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Effect of melatonin on α -synuclein self-assembly and cytotoxicity

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Abstract

α -Synuclein (α S) assembly has been implicated as a critical step in the development of Lewy body diseases such as Parkinson's disease and dementia with Lewy bodies. Melatonin (Mel), a secretory product of the pineal gland, is known to have beneficial effects such as an antioxidant function and neuroprotection. To elucidate whether Mel has an antiassembly effect, here we used circular dichroism spectroscopy, photoinduced crosslinking of unmodified proteins, thioflavin S fluorescence, size exclusion chromatography, electron microscopy and atomic force microscopy to examine the effects of Mel on the α S assembly. We also examined the effects of Mel on α S-induced cytotoxicity by assaying 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide metabolism in α S-treated, primary neuronal cells. Initial studies revealed that Mel blocked α S fibril formation as well as destabilizing preformed α S fibrils. Subsequent evaluation of the assembly-stage specificity of the effect showed that Mel was able to inhibit protofibril formation, oligomerization, and secondary structure transitions. Importantly, Mel decreased α S-induced cytotoxicity. These data suggest a mechanism of action for Mel, inhibition of assembly of toxic polymers and protection of neurons from their effect.

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Keywords: Parkinson's disease; Dementia with Lewy bodies; α -synuclein; Melatonin; Oligomers; Cytotoxicity

1. Introduction

Parkinson's disease (PD) is 1 of the most common neurodegenerative diseases affecting mainly the extrapyramidal motor system (Forno, 1996). The major lesion in PD resides in the dopaminergic neurons in the substantia nigra, as well as other brain stem nuclei including locus coeruleus and dorsal motor vagal nucleus with appearance of Lewy bodies (LBs) (Forno, 1996). Dementia with LBs (DLB) is a progressive dementing disorder of the elderly clinically characterized by fluctuation in mental decline, visual hallucina-

tions, parkinsonism, and widespread distribution of LBs in the brain (McKeith et al., 2005). LBs constitute the main histopathological features of PD and DLB, and are comprised of amyloid-like fibrils composed of a small protein (approximately 14 kDa) named α -synuclein (α S) (Baba et al., 1998; Forno, 1996; Goedert, 2001). Several transgenic animal models overexpressing human α S display varying degrees of biochemical, pathological, and clinical abnormalities reminiscent of PD (Feany and Bender, 2000; Giasson et al., 2002; Lee et al., 2002).

α S is also associated with pathological lesions in other neurodegenerative diseases, sometimes involving nonneuronal cells, such as the glial cytoplasmic inclusions found in multiple system atrophy (MSA), a sporadic, progressive neurological disorder characterized by parkinsonism, cerebellar dysfunction, autonomic impairment, and pyramidal signs (Gai et al., 1998; Gilman et al., 1999; Spillantini et al.,

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1998). A recent study in mice demonstrated that overexpression of α S in oligodendrocytes resulted in MSA-like degeneration in the central nervous system (CNS) (Yazawa et al., 2005). Convergent biochemical and genetic evidence suggests that the assembly of α S is an important and, probably, seminal step in the development of Lewy body diseases (LBD) including PD, DLB, and other α -synucleinopathies such as MSA.

Based on the nucleation-dependent polymerization model to explain the mechanism of α S assembly (Wood et al., 1999) *in vitro*, we and other groups previously reported that some antioxidants such as wine-related polyphenols, curcumin and rifampicin, inhibit formation of α S fibrils (α S), as well as destabilize preformed α S *in vitro* (Li et al., 2004; Ono and Yamada, 2006; Zhu et al., 2004).

The conversion of α S occurs via a multiple-step process involving nonfibrillar aggregates such as protofibrils or oligomers on α S assembly pathway (Caughey and Lansbury, 2003). As in the case of amyloid β -protein ($A\beta$), there is mounting evidence that protofibrils or oligomers of α S are more toxic than α S on the pathway to fibril formation (Lashuel et al., 2002; Outeiro et al., 2008; Volles and Lansbury, 2003). If so, the most efficacious therapeutic agents would target the assembly or neurotoxic activity of these structures.

Melatonin (Mel), a secretory product of the pineal gland, is involved in the regulation of circadian and seasonal rhythms, in oncogenesis, and in inducing osteoblast differentiation (Pévet et al., 2006; Reiter, 1991) (Fig. 1). Furthermore, Mel is superior to vitamin C and E in protecting from oxidative damage and in scavenging free radicals (López-

Burillo et al., 2003). Recently, Ishido reported that Mel protected the neural cells from neurotoxins by inhibition of both caspase-3/7 activation and disruption of the mitochondrial transmembrane potential (Ishido, 2007). He also reported that Mel inhibits α S assembly by using immunostaining (Ishido, 2007).

In the studies reported here, we sought to determine whether Mel affected α S conformational dynamics and assembly, and whether these effects correlated with α S cytotoxicity. We treated α S with Mel and then monitored assembly and toxicity using a combination of circular dichroism spectroscopy (CD), photoinduced crosslinking of unmodified proteins (PICUP), size-exclusion chromatography (SEC), thioflavin S (ThS) binding, electron microscopy (EM), atomic force microscopy (AFM), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) metabolism. The results show potent inhibitory effects at all stages of peptide assembly.

2. Methods

2.1. Chemicals and reagents

Chemicals were obtained from Sigma-Aldrich, Co. (St. Louis, MO, USA) and were of the highest purity available. Water was produced using a Milli-Q system (Millipore Corp., Bedford, MA, USA).

2.2. Preparation of α S and α S solutions

The α S and α S solutions were prepared as described previously (Ono and Yamada, 2006). Briefly, α S (lot numbers 121303AS and 50306AS) was purchased from Recombinant Peptide Technologies, LLC (Bogart, GA, USA). To prepare α S for study, α S peptide was dissolved at 70 μ M in 20 mM Tris buffer, pH 7.4. After sonication for 1 minute in a bath sonicator, the α S solution was centrifuged for 10 minutes at 16,000g. Fresh, nonaggregated α S was obtained by polymerizing fresh α S just before the destabilization reaction. In the following experiment, the concentration of α S in the final reaction mixture was regarded as 70 μ M. A stock solution of glutathione S-transferase (GST; Sigma-Aldrich) was prepared by dissolving the lyophilizate to a concentration of 250 μ M in 60 mM NaOH. Prior to use, aliquots were diluted 10-fold into 20 mM Tris buffer, pH 7.4.

2.3. Peptide aggregation

α S solutions were prepared as specified above and then 0.5-mL aliquots were placed in 1-mL microcentrifuge tubes. We selected trihexyphenidyl hydrochloride (Tri) (Fig. 1) which does not have inhibitory activity on α S aggregation as negative control (Ono et al., 2007). Mel and Tri were dissolved in ethanol to a final concentration of 2.5 mM and then diluted with 20 mM Tris, pH 7.4, 100 mM NaCl to produce concentrations of 50 and 500 μ M. One-half mL of each compound then was added to separate tubes of α S, yielding final α S concentrations of 70 μ M and final inhib-

Fig. 1. Structures of melatonin and trihexyphenidyl.

itor concentrations of 25 and 250 μM . Compound:peptide ratios thus were 5:14 at the lower compound concentration and 25:7 at the higher compound concentration. Control tubes with peptide alone received 0.5 mL of buffer. The tubes were incubated at 37 °C for 0–6 days with agitation. We note that for each sample at each point analyzed, aliquots used for different experiments (see below) generally all came from the same tube of αS , ensuring that valid correlations could be made among the data obtained.

2.4. Fibrils destabilizing assay

$\text{f}\alpha\text{S}$ solutions were prepared as specified above and then 0.5-mL aliquots were placed in 1-mL microcentrifuge tubes. Compound preparation was similar to that of “Peptide aggregation.” One-half mL of each compound then was added to separate tubes of αS , yielding final $\text{f}\alpha\text{S}$ concentrations of 70 μM and final inhibitor concentrations of 25 and 250 μM . Compound:peptide ratios thus were 5:14 at the lower compound concentration and 25:7 at the higher compound concentration. Control tubes with peptide alone received 0.5 mL of buffer. The tubes were incubated at 37 °C for 0–6 hours without agitation.

2.5. ThS fluorescence

The reaction mixture contained 5 mM ThS (MP Bio-medicals, LLC, Irvine, CA, USA) and 50 mM of glycine-NaOH buffer, pH 8.5. After vortexing briefly, fluorescence was determined 3 times at intervals of 10 seconds using a Hitachi F-2500 fluorometer (Tokyo, Japan). Excitation and emission wavelengths were 440 and 521 nm, respectively. Sample fluorescence was determined by averaging the 3 readings and subtracting the fluorescence of a ThS blank.

2.6. CD

CD spectra of αS :compound mixtures were acquired immediately after sample preparation or after 1–6 days of incubation. CD measurements were made by removing a 200- μL aliquot from the reaction mixture, adding the aliquot to a 1-mm path length CD cuvette (World Precision Instruments, Sarasota, FL, USA), and acquiring spectra in a J-805 spectropolarimeter (Jasco, Tokyo, Japan). The CD cuvettes were maintained on ice prior to introduction into the spectrometer. After temperature equilibration, spectra were recorded at 22 °C from 190 to 260 nm at 0.2-nm resolution with a scan rate of 100 nm/minute. Ten scans were acquired and averaged for each sample. Raw data were manipulated by smoothing and subtraction of buffer spectra according to the manufacturer’s instructions.

2.7. Protofibril formation

Prefibrillar intermediates, termed “protofibrils” (Conway et al., 1998, 2000; Wood et al., 1999) were defined as the material eluting in the void volume of the column by SEC as proposed previously (Volles et al., 2001). To study protofibril formation and the effects of compounds on it, we

incubated αS according to the aggregation protocol above. Periodically during the 6-day incubation period, solutions were centrifuged at 16,000g for 5 minutes and then 200 μL of the supernatant was fractionated by SEC at a flow rate of 0.5 mL/minute on a Superdex 75 column (GE Healthcare BioSciences AB, Uppsala, Sweden) attached to a Waters 515 HPLC pump and a Waters 2489 UV/Visible detector (Waters, Milford, MA, USA). The void volume peak of protofibrils was detected and recovered at an elution time of 14 minutes by ultraviolet absorbance at 254 nm.

2.8. EM

A 10- μL aliquot of each sample was spotted onto a glow-discharged, carbon-coated formvar grid (Okensoji, Co, Ltd, Tokyo, Japan) and incubated for 20 minutes. The droplet then was displaced with an equal volume of 2.5% (vol/vol) glutaraldehyde in water and incubated for an additional 5 minutes. Finally, the peptide was stained with 8 μL of 1% (vol/vol) filtered (0.2 μm) uranyl acetate in water (Wako Pure Chemical Industries, Ltd, Osaka, Japan). This solution was wicked off and then the grid was air-dried. Samples were examined using a JEM-1210 transmission electron microscope (JEOL Ltd., Tokyo, Japan).

2.9. AFM

Peptide solutions were characterized using a Nanoscope IIIa controller (Veeco Digital Instruments, Santa Barbara, CA, USA) with a multimode scanning probe microscope equipped with a JV (J-type vertical) scanner. All measurements were carried out in the tapping mode under ambient conditions using single-beam silicon cantilever probes. A 10- μL aliquot of each sample was spotted onto freshly cleaved mica (Ted Pella, Inc., Redding, CA, USA), incubated at room temperature for 5 minutes, rinsed with water, and then blown dry with air. At least 4 regions of the mica surface were examined to confirm the homogeneity of the structures throughout the sample. Mean particle heights were analyzed by averaging the measured values of 8 individual cross-sectional line scans from each image only when the particle structure was confirmed.

2.10. Chemical crosslinking and determination of oligomer frequency distributions

Immediately after their preparation, samples were cross-linked using PICUP, as described (Bitan et al., 2001). Briefly, to 18 μL of protein solution was added 1 μL of 1 mM tris(2,2'-bipyridyl)dichlororuthenium (II) ($\text{Ru}(\text{bpy})$) and 1 μL of 20-mM ammonium persulfate (APS). The final protein:tris(2,2'-bipyridyl)dichlororuthenium (II):ammonium persulfate molar ratios of αS were 0.32:1:20. The mixture was irradiated for 1 second with visible light and then the reaction was quenched with 10 μL of tricine sample buffer (Invitrogen, Carlsbad, CA, USA) containing 5% (vol/vol) β -mercaptoethanol. Determination of the frequency distribution of monomers and oligomers was accomplished using

sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining, as described (Bitan et al., 2001). Briefly, 20 μ L of each crosslinked sample was electrophoresed on a 10%–20% gradient tricine gel and visualized by silver staining (SilverXpress, Invitrogen). Non-crosslinked samples were used as controls in each experiment. Densitometry was performed with a luminescent image analyzer (LAS 4000 mini, Fujifilm, Tokyo, Japan) and image analysis software (Multigauge v. 3.2, Fujifilm). The intensity of each band in a lane from the SDS gel was normalized to the sum of the intensities of all the bands in that lane, according to the formula

$$R_i = I_i / \sum_{i=1}^n I_i \times 100 (\%)$$

where R_i is the normalized intensity of band i and I_i is the intensity of each band i . R_i varies from 0 to 100. To calculate the oligomer ratio, the sum of oligomer intensities of α S with 1, 2.5, 5, 10, 25, and 250 μ M Mel was divided by the sum of oligomer intensities without each compound. The effective concentration (EC50) was defined as the concentration of Mel to inhibit α S oligomerization to 50% of the control value. EC50 was calculated by sigmoidal curve fitting, using GraphPad Prism software (version 4.0a, GraphPad Software, Inc., San Diego, CA, USA).

2.11. Primary neuronal culture

Primary cultures of mesencephalon and neostriatum were obtained from embryos (E 13–14) of C57BL/6J mouse. Cultures were performed as described previously (Goto et al., 1997; Mochizuki et al., 1994), with the following modifications: the mesencephalon and neostriatum were dissected out and dissociated, then seeded at a density of 2.1×10^5 cells per well (mesencephalon: 0.25×10^5 , neostriatum: 1.85×10^5) (90- μ L total volume per well) on 96-well plates (Nalge Nunc International K.K., Tokyo, Japan) with 5% polyethylenimine (Sigma Chemical Co., St. Louis, MO, USA). The cultures were kept in a 37 °C incubator in a humidified atmosphere containing 95% O₂/5% CO₂. After incubation in F12/DMEM (Gibco, Grand Island, NY, USA) 10% fetal bovine serum for 48 hours, the medium was changed to F12/DMEM (Gibco) 5% (vol/vol) calf serum, 5% (vol/vol) horse serum to prepare cells for assay. For neuron-rich cultures, on Day 5, cytosine arabinoside (Sigma Chemical Co.) (10 μ M) was added for 48 hours to limit the growth of glial cells. F12/DMEM (Gibco) 5% (vol/vol) calf serum, 5% (vol/vol) horse serum was changed at Day 7 in vitro, at which point toxicity assays were done.

2.12. MTT assay

Mel and α S with either 0 μ M or 25 μ M Mel were incubated in 20 mM Tris, pH 7.4, 100 mM NaCl at 37 °C for 0, 2, or 6 days with agitation prior to the addition of a 10- μ L aliquot of the sample to the primary neurons of mesencephalon and neostriatum. Cells were treated for 48 hours with a final concentration of 2.5

μ M Mel alone, 7 μ M α S alone, or with α S plus 2.5 μ M Mel. Peptide:compound ratios of α S were 2.8. To determine toxicity, we used Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Rockville, MD, USA). In practice, the “zero time” samples were equivalent, as all components were mixed with cells at the same time.

To determine toxicity, we added 10 μ L of CCK-8 solution (Dojindo Molecular Technologies, Inc.) to each well of the microtiter plate and the plate was incubated in the CO₂ incubator for an additional 4 hours. After incubation, CCK-8 reduction was assessed by measuring absorption at 450 nm (corrected for background absorbance at 650 nm) using a Bio-Rad microplate reader (Bio-Rad, Vermont). Control included media with 20 mM Tris, pH 7.4, 100 mM NaCl (“negative”). Five replicates were done for each treatment group and reported as mean $\pm$ standard error. Cell viability = $(A_{\text{sample}})/(A_{\text{medium}}) \times 100$, where A_{sample} and A_{medium} were absorbance values from Mel alone or α S-containing samples, and medium, respectively.

2.13. Statistical analysis

One-way factorial analysis of variance (ANOVA) followed by Bonferroni post hoc comparisons were used to determine statistical significance among data sets. These tests were implemented within GraphPad Prism software (version 4.0a, GraphPad Software, Inc.). Significance was defined as $p < 0.05$.

3. Results

3.1. Mature fibril formation

To determine whether fibril formation was affected by Mel, we used ThS to monitor temporal changes in the β -sheet content in samples of α S in the absence or presence of Mel. ThS fluorescence is not a measure of fibril content per se, but because β -sheet formation correlates with fibril formation, ThS fluorescence is a useful surrogate marker (LeVine, 1993, 1999; Naiki and Nakakuki, 1996). The ThS studies also allowed us to determine the kinetics of peptide assembly, providing information on nucleation and elongation phases of α S assembly. We included Tri (Fig. 1) here as a negative control because, like Mel, it is an aromatic compound and we previously confirmed that Tri did not affect α S assembly (Ono et al., 2007).

In the absence of compounds, α S displayed a quasi-sigmoidal binding curve characterized by a lag time of 1 day, a period of successively increasing ThS binding for 3 days, and a binding plateau occurring after 4 days (Fig. 2A and B)—results consistent with the well-known nucleation-dependent polymerization model of α S assembly (Wood et al., 1999). When α S was incubated with Tri, either at a compound:peptide ratio of 5:14 or 25:7, the binding curves were identical to that of the untreated peptide, within experimental error (Fig. 2A). In contrast, significant effects were produced by Mel (Fig. 2B), such as a concentration-dependent increase in lag time, decrease in β -sheet growth rates, and decrease in final β -sheet levels (Table 1).

Fig. 2. Melatonin inhibits α -synuclein (α S) fibril formation. (A, B) thioflavin S (ThS) binding; 70 μ M α S was incubated for 6 days at 37 °C in 20 mM Tris buffer, pH 7.4, in the presence of 0 (○), 25 (●), or 250 (Δ) μ M trihexyphenidyl hydrochloride (Tri) (A) or melatonin (Mel) (B). Periodically, aliquots were removed and ThS binding levels were determined. Binding is expressed as mean fluorescence (in arbitrary fluorescence units [FU]) $\pm$ standard error. Each figure comprises data obtained in 3 independent experiments. (C–F) α S assembly morphology. Electron microscopy (EM) was used to determine the morphologies of assemblies of α S incubated at 37 °C for 6 days in 20 mM Tris buffer, pH 7.4. α S peptide was incubated in buffer alone (C) or in the presence of 250 μ M Tri (D), 25 μ M Mel (E), or 250 μ M Mel (F). Scale bars indicate 100 nm.

Using EM, we determined the morphology of the α S assemblies when maximal ThS binding was observed. Classical amyloid-like fibrils were observed in samples of untreated α S (Fig. 2C). The α S were nonbranched, helical filaments with diameters of 10 nm as reported before (Conway et al., 2000; Ono and Yamada, 2006). The addition of Tri (here for the higher, 250 μ M concentration) did not alter the assembly of α S (Fig. 2D). In contrast to the results with Tri, strong inhibition of fibril formation was observed in Mel-treated samples. At substoichiometric concentration (compound:peptide ratio of 5:14), treatment of α S with 25

μ M Mel clearly reduced fibril number and many short, shared fibrils were observed (Fig. 2E). Treatment of α S with 250 μ M Mel markedly reduced fibril number and increased the relative numbers of short fibrils and amorphous aggregates (Fig. 2F).

3.2. Destabilization of preformed fibrils

We used ThS to monitor temporal changes in β -sheet content in samples of preformed α S in the absence or presence of Mel.

Table 1
Kinetics of α -synuclein (α S) assembly

Sample	Lag time (d) ^a	Growth rate (FU/d) ^b	Maximum intensity (FU) ^c
α S	1.2	54.6	129.3
α S + 25 μ M Tri	1.2	58.8	131.2
α S + 250 μ M Tri	1.3	59.4	129.8
α S + 2.5 mM Tri	1.2	53.4	127.6
α S + 25 μ M Mel	1.2	43.2	99.3
α S + 250 μ M Mel	1.4	8.8	22.8
α S + 2.5 mM Mel	1.9	5.4	10.5

Key: d, day; FU, fluorescence units; Mel, melatonin; Tri, trihexyphenidyl hydrochloride.

^a Lag time was defined as the point of intersection with the abscissa of the line determined by the pseudolinear portion of the fluorescence progress curve, according to Evans et al., 1995.

^b Growth rate was determined by line fitting to the pseudolinear segment of the ascending portion of the fluorescence progress curve.

^c Determined by visual inspection.

In the absence of compounds, ThS fluorescence of α S was almost unchanged during 6 hours (Fig. 3A and B) as previously described (Ono and Yamada, 2006). After incubation of α S with Tri, either at a compound:peptide ratio of 5:14 or 25:7, the ThS fluorescence was similar to that of the untreated peptide, within experimental error (Fig. 3A). In contrast, Mel showed significant destabilization effects (Fig. 3B). These strong effects of Mel were in a concentration-dependent manner, suggesting decrease of final β -sheet levels.

Using EM, we determined the morphological change of the preformed α S with time course. Classical amyloid-like fibrils were observed in samples of fresh α S (Fig. 3C). At substoichiometric concentration (compound:peptide ratio of 5:14), treatment of α S with 25 μ M Mel for 6 hours clearly reduced fibril number and many short, shared fibrils were observed (Fig. 3D). After incubation of fresh α S with 250 μ M Mel (compound:peptide ratio of 25:7) for 1 hour, many sheared fibrils were observed (Fig. 3E). At 6 hours, the number of fibrils was reduced markedly, and small amorphous aggregates were occasionally observed (Fig. 3F). In contrast to the results with Mel, Tri did not have a destabilizing effect at 250- μ M concentration (Fig. 3G).

3.3. Protofibril formation

To determine the effect of Mel, we monitored the process of protofibril formation by SEC. Incubation of α S alone produced chromatograms containing 2 predominant peaks, the first eluting at 14 minutes and the second eluting at 18 minutes (Fig. 4A). We confirmed that the second peak shows a monomer band of α S, and called this nominal monomer fraction low molecular weight (LMW) α S. On the other hand, the first peak (void fraction) did not include the small bands because SDS-stable larger aggregates composed mainly of protofibrils could not be moved into the separating gel (Fig. 4B).

To quantitatively compare the temporal changes in protofibril and LMW contents among samples, we integrated the areas under the first and second peaks, and graphed them versus time (Fig. 4C and D). Untreated α S displayed a monotonic increase in protofibril amount until plateau levels were reached at 4 days (Fig. 4C). When α S was incubated with Tri at a compound:peptide ratio of 25:7, protofibril formation occurred with a kinetics indistinguishable from that of α S alone. In contrast, highly significant inhibition of protofibril formation was observed in the presence of Mel. Small increases in protofibril amount were observed until 3 days, at which point the amount reached plateau at a level fourfold lower than that of α S alone. On the other hand, untreated α S displayed a monotonic decrease in LMW amount until minimal levels were reached at 4 days (Fig. 4D). When α S was incubated with Tri at a compound:peptide ratio of 25:7, the LMW amount changed with a kinetics indistinguishable from that of α S alone. In contrast, highly significant inhibition of the decrease of LMW amount was observed in the presence of Mel. Small decreases in LMW amount were observed until 4 days, at which point the amount plateaued at a level fourfold higher than that of α S alone.

To determine the morphology of the assemblies present after α S incubation with or without compounds, we examined samples of void fraction after 6 days of incubation using EM. Untreated α S (Fig. 4E) produced short, relatively narrow (7–8 nm) structures displaying periodic substructure reminiscent of beaded strings. Similar structures were observed in samples that had been treated with Tri (Fig. 4F). However, grids of α S samples treated with Mel contained few structures and these structures were composed of fewer subunits than were structures formed in the presence of Tri or in the absence of added compounds (compare Fig. 4G with Fig. 4E and F).

3.4. α S oligomerization

We next determined whether Mel blocked protofibril formation by low-order α S oligomers or whether oligomerization itself was blocked. We applied PICUP, a photochemical crosslinking method that is rapid, efficient, requires no structural modification of peptide (for a review, see Bitan and Teplow, 2004), and accurately reveals the oligomerization state of $\alpha\beta$ as well as α S (Bitan and Teplow, 2004; Li et al., 2006). In the absence of crosslinking, there was only α S monomer (Fig. 5A, lane 2). After crosslinking, α S existed as a mixture of monomers and oligomers of order 2–4 (Fig. 5A, lane 3).

The oligomerization of α S in the presence of Tri produced oligomer distribution indistinguishable from that of α S alone (Fig. 5A, lane 4). A 10-fold increase in the compound:peptide ratio did not alter the distribution significantly (Fig. 5A, lane 5).

Mel mixed with α S at 25 μ M (compound:peptide ratio of 5:14) blocked oligomerization almost completely (Fig. 5A,

Fig. 3. Melatonin destabilizes preformed α -synuclein (α S) fibrils. (A, B) Thioflavin S (ThS) binding; 70 μ M α S fibrils was incubated for 6 hours at 37 °C in 20 mM Tris buffer, pH 7.4, in the presence of 0 ($\circ$), 25 ($\bullet$), or 250 (Δ) μ M trihexyphenidyl hydrochloride (Tri) (A) or melatonin (Mel) (B). Periodically, aliquots were removed and ThS binding levels were determined. Binding is expressed as mean fluorescence (in arbitrary fluorescence units [FU]) $\pm$ standard error. Each figure comprises data obtained in 3 independent experiments. (C–G) α S destabilization morphology. Electron microscopy (EM) was used to determine the morphologies of destabilization of α S fibrils incubated at 37 °C for 6 hours in 20 mM Tris buffer, pH 7.4. α S fibrils was incubated in the presence of 25 μ M Mel (D), 250 μ M Mel (C, E, F), or 250 μ M Tri (G) for 0 (C), 1 (E), or 6 hours (D, F, G). Scale bars indicate 100 nm.

lane 6). Increasing the compound:peptide ratio 10-fold produced similar levels of inhibition (Fig. 5A, lane 7). We also confirmed dose-dependency of this inhibition (Supplementary Fig. 1A and B). Mel exhibited an inhibitory effect on α S oligomerization at 2.5 μ M (compound:peptide ratios of 1:28), and almost completely inhibited it at 10 μ M (compound: peptide ratios of 2:14) (Supplementary Fig. 1A and B). EC50 of Mel for the oligomerization of α S was 2.7 μ M.

The strong inhibition of α S oligomerization could have resulted from an effect of the inhibitor on the PICUP chemistry itself. To evaluate this possibility, crosslinking reactions also were performed on GST (26 kDa), a positive control for the crosslinking chemistry (Fancy and Kodadek, 1999). Uncrosslinked GST exhibited an intense monomer band and a relatively faint dimer band (Fig. 5B, lane 2).

Crosslinking produced an intense dimer band, expected because GST exists normally as a homodimer, as well as higher order crosslinked species (Fig. 5B, lane 3). No alterations in GST crosslinking were observed in the presence of Tri at either of the 2 compound:protein ratios tested, 1:1 (Fig. 5B, lane 4) or 10:1 (Fig. 5B, lane 5). Similar distributions were observed with Mel at both 1:1 and 10:1 ratios (Fig. 5B, lanes 6 and 7). A chemistry effect cannot explain the strong inhibition of α S oligomerization, and the lack of inhibition of GST oligomerization.

To determine the morphology of the small assemblies present following PICUP of α S with or without compounds, we examined α S samples using AFM. The height of untreated α S was 0.56 ± 0.36 nm ($n = 47$) (Fig. 5C). After PICUP, the height of α S oligomers became 1.53 ± 0.77 nm

Fig. 4. Protofibril formation. α -Synuclein (α S) peptides were incubated alone (○) or in the presence of 250 μ M trihexyphenidyl hydrochloride (Tri) (●) or melatonin (Mel) (▲). Periodically during incubation, aliquots were analyzed by size-exclusion chromatography (SEC) to quantify protofibril formation. (A) SEC of α S incubated alone at 37 °C during 0–6 days reveals a protofibril peak eluting at 14 minutes, along with a low molecular weight (LMW) α S peak at 18 minutes. These 2 peaks are followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining (B). Lane 1, molecular weight markers; 2, peak (1); 3, peak (2). The areas under the protofibril (C) and LMW (D) peaks (see A) were integrated to determine temporal changes in protofibril and LMW amounts in samples. Areas are expressed as mean area $\pm$ standard error. Each figure comprises data obtained in 3 independent experiments. (E–G) Protofibrils morphology. Electron microscopy (EM) was used to determine the morphologies of protofibrils obtained by SEC after incubation of α S (E–G) at 37 °C for 6 days in 20 mM Tris buffer, pH 7.4. α S peptides were incubated in buffer alone (E) or in the presence of 250 μ M Tri (F) or Mel (G). Scale bars indicate 100 nm.

($n = 29$) (Fig. 5D). When α S was crosslinked with Tri at a compound:peptide ratio of 25:7, the height of treated α S was 1.42 ± 0.53 nm ($n = 29$) (Fig. 5E). When α S was crosslinked with Mel at a compound:peptide ratio of 5:14, the height of treated α S was 0.57 ± 0.44 nm ($n = 37$) (Fig. 5F).

3.5. α S secondary structure dynamics

The oligomerization studies revealed effects of Mel at the initial stages of peptide self-association. To probe the secondary structure of α S at this stage, and to determine if Mel affected later conformational properties of the peptide monomer or its oligomers, CD was used to monitor peptide

assembly (Fig. 6). α S, incubated alone, produced initial spectra characteristic of statistical coils (Fig. 6A). The major feature of these spectra was a large magnitude minimum centered at 198 nm. A significant conformational transition occurred during the subsequent 3 days, producing the spectra which was a substantial minimum centered at 216 nm, indicative of β -sheet structure. Similar conformational transition was observed in populations of α S in the presence of Tri (Fig. 6B). When Mel was mixed with α S at a compound:peptide ratio of 5:14, the secondary structure transition from statistical coil to mixture with predominant α -helix was observed (Fig. 6C). A 10-fold increase in the compound:peptide ratio produced almost complete

Fig. 5. α -Synuclein (α S) oligomerization. Photoinduced crosslinking of unmodified proteins (PICUP), followed by SDS-PAGE and silver staining, was used to determine the effects of melatonin (Mel) or trihexyphenidyl hydrochloride (Tri) on oligomerization of α S (A), or glutathione S-transferase (GST) (B). Lane 1, molecular weight markers; 2, proteins alone (no crosslinking); 3, proteins alone; 4, proteins plus Tri (25 μ M); 5, proteins plus Tri (250 μ M); 6, proteins plus Mel (25 μ M); 7, protein plus Mel (250 μ M). The gel is representative of 3 independent experiments. (C–F) Oligomer morphology. Atomic force microscopy (AFM) was used to determine the morphologies of oligomers obtained by PICUP of α S alone (no crosslinking) (C), α S alone (after crosslinking) (D), α S with 250 μ M Tri (after crosslinking) (E), α S with 25 μ M Mel (after crosslinking) (F). Scale bars indicate 100 nm.

inhibition (Fig. 6D), suggesting that Mel-treated α S revealed populations of conformers that were largely a statistical coil.

3.6. α S-mediated cellular toxicity

To investigate the ability of Mel to block α S-mediated cellular toxicity, we used primary mixed neurons obtained

from mesencephalon and neostriatum to perform MTT assay to probe cellular metabolism. The experimental design was the protocol: incubating Mel only, α S alone, or α S with Mel for various times prior to addition to cells (Fig. 7). When cells were exposed to the samples, the assays proceeded as described elsewhere (Abe and Saito, 1998; Storch et al., 2004).

Fig. 6. α -Synuclein (α S) secondary structure dynamics. (A–D) 35 μ M α S were incubated at 37 $^{\circ}$ C for 6 days in 20 mM Tris buffer, pH 7.4, in buffer alone (A) or in the presence of 250 μ M trihexyphenidyl hydrochloride (Tri) (B), or 25 μ M (C), or 250 μ M melatonin (Mel) (D). Spectra were acquired immediately at the start of the incubation period, Day 0 ($\circ$), and after Days 1 ($\blacktriangledown$), 2 ($\square$), 3 ($\blacksquare$), 4 ($\diamond$), and 6 ($\blacktriangle$). The spectra presented at each time are representative of those obtained during each of 3 independent experiments.

When α S alone was immediately added to the primary mixed neurons of mesencephalon and neostriatum, its cell viability was not significantly different from Mel only or buffer only (controls) (Fig. 7). The viability of cells with Mel-treated α S was also not significantly different from that of the controls. However, incubation of α S for 2 days, during which time oligomers, protofibrils, and fibril would be formed, produced aggregates that were significantly more toxic. Viability of cells with untreated α S was approximately 70%. Treatment of α S with Mel increased cell viability to approximately 86%, which was a highly significant increase relative to α S alone ($p < 0.01$) (Fig. 7). The same qualitative relationships among these experimental groups were observed after 6 days of incubation. Viability of cells with untreated α S was approximately 83%, which was significantly less toxic than that with α S after 2 days incubation ($p < 0.05$). Treatment of α S with Mel significantly increased cell viability to approximately 97% ($p < 0.01$) (Fig. 7).

4. Discussion

Mel is normally synthesized and secreted during the dark phase of the day. A primary function of Mel secretion is to convey information about daily cycles of light and darkness to body physiology (Srinivasan et al., 2005). A substantial body of evidence suggests that Mel may inhibit fibril formation by a variety of amyloidogenic proteins (Srinivasan et al., 2005). $A\beta$ studies showed that Mel strongly inhibited the spontaneous formation of β -sheets and $A\beta$ fibrils (Pappolla et al., 1998). The protective actions of Mel against $A\beta$ neurotoxicity have been repeatedly confirmed (Poeggeler et al., 2001; Shen et al., 2002a, 2002b, 2002c). Furthermore, Mel inhibited the expected time-dependent elevation of $A\beta$ in a transgenic mouse model of Alzheimer's amyloidosis (Matsubara et al., 2003).

Mel has been shown to attenuate arsenite-induced apoptosis via a reduction of aggregated α S levels in rat brain (Lin et al., 2007) by Western blot analysis. Similarly, Ishido reported that Mel inhibits maneb-induced assembly of α S in

Fig. 7. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) metabolism. α -Synuclein (α S) was incubated with or without melatonin (Mel) in 10 mM Tris, pH 7.4, at 37 °C for 0, 2, and 6 days prior to addition to primary mixed neurons comprised of mesencephalon and neostriatum. Effects of Mel only (open bars), untreated α S (cross-hatched bars), and Mel-treated α S (closed bars) on cell metabolism were determined fluorometrically using MTT 48 hours after sample addition. Each bar represents mean $\pm$ standard error. Statistical significance among groups was determined using 1-way fractional analysis of variance (ANOVA) and multiple comparison tests. Differences reaching statistical significance are noted by line segments between samples, along with their associated p values, where * signifies $p < 0.05$ and ** signifies $p < 0.01$.

rat pheochromocytoma cells investigated by immunostaining (Ishido, 2007). Thus, it is reasonable that Mel has an anti-misfolding effect for α S as well as for A β . We sought here to examine more deeply the mechanism of inhibition, a goal critical to accelerating knowledge-based strategies for inhibitor targeting and design.

We began by studying fibril formation in parallel with assessing assembly β -sheet content. EM and ThS experiments revealed that Mel strongly inhibited β -sheet and fibril formation by α S. Working backwards systematically along the α S assembly pathway, we found that Mel also was a highly effective inhibitor of protofibril formation and peptide oligomerization.

Some studies have sought to establish the relative importance of different types of α S assemblies in disease pathogenesis (for a recent review, see Caughey and Lansbury, 2003). For example, protofibrils have been linked to an A30P form of early-onset PD (Conway et al., 1998). Protofibrils forming annular structures may have pore-like properties and might damage membranes (Lashuel et al., 2002; Volles et al., 2001). A linear association/annealing of these spherical species, resembling an A β protofibril, was observed in the preparation of protofibrils (Conway et al., 2000). Morphology of void volume fraction gained by our SEC experiments was consistent with this report. The area of this fraction displayed monotonic increase until plateau

with the incubation. Taken together, our data suggested that Mel strongly inhibits the protofibril formation. Thus, the ability of Mel to inhibit both fibril and protofibril formation suggests that it may be of value for therapeutic strategies targeting these 2 assembly types.

Most recently, new studies have revealed that low-order oligomeric forms of α S are also toxic and critical species (Outeiro et al., 2008; Paleologou et al., 2009; Tsigelny et al., 2008). Outeiro et al. have shown that formation of dimeric and oligomeric α S species, both of which are thought to precede the formation of larger intracellular inclusions, are central steps toward cytotoxicity which can be targeted through the activity of molecular chaperones, such as heat shock protein 70 (Hsp70) (Outeiro et al., 2008). Consistent with this result, toxicity is seen without heavily aggregated α S, and it has been suggested that soluble species mediate toxicity (Xu et al., 2002). It was reported that soluble spheroid oligomer has 1.5–3.0 nm in height by AFM studies (Apetri et al., 2006), being consistent with our results of AFM. Very recently, it was reported that α S exists physiologically as a helically folded tetramer that precedes α S misfolding and aggregation, suggesting that stabilization of the tetramer could reduce LBD pathogenicity (Bartels et al., 2011).

Recently, annular α S oligomers have been isolated from human brain samples of MSA (Pountney et al., 2004). A novel enzyme-linked immunosorbent assay (ELISA) method revealed an elevation of α S oligomer level in plasma samples obtained from PD patients compared with controls (El-Agnaf et al., 2006), and the levels of soluble oligomers of α S were higher in the DLB brain than in the brain of patients with Alzheimer's disease and the controls (Paleologou et al., 2009), which support the idea that oligomers are the toxic species. Interestingly, EC₅₀ of Mel for the oligomerization of α S was 2.7 μ M (compound:peptide ratios of 1 to approximately 26), suggesting that Mel especially has strong inhibitory effect on oligomerization of α S. The strong ability of Mel to block formation of low-order α S oligomers in our results suggests that it might also be of value for targeting what some have argued are the proximate neurotoxins in LBD (Outeiro et al., 2008; Paleologou et al., 2009; Tsigelny et al., 2008).

Our CD studies, in concert with ThS experiments, showed that Mel produced a conformer population comprising primarily statistical coils. Whether Mel stabilizes unfolded α S conformers or destabilizes folded conformers or oligomeric or fibrillar assemblies cannot be ascertained from the data extant. However, the consequences of Mel treatment in the α S system do appear to differ from those observed in certain other inhibitor:amyloidogenic protein systems. For example, Zhu et al. (2004) reported that the flavonoid baicalein stabilized a partially folded conformer of α S that existed within oligomeric assemblies. Conway et al. (2001) showed that dopamine or levodopa inhibits the fibrillization of α S filaments, presumably through stabiliza-

tion of α S into protofibrillar structures unable to form fibrils. Taniguchi et al. (2005) reported the formation of tau oligomers in the presence of phenothiazines, polyphenols, or porphyrins. In each of these cases, the inhibitors stabilized oligomeric states in which the respective protein maintained at least a partial fold. The ability of Mel to block α S monomer folding and, especially, oligomerization thus is a particularly important aspect of a mechanism underlying its effect.

The most important biological consequence of α S association is the production of neurotoxic assemblies. In the work reported here, assemblies of α S that were added to cultures of primary neurons caused significant cellular damage, as measured by effects on MTT metabolism. There are some reports that the increase of cell viability in MTT assay was consistent with the increase of tyrosine hydroxylase activity in the cells of mesencephalon (Nobre-Júnior et al., 2009) and neostriatum (Barrachina et al., 2003). Our results that nonaggregated α S is less toxic than aggregated α S as well as that intermediate aggregates are more toxic than final aggregates were not inconsistent with reports that early intermediates of α S are toxic and critical species (Outeiro et al., 2008; Paleologou et al., 2009; Tsigelny et al., 2008). Mel substantially reduced these toxic effects after pretreatment of α S during assembly. Cellular injury caused by α S-mediated perturbation of cellular redox reactions is an important proposed disease mechanism in LBD (George et al., 2009). Prior studies have shown that Mel exhibits substantial antioxidant properties (Kotler et al., 1998; Reiter et al., 1997) so that Mel has been proposed as a potential therapeutic agent in diseases in which oxidative stress is thought to be a major pathogenic factor. There is 1 opposite report that melatonin is not always neuroprotective using the rotenone model of PD (Tapias et al., 2009). However, as shown in this report, Mel dose-dependently inhibits all steps of α S assembly process. Moreover, cell culture experiments with primary neurons suggested that Mel-treated α S assemblies might be less toxic than intact α S assemblies. Thus, it may be reasonable to speculate that Mel could delay the development of LBD, not only through scavenging reactive oxygen species, but also through directly inhibiting the assembly of α S in the brain.

The concentrations of Mel are in the low nanomolar range in the blood of human. As far as cerebrospinal fluid (CSF) is concerned, peak Mel concentrations ranged from 94 to 355 pm in human (Bruce et al., 1991; Reiter, 1991). The effective concentrations of Mel for α S assembly in our experiment may be somewhat higher compared with physiological levels of Mel in the brain. However, Mel exhibited antiassembly effects at substoichiometric concentration in all steps of α S assembly process, especially oligomerization. As Mel readily crosses the blood-brain barrier, Mel may exhibit antiassembly activities in vivo when administered in high doses and for a long time.

In conclusion, our demonstration here of the potent inhibitory effects of Mel on α S assembly, coupled with previously reported redox-based protective and ameliorative effects of Mel, suggest that Mel is worthy of consideration as a therapeutic agent for LBD.

Disclosure statement

The authors disclose no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.neurobiolaging.2011.10.015](https://doi.org/10.1016/j.neurobiolaging.2011.10.015).

References

- Abe, K., Saito, H., 1998. Amyloid β protein inhibits cellular MTT reduction not by suppression of mitochondrial succinate dehydrogenase but by acceleration of MTT formazan exocytosis in cultured rat cortical astrocytes. *Neurosci. Res.* 31, 295–305.
- Apetri, M.M., Maiti, N.C., Zagorski, M.G., Carey, P.R., Anderson, V.E., 2006. Secondary structure of α -synuclein oligomers: characterization by raman and atomic force microscopy. *J. Mol. Biol.* 355, 63–71.
- Baba, M., Nakajo, S., Tu, P.H., Tomita, T., Nakaya, K., Lee, V.M., Trojanowski, J.Q., Iwatsubo, T., 1998. Aggregation of α -synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. *Am. J. Pathol.* 152, 879–884.
- Barrachina, M., Domínguez, I., Ambrosio, S., Secades, J., Lozano, R., Ferrer, I., 2003. Neuroprotective effect of citicoline in 6-hydroxydopamine-lesioned rats and in 6-hydroxydopamine-treated SH-SY5Y human neuroblastoma cells. *J. Neurol. Sci.* 215, 105–110.
- Bartels, T., Choi, J.G., Selkoe, D.J., 2011. α -Synuclein occurs physiologically as a helically folded tetramer that resists aggregation. *Nature* 477, 107–110.
- Bitan, G., Lomakin, A., Teplow, D.B., 2001. Amyloid β -protein oligomerization: prenucleation interactions revealed by photo-induced cross-linking of unmodified proteins. *J. Biol. Chem.* 276, 35176–35184.
- Bitan, G., Teplow, D.B., 2004. Rapid photochemical cross-linking—a new tool for studies of metastable, amyloidogenic protein assemblies. *Acc. Chem. Res.* 37, 357–364.
- Bruce, J., Tamarkin, L., Riedel, C., Markey, S., Oldfield, E., 1991. Sequential cerebrospinal fluid and plasma sampling in humans: 24-hour melatonin measurements in normal subjects and after peripheral sympathectomy. *J. Clin. Endocrinol. Metab.* 72, 819–823.

- Caughey, B., Lansbury, P.T., 2003. Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders. *Annu. Rev. Neurosci.* 26, 267–298.
- Conway, K.A., Harper, J.D., Lansbury, P.T., 1998. Accelerated in vitro fibril formation by a mutant α -synuclein linked to early-onset Parkinson disease. *Nat. Med.* 4, 1318–1320.
- Conway, K.A., Harper, J.D., Lansbury, P.T., Jr., 2000. Fibrils formed in vitro from α -synuclein and two mutant forms linked to Parkinson's disease are typical amyloid. *Biochemistry* 39, 2552–2563.
- Conway, K.A., Rochet, J.C., Bieganski, R.M., Lansbury, P.T., Jr., 2001. Kinetic stabilization of the α -synuclein protofibril by a dopamine- α -synuclein adduct. *Science* 294, 1346–1349.
- El-Agnaf, O.M., Salem, S.A., Paleologou, K.E., Curran, M.D., Gibson, M.J., Court, J.A., Schlossmacher, M.G., Allsop, D., 2006. Detection of oligomeric forms of α -synuclein protein in human plasma as a potential biomarker for Parkinson's disease. *FASEB J.* 20, 419–425.
- Evans, K.C., Berger, E.P., Cho, C.G., Weisgraber, K.H., Lansbury, P.T., Jr., 1995. Apolipoprotein E is a kinetic but not a thermodynamic inhibitor of amyloid formation: implications for the pathogenesis and treatment of Alzheimer disease. *Proc. Natl. Acad. Sci. U. S. A.* 92, 763–767.
- Fancy, D.A., Kodadek, T., 1999. Chemistry for the analysis of protein-protein interactions: rapid and efficient cross-linking triggered by long wavelength light. *Proc. Natl. Acad. Sci. U. S. A.* 96, 6020–6024.
- Feany, M.B., Bender, W.W., 2000. A Drosophila model of Parkinson's disease. *Nature* 404, 394–398.
- Forno, L.S., 1996. Neuropathology of Parkinson's disease. *J. Neuropathol. Exp. Neurol.* 55, 259–272.
- Gai, W.P., Power, J.H., Blumbergs, P.C., Blessing, W.W., 1998. Multiple-system atrophy: a new α -synuclein disease? *Lancet* 352, 547–548.
- George, J.L., Mok, S., Moses, D., Wilkins, S., Bush, A.I., Cherny, R.A., Finkelstein, D.I., 2009. Targeting the progression of Parkinson's disease. *Curr. Neuropharmacol.* 7, 9–36.
- Giasson, B.I., Duda, J.E., Quinn, S.M., Zhang, B., Trojanowski, J.Q., Lee, V.M., 2002. Neuronal α -synucleinopathy with severe movement disorder in mice expressing A53T human α -synuclein. *Neuron* 34, 521–533.
- Gilman, S., Low, P.A., Quinn, N., Albanese, A., Ben-Shlomo, Y., Fowler, C.J., Kaufmann, H., Klockgether, T., Lang, A.E., Lantos, P.L., Litvan, I., Mathias, C.J., Oliver, E., Robertson, D., Schatz, I., Wenning, G.K., 1999. Consensus statement on the diagnosis of multiple system atrophy. *J. Neurol. Sci.* 163, 94–98.
- Goedert, M., 2001. Parkinson's disease and other α -synucleinopathies. *Clin. Chem. Lab. Med.* 39, 308–312.
- Goto, K., Mochizuki, H., Hattori, T., Nakamura, N., Mizuno, Y., 1997. Neurotoxic effects of papaverine, tetrahydropapaverine and dimethoxyphenylethylamine on dopaminergic neurons in ventral mesencephalic-striatal co-culture. *Brain Res.* 754, 260–268.
- Ishido, M., 2007. Melatonin inhibits maneb-induced aggregation of α -synuclein in rat pheochromocytoma cells. *J. Pineal Res.* 42, 125–130.
- Kotler, M., Rodríguez, C., Sáinz, R.M., Antolín, I., Menéndez-Peláez, A., 1998. Melatonin increases gene expression for antioxidant enzymes in rat brain cortex. *J. Pineal Res.* 24, 83–89.
- Lashuel, H.A., Petre, B.M., Wall, J., Simon, M., Nowak, R.J., Walz, T., Lansbury, P.T., Jr., 2002. α -Synuclein, especially the Parkinson's disease-associated mutants, forms pore-like annular and tubular protofibrils. *J. Mol. Biol.* 322, 1089–1102.
- Lee, M.K., Stirling, W., Xu, Y., Xu, X., Qui, D., Mandir, A.S., Dawson, T.M., Copeland, N.G., Jenkins, N.A., Price, D.L., 2002. Human α -synuclein-harboring familial Parkinson's disease-linked Ala-53 $\rightarrow$ Thr mutation causes neurodegenerative disease with α -synuclein aggregation in transgenic mice. *Proc. Natl. Acad. Sci. U. S. A.* 99, 8968–8973.
- LeVine, H., 3rd, 1993. Thioflavine T interaction with synthetic Alzheimer's disease β -amyloid peptides: detection of amyloid aggregation in solution. *Protein Sci.* 2, 404–410.
- LeVine, H., 3rd, 1999. Quantification of β -sheet amyloid fibril structures with thioflavin T. *Methods Enzymol.* 309, 274–284.
- Li, H.T., Lin, X.J., Xie, Y.Y., Hu, H.Y., 2006. The early events of α -synuclein oligomerization revealed by photo-induced cross-linking. *Protein Pept. Lett.* 13, 385–390.
- Li, J., Zhu, M., Rajamani, S., Uversky, V.N., Fink, A.L., 2004. Rifampicin inhibits α -synuclein fibrillation and disaggregates fibrils. *Chem. Biol.* 11, 1513–1521.
- Lin, A.M., Fang, S.F., Chao, P.L., Yang, C.H., 2007. Melatonin attenuates arsenite-induced apoptosis in rat brain: involvement of mitochondrial and endoplasmic reticulum pathways and aggregation of α -synuclein. *J. Pineal Res.* 43, 163–171.
- López-Burillo, S., Tan, D.X., Mayo, J.C., Sainz, R.M., Manchester, L.C., Reiter, R.J., 2003. Melatonin, xanthurenic acid, resveratrol, EGCG, vitamin C and α -lipoic acid differentially reduce oxidative DNA damage induced by Fenton reagents: a study of their individual and synergistic actions. *J. Pineal Res.* 34, 269–277.
- Matsubara, E., Bryant-Thomas, T., Pacheco Quinto, J., Henry, T.L., Poegeler, B., Herbert, D., Cruz-Sanchez, F., Chyan, Y.J., Smith, M.A., Perry, G., Shoji, M., Abe, K., Leone, A., Grundke-Ikbal, I., Wilson, G.L., Ghiso, J., Williams, C., Refolo, L.M., Pappolla, M.A., Chain, D.G., Neria, E., 2003. Melatonin increases survival and inhibits oxidative and amyloid pathology in a transgenic model of Alzheimer's disease. *J. Neurochem.* 85, 1101–1108.
- McKeith, I.G., Dickson, D.W., Lowe, J., Emre, M., O'Brien, J.T., Feldman, H., Cummings, J., Duda, J.E., Lippa, C., Perry, E.K., Aarsland, D., Arai, H., Ballard, C.G., Boeve, B., Burn, D.J., Costa, D., Del Ser, T., Dubois, B., Galasko, D., Gauthier, S., Goetz, C.G., Gomez-Tortosa, E., Halliday, G., Hansen, L.A., Hardy, J., Iwatsubo, T., Kalaria, R.N., Kaufer, D., Kenny, R.A., Korczyn, A., Kosaka, K., Lee, V.M., Lees, A., Litvan, I., Londos, E., Lopez, O.L., Minoshima, S., Mizuno, Y., Molina, J.A., Mukaetova-Ladinska, E.B., Pasquier, F., Perry, R.H., Schulz, J.B., Trojanowski, J.Q., Yamada, M., Consortium on DLB, 2005. Diagnosis and management of dementia with Lewy bodies: third report of the DLB Consortium. *Neurology* 65, 1863–1872.
- Mochizuki, H., Nakamura, N., Nishi, K., Mizuno, Y., 1994. Apoptosis is induced by 1-methyl-4-phenylpyridinium ion (MPP+) in ventral mesencephalic-striatal co-culture in rat. *Neurosci. Lett.* 170, 191–194.
- Naiki, H., Nakakuki, K., 1996. First-order kinetic model of Alzheimer's β -amyloid fibril extension in vitro. *Lab. Invest.* 74, 374–383.
- Nobre-Júnior, H.V., Oliveira, R.A., Maia, F.D., Nogueira, M.A., de Moraes, M.O., Bandeira, M.A., Andrade, G.M., Viana, G.S., 2009. Neuroprotective effects of chalcones from *Myracrodruon urundeuva* on 6-hydroxydopamine-induced cytotoxicity in rat mesencephalic cells. *Neurochem. Res.* 34, 1066–1075.
- Ono, K., Hirohata, M., Yamada, M., 2007. Anti-fibrillogenic and fibril-destabilizing activities of anti-Parkinsonian agents for α -synuclein fibrils in vitro. *J. Neurosci. Res.* 85, 1547–1557.
- Ono, K., Yamada, M., 2006. Antioxidant compounds have potent anti-fibrillogenic and fibril-destabilizing effects for α -synuclein fibrils in vitro. *J. Neurochem.* 97, 105–115.
- Outeiro, T.F., Putcha, P., Tetzlaff, J.E., Spoelgen, R., Koker, M., Carvalho, F., Hyman, B.T., McLean, P.J., 2008. Formation of toxic oligomeric α -synuclein species in living cells. *PLoS One* 3, e1867.
- Paleologou, K.E., Kragh, C.L., Mann, D.M., Salem, S.A., Al-Shami, R., Allsop, D., Hassan, A.H., Jensen, P.H., El-Agnaf, O.M., 2009. Detection of elevated levels of soluble α -synuclein oligomers in post-mortem brain extracts from patients with dementia with Lewy bodies. *Brain* 132, 1093–1101.
- Pappolla, M., Bozner, P., Soto, C., Shao, H., Robakis, N.K., Zagorski, M., Frangione, B., Ghiso, J., 1998. Inhibition of Alzheimer β -fibrillogenesis by melatonin. *J. Biol. Chem.* 273, 7185–7188.
- Pévet, P., Agez, L., Bothorel, B., Saboureaux, M., Gauer, F., Laurent, V., Masson-Pévet, M., 2006. Melatonin in the multi-oscillatory mammalian circadian world. *Chronobiol. Int.* 23, 39–51.

- Poeggeler, B., Miravalle, L., Zagorski, M.G., Wisniewski, T., Chyan, Y.J., Zhang, Y., Shao, H., Bryant-Thomas, T., Vidal, R., Frangione, B., Ghiso, J., Pappolla, M.A., 2001. Melatonin reverses the profibrillogenic activity of apolipoprotein E4 on the Alzheimer amyloid A β peptide. *Biochemistry* 40, 14995–15001.
- Pountney, D.L., Lowe, R., Quilty, M., Vickers, J.C., Voelcker, N.H., Gai, W.P., 2004. Annular α -synuclein species from purified multiple system atrophy inclusions. *J. Neurochem.* 90, 502–512.
- Reiter, R., Tang, L., Garcia, J.J., Muñoz-Hoyos, A., 1997. Pharmacological actions of melatonin in oxygen radical pathophysiology. *Life Sci.* 60, 2255–2271.
- Reiter, R.J., 1991. Pineal melatonin: cell biology of its synthesis and of its physiological interactions. *Endocr. Rev.* 12, 151–180.
- Shen, Y.X., Wei, W., Xu, S.Y., 2002a. Protective effects of melatonin on cortico-hippocampal neurotoxicity induced by amyloid β -peptide 25–35. *Acta Pharmacol. Sin.* 23, 71–76.
- Shen, Y.X., Xu, S.Y., Wei, W., Sun, X.X., Liu, L.H., Yang, J., Dong, C., 2002b. The protective effects of melatonin from oxidative damage induced by amyloid β -peptide 25–35 in middle-aged rats. *J. Pineal Res.* 32, 85–89.
- Shen, Y.X., Xu, S.Y., Wei, W., Wang, X.L., Wang, H., Sun, X., 2002c. Melatonin blocks rat hippocampal neuronal apoptosis induced by amyloid β -peptide 25–35. *J. Pineal Res.* 32, 163–167.
- Spillantini, M.G., Crowther, R.A., Jakes, R., Cairns, N.J., Lantos, P.L., Goedert, M., 1998. Filamentous α -synuclein inclusions link multiple system atrophy with Parkinson's disease and dementia with Lewy bodies. *Neurosci. Lett.* 251, 205–208.
- Srinivasan, V., Pandi-Perumal, S.R., Maestroni, G.J., Esquifino, A.I., Hardeland, R., Cardinali, D.P., 2005. Role of melatonin in neurodegenerative diseases. *Neurotox. Res.* 7, 293–318.
- Storch, A., Hwang, Y.I., Gearhart, D.A., Beach, J.W., Neafsey, E.J., Collins, M.A., Schwarz, J., 2004. Dopamine transporter-mediated cytotoxicity of β -carbolinium derivatives related to Parkinson's disease: relationship to transporter-dependent uptake. *J. Neurochem.* 89, 685–694.
- Taniguchi, S., Suzuki, N., Masuda, M., Hisanaga, S., Iwatsubo, T., Goedert, M., Hasegawa, M., 2005. Inhibition of heparin-induced tau filament formation by phenothiazines, polyphenols, and porphyrins. *J. Biol. Chem.* 280, 7614–7623.
- Tapias, V., Cannon, J.R., Greenamyre, J.T., 2009. Melatonin treatment potentiates neurodegeneration in a rat rotenone Parkinson's disease model. *J. Neurosci. Res.* 88, 420–427.
- Tsigelny, I.F., Crews, L., Desplats, P., Shaked, G.M., Sharikov, Y., Mizuno, H., Spencer, B., Rockenstein, E., Trejo, M., Platoshyn, O., Yuan, J.X., Masliah, E., 2008. Mechanisms of hybrid oligomer formation in the pathogenesis of combined Alzheimer's and Parkinson's diseases. *PLoS One* 3, e3135.
- Volles, M.J., Lansbury, P.T., Jr., 2003. Zeroing in on the pathogenic form of α -synuclein and its mechanism of neurotoxicity in Parkinson's disease. *Biochemistry* 42, 7871–7878.
- Volles, M.J., Lee, S.J., Rochet, J.C., Shtilerman, M.D., Ding, T.T., Kessler, J.C., Lansbury, P.T., Jr., 2001. Vesicle permeabilization by protofibrillar α -synuclein: implications for the pathogenesis and treatment of Parkinson's disease. *Biochemistry* 40, 7812–7819.
- Wood, S.J., Wypych, J., Steavenson, S., Louis, J.C., Citron, M., Biere, A.L., 1999. α -synuclein fibrillogenesis is nucleation-dependent. Implications for the pathogenesis of Parkinson's disease. *J. Biol. Chem.* 274, 19509–19512.
- Xu, J., Kao, S.Y., Lee, F.J., Song, W., Jin, L.W., Yankner, B.A., 2002. Dopamine-dependent neurotoxicity of α -synuclein: a mechanism for selective neurodegeneration in Parkinson disease. *Nat. Med.* 8, 600–606.
- Yazawa, I., Giasson, B.I., Sasaki, R., Zhang, B., Joyce, S., Uryu, K., Trojanowski, J.Q., Lee, V.M., 2005. Mouse model of multiple system atrophy α -synuclein expression in oligodendrocytes causes glial and neuronal degeneration. *Neuron* 45, 847–859.
- Zhu, M., Rajamani, S., Kaylor, J., Han, S., Zhou, F., Fink, A.L., 2004. The flavonoid baicalein inhibits fibrillation of α -synuclein and disaggregates existing fibrils. *J. Biol. Chem.* 279, 26846–26857.