

Beneficial effects of melatonin in a rat model of sporadic Alzheimer's disease

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Abstract Melatonin synthesis is disordered in patients with Alzheimer's disease (AD). To determine the role of melatonin in the pathogenesis of AD, suitable animal models are needed. The OXYS rats are an experimental model of accelerated senescence that has also been proposed as a spontaneous rat model of AD-like pathology. In the present study, we demonstrate that disturbances in melatonin secretion occur in OXYS rats at 4 months of age. These disturbances occur simultaneously with manifestation of behavioral abnormalities against the background of neurodegeneration and alterations in hormonal status but before

the signs of amyloid- β accumulation. We examined whether oral administration of melatonin could normalize the melatonin secretion and have beneficial effects on OXYS rats before progression to AD-like pathology. The results showed that melatonin treatment restored melatonin secretion in the pineal gland of OXYS rats as well as the serum levels of growth hormone and IGF-1, the level of BDNF in the hippocampus and the healthy state of hippocampal neurons. Additionally, melatonin treatment of OXYS rats prevented an increase in anxiety and the decline of locomotor activity, of exploratory activity, and of reference memory. Thus, melatonin may be involved in AD progression, whereas oral administration of melatonin could be a prophylactic strategy to prevent or slow down the progression of some features of AD pathology.

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Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder that causes dementia as a result of atrophic changes in the brain leading to disruption of attention, memory, and executive function (Moodly and Chan 2014). AD is characterized by increased load of amyloid- β in the brain, leading to

accumulation of amyloid- β , amyloid plaques, neurofibrillary tangles, neuronal cell death, excitotoxicity, inflammation, and oxidative stress. The factors that initiate (or affect the risk and onset) of amyloid- β accumulation in sporadic late-onset AD, which accounts for ~ 95 % of all AD cases, remain poorly understood (Castellano et al. 2012).

The decline of secretion and disruption of the rhythms of melatonin (*N*-acetyl-5-methoxytryptamine) production are being actively studied as a major mechanism of aging and of aging-related diseases, including neurodegenerative disorders such as AD. Melatonin is involved in the regulation of circadian rhythms, of visual function, the immune system, endocrine system, and central nervous system (Esposito and Cuzzocrea 2010). Therefore, the decline of the melatonin production with age leads to systemic changes in metabolic processes and negatively affects the functioning of many organs and systems of organs (Jenwitheesuk et al. 2014). Decreased melatonin levels (Hardeland 2012) and disruption of melatonin's diurnal rhythm (Lin et al. 2013), even disappearance thereof (Wu and Swaab 2007), are observed in patients with AD. It is unclear whether the decrease in melatonin level is a cause or consequence of aging and of AD, and the mechanisms of this decrease are unknown (Bubenik and Konturek 2011).

It is generally accepted that growth hormone and its main intermediary, IGF-1, take part in the regulation of functions of the central nervous system and play an important role in various behavioral patterns, such as cognitive behavior related to learning and memory. Impaired production of growth hormone or IGF-1 is associated with cognitive deficits (Nyberg and Hallberg 2013).

Neurodegeneration and neuronal damage are the characteristic features AD with hippocampus and entorhinal cortex being the most vulnerable brain regions (Sardi et al. 2011). In particular, the rate of hippocampal atrophy is highest namely at the early stages of AD (Moody and Chan 2014). Neurodegeneration contributes to AD-related cognitive decline occurs against background of alterations in neurotrophic supplementation including decrease of brain-derived neurotrophic factor (BDNF) level (Allen et al. 2013). Since BDNF performs a crucial role in neurogenesis, neuronal survival, and synaptic plasticity (Reichardt 2006), age-related alteration in BDNF level is considered as a contributing factor to progression of AD (Allen et al. 2013).

Recently, we showed that senescence-accelerated OXYS rats are a suitable nontransgenic model of sporadic AD (Kozhevnikova et al. 2013; Stefanova et al. 2014a, b). OXYS rats are characterized by progressive age-related aggregation of amyloid- β and hyperphosphorylation of the tau protein as well as mitochondrial dysfunction, loss of synapses, neuronal death, and ultimately cognitive decline (Stefanova et al. 2010, 2011, 2014a, b). We demonstrated previously that the signs of accelerated aging of the brain in OXYS rats develop simultaneously with changes in the metabolism of the neurotransmitter serotonin in the brain regions that are essential for learning and memory (Shcheglova et al. 2002). It is known that serotonin is synthesized from tryptophan, and the enzyme N-acetyltransferase converts serotonin to melatonin (Hardeland 2012). The development of cataract in OXYS rats is linked to the anomalies in tryptophan metabolism (Snytnikova et al. 2012). Assuming that these anomalies in OXYS rats are systemic, then it is possible that changes in the synthesis and secretion of melatonin contribute to some extent to the accelerated aging of these rats.

Here we explored age-dependent changes in melatonin levels in OXYS rats. Furthermore, we tested whether melatonin administration has beneficial effects on hormonal status, behavior, neurodegenerative changes, and cognitive problems. For this purpose, we treated OXYS rats with oral melatonin before progression to the AD-like pathology.

Materials and methods

Animals and diet

Male senescence-accelerated OXYS rats ($n = 114$) and age-matched male Wistar rats ($n = 114$) were obtained from the Breeding Experimental Animal Laboratory of the Institute of Cytology and Genetics, the Siberian Branch of the Russian Academy of Sciences (Novosibirsk, Russia). The OXYS rat strain was derived from the Wistar rat strain at the Institute of Cytology and Genetics as described earlier (Stefanova et al. 2010) and was registered in the Rat Genome Database (<http://rgd.mcw.edu/>). At this point, we have the 105th generation of OXYS rats, with spontaneously developing cataract and accelerated-senescence syndrome inherited in a linked manner.

At the age of 4 weeks, the pups were weaned, housed in groups of five animals per cage ($57 \times 36 \times 20$ cm), and kept under standard laboratory conditions (22 ± 2 °C, 60 % relative humidity, and 12 h light/12 h dark cycle; lights on at 9 a.m.). The animals were provided with standard rodent feed (PK-120-1, Laboratornsnab, Ltd., Russia) and water ad libitum. Rats between 1 and 22 months of age were used in the experiments, as described below.

To assess the influence of oral melatonin administration (from age 1.5 months to age 4 months) on the development of AD-like pathology, we randomly assigned 1.5-month-old male OXYS rats and Wistar rats (as a control strain) to one of two groups (25 rats per group, $2 \times 2 = 4$ groups). One group consumed a control diet (dried bread slice), and the other the same diet supplemented with 0.04 mg of melatonin (Melaxen, Unifarm, USA) per kg of body weight per day. Each rat received melatonin with dried bread slice individually daily at 8 p.m. To evaluate the influence of oral melatonin treatment on melatonin secretion, we killed 10 animals from each group by decapitation at 2 a.m. under red light; another 15 animals from each group were killed by decapitation at 2 p.m.

To detect age-related changes in melatonin secretion, we used untreated 1- and 16-month-old male OXYS and Wistar rats as well as untreated melatonin four-month-old male OXYS and Wistar rats [$n = 20$ per each age and strain (genotype)]. Ten animals from each age group and strain were killed by decapitation at 2 a.m. under red light; another 10 rats from each group were killed by decapitation at 2 p.m.

To detect age-related changes in BDNF levels in the hippocampus, we used untreated 3-, 12-, and 22-month-old male OXYS and Wistar rats ($n = 8$ per each age and genotype). All experimental procedures were in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Every effort was made to minimize the number of animals used and their discomfort.

Sample collection for protein analysis

To measure the concentration of melatonin, growth hormone and IGF-1 in serum, samples of blood were centrifuged at $3,000 \times g$ for 30 min at 4 °C, and the resulting serum was stored at -70 °C until analysis.

The measure BDNF content of the hippocampus in either four-month-old melatonin-treated and untreated

OXYS and Wistar rats or 3-, 12-, and 22-month-old untreated OXYS and Wistar rats ($n = 8-10$) was done as previously described (Stefanova et al. 2014b).

To measure melatonin content of the pineal gland, we used four-month-old melatonin-treated and untreated OXYS and Wistar rats ($n = 6$). Pineal glands were quickly separated from the brain, frozen in liquid nitrogen, and stored at -70 °C until analysis. Each pineal gland was weighed and washed in 50 mM phosphate-buffered saline (pH 7.4) prior to homogenization. The pineal glands were homogenized in cold 80 % ethanol (400 μ L per three glands) on ice. The homogenate was sonicated and then shaken (for 15 min each, for better metabolite extraction) and was then centrifuged at $13,000 \times g$ for 30 min at 4 °C. The pellet was reextracted with 80 % ethanol (200 μ L), sonicated, and centrifuged in the same way. The combined supernatants were lyophilized. For subsequent liquid chromatography analysis with mass spectrometric detection, the lyophilized extract was redissolved in 110 μ L of an aqueous solution containing 10^{-5} M *N*-acetyl-tryptophan (*N*-Ac-Trp) as an internal control and was filtered by centrifugation at $12,000 \times g$ for 4 min at 4 °C using centrifugal filter units Durapore PVDF 0.22 μ m (Millipore, USA). The resulting extract was serially diluted 10-fold with a 10^{-5} M *N*-Ac-Trp aqueous solution (one undiluted sample).

Enzyme-linked immunosorbent assays (ELISAs)

Melatonin, growth hormone, and IGF-1 levels in serum and hippocampal BDNF levels of the rats were measured using Melatonin (Rat) ELISA Kit (#40-371-25005; GenWay Biotech Inc., USA), Growth Hormone (Rat) ELISA Kit (#CDN-E2779; Creative Diagnostics, USA), IGF-1 (Mouse/Rat) ELISA Kit (#22-IG1MS-E01; ALPCO Diagnostics, Salem, NH, USA), and BDNF (Rat) ELISA Kit (#KA0330; Abnova, Taiwan), respectively, according to the manufacturer's instructions. Quantitation was carried out using optical density measurement on Multiskan TEKAN (GmbH, Austria).

Analysis of morphology of hippocampal neurons

This assay was conducted in four-month-old untreated and melatonin-treated OXYS and Wistar rats ($n = 5$ per group). Mounted tissue slices (5 μ m thick) were

incubated at 60 °C in a Cresyl Violet solution for 3 min; briefly washed in distilled H₂O; and dehydrated in 70, 95, and 100 % ethanol. All slices were immersed in xylene for 2–5 min and coverslipped using CC/Mount (cat. # 061M1324, Sigma–Aldrich). To evaluate morphology of the neurons in the granular cell layer of the dentate gyrus and in the CA1 and CA3 pyramidal layers of the hippocampus, we used a set of 4–6 serial tissue sections per animal. A 100× objective lens (Axioplan 2, Zeiss, Germany) was used to count >200 neurons per visual field. Different brain regions were defined according to the atlas of Paxinos and Watson (2007). Dead and damaged neurons were identified by their morphology. Images of the same area of the hippocampus were analyzed using the ImageJ software (NIH; Bethesda, MD, USA). The data were presented as a percentage of the normal (no change), damaged, and dead neurons among all neurons in each visual field of a hippocampus specimen.

Liquid chromatography with mass spectrometry

High-performance liquid chromatography (HPLC) was performed on an UltiMate 3000RS system (Dionex, Germany) equipped with a ternary pump, a thermostatted autosampler, a thermostatted column compartment, and a flow cell diode array UV–vis detector with the 190–800 nm spectral range. Separation was performed on the analytical column Agilent Zorbax XDB-C18 (4.6 × 150 mm, 80 Å, 5 μm) using the parameters described in the previous work (Yanshole et al. 2014). The flow rate was 200 μL/min, and sample injection volume 80 μL. The instrumental setup allowed for acquisition of data from both the diode array UV–vis detector and the mass spectrometer simultaneously.

The mass-spectrometric detection was performed on an electrospray ionization quadrupole time-of-flight (ESI-q-TOF) high-resolution hybrid mass spectrometer Maxis 4G (Bruker Daltonics, Germany) connected to the Dionex HPLC system after the diode array UV–vis detector cell. Mass spectra were recorded in positive-ion mode in the 50–700 m/z range at the 2 Hz sampling rate. The instrument parameters for positive ion mode and instrument calibration were taken from the work of Yanshole et al. (2014). Every mass spectrometric measurement contained the time segment with the mass spectrometer calibration data, which were used for postrecalibration of the sample data if necessary.

A stock solution of melatonin (2×10^{-6} M) for sample calibration was prepared in the internal control solution (10^{-5} M *N*-Ac-Trp aqueous solution). The stock solution of melatonin was then diluted with the internal control solution to obtain eight calibration samples with the melatonin concentrations ranging from 0.02 to 2 μM. The calibration samples were injected into the liquid chromatography–mass spectrometry system (injection volume 80 μL), and the data were analyzed using the QuantAnalysis software (Bruker Daltonics, Germany) with the following input settings for melatonin: retention time 37.2 min (window tolerance 0.5–1.0 min) and an extracted-ion chromatogram (EIC) with the 233.128 m/z value and the extraction window ±0.02 Da. The dependence of the peak area on the melatonin concentration was determined for every calibration solution and used for melatonin quantification in the samples. In all cases, the peak areas were normalized to the signals of *N*-Ac-Trp. The calibration curve for melatonin was constructed as a linear fit of the data.

Behavioral testing

Behavioral responses of the animals to treatment with melatonin were assessed using several tests in the following order: an anxiety assay in an elevated plus maze, the open field test of locomotor and exploratory activity, and learning and memory tasks in an eight-arm radial maze. Each rat to be tested was transported from the home cage to the experimental room in a black box. Each test was performed once for each animal. The test sessions were scheduled between 10 a.m. and 2 p.m.

The elevated plus maze test

We used this test to quantify anxiety. The plus maze apparatus was made of opaque Plexiglas and contained two opposite open arms (50 × 10 cm) and two closed arms of the same size but with 40-cm-high walls. Each arm was divided by lines into five equal squares (10 × 10 cm). The four arms were connected by a central square (100 cm²) and thus formed a plus sign. The apparatus was elevated 50 cm above the floor. Each rat was placed in the central square of the plus maze, facing one of the closed arms, and its behavior was analyzed for 5 min. We recorded the number of entries into the four arms of the elevated plus maze and the time

spent in the arms and in the center square. We calculated relative time spent in the open space (%) using the following formula: (time spent in the open arms + time spent in the center square) \div 300 s. A greater amount of time spent in the open arms indicated a reduction of anxiety-like behavior. In addition, the frequency of rearing events (when an animal stood on its hind limbs) was recorded: it indicated exploratory activity of the animals.

The open field test

The animals were subjected to this test 48 h after completion of the elevated plus maze test. The open field area consisted of an enclosed square arena made of opaque Plexiglas (100 \times 100 cm) surrounded by walls (40 cm high). The arena was divided by transverse lines into 100 equal squares. Each rat was placed into the same corner of the arena facing in the same direction and was allowed to freely explore the arena for 5 min. Every time both hind limbs entered a square, a crossing was recorded. The locomotor and exploratory activity was evaluated by counting the line crossings and the number of rearing events.

The eight-arm radial maze test

This test (R&PC Open Science, Moscow, Russia) was used to assess reference memory of the rats. The radial maze consisted of a central octagonal platform (26 cm in diameter) from which eight arms (100 cm long, 20 cm high and 10 cm wide) radiated. In addition, there were mobile septa separating the arms from the central area. The floor and walls of the maze as well as the septa were made of opaque Plexiglas. The end of each arm contained a small circular feeder (3 cm in diameter). The feeder was intended to make the food pellet invisible for a rat at the center of the maze. For 10 consecutive days, the rats were trained to find food pellets in the eight-arm radial maze. As the food pellets, we used dried cereal pellets weighing \sim 0.5 g (OJSC Lyubyatovo, Russia). During the 3 days before initiation of the radial maze training, the amount of feed in home cages was reduced by 15 % of daily food intake, and each rat received the food pellet. During the 24 h before initiation of the eight-arm radial maze training, the animals were habituated to the maze. There were two sessions of 10 min with a 2 h interval between them; during each session, food pellets were placed throughout

the maze. On training days, four arms were baited with a single food pellet. A combination of baited arms was selected randomly. This combination remained constant throughout the 10 days of the training.

The trial commenced with placement of a rat in the center of the maze, with septa lowered. After 20 s, the septa were lifted, and the rat was allowed to freely explore the maze. A trial was deemed finished when either all food pellets were found or 10 min elapsed, whichever happened sooner. The number of arm entries (NAE, meaning locomotor and exploratory activity) was recorded until the trial was finished; the entries into unbaited arms (reference memory errors, RME) were taken into account. To assess reference memory, the data were processed using the formula $RME (\%) = RME \div NAE$, where RME (%) is the reference memory errors rate. Except for the first day of training, the number of arm entries and reference memory performance of each animal were combined into three clusters of trials: days 2–4, 5–7, and 8–10.

Statistical analysis

The data were analyzed using repeated-measures analysis of variance (ANOVA) and nonparametric tests using the statistical software Statistica 6.0 (StatSoft, USA). Two-way ANOVA was used to evaluate the differences between OXYS and Wistar rats across ages (age \times strain, i.e., age \times genotype) and to evaluate the effects of treatment (melatonin \times genotype). To test the effects of oral melatonin on various parameters, the genotype and the melatonin were chosen as independent variables. The Newman–Keuls post hoc test was applied to significant main effects and interactions in order to assess the differences between particular sets of means. One-way ANOVA was used for individual group comparisons. Comparison between means was carried out using either one-way or repeated-measures ANOVA. The data were presented as mean \pm SEM. The differences were considered statistically significant if $p < 0.05$.

Results

Age-related alterations in melatonin secretion and the effects of oral melatonin

We used ELISA to measure the serum levels of melatonin in 1-, 4-, and 16-month-old OXYS and

Wistar rats. We found that nocturnal melatonin levels in serum were affected by age ($F_{2,30} = 17.2$, $p < 0.00$). Daytime melatonin levels were affected by age and by the relationship of age with the genotype ($F_{2,30} = 31.6$, $p < 0.00$, and $F_{2,30} = 6.7$, $p < 0.00$, respectively). Serum melatonin levels were highest at 1 month of age and did not differ between the strains (Fig. 1a, b). The nocturnal melatonin levels decreased with age in both strains. Alterations in intraday rhythms of melatonin secretion arose at 4 months of age in OXYS rats: statistically significantly higher melatonin levels during the day ($p < 0.01$) compared to Wistar rats. Statistically significant alteration in intraday rhythms of melatonin secretion was observed in 16-month-old OXYS rats ($p < 0.05$).

We then examined the effects of oral melatonin administration on the levels of melatonin in the serum of four-month-old OXYS and Wistar rats. The data on four-month-old OXYS and Wistar rats in Fig. 1c, d correspond to untreated OXYS and Wistar rats. We found that nocturnal levels (at 2 a.m.) of melatonin in serum were affected by the genotype and by the relationship of treatment with the genotype ($F_{1,95} = 28.2$, $p < 0.00$, and $F_{1,95} = 6.1$, $p < 0.02$, respectively; Fig. 1c).

As for the daytime serum level of melatonin (at 2 p.m.), it was affected by the genotype, treatment, and by the relationship between these two factors ($F_{1,95} = 6.5$, $p < 0.01$; $F_{1,95} = 9.9$, $p < 0.00$; and $F_{1,95} = 9.3$, $p < 0.00$, respectively). Our results showed that daytime melatonin levels in the serum of OXYS rats were statistically significantly higher compared to Wistar rats ($p < 0.01$); this finding may mean disturbances in rhythms of melatonin secretion in four-month-old OXYS rats (Fig. 1d). Oral administration of melatonin did not affect daytime serum melatonin levels in OXYS rats. Unexpectedly, daytime melatonin levels in the serum of melatonin-treated Wistar rats were almost 3-fold higher compared to untreated Wistar rats ($p < 0.00$).

Next, we examined melatonin content of the pineal gland in four-month-old melatonin-treated and untreated rats. The results of HPLC are presented in Fig. 1g. Melatonin levels in the pineal gland were higher at night than during the day (Fig. 1e, f). Daytime pineal melatonin levels did not differ between the two strains. Nocturnal melatonin levels in the pineal gland were statistically significantly lower in OXYS rats than in Wistar rats ($F_{1,20} = 7.9$,

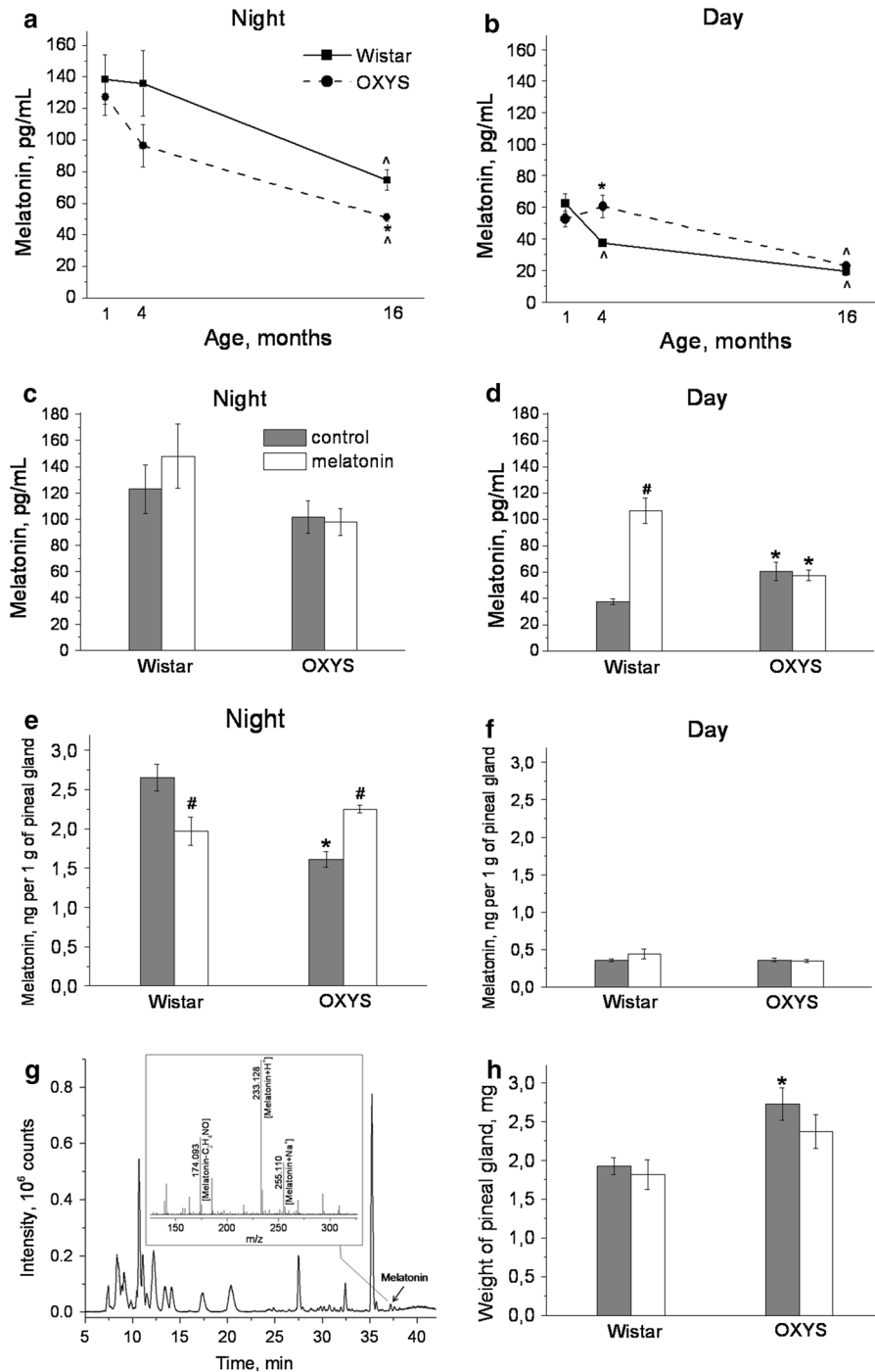
Fig. 1 Age-related alterations in serum melatonin levels of OXYS and Wistar rats at night (a) and during the day (b) according to ELISA. In OXYS rats, disturbances of intraday rhythms of melatonin secretion appeared at 4 months of age, with lower melatonin levels at night (compared to the Wistar strain; panel a) and statistically significantly higher levels during the day (b). Statistically significant alterations were observed at the age of 16 months: more pronounced disturbances of melatonin production in OXYS rats. c Serum melatonin levels in four-month-old melatonin-treated and untreated rats at night and d during the day according to ELISA. Treatment with melatonin did not affect serum melatonin levels in OXYS rats at night (c) and during the day (d) but statistically significantly increased daytime serum melatonin levels in Wistar rats (d). e Melatonin levels in the pineal gland of four-month-old melatonin-treated and untreated rats at night and f during the day according to HPLC. Compared to Wistar rats, OXYS rats had statistically significantly lower nocturnal melatonin levels in the pineal gland (e). Treatment with melatonin increased nocturnal levels of melatonin in the pineal gland of OXYS rats and reduced it in Wistar rats (e). g A typical liquid chromatography–mass spectrometry chromatogram of the protein-free extract from a rat pineal gland (melatonin assignment is shown). Inset The mass spectrum of melatonin in positive ion mode, where m/z 233.128 corresponds to the melatonin molecular ion, and m/z 255.110 corresponds to the Na^+ adduct of a melatonin fragment ($\text{C}_2\text{H}_4\text{NO}$, m/z 174.093). h Weight of the pineal gland of four-month-old melatonin-treated and untreated rats. OXYS rats had statistically significantly greater weight of the pineal gland; treatment with melatonin did not affect this variable in either strain. The data are shown as mean \pm SEM. * $p < 0.05$ for differences between the strains; # $p < 0.05$ for effects of oral melatonin; ^ $p < 0.05$ for age-related differences within a strain

$p < 0.01$). In contrast, treatment with melatonin increased nocturnal pineal melatonin levels in OXYS rats ($p < 0.00$) and decreased them in Wistar rats ($p < 0.02$).

The data on the weight of pineal glands of four-month-old melatonin-treated and untreated OXYS and Wistar rats are shown in Fig. 1h. The weight of the pineal gland was affected only by the genotype ($F_{1,20} = 13.2$, $p < 0.00$) and was higher in OXYS rats ($p < 0.01$). Treatment with melatonin did not affect the weight of the pineal gland in either strain. Nevertheless, the differences in the weight of the pineal gland between melatonin-treated OXYS rats and untreated Wistar rats were not statistically significant ($p > 0.05$).

Effects of oral melatonin on serum levels of growth hormone and IGF-1

Figure 2 shows ELISA data on serum levels of growth hormone and IGF-1 in four-month-old melatonin-



treated and untreated rats. Growth hormone levels in serum were affected by the genotype ($F_{1,44} = 17.7$, $p < 0.00$) and by melatonin treatment ($F_{1,44} = 13.5$, $p < 0.00$). *Post hoc* analysis showed that growth hormone levels were lower in OXYS rats ($p < 0.00$),

and treatment with melatonin increased growth hormone levels in the serum of both OXYS ($p < 0.00$) and Wistar rats ($p < 0.04$; Fig. 2a).

Serum IGF-1 levels were affected weakly by the genotype ($F_{1,44} = 3.3$, $p = 0.08$). *Post hoc* analysis

showed that IGF-1 protein concentration in serum was lower in OXYS rats than in Wistar rats ($p < 0.05$; Fig. 2b). Oral administration of melatonin did not affect IGF-1 levels either in OXYS or in Wistar rats. Melatonin-treated OXYS rats, however, had no statistically significant differences in serum IGF-1 levels with untreated Wistar rats.

Effects of oral melatonin on BDNF levels in the hippocampus

ELISA data on BDNF levels in the hippocampus of four-month-old melatonin-treated and untreated rats are shown in Fig. 3a. BDNF content in the hippocampus of OXYS rats was statistically significantly higher compared to age-matched Wistar rats ($p < 0.04$); after treatment with melatonin, BDNF content of the hippocampus in OXYS rats did not differ from that in Wistar rats (Fig. 3a).

This result was surprising to us; therefore, we tried to determine the reason for the higher levels of BDNF in the hippocampus of young OXYS rats (compared to young Wistar rats). For this purpose, we evaluated age-related alterations of BDNF levels in the hippocampus of the rats (Fig. 3b). We found that BDNF levels in the hippocampus were highest at 3 months of age in both strains, with higher levels in OXYS rats ($p < 0.01$). Subsequently, BDNF levels decreased with age in both strains, but OXYS rats showed a more significant decrease: BDNF protein concentration in the hippocampus of 12-month-old OXYS rats was 4-fold lower compared to three-month-old OXYS rats ($p < 0.00$).

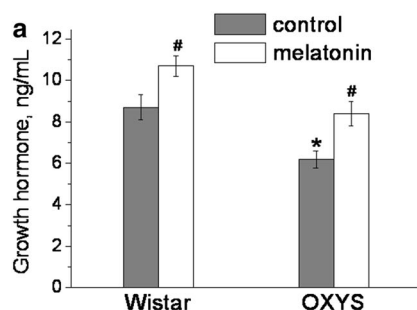


Fig. 2 Growth hormone (a) and IGF-1 (b) levels in the serum of four-month-old melatonin-treated and untreated rats according to ELISA. OXYS rats had lower serum levels of growth hormone (a) and IGF-1 (b). Oral administration of melatonin

Microscopic analysis of the state of hippocampal neurons and the effects of oral melatonin

We used light microscopy to examine hippocampal neurons in four-month-old melatonin-treated and untreated rats (Table 1); regions CA1 and CA3 and the dentate gyrus of the hippocampus were of special interest to us.

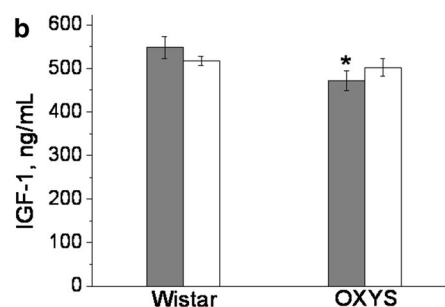
Quantitative analysis using stereological counts showed that significant structural changes developed in hippocampal neurons of four-month-old OXYS rats. The most pronounced neurodegenerative changes occurred in the CA1 region of the hippocampus of OXYS rats: 25 % of neurons were either dead or damaged, whereas almost 100 % of neurons in Wistar rats were unaffected ($p < 0.01$).

These results showed that oral administration of melatonin prevented degenerative alterations in hippocampal neurons of OXYS rats and even improved the state of neurons in the hippocampus of Wistar rats (Table 1). The most pronounced improvement was observed in the CA1 region of the hippocampus: after oral administration of melatonin, there was a 7-fold decrease in the number of affected neurons (dead + damaged) in OXYS rats and a 2.3-fold decrease of this number in Wistar rats ($p < 0.01$ and $p < 0.03$, respectively).

Effects of oral melatonin on behavior

The elevated plus maze test

The results are shown in Fig. 4. The number of entries into open arms of the elevated plus maze and the time spent there were affected by the relationship of the genotype with melatonin treatment ($F_{1,80} = 13.5$,



increased the growth hormone levels in both strains (a) and did not affect the IGF-1 levels (b). The data are shown as mean \pm SEM. * $p < 0.05$ for differences between the strains; # $p < 0.05$ for effects of oral melatonin

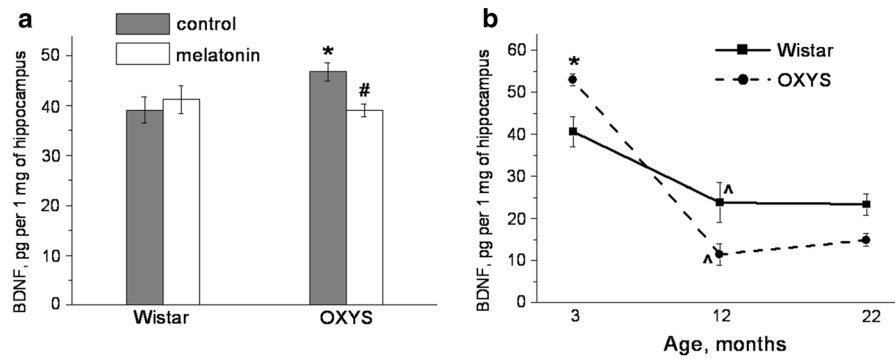


Fig. 3 BDNF levels in the hippocampus according to ELISA. **a** BDNF levels in four-month-old melatonin-treated and untreated OXYS and Wistar rats. **b** BDNF levels in the hippocampus of 3-, 12-, and 22-month-old untreated rats. Three-month-old OXYS rats showed statistically significantly higher BDNF levels compared to Wistar rats, but these levels

decreased with age (statistically significantly; panel **b**). Oral administration of melatonin prevented the increase of hippocampal BDNF levels in four-month-old OXYS rats (**a**). The data are presented as mean ± SEM. **p* < 0.05 for differences between the strains; #*p* < 0.05 for effects of oral melatonin; ^*p* < 0.05 for age-related differences within a strain

Table 1 Effects of oral melatonin administration on histological features of the dentate gyrus, CA1 region, and CA3 region in the hippocampus of OXYS and Wistar rats

Region of hippocampus	Type of neurons (%)	OXYS rats		Wistar rats	
		Control	Melatonin	Control	Melatonin
CA1	Unaffected	74.96 ± 4.70 ^a	95.86 ± 1.41 ^b	94.63 ± 1.51	92.21 ± 4.35
	Damaged	22.60 ± 2.43 ^a	2.97 ± 1.53 ^b	6.20 ± 0.88	2.16 ± 0.52 ^b
	Dead	2.44 ± 0.64	0.59 ± 0.27 ^b	0.49 ± 0.21	0.62 ± 0.11
CA3	Unaffected	89.71 ± 8.46	94.95 ± 0.81	95.59 ± 0.78	97.25 ± 0.81
	Damaged	8.60 ± 2.40	4.30 ± 0.74	4.49 ± 1.69	3.91 ± 1.30
	Dead	1.69 ± 0.49 ^a	0.68 ± 0.33	0.36 ± 0.12	0.14 ± 0.14
Dentate gyrus	Unaffected	96.76 ± 0.41	96.50 ± 0.18	95.59 ± 0.46	97.50 ± 0.21 ^b
	Damaged	0.67 ± 0.17 ^a	2.93 ± 0.15 ^b	3.51 ± 0.45	1.96 ± 0.20 ^b
	Dead	2.67 ± 0.26 ^a	0.58 ± 0.06 ^b	0.70 ± 0.23	0.55 ± 0.06

^a *p* < 0.05 for differences between the strains

^b *p* < 0.05 for effects of oral melatonin

p < 0.01, and $F_{1,80} = 8.3$, *p* < 0.01, respectively). OXYS rats made a significantly lower number of entries into the open arms compared to Wistar rats (*p* < 0.02; Fig. 4a). Likewise, OXYS rats spent 3-fold less time in the open arms (*p* < 0.01; Fig. 4b), 2.3-fold less time in the center of the elevated plus maze (*p* = 0.12; data not shown), and 2.7-fold less relative time in the open space (*p* < 0.00; Fig. 4e).

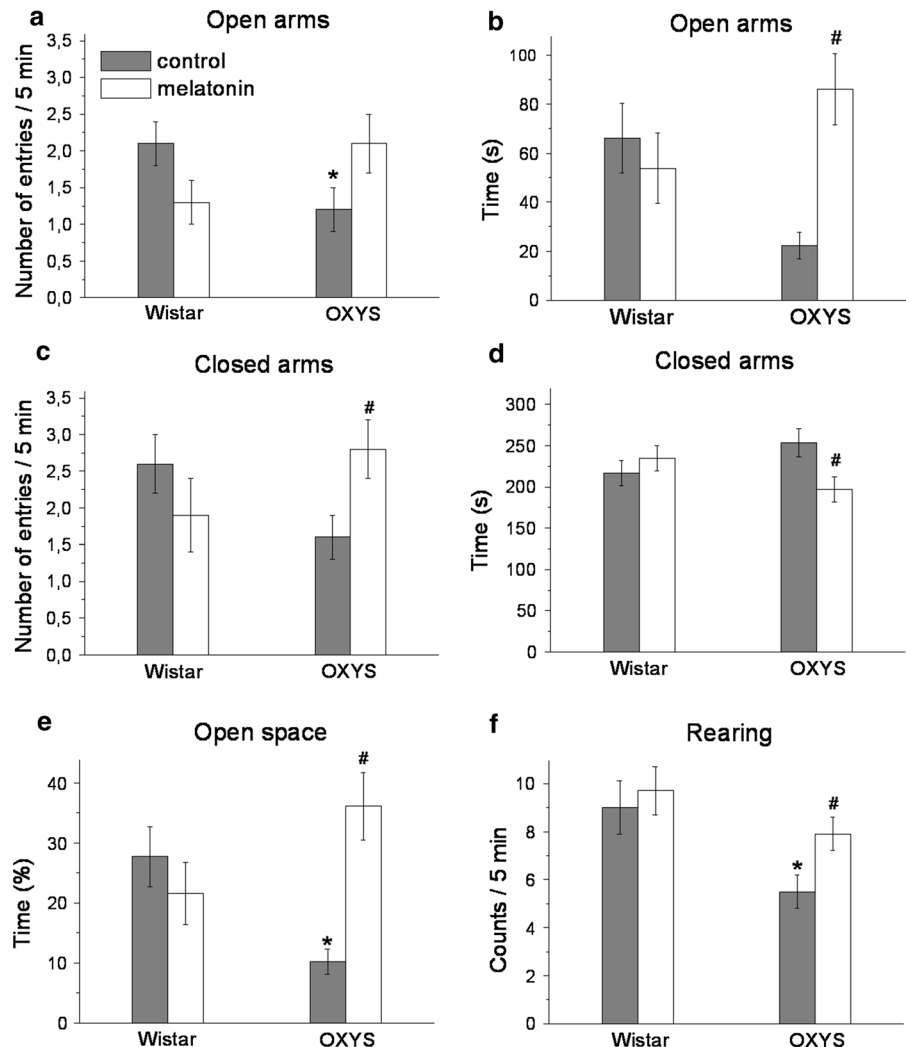
Treatment with melatonin prevented the increase of anxiety in OXYS rats according to the elevated plus maze test. Melatonin-treated OXYS rats showed a weak tendency for a higher number of entries into open arms (*p* = 0.07; Fig. 4a), and this parameter did not differ from that of Wistar rats. In contrast, the number of entries into closed arms was significantly greater in melatonin-treated OXYS rats compared to untreated animals (*p* < 0.03; Fig. 4c). After treatment

with melatonin, the time spent by OXYS rats in the open arms increased 3.8-fold (*p* < 0.00; Fig. 4b), and the relative time spent in the open space increased 3.5-fold (*p* < 0.00; Fig. 4e). In contrast, the time spent by OXYS rats in the closed arms decreased (*p* < 0.02; Fig. 4d). In addition, after treatment with melatonin, OXYS rats showed a higher frequency of rearing compared to the untreated group (*p* < 0.02; Fig. 4f).

The open field test

ANOVA showed that both the number of squares crossed and the frequency of rearing were affected only by the genotype ($F_{1,80} = 20.8$, *p* < 0.00, and $F_{1,80} = 27.3$, *p* < 0.00, respectively). The number of squares crossed and the frequency of rearing were lower in four-month-old OXYS rats than in age-

Fig. 4 Performance of four-month-old melatonin-treated and untreated rats in the elevated plus maze test. OXYS rats made a reduced number of entries into the open arms (a), spent less time there (b), and spent less of the relative time in the open space (e) in comparison with Wistar rats. Oral administration of melatonin to OXYS rats increased the time spent in the open arms (b), the relative time spent in the open space (e), and the number of entries into the closed arms (c). This treatment reduced the time spent by OXYS rats in the closed arms (d). Untreated OXYS rats showed a reduced frequency of rearing (f). Treatment with melatonin statistically significantly increased this parameter in OXYS rats. The data are shown as mean \pm SEM. * $p < 0.05$ for differences between the strains; # $p < 0.05$ for effects of oral melatonin



matched Wistar rats (Fig. 5a, b). *Post hoc* analysis showed that treatment with melatonin increased the number of squares crossed as well as the frequency of rearing in OXYS rats ($p < 0.00$ and $p < 0.01$, respectively).

The eight-arm radial maze

The results are presented in Fig. 6a, b. The animals learned how to find food pellets in the eight-arm radial maze and made fewer errors while learning. It is noteworthy that Wistar rats showed a statistically significantly lower rate of reference memory errors already during days 2–4 of testing ($p < 0.04$), whereas OXYS rats did not exhibit such a decrease. Moreover,

OXYS rats displayed a greater rate of reference memory errors compared to Wistar rats during the first 7 days of testing (Fig. 6b). Additionally, OXYS rats showed lower exploratory activity in the eight-arm radial maze: the number of entries into the maze arms was affected only by the strain (i.e., genotype; $F_{1,20} = 15.4$, $p < 0.00$, during the first day of testing; $F_{1,20} = 30.3$, $p < 0.00$, during days 2–4 of testing; $F_{1,20} = 13.3$, $p < 0.00$, during days 5–7 of testing; and $F_{1,20} = 7.6$, $p < 0.02$, during days 8–10 of testing) and was lower in OXYS rats (Fig. 6a). Treatment with melatonin did not have considerable effects on the learning ability of OXYS and Wistar rats, but there were no significant differences in the reference memory error rate between melatonin-treated OXYS

and untreated Wistar rats. Moreover, melatonin-treated OXYS rats exhibited a decreased rate of reference memory errors during days 8–10 of testing ($p < 0.00$) compared to the first day of testing. As for exploratory activity, melatonin-treated OXYS rats made a statistically significantly higher number of entries into the arms of the eight-arm radial maze during days 8–10 of testing, compared to untreated animals.

Discussion

The decrease of melatonin production is considered a risk factor of neurodegenerative diseases like AD (Bubenik and Konturek 2011). Here we demonstrate an age-dependent decline of melatonin production in OXYS and Wistar rats. However, OXYS rats exhibit the disordered melatonin secretion already at 4 months of age. These disturbances manifest themselves as lower melatonin levels at night and higher melatonin levels during the day compared to Wistar rats. In addition, we found that, compared to Wistar rats, four-month-old OXYS rats show increased weight of the pineal gland. Such enlargement to some extent may be considered a compensatory reaction that serves to maintain the correct day/night melatonin ratio. As was demonstrated, melatonin levels both in cerebrospinal fluid and in the human pineal gland (according to postmortem examination) are reduced already in people with preclinical AD, who are cognitively still intact and exhibit only the earliest signs of AD neuropathology (Lin et al. 2013).

Recently, our group showed that OXYS rats exhibit signs of AD-like pathology; in particular, amyloid- β accumulates in the hippocampus and cortex of OXYS rats with age, although substantial amyloid- β accumulation occurs only at 13 months of age (Stefanova et al. 2014a). Thus, in OXYS rats, the alterations of melatonin secretion occur before accumulation of amyloid- β .

In this study, we showed that although oral administration of melatonin fails to normalize the serum melatonin secretion in four-month-old OXYS rats, this treatment restores the normal day/night ratio of melatonin concentration in the pineal gland. The indirect antioxidant properties and anti-amyloid- β effects of melatonin are based on the maintenance of appropriate circadian phasing and anti-excitotoxic mechanisms (Lin et al. 2013). Nonetheless, it is unknown which group of AD patients and at what age should start to receive treatment with melatonin. It is difficult to evaluate the effects of any anti-aging medications on humans because of individual differences in accumulation of age-related aberrations and in quality of life (not to mention the extraordinary duration of such experiments).

As mentioned above, growth hormone exerts some of its metabolic effects in the central nervous system. Thus, impaired production of growth hormone or its main intermediary, IGF-1, is associated with cognitive deficits (Nyberg and Hallberg 2013) and may contribute in AD progression. Here we confirmed our previous results (Kolosova et al. 2012) that not only growth hormone but also IGF-1 levels are decreased in the serum of four-month-old OXYS rats compared to

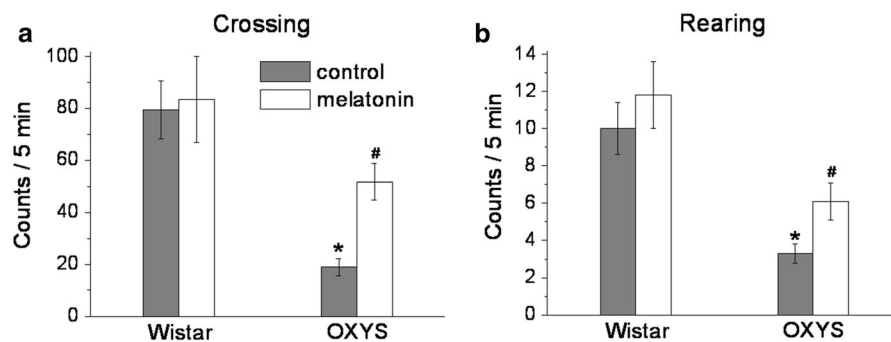


Fig. 5 Performance of four-month-old melatonin-treated and untreated rats in the open field test. OXYS rats showed a reduced number of squares crossed (a) and reduced frequency of rearing (b) compared to Wistar rats. Oral melatonin statistically

significantly increased the number of squares crossed (a) and frequency of rearing (b) in OXYS rats. The data are shown as mean \pm SEM. * $p < 0.05$ for differences between the strains; # $p < 0.05$ for effects of oral melatonin

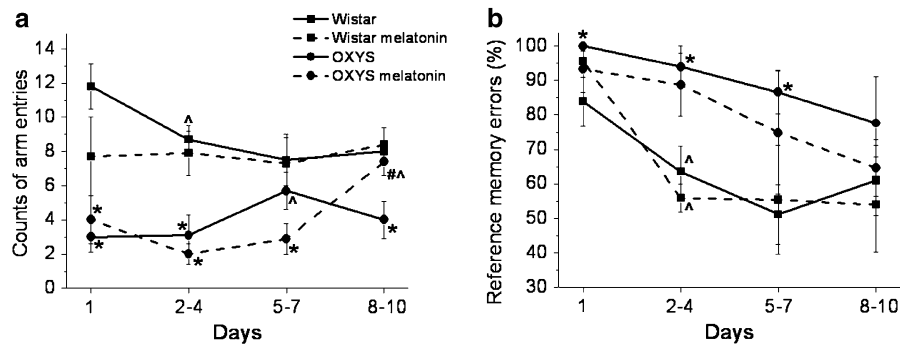


Fig. 6 Performance of four-month-old melatonin-treated and untreated rats in the eight-arm radial maze. OXYS rats made a reduced number of entries into the arms (a) and showed an increased rate of reference memory errors in the first three periods of testing (b). Neither the number of entries into the arms nor the reference memory errors rate was affected by oral

administration of melatonin. There were no statistically significant differences in the reference memory errors rate between melatonin-treated OXYS rats and untreated Wistar rats (b). The data are presented as mean \pm SEM. * $p < 0.05$ for differences between the strains; # $p < 0.05$ for effects of oral melatonin; ^ $p < 0.05$ compared to the first day of testing

age-matched Wistar rats. Our present data show that oral administration of melatonin increases serum levels of growth hormone in OXYS rats. As shown previously, melatonin may partially contribute to restoration of brain energy metabolism by increasing growth hormone and IGF-1 levels in animals with AD-like pathology (Jenwitheesuk et al. 2014).

It is generally accepted that BDNF levels are lowered in the hippocampus and cortex of patients with AD (Allen et al. 2013). The results that we obtained in the assays of BDNF levels in the hippocampus of four-month-old OXYS rats are surprising. OXYS rats show an increase of hippocampal BDNF content compared to age-matched Wistar rats. However, BDNF levels in the hippocampus of 12-month-old OXYS rats were significantly decreased. The increase of BDNF levels in four-month-old OXYS rats to some extent may be considered as a possible compensatory mechanism that is triggered under conditions of progressive neurodegeneration. Durany et al. (2000) observed a significant increase of BDNF levels in the hippocampus and parietal cortex of patients with AD. Here we demonstrated that treatment with melatonin prevents the increase of BDNF concentration in the hippocampus of four-month-old OXYS rats. The absence of such an increase may be due to the beneficial effects of melatonin on the state of neurons and, consequently, due to the inactive state of the hypothetical compensatory mechanism.

Neurodegeneration and neuronal death are among the key pathological features of AD. Particularly,

hippocampal neurons are vulnerable to AD-associated neuronal damage (Moodly and Chan 2014). Because hippocampus participates in memory consolidation and formation of emotions (Andersen et al. 2007), death of hippocampal neurons contributes to early memory loss, depression, and high anxiety observed in AD. Thus, damage to and death of hippocampal neurons in four-month-old OXYS rats may be partly responsible for the behavioral and cognitive alterations that we observed here. The oral administration of melatonin attenuates neuronal cell death in hippocampal neurons of OXYS rats. Our results are in line with the findings of Ramírez-Rodríguez et al. (2009), who demonstrated that melatonin promotes survival of hippocampal neurons.

OXYS rats exhibit altered behavior already at 4 months of age (Stefanova et al. 2010, 2014b), including increased anxiety and decreased locomotor and exploratory activity. Melatonin treatment affects the behavior of OXYS rats; it prevents the increase of anxiety and increases locomotor and exploratory activity. AD is also characterized by early memory loss and disorientation. Our results show that Wistar rats exhibit a decreased rate of reference memory errors already during days 2–4 of testing in the eight-arm radial maze; this finding is indicative of the ability to learn. In contrast, OXYS rats do not show any noticeable decrease in the rate of reference memory errors. In addition, OXYS rats display decreased locomotor activity in this test. Melatonin-treated OXYS rats do not show any significant differences

in the reference memory errors rate with Wistar rats and demonstrate fewer reference memory errors during days 8–10 of testing compared to the first day of testing. This result is suggestive of partly improvement of the memory in OXYS rats. In addition, treatment of OXYS rats with melatonin increases the locomotor activity in the eight-arm radial maze.

In conclusion, our data demonstrate that melatonin may be involved in the developing and/or progression of AD-like pathology. We also found that oral melatonin has strong neuroprotective properties: it normalizes the production of some bioactive molecules, such as BDNF and growth hormone (and its intermediary, IGF-1), prevents neuronal death in the hippocampus and, consequently, attenuates behavioral abnormalities and memory alterations in animals with AD-like pathology. Our data show that treatment with melatonin could be a good prophylactic strategy against the development of AD-like pathology at early stages of AD.

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Conflict of interest The authors declare that they have no competing financial interests.

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