

Identification and characterization of a new alpha-synuclein isoform and its role in Lewy body diseases

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Received: 5 August 2007 / Accepted: 27 September 2007 / Published online: 23 October 2007
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Abstract Alternative splicing is an important mechanism to generate a large number of mRNAs, thus increasing proteome diversity and tissue specificity. Three transcript variants of alpha-synuclein, a neuronal protein mainly involved in synapses, have been described so far. Whereas alpha-synuclein 140 is the whole and main transcript, alpha-synuclein 112 and 126 are short proteins that result from in-frame deletions of exons 3 and 5, respectively. Because the aforesaid alpha-synuclein isoforms show differential expression changes in Lewy body diseases (LBDs), in the present work, we searched for a fourth alpha-synuclein isoform and studied its expression levels in LBD brains. By using isoform-specific primers, isoform co-amplification and direct sequencing, we identified alpha-synuclein 98, which lacks exons 3 and 5. mRNA

expression analyses in non-neuronal tissue revealed that alpha-synuclein 98 is a brain-specific splice variant with varying expression levels in different areas of fetal and adult brain. Additionally, we studied alpha-synuclein 98 expression levels by real-time semi-quantitative RT-PCR in the frontal cortices of LBD patients and compared them with those of Alzheimer disease (AD) patients and control subjects. Overexpression of alpha-synuclein 98 in LBD and AD brains would indicate its specific involvement in the pathogenesis of these neurodegenerative disorders.

Keywords Alpha-synuclein · Alzheimer disease · Dementia with Lewy bodies · Differential isoform expression · mRNA expression

Electronic Supplementary Material The online version of this article (doi:10.1007/s10048-007-0106-0) contains supplementary material, which is available to authorized users.

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Abbreviations

AD	Alzheimer disease
LB	Lewy body
cLBD	common Lewy body disease
pDLB	dementia with Lewy bodies, pure form
NFTs	neurofibrillary tangles
NPs	neuritic plaques
PD	Parkinson disease

Introduction

Neuropathologically, Lewy body diseases (LBDs) are characterized by the presence of Lewy bodies (LBs) in the brain. LBDs include Parkinson disease (PD), dementia with Lewy bodies in its pure form (pDLB), and common Lewy body disease (cLBD). Because cLBD brains contain both LBs and amyloid plaques, cLBD is thought to represent a mixed form of DLBp and Alzheimer disease (AD). LBs are

intraneuronal inclusion bodies and, whereas the substantia nigra is the most affected area in PD [1, 12], widespread distribution of LBs throughout almost all brain areas is observed in DLBp and cLBD [18].

The most prominent component of LBs is alpha-synuclein and its aggregation is thought to represent a key event in LB formation [31]. Predominantly expressed in central nervous system neurons, alpha-synuclein is localized to the cytosol and presynaptic terminals at axonal termini [8, 15] with some fractions associated with synaptic-vesicle membranes [15, 41]. On the other hand, accumulating evidence suggests that alpha-synuclein may influence the size of presynaptic vesicular pools and, consequently, alpha-synuclein could play an important role in the organization and regulation of synaptic vesicles [6, 26].

Alpha-synuclein is a very conserved, small unfolded protein encoded by the six-exon SNCA (PARK1) gene, which is located on chromosome 4q21 [34]. In its primary structure, alpha-synuclein is characterized by six repeat sequences predicted to form five amphipathic helices on the N-terminal half [9, 16, 27, 40] and by an acidic, glutamate-rich C-terminal region that remains unstructured even in the presence of membranes. Alternative SNCA splicing gives rise to three major isoforms (alpha-synuclein 140, alpha-synuclein 126, and alpha-synuclein 112). Both short transcripts are the result of in-frame deletions lacking the intermediate isoform exon 3 [7], and the smaller isoform exon 5 [38]. Specific functional changes can be predicted as a result of these deletions [2, 4, 5]. Alpha-synuclein 126 shows interruption of the N-terminal protein-membrane interaction domain, and therefore a less aggregation prone protein is to be expected. In contrast, enhanced aggregation properties can be expected for alpha-synuclein 112, an isoform with a significant shortening of the unstructured C-terminal (supplementary data include the schematically representation of alpha-synuclein).

Differential roles of alpha-synuclein isoforms in pDLB pathogenesis are suggested by three facts: first, LB formation is preceded by accumulation of a specific alpha-synuclein isoform [19]; second, membrane-bound alpha-synuclein is able to form insoluble fibrils while the free cytosolic form is not [20]; and third, pDLB brains show alpha-synuclein 112 overexpression [5], in addition to alpha-synuclein 126 downregulation [4]. A further proof of the involvement of SNCA overexpression in LB formation was provided by the development of α -synuclein immunoreactive inclusions in transgenic mice [11, 25].

In the present study, we describe a new alpha-synuclein isoform lacking the sequences corresponding to exons 3 and 5. This isoform would result in a 98 amino acid protein and therefore should be named alpha-synuclein 98 or SNCA98 when referring to its mRNA. We have also determined SNCA98 expression levels in non-neuronal

tissues as well as in different areas of the fetal and adult brains. Finally, we have studied relative SNCA98 expression levels in the frontal cortices of LBD brains in comparison with those of AD and controls.

Materials and methods

Source of tissues

Frontal cortex postmortem samples were facilitated by the Institute of Neuropathology brain bank and University of Barcelona/Hospital Clinic brain bank, Barcelona, Spain. They had been obtained from six patients with pDLB, eight patients with cLBD, five patients with PD, and eight patients with AD. Whereas DLB brains showed LBs and no evidence of either neurofibrillary tangles (NFTs) or neuritic plaques (NPs), AD patients presented numerous NFTs and NPs and lacked LBs. LBs, NPs, and NFTs were present in cLBD brains. Complete characterization and pathological details are provided as [Electronic Supplementary Material](#).

As for controls, frozen postmortem prefrontal cortex samples from 13 volunteers devoid of neurological signs or symptoms and with negative neuropathological examinations were used (see “[Electronic Supplementary Material](#)”). In 4 of the 13 cases, one sample from the temporal cortex, frontal cortex, caudate nucleus, and cerebellum were also studied. Tissue was facilitated by the University of Barcelona Neurological Tissue Bank (two prefrontal cortex samples), the Bellvitge Brain Bank (four prefrontal cortex samples, and all non-prefrontal cortex samples), and the Department of Pathology, Hospital Germans Trias i Pujol (seven prefrontal cortex samples). Brains had been donated by volunteers following the guidelines of the local ethics committees.

Also included in the study was a tissue from two fetal brains (frontal and parietal cortex in each case) and two samples of each of the following adult organs: skin, lung, kidney, spleen, heart, liver, and muscle. All were obtained from the Tumor Bank of the Germans Trias i Pujol University Hospital in agreement with the requirements of the local ethics committees.

RNA isolation and reverse transcription

TRI-reagent (MRC, Cincinnati, USA) was used for RNA isolation according to the manufacturer's protocol. Briefly, 100 mg tissue samples were homogenized in a 1.5-ml tube with a sterile piston in 1.0 ml of TRI-reagent. Homogenates were incubated 5 min at room temperature and then centrifuged at 12,000 \times g for 10 min at 4°C to pellet insoluble material and high-molecular-weight DNA. After phase separation, RNA was precipitated with isopropanol

and resuspended in an appropriate volume of DEPC-treated water. RNA quantity was determined spectrophotometrically at A260, RNA purity was ascertained from optical density ratio at 260 and 280 nm and RNA integrity was checked by electrophoresis on an agarose gel. The samples were stored at -80°C until use.

First-strand cDNA synthesis was carried out using Ready-to-go™ You-Prime First-Strand Beads (Amersham Pharmacia Biotech, Uppsala, Sweden). For alpha-synuclein isoform detection, 3 μg of RNA were incubated at 37°C during 1 h with 40 pmol of the primer SNCA 6L (Table 1) and the First-Strand Beads. For relative expression analyses, 2 μg of RNA were incubated with random hexamers and the First-Strand Beads at 37°C during 1 h. In both cases, the resulting cDNA was either immediately used for PCR or stored at -20°C until use.

Specific SNCA98 amplification by PCR

Primers were designed for specific detection of SNCA 98. The forward primer was constituted by sequences of exons 2 and 4 (SNCA2/4U), and the reverse primer contained sequences of exons 4 and 6 (SNCA4/6L; Table 1). Their sequences were checked against the GenBank database to ensure lack of cross reactivity with other known human sequences. A 40-cycle PCR program with an annealing temperature of 50°C was carried out for standard reactions containing 10 pmol of each primer in a final volume of 15 μl .

PCR, gel band extraction, and sequencing

To achieve the coamplification of the four SNCA isoforms, PCR primers with very different efficiencies and high amounts of cDNA (5 μl of the RT reaction carried out with primer SNCA6L1) were used. Two PCR reactions with the

same exon 2 forward primer but different reverse primers (SNCA6L1 and SNCA6L3) were designed (Table 1). Both SNCA6L1 and SNCA6L3 were based on exon 6 sequences, but their amplification efficiency at 56°C annealing temperature differed substantially. To assure these differences, PCR amplification of various cDNA amounts (0.5, 1.0, 2.0, 4.0, and 8.0 μl) was carried out for both primer pairs. Amplification of SNCA140, the most abundant SNCA transcript, was inhibited using high cDNA amounts and the PCR efficient primer SNCA6L1. In these conditions, lesser amounts of transcripts (including SNCA98) could be amplified. In contrast, the less efficient primer SNCA6L3 amplified greater amounts of SNCA140 and allowed additional detection of SNCA112.

To verify the identity of the various PCR products, PCR fragments were separated on 2% agarose gels and extracted by the use of the NucleoSpinExtractII Band Extraction Kit (Macherey Nagel, Düren, Germany).

PCR products extracted from gels, as well as those amplified with isoform specific primers, were submitted to sequencing with the aid of the BigDye Terminator vs 1.1 Cycle Sequencing Kit (Perkin Elmer, Boston, USA) and the ABI PRISM 310 system (Perkin Elmer).

Real time PCR

Beta-actin served as housekeeping gene for the adjustment of relative expression data and the QuantumRNA™ β -actin Internal Standards with Competimer technology was used to solve the problem posed by the low SNCA98 expression levels (Ambion, Boston, USA) [4, 14]. Real-time PCR reactions were carried out on a LightCycler System (Roche Applied Science, Mannheim, Germany) with the aid of the FastStart DNA Master SYBR Green I kit (Roche Applied Science). Reaction mixes had a final volume of 10 μl and contained 3 mM MgCl_2 , 10 pmol of each primer, and 0.5 μl of cDNA obtained during reverse transcription. A standard LightCycler program set of 29 cycles and 50°C as annealing temperature was used. All assays were performed twice and independently to assure their reproducibility and minimize possible errors and a negative control was included in each run.

Non-neuronal tissue and brain area distribution of SNCA 98 mRNA

A standard PCR program with annealing temperature of 50°C and 40 cycles was carried out for samples with a final volume of 15 μl . Reaction mixes contained, in addition to 10 pmol of each isoform specific primer (SNCA-2/4U and SNCA-4/6L), 3 mM MgCl_2 and 1 μl of cDNA obtained during reverse transcription (Table 1). Additionally, the presence of SNCA140, SNCA126, and SNCA112 was also analyzed in

Table 1 Primer sequences used for SNCA isoform PCR amplification

Primer name ^{""}	Primer sequence (5'-3')	PCR use
SNCA-2U	GGGTGTTCTCT ATGTAGGC	SNCA140
SNCA-6L1	GAGCACTTGTA CAGGATGG	SNCA140, 126, and 112; high efficient sequencing
SNCA-6L2	GGATGGAACAT CTGTCAGC	low efficient
SNCA-6L3	AGGCTTCAGGT TCGTAGTC	low efficient
SNCA-2/4U	CTCTATGTAGTG GCTGAGAA	SNCA126 and 98
SNCA-4/6U	GTTGGGCAAGG AAGGGTAT	SNCA112
SNCA-4/6L	ATACCCTTCCTT GCCCTTC	SNCA98

each sample. Table 1 includes primer sequences used for the amplification of these isoforms (SNCA140: 2U + 6L; SNCA126: 2/4U + 6L; SNCA112: 4/6U + 6L). To assure that possible differences were the result of different mRNA amounts of the SNCA isoforms, a beta-actin fragment was amplified for all samples. The annealing temperature was 50°C for SNCA126 amplification and 56°C for SNCA140, SNCA112, and beta-actin amplification.

Alpha-synuclein 98 protein sequence analysis

To predict the protein sequence of all alpha-synuclein isoforms, the program “Translator” (JustBio: <http://www.justbio.com>) was used. Afterwards, obtained sequences were aligned by the “Aligner” program from the same web site.

Statistical analysis

Melting peak areas were obtained on the LightCycler instrument using polynomial analysis with background corrections. Relative expression levels for total SNCA and SNCA isoforms were obtained as ratios between the respective RNA melting peak area and the β -actin: competitor melting peak area. All data are shown as mean+SEM values. Analyses of variance (ANOVA) were used to evaluate differences among means. If ANOVA showed significant differences, pair-wise comparisons between means were tested by Tukey B post-hoc test. Null hypothesis was rejected at the 0.05 level.

Results

Identification of the new alpha-synuclein isoform SNCA98

The main hypothesis of the present study was the existence of an additional alpha-synuclein transcript variant. Because SNCA126 and SNCA112 are alpha-synuclein isoforms that

present in-frame deletions as a result of the splicing out of exon 3 or exon 5, respectively, the fourth isoform would be the shortest, lacking exons 3 and 5. With the aim of detecting this transcript, we designed specific primers. The forward primer contained sequences of exons 2 and 4 (SNCA2/4U) and the reverse primer sequences of exons 4 and 6 (SNCA4/6L). We obtained a 163-bp fragment (Fig. 1a) and direct sequencing of this PCR product confirmed deletions of both exons 3 and 5. As the known SNCA transcripts are named according to the amino acid content of the expected protein, this novel isoform should be called SNCA98.

Then we tried to coamplify the different SNCA mRNAs. Whereas the use of cDNA obtained with random primers resulted in the amplification of SNCA140 only, SNCA specific cDNA amplified with high efficiency PCR primers allowed the detection of all SNCA transcripts. Figure 1b shows the coamplification of the four SNCA isoforms. When high-efficient primers were used in addition to high cDNA concentrations, the whole transcript (SNCA140) was very poorly amplified, but the SNCA126 as well as the SNCA98 fragments were detected. In this PCR reaction, the fragment corresponding to SNCA112 was identified in pDLB samples only (Fig. 1b). Direct sequencing confirmed that the smallest fragment corresponded to SNCA98 (Fig. 1c).

SNCA140 was found to be the most abundant alpha-synuclein transcript, followed by SNCA112 and SNCA126. It is interesting to note that after coamplification, SNCA112 was detected in pDLB samples only, a result in complete concordance with our earlier finding of SNCA112 overexpression in this disease. As shown by isoform coamplification and comparative brain area expression, SNCA98 was present in the frontal cortex only at low levels.

SNCA 98 expression in non-neuronal tissue

After its identification, we analyzed SNCA98 expression in non-neuronal tissue to find out whether SNCA98 is a brain

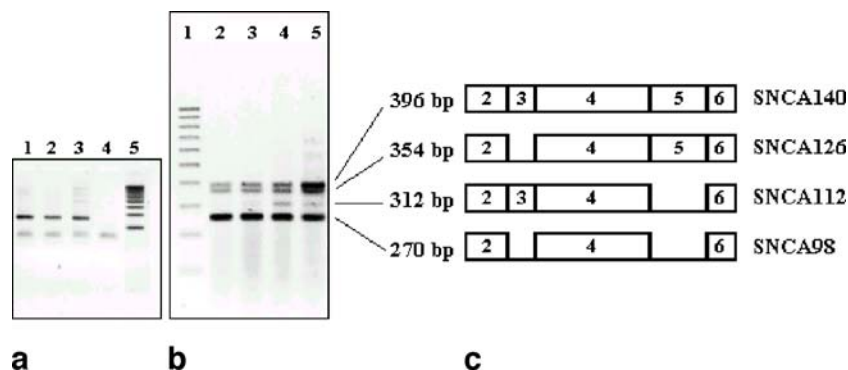


Fig. 1 Identification of SNCA 98. **a** SNCA98 amplification by the use of isoform-specific primers. Lanes 1, 2, and 3: a single band of 163 bp is observed in control brain (lane 1) and pDLB brains (lanes 2 and 3); lane 4 blank, lane 5 100-bp ladder. **b** Co-amplification of the four

SNCA isoforms. Whereas SNCA112 was not amplified in control brains (lanes 2 and 3), all four fragments were amplified in pDLB brains (lanes 4 and 5). Lane 1 contains a 100-bp ladder. Fragment length is indicated on the right. **c** Graphical representation of the SNCA isoforms

specific isoform. SNCA98 expression levels were compared with those of SNCA140, SNCA126 and SNCA112, as well as beta-actin as a housekeeping gene. As expected, the brain expressed all four SNCA isoforms (Fig. 2). SNCA140 was found in all skin, lung, kidney, spleen, heart, liver, and muscle. Although SNCA140 was present at high amounts, all skin, lung, kidney, spleen, heart, liver, and muscle samples, its brain expression levels were even higher. As for SNCA126, intermediate levels were present in the lung, kidney, spleen, heart, and muscle, but this isoform could not be detected in the skin or liver (Fig. 2). SNCA112 was expressed at very low levels in the skin, lung, kidney, heart, and muscle, but it could not be detected in the spleen or liver (Fig. 2).

In contrast to all other SNCA isoforms, SNCA98 was detected in the brain only (Fig. 2) and therefore seems to be a brain-specific SNCA isoform.

SNCA 98 expression in different brain areas

We tried to determine whether there are significant SNCA98 expression differences among various brain areas and whether SNCA98 is present in the fetal brain. SNCA140, SNCA126, SNCA112, and beta-actin expression was analyzed in the same tissue samples. Figure 3 shows that SNCA140 and beta-actin show similar high expression levels in all brain areas studied (frontal cortex, temporal cortex, parietal cortex, caudate nucleus, and cerebellum) and in the fetal brain (frontal cortex and parietal cortex). SNCA126 and SNCA112 expression levels were lower in

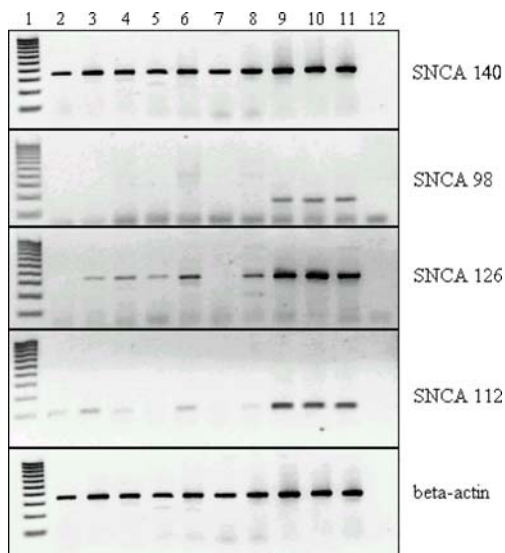


Fig. 2 SNCA isoform (SNCA 140, SNCA 98, SNCA 126, and SNCA 112) expression in non-neuronal tissue, with beta-actin expression as housekeeping gene. Lane 1 100-bp ladder as molecular weight marker; lane 2 skin; lane 3 lung; lane 4 kidney; lane 5 spleen; lane 6 heart; lane 7 liver; lane 8 muscle; lane 9 control brain; lane 10 AD brain; lane 11 DLB brain; and lane 12 blank

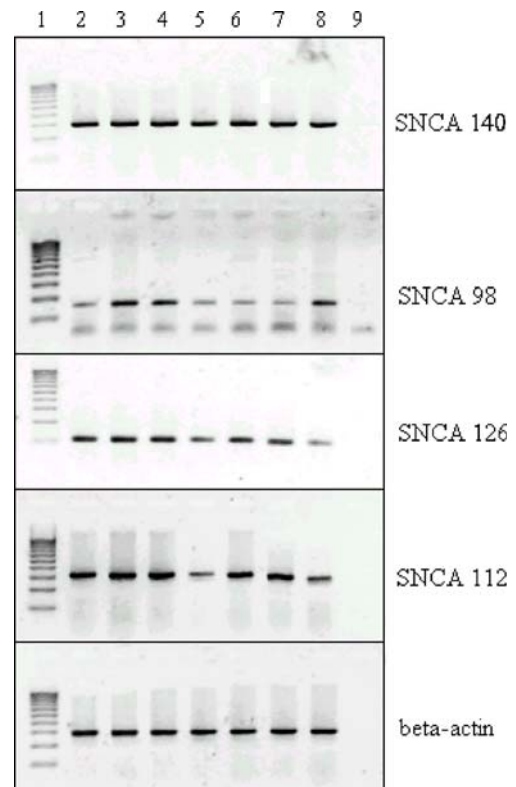


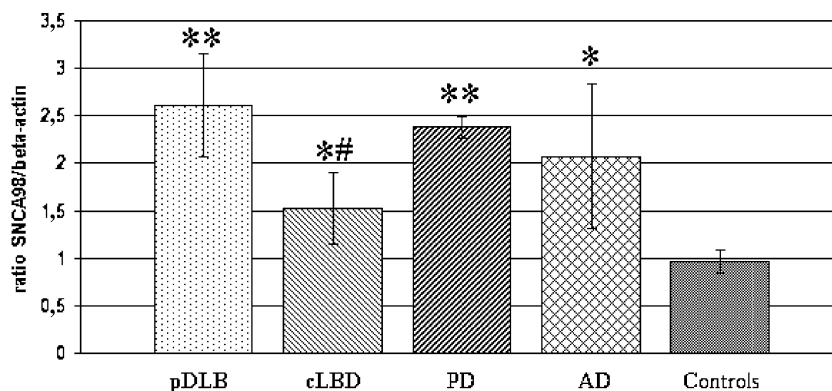
Fig. 3 SNCA isoform (SNCA 140, SNCA 98, SNCA 126, and SNCA 112) expression in different areas of adult and fetal brains, with beta-actin expression as housekeeping gene. Lane 1 100-bp ladder as molecular weight marker; lane 2 frontal cortex; lane 3 temporal cortex; lane 4 parietal cortex; lane 5 caudate nucleus; lane 6 cerebellum (all from adult brain); lane 7 frontal cortex; lane 8 parietal cortex (both from fetal brain); and lane 9 blank

the adult caudate nucleus and the fetal parietal cortex. It is interesting to note that SNCA98 showed marked expression differences in the various brain areas. Thus, whereas its expression levels were high in the adult temporal and parietal cortices and the fetal parietal cortex, only low SNCA98 amounts could be detected in all other brain areas studied (Fig. 3).

SNCA 98 expression levels in frontal cortices of LB diseases and AD

Because we have reported altered differential expression of both SNCA112 and SNCA126 in pDLB, cLBD, and AD [4, 5], in this work we have investigated SNCA98 expression in LBDs in comparison with AD and controls. It is interesting to note that both LBDs and AD showed important differences in SNCA98 brain expression in comparison with controls (Fig. 4). SNCA98 expression was markedly increased in pDLB and PD when compared with controls (2.61 ± 0.45 in pDLB, 2.38 ± 0.11 in PD vs 0.96 ± 0.12 in controls; $p < 0.001$). AD frontal cortices showed also elevated SNCA98 expression, although with a marked standard deviation between samples (2.07 ± 0.76

Fig. 4 Relative SNCA 98 expression levels in LBDs, AD and controls. Expression levels are represented as ratios between the SNCA 98 melting peak area and the beta-actin: competitor melting peak area ($*p<0.05$, $**p<0.001$), *comparison between disease and control samples, # comparison between pDLB and cLBD samples



vs 0.96 ± 0.12 in controls; $p=0.013$). Finally, cLBD cases showed only slightly increased SNCA98 expression when compared with controls (1.52 ± 0.38 vs 0.96 ± 0.12 in controls; $p=0.038$). Whereas differences in SNCA98 expression among pDLB, PD and AD were not significant, cLBD showed significantly lower SNCA98 expression levels in comparison with pDLB (1.52 ± 0.38 in cLBD vs 2.61 ± 0.45 in pDLB, $p=0.032$).

SNCA98 expression levels in relation to polyT length on intron 2

Recently, we have characterized a polyT sequence of variable length on SNCA gene intron 2 [3]. This polyT sequence consists of 5T, 7T, or 12T, and it is located in the vicinity of exon 3, excluded from SNCA126 by alternative splicing. Moreover, we have shown that the polyT length correlates with SNCA126 expression levels. Whereas the 7T/7T genotype was associated with intermediate SNCA126 expression levels, allele 5T carriers showed diminished and 12T allele carriers increased SNCA126 expression [3]. In the case of SNCA98, exon 3 is spliced out also from the pre-mRNA. Therefore, we analyzed SNCA98 expression levels in relation to polyT length. Results showed similar SNCA98 expression levels in all three 7T/7T genotype, 5T allele and 12T allele carriers (0.99 ± 0.06 , 0.97 ± 0.08 , and 0.84 ± 0.03 , respectively; Fig. 5), suggesting that SNCA98 expression is independent of polyT length.

The protein: α -synuclein 98 sequence analysis

Protein sequence alteration resulting from alternative splicing was analyzed with a translator program offered by the JustBio webpage. Results showed that α -synuclein 98 is characterised by in-frame deletions of the amino acid sequences corresponding to SNCA gene exons 3 and 5 (Fig. 6). No premature stop-codon is introduced, so that the production of a C-terminal truncated protein could be excluded. Further alignment analysis permitted to compare

alpha-synuclein 98 with the whole protein. Figure 6 shows that this isoform would be characterized by changes different from those of alpha-synuclein 126 and alpha-synuclein 112. On the one hand, the four-helical protein-membrane interacting domain is interrupted by the deletion of most of helix 3 and part of helix 4. The 60 amino acid C-terminal found in alpha-synuclein 140 would have only 46 amino acids in the case of alpha-synuclein 98. On the other hand, an important shortening of the unstructured N-terminal would take place. Instead of 45 amino acids, only 17 amino acids remain within the substantially shortened C-terminal, so that the resulting protein would consist almost only of the central region containing NAC (residues 61–96).

Discussion

In the present study, we have identified a new alpha-synuclein isoform, SNCA98. This splice variant is the smallest of all alpha-synuclein isoforms known so far. Moreover, SNCA98 shows the lowest expression levels when compared with the other alpha-synuclein isoforms. SNCA98 was found to be a brain specific isoform, present with varying amounts in different adult as well as fetal brain areas. This finding is in full agreement with the fact that alternative splicing, although observed in all tissues, is

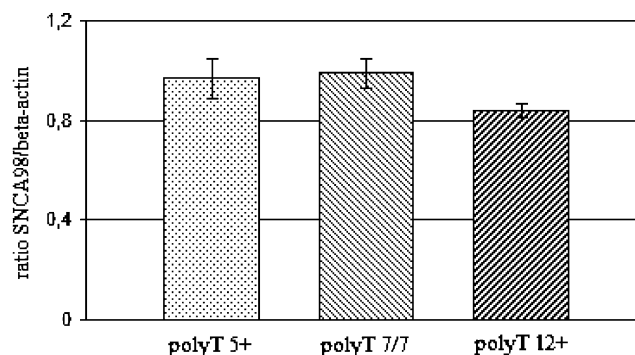


Fig. 5 PolyT length-dependent relative SNCA 98 expression levels in frontal cortices of control brains. Genotypes to which correspond the different expression levels are indicated below each bar

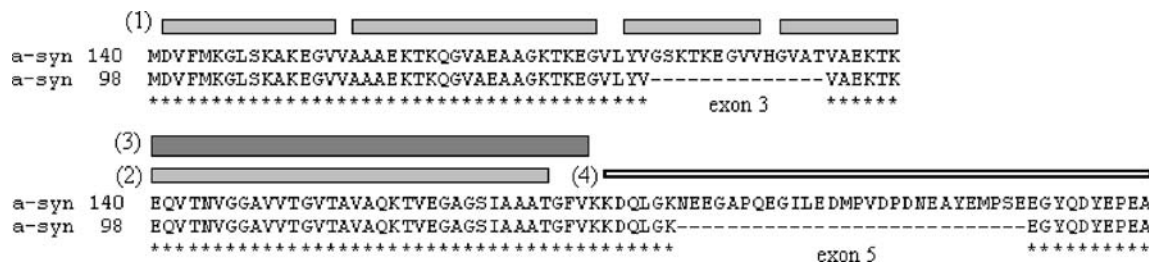


Fig. 6 Sequence alignment of alpha-synuclein 140 and alpha-synuclein 98. (1) Protein-membrane interacting domain (four alpha-helices); (2) fifth alpha helix (protein-protein interacting domain); (3) central fragment

NAC; and (4) unorganized C-terminal. Alpha-synuclein 98 is characterized by in-frame deletions of exon 3 (interruption of the protein-membrane interacting domain) and exon 5 (C-terminal shortening)

most prevalent in brain cells [36, 42]. About 75% of all human genes are alternatively spliced, with an average of two to three transcripts by gene [17]. Gene regulation through alternative splicing is more versatile than is regulation through promoter activity, because it can alter the structure of the gene product by inserting or deleting protein parts [35]. In fact, approximately 75% of alternative splicing events occur in the translated region of mRNAs, affecting the protein-coding region. The scale of structural changes evoked can range from a complete loss to a very subtle modification of function [28, 43].

In the last few years, an increasing number of genes involved in neurodegenerative disorders have been shown to express more than one splice variant differentially. This is so for the presenilin (PS) genes (PS1 and PS2); both of which undergo alternative splicing. Of the PS1 isoforms, one of them lacks exon 9 and the other two variably incorporate four amino acids (VRSQ) on exon 4 [29]. Recently, it has been shown that the four amino acid VRSQ-lacking isoform binds to the RabGDP dissociation inhibitor (rabGDI), whereas the VRSQ-containing isoform does not, a finding that is the first evidence of a specific function for the different isoforms [30]. On the other hand, one of PS2 transcript variants lacks exon 5 and results in a short, truncated protein. This isoform is differentially overexpressed in AD and, therefore, it has been proposed that it contributes to neurodegeneration [32]. Another alternatively spliced gene is tau, of which six isoforms resulting from complex splicing events are expressed in the adult brain [10, 22, 23]. The inclusion of exon 10, which codes for an additional microtubule-binding domain, causes frontotemporal dementia with parkinsonism [13]. Finally, it has been recently shown that differential expression of parkin and synphilin transcript variants is altered in LBDs [14, 21].

Transcribed alpha-synuclein 98 would be characterized by two deletions, one of 14 amino acids at the N-terminal and the other of 28 amino acids at the C-terminal. Alpha-synuclein 98 would then be characterized by a shortened C-terminal and only the central part of the protein would remain intact. As in the case of alpha-synuclein 112, deletion of exon 5 would result in an important shortening

of the alpha-synuclein C-terminal and, as in the case of alpha-synuclein 126, deletion of exon 3 would lead to the interruption of the membrane protein-binding domain. It is interesting to note that alpha-synuclein 112 is an aggregation-prone protein [2, 5] and alpha-synuclein 126 shows diminished aggregation properties [4], and alpha-synuclein 98 seems to combine structural changes of both. It may then be surmised that alpha-synuclein 98 would be unable to bind to membranes, an event necessary before alpha-synuclein aggregation [44].

At this point, it is interesting to mention that before alpha-synuclein was detected as the major LB component, its highly hydrophobic central region comprising residues 61 to 95 was isolated from AD senile plaques and named precursor protein of non-A-beta component of AD amyloid [37]. As shown in Fig. 6, alpha-synuclein 98 would preserve mainly this probably nonfunctional N-terminal fragment of the protein, flanked by a 46 amino acid, and a 17 (instead of 45) amino acid C-terminal. The remaining central part would correspond to the protein-protein interaction domain (Fig. 6) and, therefore, it seems reasonable to propose that alpha-synuclein 98 could be a protein with increased amyloidogenic properties, able to either enhance protein aggregation or strength existing aggregation nuclei. Because alpha-synuclein 98 expression is specific to the brain, a highly specific function is to be expected for this isoform. Overexpression of SNCA98 in LBDs, as well as in AD, is an additional fact in support of this hypothesis.

Recently, we have shown that the length of a variable polyT sequence located on intron 2 in the vicinity of exon 3 directly influences SNCA 126 expression levels in the normal brain [3]. Contrariwise, the polyT length does not modulate SNCA98 expression levels. These findings strongly indicate that different alternative splicing mechanisms are responsible for the generation of SNCA126 and SNCA98. The brain-specific regulation of splicing, as by Nova proteins [39], could be responsible for the generation of SNCA98.

Based on the results of the present work and two earlier studies of ours [4, 5], it can be claimed that alteration of alpha-synuclein isoform expression plays a key role in the

pathogenesis of LBDs and AD. It is important to note that the same samples, and often even the same cDNAs, have been used in all our studies, so that results are directly comparable.

In addition to the SNCA98 overexpression observed, SNCA126 expression was shown to be drastically down-regulated in the present study. It is interesting to note that both SNCA126 and SNCA98, in contrast to SNCA112, were expressed at relative low levels. Nevertheless, a shift of their normal expression levels seems to cause important changes within the cell. It has been proposed that alpha-synuclein 126, with diminished aggregation properties, could be necessary for normal brain functioning during normal aging [3, 4]. Alpha-synuclein 126 diminution, in addition to the over-representation of alpha-synuclein 98, could result in interaction with more proteins than necessary and disable those for their normal function.

In addition to SNCA126 down-regulation and SNCA98 up-regulation, we have previously reported specific SNCA112 overexpression in pDLB [5]. In contrast, cLBD frontal cortices share drastic SNCA126 down-regulation, but show only slightly high SNCA98 levels in conjunction with unaltered SNCA112 expression. These findings suggest that differential expression of alpha-synuclein isoforms could be primarily involved in LBD pathogenesis.

Very thorough studies are being performed on the structure and function of alpha-synuclein, particularly on its aggregation properties and fibril formation capacity [24, 33]. Nevertheless, the vast majority of these studies are carried out on the whole protein (alpha-synuclein 140). Most antibodies recognize alpha-synuclein 140 only and the low levels of the other isoforms would not allow their co-detection by the use of only this antibody. Consequently, there is a growing need for the design and production of isoform-specific antibodies.

In summary, SNCA98 is a new, brain-specific alpha-synuclein isoform that is overexpressed in the frontal cortices of LBD and AD brains. This finding provides further evidence of brain-specific splicing and the involvement of differential isoform expression changes in the pathogenesis of LBDs. Although minimally altered expression of only one isoform would be insufficient to produce a significant cell imbalance, the combined effect of several overregulated or downregulated isoforms would lead to the substantial protein–protein interaction changes underlying neurodegeneration.

Acknowledgments We thank the University of Barcelona and Institute of Neuropathology Brain Banks, Barcelona, Spain, for their help with tissue procurement. This work was supported by Spain's Ministry of Health FIS grants PI 030132 and PI 050867, Catalonia's AGAUR grant 2005SGR828, and MaratóTV3 grant 06/1410.

References

- Andringa G, Lam KY, Chegary M, Wang X, Chase TN, Bennett MC (2004) Tissue transglutaminase catalyzes the formation of alpha-synuclein crosslinks in Parkinson's disease. *FASEB J* 18:932–934
- Beyer K (2006) Alpha-synuclein structure, posttranslational modification and alternative splicing as aggregation enhancers. *Acta Neuropathol (Berl)* 112:237–251
- Beyer K, Humbert J, Ferrer A, Lao JI, Latorre P, Lopez D, Tolosa E, Ferrer I, Ariza A (2007) A variable poly-T sequence modulates alpha-synuclein isoform expression and is associated with aging. *J Neurosci Res* 85:1538–1546
- Beyer K, Humbert J, Ferrer A, Lao JI, Carrato C, López D, Ferrer I, Ariza A (2006) Low a-synuclein 126 mRNA levels in dementia with Lewy bodies and Alzheimer disease. *NeuroReport* 17:1327–1330
- Beyer K, Lao JI, Carrato C, Mate JL, Lopez D, Ferrer I, Ariza A (2004) Differential expression of alpha-synuclein isoforms in dementia with Lewy bodies. *Neuropathol Appl Neurobiol* 30:601–607
- Cabin DE, Shimazu K, Murphy D, Cole NB, Gottschalk W, McIlwain KL, Orrison B, Chen A, Ellis CE, Paylor R, Lu B, Nussbaum RL (2002) Synaptic vesicle depletion correlates with attenuated synaptic responses to prolonged repetitive stimulation in mice lacking alpha-synuclein. *J Neurosci* 22:8797–8807
- Campion D, Martin C, Heilig R, Charbonnier F, Moreau V, Flaman JM, Petit JL, Hannequin D, Brice A, Frebourg T (1995) The NACP/synuclein gene: chromosomal assignment and screening for alterations in Alzheimer disease. *Genomics* 26:254–257
- Dalfo E, Barrachina M, Rosa JL, Ambrosio S, Ferrer I (2004) Abnormal alpha-synuclein interactions with rab3a and rabphilin in diffuse Lewy body disease. *Neurobiol Dis* 16:92–97
- Davidson WS, Jonas A, Clayton DF, George JM (1998) Stabilization of alpha-synuclein secondary structure upon binding to synthetic membranes. *J Biol Chem* 273:9443–9449
- Ferrer I, Gomez-Isla T, Puig B, Freixes M, Ribe E, Dalfo E, Avila J (2005) Current advances on different kinases involved in tau phosphorylation, and implications in Alzheimer's disease and tauopathies. *Curr Alzheimer Res* 2:3–18
- Fleming SM, Salcedo J, Fernagut PO, Rockenstein E, Masliah E, Levine MS, Chesselet MF (2004) Early and progressive sensorimotor anomalies in mice overexpressing wild-type human alpha-synuclein. *J Neurosci* 24:9434–9440
- Forno LS (1996) Neuropathology of Parkinson's disease. *J Neuropathol Exp Neurol* 55:259–272
- Gao QS, Memmott J, Lafyatis R, Stamm S, Sreaton G, Andreadis A (2000) Complex regulation of tau exon 10, whose missplicing causes frontotemporal dementia. *J Neurochem* 74:490–500
- Humbert J, Beyer K, Carrato C, Mate JL, Ferrer I, Ariza A (2007) Parkin and synphilin-1 isoform expression changes in Lewy body diseases. *Neurobiol Dis* 26:681–687
- Iwai A, Masliah E, Yoshimoto M, Ge N, Flanagan L, de Silva HA, Kittel A, Saitoh T (1995) The precursor protein of non-A beta component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system. *Neuron* 14:467–475
- Jao CC, Der-Sarkissian A, Chen J, Langen R (2004) Structure of membrane-bound alpha-synuclein studied by site-directed spin labeling. *Proc Natl Acad Sci U S A* 101:8331–8336
- Johnson JM, Castle J, Garrett-Engele P, Kan Z, Loerch PM, Armour CD, Santos R, Schadt EE, Stoughton R, Shoemaker DD (2003) Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. *Science* 302:2141–2144
- Kosaka K (1978) Lewy bodies in cerebral cortex, report of three cases. *Acta Neuropathol (Berl)* 42:127–134

19. Kuusisto E, Parkkinen L, Alafuzoff I (2003) Morphogenesis of Lewy bodies: dissimilar incorporation of alpha-synuclein, ubiquitin, and p62. *J Neuropathol Exp Neurol* 62:1241–1253
20. Lee HJ, Choi C, Lee SJ (2002) Membrane-bound alpha-synuclein has a high aggregation propensity and the ability to seed the aggregation of the cytosolic form. *J Biol Chem* 277:671–678
21. Liani E, Eyal A, Avraham E, Shemer R, Szargel R, Berg D, Bornemann A, Riess O, Ross CA, Rott R, Engelender S (2004) Ubiquitylation of synphilin-1 and alpha-synuclein by SIAH and its presence in cellular inclusions and Lewy bodies imply a role in Parkinson's disease. *Proc Natl Acad Sci U S A* 101:5500–5505
22. Luo MH, Leski ML, Andreadis A (2004) Tau isoforms which contain the domain encoded by exon 6 and their role in neurite elongation. *J Cell Biochem* 91:880–895
23. Luo MH, Tse SW, Memmott J, Andreadis A (2004) Novel isoforms of tau that lack the microtubule-binding domain. *J Neurochem* 90:340–351
24. Marsh JA, Singh VK, Jia Z, Forman-Kay JD (2006) Sensitivity of secondary structure propensities to sequence differences between alpha- and gamma-synuclein: implications for fibrillation. *Protein Sci* 15:2795–2804
25. Mukaetova-Ladinska EB, McKeith IG (2006) Pathophysiology of synuclein aggregation in Lewy body disease. *Mech Ageing Dev* 127:188–202
26. Murphy DD, Rueter SM, Trojanowski JQ, Lee VM (2000) Synucleins are developmentally expressed, and alpha-synuclein regulates the size of the presynaptic vesicular pool in primary hippocampal neurons. *J Neurosci* 20:3214–3220
27. Norris EH, Giasson BI, Lee VM (2004) Alpha-synuclein: normal function and role in neurodegenerative diseases. *Curr Top Dev Biol* 60:17–54
28. Okazaki Y, Furuno M, Kasukawa T, Adachi J, Bono H, Kondo S, Nikaido I, Osato N, Saito R, Suzuki H, Yamanaka I, Kiyosawa H, Yagi K, Tomaru Y, Hasegawa Y, Nogami A, Schonbach C, Gojobori T, Baldarelli R, Hill DP, Bult C, Hume DA, Quackenbush J, Schriml LM, Kanapin A, Matsuda H, Batalov S, Beisel KW, Blake JA, Bradt D, Brusic V, Chothia C, Corbani LE, Cousins S, Dalla E, Dragani TA, Fletcher CF, Forrest A, Frazer KS, Gaasterland T, Gariboldi M, Gissi C, Godzik A, Gough J, Grimmond S, Gustincich S, Hirokawa N, Jackson JJ, Jarvis ED, Kanai A, Kawaji H, Kawasawa Y, Kedziński RM, King BL, Konagaya A, Kurochkin IV, Lee Y, Lenhard B, Lyons PA, Maglott DR, Maltais L, Marchionni L, McKenzie L, Miki H, Nagashima T, Numata K, Okido T, Pavan WJ, Pertea G, Pesole G, Petrovsky N, Pillai R, Pontius JU, Qi D, Ramachandran S, Ravasi T, Reed JC, Reed DJ, Reid J, Ring BZ, Ringwald M, Sandelin A, Schneider C, Semple CA, Setou M, Shimada K, Sultana R, Takenaka Y, Taylor MS, Teasdale RD, Tomita M, Verardo R, Wagner L, Wahlestedt C, Wang Y, Watanabe Y, Wells C, Wilming LG, Wynshaw-Boris A, Yanagisawa M et al (2002) Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. *Nature* 420:563–573
29. Rogaev EI, Sherrington R, Wu C, Levesque G, Liang Y, Rogaeva EA, Ikeda M, Holman K, Lin C, Lukiw WJ, de Jong PJ, Fraser PE, Rommens JM, St George-Hyslop P (1997) Analysis of the 5' sequence, genomic structure, and alternative splicing of the presenilin-1 gene (PSEN1) associated with early onset Alzheimer disease. *Genomics* 40:415–424
30. Scheper W, Zwart R, Baas F (2004) Alternative splicing in the N-terminus of Alzheimer's presenilin 1. *Neurogenetics* 5:223–227
31. Shults CW (2006) Lewy bodies. *Proc Natl Acad Sci U S A* 103:1661–1668
32. Smith MJ, Sharples RA, Evin G, McLean CA, Dean B, Pavay G, Fantino E, Cotton RG, Imaizumi K, Masters CL, Cappai R, Culvenor JG (2004) Expression of truncated presenilin 2 splice variant in Alzheimer's disease, bipolar disorder, and schizophrenia brain cortex. *Brain Res Mol Brain Res* 127:128–135
33. Sode K, Ochiai S, Kobayashi N, Usuzaka E (2007) Effect of repair of repeat sequences in the human alpha-synuclein on fibrillation ability. *Int J Biol Sci* 3:1–7
34. Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M (1998) alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. *Proc Natl Acad Sci U S A* 95:6469–6473
35. Stamm S, Ben-Ari S, Rafalska I, Tang Y, Zhang Z, Toiber D, Thanaraj TA, Soreq H (2005) Function of alternative splicing. *Gene* 344:1–20
36. Stamm S, Zhu J, Nakai K, Stoilov P, Stoss O, Zhang MQ (2000) An alternative-exon database and its statistical analysis. *DNA Cell Biol* 19:739–756
37. Ueda K, Fukushima H, Masliah E, Xia Y, Iwai A, Yoshimoto M, Otero DA, Kondo J, Ihara Y, Saitoh T (1993) Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease. *Proc Natl Acad Sci U S A* 90:11282–11286
38. Ueda K, Saitoh T, Mori H (1994) Tissue-dependent alternative splicing of mRNA for NACP, the precursor of non-A beta component of Alzheimer's disease amyloid. *Biochem Biophys Res Commun* 205:1366–1372
39. Ule J, Ule A, Spencer J, Williams A, Hu JS, Cline M, Wang H, Clark T, Fraser C, Ruggiu M, Zeeberg BR, Kane D, Weinstein JN, Blume J, Darnell RB (2005) Nova regulates brain-specific splicing to shape the synapse. *Nat Genet* 37:844–852
40. Vekrellis K, Rideout HJ, Stefanis L (2004) Neurobiology of alpha-synuclein. *Mol Neurobiol* 30:1–21
41. Weinreb PH, Zhen W, Poon AW, Conway KA, Lansbury PT, Jr. (1996) NACP, a protein implicated in Alzheimer's disease and learning, is natively unfolded. *Biochemistry* 35:13709–13715
42. Xu Q, Modrek B, Lee C (2002) Genome-wide detection of tissue-specific alternative splicing in the human transcriptome. *Nucleic Acids Res* 30:3754–3766
43. Zavolan M, Kondo S, Schonbach C, Adachi J, Hume DA, Hayashizaki Y, Gaasterland T (2003) Impact of alternative initiation, splicing, and termination on the diversity of the mRNA transcripts encoded by the mouse transcriptome. *Genome Res* 13:1290–1300
44. Zhu M, Li J, Fink AL (2003) The association of alpha-synuclein with membranes affects bilayer structure, stability, and fibril formation. *J Biol Chem* 278:40186–40197