

# Identification of Novel $\alpha$ -Synuclein Isoforms in Human Brain Tissue by using an Online NanoLC-ESI-FTICR-MS Method

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**Abstract** Parkinson's disease (PD) and Dementia with Lewy bodies (DLB) are neurodegenerative diseases that are characterized by intra-neuronal inclusions of Lewy bodies in distinct brain regions. These inclusions consist mainly of aggregated  $\alpha$ -synuclein ( $\alpha$ -syn) protein. The present study used immunoprecipitation combined with nanoflow liquid chromatography (LC) coupled to high resolution electrospray ionization Fourier transform ion cyclotron resonance tandem mass spectrometry (ESI-FTICR-MS/MS) to determine known and novel isoforms of  $\alpha$ -syn in brain tissue homogenates. N-terminally acetylated full-length  $\alpha$ -syn (Ac- $\alpha$ -syn<sub>1–140</sub>) and two N-terminally acetylated C-terminally truncated forms of  $\alpha$ -syn (Ac- $\alpha$ -syn<sub>1–139</sub> and Ac- $\alpha$ -syn<sub>1–103</sub>) were found. The different forms of  $\alpha$ -syn were further studied by Western blotting in brain tissue homogenates from the temporal cortex Brodmann area 36 (BA36) and the dorsolateral prefrontal cortex BA9 derived from controls, patients with DLB and PD with

dementia (PDD). Quantification of  $\alpha$ -syn in each brain tissue fraction was performed using a novel enzyme-linked immunosorbent assay (ELISA).

**Keywords** Cerebrospinal fluid · Immunoprecipitation · Neurodegenerative diseases · Mass spectrometry · Synuclein

## Introduction

$\alpha$ -Synuclein ( $\alpha$ -syn) is a cytosolic protein, enriched in pre-synaptic terminals associated with synaptic vesicles [1, 2]. Four known alternatively spliced isoforms of  $\alpha$ -syn exist, full-length that consist of 140 amino acids and shorter isoforms of 126 (missing amino acids 41–54), 112 (missing amino acids 103–130) and 98 (missing amino acids 41–54 and 103–130) amino acids, respectively [3, 4]. Besides  $\alpha$ -,  $\beta$ - and  $\gamma$ -syn are members of the protein family of synucleins.  $\alpha$ - and  $\beta$ -syn proteins are primarily found in brain tissue, while  $\gamma$ -syn predominates in the peripheral nervous system [5].

The first indication of involvement of  $\alpha$ -syn in the pathogenesis of neurodegenerative diseases came from the isolation of a short peptide (non-amyloid component, NAC) derived from the amyloid plaques from the brains of patients with Alzheimer's disease (AD) [6]. Amino acid sequencing revealed that this component consisted of a 35-amino acid-peptide, corresponding to residues 61–95 of  $\alpha$ -syn [6]. Subsequently, it was demonstrated that  $\alpha$ -syn was the major component of intracellular aggregates present in Lewy bodies in Parkinson's disease (PD) and dementia with Lewy bodies (DLB) and in glial cytoplasmic inclusions of multiple system atrophy [7–10]. These diseases are collectively known as the “synucleinopathies”.

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Several studies have convincingly demonstrated clinical and pathological similarities between DLB, PD and PD with dementia (PDD), the distinctive feature being the relative timing of dementia and Parkinsonism [11]. Findings of missense mutations in the gene encoding  $\alpha$ -syn (*SNCA*) [12, 13] and multiplications of *SNCA* in families with PD [14] promote the conversion of  $\alpha$ -syn from soluble monomers to aggregated, insoluble forms in the brain, which is thought to be an important event in the pathogenesis of synucleinopathies [15]. Moreover, different post-translationally modified forms of  $\alpha$ -syn have been identified, including phosphorylated, nitrated, and ubiquitinated  $\alpha$ -syn [16–18]. Truncated forms of  $\alpha$ -syn have been isolated from Lewy bodies and several studies have also shown that specific truncated forms of  $\alpha$ -syn have enhanced tendency to form aggregates [19]. This tendency of truncated  $\alpha$ -syn species to rapidly aggregate suggests that they may play a role in inducing Lewy body formation.

In AD, distinct cerebrospinal fluid (CSF) markers have been developed and validated to complement the clinical diagnosis [20]. For synucleinopathies, most assays are in the early stages of development, but there are promising preliminary results on CSF measurements of  $\alpha$ -syn [21–24] although no conclusive Lewy body-associated changes have been found. This reminds of a similar situation in AD research 20 years ago. The first reports on CSF amyloid-beta peptide ( $A\beta$ ) as a biomarker for AD were disappointing, and results ranged from a slight decrease in AD to no change. In these studies, total  $A\beta$  in the CSF was measured. After research showed that there are several C-terminally truncated forms of  $A\beta$  [25], and that one specific isoform, the 42 amino acid long  $A\beta$  peptide ( $A\beta_{1-42}$ ) is the most prone to form AD-associated senile plaques, ELISA methods were developed with antibodies specific for this form. Today, a combination of  $A\beta_{1-42}$ , total-tau, and phospho-tau is used to identify incipient AD with good sensitivity and specificity [26]. Presence of an altered variant of  $\alpha$ -syn that can reflect subtypes of PD can therefore be anticipated.

The purpose of the study was to investigate and characterize the variants of  $\alpha$ -syn in post-mortem brains from controls and patients with DLB and PDD.  $\alpha$ -Syn from human brain tissue was biochemically fractionated in absence or presence of different detergents leading to separation of  $\alpha$ -syn according to solubility. We have in this study applied a hybrid approach, where an affinity based purification is combined with two different top-down mass spectrometry approaches to analyze the selectively purified endogenous peptides and proteins [27–30]. An immunoprecipitation method was optimized to analyze  $\alpha$ -syn in the brain tissue homogenates by matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOFMS). Using this immunoprecipitation method combined with

nanoflow liquid chromatography (LC) coupled to high resolution electrospray ionization Fourier transform ion cyclotron resonance tandem MS (ESI-FTICR-MS/MS) we were able to detect and identify N-terminally acetylated full-length  $\alpha$ -syn (Ac- $\alpha$ -syn<sub>1-140</sub>) and two N-terminally acetylated truncated forms of  $\alpha$ -syn (Ac- $\alpha$ -syn<sub>1-139</sub> and Ac- $\alpha$ -syn<sub>1-103</sub>). The different forms of  $\alpha$ -syn were further studied by Western blot analysis in brain tissue homogenates from the temporal cortex Brodmann area 36 (BA36) and the dorsolateral prefrontal cortex BA9 derived from controls, patients with DLB and PDD. Quantification of  $\alpha$ -syn in each brain tissue fraction was performed using a novel enzyme-linked immunosorbent assay (ELISA).

## Experimental Procedure

### Brain Tissue Samples

This study involves seven autopsy-confirmed patients with DLB (N = 3) or PDD (N = 4) and age-matched controls (N = 6), from the Brains for Dementia Research (UK) and Stavanger Brain Bank Resources. Table 1 shows the clinical and demographic characteristics of the groups. All subjects received prospective, standardized, annual clinical evaluations during life, including clinical histories, cognitive and motor evaluations. The clinical assessment battery incorporated successive cognitive evaluations which included the Mini-Mental State Examination [31] and longitudinal clinical assessment of Parkinsonism. The patients were diagnosed clinically and neuropathologically according to consensus criteria [11, 32, 33]. Controls were selected on the basis of the absence of psychiatric or neurological disease and of no demonstrable neuropathological abnormality in the brain. The clinical and autopsy aspects of the study were fully approved by the appropriate Human Subject Research Ethic Committee in Newcastle.

All neuropathologic evaluations were performed blind to clinical assignment. Snap frozen blocks of tissue, maintained at  $-70^{\circ}\text{C}$  from the temporal cortex Brodmann area 36 (BA36) and dorsolateral prefrontal cortex BA9 were sub-dissected to remove white matter and receive representative samples of grey matter. The severity of plaques was assessed using the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) guidelines [34]. Braak and Braak staging was performed to stage tangle pathology [35] and Lewy bodies were stained using immunohistochemistry as described by Ballard et al. [36] and assessed in brainstem, limbic and neocortical areas using the principles outlined in the DLB consensus criteria [32, 33]. In addition, a 0–20 semi-quantified scale was completed following the same hierarchy as the system within the DLB consensus criteria but incorporating

**Table 1** Clinical and demographic characteristics of the groups according to timing of dementia and Parkinsonism

Sample no	Groups	Regions	Gender	Age at death (y)	Duration of PD symptoms at death (y)	Duration of dementia at death (y)	MMSE closest to death
6	DLB	BA36/BA9	Male	92	9	9	12
7	PDD	BA36/BA9	Male	81	10	6	6
8	PDD	BA36/BA9	Male	79	19	8	22
9	DLB	BA36/BA9	Male	63	8	9	
10	DLB	BA36	Female	69	10	10	19
18	PDD	BA9	Male	75	4	2	17
20	PDD	BA9	Male	77	12	2	*
4	Control	BA36/BA9	Male	64			
5	Control	BA36	Male	87			
1	Control	BA36	Male	85			
2	Control	BA36	Female	85			
3	Control	BA36	Female	63			
12	Control	BA9	Female	82			

Abbreviations used: *BA* Brodmann area, *DLB* dementia with Lewy bodies, *MMSE* Mini-mental state examination, *PDD* Parkinson's disease with dementia, *y* years

\* No MMSE score, but other cognitive screening tests suggest dementia of moderate severity

additional rating points with the neocortical range as previously described [32, 33, 36]. The scoring from 0 to 10 reflects brainstem and limbic cortex pathology, and scores from 11 to 20 provide a semi-quantitative measure, with a particular emphasis on neocortical pathology. Table 2 presents the results from the neuropathological staging.

Brain tissues (superior parietal gyrus) from patients with AD were used for the optimization of the immunoprecipitation method of  $\alpha$ -syn.

#### CSF Samples

The optimization of the immunoprecipitation method of  $\alpha$ -syn was also performed on decoded CSF samples from the routine workflow at the Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, from patients

who underwent lumbar puncture to exclude infectious disorders of the central nervous system. No other clinical data were available for these patients. Twelve milliliters CSF was collected in a polypropylene tube and gently mixed to avoid gradient effects. All samples were centrifuged at 2,000 g for 10 min to remove cells, and stored in aliquots at  $-80^{\circ}\text{C}$  pending biochemical analysis. The inclusion criteria were normal white cell count, normal blood–brain barrier function and the absence of intrathecal IgG and IgM production.

#### Homogenization of Brain Tissue

The brain extraction procedure was performed as described by Deramecourt et al. with minor modifications [37]. Briefly, 100–150 mg of brain tissue was homogenized on

**Table 2** Results from the neuropathological tests<sup>a</sup>

Sample no	Groups	Braak stage	Plaques (CERAD)	LB stage	LB disease score
6	DLB	4	Moderate	Neocortical	14
7	PDD	2	None	Neocortical	11
8	PDD	4	Scarce	Neocortical	20
9	DLB	6	Moderate	Neocortical	10
10	DLB	2	Scarce	Neocortical	11
18	PDD	3	Scarce	Neocortical	19
20	PDD	3	Moderate	Neocortical	16

<sup>a</sup> Please refer to Materials and methods for more information regarding these tests

Abbreviations used: *CERAD* Consortium to Establish a Registry for Alzheimer's Disease, *DLB* dementia with Lewy bodies, *LB* Lewy body, *PDD* Parkinson's disease with dementia

ice in 1 mL Tris-buffer (10 mM Tris-HCl, pH 6.8) containing complete protease inhibitor (Roche Diagnostics GmbH). Centrifugation of the homogenate occurred at 31,000 g for 1 h at +4°C and the supernatant was collected (Tris). One milliliter of Tris-buffer containing 0.5% Triton® X-100 (Union Carbide Corporation) was added to the pellet homogenized on ice and sonicated using a microprobe sonicator. The centrifugation step was repeated and the supernatant was collected (0.5% Triton®). The same procedure was repeated by addition of Tris-buffer containing 2% Triton® (2% Triton®) and by addition of Tris-buffer containing 0.5% sodium dodecyl sulphate (SDS) for a final centrifugation at +12°C (SDS). The final pellets were stored at -80°C pending Western blot analysis.

### Antibodies of Synucleins

The following antibodies were used; the monoclonal Syn1b (3B5) recognized amino acids 113–126 (LED-MPVDPDNEAYE), the monoclonal anti- $\alpha$ -syn antibody Clone 42 (BD Transduction Laboratories) which was raised against amino acids 15–123 of rat  $\alpha$ -syn, and the rabbit polyclonal  $\alpha/\beta/\gamma$ -syn antibody FL140 (Santa Cruz Biotechnology). The monoclonal anti- $\alpha$ -syn antibody Clone 42, also called Syn-1 [16], was epitope-mapped using a series of overlapping, linear peptides covering the C-terminal part of the  $\alpha$ -synuclein sequence. Its epitope was defined as A<sub>85</sub>GSIAAATGFFVVD<sub>98</sub> using 14-mer N-terminally biotinylated peptides overlapping with 7 amino acids.

### Cloning and Purification of Human $\alpha$ -syn

An  $\alpha$ -syn open reading frame (from ATG to stop codon) was amplified from a human brain cDNA library (HL5018; Clontech) using primers based on published sequence data ( $\alpha$ -syn: accession number L08850), cloned in pGEM-T cloning vector and completely confirmed with sequencing. The insert was subsequently sub-cloned in a PL-based expression system (ICCG 3307; Innogenetics) adding six additional histidines (His) at the amino-terminal. The His-tagged syn fusion proteins were expressed in *E. coli* and solubilized in 7 M guanidinium hydrochloride on a nickel immobilized metal affinity chromatography (Ni-IMAC). The purity of the His-tagged  $\alpha$ -syn preparation was checked by SDS-PAGE and silverstaining and was judged >98% pure. Protein concentration was determined by amino acid composition analysis on separate aliquots.

### Immunoprecipitation

The immunoprecipitation methods of brain tissue extracts were developed using the instructions from the manufacturer's product description (Invitrogen Corporation), and

using the KingFisher® magnetic particle processor (Thermo Fisher Scientific). Optimization of the immunoprecipitation method was done for MALDI-TOFMS analyses of endogenous  $\alpha$ -syn in brain tissue extracts and in most experiments by addition of standard of  $\alpha$ -syn (7.5 pmol) to phosphate-buffered saline (PBS) (10 mM Na-phosphate, 0.15 M NaCl, pH 7.4) and/or CSF samples. The following parameters were tested; two different antibodies (monoclonal anti- $\alpha$ -syn antibody Clone 42 and monoclonal syn1b antibody); the amount of antibodies (0.8 and 8  $\mu$ g) bounded to magnetic beads via anti-mouse IgG; volume of beads (50 and 100  $\mu$ L); presence/absence of n-octylglucopyranoside (nOGP; 0.5 mM) (during incubation of samples, elution of immunoprecipitated  $\alpha$ -syn or preparation of eluted  $\alpha$ -syn on the MALDI probe). Cross-linking of antibodies to beads was investigated as well as the concentrations of Triton® (0, 0.05, 0.5 and 2%) and SDS (0, 0.05 and 0.5%) in presence/absence of 0.025% Tween® 20 during the incubation of samples with antibody-bounded beads. The final method for immunoprecipitation of  $\alpha$ -syn is given below.

An aliquot (0.8  $\mu$ g) of the monoclonal anti- $\alpha$ -syn antibody Clone 42 (250 ng/L) or IgG from murine serum (1 g/L, Sigma-Aldrich) (a negative control), was separately added to 100  $\mu$ L magnetic Dynabeads® M-280 Sheep anti-mouse IgG (Invitrogen Corporation) and incubated 1 h on a rocking platform at room temperature. The beads were washed three times with 1 mL of PBS. The antibodies were cross-linked using 20 mM dimethyl pimelimidate dihydrochloride (DMP; Sigma-Aldrich) and 0.2 M triethanolamine (pH 8.2; Sigma-Aldrich) according to the manufacturer's product description. Each brain tissue extract were adjusted with 10% Triton® and Tris to a final concentration of 2% Triton®. The cross-linked beads were washed 3 times in 0.1% bovine serum albumin (BSA) in PBS and added to the diluted brain tissue extracts. Samples and magnetic beads were incubated over night on a rocking platform at +4°C.

The magnetic beads/sample solution was transferred to the KingFisher® magnetic particle processor, tube 1. The following three wash steps (tubes 2–4) were conducted for 10 s in 1 mL of each washing buffer: (tube 2) 0.025% Tween® 20 in PBS, (tube 3) PBS and (tube 4) 50 mM ammonium hydrogen carbonate (NH<sub>4</sub>HCO<sub>3</sub>, pH 8.0). Then,  $\alpha$ -syn was eluted from the beads by adding 100  $\mu$ L 0.5 mM nOGP in 0.5% formic acid (FA) (tube 5) for 4 min. The eluted fractions were transferred to 0.5 mL Protein LoBind Tube (Eppendorf AG) and dried in a vacuum centrifuge.

### MALDI-TOFMS

MALDI-TOFMS measurements were performed using an AUTOFLEX instrument (Bruker Daltonics) operating in

linear mode at 19 kV acceleration voltage. Each spectrum represents an average of 1,500 shots acquired 50 at a time. The MALDI samples were prepared with the seed layer method as described previously with  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) used as matrix [38]. All reported mass-to-charge ratios ( $m/z$ ) are the average peak of the protonated molecule  $[M + H]^+$ .

#### Nanoflow LC–ESI–MS

For the intact proteins in the top-down proteomics analysis the brain homogenate fractions were subjected to two consecutive immunoprecipitations with  $\alpha$ -syn antibody to decrease the chemical background. Subsequently, the dried samples were dissolved in 25  $\mu$ L of 20% acetonitrile (ACN) in 0.1% formic acid (FA) for 1 h and then centrifuged (16,900 g, 10 min, +4°C) to eliminate particles. The nanoflow LC–ESI–FTICR–MS/MS spectra were acquired using an LTQ–FT classic (Thermo Fischer Scientific), a hybrid linear ion trap–FTICR mass spectrometer equipped with a 7 T magnet coupled to an Ettan MDLC (GE Healthcare) multi-dimensional nanoflow chromatography system. A HotSep Tracy C4 trap column (length 5 mm, i.d. 0.3 mm, particle size 5  $\mu$ m (G&T Septech)) was used for online desalting and sample clean-up, followed by a nanoscale reversed phase HotSep Kromasil C4 column (length 150 mm, i.d. 0.075 mm, particle size 5  $\mu$ m (G&T Septech)). Mobile phases were 0.1% aqueous FA (v/v) (A) and 0.1% FA in 84% ACN in water (v/v) (B). The separation was performed at a flow rate of approximately 400 nL/min by applying a linear gradient of 0–60% B for 50 min. The LTQ–FT was operated in the data-dependent mode, where a typical scan cycle consisted of one full scan mass spectrum ( $m/z$  600–1,500), a single ion monitoring (SIM) scan and two tandem mass spectrometry (MS/MS) scans using collision-induced dissociation (CID). All full mass spectra and the first MS/MS scan were acquired in FTICR mode while the second MS/MS scan was acquired in linear quadrupole ion trap (LQIT) mode. MS/MS isolation width was set to 10  $m/z$  units, and the normalized collision energy to 35. Each scan consisted of three microscans; full scans and SIM scans were acquired at the resolution setting 100,000, while FTICR MS/MS scans were acquired at 50,000.

Mono isotopic mass determination of both intact ions and fragment ions was generated by the Xtract program in the Xcalibur software (Thermo Fisher Scientific). In-house developed software (Sequence v. 2.0) was then utilized to match obtained peak lists to theoretical peptide mass lists. Obtained ion fragment masses were compared with theoretical fragment masses with the aid of the Protein Calculator tool in the Xcalibur software. Database searches were submitted to the in-house database server by using Mascot

Deamon 2.2 (Matrix Science) in combination with Mascot Distiller v. 2.3.2.0 (Matrix Science). Database search parameters were; database (Swissprot 57.15), taxonomy (Homo sapiens), enzyme (none), variable modifications (acetyl [N-term] and oxidation [M]), fixed modifications (none), mass values (monoisotopic), peptide mass tolerance (20 ppm), fragment mass tolerance (100 mmu), and max missed cleavages (not applicable).

In the bottom-up analysis of the in gel digested peptides eluted from the 1D gels the dried samples were dissolved in 20  $\mu$ L 0.1% FA, centrifuged and analyzed by a LTQ–FT ultra coupled to an Ettan MDLC. Here a Zorbax 300 SB–C18 trap column (length 5 mm, inner diameter 0.3 mm, particle size 5  $\mu$ m (Agilent Technologies)) was used for on-line desalting and a reversed phase Zorbax 300 SBC18 column (length 150 mm, inner diameter 0.075 mm, particle size 3.5  $\mu$ m (Agilent Technologies)) was used for high-resolution separation. The separation was performed at a flow rate of approximately 250 nL/min by applying the same gradient and mobile phases as in the top-down experiment. The LTQ–FT ultra was set to acquire positive ions and operated in the data-dependent mode, where a scan cycle consisted of one full scan mass spectrum ( $m/z$  350–1500) acquired in the FTICR mode, followed by MS/MS scans of the ten most intense ions acquired in the linear ion trap. Mascot Distiller v. 2.3.2.0 was used for database search and protein identification. Specified search parameters were database (NCBI nr 20100306), taxonomy (Mammalia), enzyme (trypsin), variable modifications (acetyl [N-term] and oxidation [M]), fixed modifications (carbamidomethyl [C]), mass values (monoisotopic), peptide mass tolerance (10 ppm), fragment mass tolerance (0.5 Da), and max missed cleavages (1). Only peptides with individual ion scores >36 (indicating identity or extensive homology ( $P < 0.05$ )) were considered for identification.

#### Gel Electrophoresis and in-Gel Digestion

Dried immunoprecipitated brain homogenate fractions (Tris, Triton<sup>®</sup>, and SDS) were dissolved in 15  $\mu$ L NuPAGE<sup>®</sup> sample buffer. The samples were electrophoresed using NuPAGE<sup>®</sup> 10% Bis–Tris gels (Invitrogen Corporation) on NuPAGE mini gel system (Xcell II Mini-Cell; Novex). Each fraction (lane) was sliced into 15 equally large pieces and washed twice with 100% ACN for 10 min each. ACN was replaced with 25 mM  $\text{NH}_4\text{HCO}_3$  containing 10 mM dithiothreitol and incubated in +56°C for 30 min. After removing the solution the gel pieces were washed with ACN, replaced with 25 mM  $\text{NH}_4\text{HCO}_3$  containing 55 mM iodoacetamide (IAA) and incubated 20 min in darkness at room temperature. After removing the solution the gel pieces were washed with 50 mM

$\text{NH}_4\text{HCO}_3$  for 10 min before removing the solution. The gel pieces were then incubated for 5 min in ACN three times before digestion with 12.5 ng/ $\mu\text{L}$  trypsin (Promega Corporation) in 25 mM  $\text{NH}_4\text{HCO}_3$  at  $+37^\circ\text{C}$  overnight. The supernatants were collected in cycles in new 0.5 mL Protein LoBind Tubes. Gel pieces were then incubated for 10 min at room temperature in 25 mM  $\text{NH}_4\text{HCO}_3$  and the supernatants were then pooled with the previously collected supernatants. The remaining peptides were extracted by incubating the gel pieces for 15 min at  $+37^\circ\text{C}$  in ACN, 5% FA and ACN, subsequently. After each incubation, the supernatant was collected and pooled with the supernatants previously collected. The pooled supernatants were dried with vacuum centrifugation overnight and stored at  $-80^\circ\text{C}$ . Dried peptides were dissolved in 20  $\mu\text{L}$  0.1% FA prior to LC–ESI–MS/MS analysis.

### Western Blotting

For protein quantitation, Protein DC assay (Bio-Rad Laboratories) reagent was used. This reagent is reducing agent and detergent compatible. Aliquots of 5  $\mu\text{g}$  (Tris and Triton<sup>®</sup>) and 10  $\mu\text{g}$  (SDS) of total protein were mixed with NuPAGE<sup>®</sup> sample buffer. Pellets were dissolved in NuPAGE<sup>®</sup> sample buffer containing 5% lithium dodecyl sulphate (LDS). All samples were electrophoresed on NuPAGE<sup>®</sup> 10% Bis–Tris gel using the NuPage<sup>®</sup> mini-gel system. The proteins were transferred to Immobilon<sup>™</sup>-P PVDF membranes (Millipore Corporation) using the semi-dry blotting technique. Blocking was performed for 1 h at room temperature using Roti<sup>®</sup>-Block (Carl Roth). Incubations with the monoclonal anti- $\alpha$ -syn antibody Clone 42 (250 ng/L) diluted 1:1,000 in Roti<sup>®</sup>-Block or no primary antibody (negative control) were performed overnight at  $+4^\circ\text{C}$ . The membranes were washed for 30, 15, and  $2 \times 10$  min in PBS-T (0.05% v/v Tween<sup>®</sup> 20) and then incubated for 1 h at room temperature with biotinylated anti-mouse IgG antibody (Vector Laboratories) (1 mg/mL), diluted 1:3,000 in PBS-T. A second PBS-T wash was performed for  $3 \times 10$  min after which the membranes were incubated for 1 h at room temperature with streptavidin-biotinylated horseradish peroxidase complex (GE Healthcare) diluted 1:3,000 in PBS-T. Following  $3 \times 10$  min washes, the membranes were developed for 2 min with ECL Advanced (GE Healthcare) solution according to the manufacturer's instructions. The emitted signal was detected by a Fujifilm LAS-3000 System (FUJIFILM Corporation) and evaluated using the Multi Gauge v 2.2 software (FUJIFILM Corporation).  $\alpha$ -Syn band intensities (+(weak), ++(moderate), +++(strong)) were based on the optical density of each band subtracting the background. All samples were analyzed in duplicate on two separate gels.

### Sandwich ELISA Method for $\alpha$ -syn

A combination of rabbit polyclonal  $\alpha/\beta/\gamma$ -syn antibody FL140, as capturing antibody, and the monoclonal antibody Syn1b, as detection antibody, resulted in the most sensitive detection of  $\alpha$ -syn in human brain tissue extract. Maxisorp plates (Nunc) were coated with 100  $\mu\text{L}$  of polyclonal FL140 antibody (200 ng/L), diluted 1:500 in 50 mM carbonate buffer, pH 9.6, and incubated over night at  $+4^\circ\text{C}$ . The plates were washed four times with 300  $\mu\text{L}$  of PBS and were blocked with 250  $\mu\text{L}$  of 1% BSA in PBS for 1 h at room temperature. The plates were washed further four times with 300  $\mu\text{L}$  of 0.1% BSA in PBS. The washing procedure was repeated between every following incubation step. All samples were analyzed in duplicate. All brain tissue extracts were diluted 1:20 in 0.1% BSA, 1% Triton<sup>®</sup> in PBS. The standard of  $\alpha$ -syn was diluted in 0.1% BSA, 1% Triton<sup>®</sup> in PBS to a concentration range of 0.39–50  $\mu\text{g/L}$ . Samples (100  $\mu\text{L}$ ) of diluted brain tissue extract and standard were incubated for 2 h at room temperature. Then, 100  $\mu\text{L}$  of monoclonal Syn1b antibody (1.9 g/L) diluted 1:2,000 in 0.1% BSA, 1% Triton<sup>®</sup> in PBS was incubated for 1 h at  $+37^\circ\text{C}$ . Thereafter, 100  $\mu\text{L}$  of mouse IgG-biotin (Sigma–Aldrich) diluted 1:6,000 in 0.1% BSA in PBS was added and incubated for 1 h at  $+37^\circ\text{C}$ . Then, NeutrAvidin Horseradish Peroxidase Conjugate (1 g/L) (Thermo Scientific) diluted 1:5,000 in 1% Triton<sup>®</sup> in PBS was incubated for 1 h at room temperature, and development occurred by adding 100  $\mu\text{L}$  of 3,3',5,5'-tetramethylbenzidine substrate (TMB Peroxidase EIA Substrate Kit; Bio-Rad Laboratories). The reaction was quenched with 100  $\mu\text{L}$  of  $\text{H}_2\text{SO}_4$  (2 M). The absorbance was measured at 450 nm. The concentration of  $\alpha$ -syn in samples of brain tissue extracts was calculated from the standard curve. For each sample a ratio was calculated where the  $\alpha$ -syn level was divided by the total protein concentration.

### Statistical Analysis

All statistical analyses were performed using the Statistical Package of the Social Science (SPSS). Data are given as median (inter-quartile range). The Mann–Whitney test was used to investigate group differences.

### Ethics

The study was conducted in accordance with the provisions of the Helsinki declaration and approved by the regional ethics committees.

## Results

### Identification of Novel Fragments of $\alpha$ -syn with Mass Spectrometry

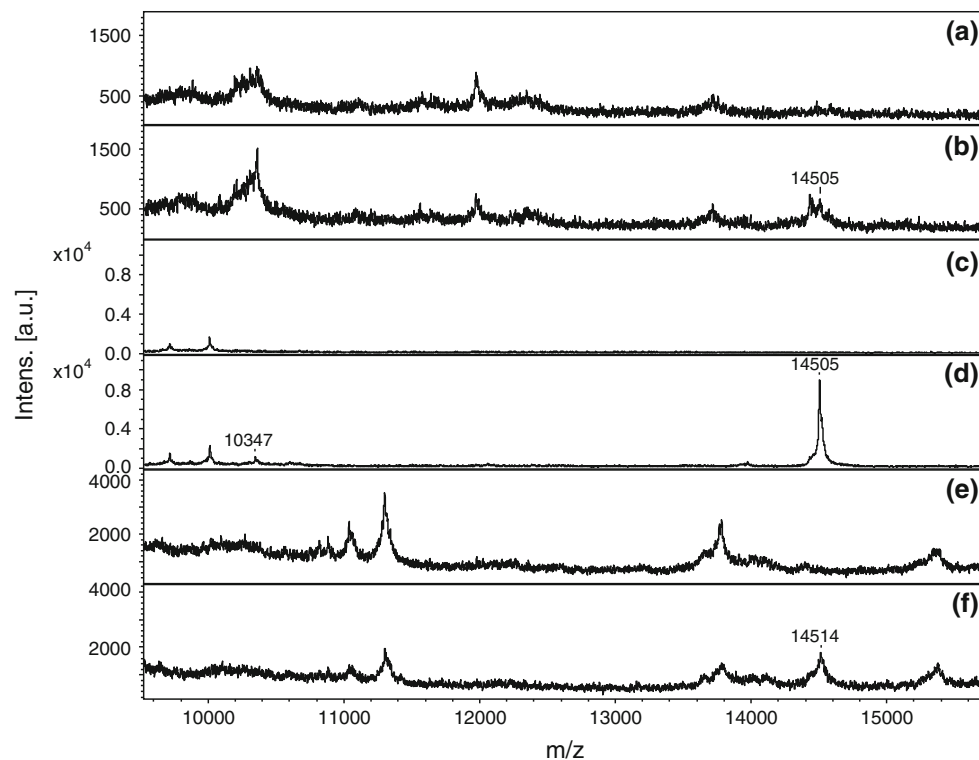
$\alpha$ -Syn was biochemically fractionated according to solubility using non-ionic and ionic detergents at different concentrations. Generally, the Tris fractions contain highly soluble proteins (mainly cytosolic proteins), while membrane-bound proteins are found in the Triton<sup>®</sup> fractions. The SDS fractions might represent raft-associated  $\alpha$ -syn [37, 39]. Triton<sup>®</sup> at a higher concentration (2%) was applied to ensure extraction of all membrane-bound  $\alpha$ -syn. Optimization of the immunoprecipitation method was done for MALDI-TOFMS analyses of endogenous  $\alpha$ -syn in brain tissue extracts and in most experiments by addition of standard of  $\alpha$ -syn to PBS and/or CSF samples. Subsequently, the optimized MALDI-TOFMS settings and sample preparation protocol were utilized to acquire mass spectra of the immunoprecipitated brain homogenate fractions. Even though the immunoprecipitation greatly reduced the complexity of the original brain homogenate samples, unspecific binding still occurred. However, by comparing the mass spectra from the samples immunoprecipitated with the  $\alpha$ -syn antibody and the respective negative controls, several peaks/compounds that were specific for  $\alpha$ -syn immunoprecipitation could be distinguished (Fig. 1). The most prominent features in the MALDI mass spectra that were specific for the  $\alpha$ -syn immunoprecipitated samples were two peaks detected at  $m/z$  14,505 and  $m/z$  7,250 (not shown), respectively (Fig. 1 b, d, f). These experimental  $m/z$  values agree with the theoretical  $m/z$  of the singly and doubly protonated full-length N-terminally acetylated  $\alpha$ -syn (Ac- $\alpha$ -syn<sub>1–140</sub>) ( $m/z$  14,503 and  $m/z$  7,252 with average mass values) within the specified mass accuracy of the method. Several other peaks could only be detected in the samples immunoprecipitated with the  $\alpha$ -syn antibody but limited mass accuracy of the MALDI-TOFMS made it difficult to interpret *in silico* searches of potential truncated forms of  $\alpha$ -syn.

To obtain accurate mass values of the tentative  $\alpha$ -syn species and if possible verify their identity with amino acid sequence information, we developed an online top-down proteomics approach where LC-ESI-MS/MS analysis of the immunoprecipitated brain homogenate fractions was utilized. The major strength of the top-down approach is that a discrepancy between the measured molecular mass and that predicted by the DNA sequence directly reveals the presence of modifications, sequence errors, or variants. Furthermore, tandem mass spectrometric analysis of the intact fully modified protein can verify the identity and determine the locations of the modifications or sequence variants. However, in general MALDI-MS has shown a

greater ability to detect proteins in complex mixtures in the presence of high concentrations of salts, buffers, detergents, and other species than ESI-MS. Unless the sample is relatively pure the ESI-MS technique requires a clean-up procedure prior to injection. This is typically accomplished by coupling one or more separation/purification step, e.g., LC, on- or offline to the ESI-source. Because of the high complexity of the samples investigated in this study, we found that it was beneficiary to add a second immunoprecipitation as a further cleaning step before the topdown LC-ESI-MS/MS analysis in order to reduce the levels of compounds that have bound non-specifically to beads and/or antibodies. With this set-up Ac- $\alpha$ -syn<sub>1–140</sub> was detected with high mass accuracy in all three brain homogenate fractions and verified with amino acid sequence data (Fig. 2a–c, f, and Table 3). It is interesting to note that the sequence tags confirmed by the MS/MS data are specific for  $\alpha$ -syn and are not present in  $\beta$ - or  $\alpha$ -synuclein. Apart from Ac- $\alpha$ -syn<sub>1–140</sub> two novel truncated forms of  $\alpha$ -syn were tentatively identified. In both the Tris and Triton<sup>®</sup> fractions a fragment corresponding to N-terminally acetylated  $\alpha$ -syn 1–103 (Ac- $\alpha$ -syn<sub>1–103</sub>) was detected and verified with amino acid sequence data (Fig. 2d, e, and Table 3). The identity of the Ac- $\alpha$ -syn<sub>1–103</sub> form was also independently confirmed with a database search using mascot distiller (Table 3). A peak with the corresponding  $m/z$  of Ac- $\alpha$ -syn<sub>1–103</sub> can also be discerned in the MALDI-TOFMS spectra of the Triton<sup>®</sup> brain homogenate fraction (Fig. 1d).

The other novel  $\alpha$ -syn species that was detected with a high mass accuracy and tentatively verified by partial top-down amino acid sequence data during the LC-ESI-MS/MS analysis was Ac- $\alpha$ -syn<sub>1–139</sub> (Fig. 2a, b and Table 3). Since Ac- $\alpha$ -syn<sub>1–139</sub> and Ac- $\alpha$ -syn<sub>1–140</sub> co-elute and have similar  $m/z$  it was difficult to specifically isolate Ac- $\alpha$ -syn<sub>1–139</sub> from Ac- $\alpha$ -syn<sub>1–140</sub>. The presented fragment ion data was obtained in the analysis of the Tris fraction. Even though this truncated  $\alpha$ -syn form only differs from the full length  $\alpha$ -syn with one amino acid (alanine), a tendency of a shift in relative concentration of Ac- $\alpha$ -syn<sub>1–139</sub> and Ac- $\alpha$ -syn<sub>1–140</sub> between the Tris and the Triton<sup>®</sup> fractions (Fig. 2a, b), suggests that Ac- $\alpha$ -syn<sub>1–139</sub> is somewhat more soluble than full-length  $\alpha$ -syn. Moreover, an MS peak with approximately the mass value of Ac- $\alpha$ -syn<sub>1–139</sub> could be discerned in the MALDI-TOFMS analysis, but the resolution of the method was too low to fully resolve Ac- $\alpha$ -syn<sub>1–139</sub> and Ac- $\alpha$ -syn<sub>1–140</sub>. The MALDI-TOFMS data also showed a tendency to a more intense Ac- $\alpha$ -syn<sub>1–139</sub> peak compared to the Ac- $\alpha$ -syn<sub>1–140</sub> peak in the analysis of the Tris fraction compared to the Triton<sup>®</sup> fraction.

A weak signal from one more tentative truncated form of N-terminally acetylated  $\alpha$ -syn (Ac- $\alpha$ -syn<sub>1–119</sub>), was detected in the Triton<sup>®</sup> brain homogenate fraction, but



**Fig. 1** MALDI TOF mass spectra of the brain homogenate fractions (Tris fractions (**a**, **b**), Triton<sup>®</sup> fractions (**c**, **d**), SDS fractions (**e**, **f**)) immunoprecipitated using the monoclonal anti- $\alpha$ -syn antibody Clone

42 (BD Transduction Laboratories) (**b**, **d**, **f**) or IgG from murine serum (*negative controls*) (**a**, **c**, **e**)

unfortunately the identity could not be confirmed with tandem MS data (data not shown).

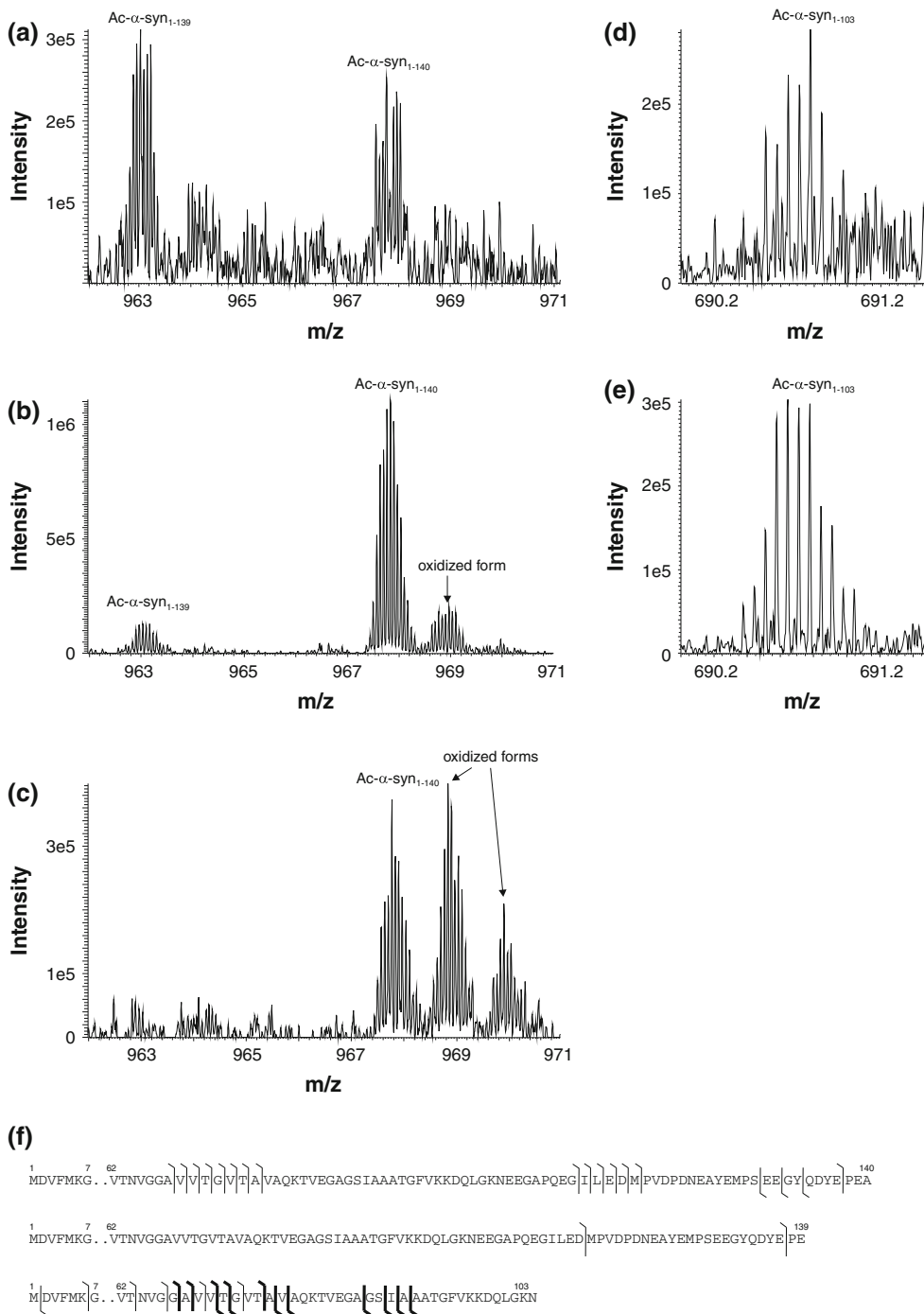
In addition, the proteins in the  $\alpha$ -syn antibody immunoprecipitated brain homogenate fractions were separated by one-dimensional gel electrophoresis, subjected to in-gel digestion with trypsin and analyzed by LC-ESI-MS/MS (Table 4). The results confirmed the presence of  $\alpha$ -syn in the brain homogenates, but despite up to 50% amino acid sequence coverage neither the N-terminal nor the C-terminal part of the amino acid sequence were identified in the data base search. Generally, the highest concentration of  $\alpha$ -syn was detected at the gel bands containing proteins of the approximate weight of  $\sim 16$  kDa for all three brain homogenate fractions. Furthermore, the data indicated that the Tris fraction contained higher molecular weight forms of  $\alpha$ -syn, possibly aggregates, and both the Tris and Triton<sup>®</sup> brain homogenate fractions contained lower molecular weight (maybe truncated)  $\alpha$ -syn forms. The truncated  $\alpha$ -syn forms at least contained amino acids 81–96.

#### Evaluation of $\alpha$ -syn by Western Blotting

The presence of full-length  $\alpha$ -syn detected at  $\sim 16$  kDa was also shown by Western blotting in all of the brain homogenate fractions (Tris, Triton<sup>®</sup>, SDS) in both brain regions—temporal cortex BA36 (Fig. 3a) and dorsolateral

prefrontal cortex BA9 (data not shown). In agreement with previous studies, there were higher contents of full-length  $\alpha$ -syn in the Tris fractions and 0.5% Triton<sup>®</sup> fractions, respectively, than in the SDS fractions (data not shown). In temporal cortex BA36, but not in dorsolateral prefrontal cortex BA9, weak additional  $\alpha$ -syn bands of  $\sim 12$  kDa were observed in most of the Tris and Triton<sup>®</sup>-soluble fractions from both controls and patients with DLB and PDD (data not shown). Overall, there was no visible difference in the content of either full-length or truncated 12 kDa  $\alpha$ -syn for the controls, the patients of DLB and PDD in any of the brain homogenate fractions (Tris, Triton<sup>®</sup> or SDS) (data not shown). However, the Western blotting of the pellet fractions from temporal cortex BA36, clearly demonstrated that brains from patients with DLB and PDD compared with control brains had a considerably higher content of full-length  $\alpha$ -syn (Fig. 3c). Moreover, most pellets from patients with DLB and PDD in temporal cortex BA36 contained additional bands of 32, 12 and 8 kDa, which were absent in all samples from controls (Fig. 3c). In contrast, in dorsolateral prefrontal cortex BA9, there was no difference in the relative content of full-length  $\alpha$ -syn between controls and patients with DLB and PDD, and the bands of  $\sim 32$ ,  $\sim 12$  and  $\sim 8$  kDa were only present in a few samples (Fig. 3e).





**Fig. 2** Nanoflow LC-ESI-FTICR full scan mass spectra showing the  $\alpha$ -syn forms Ac- $\alpha$ -syn<sub>1-139</sub> (**a**, **b**) and Ac- $\alpha$ -syn<sub>1-140</sub> (**a**–**c**). Note also the presence of mono- and dioxidized forms. The spectra are summed over a 1.5 min retention time interval around 46 min (50 min gradient). In the spectra the 15 + charge state of the Ac- $\alpha$ -syn<sub>1-139</sub> and Ac- $\alpha$ -syn<sub>1-140</sub> forms are indicated. One SIM scan (3 microscans) of the 15 + ion of the Ac- $\alpha$ -syn<sub>1-103</sub> form (**d**, **e**). Spectra from the

LC-ESI-MS analysis of the Tris fraction of the brain homogenate (**a** and **d**). Spectra from the LC-ESI-MS analysis of the Triton<sup>®</sup> fraction of the brain homogenate (**b** and **e**). Spectra from the LC-ESI-MS analysis of the SDS fraction of the brain homogenate (**c**). The detected y and b fragments are indicated in the respective sequences (**f**). Cleavage sites labeled with **bold lines** are confirmed by database search

**Quantification of  $\alpha$ -syn by ELISA**

The intra-assay variation was determined by ten measurements of two samples of brain tissue homogenate from

each fraction (Tris, 0.5% Triton<sup>®</sup>, SDS), which resulted in a CV of 9.3% (N = 6). All samples from the study of patients were analyzed at the same day. In agreement with the Western blotting analysis, the ratios ( $\alpha$ -syn/total

**Table 3**  $\alpha$ -syn forms detected by LC-ESI-FTICR-MS/MS

$\alpha$ -syn isoforms	Theoretical mass [Da] <sup>a</sup>	Mass deviation (ppm)	Fragments detected (b/y)
Ac- $\alpha$ -syn <sub>1–140</sub>	14,493.23	2	15/3
Ac- $\alpha$ -syn <sub>1–139</sub>	14,422.19	5	2/0
Ac- $\alpha$ -syn <sub>1–103</sub>	10,339.56	4	8/7

<sup>a</sup> monoisotopic mass values

protein) were significantly increased in the Tris fractions (90 (77–124) ( $P < 0.001$ ) (temporal cortex BA36) (N = 10) and 61 (59–70) ( $P < 0.001$ ) (dorsolateral prefrontal cortex BA9) (N = 8)) and Triton<sup>®</sup> fractions (45 (41–60) ( $P < 0.001$ ) (temporal cortex BA36) (N = 10) and 41 (36–46) ( $P < 0.001$ ) (dorsolateral prefrontal cortex BA9) (N = 8)) compared with the SDS fractions (7 (6–8) (temporal cortex BA36) (N = 10) and 4 (4–5) (dorsolateral prefrontal cortex BA9) (N = 8)) (Fig. 4). The ratios ( $\alpha$ -syn/total protein) were significantly increased in temporal cortex BA36 compared with dorsolateral prefrontal cortex BA9 for the Tris ( $P < 0.05$ ) and SDS ( $P < 0.01$ ) fractions (see above) (Fig. 4), which also is consistent with the Western blotting analyses. Fig. 4 shows each ratio ( $\alpha$ -syn/total protein) for controls and patients with DLB and PDD within each brain region. Unfortunately, the sample size was too low for assessing differences between controls, patients with DLB and PDD (Fig. 4).

**Table 4** Detection of  $\alpha$ -syn (NACP/alpha-synuclein NCBI Accession key gi1230575) in different brain homogenate fractions (Tris, Triton<sup>®</sup>, SDS) separated by one-dimensional gel electrophoresis combined with MS/MS for protein identification

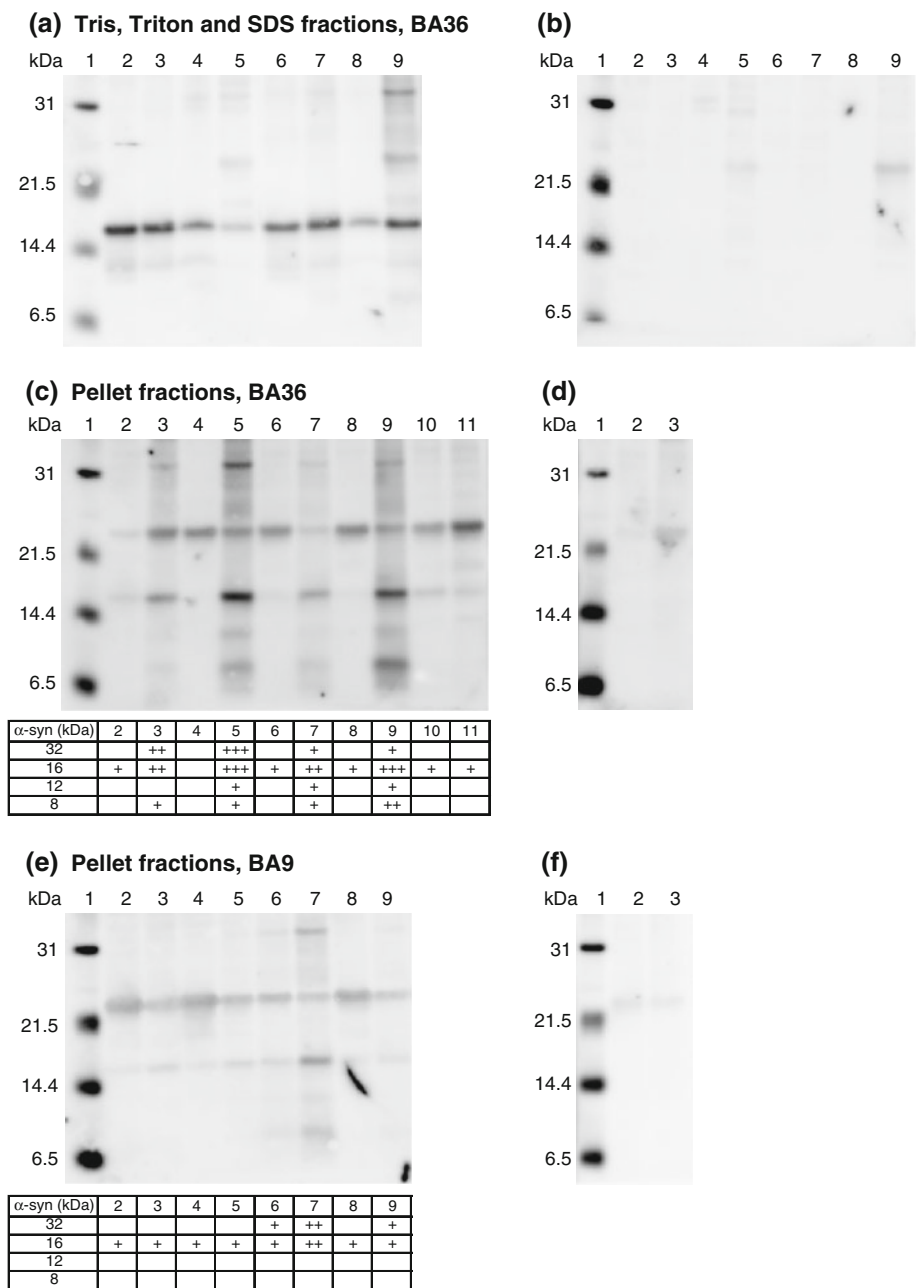
Fraction	Approx. MW [kDa]	Sequence coverage [%]	Amino acids	Unique peptides
Tris	40	11	81–96	1
	30	11	81–96	1
	25	11	81–96	1
	20	35	46–58, 61–96	3
	16	50	11–21, 33–43, 46–58, 61–96	7
	12	50	11–21, 33–43, 46–58, 61–96	10
	10	44	11–21, 44–58, 61–96	8
	6	36	46–96	4
	4	11	81–96	1
	Triton <sup>®</sup>	20	11	81–96
16		49	11–21, 35–43, 46–58, 61–96	11
12		37	11–21, 33–58, 81–96	10
10		35	46–58, 61–96	5
6		20	46–58, 81–96	2
4		11	81–96	1
3		11	81–96	1
SDS	25	11	81–96	1
	14	52	11–21, 33–58, 61–97	19
	10	20	46–58, 81–96	3

## Discussion

Gene mutations of  $\alpha$ -syn in familial PD [12, 13] and the presence of aggregated  $\alpha$ -syn protein in Lewy bodies strongly suggest that  $\alpha$ -syn plays a key role in the pathogenesis of PD and DLB. The alteration of solubility of  $\alpha$ -syn, aggregating into Lewy bodies, might be relevant for understanding the underlying pathogenic mechanism, and tentatively facilitate earlier diagnosis and the development of novel mechanism-based therapeutic interventions [40]. To our knowledge this is the first study where intact  $\alpha$ -syn from human brain tissue has been characterized with mass spectrometry. All four  $\alpha$ -syn forms detected were N-terminally acetylated. This is in agreement with data presented by Anderson et al. [16] and it is also commonly known that N-terminal acetylation of cytosolic proteins, especially in proteins initiated with a Met residue often occurs [41] and has been shown in yeast to be indispensable for the proper plasma membrane targeting of  $\alpha$ -syn [42].

An especially interesting finding was the identification of the novel truncated  $\alpha$ -syn Ac- $\alpha$ -syn<sub>1–103</sub>. This  $\alpha$ -syn species could tentatively be identical to the truncated form observed both in this and previous studies with Western blotting from the temporal cortex [43, 44]. In addition, the weak  $\alpha$ -syn band of  $\sim 12$  kDa was observed in most of the Tris and Triton<sup>®</sup>-soluble fractions, but not in the SDS-soluble fractions, which is in agreement with the mass

**Fig. 3** Western blotting of biochemically fractionated temporal cortex Brodmann area 36 (BA36) (a–d) and dorsolateral prefrontal cortex BA9 (e, f) from controls, patients with dementia with Lewy bodies (DLB) and Parkinson’s disease with dementia (PDD) using the monoclonal anti- $\alpha$ -syn antibody Clone 42 (a, c, e) and without primary antibody (b, d, f). Molecular markers (lane 1), Tris fraction (lane 2 and 6), 0.5% Triton<sup>®</sup> fraction (lane 3 and 7), 2% Triton<sup>®</sup> fraction (lane 4 and 8) and 0.5% SDS fraction (lane 5 and 9) from a control and patient with DLB, respectively (a, b). Western blotting of the final pellet (c–f) using the monoclonal anti- $\alpha$ -syn antibody Clone 42 (c, e) and without primary antibody (d, f). Molecular markers (lane 1), from controls (lane 2, 4, 6, 8 and 10), patients with DLB (lane 3, 9, 11) and PDD (lane 5, 7) (c). Molecular markers (lane 1), from controls (lane 2 and 4), patients with DLB (lane 3) and PDD (lane 5–9) (e). The table below each Western blot (c, e) shows the abundance of the  $\alpha$ -syn bands of 32, 16, 12, and 8 kDa given. The intensities of the band (+(weak), ++(moderate), +++(strong)) presented in the table panel are based on optical density for each band of  $\alpha$ -syn subtracting the background. Empty boxes meaning no visible bands

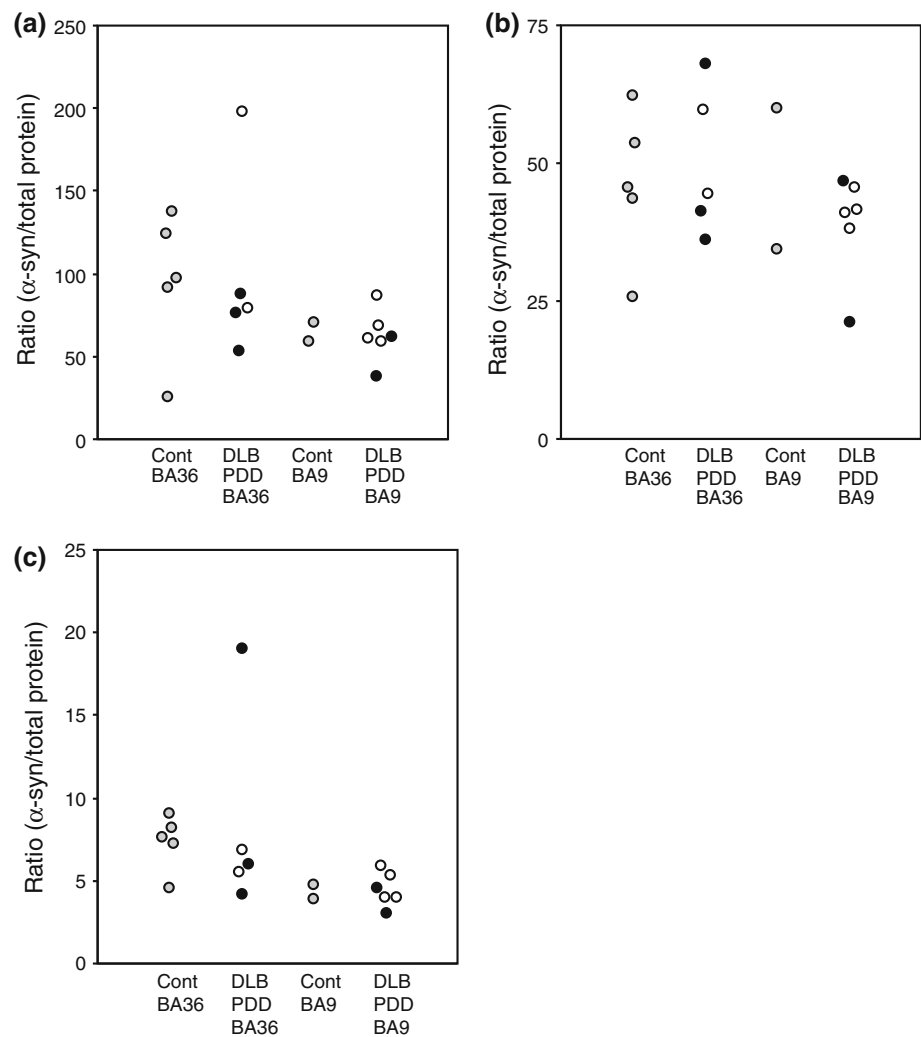


spectrometry analysis. The ~12 kDa band was only visible in brain homogenate fractions from the temporal cortex region BA36 and not from the dorsolateral prefrontal cortex. However, there were no differences in the content of either full-length or truncated 12 kDa  $\alpha$ -syn between the controls and the patients of DLB/PDD in any of the brain homogenate fractions, which is consistent with previous studies [43, 45]. Recently, it was shown that matrix metalloproteinase 3 (MMP-3) cleaves  $\alpha$ -syn at Asp<sub>103</sub> in vitro [46]. Levin et al. have also shown that limited proteolysis of  $\alpha$ -syn by MMP-3 and MMP-1 leads to fragments that increase aggregation of  $\alpha$ -syn in vitro. The presence of

the Ac- $\alpha$ -syn<sub>1–103</sub> form in the brain homogenate opens up several interesting lines of investigation. One would be to examine if Ac- $\alpha$ -syn<sub>1–103</sub> is generated by MMP-3 cleavage of  $\alpha$ -syn in vivo. Another would be to compare the amount of Ac- $\alpha$ -syn<sub>1–103</sub> found in different brain samples from controls and patients with DLB and PDD.

Tentative, but not conclusive evidence of one of the truncated  $\alpha$ -syn forms (Ac- $\alpha$ -syn<sub>1–119</sub>) that Anderson et al. detected in Lewy bodies from patients with DLB was found in this study [16]. However, we could not find evidence of the phosphorylated (P-S129)  $\alpha$ -syn form or the ubiquitinated phosphorylated  $\alpha$ -syn discovered in the same

**Fig. 4** Individual values for the ratio ( $\alpha$ -syn/total protein) in biochemically fractionated (Tris (a), 0.5% Triton<sup>®</sup> (b), and SDS fraction (c)) temporal cortex Brodmann area 36 (BA36) and dorsolateral prefrontal cortex BA9 from controls (*grey circle*), patients with dementia with Lewy bodies (DLB) (*black circle*) and Parkinson's disease with dementia (PDD) (*white circle*) using ELISA



study. Since both the examined material and the utilized methods differ between this study and the study by Anderson et al. it is difficult to speculate on the reason behind the discrepancies. One major difference between the present study and Anderson et al. is that we have focused mainly on the more soluble forms of  $\alpha$ -syn while they have concentrated on the least soluble forms.

Previous studies have shown that  $\alpha$ -syn is a highly soluble protein mainly localized in the cytosol and enriched in pre-synaptic terminals where it might be organized in lipid rafts and in association with synaptic vesicles [1, 2, 39, 47]. In accordance with those studies, ELISA analyses of the brain homogenate fractions showed that the ratios ( $\alpha$ -syn/total protein) were significantly increased in the Tris fractions (soluble  $\alpha$ -syn), and the Triton<sup>®</sup> fractions (membrane-bound  $\alpha$ -syn) compared with the SDS fractions (raft associated  $\alpha$ -syn). This finding was confirmed by the Western blot analyses of full-length  $\alpha$ -syn. In addition, our data indicate that  $\alpha$ -syn is differently expressed in brain regions, since the

ratios ( $\alpha$ -syn/total protein) were significantly increased in temporal cortex BA36 compared with dorsolateral prefrontal cortex BA9 for the fractions of Tris and SDS, which also is consistent with the Western blotting analyses.

Interestingly, we found that the pellet fractions, which contain the remaining aggregates of  $\alpha$ -syn, from brain region temporal cortex from patients with DLB and PDD, had a considerable higher content of full-length  $\alpha$ -syn (16 kDa) and also contained additional  $\alpha$ -syn bands of 32 kDa (probably dimeric  $\alpha$ -syn), 12 and 8 kDa, which were absent in all control samples. This is in agreement with previous studies finding an increase of both full-length and dimeric  $\alpha$ -syn in the temporal cortex in patients with DLB compared to controls [43, 45]. These findings extend recent reports of reduced level of total  $\alpha$ -syn in CSF in synucleinopathies compared to controls in some [23, 36] but not all [48] studies, which if confirmed would parallel the situation in AD with the  $A\alpha_{1-42}$  peptide [20]. Interestingly, cortical Lewy bodies are absent in the brains of most

normal control individuals [49], which also is consistent with the observation that negligible amounts of  $\alpha$ -syn were found in the pellets from our control samples. The different  $\alpha$ -syn pattern found in the pellet fractions from brain region temporal cortex from patients with DLB and PDD compared to controls are also consistent with pathological changes of  $\alpha$ -syn that involves presence of more lipid-associated oligomeric species and an insoluble fraction of  $\alpha$ -syn species that are not reported in non-diseased brain tissue [40]. In contrast to temporal cortex BA36, there were no differences in the content of full-length  $\alpha$ -syn in dorsolateral prefrontal cortex BA9 between controls, patients with DLB and PDD, and the  $\alpha$ -syn bands of 32, 12 and 8 kDa were only present in a few samples. These regional differences might be explained by previous studies suggesting that the hippocampus, temporal cortex, and inferior temporal gyrus are particularly vulnerable to Lewy body formation [50, 51]. Furthermore studies of PD-related pathology have shown that the dorsolateral prefrontal cortex is affected considerably later in the disease process than temporal cortex [50]. This is also consistent with recent clinical studies suggesting that early impairments in posteriorly based cognitive functions in PD progress to dementia, whereas frontally-based impairments is reversible or remains stable [52].

To our knowledge this is the first study where intact  $\alpha$ -syn from human brain tissue has been characterized with mass spectrometry. By using a combination of protein solubility fractionation, immunoprecipitation and mass spectrometric top-down sequencing with high resolution LC-MS/MS we have found at least two novel C-terminally truncated forms of  $\alpha$ -syn, Ac- $\alpha$ -syn<sub>1–103</sub> and Ac- $\alpha$ -syn<sub>1–139</sub>, and possibly Ac- $\alpha$ -syn<sub>1–119</sub>. All four identified forms of  $\alpha$ -syn including the full length  $\alpha$ -syn<sub>1–140</sub> were found to be post translationally modified by N-terminal acetylation. Hence, we have shown that it is possible by combing targeted antibody based purification with solubility fractionation and high resolution proteomics techniques to identify novel  $\alpha$ -syn forms.

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