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Melatonin prevents amyloid protofibrillar induced oxidative imbalance and biogenic amine catabolism

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ABSTRACT

Oxidative stress is one of the hypothesized pathogenic mechanisms for neurodegenerative diseases, including Alzheimer's disease (AD); numerous studies suggest that Aβ is toxic to neurons by free radical mediated mechanism. A constant feature in AD brain is selective neuronal loss, accompanied by dysfunction of several neurotransmitter systems, such as cholinergic, serotoninergic and noradrenergic systems. In the present study, we studied the neuroprotective role of melatonin against amyloid protofibrils and the toxicity of protofibrils on serotoninergic and noradrenergic systems. Mice were divided into four groups (n=8 each), control, Scrambles $A\beta_{35-25}$ treated, $A\beta_{25-35}$ injected, and melatonin treated. A single dose of $A\beta_{25-35}$ (25μg) was administered to mice via intraperitoneal injection. Melatonin (50 mg/kg body weight) was administered intraperitoneally for 3 days to the $A\beta_{25-35}$ injected mice. Control mice received only physiological saline and Scrambles receives $A\beta_{35-25}$ single intraperitoneal injection of 25 μg of $A\beta_{35-25}$. Our study showed that melatonin significantly reduces reactive oxygen species (ROS) production in the astrocytes, lymphocytes and hepatocytes of Aβ injected mice by increasing the levels of scavenging enzymes, SOD, catalase and GSH when compared to the untreated group. Immunohistochemistry study reveals that melatonin prevents the activation of GFAP in neocortex and transcription factor NF-κB in liver and neocortex of Aβ injected mice. It also prevents the elevation of dopamine depletion and its degradation products. Thus, while melatonin may be a potential therapeutic agent in the prevention of oxidative stress associated with Aβ and AD, it can also prevent dopamine turnover induced by Aβ.

Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the elderly. The classical neuropathological hallmarks in AD are the formation of β-amyloid protein (Aβ) containing senile plaques and neurofibrillary tangles composed of hyperphosphorylated tau protein in specific areas of the brain. Although great progress in understanding the etiology, pathology and neurochemistry of AD has been achieved, the pathogenic mechanism(s) underlying this disorder are still unclear, but available evidence suggests that oxidative stress plays a key role. Earlier studies indicate Aβ neurotoxicity in AD resulting from Aβ fibril induced oxidative stress (Yan et al., 1996; Zhang et al., 2007) presumably initiated either directly by Aβ generated free radicals (Kelly et al., 1996; Monji et al., 2002) or indirectly through intracellular production of ROS (Yan et al., 1996). Oligomers of Aβ, protofibril, and fibrillar forms of Aβ are highly neurotoxic (Uberti et al., 2007). Protofibrils appear transiently during Aβ fibrillogenesis (Harper et al., 1997a; Walsh et al., 1997). Several *in vitro* studies have shown that protofibrils are precursors of the longer, more rigid, amyloid-type fibrils typically produced using synthetic peptides (Harper et al., 1997b; Walsh et al., 1997). If an analogous fibril maturation mechanism operates *in vivo*, the protofibril stage could be an important therapeutic focus. *In vivo* experiments using transgenic mice overexpress Aβ protein precursor and develop characteristic Aβ deposits within the brain parenchyma (Poeggeler et al., 2001) and demonstrating the same type of oxidative damage that is found in AD (Dairam et al., 2007; Pratico et al., 2001).

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A constant feature in AD brain is selective neuronal loss, accompanied by dysfunction of several neurotransmitter systems, such as cholinergic, serotoninergic and noradrenergic systems (Bell and Claudio Cuello 2006; Lehericy et al., 1993; Szot et al., 2000). Since cortical cholinergic deficits are correlated with cognitive impairments such as learning and memory dysfunction in AD (Dairam et al., 2007; Olariu et al., 2001), most studies have focused on the cholinergic deficits (Harkany et al., 2001; Tang et al., 2002). However, little is known about the possible neurotoxic effects of *in vivo* administered Aβ on several other neurotransmitter systems like serotoninergic and noradrenergic. The noncognitive impairments, such as depression and aggressive behavior involved in AD are primarily influenced by the serotoninergic and noradrenergic systems (Dyon-Laurent et al., 1994; Steckler and Sahgal 1995; Uberti et al., 2007; Vanderwolf and Baker 1996).

Antioxidants such as vitamin E, estrogens or melatonin have demonstrated neuroprotective effects on Aβ mediated cytotoxicity (Behl, 1999; Behl et al., 1992; Huang et al., 2000; Pappolla et al., 1997; Wang et al., 2004). Melatonin has been found to inhibit neurotoxicity (Lahiri et al., 2005) associated with Aβ peptides *in vitro* (Clapp-Lilly et al., 2001) in addition to repression of progressive formation of amyloid fibrils (Skribanek et al., 2001). Here, we studied the neuroprotective role of melatonin against amyloid protofibrils and investigated the toxicity of protofibrils on serotoninergic and noradrenergic systems.

Materials and methods

Dithiobisnitrobenzoic acid (DTNB), 2–7-diacetyl dichlorofluorescein (DCFH-DH) and Amyloid β-Protein Fragment 35–25 were obtained from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals were of analytical grade or the highest purity available. Adult, healthy and virgin Swiss strain male albino mice weighing 25–30 g of age between 6 and 8 weeks were obtained from Tamilnadu veterinary and Animal Sciences University, Madhavaram, Chennai, Tamilnadu, India. The animals were maintained in the poly acrylic cages under hygienic conditions at normal room temperature (28–30 °C) on a 12 h light/dark cycle. They were fed with commercial pellet diet *ad libitum* (Hindustan Lever Ltd., Bangalore, India). The animals had free access to water. The whole surgical procedure was performed in a laminar flow hood under aseptic conditions following NIH animal ethical guidelines and following approval by the animal care committee of Central Leather Research Institute, Chennai, Tamilnadu.

Experimental design

The animals were divided in to three groups; each group consists of eight mice.

Group I (Control): Mice were given physiological saline (0.5 ml) intraperitoneally.

Group II (Scrambles $A\beta_{35-25}$ treated): Mice were administered with a single intraperitoneal injection of 25 μg of $A\beta_{35-25}$.

Group III ($A\beta_{25-35}$ injected): Mice were administered with a single intraperitoneal injection of 25 μg of $A\beta_{25-35}$ ($A\beta_{25-35}$ which was dissolved in deionized water and incubated at 37 °C for one day) (Spooner et al., 2002). $A\beta_{25-35}$ was used for the present work because it exhibits all the biological activity of the full length Aβ1–40 and Aβ1–42 (Giovannelli et al., 1995).

Group IV (Melatonin treated): $A\beta_{25-35}$ injected mice were intraperitoneally injected with melatonin (50 mg/kg body weight) for consecutive three days. The duration was fixed on the basis of the preliminary experiments capable of inhibiting Aβ induced LPO concentration by melatonin therapy. Melatonin treatment was started immediately after induction of Aβ. On the 3rd day animals were sacrificed by cervical decapitation under aseptic conditions in a laminar flow hood. Blood was collected in heparinized vial and plasma was separated by centrifugation (2000 ×*g*, 10 min) at 4 °C. The liver, neocortex and hippocampus were micro dissected and weighed. The tissues were homogenized in 0.1 M Tris buffer (pH 7.4). The homogenate was then centrifuged at 5000 ×*g* for 10 min (4 °C), and the supernatant was used for the estimation of oxidant and antioxidants.

Synthesis and purification of $A\beta_{25-35}$

$A\beta_{25-35}$ was synthesized by manual solid phase chemistry, using Fmoc as the protective group for N-terminal ends, and 1-Hydroxy benzotriazole (HOBt), and *N*,*N*-dicyclohexyl carbodiimide as coupling agent and pentafluorophenol activators of carboxylic ends. The peptides were cleaved from Wang resin (4-Hydroxymethylphenoxy acetyl) with the mixture of trifluoromethane sulphonic acid: thioanisole: ethanedithiol: trifluoroacetic acid (1:1:1:7) and precipitated with cold ether. The composition of peptides was determined by amino acid analysis using Phenyl isothiocyanate (PITC) method. The synthesized peptides were purified by RP-HPLC as previously described (Burdick et al., 1992). The purified peptide was characterized by MALDI-TOF MS analysis. The peak corresponding to the M+ ion for $A\beta_{25-35}$ peptide was observed. The derived mass of the peptide was calculated from the MALDI spectrum to be 1060.4 ($A\beta_{25-35}$) Daltons. The peptide was then characterized by 1H-NMR. All reagents used in peptide synthesis pentafluorophenol (Spectrochem Pvt. Ltd., Mumbai), *N*,*N*-dicyclohexyl carbodiimide (Sigma-Aldrich), Fmoc-AA (Novabiochem), HOBt (Sigma-Aldrich) and TFA (Sigma-Aldrich) were of the purest analytical grade. The Aβ peptide fibrils were formed by solubilizing the peptide in sterile double distilled (DD) water (1 mg/ml) and incubated for 24 h at 37 °C (Nielsen et al., 1999). The protofibrillar form thus obtained was used fresh for the present study.

Lymphocyte isolation

Lymphocytes were isolated from the experimental mice (Masilamoni et al., 2006; Moen et al., 1981). Blood samples were collected in a heparinized vial and were diluted with equal volumes of Hanks balanced salt solution (HBSS). The diluted blood samples were carefully layered over 3 ml of Ficoll gradient and centrifuged at 800×*g* for 20 min at room temperature. After completion of the centrifugation process, the lymphocytes formed a distinct band, which was removed from the interface. Approximately 8.5×10^2 10^2 cells/ml were used for ROS quantification.

Hepatocyte isolation

Hepatocytes were isolated from the liver of the experimental mice by a modified collagenase perfusion technique (Masilamoni et al., 2005a). An apical piece of liver was perfused with 100-ml preperfusion solution (Earle's balanced salt solution without calcium, with 0.7 mM EGTA) for 20 min, followed by perfusion for 20 min with 80-μl collagenase D (0.5 mg/ml; Boehringer). Cells were filtered and hepatocytes were separated from non-parenchymal cells by low-speed centrifugation. Approximately 8.5×102 cells/ml was used for ROS quantification.

Astrocyte isolation

Astrocytes were isolated from the forebrain of the experimental mice by a modified collagenase perfusion technique, which include 20% of glial and neuronal cells (Abe and Saito 1996; Jesudason et al., 2007) with slight modification. In brief, 100 mg of forebrains were removed from experimental animals (control and treated) and minced with scalpel in a solution containing 20 mg/ml DNase and 0.3% BSA in HBSS. This is centrifuged and incubated in a solution containing 0.025% trypsin, 0.1% collagenase and 0.3% BSA in HBSS in a water bath at 37 °C. After 15 min the resulting mixer is centrifuged and the pellet is agitated 5 times with a fire-polished Pasteur pipette. The contents

were left to settle for 4 min and the supernatant was collected. Approximately, 8.5×10^2 10^2 cells/ml were used for ROS quantification. The initial viability of astrocytes was greater than 90% as assessed by Trypan blue exclusion. Isolated astrocytes were used immediately for ROS quantification.

Measurement of intracellular ROS formation

Formation of intracellular peroxides was detected by fluorescence spectroscopy using a non-fluorescent compound DCFH2-DA, which is desterified within cells by endogenous esterase with the acid 2′7′-dichlorofluorescein ($DCFH^2$) as previously described (Abe and Saito, 1996). The ionized free acid is trapped within the cells and is capable of being oxidized to fluorescent 2′7′-dichlorofluorescin (DCF) by hydrogen peroxides although other mechanism of oxidation cannot be ruled out (Cathcart et al., 1983). An aliquot of the isolated cells (200 µl) was made up to a final volume of 2 ml in normal phosphate buffered saline (pH 7.4), to which 100 µl of DCFH2-DA (200 µM) was added and incubated at 37 °C for 30 min. Fluorescent measurements were done with excitation and emission filters set at 485 and 530, respectively. Results were expressed as percent increase in fluorescence by using a formula $[(Ft_{30}-Ft_0)/(Ft_0\times100)]$, where Ft_0 and Ft_{30} are the fluorescence intensities at 0 and 30 min.

Determination of lipid peroxides

Lipid peroxides were measured as malondialdehyde (MDA) products based on the acetonitrile precipitation assay, using high performance liquid chromatography (HPLC) (Masilamoni et al., 2006). Briefly, aliquots of plasma, liver, neocortex and hippocampus in buffer were mixed with equal volume of acetonitrile, homogenized and centrifuged to precipitate the proteins. The supernatants were then filtered through millipore nylon membrane (0.2 µm) and 20 µl of samples was injected by Hamilton syringe in the HPLC for detection of MDA. The MDA was identified by comparing the retention time (RT) of standard, tetra methoxy propane, run under identical conditions.

Immunohistochemical analysis of GFAP and NF-κB

Under deep anesthesia, the experimental animals were perfused transcardially with ice-cold paraformaldehyde solution (4% in phosphate buffer, pH 7.4). The neocortex was post fixed for 4 h and cryoprotected with 18% sucrose solution for 48 h. Neocortex were cut in 10 m thick sections and placed in phosphate buffered saline (PBS) containing 0.1% sodium azide, and stored at 4 °C until used for immunohistochemical evaluation for reactive astrocytes marker glial fibrillary acidic protein (GFAP) and nuclear factor-kappa B (NF-κB). Similarly, the liver of experimental animals are also sectioned and processed for immunohistochemical studies for NF-κB. GFAP was detected by means of mouse monoclonal mouse antibody (1:1000 dilution, Oncogene research products). NF-κB (p65, Rel A) (Ab-1) was detected by means of a rabbit polyclonal antibody (1:500, Oncogene research products).

Protein content

Protein contents of the plasma and homogenates were determined by the method of Lowry et al.(Lowry et al. (1951) using bovine serum albumin as standard.

Antioxidant enzyme assays

SOD

SOD activity was assayed according to the method of Misra and Fridovich (1972). The assay is based on the inhibition of epinephrine–adrenochrome transition by the enzyme.

Catalase

The activity of catalase was determined by the method of Beers and Sizer (1952). The breakdown of hydrogen peroxide is followed on addition of enzyme, which was observed through the decrease in light absorption of peroxide solution in the ultraviolet region.

Total reduced glutathione (GSH)

The method of Moron *et al.*(Moron et al. (1979) was followed to determine the total reduced glutathione. The method is based on the reaction of glutathione with DTNB and the absorbance at 412 nm.

Sample preparation for the quantification of neurotransmitters

Sample preparations were carried out according to our earlier investigation (Jesudason et al., 2005). Dissected out hippocampus and neocortex were weighed before homogenizing at 4 °C with perchloric acid (0.1 M) in Teflon/glass homogenizer. The homogenates were centrifuged at 4 °C for 20 min at 20,000 000 ×*g*. The supernatants were collected and filtered through 0.2 µm Acrodisc filter (Pall Gelman, USA) before injecting into the HPLC system using Hamilton syringe for the estimation of neurotransmitters.

Standard preparation

Standard solution each 2.5 ng/ml concentration of DA, 5-HT, NE, HVA, 5-HIAA, DOPAC and Dihydroxy benzylamine (DHBA, internal standard) were prepared in 0.17 M perchloric acid, which was previously filtered and degassed. These solutions were freshly prepared and stored at −70 °C. The prepared standard solution was filtered through 0.2 µm Acrodisc filter (Pall Gelman, USA) before use. Different concentrations were injected at a volume of 20 µl and a standard graph was prepared in order to quantify sample values after authenticating the retention time of neurotransmitters. Percent recovery was also calculated and accordingly the sample values were adjusted. Only polypropylene disposable wares were used for storage, sample preparation and standards preparation.

Mobile phase

Citric acid (MW = 210.14; 32 mmol; 13.448 g), Na_2PO_4 (MW = 141.96; 3.54 g) EDTA (MW = 372.24; 0.037 g) and octyl sodium sulphate (0.236 g) were added to 2 l of triple distilled water and mixed well. The pH of above solution was adjusted to 4.2. To this 265 ml of methanol was added and the entire solution was filtered using 0.2 µm nylon filter (Pall Gelman, USA) and then degassed just before use.

HPLC-EC detection of neurotransmitters

HPLC-EC detection of standards for DA, 5-HT, NE, HVA and 5-HIAA was made and maintained in the following working conditions: Isocratic elution; mobile phase (32 mmol citrate buffer in methanol with EDTA and octyl sodium sulphate); Phenomenex column (Reverse phase C18, particle size 3µ, 250×4.6 mm); Flow rate (1 ml/min); Glassy carbon working electrode (+0.49 V 2 *vs* Ag/AgCl electrode). The level of neurotransmitters was expressed as ng/g wet tissue.

Table 1
Total ROS production

% Change in fluorescence				
Parameters	Control	$A\beta_{35-25}$ treated	$A\beta_{25-35}$ treated	$A\beta_{25-35}$ + melatonin
Lymphocytes	9.88 ± 0.21	10.08 ± 0.33	17.63 ± 0.28*	13.73 ± 0.21
Hepatocytes	13.3 ± 1.08	16.13 ± 0.19	37.13 ± 0.41$^{\$}$	17.53 ± 0.31
Astrocytes	15.15 ± 0.30	15.23 ± 0.38	59.50 ± 0.72$^{\#}$	21.25 ± 0.43

Effect of melatonin therapy on ROS in lymphocytes, hepatocytes and astrocytes of experimental animals. Data represents the group mean ± SD for eight animals. Different superscripts show significant variation between groups (*$P<0.05$, $^{\$}P<0.01$, $^{\#}P<0.001$).

Table 2
HPLC-UV determination of MDA and conjugated dienes in plasma, liver, neocortex and hippocampus of Control, $A\beta_{35-25}$ treated, $A\beta_{25-35}$ treated and $A\beta_{25-35}$ + melatonin treated mice

Parameter	Control	$A\beta_{35-25}$ treated	$A\beta_{25-35}$ treated	$A\beta_{25-35}$ + melatonin treated
Plasma (nmols MDA/mg protein)	10.21±0.72	11.03±0.98	15.12±1.14*	12.14±1.21
Percentage variation of MDA production compared to control	–	–	48.09	18.90
Liver (nmols MDA/mg protein)	25.24±1.87	24.45±1.21	35.68±3.41$^{\$}$	26.82±2.74
Percentage variation of MDA production compared to control	–	–	41.36	6.25
Neocortex (nmols MDA/mg protein)	35.62±3.33	34.55±2.98	56.11±3.61$^{\#}$	37.99±3.5
Percentage variation of MDA production compared to control	–	–	57.52	6.65
Hippocampus (nmols MDA/mg protein)	29.68±2.31	30.32±2.55	45.58±2.68*	31.55±3.04
Percentage variation of MDA production compared to control	–	–	53.57	6.30

Values represent the group means±S.D. for eight animals; Different superscripts show significant variation between groups (*$P<0.05$, $^{\$}P<0.01$, $^{\#}P<0.001$).

Statistical analysis

Data is expressed as mean±SD for eight animals in each group. Statistical analysis of variance (ANOVA) followed by the Tukey's test was applied to determine the significant differences among the groups. Different superscripts show significant variation between groups (*$P<0.05$, $^{\$}P<0.01$, $^{\#}P<0.001$) were considered as significant.

Results

ROS

Intracellular ROS concentration was significantly higher in lymphocytes, hepatocytes and astrocytes of Aβ injected group when compared to control and melatonin groups (Table 1). Data shows that Aβ induction had 2.4, 2.2 and 3.4 fold increase of ROS in the lymphocytes, hepatocytes and astrocytes, respectively, as compared to the control group. Our results show that melatonin therapy significantly reduced (*$P<0.05$ lymphocytes, $^{\$}P<0.01$ Hepatocytes, $^{\#}P<0.001$) intracellular ROS production in the Aβ induced mice on the 3rd day. However, there were no significant changes in ROS level of the melatonin treated mice when compared to control and scrambles $A\beta_{35-25}$ treated group (Table 1).

Lipid peroxidation

Elevated concentrations of MDA were recorded in plasma, liver, neocortex and hippocampus of Aβ injected mice when compared to the control and melatonin treated groups (Table 2). The present data shows that Aβ induced mice has 148%, 141%, 157%, and 153% increase

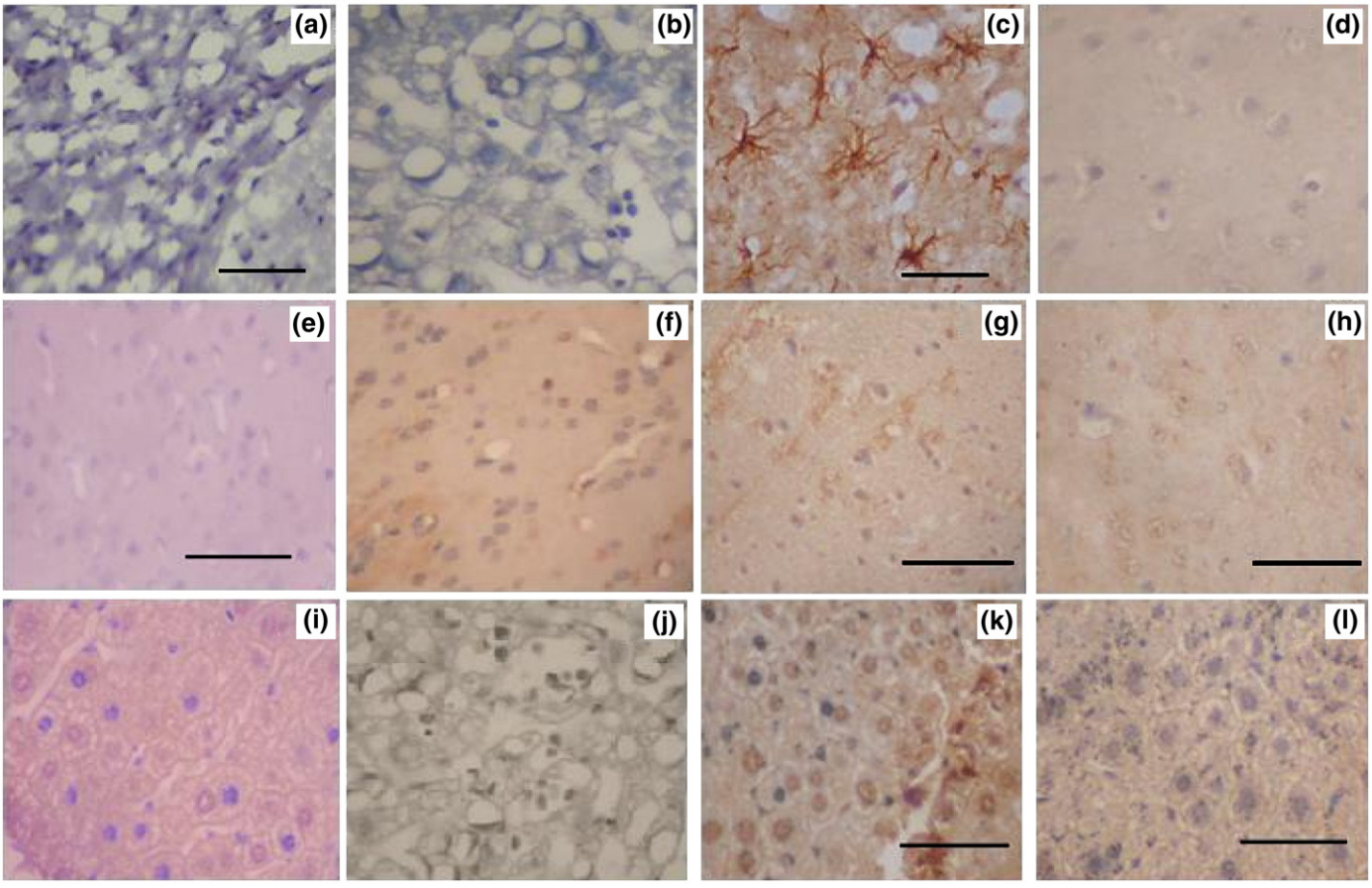

Fig. 1. (a) GFAP expression was absent in the control mice and (b) Scrambles $A\beta_{35-25}$ treated mice. (c) Intense staining with GFAP antibody was observed in the Aβ injected mice. (d) GFAP expression was prevented by melatonin treatment in the neocortex section of Aβ injected mice. Immunohistochemical analysis of neocortex and liver tissue sections revealed that NF-κB expression was absent in the control mice and Scrambles $A\beta_{35-25}$ treated mice (e, f, i and j). NF-κB expression was apparently increased in Aβ injected mice (g and k). The expression of NF-κB neocortex and liver was prevented by melatonin therapy (h and l). Immunohistochemical stain scale bar = 100 μm.

Table 3
The antioxidant status in the plasma, liver, neocortex and hippocampus of Control, $A\beta_{35-25}$ treated, $A\beta_{25-35}$ treated and $A\beta_{25-35}$+melatonin treated groups at 3rd day

Parameter	Control	$A\beta_{35-25}$ treated	$A\beta_{25-35}$ treated	$A\beta_{25-35}$+melatonin treated
SOD				
Plasma	1.38±0.11	1.32±0.13	1.02±0.10*	1.29±0.12
Liver	2.29±0.10	2.20±0.18	1.19±0.03$^{\#}$	2.19±0.18
Neocortex	0.75±0.21	0.77±0.24	0.45±0.02*	0.73±0.03
Hippocampus	0.86±0.01	0.88±0.05	0.55±0.06$^{\$}$	0.82±0.11
Catalase				
Plasma	3.29±0.03	3.22±0.06	1.56±0.04$^{\$}$	3.12±0.11
Liver	3.22±0.21	3.25±0.05	1.78±0.11*	3.24±0.14
Neocortex	2.55±0.21	2.51±0.24	1.51±0.17$^{\#}$	2.49±0.25
Hippocampus	2.54±0.13	2.55±0.15	1.35±0.14$^{\$}$	2.46±0.14
GSH				
Plasma	10.58±0.22	10.48±0.26	5.48±0.14*	9.15±0.23
Liver	11.54±1.12	11.44±0.98	6.39±0.21$^{\$}$	10.48±0.39
Neocortex	7.71±0.35	7.78±0.32	3.68±0.34$^{\#}$	7.51±0.27
Hippocampus	6.68±0.51	6.65±0.60	3.25±0.16*	6.51±0.14

Values represent the group means±S.D for eight animals; Different superscripts show significant variation between groups (*$P<0.05$, $^{\$}P<0.01$, $^{\#}P<0.001$).
(SOD: units/min/mg protein; Catalase: μmol of H_2O_2 consumed/min/mg protein; GSH: μg/mg protein).

in the concentration of MDA of plasma, liver, neocortex and hippocampus, respectively, in comparison with control group and scrambles $A\beta_{35-25}$ treated group (*$P<0.05$, $^{\$}P<0.01$, $^{\#}P<0.001$, and *$P<0.05$ respectively). The levels of MDA and diene conjugates were normalized in the melatonin treated mice on the 3rd day (Table 1). Melatonin therapy to the Aβ induced mice reduced the MDA concentration to 19%, 6%, 7% and 6% in the plasma, liver, neocortex and hippocampus, respectively, when compared to controls and scrambles $A\beta_{35-25}$ treated group (Table 2).

Immunohistochemical analysis of GFAP and NF-κB

GFAP in neocortex and NF-κB in liver, and neocortex were markedly enhanced on Aβ injected group (Fig. 1c, g and k). The GFAP immunoreactive cells appeared to be astrocyte (Fig. 1c). Melatonin therapy effectively prevents the expression of Aβ induced GFAP and NF-κB immunoreactivities (Fig. 1d, h, and l).

Antioxidant status

To prevent the cells from the harmful damage originated by free radicals and related reactants, numerous defense mechanisms operate in the cell to scavenge ROS from the intracellular environment. The activities of antioxidants such as SOD and catalase and the level of GSH in the plasma, liver, neocortex and hippocampus are presented in Table 3. The result shows a significant (*$P<0.05$, $^{\$}P<0.01$, $^{\#}P<0.001$) decrease in the activity of antioxidant enzymes and GSH level of Aβ injected group when compared to control and Scrambles $A\beta_{35-25}$ treated mice. Melatonin therapy significantly prevents Aβ induced antioxidant depletion (Table 3).

HPLC-EC detection of neurotransmitters

Aβ injected mice were used to evaluate the role of melatonin induced changes in aminergic system. The endogenous content of biogenic amines was found significantly decreased in the neocortex and hippocampus of Aβ injected mice when compared with control and scrambles $A\beta_{35-25}$ treated group (Table 4). Melatonin treatment prevents the degradation of monoamines and catecholamines content. In contrast, their metabolites, such as 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindole acetic acid (5-HIAA) and homovanillic acid (HVA) were significantly (*$P<0.05$, $^{\$}P<0.01$, $^{\#}P<0.001$) increased in the Aβ injected mice. The data highlights that DA and 5-HT turnover was significantly higher in the Aβ injected mice. This shows that the monoamines and catecholamines catabolism was significantly higher in the Aβ injected mice. Melatonin treatment successfully reduced Aβ-induced catabolism of biogenic amines.

Discussion

There is a growing body of evidence suggesting that Aβ peptide toxicity is mediated by free radical damage to cell membranes (Kraus et al., 2007; Uberti et al., 2007). Our present study demonstrates that protofibrillar Aβ causes a significant increase in ROS levels in mouse lymphocytes, hepatocytes and astrocytes (Table 1). Aβ can directly produce H_2O_2 by a mechanism that involves the reduction of the metal ion Fe (III) or Cu (II) setting up conditions for Fenton type chemistry (Huang et al., 1999). Furthermore, increasing evidence points to a central role for transport of Aβ across the BBB (Poduslo et al., 1999; Zlokovic et al., 1993) and also Aβ activate the astrocytes to release ROS (Johnstone et al., 1999). This may be the possible reason for the significant production of ROS in the astrocytes, hepatocytes and lymphocytes of the Aβ induced mice. The present results show that melatonin therapy significantly enhances the activities of oxidative scavenging enzymes such as SOD and catalase and GSH levels in the Aβ induced mice (Table 3). These findings suggest that melatonin acts as a protective agent against oxidative damage by scavenging ROS and thus by maintaining the activities of the antioxidant enzymes and regulating the levels of glutathione. Melatonin enhances gene expression of antioxidative enzymes either under basal conditions or after their inhibition by neurotoxic agents (Antolin et al., 2002; Liu et al., 2004; Reiter et al., 1997; Tang et al., 2002; Sharman et al., 2007). The stimulation of GSH synthesis by melatonin could be a major

Table 4
Effect of amyloid toxicity and melatonin therapy on the level and turnover of neurotransmitters in the neocortex and hippocampus region

Region	DA	DOPAC	HVA	DA turnover	5-HT	5- HIAA	5-HT turnover	NE
Neocortex								
Control	1934±86	413±44	938±72	0.698	1508±94	747±69	0.522±0.01	229±20
$A\beta_{35-25}$ treated	1930±72	418±32	927±45	0.696	1499±88	756±56	0.504±0.08	220±16
$A\beta_{25-35}$ treated	1457±66*	476±38*	1372±78$^{\$}$	1.264$^{\#}$	1044±55$^{\$}$	1098±95$^{\#}$	1.081±0.17$^{\$}$	159±16$^{\#}$
$A\beta_{25-35}$+melatonin treated	1885±82	422±35	948±39	0.714	1488±48	835±65	0.538±0.01	238 ± 16
Hippocampus								
Control	1851±60	527±59	849±61	0.748	1379±79	44±5	0.031±0.005	999±40
$A\beta_{35-25}$ treated	1850±98	535±49	830±76	0.737	1344±55	46±3	0.034±0.001	972±67
$A\beta_{25-35}$ treated	1388±84$^{\$}$	578±56*	1289±84$^{\$}$	1.324*	1089±87$^{\#}$	81±8$^{\$}$	0.079±0.002$^{\#}$	648±41*
$A\beta_{25-35}$+melatonin treated	1814±75	549±51	864±80	0.768	1278±84	51±4	0.040±0.001	955±59

DA: dopamine, HVA: homovanillic acid, DOPAC: 3, 4-dihydroxyphenylacetic acid 5-HT: 5-hydroxytryptamine, 5-HIAA: 5-hydroxyindoleacetic acid, NE: norepinephrine DA turnover=(DOPAC+HVA)/DA; 5-HT turnover=5-HIAA/-5HT.
Different superscripts show significant variation between groups (*$P<0.05$, $^{\$}P<0.01$, $^{\#}P<0.001$).
Values are mean±S.E.M.; ng/g wet tissue wt.

antioxidative action of melatonin. Earlier reports suggest that melatonin stimulates the synthesis of rate-limiting enzyme, γ-glutamylcysteine synthase and thereby increases intracellular GSH concentrations (Urata et al., 1999).

Our earlier investigation indicates that Aβ induces lipid peroxidation, a key component of free radical associated neurodegeneration in AD (Masilamoni et al., 2005b). The present study shows that melatonin therapy significantly reduces the Aβ induced MDA levels (Table 2). Cellular oxidative imbalance can activate astrocytes (von Bernhardi and Eugenin, 2004). The reactive astrocytes participate in the inflammatory responses by producing proinflammatory cytokines and free radicals (Wallace et al., 1997). The possible mechanism in AD is the accumulation of such free radicals during inflammation (Minghetti, 2005). In the current study the activated astrocytes were identified by the enhanced expression of GFAP (a marker of activation of astrocytes, Fig. 1c), thus support the hypothesis that the elevated GFAP content and its degradation products are the response of astrocytes to oxidative stress. A significant positive correlation between glial markers and lipid peroxidation in the brain homogenates supports this idea. In a previous report, the increased expression of GFAP during aging was found to be in relation to oxidative stress (Baydas and Tuzcu, 2005). The present result shows that the pharmacological administration of melatonin prevents the elevation of GFAP content and its degradation products induced by Aβ (Fig. 1d). Since free radical processes play a role in reactive astrocytes (Gabryel and Trzeciak, 2001), it is likely that melatonin therapy would have inhibited free radical generation.

Several studies have linked increased intraneuronal generation of ROS and nuclear factor-kappa B (NF-κB), a member of the Rel transcription factor family, activation (Pizzi and Spano, 2006). The transcription factor NF-κB has been recognized as a key regulator in the early expression of genes involved in the intracellular changes in redox status and subsequent oxidative stress (Wise et al., 2005). Recent studies have shown that Aβ treatment activates NF-κB in neurons and astrocytes, a process, which is mediated by the generation of ROS (Kaltschmidt et al., 2002). Melatonin, a radical scavenger, thereby prevents the NF-κBNFkB activation in the hepatocytes and astrocytes of Aβ injected mice (Fig. 1h and l). It has been well documented that excessive oxidant production involved in the degeneration of dopaminergic neurons in Parkinson's disease (McCormack et al., 2005). The present study supports the hypothesis that a significant positive correlation exists between free radical production and dopamine turnover in the brain. Herein, we report that the pharmacological administration of melatonin prevents the dopamine depletion and its degradation products induced by Aβ (Table 4).

Dietary supplementation with melatonin has recently been shown to result in a significant rise in levels of endogenous melatonin and slows the neurodegenerative changes associated with brain aging (Lahiri et al., 2004a,b). An interesting recent study discovered that melatonin modulates the inflammation associated gene expression and immune function with advancing age (Sharman et al., 2007). Previous studies indicate that antioxidative effect of GSH could be one mechanism behind this protective action of melatonin, since dopaminergic metabolism is a source of free radicals (Hirrlinger et al., 2002). GSH also conjugate DA and thereby counter its toxicity (Grima et al., 2003). Interestingly, several researchers found that the antioxidant enzyme SOD also prevents NO induced dopamine oxidation (Segura-Aguilar and Lind, 1989). In conclusion, we suggest that melatonin has the potential to act as an antioxidative agent thus by preventing amyloid protofibrillar induced oxidative imbalance and biogenic amine catabolism.

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