Melatonin Reverses the Profibrillogenic Activity of Apolipoprotein E4 on the Alzheimer Amyloid Aβ Peptide†

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ABSTRACT: Inheritance of apoE4 is a strong risk factor for the development of late-onset sporadic Alzheimer's disease (AD). Several lines of evidence suggest that apoE4 binds to the Alzheimer Aβ protein and, under certain experimental conditions, promotes formation of β-sheet structures and amyloid fibrils. Deposition of amyloid fibrils is a critical step in the development of AD. We report here that addition of melatonin to Aβ in the presence of apoE resulted in a potent isoform-specific inhibition of fibril formation, the extent of which was far greater than that of the inhibition produced by melatonin alone. This effect was structure-dependent and unrelated to the antioxidant properties of melatonin, since it could be reproduced neither with the structurally related indole *N*-acetyl-5-hydroxytryptamine nor with the antioxidants ascorbate, α-tocophenol, and PBN. The enhanced inhibitory effects of melatonin and apoE were lost when bovine serum albumin was substituted for apoE. In addition, Aβ in combination with apoE was highly neurotoxic (apoE4 > apoE3) to neuronal cells in culture, and this activity was also prevented by melatonin. These findings suggest that reductions in brain melatonin, which occur during aging, may contribute to a proamyloidogenic microenvironment in the aging brain.

Many of the advances in Alzheimer's disease (AD)[1] research have followed the identification of a 40- or 42-amino acid peptide called the Aβ peptide, as the essential pathologic marker of the disease (*1*, *2*). Under specific experimental conditions, Aβ peptides will aggregate spontaneously and eventually produce insoluble amyloid fibrils. In AD brains, there is widespread accumulation of amyloid fibrils mostly within senile plaques, as well as in the cerebral and meningeal vasculature (*3*).

The amyloid cascade hypothesis of AD proposes that formation of aggregated Aβ, a proteolytic cleavage product of the amyloid precursor protein, is a key step in the development of the disorder (*4*). Misprocessing of APP and inadequate clearance of Aβ have both been postulated to play important roles in amyloid accumulation (*4*, *5*). Despite recent advances in AD research, the molecular mechanisms underlying β-amyloid formation remain unknown.

Several studies have demonstrated that amyloid fibril formation requires conformational changes in Aβ peptides (random coil or α-helix → β-sheet), resulting in a larger amount of β-sheet structures (*6*−*9*). These structural alterations are modulated in vitro by interactions between the Aβ peptides and a host of chaperone proteins (*6*, *8*−*11*). Among these interactions, the binding between Aβ and apoE4 have received considerable attention (*9*, *12*) as a result of strong epidemiological and genetic data indicating that inheritance of the apoE4 allele increases the risk of developing AD (*13*, *14*). ApoE4 colocalizes with β-amyloid deposits, and the presence of one or two alleles correlates with the amyloid load in a dose-dependent manner (*15*).

We previously reported that melatonin inhibits the formation of β-sheet structures and β-amyloid fibrils in vitro (*16*). To further characterize these properties, we tested whether the activity of melatonin was of sufficient magnitude to counteract the profibrillogenic properties of apoE4. For this purpose, melatonin was added to preparations containing Aβ, Aβ and apoE3, or Aβ and apoE4, after which formation of β-sheets and amyloid fibrils was monitored at various time intervals using four different methodologies. Under profibrillogenic conditions, the presence of apoE had isoform-specific proaggregatory properties (apoE4 > apoE3) as evidenced by faster formation of β-sheet structures and amyloid fibrils compared to that in Aβ alone. Remarkably, addition of melatonin to these preparations not only reversed these proaggregatory features but also resulted in a more potent inhibition of fibril formation than when melatonin alone was employed. These findings may be relevant to amyloidogenesis.

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[1] Abbreviations: apoE4, apolipoprotein E ϵ4 allele; apoE3, apolipoprotein E ϵ3 allele; AD, Alzheimer's disease; Aβ, amyloid β protein; PBN, *N*-*tert*-butyl-α-phenylnitrone; APP, amyloid precursor protein; ThT, thioflavin T fluorescence assay; TEM, transmission electron microscopy; CD, circular dichroism; T-FTIR, transition Fourier transform infrared spectroscopy; NAS, *N*-acetylserotonin; BSA, bovine serum albumin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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EXPERIMENTAL PROCEDURES

Reagents and Proteins. The Aβ1−40 peptide was synthesized at the W. M. Keck Laboratories (Yale University, New Haven, CT). The peptide purity was evaluated by reverse-phase HPLC, gel electrophoresis, amino acid sequence analysis, and laser desorption mass spectrometry (*17*). Peptide concentrations were accurately calculated by amino acid analysis (*17*). Recombinant apoE3 and apoE4 were purchased from a commercial vendor (Panvera Corp.). Ultrapure melatonin (99.9%) was purchased from Helsinn Pharmaceuticals (Biasca, Switzerland). All other chemicals were purchased from Sigma.

Preparation of lipidated apoE particles was conducted as described elsewhere (*18*). Briefly, astrocytes were cultured from transgenic mice in which human apoE3 or human apoE4 expression was under the control of a GFAP promoter, with the mice being bred on a murine apoE knockout background (*18*). Forebrain astrocytes were prepared from individual 1−2-day-old mouse pups and cultured in T-75 flasks as described previously (*19*). ApoE-containing particles were immunopurified from the conditioned medium of the astrocyte cultures using anti-human apoE (3D12, Biodesign, Inc.) coupled to CNBr-activated Sepharose CL-4B (Pharmacia, Inc.) according to the manufacturer's instructions and as described previously (*20*). The apoE content of the immunopurified fractions was assessed by SDS−PAGE and immunoblotting (*21*, *22*).

Structural Studies. Initially, the formation of β-sheets and amyloid fibrils was monitored at multiple time points by the thioflavin T fluorescence assay (*23*) and by transmission electron microscopy (*24*). From the initial data that were obtained, representative time points were selected and the findings were verified by two additional methods, CD spectroscopy and Fourier transform infrared spectroscopy. For the ThT and TEM methods, parallel samples containing either the Aβ1−40 peptide (with and without melatonin), Aβ and apoE3 (with and without melatonin), or Aβ and apoE4 (with or without melatonin) were prepared as described in detail in a subsequent paragraph. Six independent experiments with recombinant apoE4 and apoE3 were conducted. Two similar independent experiments were also performed with lipidized apoE3 and apoE4. In addition, two independent sets of experiments were performed using recombinant lipoproteins. In one set of experiments, several concentrations of melatonin were used; in another set, several concentrations of Aβ were examined.

Thioflavin T Spectrofluorometry. The ThT was performed as previously described (*23*). Briefly, 5 μL was obtained from each sample after incubation and added to 2 mL of a glycine/NaOH buffer (50 mM, pH 9.2) containing 2 μM thioflavin T. Fluorescence intensities were measured at an excitation wavelength of 435 nm and an emission wavelength of 485 nm in a Hitachi F-2000 fluorescence spectrophotometer. A time scan of fluorescence intensity was performed, and three measurements were taken after the decay reached a plateau at 200, 220, and 240 s and averaged after subtracting the background fluorescence of 2.0 μM thioflavin T in the blank buffers. Neither melatonin nor any of the other compounds used in this study exhibited significant fluorescence within the regions of interest at any time point. All measurements were taken in triplicate. Siliconized polypropylene microcentrifuge tubes (USA Scientific) were used for these experiments. Solutions of recombinant apolipoproteins were immediately lyophilized and, prior to being used, resuspended in 0.1 M tris-phosphate-HCl buffer (pH 7.4). Aqueous stock solutions of 1 mM melatonin were made by first preparing a 10 mM suspension of the hydrochloride salt of the hormone in 1 N HCl and then by completely dissolving it in 100 mM phosphate-buffered saline at pH 7.4 (1:10, v:v) and readjusting the pH to 7.4 with 1 N NaOH. Solutions of Aβ were prepared by dissolving 2.2 mg of the peptide in 1 mL of 50 mM bicarbonate buffer at pH 9.6. Aliquots (50 μL) of this solution were lyophilized and stored at −80 °C until they were needed for the experiments. Working stock solutions of the peptide (concentration of 500 μM) were prepared in HPLC-grade water immediately prior to the experiments. The absence of aggregates and amyloid fibrils in these solutions was verified by ThT and TEM. In the experimental samples, Aβ was further diluted 1:1 with phosphate-buffered saline (pH 7.4, 100 mM) to which melatonin and/or apoE4 or apoE3 or equivalent volumes of buffer solution were added. The final concentration of Aβ in each sample was 250 μM (unless otherwise indicated), and the melatonin:Aβ:apoE4/apoE3 molar ratios were 100:100:1. For the dose−response experiments, the amount of melatonin or Aβ varied as indicated in later sections for each particular experiment. In several independent parallel experiments, the melatonin precursor NAS was substituted for melatonin as a control for the indole structure.

Because oxidative mechanisms were proposed to play a role in Aβ aggregation (*25*, *26*), we also performed experiments to determine whether the results obtained were dependent on the structural properties of melatonin or on "nonspecific" antioxidant features of this hormone. For this purpose, we used the antioxidants ascorbate, α-tocopherol, and PBN. Each of these compounds was substituted for melatonin under otherwise identical experimental conditions. PBN and the sodium salt of ascorbate were directly dissolved in phosphate-buffered saline. α-Tocopherol was dissolved in DMSO, and this solvent was then diluted in phosphate-buffered saline to a final concentration of 1%. The DMSO vehicle had no effect on Aβ aggregation. The experiments using antioxidants were conducted simultaneously in parallel with some of the melatonin−apoE experiments to minimize experimental error and were independently reproduced. The samples were incubated at 37.5 °C in a water bath incubator for various time intervals. As additional controls, bovine serum albumin was substituted for apoE in two independent experiments. In addition, the results of the ThT experiments were verified in three additional independent experiments using Aβ obtained from a different commercial source (US Peptides, Inc.).

As originally discovered by Naiki et al. (*27*), the activity of apoE can oscillate between inhibitory and profibrillogenic, depending mostly on Aβ concentrations. To verify these important opposing effects of apoE on amyloid formation, we conducted experiments using two concentrations of Aβ, since such differences in the activity of apoE could be of relevance to the melatonin-based inhibition of Aβ aggregation reported in this study. While Naiki et al. used concentrations of Aβ ranging from 50 to 300 μM, we employed two concentrations (150 and 250 μM) within the same range.

These were sufficient to observe the dual inhibitory and proaggregatory effects of apoE.

Transmission Electron Microscopy. Aliquots from the preparations used in three of the six independent ThT experiments performed with recombinant apoE4 and apoE3 were processed for TEM; samples from each of the experimental conditions and time points were ultrastructurally examined. TEM was performed using standard methods (*24*) with a Philips CM100 electron microscope. Briefly, 5 μL from each sample was loaded onto carbon-coated copper grids and stained with 0.2% methylamine tungstate (dissolved in ultrapure HPLC-grade water containing 10 μg/mL bacitracin). TEM grids were prepared at different time points from samples obtained from three independent experiments using recombinant apoE3 and apoE4.

Circular Dichroism Spectroscopy. For the CD experiments, solutions of Aβ in either the absence or presence of apoE4 or apoE4 with melatonin were prepared as described for the ThT experiments. Samples at time zero and after incubation for 144 h were centrifuged at 12 000 rpm for 5 min to verify the formation of aggregates, and 330 μL of the supernatant was loaded onto a 0.1 mm path length quartz cell (Jasco, Inc.). CD spectra in the far-ultraviolet region were recorded using a J-720 spectropolarimeter (Jasco, Inc.) as described previously (*17*). Forty scans per experimental condition were obtained at 0.2 nm intervals over the wavelength range of 190−260 nm.

Transmission Fourier Transform Infrared Spectroscopy. For T-FTIR spectroscopy, samples of Aβ in either the absence or presence of apoE4 or apoE4 with melatonin, freshly prepared or after incubation for 144 h, were lyophilized and resuspended at a concentration of 10 mg/mL in D_2O. Infrared spectra of the suspensions were collected in an FTS 6000 FT-IR spectrometer (Bio-Rad) equipped with a DTGS detector. Aliquots (25 μL) were placed in demountable cells containing CaF_2 windows separated by 50 μm Teflon spacers. For each sample, 256 interferograms were co-added and Fourier transformed to generate a spectrum with a nominal resolution of 4 cm^{-1}. The solvent spectrum was recorded under identical conditions and subtracted from the peptide spectra. Correct subtraction of residual H_2O was judged to yield an approximately flat baseline at 2100 cm^{-1}. Fourier self-deconvolution of the spectra in the amide I region was performed using a Bessel apodization function with a resolution enhancement factor *k* of 2 and peak half-width of 12 cm^{-1}, in the Win-IR Pro system (Bio-Rad). Individual components of the amide I mode were resolved from the deconvoluted spectra by least-squares iterative curve fitting using Lorentzian−Gaussian curves and Grams/32 software (Galactic, Salem, NH). Assignment of the different components of the amide I after Fourier self-deconvolution to secondary structure was performed as described previously (*28*).

Neurotoxicity Experiments. Previous studies have shown that the neurotoxic properties of Aβ are linked to β-sheet structure and the fibrillary state of Aβ (*29*, *30*). To determine whether the inhibition of amyloid formation by melatonin and apo E4 caused a corresponding decrease in Aβ-mediated neurotoxicity, we assessed the survival of cultured human neuroblastoma cells exposed to either Aβ (with and without melatonin) or Aβ preincubated with apoE4 (with and without melatonin). SK-N-SH human neuroblastoma cells were grown under conditions detailed elsewhere (*31*), and the level of survival was determined by the MTT method as previously described (*32*).

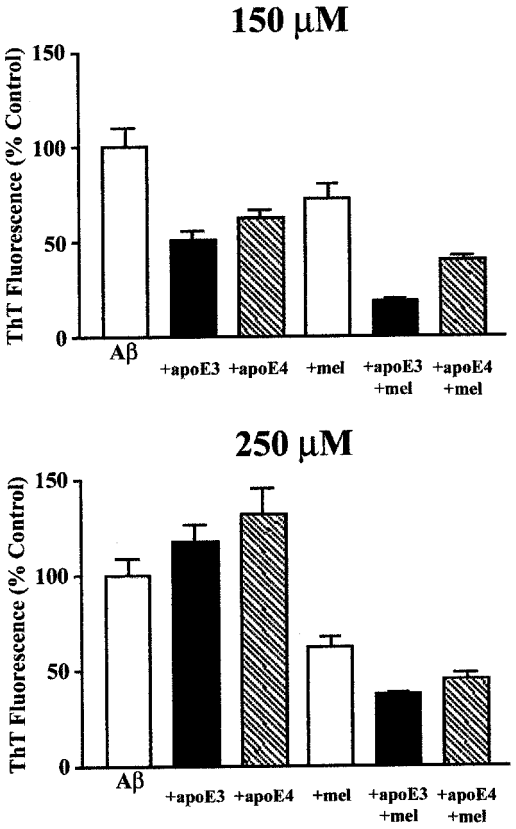


FIGURE 1: Aβ fibrillogenesis as evaluated by ThT spectrofluorometry. The graphs depict the means ± SD of six independent experiments after incubation for 288 h. Note the marked potentiation of the inhibition by the conjunct effect of melatonin and apoE. The data have been normalized as a percentage of control.

RESULTS

Potent Inhibition of Aβ Fibrillogenesis by the Conjunct Activity of Melatonin and Apolipoproteins. For all the fibrillogenesis experiments (unless otherwise noted), a melatonin:Aβ ratio of 1:1 was chosen because it is comparable to that observed under physiological conditions, although the design and sensitivity of the methods that were used required actual concentrations of melatonin and Aβ that exceeded physiological levels ("pathological levels" of these proteins occurring in the epicenters of aggregation, such as in early senile plaques in AD, are not known). As will be shown, however, it is significant that even at high concentrations of Aβ, apoE can either inhibit or promote aggregation depending on the presence or absence of melatonin, respectively.

Two well-established assays were utilized to monitor the formation of β-sheet−amyloid fibrils, which included the ThT spectrofluorometric method and TEM. The addition of either apoE3 or apoE4 to Aβ solutions resulted in either distinct inhibitory or fibrillogenic properties depending on the concentration of Aβ that was used in experiments. At an Aβ concentration of 150 μM, the presence of apoE3 or apoE4 inhibited Aβ aggregation (Figure 1A). In contrast, apoE3 or apoE4 had distinct profibrillogenic properties when Aβ was present in solution at a concentration of 250 μM

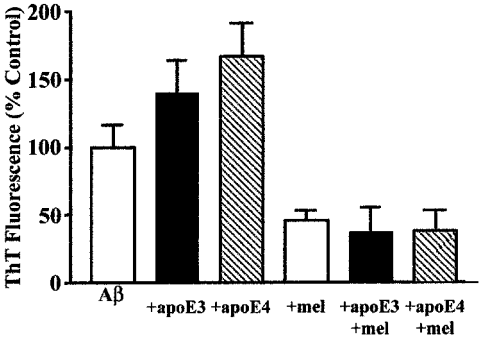


FIGURE 2: Fibrillogenesis of Aβ residues 1−40 over time using lipidated apolipoproteins in the presence or absence of melatonin. The graph depicts the means ± SD of two independent experiments after incubation for 144 h. Results were qualitatively similar to those obtained with recombinant apoE3 and apoE4. The data have been normalized as a percentage of control.

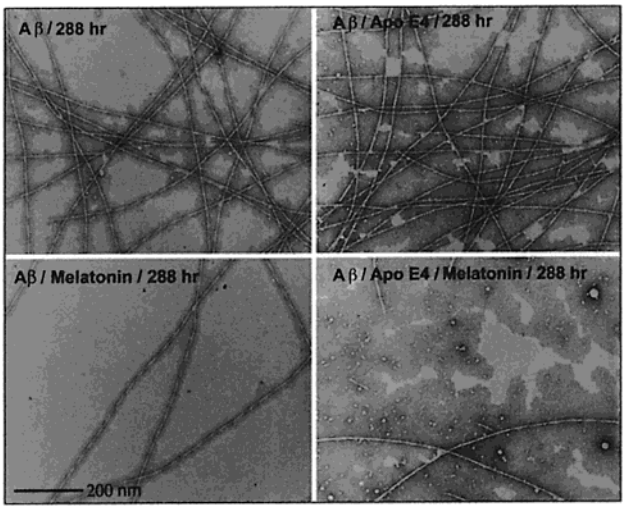


FIGURE 3: TEM of Aβ fibrillogenesis performed at 288 h under various experimental conditions (250 μM Aβ). Each panel is representative of the overall filament density observed in the preparations. Note the profuse amount of fibrils present with Aβ alone and with Aβ and apoE4. A similar phenomenon was observed using recombinant apoE3 (not shown). The bar is 200 nm long.

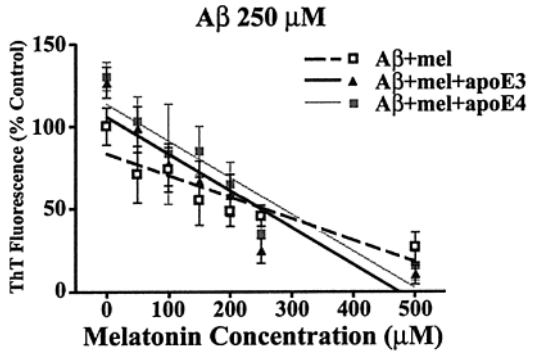


FIGURE 4: ThT fluorescence measured after incubation for 144 h using six concentrations of melatonin. The graph depicts the means ± SD of two independent experiments. The data have been normalized as a percentage of control.

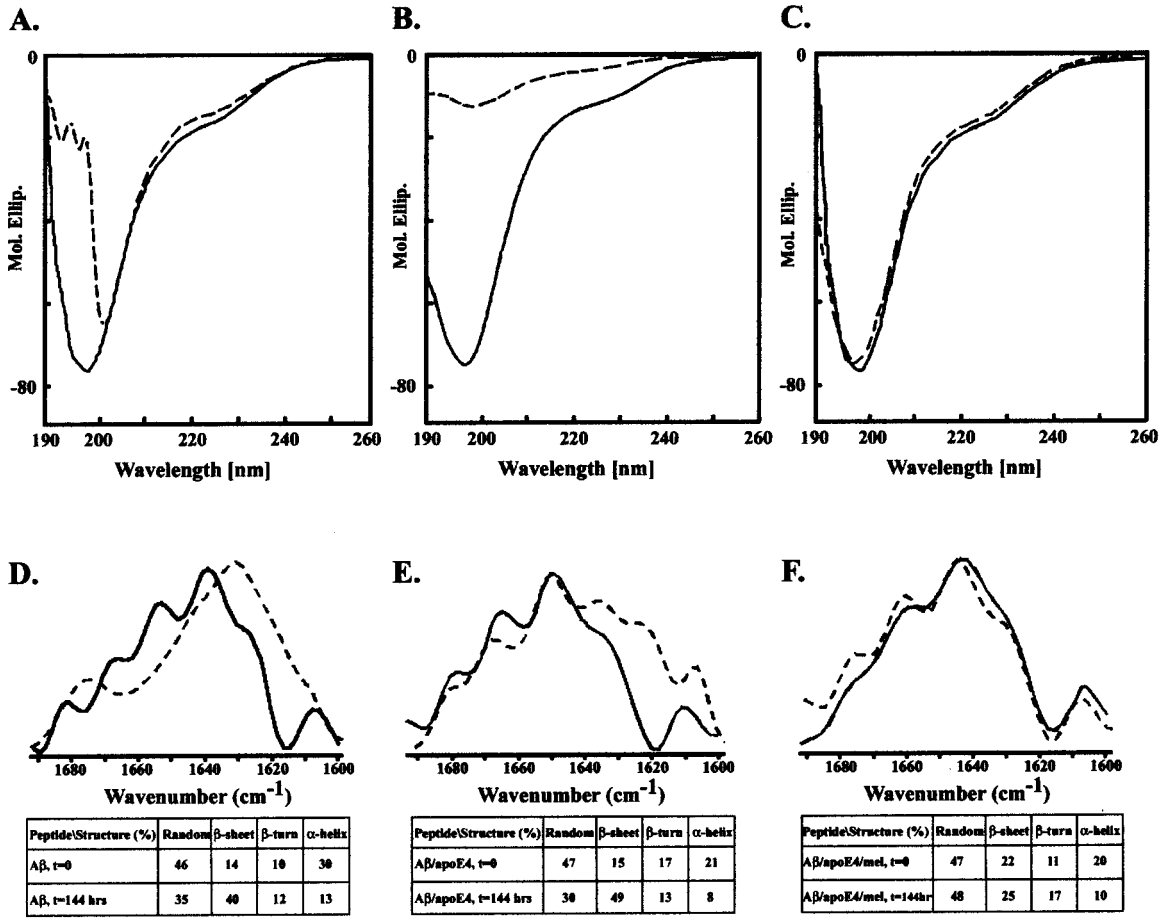
(Figure 1B). Interestingly, there were isoform-specific properties regarding both inhibition and promotion of fibril formation by apoE under the experimental conditions that were used. ApoE3 exhibited a more potent inhibitory effect on Aβ aggregation than apoE4 when Aβ was present at 150 μM; conversely, apoE4 exhibited stronger fibril-promoting effects than apoE3 at 250 μM. These results were qualitatively similar to those published by another group of investigators (*27*). When melatonin was added to identical preparations containing either apoE3 or apoE4, there was a profound level of inhibition of fibril formation that was far greater than that observed in parallel samples containing melatonin and Aβ alone (Figure 1). Remarkably, there was a paradoxical reversal of the fibril promoting properties of apoE3 and apoE4 whenever melatonin was present in the solution. These results, which were confirmed in six independent experiments with recombinant apoE lipoproteins by the ThT method, were further corroborated with a different batch of Aβ (not shown).

To further extend these findings, we studied the effect of lipidized apoE3 or apoE4 produced by astrocytes and performed the ThT fibril assay. As illustrated in Figure 2, nearly identical data were generated with the lipid particles, thus establishing the fact that the more physiologically relevant lipid particles do not influence the effects of melatonin-based inhibition of amyloid fibril formation.

With TEM, a striking inhibition of amyloid fibril formation was confirmed whenever melatonin was present in the preparations containing Aβ with apoE4 (Figure 3) or apoE3 (not shown). At a concentration of 250 μM, Aβ alone showed β-amyloid fibrils within 24 h and the density of these fibrils increased after 48 h. For solutions containing melatonin and Aβ, the onset of amyloid fibril formation was delayed up to 72 h. Interestingly, when both melatonin and either apoE isoform were present, the inhibitory effects lasted up to the time when the experiments were terminated. At this time, only small amounts of amyloid fibrils were seen after careful screening of the TEM preparations containing Aβ, melatonin, and either apoE3 or E4. In sharp contrast, amyloid fibrils were profuse in all the preparations containing Aβ and apoE4 or apoE3 without melatonin (Figure 3).

Additional verification of the results was also achieved from the dose−response experiments using six concentrations of melatonin. As indicated in Figure 4, the antiamyloidogenic effects correlated well with the dose of melatonin that was used.

Influence of ApoE and Melatonin on Aβ Secondary Structure. To gain some insight into the mechanism of this phenomenon, similar experiments using Aβ at 250 μM were analyzed by CD and T-FTIR spectroscopy. Freshly dissolved Aβ exhibited a CD spectrum characteristic of a predominantly random structure with a strong negative band at 198 nm (Figure 5A) (*33*). In the presence of apoE4 (Aβ:apoE4 molar ratio of 100:1) or apoE4 and melatonin (melatonin:Aβ:apoE4 molar ratio of 100:100:1), an almost identical pattern was obtained (Figure 5B,C). After incubation for 144 h, the CD spectrum of Aβ alone exhibited a shift to a higher wavelengths, consistent with a loss in random structure and a gain in β-sheet structure. The spectrum of the Aβ/apoE4 mixture did not change significantly (minimum still at 198 nm), although signal reduction occurred as a result of the loss of the level of protein in solution and the accumulation of insoluble protein aggregates. In contrast, the spectrum of the sample containing Aβ, apoE4, and melatonin remained relatively constant even after incubation for 144 h (Figure 5A−C).



Peptide\Structure (%)	Random	β-sheet	β-turn	α-helix
Aβ, t=0	46	14	10	30
Aβ, t=144 hrs	35	40	12	13

Peptide\Structure (%)	Random	β-sheet	β-turn	α-helix
Aβ/apoE4, t=0	47	15	17	21
Aβ/apoE4, t=144 hrs	30	49	13	8

Peptide\Structure (%)	Random	β-sheet	β-turn	α-helix
Aβ/apoE4/mel, t=0	47	22	11	20
Aβ/apoE4/mel, t=144hr	48	25	17	10

FIGURE 5: Secondary structure evaluated by circular dichroism (A−C) and T-FTIR spectra (D−F) of freshly prepared suspensions and suspensions incubated for 144 h: (A and D) fresh (—) and 144 h aged (− − −) Aβ residues 1−40, (B and E) fresh (—) and 144 h aged (− − −) Aβ residues 1−40 with apoE4, and (C and F) fresh (—) and 144 h aged (− − −) Aβ residues 1−40 with apoE4 and melatonin. (A−C) The spectrum of the sample containing Aβ with apoE4 (B) did not change its appearance (minimum at 198 nm) but underwent an important reduction in the magnitude of the signal as a result of the accumulation of protein aggregates. Analysis of these aggregares by reflection T-FTIR showed them to be composed of predominantly β-sheets (not shown). In contrast, the signal intensity and the spectrum of the samples containing Aβ with apoE4 and melatonin (C) remained nearly constant, with an only slight reduction in intensity after incubation for 144 h. (D−F) For each sample, 256 interferograms were co-added and Fourier transformed. Curve fitting results of the amide I band of the samples after Fourier self-deconvolution with a resolution enhancement factor *k* of 2. The estimated percentages of the secondary structures are indicated in the boxes.

For the T-FTIR studies, peptide suspensions freshly prepared or incubated for 144 h were analyzed. The deconvoluted amide I profile spectra of a freshly prepared suspension of Aβ exhibited a principal component at 1640 cm^{-1} corresponding to approximately 46% of random structure and other minor contributions associated with α-helix, β-sheet/aggregated strands, and β-turn structures (Figure 5D). The T-FTIR spectrum of a freshly prepared suspension of Aβ and apoE4 exhibited a principal component at 1650 cm^{-1} corresponding to approximately 47% random structure (Figure 5E). Similarly, the spectrum of Aβ with apoE4 and melatonin exhibited a principal band at 1645 cm^{-1} corresponding to approximately 47% random structure and other minor bands corresponding to other structures (Figure 5F), consistent with the largely random structure seen by CD spectroscopy. After incubation for 144 h, an increase in the intensity of bands between 1620 and 1640 cm^{-1} was noted for Aβ (Figure 5D), and for Aβ with apoE4 (Figure 5E), suggesting an increment in the content of β-sheet structures during the incubation (Figure 5D,E). The contribution of the different components of amide I after Fourier self-deconvolution suggests an increase in content of β-sheet and a comparable decrease in the level of unordered structures for the Aβ with or without apoE4. In addition, the spectrum of the sample containing apoE4 also exhibited an important band at 1608 cm^{-1} that is suggestive of the presence of aggregates and/or strands in the sample (*34*). For the Aβ incubated for 6 days with apoE4 and melatonin, the T-FTIR spectrum did not show significant changes when compared with the spectrum of the freshly prepared sample (Figure 5F).

Effects of Melatonin Are Structure-Specific. To gain additional information regarding the mechanisms of the melatonin-based inhibition of amyloid fibril formation, we performed control experiments using the structurally related indole compound, NAS, and three structurally unrelated antioxidants (ascorbate, α-tocopherol, and PBN). These experiments were also performed using 250 μM Aβ. The rationale to include antioxidants stems from the suggestion that oxidative mechanisms may play a role in Aβ aggregation (*25*, *26*). As evaluated by ThT and corroborated by TEM (not shown), the magnitude of the fibrillogenic effects of apoE4 did not decrease with any of these compounds. These findings suggest that the basis for the strong inhibition by

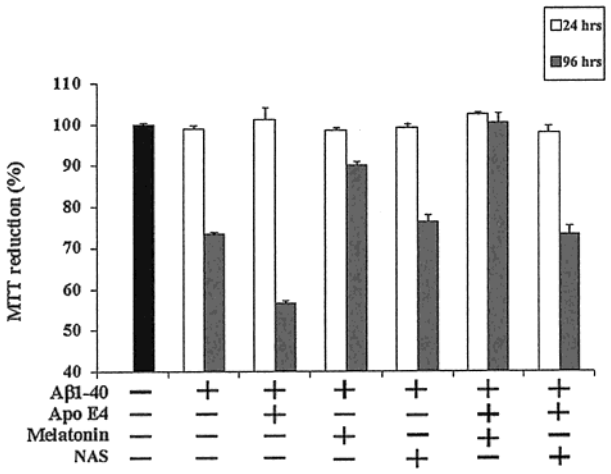


FIGURE 6: Melatonin and apoE4 prevent Aβ-mediated neurotoxicity. SK-N-SH human neuroblastoma cells were exposed to Aβ for 24 or 96 h or to Aβ that had been preincubated with apoE4 for 24 or 96 h, and the level of survival was determined. Exposure of neuronal cells to Aβ alone or with apoE4 showed a reduced level of survival; addition of melatonin to either preparation resulted in complete neuroprotection. No neuroprotection was observed in parallel control preparations when NAS was used instead of melatonin.

melatonin was related to specific structural characteristics of the indoleamine and not determined exclusively by its antioxidant activity (not shown). Also, the effects of melatonin were lost when apoE4 was replaced with BSA (not shown).

Melatonin and ApoE4 Prevent Aβ-Mediated Neurotoxicity. Exposure of neuronal cells to Aβ (with or without preincubated apoE4) showed reduced levels of survival (Figure 6), and addition of melatonin to either preparation resulted in complete neuroprotection. In agreement with the fibrillogenesis experiments, these effects were not observed in parallel control preparations when NAS was used instead of melatonin (Figure 6).

DISCUSSION

Recent studies suggest that brain tissue levels of Aβ become elevated very early in the disease process and may be responsible for the neuropathological cascade and the cognitive decline that characterizes full-blown AD (*35*). Significant research efforts are thus focused on finding approaches to reduce the rates of amyloid accumulation.

Melatonin or *N*-actyl-5-methoxytryptamine is a pineal neurohormone secreted during the dark phase of the circadian cycle in animals and humans (*36*). Its level of rhythmic synthesis and secretion declines sharply during the aging process (*36*). Of interest here are the results from various studies showing that the endogenous brain levels of melatonin are more profoundly reduced in patients with AD than in healthy elderly control patients (*37*−*39*). Significantly, the lowest levels of melatonin are found in AD patients who are homozygous for the apoE-ϵ4/4 allele, suggesting a more severe deficiency in this subpopulation of patients (*39*).

Several observations suggest that reductions in the amounts of β-sheet structures of Aβ as well as inhibition of fibril formation are important targets for interrupting the development of neuropathological changes in AD. First, it has been proposed that increases in β-sheet levels are required for seeding and nucleation of Aβ into prefibrillar intermediate amyloidogenic species (*40*). Second, evidence has been generated which indicates that the neurotoxic properties of Aβ are linked to either prefibrillar intermediates or fibrils enriched in β-sheets but not to soluble monomeric Aβ containing high percentages of unordered conformations (*29*, *30*). Last, it has been reported that Aβ peptides containing high percentages of β-sheets are partially resistant to proteolytic degradation, whereas peptides with high contents of random structures are protease sensitive (*41*). This last observation is believed to be important in amyloid accumulation. Therefore, the combined inhibitory effect of melatonin and apoE on β-sheet formation, fibril assembly, and neurotoxicity may be relevant to several key steps in the pathogenesis of AD. Interestingly, the degree of neuroprotection measured when melatonin was used along with apoE exceeded that observed when melatonin was used with Aβ alone. In agreement with the fibrillogenesis experiments, no neuroprotection was observed in parallel control preparations containing the control indole NAS instead of melatonin.

As a first step toward explaining the mechanism(s) of the melatonin-based inhibition, we first re-examined the activity of apoE on Aβ aggregation using different concentrations of Aβ peptides. These experiments were prompted by a number of previous conflicting observations from several laboratories that had reported both inhibitory and profibrillogenic effects for apoE (*27*). Our results suggest that the effects of apoE on Aβ aggregation can oscillate between inhibitory and profibrillogenic depending on the Aβ concentration. These results are very similar to those reported by another group of investigators (*27*), who also re-examined this controversial issue and similarly concluded that the activity of apoE on Aβ aggregation can fluctuate between inhibitory and profibrillogenic depending on the Aβ concentration. The investigators in this other study employed concentrations of Aβ ranging from 50 to 300 μM, which are comparable to those used in our study. These dual effects of apoE may be important in understanding the mechanism of melatonin-based inhibition for the following reasons. Lower and higher Aβ concentrations result in lower and higher contents of β-sheet structures, respectively (*6*, *7*, *17*). As already established by previous studies, apoE binds (under profibrillogenic conditions) to the β-sheet structure of Aβ peptide within the peptide region of residues 1−28, further promoting aggregation (*14*, *22*). Since melatonin increases the amount of random coil structures and decreases the β-sheet content, melatonin may decrease the number of apoE binding sites available in Aβ that are necessary for promotion of aggregation by apoE. Thus, in the presence of melatonin, a larger fraction of Aβ may be maintained in a conformation that is permissive for the inhibitory activity of apoE.

Although the precise interations between apoE, melatonin, and Aβ are under investigation, on the basis of the current results and our previously published data, we speculate about the following mechanism for the melatonin-based inhibition of β-amyloidosis. Once released from APP, Aβ is presumably monomeric and random, while unknown age-related microenvironmental changes in the brain lead to increases in the Aβ concentration and the level of β-sheet formation that result in aggregation. When the aggregation reaches a critical mass, the β-sheet structure assembles as amyloid, and at this point, reconversion back to soluble random structure may

no longer be possible. Previous NMR and CD studies suggest that melatonin disrupts the imidazole−carboxylate salt bridges between the side chains of the His^+ and Asp^- residues in Aβ (*16*) that are critical to the formation and stabilization of β-sheet structures. Therefore, melatonin may promote a β-sheet → random conversion by binding to an early-formed aggregate that is still permissive for the inhibitory activity by apolipoprotein E. Preliminary data suggests that melatonin inhibits nucleation (seeding) of Aβ, but has no effect on the polymerization phase of Aβ aggregation. The nature of the interaction between melatonin, apoE, and Aβ which leads to inhibition of aggregation by apoE is complex and is currently being investigated by NMR approaches (M. G. Zagorski et al., work in progress).

The data obtained with other antioxidants are interesting in view of previous reports suggesting that certain oxidative conditions may promote amyloid fibril formation (*25*, *26*) and, conversely, that fibril polymerization may be inhibited by antioxidant mechanisms (*25*). The results presented here suggest that the inhibition of fibril formation by melatonin and apoE is relatively structure-specific rather than being determined by nonspecific antioxidant activities of melatonin.

Further research is warranted to determine the role of melatonin in inhibiting Aβ peptide aggregation.

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