

# Synuclein Proteins of the Pufferfish *Fugu rubripes*: Sequences and Functional Characterization<sup>†</sup>

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**ABSTRACT:** In humans, three genes encode the related  $\alpha$ -,  $\beta$ -, and  $\gamma$ -synucleins, which function as lipid-binding proteins in vitro. They are being widely studied, mainly because of the central involvement of  $\alpha$ -synuclein in a number of neurodegenerative diseases, including Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy. In these diseases, the normally soluble  $\alpha$ -synuclein assembles into abnormal filaments. Here, we have identified and characterized the synuclein gene family from the pufferfish *Fugu rubripes*. It consists of four genes, which encode  $\alpha$ -,  $\beta$ -,  $\gamma 1$ -, and  $\gamma 2$ -synucleins. They range from 113 to 127 amino acids in length and share many of the characteristics of human synucleins, including the presence of imperfect amino-terminal repeats of 11 amino acids, a hydrophobic middle region, and a negatively charged carboxy-terminus. All four synucleins are expressed in the *Fugu* brain. Recombinant *Fugu* synucleins exhibited differential liposome binding, which was strongest for  $\alpha$ -synuclein, followed by  $\beta$ -,  $\gamma 2$ -, and  $\gamma 1$ -synucleins. In assembly experiments, *Fugu*  $\alpha$ -,  $\gamma 1$ -, and  $\gamma 2$ -synucleins formed filaments more readily than human  $\alpha$ -synuclein. *Fugu*  $\beta$ -synuclein, by contrast, failed to assemble in bulk. Filament assembly of synucleins was directly proportional to their degree of hydrophobicity and their tendency to form  $\beta$ -sheet structure, and correlated inversely with their net charge.

Synucleins are abundant nervous system proteins that are localized to presynaptic nerve terminals. In birds and mammals, three genes encode the related proteins  $\alpha$ -synuclein,  $\beta$ -synuclein, and  $\gamma$ -synuclein (1–8). The human proteins range from 127 to 140 amino acids in length and are 55–62% identical in amino acid sequence, with a similar domain organization. The amino-terminal region of each synuclein is taken up by imperfect repeats that partially overlap with a hydrophobic middle region with the carboxy-terminal region being negatively charged. Synucleins are natively unfolded proteins, with little or no ordered secondary structure, that bind to acidic phospholipids and micelles though their amino-terminal repeats, indicating that they are lipid-binding proteins (9–13). Upon binding to lipid membranes and detergent micelles, synucleins adopt structures rich in  $\alpha$ -helical character (10, 12–17).

$\alpha$ -Synuclein is being widely studied, because of its central role in a number of common movement and demential disorders (18). Missense mutations (A30P, E46K, and A53T) in human  $\alpha$ -synuclein and duplications of the region encompassing the  $\alpha$ -synuclein gene cause dominantly in-

herited forms of Parkinson's disease (PD)<sup>1</sup> and dementia with Lewy bodies (DLB) (19–22). Furthermore,  $\alpha$ -synuclein is the major component of the abnormal filamentous inclusions of Lewy bodies and Lewy neurites in idiopathic PD and DLB (23–25). The filamentous glial and neuronal inclusions of multiple system atrophy (MSA) are also made of  $\alpha$ -synuclein (26–28).

Recombinant mammalian  $\alpha$ -synuclein readily assembles into filaments that share many of the morphological and ultrastructural characteristics of the filaments present in the human brain (29–32). Upon assembly,  $\alpha$ -synuclein adopts structures rich in  $\beta$ -sheets (32, 33). Assembly is a nucleation-dependent process and occurs through sequences located in the amino-terminal 100 amino acids of  $\alpha$ -synuclein (34, 35). The carboxy-terminal region, in contrast, inhibits assembly (29, 32), probably through long-range interactions with part of the hydrophobic region (36, 37). Mutations E46K and A53T in  $\alpha$ -synuclein accelerate the rate of filament assembly (31, 38, 39). The A30P mutation has been reported to increase the total aggregation of  $\alpha$ -synuclein (40–42) but slow the rate of mature filament formation (43). It reduces the binding of  $\alpha$ -synuclein to natural lipid membranes (11, 44–46), suggesting that this may lead to its progressive

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<sup>1</sup> Abbreviations: CBB, Coomassie brilliant blue; DiO, 3,3'-diethyloxycarbocyanine; DLB, dementia with Lewy bodies; GST, glutathione S-transferase; MOPS, 3-[N-morpholino]propanesulfonic acid; MSA, multiple system atrophy; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PCR, polymerase chain reaction; PD, Parkinson's disease; PS, phosphatidylserine; SDS, sodium dodecyl sulfate; ThT, thioflavin T.

accumulation in the cytoplasm, thus facilitating aggregation and filament formation. Mutation E46K increases phospholipid binding (38), whereas mutation A53T has no significant effect on the ability of  $\alpha$ -synuclein to bind to lipid membranes (11, 44–47). In parallel experiments, human  $\beta$ - and  $\gamma$ -synucleins failed to assemble into filaments and remained in a natively unfolded conformation (32, 48, 49). This behavior is consistent with their absence from the filamentous lesions of the  $\alpha$ -synuclein diseases (23, 25). When incubated with  $\alpha$ -synuclein,  $\beta$ -, and  $\gamma$ -synucleins inhibited the fibrillation of  $\alpha$ -synuclein (50–52), suggesting that they may indirectly influence the pathogenesis of Lewy body diseases and MSA.

Little is known about the evolutionary and functional conservation of synucleins. They appear to be vertebrate-specific proteins, with no counterparts in *Caenorhabditis elegans*, *Drosophila melanogaster*, or *Ciona intestinalis* (8). Although the first synuclein sequence was obtained from the Pacific electric ray *Torpedo californica* (1), nothing is known about the number of teleost synucleins, their sequences, or functional characteristics. Here we have identified and characterized the complement of four synucleins from the pufferfish *Fugu rubripes*.

## EXPERIMENTAL PROCEDURES

**Isolation of cDNA Clones Encoding *Fugu* Synucleins.** The genomic sequence of *Fugu rubripes* (53) was searched with the human  $\alpha$ - and  $\beta$ -synuclein amino acid sequences using tblastn. RNA from *Fugu* brain cells was obtained from mrc geneservice (Babraham Institute, Cambridge, U.K.), and cDNA was produced with the Advantage RT kit (Clontech, Palo Alto, CA). Polymerase chain reaction (PCR) was used to amplify parts of the coding regions of *Fugu* synucleins from the brain cDNA. Following sequencing, these partial sequences were labeled by random priming and used to screen a gridded *Fugu* brain cDNA library on high-density filters (mrc geneservice). Positive clones in pBluescript II KS were obtained from mrc geneservice, and their inserts were sequenced. The entire coding region of each synuclein was then amplified by PCR from *Fugu* brain cDNA, followed by DNA sequencing.

**Identification and Comparison of Synuclein Gene Sequences.** Tblastn and blastn searches of all nucleotide sequences at NCBI (<http://www.ncbi.nlm.nih.gov>) were used to detect synuclein homologues. Synuclein transcript sequences were assembled into a Staden gap 4 database (54). Full-length coding sequences were derived from transcript sequences representing 52 genes from 21 different species. Five additional full-length sequences were predicted from the genomic sequences of five species. The amino acid sequences derived from these genes, together with those from *F. rubripes*, were then compared (55). See Supporting Information for amino acid sequences.

**Characterization of Synuclein Sequences.** To determine the ratio of nonhydrophilic to hydrophilic residues in synucleins, the hydrophilicity scale of Radzicka and Wolfenden was used (56). Amino acids less hydrophilic than threonine were considered to be nonhydrophilic. They included methionine, proline, phenylalanine, alanine, valine, isoleucine, leucine, glycine, and cysteine. The ratio of  $\beta$ -sheet favorable to nonfavorable residues was also calculated. Residues

selected as  $\beta$ -sheet favorable were those whose Chou and Fasman  $\beta$ -sheet preference scores were higher than either their  $\alpha$ -helix or  $\beta$ -turn numbers (57). They included valine, isoleucine, threonine, tyrosine, and phenylalanine. The net charge per residue was calculated by subtracting the number of aspartate and glutamate residues from the number of lysines and arginines, divided by the total number of amino acids in the protein.

**Expression and Purification of Recombinant *Fugu* Synucleins.** The constructs encoding *Fugu* synucleins were prepared by PCR amplification using full-length cDNA clones as the template. They were subcloned into the prokaryotic expression vector pRK172, as NdeI/EcoRI fragments, followed by DNA sequencing. For *Fugu*  $\beta$ -synuclein, the internal NdeI site was removed by site-directed mutagenesis, prior to PCR amplification. The *Fugu* synucleins and human  $\alpha$ -synuclein were expressed in *E. coli* BL21(DE3) and purified as described (4). Protein concentrations were determined by quantitative amino acid analysis. To assess heat stability, purified human  $\alpha$ -synuclein and the four *Fugu* synucleins were boiled for 5 min and spun for 20 min at 543 000g. Supernatants and pellets were run on SDS-PAGE and protein bands visualized with Coomassie brilliant blue (CBB).

For constructs encoding *N*-terminal glutathione *S*-transferase (GST)-fusion tags, PCR was used to amplify the coding region of each *Fugu* synuclein in pRK172, and the inserts were cloned into the EcoRI site of pGEX-5X-1 (Amersham Biosciences, Little Chalfont, U.K.). Constructs were verified by DNA sequencing. GST-tagged human  $\alpha$ -synuclein was expressed and purified in parallel. Bacterial expression and fusion protein purification were done as described (38), except that the purified proteins were dialyzed twice for 3 h against phosphate-buffered saline (PBS) at 4 °C, followed by overnight dialysis against PBS. Protein concentrations were determined using the Coomassie Plus reagent (Pierce, Rockford, IL) following the manufacturer's instructions.

**Tissue Extraction and Immunoblotting.** *Fugu* brain tissue (0.2 g) was homogenized in 0.4 mL tris-buffered saline (50 mM trisHCl, pH 7.4, 150 mM sodium chloride, 1 mM EDTA, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM  $\beta$ -glycerophosphate, and a cocktail of protease inhibitors (Complete, Roche, Penzberg, Germany)), followed by centrifugation at 543 000g for 20 min at 4 °C. Protein concentrations were determined using the BCA assay kit (Pierce, Rockford, IL). Aliquots of the supernatant were subjected to SDS-PAGE and immunoblotting as described (58). In some experiments, the supernatant was boiled for 5 min, followed by a 20 min centrifugation at 543 000g. The resulting supernatant was used as the heat stable fraction. Polyclonal antibodies PER8, PER9, and PER10 were produced by immunizing white Dutch rabbits with purified recombinant *Fugu*  $\alpha$ -synuclein,  $\gamma$ 1-synuclein, and  $\gamma$ 2-synuclein, respectively. Antibody PER4 has been described previously (25). Each antibody was used at a working dilution of 1:500.

**Preparation of Fluorescence-Labeled Liposomes.** Brain phosphatidylcholine (PC) and cholesterol were purchased from Avanti Polar Lipids (Birmingham, AL). Brain phosphatidylserine (PS) was obtained from Sigma-Aldrich. The fluorescent dye 3,3'-dioctadecyloxycarbocyanine perchlorate

Fu $\alpha$ Syn	MDAFMKCF <sup>3</sup> SKAKDGVVA <sup>4</sup> AAEKTQGV <sup>4</sup> TGA <sup>4</sup> EMTKDGV <sup>4</sup> MFVGT <sup>4</sup> TK <sup>4</sup> KG <sup>4</sup> VT <sup>4</sup>	50
Fu $\beta$ Syn	MDVFMKGLSKAKEGM <sup>4</sup> AVAAEKTKEGV <sup>4</sup> AAEKTKEGV <sup>4</sup> MFVGNK <sup>4</sup> AKDSV <sup>4</sup> GT	50
Fu $\gamma$ 1Syn	MDVFMKCF <sup>3</sup> SMAKEGVVA <sup>4</sup> AAEKTAGME <sup>4</sup> EAAKTKEGV <sup>4</sup> MYVGNKTME <sup>4</sup> GV <sup>4</sup> VS	50
Fu $\gamma$ 2Syn	MDVLKKCF <sup>3</sup> SMAKDGVVA <sup>4</sup> AAEKTAGVEGA <sup>4</sup> ATKTKEGV <sup>4</sup> IYVGNKTME <sup>4</sup> GV <sup>4</sup> VT	50
Fu $\alpha$ Syn	VA---GKT <sup>4</sup> VS <sup>4</sup> GV <sup>4</sup> SQVGGAM <sup>4</sup> VTG <sup>4</sup> VTAV <sup>4</sup> AQKT <sup>4</sup> VE <sup>4</sup> SAGSIA <sup>4</sup> AAATGLV <sup>4</sup> KKEPG	96
Fu $\beta$ Syn	VA---EKT <sup>4</sup> TG---AMGN <sup>4</sup> IVAATGLV <sup>4</sup> KKDEF	74
Fu $\gamma$ 1Syn	SVNTV <sup>4</sup> TNKTVD <sup>4</sup> QTNIVGDA <sup>4</sup> VVGANEV <sup>4</sup> SQAT <sup>4</sup> VEGVENMA <sup>4</sup> ASSGVLGQGEY	100
Fu $\gamma$ 2Syn	SVNTVAHKT <sup>4</sup> TEQANI <sup>4</sup> IADTAV <sup>4</sup> SGANEVA <sup>4</sup> QSAVEGV <sup>4</sup> ENAAVASGLV <sup>4</sup> SLEEA	100
Fu $\alpha$ Syn	KQGDDAAAPENMAESP <sup>4</sup> VDTPAEATEEDADD	127
Fu $\beta$ Syn	PTDMNP <sup>4</sup> E <sup>4</sup> Y <sup>4</sup> Q <sup>4</sup> EAMEGQGEAMLDPEGETY <sup>4</sup> ESQQESQDYEP <sup>4</sup> EA	117
Fu $\gamma$ 1Syn	GGTEQGGEGEGY	113
Fu $\gamma$ 2Syn	GPVSEKAGVPNT <sup>4</sup> EAEAESEQAVQ	124

FIGURE 1: Amino acid sequences of *Fugu* synucleins. Amino acids that are identical between two, three, and four synucleins are indicated by purple, yellow, and blue bars, respectively. Dashes denote gaps introduced into the sequences to maximize the alignment.

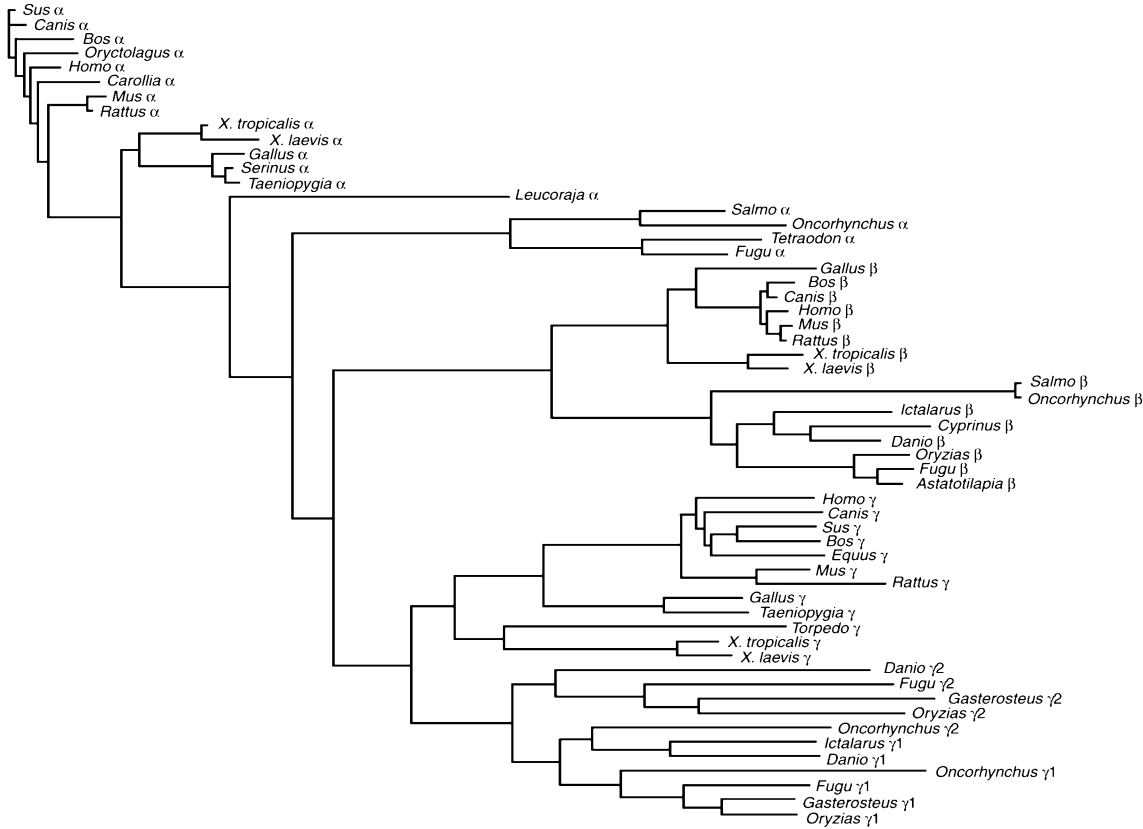


FIGURE 2: Phylogenetic tree of synuclein proteins. The tree was generated using 57 full-length synuclein protein sequences from 26 species, including the four proteins from *Fugu rubripes* (see Supporting Information for amino acid sequences).

(DiO) was purchased from Molecular Probes (Eugene, OR). Lipids and DiO were dissolved in chloroform-methanol (1:1, v/v), and a lipid mixture with the composition PC/PS/Cholesterol 52.5:17.5:30 mol % was prepared, with DiO being added to 1% (w/v). Mixed lipids were dried under a stream of nitrogen and resuspended in HBSE buffer (20 mM HEPES, pH 7.3, 100 mM NaCl, 1 mM EDTA). They were then serially extruded (20 passages) at room temperature through 50-nm-pore-diameter nucleopore polycarbonate membranes (Whatman). The extruded liposomes were stored at 4 °C and used within 24 h.

**Liposome Pull-Down Assay.** The liposome pull-down assay was done essentially as described (38). Briefly, a recombinant GST-fusion protein (30  $\mu$ g) was bound to glutathione-agarose beads (150  $\mu$ L of a 25% bead solution). Following one wash in 0.1% Triton X-100 in HBSE and three washes in HBSE, the beads were resuspended in

150  $\mu$ L of HBSE and mixed with 150  $\mu$ L of diluted liposomes (75  $\mu$ M lipid), followed by a 20 min incubation at 37 °C with vigorous shaking. The incubation was terminated by pelleting, and the beads were washed twice with HBSE at 4 °C. Bound lipids were solubilized with 200  $\mu$ L of 0.1% Triton X-100 in HBSE and transferred to 96-well microtiter plates. Fluorescence was measured (460 nm excitation/538 nm emission) using a microplate fluorimeter (Fluoroskan, Labsystems).

**Filament Assembly.** Untagged synuclein proteins were used at 400  $\mu$ M in 30 mM 3-[N-morpholino]propanesulfonic acid (MOPS), pH 7.2, containing 0.02% sodium azide and 20  $\mu$ M thioflavin T (ThT, Sigma-Aldrich) and placed in a shaking incubator at 37 °C as described (32, 38). For a quantitative assessment of filament formation, ThT fluorescence was used. Aliquots (10  $\mu$ L) were removed at various time points and brought to 400  $\mu$ L with 20  $\mu$ M ThT in 50 mM glycine



			1	2	3	4	5	6	7	8	9	10	11
Fugu $\alpha$	I	9	S	K	A	K	D	G	V	V	A	A	A
	II	20	E	K	T	K	Q	G	V	T	G	A	A
	III	31	E	M	T	K	D	G	V	M	F	V	G
	IV	42	T	K	T	K	D	G	V	T	V	V	A
	V	53	G	K	T	V	S	G	V	S	Q	V	G
	VI	64	G	A	M	V	T	G	V	T	A	V	A
	VII	75	Q	K	T	V	E	S	A	G	S	I	A
Fugu $\beta$	I	9	S	K	A	K	E	G	M	A	V	A	A
	II	20	E	K	T	K	E	G	V	A	V	A	A
	III	31	E	K	T	K	E	G	V	M	F	V	G
	IV	42	N	K	A	K	D	S	V	G	T	V	A
	V	53	E	K	T	T	G	A	M	G	N	I	V
Fugu $\gamma 1$	I	9	S	M	A	K	E	G	V	V	A	A	A
	II	20	E	K	T	K	A	G	M	E	E	A	A
	III	31	A	K	T	K	E	G	V	M	Y	V	G
	IV	42	N	K	T	M	E	G	V	V	S	S	V
	V	57	N	K	T	V	D	Q	T	N	I	V	G
	VI	68	D	A	V	V	G	G	A	N	E	V	S
	VII	79	Q	A	T	V	E	G	V	E	N	M	A
Fugu $\gamma 2$	I	9	S	M	A	K	D	G	V	V	A	A	A
	II	20	E	K	T	K	A	G	V	E	G	A	A
	III	31	T	K	T	K	E	G	V	I	Y	V	G
	IV	42	N	K	T	M	E	G	V	V	T	S	V
	V	57	H	K	T	T	E	Q	A	N	I	I	A
	VI	68	D	T	A	V	S	G	A	N	E	V	A
	VII	79	Q	S	A	V	E	G	V	E	N	A	A

FIGURE 3: Repeats in *Fugu* synucleins. The amino acid sequences of repeats I–VII of  $\alpha$ -synuclein (residues 9–85), repeats I–VII of  $\gamma 1$ - and  $\gamma 2$ -synucleins (residues 9–89), and repeats I–V of  $\beta$ -synuclein (residues 9–63) are shown. Repeat positions (1–11) are indicated above the alignments. Note that the repeats of  $\alpha$ - and  $\beta$ -synucleins are continuous, with a four amino acid linker between repeats IV and V of  $\gamma 1$ - and  $\gamma 2$ -synucleins.

buffer, pH 8.5. Fluorimetry was performed as described (38). For a semiquantitative assessment of filament formation, electron microscopy was used as described (32). Briefly,

aliquots of assembly mixtures were placed on carbon-coated 400-mesh grids and stained with 1% potassium phosphotungstate and micrographs recorded at a nominal magnification of 20 000 on a Philips model EM208S microscope. In some experiments, aliquots were removed at each time point and centrifuged at 279 000g for 15 min at 4 °C. Supernatants and pellets were reconstituted in an SDS sample buffer containing 5M urea and subjected to SDS–PAGE. Protein bands were visualized with CBB.

# RESULTS

**Sequencing of cDNA Clones Encoding *Fugu* Synucleins.** A tblastn search of the genomic sequence of *Fugu rubripes* (53) revealed the presence of four synuclein loci. PCR amplification of *Fugu* brain cDNA was used to generate parts of the coding regions of the four synucleins, which were then used as probes to screen a *Fugu* brain cDNA library. Clones corresponding to each synuclein were isolated and sequenced. To confirm these sequences, we used PCR to amplify the entire coding region of each synuclein from *Fugu* brain cDNA, followed by DNA sequencing. *Fugu* synucleins encode related proteins ranging from 113 to 127 amino acids in length, with many hallmarks of synucleins from other species (Figure 1). Comparison of *Fugu* synucleins with a collection of 53 full-length synuclein sequences from fishes, frogs, birds, and mammals (Supporting Information) showed that they can be assigned to  $\alpha$ -,  $\beta$ -, and  $\gamma$ -synuclein groups (Figure 2). The 127 amino acid protein (molecular mass 12 581) belongs to the  $\alpha$ -synuclein group, the 117 amino acid protein (molecular mass 12 447) belongs to the  $\beta$ -synuclein group, with the 113 and 124 amino acid proteins (molecular masses of 11 375 and 12 462, respectively) belonging to the  $\gamma$ -synuclein group. We therefore refer to the four proteins as *Fugu*  $\alpha$ -,  $\beta$ -,  $\gamma 1$ -, and  $\gamma 2$ -synucleins. The latter two are approximately 60% identical in sequence, with the identities between the other synucleins ranging from

	10	20	30	40	50	60	70	80	90	100	110	120	130	140
Fugu $\alpha$	MDAFMKGFSKAKDGVAAAEKTKQGVTAAGTMDGVMFYGTGKTKDGVTVVA-----GKTVSGVSGVGGAMVTGVTAVAQKTVESAGSIAAATGLVKKKPGKQSDAAAPENMAESPDVTDPAEATFEDADD-----													
Fugu $\beta$	MDVFMKGLSKAKEGMAVAAEKTKGVAAGTMDGVMFYGNKAKDSVGTVA-----EKTTC-----AMGNIVAATGLVKKDEFPTDMFPEYQGEAMEGQSEAMLDPEGETYDES-QQES-QDYEF													
Fugu $\gamma 1$	MDVFMKGLSKAKEGVAAAEKTKAGVEAAKTKGVMFYGNKTKMEGVVTSVNTVNTKTTQNTIIVGDVAVGGANEVSAQTEGVENMAASSGVLCDEYGGTEGGGGGGGYY-----													
Fugu $\gamma 2$	MDVLMKGLFSMAKDGVAAEKTKAGVEAAKTKGVMFYGNKTKMEGVVTSVNTVAHKTKEQNTIIVADTVAGSANEVSAQTEGVENMAASSGVLCDEYGGTEGGGGGGGYY-----													
Homo $\alpha$	MDVFMKGLSKAKEGVAAAEKTKQGVTAAGTMDGVMFYGNKTKMEGVTVHGVATVAEKTKEQNTNVGGAVVTGVTAVAQKTVESAGSIAAATGLVKKDQLGQ-----NEEAPQEGILE-----DMPYDFPNEAYEMPSPEYQDYEF													
Homo $\beta$	MDVFMKGLSKAKEGVAAAEKTKQGVTAAGTMDGVMFYGNKTKMEGVTVHGVATVAEKTKEQASHLGGAVFS-----GAGNIAAATGLVKKDEFPTDLFPEETAGAAEFLIEFLMEFEGESYEDFFPEYQDYEF													
Homo $\gamma$	MDVFMKGLSKAKEGVAAAEKTKQGVTAAGTMDGVMFYGNKTKENVVQSVTSVAEKTKEQANAVSEAVVSSVNTVATRTTEAENIAATGLVKKDEFPTDLFPEETAGAAEFLIEFLMEFEGESYEDFFPEYQDYEF													

FIGURE 4: Exon boundaries in *Fugu* and human synucleins. Amino acid sequences are shown, with exon boundaries indicated in black. To identify the exon/intron boundaries of *Fugu* synucleins, cDNA and genomic sequences were compared. The boundary between exons 4 and 5 of  $\gamma 1$ -synuclein is derived from the sequence of the pufferfish *T. nigroviridis* because the corresponding sequence was missing from the genomic sequence of *F. rubripes*.

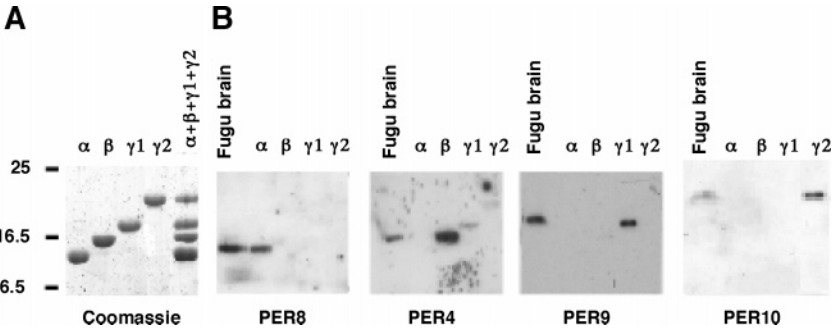


FIGURE 5: Expression of synucleins in the *Fugu* brain. (A) Recombinant  $\alpha$ -,  $\beta$ -,  $\gamma 1$ -, and  $\gamma 2$ -synucleins gave a characteristic ladder of four bands ranging from 14 to 21 kDa apparent molecular mass. The proteins were run on 15% SDS–PAGE and stained using Coomassie brilliant blue. (B) *Fugu* brain extract was run alongside recombinant synucleins and immunoblotted with antibodies PER8, PER4, PER9, and PER10. Note the presence of synuclein bands in the brain extract that comigrated with recombinant  $\alpha$ -,  $\beta$ -,  $\gamma 1$ -, and  $\gamma 2$ -synucleins. Brain extract (10  $\mu$ g) was used for immunoblotting with PER8, PER4, and PER9, and 50  $\mu$ g of brain extract was used for labeling with PER10.

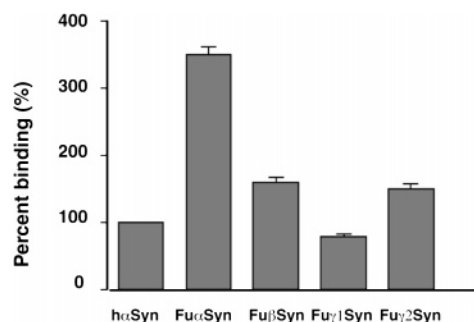


FIGURE 6: Binding of human  $\alpha$ -synuclein (h $\alpha$ Syn) and *Fugu*  $\alpha$ -,  $\beta$ -,  $\gamma$ 1-, and  $\gamma$ 2-synucleins to unilamellar liposomes. Lipid vesicles of 50 nm diameter with the composition PC/PS/cholesterol 52.5:17.5:30 mol % were used. The results are presented as percent binding of human  $\alpha$ -synuclein (taken as 100%) and expressed as means  $\pm$ SEM of the measurements obtained from four independent protein preparations, each run in triplicate.

40 to 50%. *Fugu* synucleins are most similar in the amino-terminal half, which consists of imperfect repeats of 11 amino acids (Figure 3). Alpha-Synuclein has 7 repeats (amino acids 9–85), and  $\beta$ -synuclein has 5 repeats (amino acids 9–63), whereas  $\gamma$ 1- and  $\gamma$ 2-synucleins have 7 repeats each (amino acids 9–89). The repeats are continuous in  $\alpha$ - and  $\beta$ -synucleins, with a four amino acid linker between repeats 4 and 5 in  $\gamma$ 1- and  $\gamma$ 2-synucleins. They partially overlap with a hydrophobic region, which is followed by a poorly conserved and negatively charged carboxy-terminus. Unlike  $\alpha$ -,  $\gamma$ 1-, and  $\gamma$ 2-synucleins, *Fugu*  $\beta$ -synuclein lacks a large part of the hydrophobic region. Comparison of the cDNA sequences with the genome sequences of *Fugu rubripes* (53) and *Tetraodon nigroviridis* (59) showed that each synuclein gene contains five coding exons (Figure 4). The exon sizes and exon/intron organization of *Fugu* and human (19, 59, 60) synuclein genes are similar. Exons 1 and 2 encode much of the amino-terminal repeat region, exon 3 encodes part of the repeats and most of the hydrophobic region, and exons 4 and 5 encode the carboxy-terminal region. At the amino acid level, *Fugu* synucleins are 54–64% identical to their human counterparts.

**Expression of Synucleins in the *Fugu* Brain.** The four *Fugu* synucleins were expressed in *E. coli* and purified. They were heat stable, as was the human  $\alpha$ -synuclein. By SDS–PAGE, *Fugu* synucleins gave rise to a characteristic ladder of protein bands ranging from 14 to 21 kDa apparent molecular mass (Figure 5). By immunoblotting, antibody PER8 recognized  $\alpha$ -synuclein at 14 kDa, PER4 labeled  $\beta$ -synuclein at 16 kDa, and PER9 recognized  $\gamma$ 1-synuclein at 18 kDa and PER10 labeled  $\gamma$ 2-synuclein at 21 kDa. In the *Fugu* brain extract, PER8, PER4, PER9, and PER10 labeled bands aligned with the corresponding recombinant synuclein proteins (Figure 5). *Fugu*  $\alpha$ -,  $\beta$ -, and  $\gamma$ 1-synucleins were found to be present at similar levels, with  $\gamma$ 2-synuclein being less abundant.

**Liposome Binding of *Fugu* Synucleins.** A pull-down assay was used to investigate the lipid binding properties of *Fugu* synucleins relative to those of human  $\alpha$ -synuclein. Liposomes of 50 nm diameter with the lipid composition PC/PS/cholesterol 52.5:17.5:30 mol % and synuclein proteins with N-terminal GST-fusion tags were used. We previously showed that human  $\alpha$ -synuclein with a GST-fusion tag at either N- or C-terminus has similar lipid binding properties (38). *Fugu* synucleins exhibited differential liposome binding,

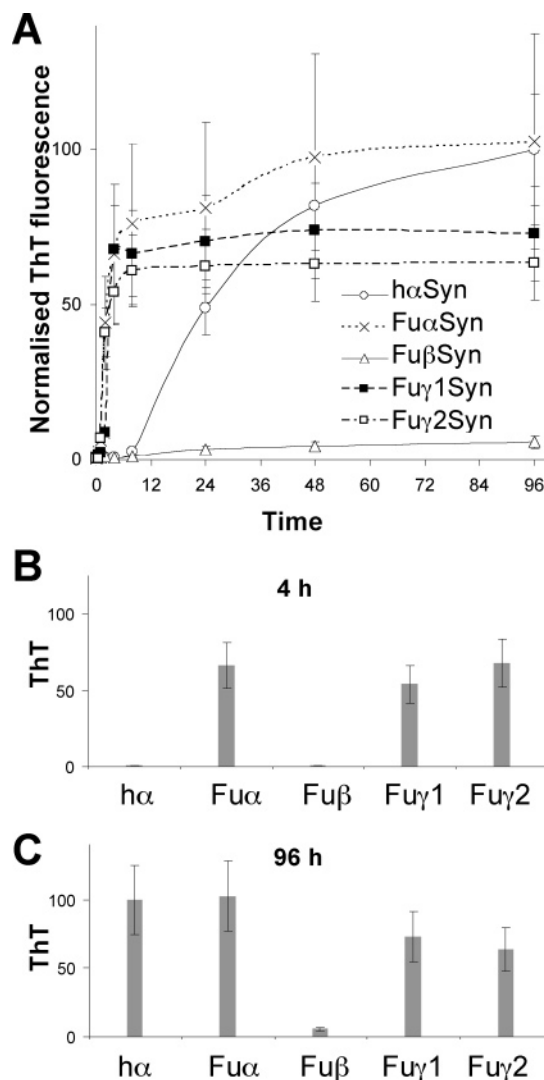
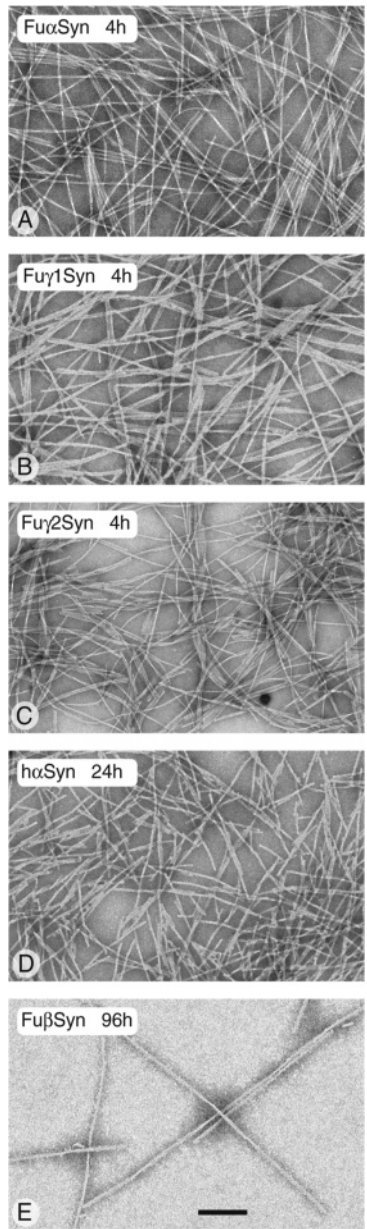


FIGURE 7: Growth curve of the filament formation of human  $\alpha$ -synuclein (h $\alpha$ Syn) and *Fugu*  $\alpha$ -,  $\beta$ -,  $\gamma$ 1-, and  $\gamma$ 2-synucleins. Assembly was monitored by the enhancement of thioflavin T (ThT) fluorescence intensity over time. The results are presented as normalized fluorescence (with the value for wild-type  $\alpha$ -synuclein at 96 h taken as 100) and expressed as the means  $\pm$ SEM of the measurements obtained from five independent protein preparations. (A) The full time course; (B and C) the normalized ThT fluorescence at 4 and 96 h, respectively.

which was strongest for  $\alpha$ -synuclein, followed by  $\beta$ -, and  $\gamma$ 2-synucleins (Figure 6). All three proteins gave stronger binding than that of human  $\alpha$ -synuclein. The values were  $352 \pm 15\%$  for  $\alpha$ -synuclein,  $165 \pm 8\%$  for  $\beta$ -synuclein, and  $159 \pm 12\%$  for  $\gamma$ 2-synuclein (with human  $\alpha$ -synuclein taken as 100%). Only *Fugu*  $\gamma$ 1-synuclein ( $76 \pm 5\%$ ) bound less strongly to liposomes than did human  $\alpha$ -synuclein (Figure 6).

**Filament Assembly of *Fugu* Synucleins.** Self assembly of recombinant *Fugu* synuclein proteins was monitored quantitatively by ThT fluorescence and semiquantitatively by electron microscopy. Both methods were used in parallel in all experiments, and a close correspondence was observed between levels of ThT fluorescence and filament numbers. The time-dependent changes in ThT fluorescence during incubation of untagged *Fugu* synucleins and human  $\alpha$ -synuclein in a shaking incubator at 37 °C are shown in Figure 7. Of the four *Fugu* proteins,  $\alpha$ -synuclein assembled the



**FIGURE 8:** In vitro assembly of synuclein filaments. The micrographs show representative views of human  $\alpha$ -synuclein (h $\alpha$ Syn) and *Fugu*  $\alpha$ -,  $\beta$ -,  $\gamma$ 1-, and  $\gamma$ 2-synuclein filaments to demonstrate the different time courses of assembly. (A) *Fugu*  $\alpha$  at 4 h; (B) *Fugu*  $\gamma$ 1 at 4 h; (C) *Fugu*  $\gamma$ 2 at 4 h; (D) human  $\alpha$  at 24 h; and (E) *Fugu*  $\beta$  at 96 h. The samples all come from one parallel experiment, and the numbers of filaments seen match the levels of assembly monitored by ThT fluorescence. *Fugu*  $\alpha$ -,  $\gamma$ 1-, and  $\gamma$ 2-synucleins have a shorter lag time than human  $\alpha$ -synuclein, but *Fugu*  $\beta$ -synuclein has a longer lag time and a much lower level of assembly compared with that of human  $\alpha$ -synuclein. Scale bar, 200 nm.

fastest, followed closely by  $\gamma$ 1- and  $\gamma$ 2-synucleins. *Fugu*  $\beta$ -synuclein, by contrast, failed to assemble significantly. The lag phase for the assembly of *Fugu*  $\alpha$ -,  $\gamma$ 1-, and  $\gamma$ 2-synucleins was less than 1 h, whereas it was 8 h for human  $\alpha$ -synuclein. After 4 days of incubation, as judged by ThT fluorescence, the amount of assembly was greater for human and *Fugu*  $\alpha$ -synucleins than that for *Fugu*  $\gamma$ 1- and  $\gamma$ 2-synucleins. By electron microscopy, incubation with shaking led to the bulk assembly of *Fugu*  $\alpha$ -,  $\gamma$ 1-, and  $\gamma$ 2-synucleins, as well as human  $\alpha$ -synuclein, into filaments (Figure 8). The filaments were about 10 nm wide with a variably twisted

**Table 1**

synuclein	ratio of nonhydrophilic to hydrophilic residues ( $\times 100$ )	ratio of $\beta$ -sheet favorable to nonfavorable residues ( $\times 100$ )	net charge per residue ( $\times 100$ )
human $\alpha$	109	36	-6.4
<i>Fugu</i> $\alpha$	123	37	-5.5
<i>Fugu</i> $\beta$	95	27	-11.1
<i>Fugu</i> $\gamma$ 1	113	40	-8
<i>Fugu</i> $\gamma$ 2	110	38	-7.3

appearance. *Fugu*  $\beta$ -synuclein, by contrast, failed to assemble in bulk. Only occasional filaments were observed after 4 days of incubation (Figure 8E). The kinetics and amount of assembly seen by electron microscopy mirrored those observed using the ThT fluorescence assay. Overall, the amount of pelletable material corresponded to the level of ThT fluorescence and the number of filaments seen by electron microscopy. By 96 h, with the exception of *Fugu*  $\beta$ -synuclein, all the material was pelletable, with no synuclein present in the supernatant. *Fugu*  $\gamma$ 1- and  $\gamma$ 2-synucleins became extremely viscous upon assembly. This may have reflected bundling of filaments and may explain the lower level of ThT fluorescence at 96 h when compared to that of human and *Fugu*  $\alpha$ -synucleins.

The rates of assembly of *Fugu*  $\alpha$ -,  $\gamma$ 1-, and  $\gamma$ 2-synucleins were faster than the assembly rate of human  $\alpha$ -synuclein. These findings correlated with the ratios of nonhydrophilic/hydrophilic and  $\beta$ -sheet favorable/nonfavorable residues as well as with the net charge of the synucleins (Table 1).

# DISCUSSION

We have sequenced cDNAs encoding the four synuclein proteins of the pufferfish *Fugu rubripes* and measured the lipid binding and self-assembly properties of the recombinant proteins. *Fugu* is the first species to be found with a complement of four synuclein genes, as opposed to the three genes characteristic of birds and mammals. This gene number is probably the result of the duplication of the whole genome that took place in the ray-finned fish lineage some 230 million years ago and the subsequent loss of duplicated genes (59, 60).

*Fugu* synucleins have many characteristics in common with their avian and mammalian counterparts, including amino-terminal repeats of 11 amino acids, a hydrophobic middle region that partially overlaps with these repeats, and a negatively charged carboxy-terminus. When compared with a collection of full-length sequences (Supporting Information), they fall into  $\alpha$ -,  $\beta$ -, and  $\gamma$ -synuclein groups, with two proteins belonging to the  $\gamma$ -synuclein group. We therefore refer to the *Fugu* proteins, which range from 113 to 127 amino acids in length, as  $\alpha$ -,  $\beta$ -,  $\gamma$ 1-, and  $\gamma$ 2-synucleins. As in human synucleins (18), *Fugu*  $\alpha$ - and  $\gamma$ -synucleins have 7 repeats each, with only 5 repeats in  $\beta$ -synuclein. Like their human counterparts (19, 61, 62), the *Fugu* synuclein genes contain five coding exons, with good conservation of exon lengths and exon/intron positions. *Fugu* and human synucleins are most similar in the amino-terminal repeat region, where the degree of sequence identity is of the order of 70%. One additional difference is that *Fugu*  $\alpha$ - and  $\beta$ -synucleins lack a four amino acid sequence in the region linking repeats 4 and 5. The hydrophobic region of *Fugu*  $\alpha$ -synuclein is



80% identical to the corresponding region in human  $\alpha$ -synuclein, with poorer sequence conservation in the corresponding regions of  $\beta$ -,  $\gamma$ 1-, and  $\gamma$ 2-synucleins. A feature that distinguishes avian and mammalian  $\beta$ -synucleins from  $\alpha$ - and  $\gamma$ -synucleins is the absence of an 11 amino acid stretch in the hydrophobic region (GVTAVAQKTVE in human  $\alpha$ -synuclein and SVNTVATKTVE in human  $\gamma$ -synuclein). This sequence is 100% conserved between *Fugu*  $\alpha$ - and human  $\alpha$ -synucleins and is 45% identical between *Fugu*  $\gamma$ - and human  $\gamma$ -synucleins. In *Fugu*  $\beta$ -synuclein, a continuous sequence of 22 amino acids, including the 11 amino acid stretch, is missing. Unlike the repeat and hydrophobic regions, the carboxy-terminal regions of *Fugu* and human synucleins are only poorly conserved, except for being negatively charged overall.

Three missense mutations (A30P, E46K, and A53T) in human  $\alpha$ -synuclein cause autosomal dominantly inherited forms of PD and DLB (19, 20, 22). In *Fugu* synucleins, an alanine residue is present at position 30, and a negatively charged amino acid (D or E) is present at position 46. By contrast, the residue at position 53 is not conserved, which is consistent with previous findings in other species (8). This is in line with work showing that of the three mutations only A30P and E46K influence the binding of human  $\alpha$ -synuclein to lipid membranes (11, 38). Prior to the present work, only one fish synuclein had been studied (1). As noted previously (63), this protein from *T. californica* belongs to the  $\gamma$ -synuclein group. It is 55–60% identical to *Fugu*  $\gamma$ 1- and  $\gamma$ 2-synucleins and carries an extra repeat between repeats 2 and 3 (1). It remains to be seen whether this extra repeat, which has not been observed in any other synuclein, is genuine, or whether it was the result of a cloning artifact.

By immunoblotting, the *Fugu* brain expressed all four synuclein proteins, with similar levels of  $\alpha$ -,  $\beta$ -, and  $\gamma$ 1-synucleins and lower levels of  $\gamma$ 2-synuclein. Recombinant *Fugu* synucleins gave a characteristic ladder of four protein bands ranging from 14 to 21 kDa apparent molecular mass, indicating that they migrate abnormally on SDS–PAGE, as has been observed previously for mammalian synucleins (4). In the central nervous system and the electric organ of *T. californica*, a similar ladder of bands has been described following immunoblotting with an antibody raised against a  $\beta$ -galactosidase/synuclein fusion protein (1), suggesting expression of multiple synucleins in *Torpedo*.

Synucleins bind to acidic phospholipids and detergent micelles through their amino-terminal repeats (10–13). We therefore investigated the binding of recombinant, GST-tagged *Fugu* synucleins to unilamellar liposomes. All four proteins exhibited liposome binding, which was strongest for  $\alpha$ -synuclein, followed by  $\beta$ -,  $\gamma$ 2-, and  $\gamma$ 1-synucleins. *Fugu*  $\alpha$ -synuclein bound to liposomes 3.5 times more than its human counterpart. The two proteins are 71% identical in the repeat region, and of the 22 amino acid differences, 9 are conservative substitutions. When bound to detergent micelles (17), human  $\alpha$ -synuclein forms two  $\alpha$ -helices, which extend from residues 3 to 37 (helix-N) and 45 to 92 (helix-C) and are connected by an extended linker (residues 38–44). Helix-C is followed by a short extended region (residues 93–97) and an unstructured tail (residues 98–140). *Fugu*  $\alpha$ -synuclein is 77% identical to its human counterpart in helix-N and 65% identical in helix-C, with 57% sequence identity in the linker region and 80% in the short extended

region following helix-C. Helix-C of human  $\alpha$ -synuclein is four amino acids longer than the corresponding region in the *Fugu* protein. It is unclear, at present, how these differences account for the greater lipid binding of *Fugu*  $\alpha$ -synuclein.

The ordered in vitro assembly of human  $\alpha$ -synuclein into abnormal filaments is a property that is closely linked to its central role in Lewy body disorders and MSA (18). Under the same experimental conditions, human  $\beta$ - and  $\gamma$ -synucleins fail to form filaments (32, 48, 49), consistent with their absence from the deposits that characterize the human diseases (23, 25). We found here that *Fugu*  $\alpha$ -,  $\gamma$ 1-, and  $\gamma$ 2-synucleins readily assemble into filaments. In contrast, *Fugu*  $\beta$ -synuclein, like its human counterpart, failed to assemble in bulk. *Fugu*  $\alpha$ -,  $\gamma$ 1-, and  $\gamma$ 2-synucleins assembled with shorter lag times and faster rates than those of human  $\alpha$ -synuclein. The lag period for assembly was 8 h for human  $\alpha$ -synuclein and less than 1 h for the three *Fugu* proteins.

In unfolded proteins, increasing hydrophobicity, a propensity for  $\beta$ -sheet structure, and low net charge have been associated with amyloid formation (64–66). We therefore estimated hydrophilicity, net charge, and  $\beta$ -sheet propensity of human  $\alpha$ -synuclein and the *Fugu* synucleins. Overall, *Fugu*  $\alpha$ -,  $\gamma$ 1-, and  $\gamma$ 2-synucleins are less hydrophilic, have a lower net charge, and contain a higher percentage of  $\beta$ -sheet favorable amino acids than human  $\alpha$ -synuclein. By contrast, *Fugu*  $\beta$ -synuclein, like human  $\beta$ -synuclein, is more hydrophilic, carries a higher net charge, and a smaller percentage of  $\beta$ -sheet favorable amino acids than the other synucleins. These characteristics help to explain the markedly higher propensity of *Fugu*  $\alpha$ -,  $\gamma$ 1-, and  $\gamma$ 2-synucleins to fibrillate and the inability of  $\beta$ -synuclein to assemble in bulk.

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## SUPPORTING INFORMATION AVAILABLE

Accession numbers of nucleotide sequences of the deduced amino acid sequences and synucleins from 26 species. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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