies that specifically recognize PTMs are regularly used, the availability of such antibodies toward α-syn specific PTMs is limited.

Provided that the extraction and enrichment of α-syn is achieved, mass spectrometry analysis can be employed as a powerful approach for the identification of α-syn PTMs (Jakes *et al.* 1994; Weinreb *et al.* 1996). To that purpose, we used a straightforward strategy for the partial purification and enrichment of α-syn from human, rat, and mouse brains as well as from yeast and mammalian cell lines expressing human α-syn. This strategy, based on the known thermostability of α-syn (Jakes *et al.* 1994), overcomes existing technical limitations enabling the subsequent analysis of α-syn PTMs.

Materials and methods

Tissue protein extraction

Three brains from Wistar adult female rats aged 32 weeks (Harlan, Barcelona, Spain) were dissected and the different brain regions (cerebellum, cortex, hippocampus, and midbrain) were stored at −80°C prior to use.

Human brain tissue of parietal, frontal, temporal, and occipital regions from two subjects with frontotemporal dementia were obtained from the Pathology Department, Hospital de Santa Maria.

Mouse midbrain tissues were collected from four C57BL/6 male mice (WT) at 12 weeks of age. Midbrain samples from five A30P transgenic (Kahle *et al.* 2000) and five non-transgenic mice at 8 weeks of age were also collected. Animals were housed and handled according to institutional and national guidelines.

Brain samples were homogenized in ristocetin-induced platelet agglutination buffer [50 mM Tris/HCl pH7.4, 150 mM NaCl, 2 mM EDTA, 1% (v/v) NP-40, 0.1% (w/v) sodium dodecyl sulfate] in the presence of proteases and phosphatases inhibitors in a douncer homogeneizer Potter-Elvehjam (Thomas Scientific, Swedesboro, NJ, USA) at 4°C. Samples were then rotated end-over-end for 60 min at 4°C and centrifuged at 18 000 *g* for 30 min at 4°C. Soluble fraction was collected. To assess the insoluble fraction of human brain lysates, the resulting pellet was re-suspended in Radioimmunoprecipitation assay buffer, sonicated three times for 30 s at 10 μm amplitude with 1 min incubation on ice between each sonication step (Soniprep 150 MSE, MSE, London, UK), and centrifuged at 18 000 *g* for 30 min at 4°C. The resulting supernatant was isolated. After protein quantification (BCA protein assay), 1 mg total protein was heated at 50, 70, and 90°C for 15, 30, and 45 min and centrifuged at 18 000 *g* for 15 min at 4°C, and both the supernatant and pellet collected. For the other brain regions, lysates were heated at 90°C for 15 min. For A30P transgenic mice, 0.5 mg of total protein were heated at 90°C for 15 min. After heating, protein concentration was determined. Tissue collection and processing was performed according to the approval of the Ethics/Animal Committee of Instituto de Medicina Molecular.

Yeast α-syn transformation and protein extraction

The *Saccharomyces cerevisiae* strain BY4741 (genotype BY4741 *MATa*; *his3*Δ1; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) from Euroscarf collection (Frankfurt, Germany) was used. The strain was kept in YPGlu [0.5% (w/v) yeast extract, 1% (w/v) peptone and 2% (w/v) D-glucose] agar plates [2% (w/v) agar] at 4°C and cultured in liquid YPGlu medium at 30°C. *S. cerevisiae* strain expressing α-syn-containing plasmid was cultured in synthetic medium without uracil (YNB-U) [0.67% (w/v) yeast nitrogen base, 2% (w/v) D-glucose and 0.025% (w/v) L-methionine, L-histidine, L-leucine]. The plasmid was introduced using the lithium acetate method and transformants selected on YNB-U agar plates, following the procedure described in the Yeast Protocols Handbook ('Small-scale LiAc Yeast Transformation Procedure', from Clontech, Saint-Germain-en-Laye, France). For protein expression, transformants were grown in YNB-U selective medium. Total yeast protein extraction was performed by glass bead lysis as previously described (Ausubel *et al.* 1990; Gomes *et al.* 2005) followed by 3 cycles of sonication for 30 s at 10 μm amplitude with 1 min incubation on ice between each sonication step (Soniprep 150 MSE), and centrifuged at 18 000 *g* for 30 min at 4°C. After protein quantification (BCA protein assay), lysates with 1 mg total protein were heated at 90°C for 15 min and centrifuged at 18 000 *g* for 15 min at 4°C, and the supernatant isolated. Protein concentration in the supernatants was determined.

H4 cell line α-syn transfection and protein extraction

Human H4 neuroglioma cells (gift from Dr. Bradley T. Hyman, Harvard Medical School) were maintained at 37°C in OPTI-MEM I (Gibco, Invitrogen, Barcelona, Spain) supplemented with 10% fetal bovine serum and seeded onto 10 cm dishes (Techno Plastic Cultures AG, Trasadingen, Switzerland) 24 h prior to transfection. Cells were transfected with pSI-α-syn, a plasmid encoding for α-syn (gift from Dr. Bradley T. Hyman) using FuGENE 6 (Roche diagnostics, Mannheim, Germany) and collected 48 h after transfection. H4 cells were also transfected with pcDNA3.1 α-syn-V5, pcDNA3.1 β-syn-V5 or co-transfected with both plasmids (β-syn – beta-synuclein). pcDNA3.1 α-syn-V5 was already described (Klucken *et al.* 2006). β-syn cDNA was amplified by PCR and cloned into pcDNA3.1, replacing α-syn from the pcDNA3.1 α-syn-V5.

For total protein extraction, cells were lysed in NP-40 buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40) supplemented with protease inhibitor cocktail tablet. Samples were sonicated three times for 30 s at 10 μm amplitude with 1 min incubation on ice between each sonication step (Soniprep 150 MSE). Proteins were separated from debris by centrifugation (18 000 *g* for 30 min at 4°C). After protein quantification (BCA protein assay), 1 mg total protein was heated at 90°C for 15 min and centrifuged at 18 000 *g* for 15 min at 4°C, and the supernatant isolated. Protein concentration in the supernatants was determined.

Human recombinant α-syn expression and purification

The expression and purification of human α-syn was a modified version of a previously described method (Kessler *et al.* 2003). Briefly, *E. coli* BL21 (DE3) pLysS competent cells (Novagen, San Diego, CA, USA) were transformed with a human α-syn PT7-7 construct (gift from Dr. Hilal Lashuel, EPFL, Lausanne) and expression was induced for 3 h with isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. Cells were pelleted, frozen at −80°C, and re-suspended in lysis buffer (10 mM Tris/HCl pH 8, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). Cells were lysed by three freeze/thaw cycles and sonication on ice (five times, 1 min, Branson Sonifier B12, Danbury, CT, USA), heated at 100°C for 20 min and then centrifuged at 22 000 *g* for 30 min. The supernatant was further precipitated in ammonium sulfate (360 mg/mL), followed by a centrifugation at 22 000 *g* for 30 min. The resulting α-syn-containing pellet was resuspended in 30 mM Tris/HCl, pH 7.4. After removing the ammonium sulfate by a PD-10 desalting column (GE Healthcare, Buckinghamshire, UK) α-syn was purified on a Resource-Q 6 mL anion exchange column (GE Healthcare, Uppsala, Sweden) in 30 mM Tris/HCl pH 7.4 with a NaCl gradient from 0 to 750 mM. Resulting α-syn fractions, as judged by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), were collected and concentrated by centrifugation through a Vivaspin column (Sartorius Stedim Biotech, Göttingen, Germany). α-syn was further purified by size-exclusion chromatography (SuperdexTM 75 10/300, GE Healthcare, Uppsala, Sweden). SDS-PAGE, followed by western blotting analysis (using standard procedures), confirmed the monomeric purification of α-syn (Syn-1 BD dilution 1 : 2000; Transduction Laboratories, San Jose, CA, USA).

α-syn nitration and phosphorylation

α-syn nitration was carried out by incubating human recombinant α-syn with 10–20 mM tetranitromethane (TNM), an effective reagent for protein nitration (Cuatrecasas *et al.* 1968), at 20–23°C for 3–4 h. Remaining TNM was removed by microcon centrifugal filter devices (membrane NMWL 3000; Millipore, Bedford, MA, USA). To induce phosphorylation, α-syn was treated with 8U Casein kinase 1 (New England BioLabs, Inc, Beverly, MA, USA) in the presence of 1 mM ATP per μg of α-syn at 30°C for 24 h. The reaction was ended with the addition of 5 mM EDTA (Paleologou *et al.* 2008).

Solubility of modified α-syn upon heat treatment

Unmodified and modified (nitrated or phosphorylated) α-syn were incubated at 90°C for 30 min, and subsequently centrifuged at 15 000 *g* for 30 min at 4°C. The supernatants were mixed with one volume of SDS sample buffer (0.125 M Tris/HCl pH 6.8, 4% SDS, 20% glycerol, 0.004% bromophenol blue). The pellets were dissolved with the same volume of SDS sample buffer as the SDS-treated supernatants. The samples were subsequently analyzed using SDS-PAGE and western blotting.

SDS-PAGE and western blotting analysis

In this study, 5 μg of total protein from brain and cell extracts, 0.5 μg of recombinant α-syn, or 5 or 40 μg of cell extracts from cells transfected with α-syn and/or β-syn were separated by SDS/PAGE using a Tetra cell (Bio-Rad, Hercules, CA, USA) in 15% polyacrylamide separation gel and a 6% polyacrylamide stacking gel and applying a constant voltage of 100 V. Pre-stained standard proteins were also loaded onto the gel. Proteins were transferred to nitrocellulose membranes, using standard procedures with a Mini Trans-Blot system (Bio-Rad). Western blotting was performed using standard procedures using a mouse anti-alpha-synuclein-1 (S63320 BD Transduction Laboratories, 1 : 5000 dilution); a mouse antibody against phosphorylated S129-α-syn (01-2028, dilution 1 : 3000, Wako pure chemicals, Osaka, Japan); and a rabbit anti-nitrotyrosine antibody (dilution 1 : 500; Sigma-Aldrich, Steinheim am Albuch, Germany). Corresponding secondary goat anti-rabbit or anti-mouse antibodies coupled to horseradish peroxidase (dilution 1 : 10 000; Dianova, Hamburg, Germany or GE Healthcare) were used. Protein densitometry was assayed using ImageJ – Image Processing and Analysis in Java (Abramoff *et al.* 2004). Each immunoblot was repeated at least three times in independent experiments. To determine the fold-increase changes upon enrichment, the ratio between the enriched and its corresponding non-enriched fraction was determined.

Two-dimensional electrophoresis (2D-E)

Total protein extracts from brain tissues and cells were concentrated and buffer exchanged to 25 mM NH_4HCO_3 by centrifugal filtration using 3 kDa MWCO spin concentrators. Two hundred micrograms of SpeedVac (Savant) lyophilized proteins were re-suspended in rehydration buffer [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) pharmalyte, 0.002% (w/v) bromophenol blue and 1.2% (v/v) DeStreak reagent™] (GE Healthcare, Uppsala, Sweden) to a final volume of 250 μL. In-gel rehydration took place onto 13-cm-long IPG strips either with a non-linear wide range pH gradient (pH 3–10) or a linear pH gradient (pH 4–7) for 12 h. Proteins were focused for a total of ≈21 kVh, during which the voltage was gradually increased up to 8000 V for a total of 8 h. After focusing, proteins in the IPG strips were reduced and then alkylated by soaking at 20–23°C for 15 min, respectively, with 1% (w/v) dithiothreitol and then 4% (w/v) iodoacetamide in 75 mM Tris/HCl, pH 8.8; 6 M urea; 30% (v/v) glycerol; and 2% (w/v) SDS. Equilibrated strips were then horizontally applied on top of a 13% SDS-PAGE midi gel and proteins separated vertically at 10°C, using the SE 600 Ruby system (GE HealthCare) with constant current (50 mA/gel). Gels were prepared in triplicates corresponding to independent experiments. Gels were transferred with tank transfer system from Hoefer using nitrocellulose membranes and analyzed using western blotting. Gels and membranes were, respectively, stained with SimplyBlue™ (Invitrogen, Barcelona, Spain) SafeStain and Ponceau S solution for reversible staining to verify transfer efficiency. Coomassie-stained 2-DE gels, blotted membranes and

immunoblots were scanned on ImageScanner (GE HealthCare) and the obtained images converted into gray scale 14-bit TIFF files, which were then analyzed on Progenesis SameSpotst™ version 3.0 (NonLinear Dynamics, Newcastle upon Tyne, UK) image analysis program.

α-syn and β-syn digestion products

α-syn and β-syn in silico trypsin and Glu-C digestion products were obtained using mMass which is an open source multifunctional mass spectrometry software (Strohalm *et al.* 2008, 2010; Niedermeyer and Strohalm 2012). 2 miss cleavages were allowed with a mass range between 300 and 6000 Da.

In-solution digestion and PTM analysis by MALDI-TOF-MS

For tryptic proteolysis, recombinant α-syn was dried in a speed vac (Eppendorf, Hamburg, Germany) and dissolved in 25 mM NH_4HCO_3 containing 20 ng/μL porcine modified sequencing grade trypsin (Promega, Madison, WI, USA). The trypsin to protein ratio was adjusted to 1 : 50 (w/w) and the digestion carried out at 37°C overnight. Digested peptides were purified by Ziptip C18 (Millipore), mixed with MALDI matrix (2,5–dihydroxyacetophenone), spotted on a stainless steel target, and measured by a Bruker Autoflex (Bruker Daltonik, Bremen, Germany). Positive ions were analyzed in reflector mode after 20 kV acceleration. External calibration was performed using a peptide calibration standard (Bruker Daltonik). Each displayed mass spectrum was produced by six individual spectra, which were generated by 50 shots/individual spectrum recorded from several positions on a spot. Spectra were analyzed using Flex Analysis software (Bruker Daltonik).

In-gel digestion and protein identification by combined MS + MS/MS

α-syn positive spots identified by western blotting were excised from the gel and submitted to in-gel digestion with sequence grade modified trypsin (Promega, Mannheim, Germany) or endoproteinase Glu-C (Sigma, St. Louis, MO, USA) as described (Shevchenko *et al.* 1996; Bensalem *et al.* 2007) prior to identification by MS. Peptides, prepared in 50% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid (TFA) were directly deposited on a 192-well MALDI plate with 5 mg/mL CHCA (1 : 1) prepared in 0.1% TFA/60% ACN (v/v) and allowed to co-crystallize at ambient temperature.

Peptides were analyzed on an Applied Biosystems 4700 Proteomics Analyzer with TOF/TOF ion optics. Data were acquired in positive MS reflector mode with six spots of standard (Calibration Mixture 2; Applied Biosystem, Foster City, CA, USA) used for calibration (4000 Series Explorer Software v3.0 RC1). MS spectra were obtained by 1000 shot/subspectrum accumulations. Five precursor peaks with the best signal to noise ratio (S/N) were selected from each spectrum for MS/MS analysis. For MS/MS spectra, a maximum of 5,200 laser shots were accumulated. Data interpretation was carried out using MASCOT MS ion Search (www.matrixsciences.com).

For combined MS+MS/MS analysis, all peptide mass values were considered monoisotopic, a MS mass tolerance was set at 80 ppm and a MS/MS fragment tolerance set at 0.25 Da. Trypsin and endoproteinase Glu-C were assigned as the digestion enzymes, a double miss cleavage was allowed and carbamidomethylation of cysteinyl and oxidation of methionyl residues were assumed as fixed and variable modifications, respectively. A taxonomic restriction to *Rattus*, *Mus muscullus,* and *Homo sapiens* protein sequences were included for the respective samples. For MS and MS/MS, all peaks with S/N greater than 5 were included for searching against the Swiss-Prot database. For PTMs search, online MASCOT MS/MS ion Search (www.matrixsciences.com) was performed defining a peptide tolerance of 100 ppm, peptide charge +1, MS/MS tolerance of 0.6 Da, restricting taxonomically to *Rattus*, *Mus muscullus* and *Homo sapiens* protein sequences in SwissProt database accordingly to the analyzed sample with protein N-terminal acetylation and phospho (ST)/(Y) as variable modifications. The criteria used to accept the identification were significant homology scores achieved in Mascot ($p < 0.05$).

Size-exclusion chromatography (SEC)

α-syn samples were centrifuged at 100 000 g for 60 min and loaded on a SuperdexTM 75 10/300 column (GE Healthcare) using 30 mM Tris/HCl (pH 7.4, with 0.2 M NaCl) as an eluent at a flow rate of 0.5 mL/min and monitoring the UV absorbance at 215 nm. To control for reproducibility between SEC runs, Gel Filtration Standard (Bio-Rad Laboratories) were used prior to each set of analysis.

Results

α-syn enrichment in tissue samples

To investigate the effect of heating (90°C for 15 min) on the solubility of α-syn, α-syn levels were measured by western blotting in soluble fractions from different regions of the rat brain. This treatment resulted in a marked increase of α-syn levels (Fig. 1a). Applying the same heat treatment to human brain samples also resulted in α-syn enrichment (Fig. 1b). In addition, we investigated α-syn thermostability of human brain NP-40 insoluble proteins and observed a similar increase in α-syn protein levels (Fig. 1b). Upon enrichment, a different proteome pattern was obtained, as observed in the coomassie staining (Fig. 1b). To verify whether α-syn thermostability could be exploited to enrich samples generated from model systems other than mammalian brain tissues, the same treatment was also performed in human H4 cells and in yeast cells expressing human α-syn. As expected, we also observed the proportional increase in α-syn levels in the soluble fraction (Fig. 1c). To rule out the possibility that α-syn was also present in the post-heating pellet, the respective pellet fractions were analyzed using western blotting and α-syn was barely detectable (Fig. 1c). Taken together, these data suggest that heat treatment can be applied to a broad range of biological samples to substantially enrich for α-syn protein.

To further analyze the thermostability properties of α-syn, we tested three different temperatures and heat incubation times. At 50°C no apparent enrichment was observed for the three incubation times tested. At both 75°C and 90°C, clear enrichment was achieved. In addition, we did not observe further enrichment with greater incubation times up to 45 min (Fig. 1d). Again, we did not detect α-syn in the pellet fractions using western blotting, independent of both

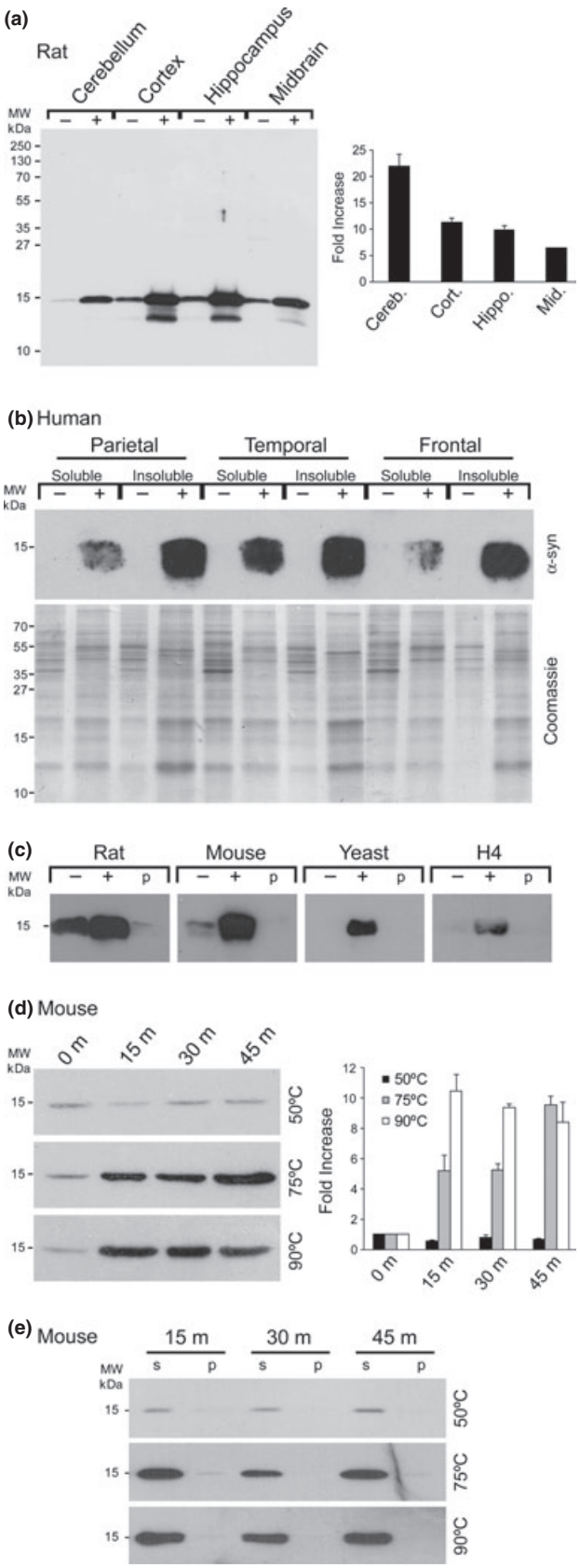

Fig. 1 α-syn enrichment upon heat treatment. Western blots probed with anti-α-syn antibody of (a) non-heated (−) and heated (+) protein extracts (5 μg) from rat cerebellum, cortex, hippocampus, and midbrain and corresponding densitometry analysis; (b) non-heated (−) and heated (+) protein extracts from human parietal, temporal, and frontal soluble and insoluble fractions and corresponding coomassie staining; (c) non-heated (−), heated (+) soluble fractions and post-heat-resulting pellet (p) of rat and mouse mid-brain; α-syn transformed yeast and transfected H4 protein extracts; (d) 0, 15, 30, and 45 m heated mouse midbrain protein extracts at 50, 75, and 90°C and corresponding densitometry analysis; and (e) 15, 30, and 45 m heated mouse midbrain protein extracts and heat-resulting pellet at 50, 75, and 90°C. We observed α-syn enrichment after heat treatment both in the soluble and in the insoluble brain samples fractions, and also in mammalian and yeast cells. The highest enrichment was observed at 90°C for 15 min incubation and no pellet formation was observed for the conditions tested. The images are representatives of three independent experiments.

temperature and incubation times in study (Fig. 1e). Given these observations, all the subsequent studies were performed by heating the samples at 90°C for 15 min.

To validate the applicability of the thermo-enrichment toward other amyloidogenic proteins, we analyzed tau protein, an important player in the context of Alzheimer's disease (Ghiso *et al.* 1994). Interestingly, we detected a clear enrichment of tau protein after heating mouse brain protein at 75°C for 15 min (Vicente Miranda, H. and Outeiro, TF, unpublished results).

Solubility of modified α-syn and stability of PTMs upon heating

Next, we addressed whether heat treatment would alter the solubility and stability of post-translationally modified α-syn. To that purpose, nitrated and phosphorylated α-syn were generated *in vitro* by incubation of human recombinant α-syn with TNM or CK1, respectively. Heat treatment did not induce precipitation of recombinant α-syn, demonstrating that heating did not alter the solubility of α-syn (Fig. 2) similar to what was observed in both brain and cell extracts (Fig. 1c and e). Furthermore, there was no alteration in the immunoreactivity of both unmodified and nitrated/phosphorylated α-syn upon heating, indicating that heating did not uncover otherwise hidden antigenic sites (Fig. 2).

For nitrated α-syn, we found SDS-resistant higher molecular weight bands, suggesting the formation of dimers, trimers, and multimers (Fig. 2). This result was consistent with the increased oligomerization propensity of nitrated α-syn at neutral pH (Uversky *et al.* 2005). We observed no increase of α-syn precipitation neither for nitrated nor phosphorylated protein (Fig. 2).

Importantly, when analyzing the nitration and S129 phosphorylation patterns using specific antibodies toward these PTMs, we did not observe changes in the levels of modified α-syn after treatment (Fig. 2, middle and lower panels).

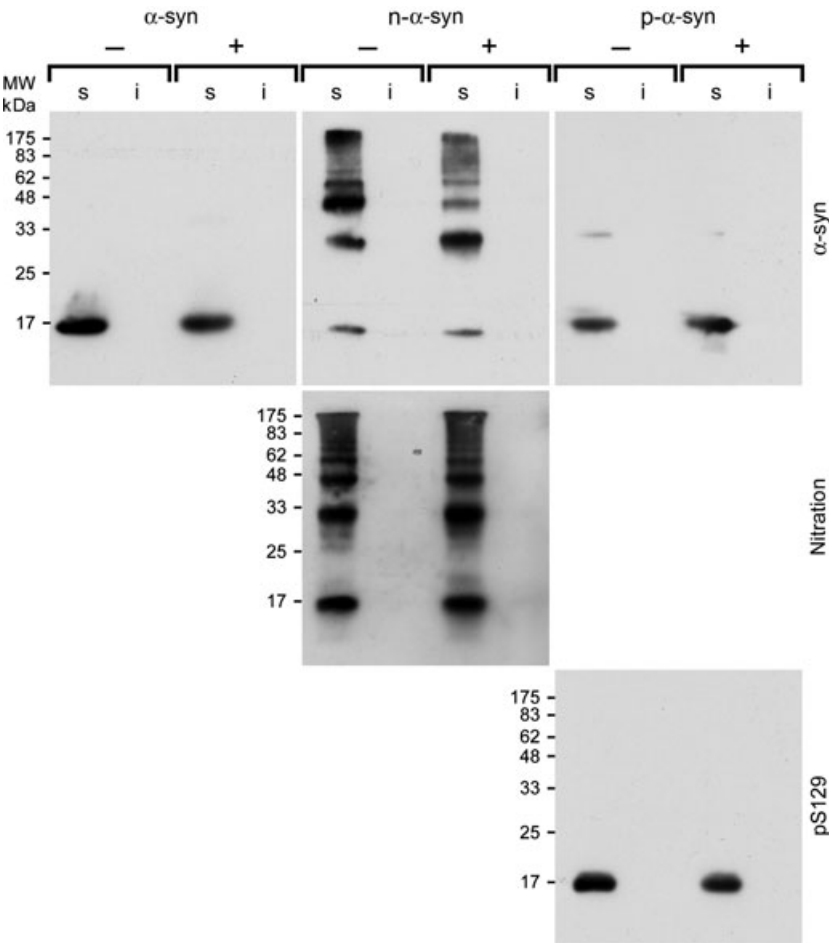

Fig. 2 Heat treatment does not affect the solubility of modified α-syn nor the stability of PTMs. Western blots of non-heated (–) and heated (+), soluble (s) and insoluble (i) unmodified (α-syn), nitrated (n-α-syn) and phosphorylated (p-α-syn) recombinant α-syn protein (0.5 μg) probed with anti-α-syn, anti-nitration and anti-pS129 antibodies. No increased α-syn precipitation is observed upon heating of all evaluated PTM forms as seen in the resulting insoluble fractions. The α-syn nitration and phosphorylation profiles are not altered. Images shown are representatives of three independent experiments.

Concerning n-α-syn, we did not detect differences in α-syn oligomerization using western blotting (Fig. 2, middle panel).

We also verified that heating at 90°C did not affect the stability of α-syn PTMs. To this purpose, we analyzed unmodified, nitrated, and phosphorylated recombinant α-syn by MALDI–TOF before and after heat treatment. Upon trypsin digestion, the m/z of resulting α-syn peptides was measured. We focused on tryptic peptides carrying candidate target amino acid residues, for example, tyrosine residues in nitrated samples and serine residues in phosphorylated samples. Mass increments of 45 Da (nitration) and 80 Da (phosphorylation) were detected in some peptides of interest in the nitrated and phosphorylated α-syn samples, respectively. Three α-syn peptides containing S87, S129, and Y39, whose modifications (pS87, pS129, and nY39) have been described previously, were analyzed with further detail. For all these peptides, no differences in the MS patterns of unmodified and modified peptides were observed upon heating (Fig. 3a). Moreover, by normalizing the intensity of modified peptides with their corresponding unmodified ones, no significant differences in these ratios were observed upon heating (Fig. 3b). Taken together, these results indicate that thermo-enrichment does neither alter the solubility of modified α-syn nor PTM stability.

Stability of n-α-syn oligomerization upon heating

To evaluate if boiling could alter the oligomerization status of α-syn, we analyzed recombinant n-α-syn. As previously mentioned, the SDS-PAGE pattern of n-α-syn was not significantly altered upon treatment (Fig. 2). To confirm this observation we analyzed n-α-syn oligomerization by SEC. For untreated n-α-syn, two clear peaks were observed, corresponding to monomeric and oligomeric n-α-syn (19.6 m and 15.7 m, respectively). Upon treatment, we observed a non-significant increase in the amount of monomeric fraction, and concomitant decrease in oligomeric n-α-syn (19.7 m and 15.8 m, respectively) (Fig. 4a). By analyzing the ratio between oligomeric and monomeric n-α-syn, only a slight decrease was detected (Fig. 4b).

α-syn identification by 2D-PAGE followed by mass spectrometry

To characterize α-syn from either brain homogenates or cell lysates, a good separation of the protein is required. To that purpose, we tested both one and two dimension (2-DE) denaturing electrophoresis separation. We observed that in one dimension SDS-PAGE α-syn co-migrates with several other proteins of similar MW including myelin (Figure S1, Tables S1

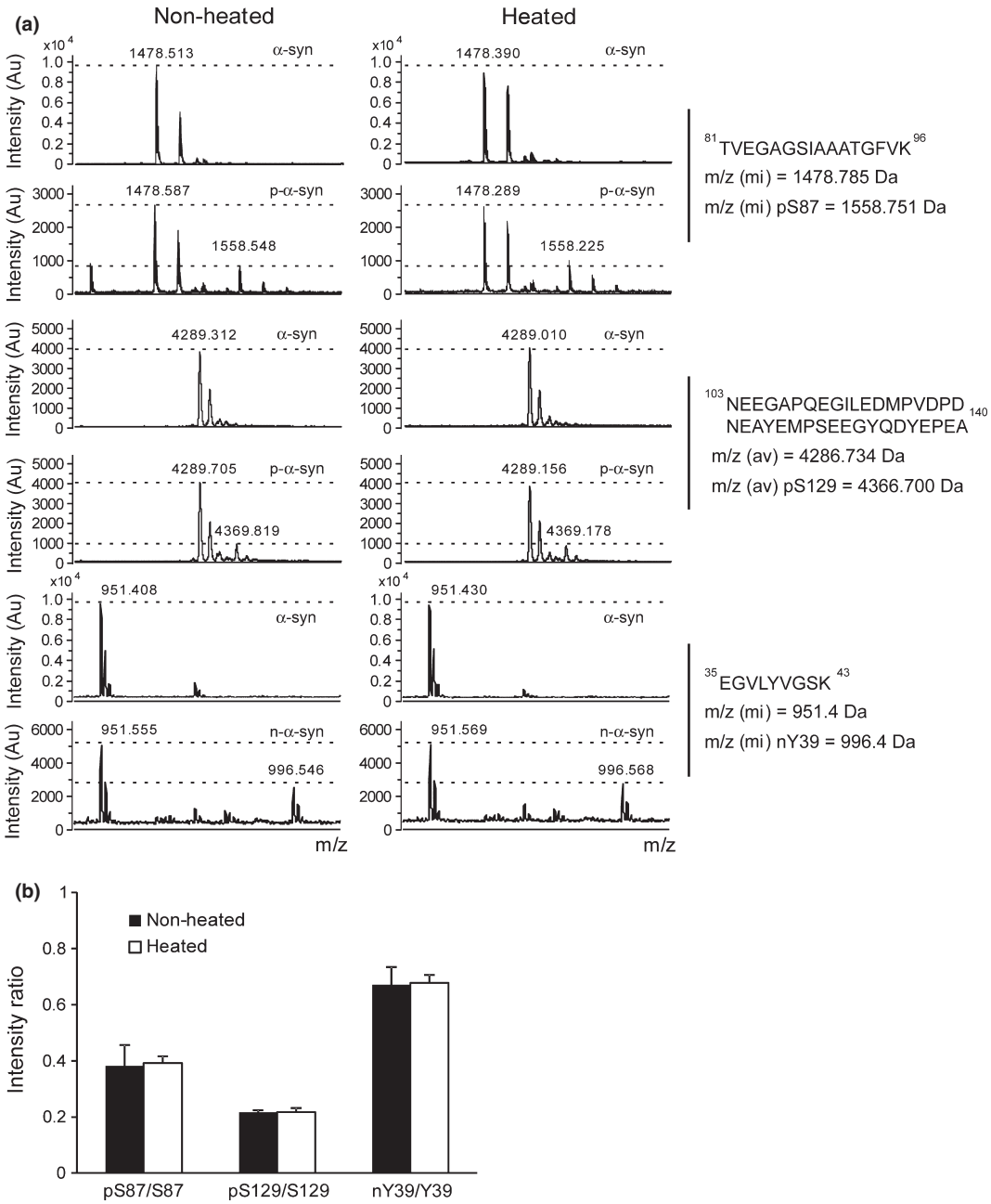

Fig. 3 Heat treatment does not affect the stability of the studied PTMs. (a) Representative mass spectra of tryptic peptides from soluble unmodified, nitrated, and phosphorylated α-syn. The spectra on the left show the peptides of interest from non-heated samples, and on the right the same peptides from heated samples. Mass spectra of two serine carrying peptides, 81–96 and 103–140, together with one tyrosine carrying peptide, 35–43, are displayed. Phosphorylation and nitration results in a mass increment of 80 and 45 Da, respectively. (b) Intensity ratio between phosphorylated/nitrated and corresponding unmodified peaks. All modifications remain assessable upon treatment with no significant changes in the ratio of peak intensities. All spectra are representative of three independent experiments.

and S2). 2-DE further resolved α-syn avoiding the co-migration issue which precludes the analysis (Fig. 5). Moreover, using 2-DE we confirmed that heat treatment remarkably enriched thermal-resistant proteins such as α-syn in the protein supernatants, by reducing the number of temperature-sensitive proteins (Fig. 5, upper images). Western blotting analysis confirmed α-syn enrichment by identifying α-syn in the heated samples (arrow in Fig. 5, lower images).

Excision from a coomassie blue stained gel of the correspondent α-syn immunoreactive spots identified by western blotting, followed by peptide mass fingerprinting analysis of either tryptic or endoproteinase Glu-C resulting

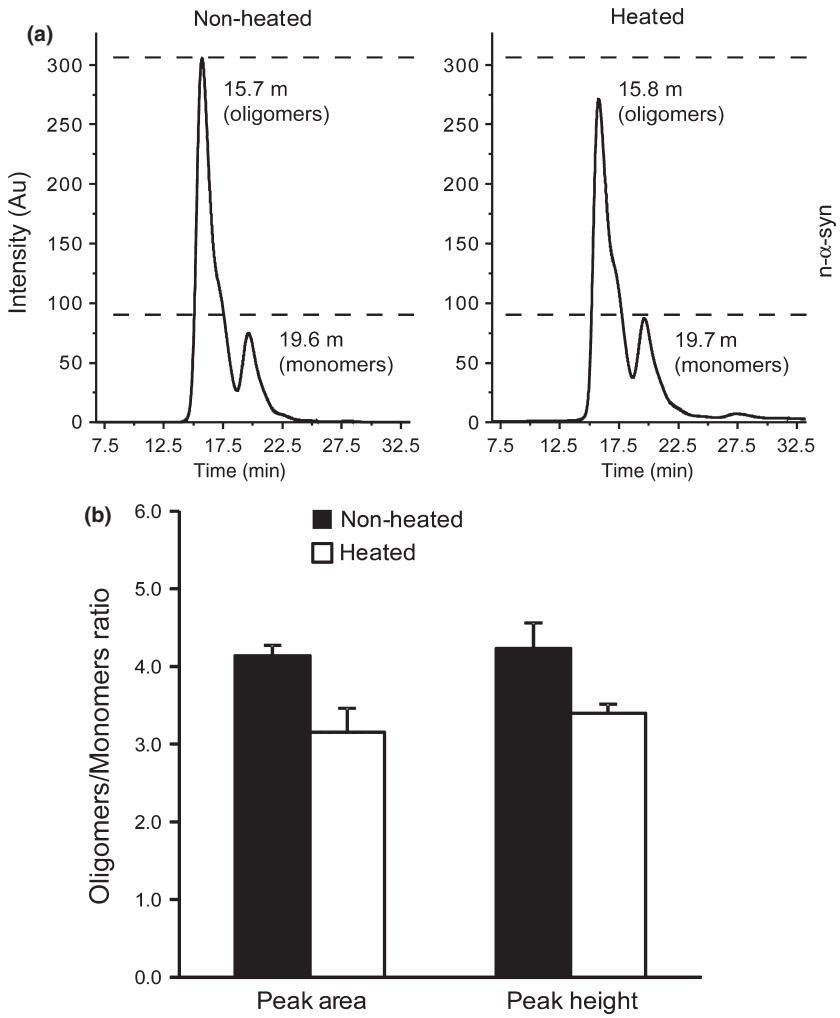

Fig. 4 n-α-syn oligomerization upon heat treatment. (a) Size-exclusion chromatograms of non-heated and heated n-α-syn. (b) Quantitative analysis of the ratio between oligomeric and monomeric peak area and height. Upon heating, a slight decrease in the levels of n-α-syn oligomers was observed.

peptides, confirmed the presence of α-syn in these spots with sequence coverage of 98% (Tables S3, S4, S5 and S6).

α-syn and β-syn migrate differently on SDS-PAGE

Given the high degree of homology [58% identity, 70% similarity, 12% gaps – BLASTP (Altschul *et al.* 1997, 2005)] and similar molecular weights between α-syn and β-syn (14460 and 14287 Da, respectively), it was possible that both proteins co-migrate and are undistinguishable in the gel. To ensure we were analyzing α-syn alone, we first investigated the migration pattern of both proteins. To that purpose, H4 cells were transfected with either α-syn or β-syn with a C-terminal V5 tag, or co-transfected with both constructs. On 15% SDS-PAGE we were able to detect a different migration pattern for both proteins, where α-syn migrated slightly slower than β-syn. This finding was confirmed by using a specific antibody anti-α-syn that did not recognize β-syn (Fig. 6, middle panel). Interestingly, both proteins were thermo-enriched (Fig. 6, upper panel). GAPDH, used as a loading control, was not present in the heat-treated fraction, precipitating upon treatment (Fig. 6, bottom panel).

Differential digestion products of α-syn and β-syn with trypsin and Glu-C

To further confirm that both proteins could also be distinguished by mass spectrometry, we analyzed trypsin and endoproteinase Glu-C in silico digestion products. Although the sequences have high similarities, a different digestion profile was observed for both trypsin and endoproteinase Glu-C digestions (Tables S3, S4, S5 and S6). For trypsin digestion, only 5 of 39 resulting peptides are equal between both proteins, corresponding to 20% of α-syn sequence. Importantly, only 4% of the α-syn sequence is undistinguishable from β-syn as unique α-syn peptides could distinguish both proteins (Tables S3 and S4). For endoproteinase Glu-C digestion, only 2 out of 64 resulting peptides were equal, corresponding to 8% of the α-syn sequence. In this case, α-syn unique peptides totally distinguish the proteins (Tables S5 and S6).

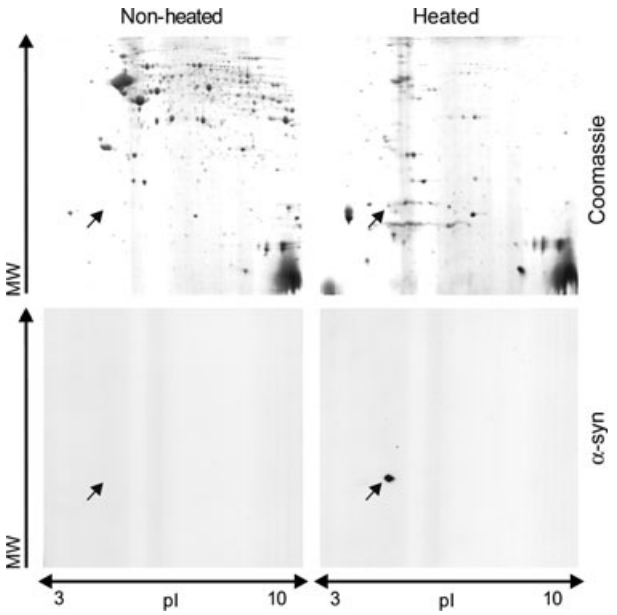

Fig. 5 Rat brain α-syn is thermo-enriched. Two hundred micrograms of rat brain protein extracts were resolved by 2-DE. Coomassie staining of soluble non-heated extracts (upper left) and heated extracts (upper right) show a clear decrease in the number of proteins after heating. The western blot, probed with anti-α-syn antibody, shows clear α-syn enrichment after heating (lower right). Arrow indicates the α-syn positive spots (absent for non-heated samples). All images are representative of three independent experiments.

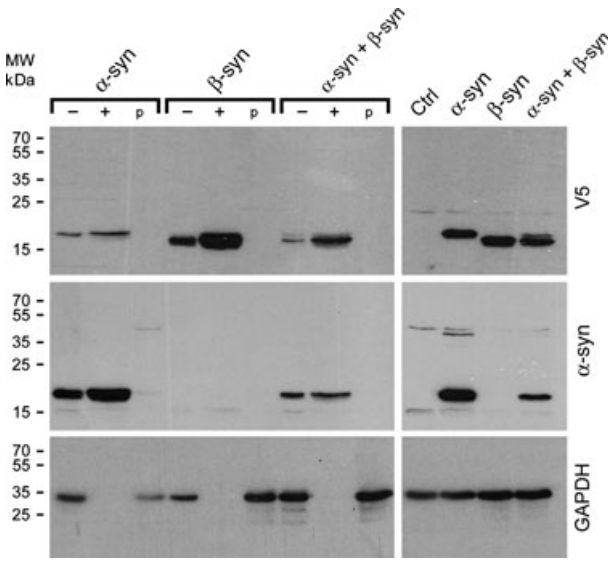

Fig. 6 α-syn and β-syn resolved by SDS-PAGE. Representative western blots of non-heated (−) heated (+) and resulting pellet (p) protein extracts (5 μg, left panel) or non-heated protein extracts (40 μg, right panel) from H4 cells transfected with α-syn-V5, β-syn-V5 or co-transfected with both constructs probed with anti-V5 tag, anti-α-syn and anti-GAPDH antibodies. Enrichment of both α-syn and β-syn after heat treatment was detected. Proteins were resolved and detected as two distinct migrating bands when probed with an antibody against the V5 epitope tag (upper panels). The anti-α-syn antibody does not recognize β-syn (middle panel). Enriched fractions are devoid of GAPDH as it is present in the total and resulting pellet fractions. The images are representatives of three independent experiments.

Analysis of α-syn PTMs in cells and tissues

Although heat treatment of recombinant α-syn did not modify the studied PTMs, phosphorylation and nitration, it remained possible that applying the treatment to biological samples would elicit changes in other α-syn PTMs. To investigate whether this was the case, we used α-syn heat-enriched from rat and mouse tissues or from yeast and H4 cells and isolated α-syn-containing spots from 2-DE gels. As a combination of various proteases generates different peptides, which improves the sequence coverage of α-syn, spots were digested either with trypsin or Glu-C. Mascot MS/MS Ions search defined for phosphorylation and N-terminal acetylation, two previously well described PTMs in α-syn (Anderson *et al.* 2006; Zabrocki *et al.* 2008; Ohrfelt *et al.* 2011), revealed the occurrence of phosphorylated and N-terminal acetylated α-syn variants (Tables S7 to S10). These data strongly suggest that heat treatment does not disrupt α-syn PTMs, validating its usefulness for the identification of PTMs.

Analysis of α-syn from a transgenic mouse model of synucleinopathy

To evaluate the applicability of the thermo-enrichment of α-syn directly in a mouse model of synucleinopathy, we compared non-transgenic and transgenic α-syn A30P mice. We observed α-syn enrichment in both control and transgenic A30P mice upon treatment (Fig. 7, top panel). Interestingly, we observed a different pattern of α-syn S129 phosphorylation. Only the heat-treated samples from transgenic mice showed α-syn phosphorylation in S129 (Fig. 7, middle panel). GAPDH was not found in the heat-treated fraction, as it precipitated upon heating (Fig. 7, bottom panel).

Discussion

Post-translationally modified α-syn is present in LBs in the brain of patients suffering from synucleinopathies and also in α-syn inclusions formed in different α-syn transgenic mouse models (Castellani *et al.* 1996; Giasson *et al.* 2000; Fujiwara *et al.* 2002; Hasegawa *et al.* 2002; Anderson *et al.* 2006; Beyer 2006). Previous *in vitro* and *in vivo* studies showed that PTMs may impact the structural and functional properties of α-syn (Beyer 2006; Oueslati *et al.* 2010). However, there is still controversy about the role of PTMs on the aggregation or in the normal biology of α-syn. Thus, further studies are required to elucidate the pathophysiological role of α-syn PTMs. For example, a systematic study of the presence of α-syn PTMs in models of synucleinopathies would provide insight into the pathological mechanisms.

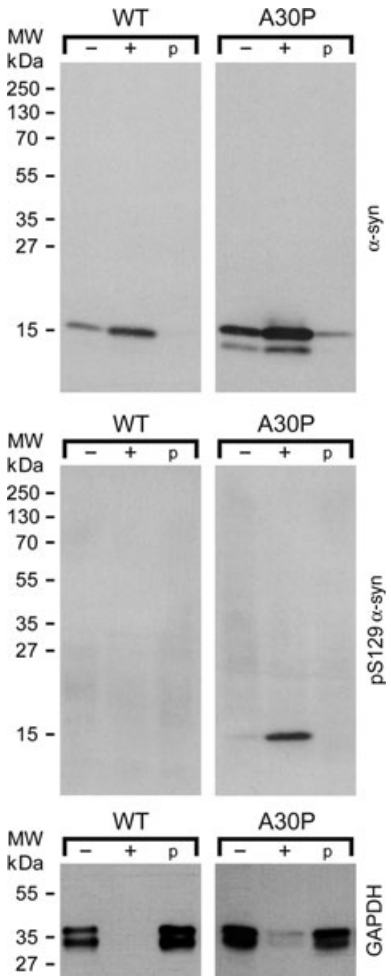

Fig. 7 α-syn is phosphorylated at S129 in A30P transgenic mice. Western blots of non-heated (–), heated (+), and resulting pellet (p) protein extracts (5 μg) from two-month-old non-transgenic and A30P transgenic mice midbrains probed with anti-α-syn (top panel), anti-pS129 α-syn (middle panel) and anti-GAPDH (bottom panel) antibodies. α-syn is enriched upon heating, and present at higher levels in A30P transgenic mice. Only transgenic mice display phosphorylated α-syn at residue S129. The enriched fractions are devoid of GADPH since it precipitates. The images are representatives of three independent experiments of $n = 5$ WT and A30P transgenic mice.

α-syn is a thermostable protein because of its highly negatively charged C-terminal domain, composed of 10 glutamate and 5 aspartate residues whose deletion compromise its thermosolubility (Park *et al.* 2002). Moreover, α-syn is able to affect other proteins solubility as seen in *E. coli*. In these bacteria, the proteome solubility is increased upon α-syn expression and reduced when a C-terminal truncated mutant is expressed (Kim *et al.* 2004). These effects are likely to be related to α-syn putative chaperone function, since it is able to prevent the aggregation of different proteins *in vitro* upon heat shock (Kim *et al.* 2000, 2002; Ahn *et al.* 2006).

Here, we showed that α-syn is stable and remains soluble when brain or cell lysates are heated (Fig. 1). Heat treatment increased the percentage of α-syn in the soluble fraction while the majority of other proteins precipitate (Fig. 5). In addition, we demonstrate that partial purification and enrichment of α-syn by applying heat treatment facilitates α-syn characterization by western blot analysis as well as by mass spectrometry (supporting information). Our findings are of high importance since the amount of α-syn is critical for the adequate characterization of its PTMs either by western blotting or mass spectrometry-based techniques. With such procedure, it is possible to avoid loading high amounts of protein extracts that could compromise the analysis of α-syn via gel-based techniques. Moreover, by confirming our findings using recombinant α-syn, we ensured that the pattern of PTMs is not altered upon heating (Figs 2 and 3). Given that α-syn nitration induces its oligomerization, we further tested the effects of heating on the oligomerization of n-α-syn. By analyzing n-α-syn oligomerization by size-exclusion chromatography, we only found a slight decrease in the levels of oligomeric fraction (Fig. 4). These findings are expected as heating may slightly affect unnstable oligomeric structures. Nevertheless, our findings suggest heating does not disrupt PTMs and does not reduce their levels.

Given the strong similarity between α-syn and β-syn proteins, it was conceivable that these proteins could co-migrate, leading to the incorrect assignment of specific α-syn modifications. We showed these proteins can be easily resolved by one dimension SDS-PAGE (Fig. 6) and that even if co-migration occurred, the specific trypsin and endoproteinase Glu-C digestion profiles are distinct for the two proteins, enabling their unequivocal identification by mass spectrometry analysis (supporting information).

Finally, to validate the usefulness of the proposed method to samples from mouse models of PD, we investigated the profile of α-syn S129 phosphorylation in transgenic A30P mice. Transgenic, but not control mice, clearly display phosphorylated α-syn at S129. As expected, heat treatment of mouse brain protein extracts not only enriched total α-syn but also phosphorylated α-syn at S129, without α-syn precipitation. The results observed are in agreement with previous studies showing that α-syn is phosphorylated in S129 in young transgenic mice but not in control mice (Freichel *et al.* 2007; Schell *et al.* 2009).

It is widely accepted that α-syn is an intrinsically disordered monomeric protein. However, it was recently shown that, under some circumstances, α-syn may exist as a α-helical tetramer (Bartels *et al.* 2011; Wang *et al.* 2011). Furthermore, it was also shown that native human α-syn tetramers do not undergo amyloid-like aggregation in a similar way to what happens with the natively unfolded monomer, suggesting that destabilization of the tetramer might act as a trigger for amyloidogenesis. More recently, the systematic analysis of α-syn from different brain areas as

well as that expressed in mammalian cell lines and *E. coli*, revealed that the unstructured monomer is the predominant form of α-syn (Fauvet *et al.* 2012). Despite the intense debate, one cannot discard a possible association of α-syn into higher molecular weight structured forms upon interaction with other biological partners. It is known that certain protein–protein interactions depend on specific modifications such as phosphorylation or acetylation. How these modifications affect protein assembly/disassembly is still unknown. PTMs may act as molecular switches to regulate α-syn assembly into higher structured oligomers. Using the procedure we reported, one can gain novel insight into the biology/pathobiology of α-syn and, ultimately, this might lead to the development of novel therapeutic strategies for PD and other synucleinopathies.

Acknowledgements

HVM, RMO, and TS were supported by Fundação para a Ciência e Tecnologia (FCT), Portugal (SFRH/BPD/64702/2009, SFRH/BPD/41416/2007, and SFRH/BPD/31209/2006, respectively). HVM was also supported by the EU FP7 project MEFOPA. WX was supported by Deutsche Forschungsgemeinschaft (SFB539/A3). TFO was supported by an EMBO Installation Grant, by a Marie Curie IRG (Neurofold) and by an FCT Grant PTDC/SAU-NEU/105215/2008. JK was supported by the Albert-Raps Foundation, a grant ("Elan"-Fonds) of the University Hospital, Erlangen (ELAN No. 08.11.05.1). This study was partially supported by FCT and FEDER-Saúde XXI. Ingmar Henz, Annette Serwotka, and Petra Wenzeler are gratefully acknowledged for their excellent technical assistance.

Author contribution

HVM; RMO and TFO conceived, carried out experiments and analyzed data. TS and WX carried out experiments and analyzed data. TFO, JK, DP, and JP contributed with the analytic tools. All authors were involved in writing the manuscript and gave final approval for the submitted version.

The authors have no conflicts of interest to declare.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. SDS-PAGE (15%) analysis of 5 g of non-heated (-) and heated rat brain protein extract.

Table S1. Myelin basic protein identification by peptide mass fingerprinting trypsin digestion of α-syn positive rat enriched SDS-PAGE spot.

Table S2. α-syn identification by peptide mass fingerprinting trypsin digestion of α-syn positive rat enriched 1D SDS-PAGE spot.

Table S3. α-syn in silico trypsin digestion. Start-end peptides; peptide sequences; number of miscleavages; mass average (Da).

Table S4. β-Syn in silico trypsin digestion. Start-end peptides; peptide sequences; number of miscleavages; mass average (Da).

Table S5. α-Syn in silico Glu-C digestion. Start-end peptides; peptide sequences; number of miscleavages; mass average (Da).

Table S6. β-Syn in silico Glu-C digestion. Start-end peptides; peptide sequences; number of miscleavages; mass average (Da).

Table S7. Peptide mass fingerprinting results from α-Syn trypsin digestion.

Table S8. Peptide mass fingerprinting results from α-Syn Glu-C digestion.

Table S9. Peptide mass fingerprinting results from α-Syn trypsin digestion.

Table S10. Peptide mass fingerprinting results from α-Syn Glu-C digestion.

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