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Reduced oxidative damage in ALS by high-dose enteral melatonin treatment

Abstract: Amyotrophic lateral sclerosis (ALS) is the collective term for a fatal motoneuron disease of different etiologies, with oxidative stress as a common molecular denominator of disease progression. Melatonin is an amphiphilic molecule with a unique spectrum of antioxidative effects not conveyed by classical antioxidants. In preparation of a possible future clinical trial, we explored the potential of melatonin as neuroprotective compound and antioxidant in: (1) cultured motoneuronal cells (NSC-34), (2) a genetic mouse model of ALS ($SOD1^{G93A}$-transgenic mice), and (3) a group of 31 patients with sporadic ALS. We found that melatonin attenuates glutamate-induced cell death of cultured motoneurons. In $SOD1^{G93A}$-transgenic mice, high-dose oral melatonin delayed disease progression and extended survival. In a clinical safety study, chronic high-dose (300 mg/day) rectal melatonin was well tolerated during an observation period of up to 2 yr. Importantly, circulating serum protein carbonyls, which provide a surrogate marker for oxidative stress, were elevated in ALS patients, but were normalized to control values by melatonin treatment. This combination of preclinical effectiveness and proven safety in humans suggests that high-dose melatonin is suitable for clinical trials aimed at neuroprotection through antioxidation in ALS.

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Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease, affecting predominantly motoneurons in the cerebral cortex and anterior horn of the spinal cord. Dysfunction and premature death of these neurons causes spasticity, hyperreflexia, muscular atrophy, and generalized paralysis. Respiratory failure is the main cause of death within 2–5 yr after diagnosis. Most ALS cases occur sporadic. Among the 5–10% familial forms, about 20% are associated with mutations in the gene for superoxide dismutase (SOD1) [1]. The disease is incurable and practically without treatment.

Different pathological mechanisms have been suggested to contribute to cell death in motoneuron diseases, independent of the underlying molecular/genetic defect. These include impaired axonal transport, mitochondrial dysfunction, neurofilament disorganization, protein aggregation, and impaired proteasome function [2–4]. Also excitotoxic mechanisms contribute to ALS. Indeed, the only therapeutic drug with a marginal effect on patient survival is riluzole, an antiexcitotoxin [5, 6]. Increased levels of glutamate were found in the cerebrospinal fluid of ALS patients [7]. In the spinal cord, a decreased glutamate uptake has been attributed to decreased glutamate transporter expression in the anterior horn, both in sporadic

cases of ALS and in SOD1 transgenic models [8–10]. Transgenic overexpression of the glutamate transporter EAAT2 delayed symptoms in mutant SOD1 transgenic mice [11]. Recently, a defect in AMPA-type glutamate receptor editing, leading to enhanced Ca^{2+} permeability, has been reported for a subset of ALS patients [12]. Interestingly, ocular motoneurons that are intrinsically resistant to ALS have higher calcium buffering capacities [13, 14].

It is well known that excitotoxicity by glutamate includes the generation of reactive oxygen species (ROS) [15]. For example, elevated intracellular calcium causes mitochondrial dysfunction, impairment of the respiratory chain, activation of NO synthases (NOS), and the generation of toxic radicals. An upregulation of iNOS was found in $SOD1^{G93A}$-transgenic mice [16]. It has also been proposed that aberrant enzymatic activity of mutant SOD1 leads to the production of toxic hydroxyl radicals and nitrotyrosine [4, 17]. By oxidative stress, in turn, free radical species can enhance monomer formation and aggregation of SOD1 [18]. Similarly, ROS alter the properties of neurofilaments that play a key role in axonal transport which is impaired in models of motoneuron disease [19]. Previously tested compounds with 'simple' antioxidative properties, such as vitamin C or E, have failed to prolong survival in ALS clinical trials [20–22].

Melatonin, a derivative of the essential amino acid tryptophan, is best known for its secretion by the pineal gland in the regulation of light–dark cycle [23]. It exhibits an unusually broad spectrum of antioxidative properties [24–26]. These include scavenging of hydroxyl carbonate, alkoxyl, peroxyl, and aryl cation radicals, stimulation of glutathione peroxidase, and suppression of NOS [26]. In particular, the interference of melatonin with NO metabolism is expected to have a major neuroprotective potential, because it counteracts both, cellular damage by peroxynitrite-related radicals and Ca^{2+}-dependent excitotoxicity [25, 26]. The amphiphilic chemical structure of melatonin and its rapid transfer through the blood–brain barrier [27] argue for its usefulness as a neuroprotective drug.

The present work has been designed in preparation of a possible future clinical trial of melatonin in ALS. We show that: (1) melatonin attenuates glutamate-induced death of motoneuronal cells in a dose-dependent fashion, and concomitantly reduces generation of ROS, and that (2) high-dose melatonin treatment of $SOD1^{G93A}$-transgenic mice improves outcome with respect to disease progression and survival. Importantly, long-term high-dose treatment of human ALS patients, using suppositories as a new route of melatonin application, is well tolerated and safe. A major therapeutic effect of this antioxidant is demonstrated by serum protein carbonyl levels that are initially elevated in all ALS patients and corrected to control values by melatonin treatment.

Materials and methods

NSC-34 cell culture experiments

Motoneuronal NSC-34 cells were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. To differentiate NSC-34 cells into a motoneuronal and glutamate-responsive phenotype [28], DMEM was replaced by DMEM/Ham's F12 supplemented by 1% FCS, 1% penicillin/streptomycin and 1% modified Eagle's medium nonessential amino acids. Cells were allowed to differentiate for 8 wk under reduced serum conditions and then seeded in 96-well plates at a density of 15 000 cells/well for the following experiments.

Glutamate toxicity assay

For melatonin treatment, melatonin (Sigma, Seelze, Germany) was dissolved in dimethylsulfoxide (DMSO; Sigma) and diluted in culture-medium to a final concentration of 10 and 50 μM. The final DMSO concentration was 0.2%, and this was used as the corresponding vehicle control. Glutamate was dissolved in phosphate-buffered saline (PBS), and added to cultures at concentrations of 2 or 10 mM, 3 hr after the start of melatonin treatment. 72 hr after start of glutamate exposure, cell viability was tested with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. MTT was added to culture wells (final concentration of 0.5 mg/mL) and incubated at 37 °C for 2 hr. Viability of motoneurons was assessed by counting MTT-positive cells in six fields within a culture well with a 20× objective. Mean values of at least four independent wells from three experiments were calculated, and cell survival expressed as percent MTT-positive cells compared with untreated control conditions.

Detection of oxidative stress in NSC-34 cells

Formation of intracellular ROS following glutamate exposure for 12 hr, or treatment with 200 μM of H_2O_2 for 30 min as positive control, was detected by loading the cells with the fluorescent oxidative stress indicator dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes; final concentration 20 μM) for 30 min at 37 °C. Fluorescence intensity of the oxidized dye was assessed using a LEICA TCS confocal laser scanning microscope with a 40× oil immersion objective at 496 nm excitation and 510 to 530 nm emission wavelength. Quantification of fluorescence intensity was achieved by subtraction of background fluorescence using ImageJ software. Intensity values were calculated as mean pixel intensity expressed in arbitrary fluorescence units. For each experimental condition, mean pixel intensity of at least 150 cell bodies from at least four independent wells of three experiments and seven independently taken laser scanning microscope frames was calculated.

Transgenic mice, melatonin treatment and behavioral testing

All animal experiments were approved by and conducted in accordance with the regulations of the local Animal Care and Use Committee. Mice transgenic for human $SOD1^{G93A}$ [TgN(SOD1-G93A)1 GUR] were purchased from Jackson Laboratory and bred at the University of Göttingen Medical School and Max-Planck animal facilities. A total

of 120 mice (group size 15–30, dependent on the experiments, if not indicated otherwise in the text) were used. Transgenic mice were identified using polymerase chain reaction (PCR) according to Gurney et al. [29]. Melatonin was dissolved in ethanol, then diluted in drinking water. Final ethanol concentration was 1% in all experimental groups, and vehicle controls received 1% ethanol in drinking water. To protect melatonin from light, drinking bottles were wrapped with aluminium foil at all times, and drinking water was replaced weekly. Fluorometric determination of melatonin concentrations in drinking water showed that melatonin remained completely stable under these conditions (data not shown). Depending on the experiment, treatment started at the age of 28 days or at onset of first symptoms. Locomotor function was assessed three times per week using a mouse rotarod (TCD, Germany), starting at the age of 6 wk. A constant velocity of 10 rounds/min was used. Onset of rotarod failure was defined as the first day when animals failed to perform on the rotarod for at least 5 min when three trials were allowed. In accordance with guidelines for the care and use of laboratory animals, mice were killed when they had lost more than 15% of their weight or were unable to move upright within 30 s when laid on one side. This timepoint was defined as the age of death.

Western blot analysis

Western blot experiments were performed using early symptomatic (100 days old) mice. Lumbar or cervical spinal cords were rapidly prepared and homogenized in lysis buffer containing 50 mM of Tris-HCl, 150 mM of NaCl, 1% Triton-X 100, 0.1 mM of polymethylsulfonyl fluoride (PMSF) and 2 μL/mL of pepstatin, leupeptin, and aprotinin, pH 8.3. Lysates were centrifuged at 14 000 rpm and the protein concentration of the supernatant was determined using the BCA reagent (Pierce, Rockford, IL, USA). After separating the lysates by reducing SDS-PAGE (15% gel, 20 μg protein per lane), proteins were transferred to polyvinylidene difluoride (PVDF) membrane and blocked in 5% skim milk in PBS-T (0.1% Tween 20). Proteins were detected by incubating with the following primary antibodies directed against: human SOD1 (SOD-100; Stressgen, Victoria, Canada), beta-tubulin (Sigma), phospho-ERK1/2, and phospho-AKT (Cell Signaling Technology, Beverly, MA, USA). Primary antibodies were visualized by incubation with respective HRP-conjugated secondary antibodies (Dianova, Hamburg, Germany). For densitometrical analysis, imagequant software was used and density normalized to background values. Ratios between corresponding melatonin- and vehicle-treated animals were then calculated for individual Western blot experiments after normalization to a tubulin standard. For standardization to tubulin, Western blot membranes were stripped after SOD1 staining and re-probed with a tubulin primary antibody followed by the respective HRP-conjugated secondary antibody. Lysates from at least three different animals per experimental group were used.

Immunohistochemistry

Coronal sections (20 μm) of the freshly frozen cervical spinal cord tissue were cut in a freezing cryostat (Jung CM3000; Leica Instruments, Nussloch, Germany). The tissue was postfixed for 30 min in 4% formaldehyde in PBS and rinsed with PBS. For immunoperoxidase-labeling of microglial cells with avidin-biotin, we used the following antibodies: rabbit anti-IBA-1 (Wako-Chemicals, Neuss, Germany, 1:5000), rat anti-MAC-3 (Pharmingen-BD Biosciences, Heidelberg, Germany, 1:1000), rat anti-F4/80 (Serotec, Oxford, UK, 1:1000).

Patients

Following announcement of the safety trial to the local Ethical Committee, 31 patients were included after informed consent. Inclusion required probable or definite ALS, according to El Escorial Criteria and disease duration of not more than 6 yr. Upon entry into the study, the ALS patients were admitted to a neurology ward for 5 days. Diagnosis was confirmed and treatment initiated. If patients were on riluzole (n = 25), vitamin E (n = 23), creatine (n = 4), or amitriptylin (n = 15) at admission to the study, they were allowed to continue this medication in addition to high-dose melatonin (see Table 1). Blood samples were taken before and during the initial days of treatment, and every 3–4 months during follow-up visits. ALS functional rating score (ALSFRS) was performed regularly, together with an extensive semi-structured interview of patients and relatives including questions for adverse event monitoring.

Preparation of melatonin suppositories

Melatonin suppositories were prepared through pouring of a melted cream to avoid heat destruction of the active compound. An equivalent of 300 mg pulverized high-performance liquid chromatography (HPLC)-grade melatonin (Synopharm, Barsbuettel, Germany; BUFA, Uitgeest, Netherlands) per suppository was mixed with an

Table 1. Amyotrophic lateral sclerosis baseline medication in melatonin-treated (n = 31) patients with and without continuous high-dose vitamin E (400–5000 IU/day) treatment

	Riluzole	Amitriptylin	Baclofen	Magnesium	Vitamin C	Creatine
No vitamin E premedication (n = 8)	6 (75)	5 (63)	1 (13)	4 (50)	2 (25)	1 (13)
Vitamin E premedication started 8.2 ± 2.4 months before melatonin (n = 23)	19 (83)	10 (43)	7 (30)	12 (52)	5 (22)	3 (13)

Absolute numbers of patients given, % of the respective cohort in brackets.

equivalent amount of hard fat, warmed to a cream-like consistency, mixed to homogeneity, and poured into polyethylene wrapped shapes with a 'torpedo' design (Iphas, Wuerselen, Germany). After the mixtures cooled and solidified, the suppositories were removed with warmed spatulas, and placed in special dispensing boxes (2×5). Each package was labeled as required by German laws and rules (§14 ApoBetrO, rules of AMG for clinical trials), indicating formulation of each suppository.

Melatonin radioimmunoassay

Melatonin levels in plasma and urine were determined by radioimmunoassay (RIA), as described previously [30]. In brief, plasma melatonin was measured by a direct charcoal-based RIA using tritium-labeled melatonin and a highly specific antibody (GS 704–6483) from Guildhay Antisera (Guildford, UK). The detection limit of this assay was 1 pg/300 μL. Intra- and interassay coefficients of variation were 6% and 12%, respectively. Melatonin from urine samples was extracted into chloroform under addition of acetate buffer (pH 4.0) and 0.1 mol of NaOH. The chloroform phase was evaporated to dryness and redissolved in assay buffer for RIA measurements.

Protein carbonyl determination

Protein carbonyl measurements were performed in the serum of 19 consecutively admitted ALS patients (61.7 ± 1.9 yr, 12 males, seven females) before and after ≥4 months of melatonin treatment and of ten healthy controls (61.2 ± 2.0 years, five males, five females). Protein carbonyls were determined by a variant of the 2,4-dinitrophenylhydrazine method [31]. Serum was diluted 1:10 with ice-cold 5-mM pf potassium phosphate buffer, pH 7.5, containing leupeptin (0.5 μg/mL), aprotinin (0.5 μg/mL), and pepstatin A (0.7 μg/mL); 300 μL was used for a single determination. Deviations from the original procedure concerned elimination of the chromogen: protein pellets were washed three times with 1 mL of ethanol/ethylacetate (1:1); particular attention was given to thorough resuspension (vortexing) and complete removal of the supernatant using Pasteur pipettes, and thereafter, stripes of blotting paper. Protein was measured according to Lowry et al. [32].

Chemical reagents

Laboratory chemicals were obtained from Sigma, unless otherwise stated in the text.

Statistical analysis

All numerical results are presented as mean ± S.E.M. Mean values between two groups were compared using Student t test (two-tailed unpaired t test in interindividual, dependent t test in intraindividual comparisons) in most instances, unless otherwise indicated. Statistical significance was set at $P \leq 0.05$ for all analyses.

Results

Melatonin modulates glutamate toxicity in NSC-34 motoneuronal cells

We screened candidate drugs for their motoneuron-protective potential, based on the hypothesis that excitotoxicity and the generation of ROS are major contributors to neurodegeneration in ALS. We employed NSC-34 cells, a widely used motoneuron-neuroblastoma fusion line [28], which can be differentiated by serum deprivation. After exposure to 2 or 10 mM of glutamate for 3 days, we determined a loss of 29.2% and 52.1% of cells, respectively, compared with the survival of untreated neurons. In these experiments, all viable cells were counted after staining with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). Interestingly, when such cultures were pretreated for 3 hr with melatonin (10 or 50 μM), we observed a small but significant rescue effect that appeared dose dependent (Fig. 1). Some protection from cell death

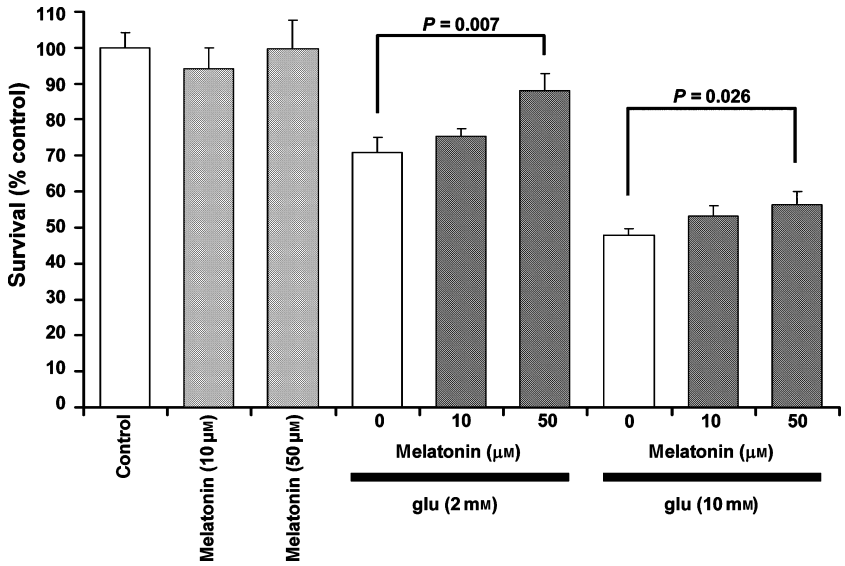

Fig. 1. Melatonin protects cultured motoneuronal cells against glutamate toxicity. Quantification of cell survival upon glutamate exposure (2 or 10 mM) for 3 days in the absence or presence of melatonin (10 or 50 μM). Number of viable cells, determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide staining, is expressed in percent of the respective untreated control cultures; n = 3–4 independent experiments; mean ± S.E.M. presented.

was even detectable for the 10 mM of glutamate challenge (8.5% more surviving cells than in unprotected cultures), but the effect was more obvious at the 'physiological' concentration of 2 mM of glutamate (17.2% more surviving cells with 50 μM of melatonin). As cell lines have acquired poorly defined antiapoptotic mechanisms, we turned to in vivo systems.

High-dose oral melatonin treatment prolongs survival of ALS mice

To determine possible neuroprotective properties of melatonin in ALS, we tested a widely used preclinical model, the SOD1^{G93A}-transgenic mouse line G1H [29]. Melatonin was given orally to female mutants at a concentration of 0.5 mg/mL in drinking water, starting at postnatal day 28. Age-matched transgenic female littermates served as respective controls. To quantify melatonin and water intake, we determined the weekly drinking volume for both experimental groups. No difference regarding body weight or fluid intake was detected (suppl. Fig. 1A,B). At about 3 months of age, SOD1^{G93A}-transgenic mice became symptomatic, starting with a fine tremor of the hindlimbs that progressed over 6–8 wk to severe paresis and premature death. Consequently, during later disease stages, drinking volumes and melatonin uptake declined (suppl. Fig. 1B,C). Between 8 and 15 wk of age, mean daily melatonin uptake was 88.3 ± 2.1 mg/kg body weight, decreasing to 56.9 ± 2.3 mg/kg between 16 and 20 wk of age, i.e., in more severely affected animals.

In this blinded study, we observed a significant benefit from melatonin treatment, with regard to disease progression and overall survival. According to national guidelines, animals had to be sacrificed at end stage (for definition, see material and methods). Mean survival time was extended by 5.9 days in melatonin-treated SOD1^{G93A}-transgenic mice as compared with untreated transgenic controls (136.9 ± 1.7 days (n = 29) versus 131.0 ± 1.2 days (n = 25) in vehicle-treated animals; Fig. 2A). The onset of symptoms, i.e., the appearance of hindlimb tremor, was not significantly changed (85.4 ± 2.3 versus 89.7 ± 2.3 days). However, disease progression, defined by the time span between the onset of tremor and premature death (41.3 ± 2.4 days in untreated mutants), was delayed by 25% in melatonin-treated mice (51.5 ± 2.7 days) (Fig. 2B). Even after failing the rotarod test, disease progression to death was prolonged (by 73.2%) in the melatonin-treated group [17.2 ± 3.6 days (n = 15) versus 9.9 ± 1.1 days (n = 15) in vehicle-treated animals; Fig. 2C]. In addition, the peak, i.e., the age at which most mutants died, was shifted by 1 wk, and a small subgroup of mice seemed to benefit overproportionally from melatonin treatment (see shoulder of red line in Fig. 2D).

Protective effects of melatonin require high dosage and early treatment

Owing to its very low solubility, the melatonin concentration in drinking water could not be further increased. We thus investigated the dose-dependency by reducing melatonin in drinking water to 10% of the tested concentration,

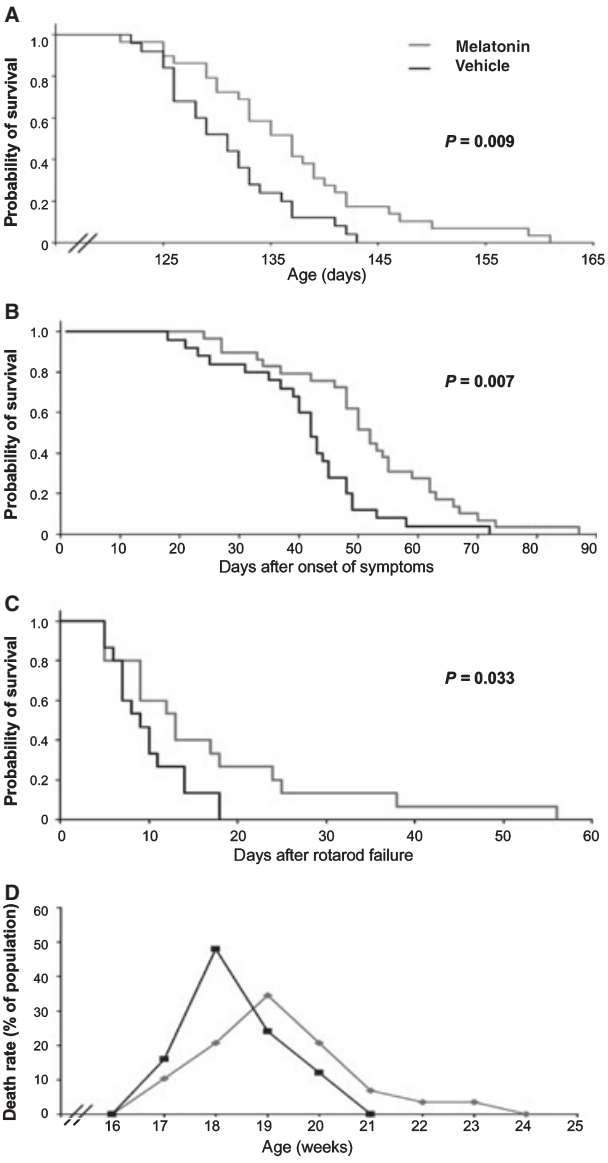

Fig. 2. Melatonin reduces disease progression and prolongs survival of SOD1^{G93A}-transgenic mice. Kaplan–Meyer curves illustrate significant benefits from melatonin treatment (0.5-mg/mL drinking water) regarding overall survival (n = 25–29 per group) (A), and disease progression from the onset of tremor (n = 25–29 per group) (B) or from rotarod failure (n = 15 per group) (C) to death in SOD1^{G93A}-transgenic mice. (D) Percentage of mouse population that reached endpoint criteria/death at the indicated age. The peak was reached later in melatonin-treated mice, and the curve is broadened to the right compared with the respective curve of the control population.

i.e., 0.05 mg/mL. This resulted in a mean melatonin uptake of 9.0 ± 0.5 mg/kg/day between 8 and 20 wk of age. At this dose, we only noticed a tendency toward increased survival times (134.6 ± 2.9 versus 132.7 ± 3.0 days) and a delay in disease progression between tremor and death (52.6 ± 6.9 versus 45.7 ± 6.0 days), without statistical significance ($P = 0.52$ and 0.15, respectively; n = 7 in each experimental group).

Similarly, when treatment was started at the onset of symptoms, which reduces the total time of treatment to

40.5 ± 2.5 days, significant differences were lost for mean survival time and disease progression (survival of 132.8 ± 2.1 days versus 134.4 ± 2.4 days in vehicle-treated control animals; $P = 0.602$; disease duration from first tremor to death 40.6 ± 2.5 versus 41.7 ± 2.8 days; $P = 0.763$).

Melatonin reduces ROS in motoneuronal cells

Although melatonin has antioxidant activities, the mechanism of neuroprotection in $SOD1^{G93A}$-transgenic mice is not known. Theoretically, melatonin might have altered the expression of the toxic SOD1-transgene. However, by Western blot analysis of spinal cord homogenates from melatonin- versus vehicle-treated mice, we were unable to detect quantitative differences at the protein level for the mutant (transgene-derived) or the endogenous SOD1 protein (suppl. Fig. 2).

As melatonin has been reported to stimulate glial-derived neurotrophic factor (GDNF) expression in the central nervous system [33], we considered the theoretical possibility that its neuroprotective effect is GDNF mediated. However, by Western blot analysis of spinal cord lysates, we found no changes in GDNF levels in mutant mice with melatonin treatment compared with untreated controls (data not shown).

Similarly, Western blot analysis of spinal cord protein lysates failed to detect differences in total amount or phosphorylation status of AKT or ERK1/2, kinases involved in the modulation of neuronal cell death [34]. Finally, melatonin treatment did not reduce microglial activation in mutant mice. Immunostaining of the cervical spinal cord, using microglial markers, was increased in all transgenic mice, when compared with wildtype mice at age P100, independent of melatonin treatment (data not shown).

Thus, the known antioxidative power of melatonin remains as the major candidate to explain the therapeutic effects observed in vivo. We focused on this potential mechanism, by studying differentiated NSC-34 motoneuronal cells in combination with dichloro-dihydro-fluoresceindiacetate (H_2DCFDA) as a fluorescent cell-permeant indicator for ROS. H_2DCFDA is nonfluorescent until the acetate groups are removed by intracellular esterases, and oxidation occurs within the cell [35]. The H_2DCFDA fluorescence in NSC-34 cells after exposure to glutamate was clearly enhanced (using H_2O_2 as positive control), but attenuated by treatment with melatonin (Fig. 3A–E). This finding is in good agreement with our hypothesis that melatonin acts as a free radical scavenger in motoneurons under distress.

Clinical safety trial

Although melatonin has been in use for almost two decades to treat jet lag at doses of 3–6 mg/day, high-dose treatments over years have not been reported. Daily melatonin uptake by SOD1-transgenic mice was 56.9 mg/kg during the symptomatic period. Because mice presumably metabolize most pharmacological compounds at least one order of magnitude faster than humans [36], we chose a dose of about 5 mg/kg for a safety study in ALS patients (corresponding to 300 mg per day). We decided to apply melatonin as suppositories at bedtime. This new form of application offered several advantages, including (1) intake

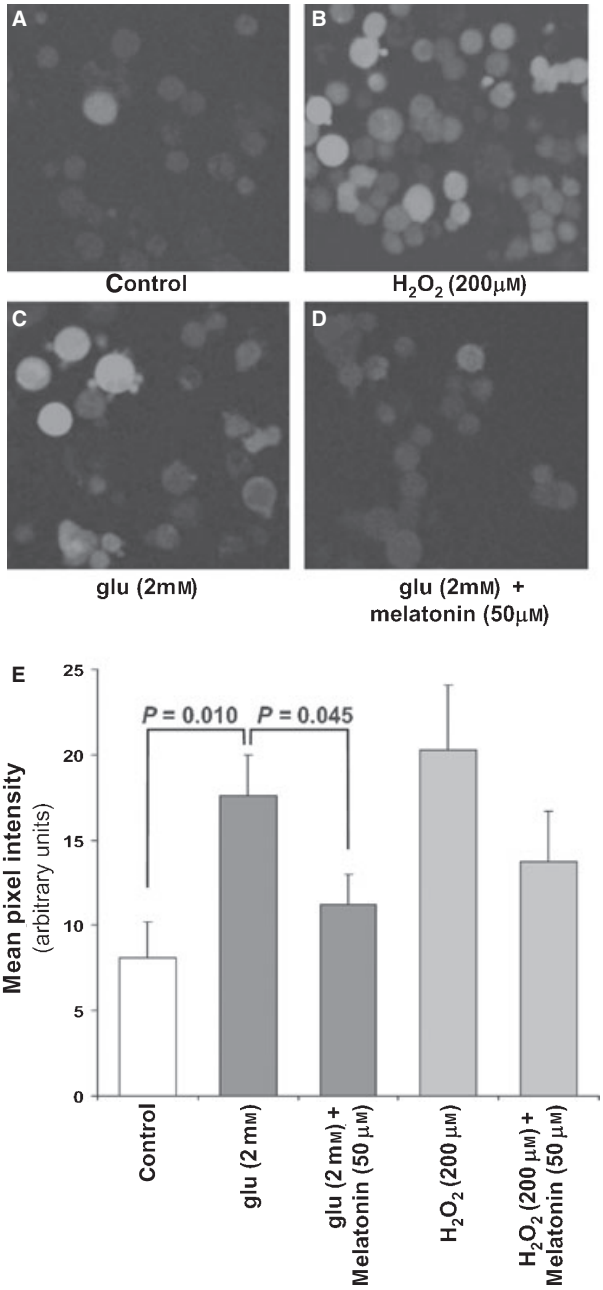

Fig. 3. Melatonin reduces the formation of reactive oxygen species in motoneuronal cells. NSC-34 cells (A, unchallenged control cultures) were exposed to 200-μM H_2O_2 for 30 min (B, positive control) or 2-mM glutamate for 12 hr (C). Cells shown in (D) were co-treated with glutamate and melatonin (50 μM). Free radical formation was assessed with the green fluorescent oxidative stress indicator H_2DCFDA. (E) Results of quantitative densitometric evaluation of confocal microscope pictures: fluorescence is given as mean pixel intensity after subtraction of background values of individual confocal pictures. Confocal laser scanning images were taken with a 40× oil immersion objective; n = 3 independent experiments; mean ± S.E.M. presented.

of high doses via an enteral route, (2) circumventing liver first-pass metabolism, and (3) avoiding swallowing that is frequently compromised in ALS patients.

A total of 31 patients (19 males, 12 females) with probable or definite ALS, according to El Escorial Criteria, but without genetic diagnosis, were enrolled over 24 months into the safety trial. Mean age at inclusion was 59.8 ± 1.9 (range 32–79) yr. The age at presumed disease onset was 58.0 ± 1.9 (28–77) yr. The latency from first symptoms to diagnosis amounted to 11.7 ± 1.2 (3–24) months. Upon entry into the study, mean duration of disease was almost 2 yr (22.1 ± 2.9 months) (range 5–76 months), the ALSFRS was 27.0 ± 1.2 (12–38; maximum = healthy 40), and forced vital capacity 62.3 ± 5.5% (13–100%), revealing that most patients were in a progressed disease state. The predominant clinical symptoms at disease onset were distributed 21:6:4 for the spinal:bulbar:mixed forms of ALS. Severe comorbidities in our patient population included pre-existing epilepsy (n = 2), prostate carcinoma (n = 1), mamma carcinoma (n = 1), cardiac diseases (n = 6), colitis ulcerosa (n = 1), bipolar disorder (n = 1), and alcoholism (n = 1).

Patients were treated for 11.5 ± 1.2 (2–24) months. Rectal application immediately achieved high plasma and urinary melatonin levels, but still maintained–at a higher niveau–a day/night pattern (Fig. 4A and suppl. Fig. 3). Follow-up analysis after 2 months showed that melatonin plasma levels stayed in the expected range, indicating that continuous melatonin administration does not lead to accumulation or increased metabolism. No adverse effects were observed nor reported. Mean routine laboratory data remained essentially unchanged. Several parameters showed typical fluctuations, e.g., associated with physical stress or intercurrent infections (creatine kinase or leucocytes), but none of them likely to be related to melatonin. In two cases, the discontinuation of riluzole revealed that temporarily increased transaminase values were the result of riluzole rather than melatonin. Some patients reported initially faster sleep onset (n = 5), improved sleep quality (n = 15), and better sleep continuation (n = 3), while others did not find their sleep changed (n = 14). No signs of hangover or increased fatigue during daytime were noted. Melatonin medication was well accepted by patients, and only eight discontinued treatment: five at the end stage, three with extremely rapidly progressing disease, who refused *any* medication. A total of 13 patients died (respiratory failure), most of them entering the safety study at an advanced disease stage (mean ALSFRS upon entry 23.6 ± 1.8; range 12–31). The ALSFRS scores from study entry to 1 yr of follow-up for all patients (n = 31) are presented in Fig. 4B.

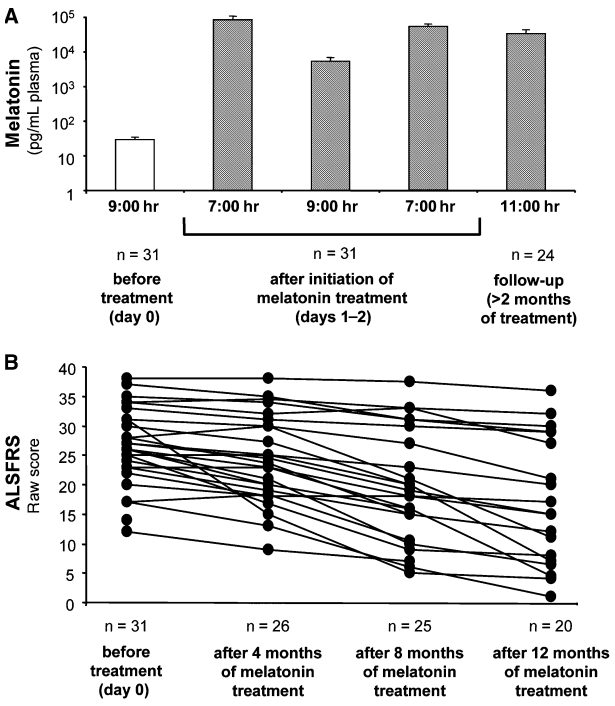

Fig. 4. Clinical safety trial in amyotrophic lateral sclerosis (ALS) patients. (A) Pharmacokinetic aspects: high-dose (300 mg) rectal melatonin treatment daily in the evening immediately potentiates plasma levels, while maintaining a day–night pattern. Plasma levels remain stable after a mean treatment time of 2.23 ± 0.23 months. (B) Clinical course: individual ALS functional rating score (ALSFRS) scores of ALS patients (n = 31) from study entry to 12 months of melatonin treatment.

Melatonin treatment reduces biochemical markers of oxidative stress in ALS

ALS autopsy material contains increased levels of protein carbonyls, representing protein modifications caused by oxidative stress [37, 38]. Recently, elevated lipid peroxides have been detected in serum from ALS patients [39]. Here, we found that an elevation of protein carbonyls can be monitored in peripheral blood samples from ALS patients, and can be used as a biochemical readout for a therapeutic effect. We measured small but significantly elevated protein carbonyl concentrations in serum of ALS patients compared with the serum of matched healthy controls. Significantly, by follow-up of the same ALS patients more than 4 months after the start of daily melatonin treatment, we found protein carbonyl levels fully reverted to control levels (Fig. 5). This is remarkable, as most of these patients (n = 12) had already been on high-dose vitamin E, another antioxidant, for 10.4 ± 2.5 months before starting

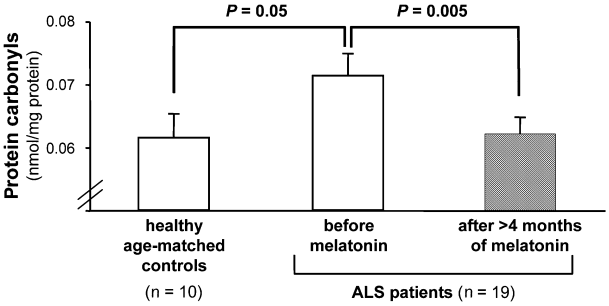

Fig. 5. Laboratory efficacy of melatonin in amyotrophic lateral sclerosis (ALS). Elevated protein carbonyls in the serum of 19 untreated ALS patients decrease to levels of matched healthy controls upon melatonin treatment (mean treatment time: 4.68 ± 0.22 months). Mean ± S.E.M. presented.

melatonin. In fact, a comparison of the clinical outcome (ALSFRS after 1 yr of melatonin treatment) of ALS patients with or without continuous high-dose vitamin E (see Table 1), did not reveal any beneficial effect of vitamin E ($P = 0.901$, $F = 0.016$).

It has been suggested that melatonin influences antioxidant enzymes by stimulating their gene expression [40]. To detect such consequences, we obtained RNA expression profiles from peripheral blood mononuclear cells (PBMC). We compared the RNA of ALS patients, just prior to melatonin treatment, with the RNA of the same patients after 1 day and after 4 months of continuous treatment. These experiments yielded no evidence for the upregulation of genes encoding antioxidant enzymes (data not shown). By quantitative reverse transcriptase polymerase chain reaction (RT-PCR), we only confirmed changes of mRNA levels for proteins associated with proteasome function, the unfolded protein response, and protein sorting (primary data and experimental details available upon request). Although these findings were negative with respect to the induction of antioxidant enzymes in PBMC, they demonstrate gene expression changes in humans triggered by melatonin. Moreover, these changes may turn out to be relevant in light of some features of ALS resembling an 'aggregopathy'. Assessing the possible contribution of melatonin-regulated genes to neuroprotection, in general, and to the formation/processing of insoluble intracellular protein aggregates, in particular, awaits the analysis of neural tissue samples.

Discussion

Radical damage is a final common pathway in the pathology of human ALS, and we have shown that melatonin treatment reduced oxidative stress in vitro and in vivo. High-dose melatonin prolonged the survival of $SOD1^{G93A}$-transgenic mice, a widely used ALS model. In an initial safety study with ALS patients, enteral high-dose application of melatonin was well tolerated and safe. Moreover, melatonin led to the normalization of serum protein carbonyls, markers for oxidative stress, which were significantly elevated prior to treatment in a diverse group of sporadic ALS patients. We do not believe that elevated peripheral protein carbonyls are derived from the central nervous system, but they appear to reflect systemic oxidative stress in ALS patients. The observations that high-dose melatonin (1) improves neuronal survival in vitro, (2) prolongs central nervous system functions in ALS mice, and (3) corrects human ALS laboratory data on oxidative damage, strongly suggest, when combined, that high-dose melatonin also provides neuroprotection in ALS patients. The neuroprotective potential of melatonin in ALS can now be addressed in a clinical proof-of-concept trial.

Till date, there is no causal or convincing symptomatic treatment for ALS. The only compound with borderline efficacy in ALS patients is riluzole, an antiexcitotoxic drug that marginally prolonged survival in clinical trials [5, 6], but lacked benefit with respect to other important outcome measures. Owing to its side effects (including asthenia, nausea, anorexia, diarrhea, headache, and increase in liver transaminases), riluzole is frequently discontinued. It also enhances catabolism in this already wasting disease [41]. Thus, when screening for new drugs in ALS, it is important to search for well-tolerated compounds that can be rapidly transferred to a clinical setting.

All mechanisms believed to explain neuronal cell death or dysfunction in ALS involve oxidative stress, either as primary insult or as part of the final common pathway of disease [4, 25, 26]. A recent 10-yr prospective study with over 900,000 individuals showed a decreased risk of developing ALS that was associated with the regular intake of vitamin E [42]. Nevertheless, none of the antioxidative compounds tested, including vitamin E, proved to be effective in the clinical setting [1, 20, 21]. This suggests that for ALS, early intervention is critical and that better compounds against oxidative stress need to be defined.

Melatonin is distinct from classical antioxidants. First, it acts on a unique broad spectrum of free radical targets by direct scavenging [24–26]. Second, its antioxidative profile extends to the activation of other antioxidative systems, such as glutathione peroxidase [24–26]. Third, melatonin is amphiphilic, and in contrast to standard antioxidants, enters both lipophilic and hydrophilic cellular environments [26].

With respect to the mechanisms underlying melatonin-mediated neuroprotection in cultured motoneuronal cells and in $SOD1^{G93A}$-transgenic mice, we could exclude alternative explanations, including GDNF induction and reduced transgene expression. Thus, the antioxidative properties are indeed the most likely reason for the observed beneficial effects of melatonin on survival in mice. Interestingly, increased protein carbonyl levels have been reported earlier in brain and spinal cord tissue samples from sporadic ALS patients [37, 38, 43, 44]. We found serum protein carbonyls, as indicators of oxidative stress, to be reduced to control levels upon chronic melatonin treatment in ALS patients. In the absence of clinical efficacy data, this finding provides indirect biochemical evidence of a therapeutic effect in human ALS.

Another potentially important mechanism of action of melatonin in ALS may be its mitigating effect on mitochondrial malfunction. Hence, melatonin-induced enhancement of ATP availability could provide additional benefits in ALS [45].

The daily dose of 300 mg melatonin for the safety study was chosen with respect to our preclinical data in mice, and taking into account up to tenfold higher metabolism rates of most drugs in rodents [36]. Jet lag in humans is usually treated with 3 to 6 mg melatonin per day, and short-term treatments with up to 2000 mg daily in cancer patients are tolerated without side effects [46]. Suppositories were effective as a new route of melatonin administration and well accepted by our patients. No unwanted effects were observed in clinical and laboratory follow-up. Some patients noted faster sleep onset and improved sleep quality, but never complained about daytime sleepiness. This novel way of melatonin application, with respect to route and dose, could prove useful in other diseases associated with the generation of ROS. We note that similar to ALS, elevated tissue protein carbonyls have been detected in Alzheimer's and Parkinson's disease [37], but the causal role of ROS in the progression of these diseases is less clear.

Similar to other experimental therapies of $SOD1^{G93A}$-transgenic mice [47], no benefit was demonstrated when melatonin was given *after* the onset of symptoms. This does not necessarily predict a lack of efficacy in ALS, even though treatment of patients typically starts many months after the onset of first symptoms. It is questionable whether the clinical stages of a motoneuron disease in mice can be quantitatively compared with that in humans. The disease course in the chosen mouse model, carrying 18 copies of a mutant SOD1 transgene [29], is extremely compressed (onset of symptoms at age 90 days), relative to human ALS (average onset at age 60 yr), independent of the shorter lifespan of mice. In addition, disease duration in mice (6–8 wk) is a small fraction of the human course (2–5 yr). Thus, *treatment-at-disease-onset* protocols may come too late in mice, but still be effective in human ALS, which develops over years. Similar differences of disease dynamics have been noted between other transgenic models and their respective human disorders, including Alzheimer's disease and multiple sclerosis [48, 49].

Only 1–2% of all ALS cases are caused by SOD1 mutations. Thus, novel therapeutic strategies should be directed against common events that contribute to all forms of ALS. Recent publications that reported delayed disease progression or improved survival in mutant $SOD1^{G93A}$-transgenic mice were, for example, based on transgenic expression of glial glutamate transporters [11] or viral delivery of neurotrophins or siRNA [50–53]. These studies have helped to understand basic disease mechanisms of ALS, but cannot be easily translated to the clinic. The search for low molecular drugs as therapeutics in ALS mouse models [54, 55] has provided compounds that failed in clinical studies (e.g., creatine [56] or vitamin E [20, 21]) or for which no safety data are available in ALS patients (e.g., minocycline [57–59] and cephalosporines [60]).

With respect to the clinical setting, disease progression and duration are more relevant parameters than overall survival time. Minocycline, which is currently under clinical investigation, postponed disease onset and death of $SOD1^{G93A}$-transgenic mice [57, 58]. However, a decreased time span (−21%) between onset of symptoms and premature death was reported [57]. Similar negative results on disease duration were observed upon vitamin E treatment [54]. In contrast, high-dose melatonin slowed disease progression by 25% or 73%, depending on the definition of 'disease onset', by the appearance of first tremor or failure on the rotarod, respectively.

Translating these findings to human ALS, we are aware that a cure by melatonin is unlikely. An increase in life expectancy, however, is a worth-while goal, specifically during the early stages of this disease. Oxidative stress is a common denominator of cell death pathways in sporadic ALS. Thus, melatonin can be expected not only to be effective as single drug treatment, but also to exert synergistic therapeutic effects, e.g., in combined treatment schemes with riluzole [5, 6], minocycline [57, 58], cephalosporines [60], or possibly COX-2 inhibitors [61]. Treatment cocktails of compounds with individually established safety records may play an important role in future ALS treatment regimes [59].

To conclude, despite these promising preclinical results with melatonin in mice, we have to stress that there are no clinical efficacy data available, and that we have only laboratory data to suggest an efficacy of melatonin in human ALS. The human safety data and unexpected findings of complete normalization of circulating protein carbonyls by melatonin treatment distinguish this study, and are encouraging to conduct a randomized double-blind trial.

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Supplementary Material

The following material is available for this article online:

Supplementary Fig. 1. Body weight, drinking water, and melatonin intake in amyotrophic lateral sclerosis (ALS) mice. No significant difference regarding body weight (A) or water intake (B) was observed between melatonin- or vehicle-treated mice. Mean body weight was 19.1 ± 0.2 and 18.8 ± 0.3 g between 6 and 19 wk of age in control and treatment groups, respectively ($P = 0.313$). Mean daily fluid intake per kg body weight was found to be 154.8 ± 9.8 and 158.9 ± 8.8 mL between 8 and 19 wk of age, respectively ($P = 0.754$). As a consequence of reduced fluid intake in later disease stages melatonin intake declined (C).

Supplementary Fig. 2. Melatonin treatment does not change SOD1 protein expression in mouse spinal cord. (A) Melatonin treatment did not change protein expression of endogenous mouse superoxide dismutase (SOD1) and transgenic human mutant SOD1 in cervical spinal cord lysates of transgenic (tg) and wildtype (wt) 100-day-old mice. (B) Densitometric quantification of protein expression levels of endogenous (wildtype) and mutant SOD1 in mutant SOD1-transgenic mice. Shown are respective ratios between melatonin-treated animals and vehicle controls, demonstrating that melatonin did not influence the expression levels of mutant or wildtype SOD1. Before calculating ratios, SOD1 protein levels were normalized to a tubulin standard ($n = 12$ per condition).

Supplementary Fig. 3. Urinary melatonin excretion of amyotrophic lateral sclerosis (ALS) patients before and after the first and second 300 mg suppository. Columns represent total amount of melatonin measured in 10-hr urine (21:00–7:00 hr) (mean ± S.E.M.).

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