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β -Synuclein occurs in vivo in lipid associated oligomers and forms hetero oligomers with α -synuclein

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Abstract

α -synuclein (α S) and β -synuclein (β S) are homologous proteins implicated in Parkinson's disease and the related synucleinopathies. While α S is neurotoxic and its aggregation and deposition in Lewy bodies is related to neurodegeneration, β S is considered a potent inhibitor of α S aggregation and toxicity. No mechanism for the neuroprotective role of β S has been described before. Here we report that similar to α S, β S normally occurs in lipid-associated, soluble oligomers in wt mouse brains. We partially purified β S and α S proteins from whole mouse brain by size exclusion followed by ion exchange chromatography and found highly similar elution profiles. Using this technique, we were able to partially separate β S from α S and further separate β S monomer from its own oligomers. Importantly, we show that although α S and β S share high degree of similarities, β S oligomerization is not affected by increasing cellular levels of PolyUnsaturated Fatty Acids (PUFA), while α S oligomerization is dramatically enhanced by PUFA. We show the in vivo occurrence of hetero oligomers of α S and β S and suggest that β S expression inhibits PUFA-enhanced α S oligomerization by forming hetero-oligomers up to a quatramer that do not further propagate.

Keywords

alpha synuclein; beta synuclein; protein oligomerization and aggregation; polyUnsaturated Fatty Acids (PUFA)

Introduction

The synuclein family of proteins consists of α -Synuclein (α S), β -Synuclein (β S) and γ -Synuclein (γ S), cytoplasmic proteins expressed primarily in neurons. While α S and β S are co-localized in presynaptic nerve terminals of the central nervous system (Maroteaux et al. 1988), γ S is expressed in the peripheral nervous system (reviewed in (Clayton and George 1998)). All three members of the synuclein family have in their N-terminal region a repetitive, highly conserved α -helical binding motif that is similar to the class-A2 lipid-binding domains of the exchangeable apolipoproteins, which mediates binding to membrane phospholipids (George 2002).

Synucleinopathies are a group of neurodegenerative diseases including Parkinson's disease (PD), Dementia with Lewy Bodies (DLB) and Multiple system atrophy (MSA) (reviewed in (Duda et al. 2000)). Abnormal α S cytoplasmic aggregation and accumulation in Lewy bodies and Lewy neurites has been implicated as the key pathogenic event in synucleinopathies.

Nevertheless, growing evidence point to a neuroprotective role for its homolog β S in synucleinopathies.

The findings that β S protein inhibit α S aggregation and fibril formation in vitro (Hashimoto et al. 2001; Uversky et al. 2002; Park and Lansbury 2003) and furthermore, that β S expression reduced α S aggregation, Lewy body formation and toxicity in vivo in mouse brains bigenic for α S and β S (Hashimoto et al. 2001; Fan et al. 2006), have suggested a protective role for β S in PD and the related synucleinopathies. Two mutations in β S i.e., V70M and P123H, were then found in association with DLB (Ohtake et al. 2004). Over expression of the mutated β S proteins in B103 neuroblastoma cells, resulted in the formation of β S-immunoreactive, eosinophilic cytoplasmic inclusions and enhanced lysosomal pathology (Wei et al. 2007). In this regard, β S accumulations were documented in some synucleinopathies (Galvin et al. 1999). β S is considered non-amyloidogenic (Masliah and Hashimoto 2002), natively unfolded protein (Bertoncini et al. 2007). Nevertheless, it was recently shown that certain factors such as metals and pesticides cause its rapid fibrillation (Yamin et al. 2005).

The physiological role of all synucleins and β S in particular is unknown. It was suggested that β S expression induce cell protectivity. β S was shown to act as an anti apoptotic agent (da Costa et al. 2003) and induce cell protection via increased AKT activity (Hashimoto et al. 2004). Alternatively, purified β S was shown to restore proteosomal activity inhibited by α S (Snyder et al. 2005).

We have recently reported that α S normally occurs in lipids-associated oligomers (Sharon et al. 2001; Sharon et al. 2003b) and that elevated cellular levels of polyunsaturated Fatty Acids (PUFA) induce α S oligomerization, aggregation and its deposition in Lewy bodies (Sharon et al. 2003b; Assayag et al. 2007). We now report that, similar to α S, β S occurs in lipid-associated oligomers in the cytoplasm. However, unlike α S, β S oligomerization is not affected by the cellular fatty acids (FA) content. We further show the in vivo occurrence of hetero-oligomers consisting of α S and β S and suggest that, β S expression inhibits PUFA-induced α S oligomerization through the formation of hetero-oligomers that do not further propagate.

Materials and Methods

Mice

The wt C57Bl/6J (Jackson Laboratories, Maine, USA) and α S null mice C57Bl/6J (Specht and Schoepfer 2001) (Harlan Laboratories, Rehovot, Israel) mouse lines were used. Holding and breeding was carried out at the Specific Pathogen Free (SPF) animal facility. All protocols for animal use and experiments were reviewed and approved by the committee for animal care and use of the Hebrew University.

Frozen brains of β S transgenic mice and their wt controls (C57BL/6 x DBA/2) (Hashimoto et al. 2001) were generously provided by Prof. Eliezer Masliah (University of California).

Whole mouse brain fractionation was as previously described (Sharon et al. 2001). Briefly, whole mouse brains were homogenized in 1:10 (wt/vol) homogenization buffer [20 mM Hepes, pH 7.4/1 mM MgCl₂/ 0.32 M sucrose/ 43 mM 2-mercaptoethanol/ 1X protease inhibitor mix (Sigma)]. The homogenate was centrifuged at 170 xg for 10 min. The resultant pellet (P1) was washed and respun at 370 xg for 15 min. The washed pellet was resuspended in homogenization buffer, and the suspension was brought to 2.1 M sucrose and spun at 175,000 xg for 1 h. The pellet (consisting of nuclei) and the lipid-rich fraction floating to the top of this sucrose cushion were collected. S1 supernatant was centrifuged at 8,000 x g for 15 min to obtain P2 and S2. S2 supernatant was spun at 180,000 xg. Protein concentrations were assayed by the Bradford method (Bradford 1976).

Chloroform:methanol (2:1) extraction (Folch et al. 1957), of high speed supernatant (post 180,000 g) was performed as previously described (Sharon et al. 2001), with β S present at the interface between organic and aqueous phases. For Hexane extraction, one volume of high speed supernatant (20 μ g protein) and three volumes of Hexane (100%) were mixed and rotated for one hour and then centrifuged at 2,500xg for 10 min to separate phases. β S was present at the organic phase. The organic solvent was dried under stream of N_2 and the sample was re-suspended in 1x Laemmli buffer and analyzed by 12% SDS-PAGE or 10% native-PAGE.

Cells

The mesencephalic cell lines MES 23.5 and MN9D which have dopaminergic properties (Choi et al. 1991; Crawford et al. 1992) were stably transfected with human wt β S cDNA [provided by Prof. George GM, University of Illinois] in the pCDNA 3.1. Alternatively, cells were viral transduced with Lentivirus expressing either α S or β S or with both. For the expression of either protein, α S or β S, we transduced with 25,000 PFUs of α S virus and additional 25,000 PFUs of a GFP expressing virus. For the co-transduction of α S together with β S, each virus was transduced at 25,000 PFUs, altogether, 50,000 PFUs per 1×10^7 cells. Thirty two hours post transductions, the conditioning medium was replaced and cells were incubated for 16 hours with or without FAs as previously described (Sharon et al. 2003b). Cells were then (48 hours post transduction) collected and processed for analyses.

Conditioning living cells with FAs was as previously described (Sharon et al. 2001; Sharon et al. 2003b). Briefly, Subconfluent (75%) cultures of MES cells expressing human wt α S or β S or both were cultured in DMEM containing the essential nutrients for MES cells (Crawford et al., 1992) but without serum. BSA/FA complexes were added to the medium just before applying it to the cultures. These complexes were prepared by mixing BSA (FA-free; Sigma) with the indicated FA (at a molar ratio of 1:5) in binding buffer (10 mM Tris HCl [pH 8.0], 150 mM NaCl) at 37 C for 30 min. Sister cultures, used as controls, were incubated in parallel with BSA but without FA. Cells were then collected and high speed supernatant (post 180,000 xg) was prepared as above.

Oligomers detection and Western blotting: Protein samples of high speed supernatant (post 180,000 g) were incubated at 65°C for 16-18 hours (Sharon et al. 2003b) prior to loading on an 8-16% NuPAGE Bis-Tris (Invitrogen) or 12% SDS-PAGE. For native electrophoresis we used the same buffers, omitting SDS and β -mercaptoethanol throughout. Immunoblots reacted with anti β S antibodies: monoclonal anti β S (612508, BD transduction laboratories) 1:5000; polyclonal anti β S (AB5086, Chemicon, CA USA) 1:1000; monoclonal anti β S (328500, Zymed) 1:1000. For α S immuno-detection monoclonal Syn1 (BD transduction laboratories) 1:1000; and monoclonal LB509 (18-0215, Zymed - Invitrogen) 1:1000. Immuno blots normalized with actin or tubulin levels on the same blots.

Chromatography

Partial purification of α S and β S from wt and α S null mouse brains was performed as described (Lee et al. 2002) with slight modifications. Briefly, 5mg of post 180,000g supernatant in physiological buffer (Sharon et al. 2001) were loaded on size exclusion column (Sephacryl S-100 16 / 60 cm (Amersham); The column was equilibrated with Tris-Cl 20 mM, pH 7.5 and 1ml fractions were collected at a rate of 0.2 ml/minute. The fractions were tested for the presence of α S and β S by western blot and the positive fractions were combined and further purified on anion exchange column (DEAE 1ml column, Amersham; equilibration buffer: Tris-Cl 20mM, pH 7.5; elution at 0.1-0.4 M NaCl at 1ml/min). The elution profiles of α S and β S to the specific fractions were determined by western blotting reacted with specific anti α S or β S antibodies.

Immunoprecipitation (IP)

Samples of semi purified α S and β S high speed cytosols of whole wt or α S null mouse brains subjected to purification by Sephacril-100 SEC (fractions 56-95-pooled) followed by DEAE fractionation (fractions 154-168) were used. Samples containing 200 μ g protein in 1 ml IP buffer (Tris-Cl 20 mM pH 7.4; 1 $\times$ protease inhibitor mix (Sigma); sodium fluoride 20 mM; PMSF 1 mM; EDTA 1 mM, EGTA 1 mM], were incubated at 65°C for 16 hours. Next, 0.5 μ g anti α S antibody (Syn1, Transduction Labs, Lexington, KY) or anti β S antibody (Chemicon), were added to the sample and rotated for two hours at room temperature. Protein A/G PLUS-Agarose beads (Santa Cruz, CA, USA), were pre-incubated in 10 μ g rat liver protein extract for 16-18 hours at 4°C to block non-specific binding. The beads were then washed twice in Tris-Cl 20 mM pH 7.4. Binding was allowed for four hours at room temperature and the A/G protein beads were washed in IP buffer containing 0.05% SDS (wash 1) and 0.025% Tween 20 (wash 2). The proteins bound to the beads were eluted in Laemmli buffer and boiled for 10 minutes at 100 °C. The eluent was loaded on a 12% SDS-PAGE and analyzed by western blotting using anti α S or anti β S antibodies in a reciprocal order to the IP.

Results

β -Synuclein fractionation in whole mouse brain

Due to the high homology between β S and α S (reviewed in (Clayton and George 1998; George 2002)) and potential interactions between the two proteins, we initially used α S $-/-$ mouse brains to study β S protein. Whole α S $-/-$ mouse brain was fractionated (see methods) and the distribution of β S in the sub-cellular fractions was determined by quantitative western blotting with anti β S antibodies (Chemicon (polyclonal) and Zymed (monoclonal) with similar results). Approximately 55% of the total β S was found cytosolic, i.e., was detected in the high speed supernatant (post 180,000g). A low portion of β S, ~5%, was found in plasma membrane enriched fraction and only traces of β S (~0.15%) in the high speed membrane fraction. The remaining ~40% was detected in the lipid-rich fractions. No β S immunoreactivity was detected in the nuclei (Figure 1a).

β S normally occurs in lipid-associated high molecular weight forms

We next analyzed the soluble fraction of whole wt, α S $-/-$ and β S $+/+$ mouse brains by non-denaturing gel electrophoresis followed by western blotting and probed with an anti β S ab (see methods). Using these native conditions we detected various β S-immunoreactive forms. A monomer β S band migrating slightly lower than monomer α S band; higher MW β S-immunoreactive bands detected in β S tg and α S $-/-$ but not in wt mouse brain, and lower MW bands (lower than the main β S monomer) detected in the β S tg mouse brain, representing potential truncated β S forms (Figure 1b). To test whether similar to α S, β S occurs as lipid-associated high MW forms, we next applied the methods used for α S oligomer detection, i.e., delipidation with organic solvents or heat treatment at 65 °C (Sharon et al. 2001; Sharon et al. 2003b; Sharon et al. 2003a; Assayag et al. 2007). Heat delipidation at 65°C of the soluble fraction of α S $-/-$ mouse brain dramatically enhanced the detection of various β S immunoreactive forms on a western blot probed with four different specific anti β S antibodies (see methods), with indistinguishable result (Figure 1c). The immunoreactivity appeared throughout the lane as a smear representing various β S species, with several main bands that according to their calculated MW may represent β S oligomers. Specifically, the monomer β S migrating at ~19 kDa, the putative dimer at ~39 kDa, trimer at ~51 kDa and tetramer at ~77 kDa. Quantifying β S signal detected by the western blot indicated ~ two fold increase in β S monomer amount with the 65°C treatment and a dramatic increase in high MW β S forms. This immunoreactive profile was β S-specific, no immuno reactivity was detected with syn-1 anti α S antibody in the α S $-/-$ mouse brain (not shown).

Enhanced detection of β S monomer and high MW forms was also observed upon extraction in organic solvents such as chloroform/methanol (2:1) (Folch et al. 1957) or Hexane. β S monomer and high MW forms were detected in the interface between the organic and the aqueous phases in chloroform/methanol extraction and in the organic phase in Hexane extraction on an SDS-PAGE (Figure 1c). Further, extraction in Hexane affected the mobility of β S monomer from wt, α S^{-/-} and β S^{+/+} mouse brains on a native gel appearing slightly higher after the Hexane extraction (Figure 1d). Interestingly, in contrast to its effect on α S oligomers detection, incubating the soluble fraction with the fatty acid binding resin Lipidex-1000, did not affect the detection of β S high MW forms on a western blot (not shown). We next detected higher β S levels and higher oligomers to monomer ratio in β S tg (Hashimoto et al. 2001) than in wt mouse brains (Figure 1e, f). Together, the results suggest that similar to α S, β S occurs in vivo in soluble assemblies that may represent β S oligomers and their detection is dramatically improved upon applying certain methods for delipidation.

β S oligomers co-purify with α S oligomers

In an attempt to further characterize β S oligomers in comparison with α S oligomers, we performed size exclusion chromatography (SEC) of the high speed soluble fraction (post 180,000g) of wt and α S^{-/-} fresh mouse brains (for α S and β S vs. β S alone in wt and α S^{-/-} respectively), under non-denaturing conditions i.e., physiologic buffers. Using a Sephacril S-100 column we obtained a linear elution profile of standard proteins with Mr of ~6-77 kDa (Figure 2a and b). The elution profiles of α S and β S extracted from the cytosol of wt mouse brains were similar to one another. Specifically, α S and β S eluted to the same pick (fractions 56-95), recurrently appearing as a double pick (Figure 2c). The elution profile of β S from wt vs. α S^{-/-} brains was identical (not shown), indicating that α S expression does not affect the elution profile of β S. We next analyzed the eluted fractions for the presence of monomer and oligomers of β S on native gel without delipidation treatments. Using these native conditions we detected β S monomer alone at pick S1 and β S monomer together with oligomeric forms at pick S2 (Figure 2d). The monomer β S at pick S1 appeared slightly higher than the monomer at pick S2. We next applied the 65 ° C treatment followed by SDS PAGE electrophoresis to enhance the detection of β S and found similar results, i.e., β S monomer alone was detected at pick S1, while β S monomer and oligomers were detected at the S2 pick. The parallel profile of α S indicated the elution of α S monomer and oligomers to the same fraction, in agreement with previous purification attempts (Sharon et al. 2003b). Yet, lower amounts of α S oligomers are present at pick S1 than pick S2 (Figure 2e). Therefore, although α S and β S eluted to the same fractions, the elution pattern was different, enabling the separation of β S (but not α S) monomer (S1) from its oligomeric forms (S2).

We next pooled fractions 56-95 (the entire fractions of picks S1 and S2) of wt mouse brains and subjected them to DEAE anion exchange chromatography (Figure 3a). Overall, four picks were eluted from the DEAE column and both α S and β S were eluted to the third pick (fractions 154-168, pick SD3). Importantly, β S, but not α S, was detected at the forth pick, i.e., fractions 168-180 (SD4), therefore, the DEAE column enabled the separation of β S from α S. Treating samples of the SD3 and SD4 picks eluted from DEAE column for oligomers detection, again revealed the elution of monomers and oligomers to the same fractions (Figure 3b). Similar elution profile for β S was obtained in α S^{-/-} mouse brain. In conclusion, α S and β S share a highly similar elution profile on a Sephacril S-100 and DEAE columns. However, some differences in elution profiles enables the separation of β S monomer from its oligomers and from α S various forms.

18:3 α -linolenic acid induce oligomerization of purified β S in vitro

The similarities between α S and β S in chromatography (see above) and the finding that their expression levels are associated, led us to ask whether similar to α S, β S oligomerization is

affected by fatty acids (FA) (Sharon et al. 2003b). Purified recombinant human β S protein (2 μ M) were incubated at 37°C with increasing concentrations of 18:3 α Linolenic acid (ALA) for one hour and an aliquot was analyzed by SDS-PAGE (Figure 4a) followed by immunoblot probed with anti β S ab. A β S monomeric band was detected in the absence of FA. However, in the presence of 10 μ M of ALA, oligomerization of pure β S was promoted enabling the detection of β S dimer and trimer. Higher ALA concentrations of 25 and 50 μ M affected pure β S oligomerization to the same extent as the low 10 μ M concentration and 100 μ M ALA further enhanced β S oligomerization. Importantly, no heat treatment or organic solvents were applied to detect the purified β S oligomers. Incubation of pure β S with ALA not only enhanced oligomerization but also increased the total immunoreactivity, without affecting the monomer β S levels (Figure 4a). The similarities in mobility on SDS-PAGE gels of purified recombinant and mouse endogenous β S, reinforced the conclusion that β S high MW forms are oligomers.

PUFAs do not affect β S oligomerization in living mesencephalic neurons

We next tested whether altered cellular FAs content affects β S oligomerization as previously done for α S (Sharon et al. 2003b). For this aim, we have conditioned naïve, β S or α S over expressing MES (stable) cells under standard serum-supplemented conditioning medium. Cells were then transferred to serum-free medium supplemented with BSA only (at 50 μ M) or with BSA and FA (at 50 and 250 μ M for BSA and FA respectively) for sixteen hours and then harvested and fractionated. Samples of high speed supernatant were processed for β S and α S oligomer detection using 65°C treatment and SDS-PAGE western blotting. In accord with our previous observations concerning FA-induced α S oligomerization (Sharon et al. 2003b; Assayag et al. 2007), conditioning α S over expressing MES cells in DMEM supplemented with MonoUnsaturated (MU)FA 18:1 had no detectable effect on α S oligomerization, however, induced α S oligomerization was detected with PUFAs 18:2; 18:3 or 20:4. Interestingly, none of the FAs tested affected β S oligomerization in β S over expressing cells cultured and treated in parallel (Figure 4b). Specifically, we tested the effects of 18:0 Saturated (S)FA (not shown), 18:1 (MUFA) and 18:2, 18:3 and 20:4 (PUFA) with no detectable effect on β S oligomerization (Figure 4b). We therefore conclude that while purified β S oligomerization is enhanced by 18:3 PUFA in vitro (Fig. 4a), altered cellular PUFA levels do not affect β S oligomerization in living mesencephalic cells.

β S expression inhibits PUFA-induced α S oligomerization in living mesencephalic cells

Following upon the recent findings that β S expression inhibits α S oligomerization and aggregation (Hashimoto et al. 2001; Uversky et al. 2002; Park and Lansbury 2003), we now asked whether β S expression affects PUFA-induced α S oligomerization. For this aim, we transduced naïve MN9D cells either with an α S or β S expressing viral vectors or both at 1:1 PFU ratio (see methods). Following transduction, we replaced the conditioning medium and treated the cells in serum-free medium, supplemented with BSA with or without 18:3 PUFA (at 50 and 250 μ M for BSA and 18:3 respectively) for 16 hours. Cells were collected 48 hours post transduction and high speed cytosols were processed for the detection of α S and β S oligomers by western blotting probed with anti α S and anti β S antibodies, respectively. Induced α S oligomerization was detected in α S-transduced cells treated with 18:3 PUFA, with two-fold increase in oligomers to monomer ratio. Note that 18:3 treatment enhanced the levels of both, α S monomer and oligomers (Figure 5a). The PUFA-induced α S oligomerization was dramatically inhibited by β S expression (i.e., α S+ β S transduced cells), with ~85% inhibition (Figure 5b). Importantly, in cells transduced with α S + β S, the 18:3 treatment enhanced the formation of β S-immunoreactive, high MW species, that according to their molecular mass may represent dimer, trimer and tetramer. These high molecular mass β S-immunoreactive bands are detected in the α S + β S cells, but not in the β S only cells (Figure 5a). Therefore, these bands may represent the putative hetero-oligomers consisting on α S and β S.

β S occurs in hetero-oligomer with α S in vivo

We next sought to find potential hetero-oligomers composed of α S and β S in vivo, in wt mouse brains. The occurrence of such hetero-oligomers could underlie the mechanism by which β S inhibits α S oligomerization in general and PUFA-induced α S oligomerization in particular (Figures 4b and 5a). For this aim we used pooled fractions of the entire SD3 pick (see Figure 3 a and b) containing both α S and β S of wt mouse brain and the corresponding fractions from α S $-/-$ mouse brain and performed co-immunoprecipitation, using either anti α S or anti β S antibodies for the immunoprecipitation step and the reciprocal antibody for the western blotting. Three specific bands were detected on the western blot, migrating at ~36, ~51 and ~70 kDa in wt but not in the corresponding fractions from α S $-/-$ mouse brain. The molecular weight of these bands suggests that these bands represents hetero-dimer, hetero-trimer and hetero-tetramer composed of α S and β S. The three specific bands were detected either with anti α S ab or anti β S ab for immunoprecipitation. Importantly, a portion of α S and β S protein is non-specifically attached to the agarose beads (lanes 5, 6 and 11, Figure 6). This may explain the presence of a β S monomer in α S $-/-$ mouse brain IP'ed with α S ab (lane 4, Figure 6). We therefore conclude that β S normally forms hetero-oligomers with α S in vivo.

Discussion

Using biochemical methods for fractionation in whole wt, α S $-/-$ and β S $+/+$ mouse brains we detected various species of β S that according to their molecular mass and similarities to α S oligomers, may represent β S oligomers. The β S oligomers are enriched in the high speed supernatant (post 180,000g) of mouse brains and can be detected using physiological buffers and none-denaturing gel electrophoresis followed by western blotting. However, the immunodetection of the various β S species, including monomer and oligomers, is dramatically enhanced by heat treatment or extraction in organic solvents such as chloroform /Methanol or Hexane, indicating that β S oligomers may associate with lipids. The detection of mouse endogenous β S as well as human transgenic β S in the forms of oligomers in mouse brains suggests that β S normally occurs in vivo in soluble, lipid-associated oligomers.

The purification attempts using Sephacril-100 SEC followed by DEAE ion exchange chromatography resulted in highly similar elution profiles for α S and β S proteins. Both proteins co-eluted to the same fractions on the Sephacril-100 column and the following DEAE column, with the exception of pick "SD4" of the DEAE column elution profile, where β S but not α S is detected. This result further emphasizes the high degree of similarities between the two proteins, not only at the level of sequence homologies but also at the levels of biochemical and biophysical properties. Importantly, a critical difference between the elution profiles of α S and β S was found. That is the ability to separate between the different β S forms, i.e., monomer and oligomers. Specifically, only β S monomer was detected at the S1 fractions while β S monomer +oligomers were detected at S2 fractions. This finding is important for two main aspects: 1. The purification method herein provided a way by which β S monomer can be separated from its oligomers; and 2) The absence of β S oligomers in the S1 pick, after applying the protocol for enhanced oligomers detection using 65°C treatment, strongly argues against the claim that the treatments used to enhance the oligomers detection actually enhance artificial, in vitro oligomerization of the denatured protein.

In our recent publications we reported different aspects of α S interactions with FAs (Sharon et al. 2001; Sharon et al. 2003b; Sharon et al. 2003a; Assayag et al. 2007) and related α S interactions with PUFAs to PD pathogenesis. Specifically, we showed that within the physiologic range of concentrations, PUFAs but not MUFAs or SFAs induce α S oligomerization, aggregation and Lewy-like inclusion formation in dopaminergic cells (Sharon et al. 2001; Sharon et al. 2003b; Sharon et al. 2003a; Assayag et al. 2007). In accord, we detected higher long chain PUFA levels in PD and DLB brains than normal, age-matched controls

(Sharon et al. 2003b). These high PUFA levels correlated with the accumulation of α S soluble oligomers in the same brain regions. Based on these and additional results, we suggested that high PUFA levels in the brain may act as a risk factor, inducing α S cytotoxicity. In this study we asked whether similar to α S, β S responds to the presence of PUFA by induced oligomerization and aggregation. Based on the high degree of similarities between the two proteins and the finding that β S, like α S, normally occurs in lipid-associated soluble oligomers, we expected to find that β S is also oligomerizes in the presence of PUFA. The findings that β S does not oligomerizes in the presence of PUFAs or other FAs, may underlie the biochemical difference between the two proteins resulting in two highly similar proteins with opposing effects on PD and the related synucleinopathies.

We show that β S occurs in vivo in lipid-associated oligomers and that lipid extraction dramatically enhances the detection of β S oligomers. Yet, the lipid binding domain of β S differ from that of α S. The deletion of 11 residues in the β S sequence dramatically affects the helical structure of β S (Sung and Eliezer 2006). This may reduce the stability of lipid binding, including membrane phospholipids binding. For example, the missing 11 residues affects the degree of homology to the apolipoprotein A1 in the N' terminal region of the β S protein, resulting in five apolipoprotein A1 like repeats while six such repeats are present in the α S sequences (Clayton and George 1998). Further, the sequence differences between α S and β S results in a lower degree of similarities to the Fatty Acid Binding Protein (FABP) signature motif (Sharon et al. 2001). In this regard, the detection of β S in purified "heavy" myelin in mouse brains (Mor et al. 2003) and in Lipidex-1000 extracts of DLB brains (Salem et al. 2007) suggest that indeed β S interacts with lipids in vivo. Therefore, the differences in lipid binding domains in β S and α S sequence may indicate different affinities towards specific lipids and represent the core difference between α S and β S proteins.

In this regard, the finding that exposure to PUFAs enhances in vitro but not in vivo oligomerization of β S is worthy of note. This result highlights the potential of β S to nonspecifically react with lipid surface, represented by the fatty acid micelles and oligomerize upon this interaction. Yet, further studies are needed in order to identify the physiological lipid preferences of β S and the potential role of its post translational modifications in lipid interactions.

The occurrence of a hetero-dimer consisting of α S and β S was suggested before, based on molecular modeling and dynamic simulation. It was further suggested that β S occurs as a dimer that do not propagate. Using a cell free system, β S was shown to interfere with α S oligomerization (Tsigelny et al. 2007). The results presented herein support these predictions and observations. Specifically, reduced levels of PUFA-induced α S oligomerization were detected upon expression of β S at a 1:1 ratio with α S. While a 1:1 ratio is closer to the physiological mouse brain levels (R.S and E.I unpublished data), it is interesting to note that, a complete inhibition of α S fibrillation was previously reported at a 4:1 molar excess of β S (Uversky et al. 2002).

The formation of hetero-oligomers composed of α S and β S was enhanced upon exposure to PUFA. Based on the results herein, we suggested that β S inhibits PUFA-induced α S oligomerization by the formation of hetero-oligomers, up to quatramer that do not further propagate. It is interesting to note that hetero-oligomers composed of endogenous α S and β S proteins were also detected in wt mouse brains. Importantly, these hetero-oligomers were found in vivo, with endogenous α S and β S proteins and therefore suggest the presence of such hetero-oligomers is normal, healthy animal conditions. This finding may represent the normal function of β S that continually restrains α S oligomerization rate and therefore, its toxicity. However, since both α S and β S oligomers interact with lipids, this result may also suggest specific lipids preferences for the hetero-oligomers that may differ from α S or β S oligomers.

In light of the critical role of α S in neurodegeneration, we have tested the effect of β S, the intrinsic, physiological inhibitor of α S toxicity. Indeed, increasing number of evidences now suggests that β S interacts and regulates α S. The regulatory effect of β S on α S toxicity may result from the high degree of similarities between the two proteins. For example, the high sequence homologies and a nearly identical subcellular distribution including enrichment in presynaptic vesicles (reviewed in (Clayton and George 1999; George 2002)). Therefore, the two proteins may share similar, but not identical, biochemical properties and under certain conditions may compete for interacting molecules. Our discovery that similar to α S, β S normally occurs in soluble, lipid-associated oligomers highlights the potential role of lipids in PD and the related synucleinopathies and may have important implications towards our understanding of the physiological role of these highly similar proteins.

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Figure 1. β S occurs in lipid-associated high molecular mass forms in wt, α S $^{-/-}$ and β S $^{+/+}$ mouse brains

a. Endogenous β S distribution in α S $^{-/-}$ mouse brain. Whole α S $^{-/-}$ mouse brain was fractionated as in (Sharon et al. 2001) and subjected to quantitative Western blotting with anti β S antibody (Chemicon, CA USA). Results are presented as the ratio of the amounts of β S monomer (17 kDa) in each fraction to that of total β S monomer in the starting homogenate (100%) run in

parallel on each gel (means $\pm$ SD of three brains). **b.** mouse endogenous and human transgenic β S occurs in various MW species on native gel. Whole wt, α S^{-/-} and β S^{+/+} mouse brains were fractionated as in (a). Sample (15 μ g protein) of the high speed supernatant (post 180,000 g) was analyzed by 10% native PAGE. Western blot reacted with anti β S antibody (Zymed), in parallel, a sample of wt mouse brain, reacted with anti α S ab (syn-1). **c.** The detection of the various β S forms is enhanced by treatments that remove lipids. Sample (15 μ g protein of α S^{-/-} mouse brain) as in (b) were incubated at 65°C for 16 hours; extracted in chloroform/methanol (2:1) or in hexane (see methods) and analyzed by 12% SDS-PAGE. Western blot reacted with anti β S antibody. **d.** β S monomer electrophoresis is affected by hexane. Protein samples of high speed cytosols from whole wt, α S^{-/-} or β S^{+/+} mouse brains were extracted in hexane and analyzed by 10% native PAGE. A shift in the mobility of the β S monomer is detected upon hexane extraction. **e.** The relative amount of high MW β S forms is higher in β S^{+/+} mice than in control mice. High speed cytosols of whole β S^{+/+} (Hashimoto et al. 2001) and control mouse brains were treated at 65°C to enhance β S detection as in (b). **f.** Densimetric quantitation of western blot in (e), presented in arbitrary units, as the ratio of β S high MW forms to monomer.

Figure 2. The elution profiles of β S and α S is highly similar on Sephacril-100 column

(a) Elution pattern of a Sephacril-100 SEC column established using the indicated proteins as size markers. The column was eluted with Tris-Cl 20 mM, pH 7.5 at 0.2 ml/minute. Fractions containing protein (One milliliter) were collected according to the UV detector. Protein size markers: conalbumin 77 kDa, fractions 49-89; bovine serum albumin (BSA) 66 kDa, fractions 55-92; pepsin 34 kDa, fractions 77-113 and insulin 6 kDa, fractions 135-180; (b) The elution

pattern of the protein size markers was linear on a semi-log plot, indicating efficient column separation. SEC conditions and protein size markers as in (a). Graph presented as log MW to Kav. **c.** The elution profile of high speed supernatant (post 180,000g) of whole wt mouse brain. (5 mg protein starting sample). **d.** Fractions from (c) were loaded on native 10% PAGE without any further treatment and the PVDF blot was stained with anti β S ab (Chemicon). Note that the β S monomer appears as a dublet **e.** β S and α S reactivity of the equivalent SEC fractions as in (d), treated or untreated at 65°C before loading on 12% SDS-Page. Western blot reacted with anti β S antibody. Note, β S monomer alone is eluted at pick S1 and monomer+oligomers are eluted at S2.

Figure 3. β S is separated from α S on a DEAE column

a. The elution profile of the entire S1 and S2 picks (fractions 56-95) were loaded on a DEAE ion exchange column. **b.** Fractions of the SD3 and SD4 DEAE picks were treated at 65°C before loading on a 12% SDS-PAGE. Western blot reacted with anti β S antibody (chemicon) and anti α S antibody (Syn-1). Note, β S is eluted at both SD3 and SD4 picks, while α S is eluted at SD3 only.

Figure 4. Polyunsaturated FAs Induce the Oligomerization of human Wild-Type β S in vitro but not in living mesencephalic cells

a. 2.0 μ M of purified β S were incubated without or with α -linolenic acid (ALA, 18:3) at the indicated concentrations at 37 °C for one hour. Samples (75 ng protein) were Western blotted with H3C without any further treatment. Note that the presence of FA increased the level of β S detection. **b.** β S oligomerization is not affected by FA in living mesencephalic cells. High-speed cytosols (15 μ g protein of post 180,000g supernatant) of human β S stable MES cells conditioned in the presence of the indicated FA/BSA complexes (250/50 μ M) in serum-free medium. Samples were treated at 65°C overnight prior to gel loading and blotting with anti α S or β S antibodies. Sister cultures treated and processed in parallel. 18:1, oleic acid; 18:2, linoleic acid; 18:3, α -linolenic acid; 20:4, arachidonic acid.

Figure 5. β S inhibits PUFA-induced α S oligomers

a. High-speed cytosols (15 μ g protein) of MES cells transduced with either a lentiviral vector expressing α S, β S or both, conditioned and maintained in parallel, in the presence of BSA or BSA/18:3 complexes (250/50 μ M) in serum-free medium. Samples were treated at 65°C overnight prior to gel loading and blotting with anti α S or anti β S antibodies. **b.** Densitometric quantifying of α S signal detected at the blot on the left, presented as the ratio of α S oligomers to monomer. A representative blot out of n=3. Note, the β S high MW bands appearing at α S + β S and not in β S only transduced cells, these bands may represent hetero oligomers composed of α S and β S.

Figure 6. Hetero oligomers composed of α S and β S normally occur in wt mouse brain

Immunoprecipitation of 2 ml of the pooled fractions of the entire SD3 pick (see Figure 3 a and b) containing both α S and β S of wt mouse brain and the corresponding fractions from α S -/- mouse brain. The sample was heat treated at 65°C treated prior to loading on protein A/G PLUS-Agarose beads (Santa Cruz, CA, USA) that were pre-incubated in 10 μ g rat liver protein extract for 16-18 hours at 4°C to block non-specific binding. con= sample of high speed cytosol (15 μ g) of whole wt mouse brain treated at 65°C for oligomers detection; w/o (without) ab represents the non specific binding to the agarose beads; ab- the antibody used for the IP step loaded on the gel. > indicates specific bands detected with anti α S and anti β S antibodies representing hetero dimer, trimer and quatramer, respectively.