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Review

Beneficial effects of melatonin in experimental models of Alzheimer disease[1]

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Key words

melatonin; Alzheimer disease; beta-amyloid protein; calcium overload; APP transgenic mice; ovariectomized rats; tau protein hyperphosphorylation

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Abstract

Alzheimer's disease (AD), a progressive degenerative disorder, is characterized by the presence of amyloid deposits, neurofibrillary tangles and neuron loss. Emerging evidence indicates that antioxidants could be useful either for the prevention or treatment of AD. It has been shown that melatonin is a potent antioxidant and free radical scavenger. Additionally, melatonin stimulates several antioxidative enzymes and improves mitochondrial energy metabolism. These findings led us to study amyloid precursor protein transgenic mice, ovariectomized rats, and pheochromocytoma and astroglioma cell lines, to observe whether melatonin had any effect on Alzheimer's symptoms or pathological changes. We found that melatonin had many beneficial effects in experimental models of AD, including improvement of cognitive function, anti-oxidative injury, anti-apoptosis, inhibition of β-amyloid (Aβ) deposition and Aβ fiber formation. Several groups have shown that melatonin has an inhibitory effect on tau protein hyperphosphorylation. These actions may potentially slow down or stop the progression of dementia.

Introduction

N-Acetyl-5-methoxytryptamine (melatonin) is a lipophilic hormone that is mainly produced and secreted at night by the pineal gland. Melatonin was first reported to be an efficient endogenous antioxidant in 1993 by Reiter and colleagues[1–3]. It is found in all organisms, including bacteria, plants, insects, and vertebrates[4,5]. Because melatonin is also ingested in foodstuffs such as vegetables, fruits, and herbal medicines, from a nutritional point of view, melatonin can also be classified as a vitamin[3,6,7].

Alzheimer's disease (AD), a progressive degenerative disorder of the brain, is the most common cause of dementia amongst elderly people. AD is characterized by the presence of β-amyloid (Aβ) deposits and neurofibrillary tangles (NFT) in the brains of afflicted individuals. The development of early diagnostic tools and of quantitative markers are crucial for exploring promising therapeutic strategies[8]. Recent research findings have more systemically defined the molecular pathogenesis of AD, and are generating new approaches for treatment. Anti-inflammatory agents, antioxidants, vaccinations, cholesterol-lowering agents and hormone therapy are examples of new approaches that are being developed for treating or delaying the progression of AD. Additionally, nutritional, genetic, and environmental factors highlight some effective preventive strategies for AD[8–10].

One approach being pursued is to prevent the formation of senile plaques. One of the theories regarding the etiology of AD is the "Aβ toxicity" hypothesis[11]. Aβ mediates neurodegeneration by a complex series of interacting neurodegenerative processes that involve increasing extracellular concentrations of glutamate, increasing intracellular Ca^{2+} concentrations, and apoptosis[12]. Aggregated Aβ produces even more free radicals, whereas Aβ toxicity is eliminated by free radical scavengers.

Although the etiology of AD is not yet fully understood, Aβ-related oxidative stress and NFT are believed to be con-

tributing causative factors. In addition to conventional therapies, antioxidant strategies for protecting against AD have been increasingly explored, as evidenced by an increasing number of animal studies, clinical reports and patents related to antioxidant therapies for AD in recent years. Recent evidence indicates that melatonin reduces the neuronal damage mediated by oxygen-based reactive species in experimental models of AD by acting as a free radical scavenger and antioxidant[13,14]. Several clinical studies have also indicated that melatonin levels are decreased in AD patients[15]. Recently, Zhou *et al* reported that cerebrospinal fluid (CSF) melatonin levels were significantly decreased in aged individuals with early neuropathological changes in the temporal cortex[16]. The implication is that a decrease in CSF melatonin levels may be an early event in the development of AD, possibly occurring even before clinical symptoms appear.

Pappolla *et al* recently demonstrated that the neuroprotective action of melatonin against Aβ-mediated toxicity did not require the binding of melatonin to a membrane receptor, and was likely related to the antioxidant properties of melatonin[17]. Thus, both melatonin's direct receptor-independent scavenging effects and its receptor-mediated influences on enzyme activities may account for its possible beneficial effects in AD.

Previous research carried out by our group has demonstrated that melatonin can reduce glutamate release, alleviate its excitotoxicity, prevent abnormal nitric oxide (NO) elevation in the cerebral cortex, inhibit intracellular calcium overload, and enhance the expression of neurotrophin in several *in vivo* or *in vitro* experimental models of aging[18–24]. Importantly, recent findings from our group further indicate that melatonin has a pronounced neuroprotective effect against Aβ-induced neurotoxicity in both *in vivo* and *in vitro* experimental models of AD[25–29]. In contrast to classical antioxidants, melatonin, because of its amphiphilicity, readily crosses the blood-brain barrier and has a widespread intracellular distribution. Therefore, validation of its beneficial effects in the AD is necessary.

The exact mechanism by which melatonin contributes to neuroprotection is still unclear. Therefore, it is likely that determining the mechanisms by which Aβ induces neuronal cell death will lead to identification of potential molecular targets for the development of therapies for AD. Thus, interest in the protective role of antioxidants such as vitamin E, melatonin and estrogens in AD is expanding. Several clinical trials have demonstrated that melatonin is effective in treating mild-to-moderate dementia in AD patients. A scientific rationale for using multiple antioxidants in clinical trials for the prevention of AD in high-risk populations and as an adjunct to standard therapy in the treatment of this disease is worth considering. Melatonin, which has the characteristics of an antioxidant, is one of the candidates for the prevention and treatment of Alzheimer's disease.

Nevertheless, whether exogenous melatonin has any effect on the neuropathological processes of AD remains unknown. We hypothesized that any therapeutic effect of melatonin in a transgenic mouse model of AD might be due to its protective effect on neurons, as well as on glial cells. Therefore, we observed the effects of melatonin in experimental models of AD, and we summarized our results in the present review. Our results demonstrate that, in addition to the beneficial effects of providing direct antioxidant protection to neurons, melatonin may enhance neuroprotection against Aβ-related neurotoxicity by promoting the survival of neuronal or glial cells. Furthermore, hyperphosphorylation of microtubule-associated protein tau is the main cause of NFT, another neuropathological characteristic of AD. We also summarized the inhibitory effects of melatonin on the hyperphosphorylation of tau in the present review.

Effects of melatonin on amyloid precursor protein (APP) transgenic mouse

Melatonin alleviates behavioral deficits associated with apoptosis and cholinergic system dysfunction in an APP transgenic mouse model of AD Animal models are extremely valuable for the discovery and development of new treatments for AD. A valid animal model for AD should exhibit (i) progressive AD-like neuropathology, (ii) cognitive deficits, and (iii) should be verified in several laboratories[30,31]. Importantly, many of these transgenic animal models develop age-dependent deficits in some relevant behavioral tests and thus provide an animal model not only for amyloidosis but also for the cognitive deficits of AD patients. Investigations using the presently available transgenic models will help to define the relationships between impaired behavioral performance and pathological/biochemical abnormalities in the brain, to clarify pathogenic mechanisms *in vivo* and lead to the identification of new therapeutic targets[31].

A transgenic mouse model for AD mimicking the accumulation of senile plaques, neuronal apoptosis and memory impairment was used in our studies. Step-down and step-through passive avoidance tests showed that 8-month-old transgenic mice had decreases in step-down latency (SDL) and step-through latency (STL), and increases in count of error (CE) throughout the entire learning trial and memory session, which suggests learning and memory impairment. However, long-term administration of melatonin (10 mg/kg

for 4 months) significantly ameliorated learning and memory deficits[25]. Both Congo red staining and Bielschowsky silver impregnation showed apparent extracellular Aβ deposition in the frontal cortex of transgenic mice, but melatonin supplementation decreased the Aβ deposits[25]. In addition to memory loss, AD is also characterized by progressive neuronal degeneration. Recently, Matsubara *et al* reported that early (starting at 4 months of age) and long-term (lasting from 4 months to 11.5 months of age) administration of melatonin partially inhibited the expected time-dependent elevation of Aβ, reduced the abnormal nitration of proteins and increased survival in the treated Tg2576 transgenic mice, which overexpress APP695 containing the "Swedish" mutation[32]. The results of our neuropathological studies are consistent with those of Matsubara *et al*, and results from both groups provide supporting evidence for the potential clinical application of melatonin.

Studies in the human brain indicate that the cholinergic synaptic loss and dystrophy visible in transgenic animal models appear in more advanced stages of amyloid pathology in the human brain[33]. The decrease in cortical choline acetyltransferase (ChAT) activity may partly explain this. In line with the histological results, the congophilic plaques may be accompanied by dysfunction of the cholinergic system[25]. ChAT activity has been found to be reduced in the frontal cortex and hippocampus of transgenic mice compared with their non-transgenic littermates. Melatonin supplementation increased ChAT activity in the frontal cortex and hippocampus[25]. DNA fragmentation was present in the frontal cortex of the transgenic mice, and melatonin reduced the number of apoptotic neurons[25].

Neurons in the vulnerable brain regions of AD patients exhibit several alterations that are suggestive of apoptosis, including caspase upregulation[34,35], increased levels of prostate apoptosis response-4 (Par-4)[36], and increased expression of Bax[37]. In particular, caspase-3 has been shown to be significant in the development of the nervous system, as well as in AD brains[38]. We found caspase-3 upregulation in AD model transgenic mice. Par-4 has been shown to interact with several proteins that are known to modulate apoptosis, including Bcl-2 and caspase-8. Par-4, which can be induced at the translational level, acts at an early stage of the apoptotic cascade prior to caspase upregulation and mitochondrial dysfunction. Our previous results in these transgenic mice with AD indicated that cognitive impairment and apoptosis developed in mice as young as 8 months of age[25]. Apoptosis and cholinergic system dysfunction most likely contributed to behavioral impairments in the transgenic mice with AD[25]. The apoptotic markers Bax, caspase-3 and Par-4 were detected in neurons of the transgenic mice but not in the wild type mice. Long-term melatonin administration prevented the abnormal upregulation of apoptotic markers to an extent. Although it is difficult to state whether these are the primary or secondary effects of melatonin, it is reasonable to infer that melatonin exerts its neuroprotective effects through multiple direct and indirect mechanisms.

It is worth emphasizing that long-term application of melatonin alleviates memory impairments in transgenic mice. The neuropathological and biochemical findings also support this conclusion. Experimental data suggest that early, long-term administration of melatonin can significantly prevent, or at least slow the development of AD. These data provide some convincing *in vivo* evidence to support the potential clinical applications of melatonin[25].

Early melatonin supplementation alleviates oxidative stress in a transgenic mouse model of AD Increased awareness of the part oxidative stress plays in the pathogenesis of AD has highlighted the issue of whether oxidative damage is a fundamental step in pathogenesis or instead results from disease-associated pathology[39–42]. Several studies have demonstrated the presence of lipid, protein, and DNA oxidation products in postmortem examinations of the brains of AD patients[43–45]. In AD clinical trials, molecules with antioxidant properties such as vitamin E and *Ginkgo biloba* extract produce a modest benefit. Treatment with antioxidants is a promising approach for slowing disease progression given that oxidative damage may be responsible for the cognitive and functional decline observed in AD[46–48]. Through observing the specific markers of *in vivo* oxidative stress, as well as the expression of apoptotic-related factors, we demonstrated that melatonin suppresses brain lipid peroxidation in transgenic mice, and reduces the expression of apoptosis-related factors *in vivo*.

Studies in AD transgenic mice (Tg2576), which overexpress the APP695 containing the "Swedish" mutation, revealed that elevated peroxidation occurred several months before detectable Aβ accumulation and amyloid plaque formation[32,49]. We found that there were decreases in total superoxide dismutase (SOD) activity in transgenic mice, coupled with an increase in TBARS content. Our observations show that brain lipid peroxidation is present in transgenic mice, suggesting that brain oxidative damage is an early event, and might contribute to an AD-like phenotype early in the lives of these animals. Matsubara *et al* reported that early (beginning at 4 months of age), long-term (lasting from 4 months to 11.5 months of age) administration of melatonin partially inhibited the expected time-dependent elevation of Aβ and reduced the abnormal nitration of proteins in the treated

Tg2576 transgenic mice[32]. Our results are partly consistent with theirs. Importantly, we demonstrated that long-term treatment with a physiological dose of melatonin inhibited increase in lipid peroxidation in transgenic mice and increased SOD activity as well.

Further supporting a causal role for oxidative stress in amyloid-induced pathology is the fact that administration of melatonin in these mice led to reduced oxidative stress and, consequently, to reduced neuronal apoptosis. These results further suggest that melatonin can provide a combination of antioxidant and anti-amyloidogenic features that can be explored either as a means of preventive or therapeutic treatment for AD, or as a model for the development of anti-amyloidogenic indole analogs.

Effects of melatonin on ovariectomized adult rats

Long-term effects of melatonin or 17β-estradiol on improving spatial memory performance in cognitively impaired ovariectomized adult rats[26] The prevalence of AD in women is double that in men[50]. Degeneration of the cholinergic innervations from the basal forebrain to the hippocampal formation in the temporal lobe is thought to be one of the factors determining the progression of memory decay, both during normal aging and AD. Estrogen deprivation has been implicated as a risk factor in AD, and estrogen-mediated neuroprotection has been described in several *in vitro* model systems, which include Aβ induced toxicity and the associated oxidative stress[51].

Ovariectomized (OVX) female Sprague-Dawley rats are characterized by progressive memory deficits, central cholinergic nerve system degeneration and differentiation/apoptosis imbalance[52]. OVX rats typically have estrogen-reversible impairments of learning/memory behavior[53,54]. This *in vivo* model has been widely used to mimic postmenopausal pathophysiological changes in women[53]. The Morris water maze is used to investigate spatial learning and memory in rats, and lesions in various brain regions are known to impair performance in this test[55].

We carried out a study in which OVX Sprague-Dawley rats received daily injections of melatonin (5, 10, or 20 mg/kg) or 17β-estradiol (80 μg/kg) for 16 weeks. Morris water maze results indicated that ovarian steroid deprivation resulted in spatial memory impairment, but melatonin and 17β-estradiol significantly ameliorated spatial memory deficits in OVX rats[26], suggesting that long-term melatonin or 17β-estrodiol treatment prevents impaired spatial learning/memory in OVX rats. Four months after OVX, ChAT activity in the frontal cortex and hippocampus were greatly decreased in comparison with the controls. Melatonin and 17β-estradiol antagonized the effects induced by OVX. DNA fragmentation was present in the frontal cortex of the OVX rats. Melatonin and 17β-estradiol reduced the number of apoptotic neurons[26].

These results show that melatonin exerts beneficial effects on cholinergic neurotransmission in the brain by increasing ChAT activity in the frontal cortex and hippocampus. Previous data also suggests that there exists an interaction between melatonin and the cholinergic system in a transgenic mouse AD model[25]. On the basis of the results described here and TUNEL data, it is likely that apoptosis accounts for behavioral impairments in the OVX rats[26]. Melatonin and 17β-estradiol alleviated the learning and memory deficits in OVX rats and reduced apoptosis.

These findings demonstrate the important effects of melatonin and 17β-estradiol on cholinergic neurons and support the potential use of melatonin in the treatment of dementia in postmenopausal women. Our results indicate that neuroprotection by melatonin partly correlates with the modulation of apoptosis and protection of the cholinergic system. Early long-term application of melatonin is a promising strategy that could potentially be applied in a clinical setting. Collectively, the *in vivo* data show that melatonin alleviates learning and memory impairments in OVX rats, and provide some supporting evidence for the long-term use of melatonin in a clinical setting.

Long-term melatonin or 17β-estradiol supplementation alleviates oxidative stress in OVX adult rats[27] Estradiol deprivation has been implicated as a risk factor in AD, and estradiol-mediated neuroprotection has been described in several *in vitro* model systems, including Aβ-induced toxicity and associated oxidative stress[56–68]. Estradiol can interact with neuroprotective intracellular signaling pathways and is itself a neuroprotective antioxidant. Estradiol serves as a free-radical scavenger [ie an estrogen receptor (ER)-independent mechanism] in preventing nerve cell death induced by various oxidative insults[59]. Evidence from epidemiological studies supports enhanced cognitive function in women with AD taking estrogen replacement therapy (ERT) as well as a reduced risk for developing AD in healthy women receiving ERT. Additional clinical evidence suggests that estrogen may modulate specific cognitive functions such as working memory and verbal learning and memory. However, results from more recent controlled trials have not consistently shown a beneficial effect of estrogen on the cognitive function of women with AD[56–60]. ERT appears to be warranted only for short-term treatment of menopausal symptoms. Given this, it is important to find an alternative treatment for

postmenopausal-related dementia and AD. Thus, the beneficial effects of melatonin need to be confirmed in large-scale clinical trails using AD patients.

There is evidence that pineal melatonin is an anti-aging hormone and that menopause is associated with a substantial decline in melatonin secretion and an increased rate of pineal calcification. Melatonin might be involved in menopause-associated processes such as insomnia, breast cancer, and general aging[61]. Its beneficial effect on sleep has been demonstrated in controlled clinical trials; however, melatonin replacement therapy for all postmenopausal women is currently unjustified[62]. We explored the role of oxidative stress in the brains of OVX Sprague-Dawley rats and found some evidence to justify the use of melatonin in clinical trials to treat postmenopausal women[27].

The mitochondrial respiratory chain is composed of 5 enzyme complexes: NADH-CoQ reductase (complex I), succinate CoQ reductase (complex II), ubiquinol-cytochrome c reductase (complex III), COX (complex IV), and F1F0-ATPase (complex V). An impairment of the mitochondrial respiratory chain is a characteristic consequence of oxidative stress[63]. We demonstrated decreased activity of mitochondrial complex I and mitochondrial complex IV in the OVX rat brain, which suggested mitochondrial dysfunction in the OVX brain. Our results are consistent with the results of other groups. Moreover, a recent study has shown that melatonin enhances the activity of mitochondrial respiratory chain complexes I and IV in rat brain and liver[64]. Oxidative damage to mitochondria, in particular, may play a key role in aging.

SOD plays a protective role in all aerobic organisms by detoxifying the superoxide anion in a dismutase reaction, producing hydrogen peroxide. Glutathione (GSH) is a non-specific hydroxyl radical scavenger. Being the major source of reactive oxygen species (ROS), mitochondria are subjected to direct attack by large numbers of ROS in the cell and therefore might be particularly susceptible to oxidative damage. In OVX rats we found increases in brain mitochondrial malondialdehyde (MDA) levels, decreases in mitochondrial GSH content and mitochondrial SOD activity, and accelerated activation of apoptotic-related factors such as Bax, caspase-3, and Par-4.

It is well known that estrogen has antioxidant properties *in vitro*. However, there are conflicting results regarding the effect of estradiol *in vivo*[65, 66]. A short-term experiment in OVX rats suggested that oxidative stress in brains of female rats might be modulated by the level of progesterone[65]. Recently, an increase in MDA levels in the brains of OVX rats for 12 weeks post-ovariectomy has been demonstrated. Furthermore, estrogen and raloxifene have been found to exert antioxidative effects in the brain. We found a significant increase in brain mitochondrial MDA, which is consistent with the findings of a previous study[65]. In addition, we noted a decrease in mitochondrial SOD activity and mitochondrial GSH content 16 weeks after ovariectomy. Therefore, it is reasonable to expect that oxidative stress is present in the OVX rat brain. Furthermore, we found that long-term administration of melatonin prevented oxidative stress in OVX rats.

There have been several studies emphasizing the importance of postmenopausal estradiol replacement therapy for protection against the neuronal death induced by global ischemia associated with cardiac arrest or stroke[67]. A number of studies have reported that estradiol has neuroprotective effects in experimental models of stroke, although the effectiveness of long-term estradiol replacement at the levels used in ERT in humans is less clear[27]. However, the specific pathology in the brains of OVX rats remains largely unknown. Results from the OVX rats suggest that exogenous estrogen positively regulates Bcl-2 levels in rat brain, and OVX rats appeared more vulnerable to ischemia. We found that cognitive impairment and apoptosis in the frontal cortex developed 16 weeks after ovariectomy in OVX rats[26]. Apoptosis and cholinergic system dysfunction most likely contribute to behavioral impairments in OVX rats[26]. We detected the apoptotic markers Bax, caspase-3 and Par-4 in the neurons of the OVX rats, and long-term melatonin administration prevented the abnormal activation of the apoptotic markers to an extent. These results also provide some convincing *in vivo* support for the clinical application of melatonin[27]. Our findings extended those of previous studies in that we showed for the first time that long-term melatonin or estradiol administration afforded robust protection against OVX-induced neuronal apoptosis and that neuroprotection by melatonin or estradiol was associated with blocking of caspase-3 activation and Par-4 upregulation[27].

At this point, it is worth noting the possible relationship between melatonin and estradiol. However, there have been no extensive studies of the interaction of estradiol and melatonin in the central nervous system. It was found that in OVX rats, total overnight melatonin was reduced and could be restored to intact levels by administration of estradiol. We therefore concluded that estradiol could modulate melatonin production throughout the estrous cycle. Also, clinical data indicates that in postmenopausal women cortisol levels are enhanced by melatonin. In young men and women, melatonin influences vascular reactivity and reduces blood pressure and norepinephrine levels. The circulatory response to melatonin is conserved in postmenopausal women with

but not without hormone replacement therapy[68,69]. Maintenance of the cardiovascular response to melatonin may be implicated in the reduced cardiovascular risk of postmenopausal women who undergo hormone replacement therapy. Furthermore, based on our studies, it appears that oxidative stress involves OVX-related neurological abnormality, and that the neuroprotective effects of melatonin and 17β-estradiol in the OVX model mainly depend on their anti-oxidative functions.

Significantly, we reported that long-term melatonin administration preserved mitochondrial function in OVX rats, which provided further convincing evidence for the possible clinical applications of melatonin in the treatment of postmenopausal women. Our findings in general indicate that melatonin has an important role in the progress of postmenopausal neuropathy. Further supporting a causal role for oxidative stress in postmenopausal neuropathy, administration of melatonin in OVX rats led to reduced oxidative stress and consequently reduced neuronal apoptosis. On the basis of these investigations, it could be expected that melatonin would exert neuroprotective effects *in vivo* and therefore might reduce the risk of AD in postmenopausal women[27]. A definitive test of this prediction requires a randomized placebo-controlled double-blind clinical trial[26,27].

Melatonin reduces Aβ-induced apoptosis in pheochromocytoma (PC12) cells and rat astroglioma (C6) cells In AD, neuronal loss is prominent in the cerebral cortex and the limbic lobe, whereas different neuronal populations are vulnerable to various insults in other neurodegenerative diseases[28]. The finding that Aβ has neurotoxic properties and that such effects are partly mediated by free radicals has provided insights into the mechanisms of cell death in AD and an avenue to explore new therapeutic approaches. Therefore, it is likely that knowledge of the mechanisms by which Aβ induces neuronal cell death will help to identify potential molecular targets for the development of therapies for AD[28,70,71].

PC12 sympathetic nerve cells mimic the behavior of neurons, including differentiation, synapse formation and growth cone expansion[28]. Therefore, this peripheral cell line provides a useful approach for studying the cellular pathophysiology of AD, including calcium alterations and associated phenomena.

PC12 cells treated with either Aβ25-35 or Aβ1-42 underwent apoptosis. Melatonin pretreatment (at concentrations of 1×10^{-5}, 1×10^{-6} or 1×10^{-7} mol/L) significantly attenuated Aβ25-35- or Aβ1-42-induced apoptosis in PC12 cells. The anti-apoptotic effects of melatonin were highly reproducible and were corroborated by multiple quantitative methods, including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) cell viability assay, Hoechst 33342 nuclei staining, DNA fragmentation analysis and flow cytometric analysis[28,72,73]. In addition, melatonin effectively suppressed Aβ1-42-induced nitric oxide formation, potently prevented Aβ1-40-induced intracellular calcium overload and significantly alleviated Aβ1-40-induced membrane rigidity[28].

These results suggest a special link between ROS and apoptosis in the pathogenesis of AD. The protective effect of melatonin on apoptosis is due, at least in part, to its antioxidant properties and appears to be mediated either by a bona fide scavenging activity, which substitutes for decreased GSH, or by modulating calcium and signal transduction[28]. We noted a decrease in the percentage of apoptotic cells as well as DNA fragmentation when PC12 cells were treated with melatonin, and thus concluded that melatonin protected PC12 cells from Aβ-induced apoptosis. The use of melatonin or its analogs could be explored as a therapeutic approach in AD[28].

Astrocytes, the most abundant glial cell type in the brain, provide metabolic and trophic support to neurons and modulate synaptic activity. Astrocytosis is a common feature of amyloid plaques, a hallmark of AD, along with activated microglia, NFT and Aβ deposition. The Aβ-astrocyte interaction produces a detrimental effect on neurons, which may contribute to neurodegeneration in AD. The regulation of astrocyte apoptosis is essential to physiological and pathological processes in the central nervous system. Recently, Paradisi *et al* reported that astrocytes protected neurons from Aβ neurotoxicity, but that when they interacted with Aβ, this protection was undermined and neurotoxicity was enhanced[74]. Thus, the regulation of astrocyte apoptosis was essential to both physiological and pathological processes of in the central nervous system[75–77].

We found that rat astroglioma (C6) cells treated with Aβ25-35 or Aβ1-42 underwent apoptosis, and that melatonin pretreatment at concentrations of 1×10^{-5}, 1×10^{-6} and 1×10^{-7} mol/L significantly attenuated Aβ25-35- or Aβ1-42-induced apoptosis[29]. In addition, melatonin effectively suppressed Aβ1-42-induced nitric oxide formation, markedly prevented Aβ1-40-induced intracellular calcium overload, and significantly alleviated Aβ1-40-induced membrane rigidity[29].

The precise mechanisms behind the effects of anti-apoptotic drugs in astrocytes remain unknown. Advances in our knowledge of the molecular mechanisms of astrocyte apoptosis may lead to the development of novel therapeutic strategies for neurodegenerative disorders[29,78–85]. The study proposes a novel cellular mechanism underlying the beneficial effects of melatonin against degeneration of the central

nervous system. The significance of our findings is that, in addition to the beneficial effects of providing direct antioxidant protection to neurons as reported, melatonin may also provide neuroprotection through the suppression of glial reactivity and the promotion of the antioxidant defense system of glial cells[29,79–85]. Melatonin treatment exerted neuroprotective effects against injury by promoting the survival of both glial cells and neurons. These data highlight the potential for protecting the central nervous system from AD by preserving glial cells in addition to neurons. These results suggest a potential strategy directed at enhancing glial cell survival as an alternative protective approach for protection against AD damage.

Inhibitory role of melatonin in tau protein hyperphosphorylation

The cytoskeleton plays a crucial role in maintaining the highly asymmetrical shape and structural polarity of neurons that are essential for neuronal physiology. In AD, the cytoskeleton is abnormally assembled into NFT, and impairment of neurotransmission occurs. Microtubule-associated protein tau is capable of binding to tubulin to form the microtubules that are essential structures for neuronal viability. NFT are histopathological lesions that occur in AD, and lead to cytoskeletal loss and cell death. NFT are made from paired helical filaments (PHF). The core components of PHF result from the abnormal hyperphosphorylation of tau. The microtubule-stabilizing function of tau is greatly diminished by its hyperphosphorylation to PHF-tau, which binds poorly to tubulin[86], so inhibitory action on tau protein hyperphosphorylation could be a target for AD drug therapy.

It is widely accepted that hyperphosphorylation of the tau protein is due to an imbalance between the activities of the phosphorylating enzymes and the dephosphorylating enzymes[87,88]. Glycogen synthase kinase-3 (GSK-3), a downstream element of phosphoinositol-3 kinase (PI-3K), is one of the most active enzymes in phosphorylating tau *in vivo*. Wortmannin is a specific inhibitor of PI-3K. *In vivo*, inhibition of PI-3K results in overactivation of GSK-3 and tau hyperphosphorylation. The level of phosphorylated tau at a paired helical filament-1 (PHF-1) epitope was elevated after injection of wortmannin into the lateral ventricle. Increases in tau phosphorylation at a particular epitope were arrested by preinjection of melatonin. These results indicate that melatonin partially inhibits the pathological processes of AD[89–90].

Protein kinase A (PKA) is another crucial kinase in AD-like tau hyperphosphorylation. Isoproterenol (ISO), the specific PKA activator, can induce tau hyperphosphorylation, and hippocampal injection of ISO induces PKA overactivation and tau hyperphosphorylation at both PHF-1 and tau-1 sites. ISO injection also results in the activation of SOD and elevation of MDA, which suggests elevated oxidative stress. Preinfusion of melatonin intraperitoneally partially reverses ISO-induced tau hyperphosphorylation at the PHF-1 epitope and tau-1 epitope. Furthermore, melatonin obviously antagonizes ISO-induced PKA overactivation, enhances SOD activity and decreases the level of MDA. It has been suggested that ISO may induce abnormal hyperphosphorylation of tau through not only the activation of PKA but also by increasing oxidative stress. Melatonin may protect against ISO-induced tau hyperphosphorylation through suppression of both PKA overactivation and oxidative stress[91].

Decreases in the activities of protein phosphatase-2A (PP-2A) and protein phosphatase-1 (PP-1) also play important roles in the pathogenesis of AD. By using human neuroblastoma cells, Li *et al* found that calyculin A (CA), a selective inhibitor of PP-2A and PP-1, significantly increased phosphorylation and accumulation of neurofilaments (NF) in the cells[92]. Additionally, CA led to decreased cell viability. Melatonin efficiently protects the cells from CA-induced alterations in NF hyperphosphorylation and accumulation, and suppresses NF gene expression as well as decreasing cell viability[92]. In the same human neuroblastoma cells, melatonin protect against a series of pathological lesions including abnormal phosphorylation of cytoskeletal proteins, microtubule disassembly and mitochondrion-initiated cell toxicity induced by okadaic acid, a potent protein phosphatase PP-2A and PP-1 inhibitor[93].

To further investigate the effect of the *in vivo* inhibition of melatonin biosynthesis on spatial memory retention and tau phosphorylation in rats (and the underlying mechanisms), Zhu *et al* injected haloperidol, a specific inhibitor of 5-hydroxyindole-*O*-methyltransferase (a key enzyme for melatonin biosynthesis), into the lateral ventricle and into peritoneal cavity. This treatment compromised the spatial memory retention of rats and induced hyperphosphorylation of tau at tau-1 and PHF-1 epitopes. Furthermore, the activities of PP-2A and SOD decreased with an elevated level of MDA. Supplementation with melatonin by prior injection and reinforcement during haloperidol administration significantly improved memory retention deficits, arrested tau hyperphosphorylation and oxidative stress, and restored PP-2A activity. These results strongly support the involvement of decreased melatonin in Alzheimer-like spatial memory impairment and tau hyperphosphorylation. PP-2A may play a role in mediating aberrant melatonin-induced lesions[94].

Conclusion

AD is a heterogeneous disease involving a number of genetic components, risk factors and other poorly defined elements that all impact on the accumulation of Aβ. Importantly, the parallel development of early biological markers should enable intervention in pre-symptomatic disease stages. Drug development for AD should focus on the pathological events associated with neurodegeneration, such as oxidative stress, inflammation or disturbances in growth factor signaling.

Melatonin has an effect in reducing oxidative damage in the central nervous system and it can cross the blood-brain barrier. This combination of actions makes melatonin a highly effective pharmacological agent against free radical damage in brain. This property and the possibility of its interaction with the mitochondrial genome should be considered in subsequent studies related to the interaction of melatonin with mitochondria[14,27,39]. Therefore, using combinations of antioxidants with different subcellular distributions and different properties for prophylaxis or treatment would probably improve therapeutic outcomes. The development of novel antioxidants with anti-apoptotic properties and the ability to improve metabolism, for example by increasing the formulation of antioxidants with other agents, which have different functions, will become a requirement in strategies for protection against AD[40,41,44,46–48].

As in many other studies and our results summarized here, melatonin was found to have an essentially neuroprotective effect against Aβ-induced neurotoxicity. This being the case, its potential clinical application to forestall AD should be further considered. Although the clinical value of these agents for AD prevention and treatment is unknown, it is worth considering the use of melatonin in clinical trials for the prevention of AD in high-risk populations and as an adjunct to standard therapy in the treatment of this disease. The use of melatonin or its derived analogs could be explored as a therapeutic approach in AD.

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