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Melatonin inhibits the caspase-1/cytochrome c/caspase-3 cell death pathway, inhibits MT1 receptor loss and delays disease progression in a mouse model of amyotrophic lateral sclerosis

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Abstract

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Caspase-mediated cell death contributes to the pathogenesis of motor neuron degeneration in the mutant SOD1^{G93A} transgenic mouse model of amyotrophic lateral sclerosis (ALS), along with other factors such as inflammation and oxidative damage. By screening a drug library, we found that melatonin, a pineal hormone, inhibited cytochrome *c* release in purified mitochondria and prevented cell death in cultured neurons. In this study, we evaluated whether melatonin would slow disease progression in SOD1^{G93A} mice. We demonstrate that melatonin significantly delayed disease onset, neurological deterioration and mortality in ALS mice. ALS-associated ventral horn atrophy and motor neuron death were also inhibited by melatonin treatment. Melatonin inhibited Rip2/caspase-1 pathway activation, blocked the release of mitochondrial cytochrome *c*, and reduced the overexpression and activation of caspase-3. Moreover, for the first time, we determined that disease progression was associated with the loss of both melatonin and the melatonin receptor 1A (MT1) in the spinal cord of ALS mice. These results demonstrate that melatonin is neuroprotective in transgenic ALS mice, and this protective effect is mediated through its effects on the caspase-mediated cell death pathway. Furthermore, our data suggest that melatonin and MT1 receptor loss may play a role in the pathological phenotype observed in ALS. The above observations indicate that melatonin and modulation of Rip2/caspase-1/cytochrome *c* or MT1 pathways may be promising therapeutic approaches for ALS.

Keywords

Melatonin; caspases; cytochrome *c*; apoptosis; melatonin receptor 1A; amyotrophic lateral sclerosis

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by a progressive and selective loss of motor neurons, resulting in progressive paralysis and death (Rowland and Shneider, 2001). Although the exact pathophysiological mechanisms are not fully understood, the activation of caspase-mediated cell death pathways plays an important role in neuronal death in ALS patients and animal models (Friedlander, 2003; Li et al., 2000). The potential importance of apoptotic pathways in ALS is suggested by several observations: altered Bcl-2-family proteins result in a predisposition towards cell death; increased expression or activation of caspase-1 and caspase-3 in ALS; and appearance of morphological features in dying motor neurons that are reminiscent of apoptosis (Pasinelli et al., 2000; Sathasivam et al., 2001; Vukosavic et al., 1999). Further supporting evidence is the occurrence of apoptosis-related mitochondrial dysfunction in the spinal cord associated with disease progression (Dupuis et al., 2004; Shi et al., 2010). Consistent with the morphological/functional abnormalities of mitochondria in ALS, cytochrome *c* and other pro-apoptotic proteins are released into the cytoplasm. Moreover, in ALS animal models, disease onset can be delayed and lifespan prolonged by transgenic inhibition of caspase-1, by intracerebroventricular administration of specific and broad caspase inhibitors, by overexpression of Bcl-2, or by administering drugs that inhibit the release of cytochrome *c* (Friedlander et al., 1997a; Kostic et al., 1997; Li et al., 2000; Zhang et al., 2003a; Zhu et al., 2002).

Melatonin, a naturally occurring pineal hormone, is best known for its role in regulating the light-dark cycle. Many biological effects of melatonin depend upon its binding to specific receptors, although the antioxidant activity is also important to its physiological functions (Hardeland, 2005). To date, three types of melatonin receptors have been cloned, although only two, melatonin receptor 1A (MT1) and melatonin receptor 1B (MT2), are detected in mammalian neurons (Mazzucchelli et al., 1996; Naji et al., 2004). Abnormal expression of the MT1/MT2 receptor has been reported in several neurological diseases (Sanchez-Hidalgo

et al., 2009; Savaskan et al., 2001; Wang et al., 2011), however, there are no reports evaluating the importance or the role of melatonin receptors in ALS.

We recently identified melatonin as an anti-apoptotic agent. As previously described (Wang et al., 2008), a library of 1040 FDA-approved drugs and bioactive chemicals were screened for their ability to inhibit calcium-mediated cytochrome *c* release from purified mitochondria. Twenty-one compounds were further tested in a neuronal cell death model. Melatonin was one of the most effective candidates at inhibiting cytochrome *c* release and preventing neuronal death. In the present study, we evaluated the protective effects of melatonin in a transgenic mouse model of ALS, which expresses mutant-human copper-zinc superoxide dismutase (mSOD1^{G93A}). Melatonin was indeed beneficial in this animal model, and its neuroprotection was associated with its inhibition of the caspase-1/cytochrome *c*/caspase-3 pathways. Furthermore, for the first time, we detected a significant increase of receptor interacting protein-2 (Rip2), reduction of melatonin levels, and a down-regulation of MT1 in the spinal cord of ALS mice. These disease-associated changes were ameliorated in ALS mice by exogenous melatonin supplementation.

Materials and Methods

Animals and drug treatment

Transgenic ALS littermate mice expressing mutant human SOD1 G93A in the B6SJL genetic background were randomly assigned among experimental groups (Jackson Laboratory, Bar Harbor, ME), while controlling each group so that the gender ratio was always the same (Ryu et al., 2005; Veldink et al., 2003). Mice were maintained in a pathogen-free environment and provided food and water *ad libitum*. All of the animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Harvard Medical School.

Melatonin was dissolved in 0.9% saline after being dispersed by dimethyl sulphoxide (DMSO) and prepared fresh daily. Melatonin (30mg/kg, 10 µl/g body weight) was injected intraperitoneally (i.p.) daily beginning at six weeks of age until one day before sacrifice. Vehicle-control ALS mice were injected in the same manner with the DMSO/saline vehicle.

Clinical assessment

Beginning at 6 weeks of age, mice were weighed and assessed using neurobehavioral tests (n=15 animals in each group). Muscle strength and coordination deficits were evaluated weekly using a Rotarod apparatus (Columbus Instruments, Columbus, OH) as previously described (Li et al., 2000; Wang et al., 2007; Zhu et al., 2002). Testing was terminated either when the mice fell from the rod or at 7 minutes if the mouse remained on the rod. Disease onset was defined as the first day when the mouse could not remain on the Rotarod for 7 minutes at the speed of 15 rpm. Mortality was scored at the age of natural death or the age when the mouse was unable to right itself within 30 seconds (surrogate death). Three times per week, the mice were evaluated for signs of motor deficits according to a four-points neurological scoring system (Shefner et al., 2001; Veldink et al., 2003; Weydt et al., 2003): no sign of motor dysfunction (0); evident tremor and inability to extend hind limbs when suspended by the tail (1); paresis of one or both hind limbs and presence of gait abnormalities (2); paralysis of one or both hind limbs and gait with dragging of at least one hind limb (3); inability to right itself within 30 seconds (4). Body weight was monitored weekly. All studies were performed by investigators blinded to treatment.

Histopathological evaluation and immunohistochemistry

At 120 days of age, a separate cohort of mice ($n=5$ animals in each group) was anesthetized, transcardially perfused with 4% paraformaldehyde, and spinal cords were harvested. After post-fixation and cryoprotection, serial sections of the frozen tissue were cut in the coronal plane at 50 μ m intervals as described previously (Wang et al., 2007). Motor neuron survival was evaluated by Nissl staining. The absolute numbers of motor neurons with visible nucleoli were counted in the ventral horns of all sections from lumbar regions at the L2-3 level. The observer was blinded to the identity of each preparation. The numbers reported for each experimental group were average counts of motor neurons per section (including right and left ventral horns) among 20 continuous sections from each of five animals. The area of grey and white matter and the total cross-sectional area were also measured in the same Nissl-stained preparations of lumbar spinal cord using Spot RT Software (Diagnostic Instrument, Inc., Sterling Heights, MI). Subsequently, the volume per section was calculated as area $\times$ thickness. An average volume per section was calculated for mice in each experimental group.

In parallel experiments, serial sections of spinal cord were immunostained with antibodies specifically against cytochrome *c* (Zymed Laboratories, South San Francisco, CA), activated caspase-3 (BD Pharmingen, San Jose, CA), Ricinus communis agglutinin-1 (RCA-1, Sigma), glial fibrillary acidic protein (GFAP, BD Pharmingen, San Jose, CA), or MT1 (Santa Cruz Biotechnology, Inc, CA) respectively followed by conjugated secondary antibody as previously described (Ferrante et al., 1997; Ryu et al., 2005).

Tissue fractionation and western blotting

Mice were sacrificed at the age of 120 days, and samples of total lysate and cytosolic fraction were prepared from spinal cords as described previously (Li et al., 2000; Zhang et al., 2011; Zhu et al., 2002). Briefly, for the samples of total protein lysates, tissue samples (mouse or human spinal cord specimens) were homogenized on ice in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 142.5 mM KCl, 5 mM MgCl₂, 10 mM Hepes, pH 7.4) with protease inhibitor cocktail (Sigma, St. Louis, MO) and PMSF (0.1 mg/ml; Fluka, Switzerland). Lysates were centrifuged twice at 10,000 $\times$ g for 20 min at 4 °C. The resulting supernatants were analyzed by western blotting with antibodies to caspase-1, caspase-3, caspase-9, Rip2, MT1 and MT2. To prepare cytosolic fractions, tissue samples were gently homogenized in cold buffer (250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 2 mM EDTA, 1 mM DTT, 10 mM Hepes, pH 7.4, plus protease inhibitor cocktail and 0.1 mg/ml PMSF) and clarified by a 700 $\times$ g centrifugation for 5 min at 4 °C. The supernatant was centrifuged again at 15,000 $\times$ g for 25 min at 4 °C and used as the cytosolic component for the determination of released cytochrome *c* and Smac/Diablo by western blotting.

Protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA). Proteins in total lysates or in cytosolic fractions (50–100 μ g) were resolved by electrophoresis on 12% or 15% SDS-PAGE gels and transferred to a PVDF membrane. Transfer membranes were probed using antibodies to caspase-1 (provided by Dr. Junying Yuan, Harvard Medical School), pro- or active caspases-3 and caspase-9 (Cell Signaling Technology, Beverly, MA), Rip-2 (ΨProSci, Poway, CA), MT1 and MT2 (MBL, Woburn, MA), cytochrome *c* (BD PharMingen, San Diego, CA), or Smac/Diablo (Novus Biologicals, Littleton, CO). To control for variations in loading, membranes were re probed with an antibody to β -actin (Sigma).

ELISA analysis of intraspinal levels of melatonin

Melatonin levels in the spinal cord were determined using a Melatonin ELISA kit (BlueGene Biotech, Shanghai, China) performed in accordance with manufacturer

instructions. Mice were sacrificed at the age of 120 days, and spinal cord samples were collected between 1 PM and 3 PM. Spinal cord was flash frozen in liquid nitrogen and stored at -80°C until homogenization. At the time of homogenization, spinal cords were placed in 0.25 ml of ice cold homogenization buffer containing protease inhibitor cocktail. Tissue was homogenized with a tissue homogenizer set on 700 rpm. Samples were homogenized on ice using 10 to 14 up and down strokes. Homogenate was taken through one thaw-freezing cycle and spun at $1500 \times g$ for 15 minutes at 4°C . Protein concentration was measured and the supernatant was assayed at the same protein concentration.

Statistical analyses

All data are presented as the mean $\pm$ S.E.M. for groups. Behavior score of motor deficient and cumulative probability of onset and survival data were analyzed by ANOVA with Tukey's post-test, and all other analyses were compared using student's *t*-test. Significance was set at $P < 0.05$.

Results

Melatonin delays disease progression in ALS mice

ALS-mSOD1^{G93A} transgenic mice suffer from a progressive motor weakness and paralysis reminiscent of the human ALS. These animals were injected daily with 30 mg/kg melatonin or the DMSO/saline vehicle beginning at 6 weeks of age. Body weight and neurobehavioral tests, which included the Rotarod test and the motor deficit test, were evaluated to gauge the progression of disease as described in *Materials and Methods*. ALS mice began to lose weight around 16 weeks, a time when their wild-type littermates continued to gain weight. There was no difference in the time course of weight loss between the melatonin- and vehicle-treated groups (Figure 1A).

In the ALS mice, hind-limb tremor is the first detected sign of the motor-neuron disease (Weydt et al., 2003). In this study, tremors were first observed around 11–12 weeks of age in ALS mice when suspended by the tail. Mice treated with melatonin showed significant delay in the appearance of disease-related motor behavior (Figure 1B), including a delay in the presence of hind-limb tremor and gait abnormalities.

The Rotarod test is an objective method for the quantitative assessment of motor strength and coordination. The ability of mice to remain on a Rotarod turning at the speed of 15 or 5 rpm, respectively, measures behaviors relevant to the early or late disease stage of ALS. Using the Rotarod test at 15 rpm and 5rpm, we demonstrated that melatonin was beneficial to mice during both the early and late stages of the disease (Figure 1C, 1D). Moreover, treatment with melatonin significantly delayed disease onset and mortality (Figure 1E, 1F, 1G). Mice treated with melatonin exhibited disease onset at 121.4 ± 2.8 days, a statistically-significant 10.1% delay over the value of 110.3 ± 3.1 days observed with vehicle-treated mice ($n = 15$, $P < 0.01$). Melatonin also prolonged lifespan by 7.4% from 136.7 ± 2.5 days to 146.8 ± 4.1 days ($n = 15$, $P < 0.05$) (Figure 1G).

Melatonin ameliorates motor-neuron loss and spinal-cord atrophy in ALS mice

The hallmark of ALS pathology and the cause of progressive paralysis is the degeneration of motor neurons. As such, we investigated melatonin's ability to inhibit motor-neuron loss. We counted the number of neurons in the ventral horns of spinal cords at the lumbar level (L2-3) when the mice were 120 days old. Melatonin significantly inhibited the extent of motor-neuron loss by 51.9% in treated ALS mice as compared to vehicle-treated ALS mice of the same age (wild-type mice, 175 ± 11 ; vehicle-treated ALS mice, 79 ± 29 ; melatonin-treated ALS mice, 120 ± 28 ; $P < 0.01$ between melatonin and vehicle-treated ALS mice;

Figure 2). Gross atrophy of the spinal cord was calculated from the volume of equivalent anatomical structures at the lumbar level in ALS mice (melatonin- and vehicle-treated) and in age-matched wild-type animals. Unlike wild-type controls, ALS mice exhibited loss of both grey and white matter as well as a reduction in the total cross-sectional area of the spinal cord. The extent of tissue loss was reduced in melatonin-treated ALS animals compared to their vehicle-treated counterparts, and gross atrophy in grey matter, white matter and entire spinal cord tissue section at the lumbar level were significantly reduced by 13.9%, 13.7%, and 13.4%, respectively ($P < 0.01$; Figure 2A–2G). These results demonstrate that melatonin treatment ameliorates the neuropathologic features of ALS.

Reduction of endogenous melatonin in the spinal cord of ALS mice is restored by long-term treatment with melatonin

Melatonin, a naturally occurring pineal hormone found in most animals, is itself a free radical scavenger and an indirect antioxidant that acts to stimulate and/or activate antioxidant enzymes, *e.g.*, SOD, catalase, and glutathione peroxidase (Hardeland, 2005; Rodriguez et al., 2004). In order to investigate the changes of endogenous melatonin level in ALS mice and the effects of long-term treatment with melatonin, an ELISA analysis of intra-spinal melatonin levels was conducted in this study (Figure 2H). Our results showed that endogenous melatonin level in the spinal cord of ALS mice at 120 days old was significantly decreased by ~16% as compared to their age-matched wild-type littermates. Daily systemic injection of melatonin for 11 weeks completely restored the intra-spinal level of melatonin comparable to wild-type mice.

Melatonin inhibits the release of mitochondrial apoptogenic factors and the subsequent activation of downstream caspases in ALS mice

Having established a connection between melatonin's ability to ameliorate ALS pathological features and slowing of disease progression in ALS mice, we next investigated the underlying molecular mechanism of melatonin-mediated neuroprotection. The activation of programmed cell death pathways plays an important role in ALS. The release of mitochondrial apoptogenic factors is an important trigger and commits the affected neuron to activation of the cell death cascade. In particular, the release of cytochrome *c* has been implicated as a cause of neuronal cell death in ALS mice (Guegan et al., 2001; Ryu et al., 2005; Zhu et al., 2002). Immunoblotting was used to evaluate the protein levels in gross spinal cord tissue from ALS and wild-type littermate mice at the age of 120 days. Consistent with previous reports (Wang et al., 2007; Zhu et al., 2002), we detected a significant increase in the release of cytochrome *c* and Smac/Diablo into the cytoplasm in ALS mice, as compared to equivalent samples from wild-type mice. Moreover, treatment with melatonin inhibited the release of these mitochondrial apoptogenic proteins into the cytoplasm of spinal cord samples from ALS mice (Figure 3A).

Caspases play a prominent role in motor neuron cell death in ALS (Friedlander, 2003; Friedlander et al., 1997a; Li et al., 2000; Pasinelli et al., 2000). Cytochrome *c*, released from the mitochondria, binds to Apaf-1 and procaspase-9 in the presence of dATP to form the "apoptosome". Autocatalysis occurs, leading to the production of active caspase-9, which results in the activation of the downstream "executioner" caspase-3, causing cell death (Jemmerson et al., 2005; Liu et al., 1996; Yang et al., 1997). Melatonin inhibited cytochrome *c* release from purified mitochondria (Wang et al., 2009; Wang et al., 2008), and also led to a reduced release of mitochondrial apoptogenic factors in ALS mice. We therefore investigated the ability of melatonin to inhibit caspase activation. We measured the levels of pro- and active caspases in total lysates of spinal cord samples from age-matched (120-day-old) ALS and wild-type littermates by immunoblotting (Figure 3B, 5). First, we examined the expression of caspase-9, as well as that of the critical "executioner" caspase-3,

two enzymes of the cell death pathway which are activated downstream of mitochondrial events (Figure 3B). Our results confirmed a significant increase in total and active caspase-3 protein expression in ALS. Total and active caspase-9 levels suggested a similar trend, although this increase was not statistically significant. In the spinal cord tissue of ALS animals treated with melatonin, the expression and processing of apoptotic proteins was no longer significantly different from the non-ALS control.

Immunostaining for cytochrome *c* produced a punctate pattern in ventral-horn neurons of wild-type mice. By contrast, there was a diffuse homogeneous pattern of cytochrome *c* staining in neurons of vehicle-treated ALS mice, an indication that cytochrome *c* had been released into the cytoplasm. This observation is consistent with earlier reports (Guegan et al., 2001; Ryu et al., 2005). Release of mitochondrial cytochrome *c* is observed in the ventral-horn neurons of all ALS mice, and is attenuated in animals treated with melatonin (Figure 4A–4C). Parallel observations were made when spinal cord tissue was immunostained for active caspase-3. The level of activated enzyme was noticeably greater in tissue from ALS mice than in equivalent samples from wild-type animals. Again, treating ALS mice with melatonin reduced the extent of activated caspase-3 as compared to vehicle-treated animals (Figure 4D–4F). Neuroprotection by melatonin, manifested as increased survival of motor neurons, correlated with its inhibition of cytochrome *c* release and caspase-3 activation in those cells.

Melatonin attenuates astrocyte and microglial activation in ALS mice

GFAP is an intermediate-filament protein expressed abundantly and almost exclusively in astrocytes of the central nervous system. Astrocyte activation with increased levels of GFAP in the spinal cord correlates with motor-neuron degeneration. Indeed, astrogliosis may precede and drive the deterioration of motor activities in this animal model of ALS (Fujita et al., 1998; Keller et al., 2009). In addition, activated microglia is implicated in the pathogenesis of ALS (Henkel et al., 2009; McGeer and McGeer, 2002). Moreover, drugs aimed at inflammatory pathways have been detected in animal models of ALS that demonstrate beneficial effects on survival (Drachman et al., 2002). Immunostaining histological sections of spinal cord revealed a robust increase in GFAP, a marker for astrocytes, and RCA-1, a marker for microglia/macrophages, immunoreactivities in ALS mice at 120 days of age over that observed in age-matched wild-type mice. These pathological changes were reduced by melatonin-treatment in ALS mice (Figure 4G–4L).

Melatonin inhibits release of mitochondrial apoptogenic factors by suppressing Rip2 overexpression and caspase-1 activation

Caspase-1 is an upstream activator of neuronal cell death pathways (Zhang et al., 2003b), as well as an important mediator leading to inflammation. In fact, the activation of caspase-1 is the earliest cell-death signal detected during ALS progression, and is evident in the early symptomatic stage of the disease (Friedlander, 2003; Li et al., 2000; Pasinelli et al., 2000). Moreover, caspase-1 plays a key role in the cleavage of Bid and generation of tBid to regulate mitochondrial signaling in ALS (Guegan et al., 2002). Therefore, we investigated the expression of caspase-1 by western blotting using an antibody which recognizes both full-length and activated caspase-1 (*i.e.*, p20) (Li et al., 2000; Zhang et al., 2003b). Compared with wild-type mice, levels of pro- and active caspase-1 p20 were both significantly increased in ALS mice, and treatment with melatonin decreased the level of activated caspase-1 (Figure 5).

Rip2 is a serine/threonine kinase containing a C-terminal caspase recruitment domain (CARD). Rip2 binds to procaspase-1 via an interaction between their respective CARD domains, resulting in procaspase-1 oligomerization and activation (Thome et al., 1998). We

demonstrate for the first time the increase of Rip2 in spinal cords of ALS mice, as compared to wild-type control littermates. Melatonin significantly inhibited overexpression of Rip2 relative to saline-treated ALS animals (Figure 5). This result provides mechanistic insight explaining melatonin's suppression of caspase-1 activation.

Melatonin inhibits loss of MT1 expression in ALS mice

Specific cell surface melatonin receptors are expressed in neurons, which are members of the superfamily of G protein-coupled receptors with characteristic seven-transmembrane-helix topology. MT1 and MT2 are two homologous melatonin-receptor proteins. They are the only types found in humans and other mammals and are expressed both in brain and in peripheral tissues (Mazzucchelli et al., 1996; Naji et al., 2004). As is evident from Figure 6A, there was a significant reduction of MT1 receptor in spinal tissue from 120-day-old ALS mice as compared with age-matched littermate wild-type mice. Melatonin inhibited the ALS-associated reduction of MT1 receptor in the spinal cord of the diseased mice. In contrast, the level of MT2 was unchanged in spinal cord tissue from ALS and wild-type mice and was not altered by treatment with melatonin.

Changes in MT1 protein level and distribution in the spinal cord of ALS mice were further confirmed by MT1 immunostaining (Figure 6B–6H). As compared to wild-type control mice, MT1 staining and quantification demonstrated a decreased MT1 level in motor neurons in the spinal cord of ALS mice, and a significant recovery of MT1 protein level with melatonin treatment, suggesting that the reduction of MT1 in the spinal cord of ALS mice may be due to both the loss of motor neurons and a decrease of MT1 in the remaining motor neurons.

Discussion

Missense mutations of the SOD1 (mSOD1) gene, a well-documented cause of familial ALS, has been identified in about 20% of familial patients (Rowland and Shneider, 2001). Several mutant human SOD1 genes have been used to construct transgenic mouse models of ALS. The most commonly used model is the mSOD1^{G93A} mouse, an animal that expresses mutant human SOD1 genes with a substitution of glycine to alanine at position 93. These mice exhibit an age-dependent phenotype of progressive neuronal loss with declining motor strength, symptoms resembling those of ALS patients. In addition to inflammation and oxidative damage, strong evidence suggests that another disease mechanism, apoptotic cell death, is also involved in mSOD1-induced demise of spinal motor neurons (Cleveland and Rothstein, 2001; Friedlander, 2003; Julien and Beaulieu, 2000; Martin et al., 2000). Several studies, including our own, have shown that the mSOD1-mediated death signal upsets the balance between pro-apoptotic and anti-apoptotic signaling by proteins of the Bcl-2 family, and mitochondrial dysfunctions observed both in mSOD1 transgenic mice and ALS patients presumably results, at least in part, from disruption of this pathway (Friedlander et al., 1997b; Guegan et al., 2002; Kostic et al., 1997; Li et al., 2000; Martin, 1999; Pasinelli et al., 2004; Vukosavic et al., 1999; Zhu et al., 2002). Mitochondria play a central role in apoptotic signaling pathways. They transduce information from a variety of apoptotic stimuli by releasing cytochrome *c* and other pro-apoptotic proteins into the cytoplasm. These molecular events are under the regulation of Bcl-2 family proteins (Liu et al., 1996; Yang et al., 1997). In the present study, we found that melatonin inhibited the release of cytochrome *c* from the mitochondria to cytoplasm of motor neurons in ALS mice, and consequently decreased mSOD1-induced activation of downstream caspase-3. These results suggest that melatonin exerts its protective effects, at least in part, by inhibiting the release of cytochrome *c* and subsequent caspase activation.

Not only does melatonin inhibit activation of the downstream caspases-3, it also inhibits activation of caspase-1. Caspase-1 is known to be a key upstream signal in the neuronal cell death pathway and to mediate early disease processes in ALS (Friedlander, 2003). A prolonged period of caspase-1 activation is observed starting in the pre-symptomatic and early symptomatic stage of transgenic ALS mice (Cleveland and Rothstein, 2001; Li et al., 2000; Pasinelli et al., 2000). Moreover, caspase-1 participates in the regulation of mitochondrial signaling pathway in ALS via the cleavage of Bid. Dominant-negative inhibition of caspase-1 could inhibit the release of cytochrome *c*, thereby delaying disease onset and prolonging survival time of ALS mice (Friedlander et al., 1997a; Guegan et al., 2002). Furthermore, Rip2 is a stress-inducible upstream modulator of caspase-1 activation, and Rip2 overexpression-induced neuronal death is dependant on caspase-1 (Thome et al., 1998; Zhang et al., 2003b). In this study, we found that the anti-apoptotic properties of melatonin may arise from its inhibition of the Rip2/caspase-1 pathway, an upstream mechanism that affects and regulates downstream mitochondrial function and cytochrome *c* release.

As described above, abnormal expression of MT1 and MT2 receptors has been reported in models of Alzheimer's disease, Parkinson's disease, Huntington's disease, normal aging, and in senescence-accelerated mice (Caballero et al., 2008; Sanchez-Hidalgo et al., 2009; Savaskan et al., 2005; Savaskan et al., 2001; Wang et al., 2011). In addition, MT1 has been shown to protect C6 astroglial cells from oxidative damage and excitotoxicity (Das et al., 2008). However, to date, alteration of melatonin receptors in ALS has not been reported. The current study is the first to demonstrate significant reduction of MT1 expression in the spinal cord of ALS patients and transgenic ALS mice. Moreover, administering melatonin to ALS mice partially restored MT1 levels. These results suggest that dysfunction of MT1 expression may play an important role in the pathogenesis of ALS, and the protective properties of melatonin in ALS mice are related to its ability to partly rescue MT1 expression, as well as saturating the remaining MT1 receptors. Thus, MT1 and its pathways may be a promising target for future investigation into the pathogenesis and treatment of ALS.

ALS is an untreatable and inevitably fatal disease. Currently, the only therapeutic drug with a marginal effect on patient survival is riluzole, a glutamate antagonist aimed at symptomatic relief (Bensimon et al., 1994; Miller et al., 1999; Morrison, 2002). In spite of great efforts to identify protective drugs for the treatment of ALS, discovery of an effective agent remains elusive. The challenges in finding chemical-based treatments for neurological disorders, especially in ALS, have been fraught with scientific issues surrounding the exact cascade of pathophysiological mechanisms, experimental murine models used for translation to patients, and in the design and implementation of clinical trials, all of which may have contributed to multiple drug failures in patients. Translational failure in general, and in neurological disease specifically, may largely be the consequence of great clinical variability in the patient population under study. This raises the issue regarding the critical need for identifying biomarkers of disease onset and progression, as well as biomarkers in response to therapy. The latter would certainly improve the power of clinical trials, reducing both costs and the number of patients enrolled in drug studies.

Recently, it was reported that melatonin benefited ALS mice as a consequence of its broad spectrum of antioxidative properties, and moreover, in ALS patients, high doses of melatonin were well tolerated and to some extent oxidative damage was reduced (Jacob et al., 2002; Weishaupt et al., 2006). With this report, taken in context with previous reports on apoptotic signaling in ALS, we provide evidence that apoptotic signaling is pathogenic in ALS and that melatonin is neuroprotective in transgenic ALS mice. We also demonstrate that this protection is likely mediated, at least in part, through the MT1 receptor. We have

previously reported that melatonin binding of the MT1 receptor is beneficial in Huntington's disease (Wang et al., 2011). We now demonstrate that melatonin has a neuroprotective effect in ALS and the mechanism of this effect may be due to its ability to inhibit the caspase-1/cytochrome c/caspase3 pathway and to rescue MT1 expression.

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References

- Bensimon G, et al. A controlled trial of riluzole in amyotrophic lateral sclerosis. ALS/Riluzole Study Group. *N Engl J Med.* 1994; 330:585–91. [PubMed: 8302340]
- Caballero B, et al. Favorable effects of a prolonged treatment with melatonin on the level of oxidative damage and neurodegeneration in senescence-accelerated mice. *J Pineal Res.* 2008; 45:302–11. [PubMed: 18410310]
- Cleveland DW, Rothstein JD. From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS. *Nat Rev Neurosci.* 2001; 2:806–19. [PubMed: 11715057]
- Das A, et al. Cytoprotective effects of melatonin on C6 astroglial cells exposed to glutamate excitotoxicity and oxidative stress. *J Pineal Res.* 2008; 45:117–24. [PubMed: 18373557]
- Drachman DB, et al. Cyclooxygenase 2 inhibition protects motor neurons and prolongs survival in a transgenic mouse model of ALS. *Ann Neurol.* 2002; 52:771–8. [PubMed: 12447931]
- Dupuis L, et al. Mitochondria in amyotrophic lateral sclerosis: a trigger and a target. *Neurodegener Dis.* 2004; 1:245–54. [PubMed: 16908975]
- Ferrante RJ, et al. Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. *J Neurochem.* 1997; 69:2064–74. [PubMed: 9349552]
- Friedlander RM. Apoptosis and caspases in neurodegenerative diseases. *N Engl J Med.* 2003; 348:1365–75. [PubMed: 12672865]
- Friedlander RM, et al. Inhibition of ICE slows ALS in mice. *Nature.* 1997a; 388:31. [PubMed: 9214497]
- Friedlander RM, et al. Expression of a dominant negative mutant of interleukin-1 beta converting enzyme in transgenic mice prevents neuronal cell death induced by trophic factor withdrawal and ischemic brain injury. *J Exp Med.* 1997b; 185:933–40. [PubMed: 9120399]
- Fujita K, et al. Increase of glial fibrillary acidic protein fragments in the spinal cord of motor neuron degeneration mutant mouse. *Brain Res.* 1998; 785:31–40. [PubMed: 9526038]
- Guegan C, et al. Recruitment of the mitochondrial-dependent apoptotic pathway in amyotrophic lateral sclerosis. *J Neurosci.* 2001; 21:6569–76. [PubMed: 11517246]
- Guegan C, et al. Instrumental activation of bid by caspase-1 in a transgenic mouse model of ALS. *Mol Cell Neurosci.* 2002; 20:553–62. [PubMed: 12213439]
- Hardeland R. Antioxidative protection by melatonin: multiplicity of mechanisms from radical detoxification to radical avoidance. *Endocrine.* 2005; 27:119–30. [PubMed: 16217125]
- Henkel JS, et al. Microglia in ALS: the good, the bad, and the resting. *J Neuroimmune Pharmacol.* 2009; 4:389–98. [PubMed: 19731042]
- Jacob S, et al. Melatonin as a candidate compound for neuroprotection in amyotrophic lateral sclerosis (ALS): high tolerability of daily oral melatonin administration in ALS patients. *J Pineal Res.* 2002; 33:186–7. [PubMed: 12220335]
- Jemmerson R, et al. Cytochrome C release from CNS mitochondria and potential for clinical intervention in apoptosis-mediated CNS diseases. *Antioxid Redox Signal.* 2005; 7:1158–72. [PubMed: 16115019]
- Julien JP, Beaulieu JM. Cytoskeletal abnormalities in amyotrophic lateral sclerosis: beneficial or detrimental effects? *J Neurol Sci.* 2000; 180:7–14. [PubMed: 11090858]

- Keller AF, et al. Live imaging of amyotrophic lateral sclerosis pathogenesis: disease onset is characterized by marked induction of GFAP in Schwann cells. *Glia*. 2009; 57:1130–42. [PubMed: 19115383]
- Kostic V, et al. Bcl-2: prolonging life in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Science*. 1997; 277:559–62. [PubMed: 9228005]
- Li M, et al. Functional role of caspase-1 and caspase-3 in an ALS transgenic mouse model. *Science*. 2000; 288:335–9. [PubMed: 10764647]
- Liu X, et al. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell*. 1996; 86:147–57. [PubMed: 8689682]
- Martin LJ. Neuronal death in amyotrophic lateral sclerosis is apoptosis: possible contribution of a programmed cell death mechanism. *J Neuropathol Exp Neurol*. 1999; 58:459–71. [PubMed: 10331434]
- Martin LJ, et al. Mechanisms for neuronal degeneration in amyotrophic lateral sclerosis and in models of motor neuron death (Review). *Int J Mol Med*. 2000; 5:3–13. [PubMed: 10601567]
- Mazzucchelli C, et al. The melatonin receptor in the human brain: cloning experiments and distribution studies. *Brain Res Mol Brain Res*. 1996; 39:117–26. [PubMed: 8804720]
- McGeer PL, McGeer EG. Inflammatory processes in amyotrophic lateral sclerosis. *Muscle Nerve*. 2002; 26:459–70. [PubMed: 12362410]
- Miller RG, et al. Practice parameter: the care of the patient with amyotrophic lateral sclerosis (an evidence-based review): report of the Quality Standards Subcommittee of the American Academy of Neurology: ALS Practice Parameters Task Force. *Neurology*. 1999; 52:1311–23. [PubMed: 10227612]
- Morrison KE. Therapies in amyotrophic lateral sclerosis-beyond riluzole. *Curr Opin Pharmacol*. 2002; 2:302–9. [PubMed: 12020475]
- Naji L, et al. Expression of membrane and nuclear melatonin receptors in mouse peripheral organs. *Life Sci*. 2004; 74:2227–36. [PubMed: 14987948]
- Pasinelli P, et al. Amyotrophic lateral sclerosis-associated SOD1 mutant proteins bind and aggregate with Bcl-2 in spinal cord mitochondria. *Neuron*. 2004; 43:19–30. [PubMed: 15233914]
- Pasinelli P, et al. Caspase-1 and -3 are sequentially activated in motor neuron death in Cu, Zn superoxide dismutase-mediated familial amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A*. 2000; 97:13901–6. [PubMed: 11095709]
- Rodriguez C, et al. Regulation of antioxidant enzymes: a significant role for melatonin. *J Pineal Res*. 2004; 36:1–9. [PubMed: 14675124]
- Rowland LP, Shneider NA. Amyotrophic lateral sclerosis. *N Engl J Med*. 2001; 344:1688–700. [PubMed: 11386269]
- Ryu H, et al. Sodium phenylbutyrate prolongs survival and regulates expression of anti-apoptotic genes in transgenic amyotrophic lateral sclerosis mice. *J Neurochem*. 2005; 93:1087–98. [PubMed: 15934930]
- Sanchez-Hidalgo M, et al. Decreased MT1 and MT2 melatonin receptor expression in extrapineal tissues of the rat during physiological aging. *J Pineal Res*. 2009; 46:29–35. [PubMed: 18513209]
- Sathasivam S, et al. Apoptosis in amyotrophic lateral sclerosis: a review of the evidence. *Neuropathol Appl Neurobiol*. 2001; 27:257–74. [PubMed: 11532157]
- Savaskan E, et al. Reduced hippocampal MT2 melatonin receptor expression in Alzheimer's disease. *J Pineal Res*. 2005; 38:10–6. [PubMed: 15617532]
- Savaskan E, et al. Cerebrovascular melatonin MT1-receptor alterations in patients with Alzheimer's disease. *Neurosci Lett*. 2001; 308:9–12. [PubMed: 11445273]
- Shefner JM, et al. Effect of neurophilin ligands on motor units in mice with SOD1 ALS mutations. *Neurology*. 2001; 57:1857–61. [PubMed: 11723276]
- Shi P, et al. Mitochondrial dysfunction in amyotrophic lateral sclerosis. *Biochim Biophys Acta*. 2010; 1802:45–51. [PubMed: 19715760]
- Thome M, et al. Identification of CARDIAK, a RIP-like kinase that associates with caspase-1. *Curr Biol*. 1998; 8:885–8. [PubMed: 9705938]

- Veldink JH, et al. Sexual differences in onset of disease and response to exercise in a transgenic model of ALS. *Neuromuscul Disord.* 2003; 13:737–43. [PubMed: 14561497]
- Vukosavic S, et al. Bax and Bcl-2 interaction in a transgenic mouse model of familial amyotrophic lateral sclerosis. *J Neurochem.* 1999; 73:2460–8. [PubMed: 10582606]
- Wang H, et al. Nortriptyline delays disease onset in models of chronic neurodegeneration. *Eur J Neurosci.* 2007; 26:633–41. [PubMed: 17686041]
- Wang X, et al. Methazolamide and melatonin inhibit mitochondrial cytochrome C release and are neuroprotective in experimental models of ischemic injury. *Stroke.* 2009; 40:1877–85. [PubMed: 19299628]
- Wang X, et al. The Melatonin MT1 Receptor Axis Modulates Mutant Huntingtin-Mediated Toxicity. *J Neurosci.* 2011; 31:14496–14507. [PubMed: 21994366]
- Wang X, et al. Inhibitors of cytochrome c release with therapeutic potential for Huntington's disease. *J Neurosci.* 2008; 28:9473–85. [PubMed: 18799679]
- Weishaupt JH, et al. Reduced oxidative damage in ALS by high-dose enteral melatonin treatment. *J Pineal Res.* 2006; 41:313–23. [PubMed: 17014688]
- Weydt P, et al. Assessing disease onset and progression in the SOD1 mouse model of ALS. *Neuroreport.* 2003; 14:1051–4. [PubMed: 12802201]
- Yang J, et al. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science.* 1997; 275:1129–32. [PubMed: 9027314]
- Zhang W, et al. Additive neuroprotective effects of minocycline with creatine in a mouse model of ALS. *Ann Neurol.* 2003a; 53:267–70. [PubMed: 12557297]
- Zhang WH, et al. Fundamental role of the Rip2/caspase-1 pathway in hypoxia and ischemia-induced neuronal cell death. *Proc Natl Acad Sci U S A.* 2003b; 100:16012–7. [PubMed: 14663141]
- Zhang Y, et al. Dipyrone inhibits neuronal cell death and diminishes hypoxic/ischemic brain injury. *Neurosurgery.* 2011; 69:942–56. [PubMed: 21552169]
- Zhu S, et al. Minocycline inhibits cytochrome c release and delays progression of amyotrophic lateral sclerosis in mice. *Nature.* 2002; 417:74–8. [PubMed: 11986668]

Highlights

- Melatonin treatment is beneficial in a transgenic mouse model of ALS.
- Melatonin improves motor behavior and delayed disease onset and mortality.
- Melatonin inhibits the caspase-1/cytochrome c/caspase-3 cell death pathway.
- Disease-related overexpression of Rip2 is prevented by melatonin treatment.
- Melatonin rescues down-regulated expression of MT1 receptor.

Figure 1.

Melatonin delays disease progression in mSOD1^{G93A} ALS mice. Melatonin (30mg/kg) was injected i.p. every day from 6 weeks of age until the end of life. Disease progression was followed by assessment of behavioral tests and determination of body weight, beginning at 6 weeks and continuing throughout the study. **A**, Average body weight as a function of age in melatonin- and vehicle-treated ALS and wild-type mice. ALS mice began to lose weight at 16 weeks of age when wild-type mice were continuing to gain weight. **B**, Melatonin decreased the measure of motor deficits as defined in *Materials and Methods*, indicating that it slows the development and progression of symptoms in ALS mice ($P < 0.05$ vs. ALS-vehicle group; two-way ANOVA). **C**, **D**, Deficits in muscle strength and coordination, as evaluated by Rotarod test at constant speeds of 15 rpm (**C**) and 5 rpm (**D**), were significantly improved with melatonin treatment at both early and late stage disease in ALS mice. **E**, **F**, Cumulative probability of onset of disease (**E**) was significantly delayed and survival (**F**) was prolonged in ALS mice treated with melatonin compared to mice treated with vehicle ($P < 0.05$; ANOVA). **G**, Onset of disease and mortality were presented analytically for age (in days). The above regimen causes statistically-significant changes in both disease parameters. $n = 15/\text{group}$ (A-G) (the gender ratio of animals for different treatment groups was the same); *, $P < 0.05$; **, $P < 0.01$; P value, melatonin-treated ALS mice vs. vehicle-treated ALS mice.

Figure 2.

Melatonin ameliorates loss of motor neurons and atrophy of the spinal cord in mSOD1^{G93A} ALS mice. Mice were sacrificed at the age of 120 days, and spinal cords were dissected after fixation. Serial sections of frozen tissue at the lumbar level were cut in the coronal plane at 50 μ m intervals and stained with Nissl substance. *A–F*, Representative Nissl-stained sections of spinal cord revealed the gross cross-sectional area (*A–C*) and motor-neuron content (*D–F*) for melatonin- and vehicle-treated ALS mice as well as for age-matched wild-type animals. Compared with wild-type littermate controls (*A, D*), gross spinal cord atrophy and ventral motor neuron loss were observed in vehicle-treated ALS mice (*B, E*), both of which were reduced by daily administration of 30 mg/kg melatonin (i.p.) (*C, F*). Scale bars in panels *A–C* and *D–F* correspond to 300 μ m and 50 μ m, respectively. *G*, Motor neuron counts and atrophy of gross areas grey matter, white matter and total area of spinal cord at the lumbar level were significantly rescued in melatonin-treated ALS mice. $n = 5/\text{group}$; ###, $P < 0.001$ vs. wild-type mice; **, $P < 0.01$; ***, $P < 0.001$ vs. vehicle-treated ALS mice. *H*, systemic injection of melatonin restored decreased levels of endogenous melatonin in the spinal cord of ALS mice. All values of intra-spinal melatonin levels were normalized to control values in wild-type mice. $n = 6/\text{group}$; #, $P < 0.05$ vs. wild-type mice; *, $P < 0.05$ vs. vehicle-treated ALS mice.

Figure 3.

Melatonin inhibits release of apoptogenic factors from mitochondria and suppresses activation of caspases in mSOD1^{G93A} ALS mice. 30 mg/kg of melatonin was administered by daily i.p. injection beginning at 6 weeks of age until the end of life. At the age of 120 days, mice were sacrificed and their spinal cords were removed. Cytosolic fractions or total lysates were prepared by homogenization and centrifugation as described in *Materials and Methods*. *A*, Samples of the cytosolic fractions were analyzed by western blotting using antibodies to cytochrome *c* and Smac ($n = 4$). *B*, Samples of total lysate were analyzed by western blotting with antibodies to pro- and active caspase-3, caspase-9 ($n = 4$). β -actin staining was used as an internal loading control. Each lane in these blots represents a different mouse. The bar graphs to the right are generated by densitometry. #, $P < 0.05$; ##, $P < 0.01$ vs. wild-type mice; *, $P < 0.05$ vs. vehicle-treated ALS mice.

Figure 4.

Melatonin decreases cytochrome *c* release, caspase-3 activation, and gliosis in mSOD1^{G93A} ALS mice. Mice were sacrificed at the age of 120 days, and their spinal cords were removed after fixation. 20- μ m coronal cryosections were prepared and immunostained with antibodies to cytochrome *c* (A–C), activated caspase-3 (D–F), RCA-1 (G–I) or GFAP (J–L) (dark stained). Cytochrome *c* and activated caspase-3 are key mediators in mitochondria-related cell death pathways in ALS. The increased RCA-1 or GFAP expression corresponds to severity of microglia or astrocyte activation. The background immunostaining in the tissue samples are different as a consequence of cell arbors in the neuropil, where one would observe little of no background staining in wild-type mice. Immunostaining results demonstrated that cytochrome *c* release, caspase-3 activation, and levels of reactive RCA-1 and GFAP increased in the vehicle-treated ALS mice (B, E, H, K) compared with wild-type mice (A, D, G, J). These pathophysiologic changes were reduced by treating the ALS mice with melatonin (C, F, I, L). Scale bars in (A–I) and (J–L) correspond to 150 μ m and 50 μ m, respectively.

Figure 5.

Melatonin inhibits Rip2 overexpression and therefore prevents activation of caspase-1 in mSOD1^{G93A} ALS mice. 30 mg/kg of melatonin was administered by daily i.p. injection beginning at 6 weeks of age until the end of life. At the age of 120 days, mice were sacrificed and their spinal cords were removed. Samples of total lysate fractions were analyzed by western blotting with antibodies to caspase-1 and Rip2 (wild-type groups, $n = 4$; ALS groups, $n = 5$). β -actin was used as an internal loading control. Each lane in the blots represents a different mouse. The bar graphs to the right are generated by densitometry. ^{##}, $P < 0.01$ vs. wild-type mice; *, $P < 0.05$; **, $P < 0.01$ vs. vehicle-treated ALS mice.

Figure 6.

A significant down-regulated expression of MT1 in the spinal cord of ALS mSOD1^{G93A} mice, and melatonin treatment protects against the depletion of MT1. **A**, Melatonin rescues MT1 expression in ALS mice. Samples of total lysates were analyzed by western blotting with antibodies to melatonin receptors MT1 and MT2 (wild-type groups, $n = 4$; ALS groups, $n = 5$). Each lane in the blots represents a different sample. The bar graphs are generated by densitometry. **B–G**, **H**, MT1 expression and distribution in the spinal cord were analyzed by immunostaining. 20- μ m coronal cryosections were prepared and immunostained with antibodies to MT-1 in wild-type mice (**B**, **E**), vehicle (**C**, **F**), and melatonin (**D**, **G**) treated ALS mice. Scale bars in (**B–D**) and (**E–G**) correspond to 150 μ m and 50 μ m, respectively. $n = 5$ /group; #, $P < 0.05$; ##, $P < 0.01$ vs. wild-type mice. *, $P < 0.05$; **, $P < 0.01$ vs. vehicle-treated ALS mice.