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

Permalink
https://escholarship.org/uc/item/6s07s5xq

Journal
Neurobiology of Aging, 33(9)

ISSN
0197-4580

Authors
Ono, K
Mochizuki, H
Ikeda, T
et al.

Publication Date
2012-09-01

DOI
10.1016/j.neurobiolaging.2011.10.015

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

Kenjiro Ono, Hideki Mochizuki, Tokuhei Ikeda, Tomoko Nihira, Jun-ichi Takasaki, David B. Teplow, Masahito Yamada

Abstract

α-Synuclein (αS) 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 αS assembly. We also examined the effects of Mel on αS-induced cytotoxicity by assaying 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide metabolism in αS-treated, primary neuronal cells. Initial studies revealed that Mel blocked αS fibril formation as well as destabilizing preformed αS 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 αS-induced cytotoxicity. These data suggest a mechanism of action for Mel, inhibition of assembly of toxic polymers and protection of neurons from their effect.

© 2012 Elsevier Inc. All rights reserved.

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 hallucinations, 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 (αS) (Baba et al., 1998; Forno, 1996; Goedert, 2001). Several transgenic animal models overexpressing human αS display varying degrees of biochemical, pathological, and clinical abnormalities reminiscent of PD (Feany and Bender, 2000; Gasson et al., 2002; Lee et al., 2002).

αS 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.,...
1998). A recent study in mice demonstrated that overexpression of αS 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 αS 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 αS 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 αS fibrils (fαS), as well as destabilize preformed fαS in vitro (Li et al., 2004; Ono and Yamada, 2006; Zhu et al., 2004).

The conversion of αS occurs via a multiple-step process involving nonfibrillar aggregates such as protofibrils or oligomers on αS assembly pathway (Caughey and Lansbury, 2003). As in the case of amyloid β-protein (Aβ), there is mounting evidence that protofibrils or oligomers of αS are more toxic than fαS 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 oncostasis, 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 neurotoxicity by inhibition of both caspase-3/7 activation and disruption of the mitochondrial transmembrane potential (Ishido, 2007). He also reported that Mel inhibits αS assembly by using immunostaining (Ishido, 2007).

In the studies reported here, we sought to determine whether Mel affected αS conformational dynamics and assembly, and whether these effects correlated with αS cytotoxicity. We treated αS 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 αS and fαS solutions

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

2.3. Peptide aggregation

αS 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 αS 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 μM. One-half mL of each compound then was added to separate tubes of αS, yielding final αS concentrations of 70 μM and final inhib-

![Fig. 1. Structures of melatonin and trihexyphenidyl.](image-url)
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

αS 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 αS 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 Biomedicals, 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. Hitachi F-2500 fluorometer (Tokyo, Japan). Excitation and emission wavelengths were 440 and 521 nm, respectively.

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 (Okenshoji, 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) (Ru(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 μL 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

\[ R_i = I_i / \sum I_i \times 100 \ (\%) \]

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 αS with 1, 2.5, 5, 10, 25, and 250 μM 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 αS 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-μL total volume per well) on 96-well plates (Nalge Nunc International K.K., Tokyo, Japan) with 5% polyethyleneimine (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 μM) 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 to F12/DMEM (Gibco) 5% (vol/vol) calf serum, 5% (vol/vol) horse 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 αS with either 0 μM or 25 μM 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-μL 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 μM Mel alone, 7 μM αS alone, or with αS plus 2.5 μM Mel. Peptide:compound ratios of αS 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 μL 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 ± standard error. Cell viability = (A_sample/(A_medium)) × 100, where A_sample and A_medium were absorbance values from Mel alone or αS-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 αS 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 αS 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 αS assembly (Ono et al., 2007).

In the absence of compounds, αS 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 αS assembly (Wood et al., 1999). When αS 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).
Using EM, we determined the morphology of the αS assemblies when maximal ThS binding was observed. Classical amyloid-like fibrils were observed in samples of untreated αS (Fig. 2C). The αS 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 μM concentration) did not alter the assembly of αS (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 αS with 25 μM Mel clearly reduced fibril number and many short, shared fibrils were observed (Fig. 2E). Treatment of αS with 250 μM 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 αS in the absence or presence of Mel.

Fig. 2. Melatonin inhibits α-synuclein (αS) fibril formation. (A, B) thioflavin S (ThS) binding: 70 μM αS was incubated for 6 days at 37 °C in 20 mM Tris buffer, pH 7.4, in the presence of 0 (○), 25 (●), or 250 (△) μM 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]) ± standard error. Each figure comprises data obtained in 3 independent experiments. (C–F) αS assembly morphology. Electron microscopy (EM) was used to determine the morphologies of assemblies of αS incubated at 37 °C for 6 days in 20 mM Tris buffer, pH 7.4. αS peptide was incubated in buffer alone (C) or in the presence of 250 μM Tri (D), 25 μM Mel (E), or 250 μM Mel (F). Scale bars indicate 100 nm.
In the absence of compounds, ThS fluorescence of αS was almost unchanged during 6 hours (Fig. 3A and B) as previously described (Ono and Yamada, 2006). After incubation of αS 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 αS with time course. Classical amyloid-like fibrils were observed in samples of fresh αS (Fig. 3C). At substoichiometric concentration (compound:peptide ratio of 5:14), treatment of αS with 25 μM Mel for 6 hours clearly reduced fibril number and many short, shared fibrils were observed (Fig. 3D). After incubation of fresh αS with 250 μM 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-μM concentration (Fig. 3G).

### 3.3. Protopilbril formation

To determine the effect of Mel, we monitored the process of protopilbril formation by SEC. Incubation of αS 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 αS, and called this nominal monomer fraction low molecular weight (LMW) αS. On the other hand, the first peak (void fraction) did not include the small bands because SDS-stable larger aggregates composed mainly of protopilbrils could not be moved into the separating gel (Fig. 4B).

To quantitatively compare the temporal changes in protopilbril 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 αS displayed a monotonic increase in protopilbril amount until plateau levels were reached at 4 days (Fig. 4C). When αS was incubated with Tri at a compound:peptide ratio of 25:7, protopilbril formation occurred with a kinetics indistinguishable from that of αS alone. In contrast, highly significant inhibition of protopilbril formation was observed in the presence of Mel. Small increases in protopilbril amount were observed until 3 days, at which point the amount reached plateau at a level fourfold lower than that of αS alone. On the other hand, untreated αS displayed a monotonic decrease in LMW amount until minimal levels were reached at 4 days (Fig. 4D). When αS was incubated with Tri at a compound:peptide ratio of 25:7, the LMW amount changed with a kinetics indistinguishable from that of αS 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 αS alone.

To determine the morphology of the assemblies present after αS incubation with or without compounds, we examined samples of void fraction after 6 days of incubation using EM. Untreated αS (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 αS 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. αS oligomerization

We next determined whether Mel blocked protopilbril formation by low-order αS 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 Aβ as well as αS (Bitan and Teplow, 2004; Li et al., 2006). In the absence of crosslinking, there was only αS monomer (Fig. 5A, lane 2). After crosslinking, αS existed as a mixture of monomers and oligomers of order 2–4 (Fig. 5A, lane 3).

The oligomerization of αS in the presence of Tri produced oligomer distribution indistinguishable from that of αS 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 αS at 25 μM (compound:peptide ratio of 5:14) blocked oligomerization almost completely (Fig. 5A,

---

**Table 1**

Kinetics of α-synuclein (αS) assembly

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lag time (d)</th>
<th>Growth rate (FU/d)</th>
<th>Maximum intensity (FU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>αS</td>
<td>1.2</td>
<td>54.6</td>
<td>129.3</td>
</tr>
<tr>
<td>αS + 25 μM Tri</td>
<td>1.2</td>
<td>58.8</td>
<td>131.2</td>
</tr>
<tr>
<td>αS + 250 μM Tri</td>
<td>1.3</td>
<td>59.4</td>
<td>129.8</td>
</tr>
<tr>
<td>αS + 2.5 mM Tri</td>
<td>1.2</td>
<td>53.4</td>
<td>127.6</td>
</tr>
<tr>
<td>αS + 25 μM Mel</td>
<td>1.4</td>
<td>43.2</td>
<td>99.3</td>
</tr>
<tr>
<td>αS + 250 μM Mel</td>
<td>1.4</td>
<td>8.8</td>
<td>22.8</td>
</tr>
<tr>
<td>αS + 2.5 mM Mel</td>
<td>1.9</td>
<td>5.4</td>
<td>10.5</td>
</tr>
</tbody>
</table>

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.
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 \( \alpha \text{-synuclein (} \alpha \text{S)} \) oligomerization at 2.5 \( \mu \text{M (compound:peptide ratios of 1:28), and almost completely inhibited it at 10 \( \mu \text{M (compound:peptide ratios of 2:14) (Supplementary Fig. 1A and B). EC50 of Mel for the oligomerization of } \alpha \text{S was 2.7 } \mu \text{M.}

The strong inhibition of } \alpha \text{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 } \alpha \text{S oligomerization, and the lack of inhibition of GST oligomerization.}

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

---

**Fig. 3.** Melatonin destabilizes preformed \( \alpha \)-synuclein (\( \alpha \text{S)} \) fibrils. (A, B) Thioflavin S (ThS) binding; 70 \( \mu \text{M } \alpha \text{S fibrils was incubated for 6 hours at 37 °C in 20 mM Tris buffer, pH 7.4, in the presence of 0 (○), 25 (●), or 250 (Δ) } \mu \text{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]) ± standard error. Each figure comprises data obtained in 3 independent experiments. (C–G) } \alpha \text{S destabilization morphology. Electron microscopy (EM) was used to determine the morphologies of destabilization of } \alpha \text{S fibrils incubated at 37 °C for 6 hours in 20 mM Tris buffer, pH 7.4. } \alpha \text{S fibrils was incubated in the presence of 25 } \mu \text{M Mel (D), 250 } \mu \text{M Mel (C, E, F), or 250 } \mu \text{M Tri (G) for 0 (C), 1 (E), or 6 hours (D, F, G). Scale bars indicate 100 nm.}
3.5. αS secondary structure dynamics

The oligomerization studies revealed effects of Mel at the initial stages of peptide self-association. To probe the secondary structure of αS 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). αS, 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 αS in the presence of Tri (Fig. 6B). When Mel was mixed with αS 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
inhibition (Fig. 6D), suggesting that Mel-treated αS revealed populations of conformers that were largely a statistical coil.

3.6. αS-mediated cellular toxicity

To investigate the ability of Mel to block αS-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, αS alone, or αS 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).
When αS 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 αS was also not significantly different from that of the controls. However, incubation of αS 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 αS was approximately 70%. Treatment of αS with Mel increased cell viability to approximately 86%, which was a highly significant increase relative to αS 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 αS was approximately 83%, which was significantly less toxic than that with αS after 2 days incubation (p < 0.05). Treatment of αS 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). Aβ studies showed that Mel strongly inhibited the spontaneous formation of β-sheets and Aβ fibrils (Pappolla et al., 1998). The protective actions of Mel against Aβ neurotoxicity have been repeatedly confirmed (Poeggeler et al., 2001; Shen et al., 2002a, 2002b, 2002c). Furthermore, Mel inhibited the expected time-dependent elevation of Aβ 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 αS levels in rat brain (Lin et al., 2007) by Western blot analysis. Similarly, Ishido reported that Mel inhibits maneb-induced assembly of αS in
of this fraction displayed monotonic increase until plateau
morning. Morphology of void volume fraction gained by our
of different types
highly effective inhibitor of protofibril formation and pep-
tide oligomerization.
ments revealed that Mel strongly inhibited
assessing assembly
for inhibitor targeting and design.

Fig. 7. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) metabolism. α-Synuclein (αS) was incubated with or without melato-
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 neostri-
atum. Effects of Mel only (open bars), untreated αS (cross-hatched bars), and Mel-treated αS (closed bars) on cell metabolism were determined
fluorometrically using MTT 48 hours after sample addition. Each bar
represents mean ± 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 immunostain-
ing (Ishido, 2007). Thus, it is reasonable that Mel has an
anti-misfolding effect for αS as well as for Aβ. We sought
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 experi-
ments revealed that Mel strongly inhibited β-sheet and fibril
formation by αS. Working backwards systematically along
the αS assembly pathway, we found that Mel also was a
highly effective inhibitor of protofibril formation and pep-
tide oligomerization.

Some studies have sought to establish the relative im-
portance of different types of αS assemblies in disease
pathogenesis (for a recent review, see Caughey and Lans-
bury, 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 Aβ 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 αS 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 αS 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
αS, and it has been suggested that soluble species mediate
toxicity (Xu et al., 2002). It was reported that soluble spher-
oid 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 αS exists physi-
ologically as a helically folded tetramer that precedes αS
misfolding and aggregation, suggesting that stabilization of
the tetramer could reduce LBD pathogenicity (Bartels et al.,
2011).

Recently, annular αS 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 αS oligomer level in plasma samples obtained from PD patients compared with
controls (El-Agnaf et al., 2006), and the levels of soluble
oligomers of αS 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 oli-
gomers are the toxic species. Interestingly, EC50 of Mel for
the oligomerization of αS was 2.7 μM (compound:peptide
ratios of 1 to approximately 26), suggesting that Mel espe-
sially has strong inhibitory effect on oligomerization of αS.
The strong ability of Mel to block formation of low-order
αS oligomers in our results suggests that it might also be of
value for targeting what some have argued are the proximi-
ate 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 compris-
ing primarily statistical coils. Whether Mel stabilizes un-
folded αS 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 αS 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 αS that existed within oligomeric assemblies. Conway
et al. (2001) showed that dopamine or levodopa inhibits
the fibrillization of αS filaments, presumably through stabiliza-
tion of αS 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 αS monomer folding and, especially, oligomerization thus is a particularly important aspect of a mechanism underlying its effect.

The most important biological consequence of αS association is the production of neurotoxic assemblies. In the work reported here, assemblies of αS 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 αS is less toxic than aggregated αS as well as that intermediate aggregates are more toxic than final aggregates were not inconsistent with reports that early intermediates of αS are toxic and critical species (Outeiro et al., 2008; Paleologou et al., 2009; Tsigelnny et al., 2008). Mel substantially reduced these toxic effects after pretreatment of αS during assembly. Cellular injury caused by αS-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 αS assembly process. Moreover, cell culture experiments with primary neurons suggested that Mel-treated αS assemblies might be less toxic than intact αS 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 αS 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 αS 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 αS 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 αS 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.

Acknowledgements
We acknowledge the support of a grant for the Knowledge Cluster Initiative (High-Tech Sensing and Knowledge Handling Technology [Brain Technology]) (MY), a grant to the Amyloidosis Research Committee from the Ministry of Health, Labor, and Welfare, Japan (MY and KO), Kanae Foundation for the Promotion of Medical Science (KO), Alumni Association of the Department of Medicine at Showa University (KO), and Nagao Memorial fund (KO). Support from the Jim Easton Consortium for Alzheimer’s Drug Discovery and Biomarkers at UCLA (DBT) is also acknowledged.

Appendix A. Supplementary data

References


Poeggeler, B., Miravalle, L., Zagorski, M.G., Wisniewski, T., Chyan, Y.J.,
Zhang, Y., Shao, H., Bryant-Thomas, T., Vidal, R., Frangione, B.,
activity of apolipoprotein E4 on the Alzheimer amyloid Aβ peptide.
Biochemistry 40, 14995–15001.
Pountney, D.L., Lowe, R., Quilty, M., Vickers, J.C., Voelcker, N.H., Gai,
W.P., 2004. Annular-synuclein species from purified multiple system
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
Shen, Y.X., Wei, W., Xu, S.Y., 2002a. Protective effects of melatonin on
cortico-hippocampal neurotoxicity induced by amyloid β-peptide 25–
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
32, 85–89.
Melatonin blocks rat hippocampal neuronal apoptosis induced by amyl-
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
Srinivasan, V., Pandi-Perumal, S.R., Maestroni, G.J., Esquifino, A.I., Hard-
eland, R., Cardinali, D.P., 2005. Role of melatonin in neurodegenerative
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
Taniguchi, S., Suzuki, N., Masuda, M., Hisanaga, S., Iwatsubo, T., Goed-
ert, M., Hasegawa, M., 2005. Inhibition of heparin-induced tau fila-
ment formation by phenothiazines, polyphenols, and porphyrins.
J. Biol. Chem. 280, 7614–7623.
potentiates neurodegeneration in a rat rotenone Parkinson’s disease
Tsigelny, I.F., Crews, L., Desplats, P., Shaked, G.M., Sharikov, Y.,
Mizuno, H., Spencer, B., Rockenstein, E., Trejo, M., Platschyn, O.,
Yuan, J.X., Masliah, E., 2008. Mechanisms of hybrid oligomer forma-
tion in the pathogenesis of combined Alzheimer’s and Parkinson’s
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
Volles, M.J., Lee, S.J., Rochet, J.C., Shiltlerman, M.D., Ding, T.T., Kessler,
J.C., Lansbury, P.T., Jr., 2001. Vesicle permeabilization by protofibril-
lar α-synuclein: implications for the pathogenesis and treatment of
Wood, S.J., Wypych, J., Steavenson, S., Louis, J.C., Citron, M., Biere,
A.L., 1999. α-synuclein fibrillogensation is nucleation-dependent. Impli-
cations for the pathogenesis of Parkinson’s disease. J. Biol. Chem. 274,
19509–19512.
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.,
atrophy α-synuclein expression in oligodendrocytes causes glial and
flavonoid baicalein inhibits fibrillation of α-synuclein and disaggre-
gates existing fibrils. J. Biol. Chem. 279, 26846–26857.