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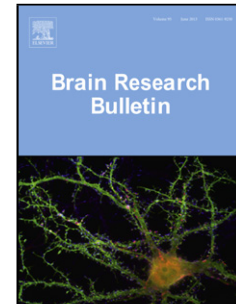

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Pre- and post-natal melatonin administration partially regulates brain oxidative stress but does not improve cognitive or histological alterations in the Ts65Dn mouse model of Down syndrome

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HIGHLIGHTS

- Neuroprotective effects of melatonin at early stages were evaluated in the TS mice
- Melatonin administered at early stages did not improve cognition in the TS mice
- At early stages, melatonin did not increased hippocellularity in the TS mice
- At early stages, melatonin partially regulates brain oxidative stress in the TS mice.

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ABSTRACT

Melatonin administered during adulthood induces beneficial effects on cognition and neuroprotection in the Ts65Dn (TS) mouse model of Down syndrome. Here, we investigated the effects of pre- and post-natal melatonin treatment on behavioral and cognitive abnormalities and on several neuromorphological alterations (hypocellularity, neurogenesis impairment and increased oxidative stress) that appear during the early developmental stages in TS mice. Pregnant TS females were orally treated with melatonin or vehicle from the time of conception until the weaning of the offspring, and the pups continued to receive the treatment from weaning until the age of 5 months. Melatonin administered during the pre- and post-natal periods did not improve the cognitive impairment of TS mice as measured by the Morris Water maze or fear conditioning tests. Histological alterations, such as decreased proliferation (Ki67+ cells) and hippocampal hypocellularity (DAPI+ cells), which are typical in TS mice, were not prevented by melatonin. However, melatonin partially regulated brain oxidative stress by modulating the activity of the primary antioxidant enzymes (superoxide dismutase in the cortex and catalase in the cortex and hippocampus) and slightly decreasing the levels of lipid peroxidation in the hippocampus of TS mice. These results show the inability of melatonin to prevent cognitive impairment in TS mice when it is administered at pre- and post-natal stages. Additionally, our findings suggest that to induce pro-cognitive effects in TS mice during the early stages of development, in addition to attenuating oxidative stress, therapies should aim to improve other altered processes, such as hippocampal neurogenesis and/or hypocellularity.

Key words: Down syndrome; Melatonin; Ts65Dn; Memory; Neurogenesis; Oxidative stress

1. INTRODUCTION

Ts65Dn mice (TS), the most commonly used model of Down syndrome (DS), exhibit numerous phenotypes similar to those found in patients with DS, including motor alterations, developmental delays and alterations in behavior and in different cognitive processes, such as hippocampal-dependent learning and memory [1].

The cognitive impairments that characterize DS individuals and TS mice have been partially attributed to hypocellularity within different areas of the brain, including the hippocampus, due to

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the impairment of neurogenesis that starts during the early developmental stages [2,3] and to other neuromorphological (abnormal dendritic tree arborization, spine density and morphology and synaptic density) and electrophysiological alterations [4] that disturb synaptic plasticity and prevent proper brain function.

Another mechanism that contributes to the cognitive and neuronal function deficits in DS is increased oxidative stress (OS) produced by the overexpression of several genes on chromosome 21 [5]. One of the overexpressed genes in individuals with DS and TS mice is *SOD1/Sod1*, which is responsible for the expression of superoxide dismutase (SOD), an enzyme that dismutates superoxide anions into hydrogen peroxide and leads to over-production and OS. In DS, OS is already present in the early stages of life and negatively influences neurogenesis, differentiation, migration, net connectivity and neuronal survival [6,7]. In the later stages of life, OS contributes to the age-related progression of cognitive and neuronal degeneration associated with DS [8].

Melatonin is an indoleamine synthesized and secreted mainly by the pineal gland and is important for normal neurodevelopment [9,10]. The fetus does not produce melatonin, but maternal melatonin crosses the placental barrier during gestation [11]. During lactation, melatonin present in maternal milk [12] reaches the brains of pups [13]. Melatonin is involved in modulating complex processes, such as learning and memory [14], as well as stress- and anxiety-related behaviors [15] . Exogenous administration of melatonin exerts neuroprotective effects [16-18]. Melatonin also facilitates memory performance in different experimental paradigms [19-22] and improves cognitive deficits in mouse models of various neuropathologies [17,18,21]. All these effects of melatonin have been attributed, at least in part, to its antioxidant- [23] and neurogenesis-promoting properties [16,18].

We previously demonstrated that chronic melatonin treatment from mid to old age improved spatial learning and memory and hippocampal long term potentiation (LTP) in TS mice. These functional benefits were associated with the prevention of cholinergic neuron degeneration and the attenuation of hippocampal oxidative stress [24,25] . In addition, melatonin increased the density of proliferating cells and differentiating neuroblasts and improved the excitatory/inhibitory balance in the hippocampus of TS mice [26]. Because DS can be diagnosed prenatally, and considering that increases in oxidative stress, impairment of neurogenesis and hypocellularity are initiated during neurodevelopment and play important roles in cognitive dysfunction, intervention during pregnancy to improve these altered phenotypes is an attractive option for ameliorating or

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restoring the intellectual disability that characterizes DS. Given the ability of melatonin to cross the placental barrier and its pro-cognitive and neuroprotective effects in adult TS mice, the aim of the present study was to evaluate whether melatonin administered from early prenatal stages to adulthood could prevent or attenuate the neurodevelopmental alterations that characterize the brain of the TS mouse and therefore reduce their behavioral and cognitive deficits.

2. EXPERIMENTAL PROCEDURES

2.1. Animals

This study was approved by the University of Cantabria Institutional Laboratory Animal Care and Use Committee and was carried out in accordance with the Declaration of Helsinki and the European Community Council Directive (86/609/EEC). Mice were mated, generated and karyotyped as previously described by Corrales et al. [24]. In all experiments, TS mice were compared to euploid littermates (CO).

2.2. Housing, melatonin treatment, and experimental groups

Mice were housed in clear Plexiglass cages (20 x 22 x 20 cm) in standard laboratory conditions, with a temperature of 22 $\pm$ 2 °C, 12 h light/ dark cycle and free access to food and water. The light/dark cycle was inverted so that the behavioral studies were conducted during the active period of the mice.

Melatonin (100 mg/L; Sigma-Aldrich, Madrid, Spain) and its diluent (vehicle) were dissolved in absolute ethanol and added to the drinking water at a final ethanol concentration of 0.06%.

To assess the effects of melatonin administration during the neurodevelopmental period, TS females (housed individually) were administered melatonin or vehicle in the drinking water from the day after conception to the 21st day postpartum. The new-born TS and CO male mice were maintained with their mothers until weaning. Post-weaning, the offspring (housed in groups of 3-4 animals) continued to receive the same treatment (melatonin or vehicle in the drinking water) as their progenitors and were karyotyped and randomly assigned to one of four experimental groups: TS-Mel (n=13), CO-Mel (n=13), TS-vehicle (n=13), and CO-vehicle (n=13). TS and CO mice in the vehicle groups received tap water containing 0.06% ethanol. The animals were treated until they were 16-18 weeks of age, followed by an additional 4 weeks of treatment during behavioral assessments. Based on an average daily water consumption rate of 5 ml/day, as estimated in

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previous studies by us and other groups [21, 24-27], the daily dose of melatonin for each mouse was ~0.5 mg.

All mice were between 4.5 and 5 months of age at the time of the behavioral assessment. The behavioral assessment was performed in 13 animals in each experimental group. Six animals per group were used to evaluate the effects of melatonin administration on oxidative stress, while 7 animals per group were used to perform the histological studies. The experimenters were blinded to the genotype and pharmacological treatment throughout the entire behavioral assessment and the other neuromorphological experiments.

2.3. Determination of melatonin concentration in plasma

Plasma melatonin concentration was measured in 6 pups from each group before weaning, at postnatal day 17, using a melatonin ELISA kit (Cloud-Clone, Ref: CEA908Ge, TX, USA). Blood was collected two hours after the beginning of the light and dark phases of the daily cycle, that is, at 10:00 p.m. and 10:00 a.m., respectively. The collected whole blood was centrifuged for 15 min at 4 ºC at 1000 × g. The supernatants were collected and stored at -80 ºC until use. The melatonin concentration was determined (in pg/ml) in duplicates according to the guidelines provided by the manufacturer.

2.4. Behavioral assays

The most invasive tests were performed last to decrease the chances of altering the behavioral responses. These tests were performed in the following order: sensorimotor test, actimetry, rotarod, hole board, open field, elevated plus maze, Morris water maze and Contextual Fear Conditioning test.

2.4.1. Spontaneous activity: actimetry. During a complete 12/12 h light/dark cycle, the daily variations in the animals' spontaneous locomotor activities were assessed using the Acti-system II device (Panlab, Barcelona), which detects the movement of the animals through the changes in the magnetic field.

2.4.2. Sensorimotor test battery. A battery of sensorimotor tests, including the visual-placing reflex, auditory sensitivity, the vibrissa placing reflex, equilibrium, grip strength and the prehensile reflex, was performed on the four groups of animals. Each parameter was measured and scored as previously described (for a detailed description, see [24]).

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2.4.3. Motor coordination: rotarod. Motor coordination was evaluated in a Rotarod apparatus (Ugo Basile; Comerio, Italy) using the protocol previously described by Corrales et al. [24]. In this test, we measured the latency to fall from a plastic rod that rotated during 60 seconds at different constant speeds (5, 20 and 40 revolutions per minute (r.p.m.)) or at progressively increasing speeds (acceleration cycle).

2.4.5. Exploratory activity: hole board test. To evaluate exploratory activity and attention, we used the same hole board apparatus and protocol previously described by Corrales et al. [24]. The exploratory activity was assessed by quantifying the horizontal and vertical (rearings) activity of the animals, the time spent exploring each hole and the number of explorations. Attention was analyzed by calculating a repetition index (ABA index) of the number of explorations of recently explored holes.

2.4.6. Open field. General activity and anxiety were assessed and quantified using the same open field apparatus and protocol described by Rueda et al. [28]. In single 5-min trials, the vertical (rearings) and horizontal (distance in the center and periphery) activity was videotaped and analyzed using the Anymaze Video Tracking System (Stoelting, Wood Dale, IL, USA).

2.4.7. Plus maze. To analyze the motor and cognitive components of anxiety in the four groups of animals, we used the same protocol described by Corrales et al. [24]. During a single 5 min trial, the time spent in the open and closed arms, the number of arm entries, and the number of head dippings (HDs) and stretch attend postures (SAPs) were registered. For each animal, the trial was videotaped, and the distance traveled in the open and closed arms and the speed of movement were analyzed using the Anymaze software.

2.4.8. Morris water maze (MWM). The MWM was used to evaluate spatial learning and memory using the same protocol and apparatus as previously described [24]. Powdered milk was added to the water to achieve opaqueness.

Animals were tested for 12 consecutive days, including eight acquisition sessions (S1-S8; platform submerged 1 cm below the water level) and four cued sessions (S9-S12; platform visible).

During the acquisition sessions, the platform was placed in a different location every day. Each session consisted of four pairs of trials, 30-45 min apart. For each trial pair, each mouse was randomly placed in one of four starting positions (North, South, East, West). Each trial lasted a maximum of 60 seconds, after which the animals were allowed to remain on the platform for 20

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seconds to help them remember its location. To allow them to develop a spatial map of the environment and to learn the platform position, fixed environmental cues were visible from within the pool. The "between-session'' performance analysis provides a measure of "spatial reference memory'' (the ability to learn and remember a spatial map of the environment).

During the four cued sessions, the platform was placed 1 cm above the water level, and its position was indicated with a flag. The experimental procedure during these trials was identical to the one following the acquisition sessions.

All trials were videotaped with a camera located 2 meters above the water level. The Anymaze video tracking system was used to analyze the swimming trajectories, escape latencies and swimming speed of each animal in each trial.

2.4.9. Contextual Fear Conditioning Test (CFC). Contextual and tone-cued fear conditioning tests were performed using a fear conditioning apparatus (Stoelting, Wood Dale, Il, USA) and the Anymaze software. The protocol described by García-Cerro et al. [29] was consistently followed. All mice underwent a training day, a tone-cued novel context testing day, and a contextual testing day. Briefly, on the first day (training day), each mouse had 3 min to explore the chamber (baseline activity) and then received five tone-shock pairings. The second day (tone-cued testing day) consisted of 3 trials of 80 s in which the mice were placed in a novel context (new visual cues) and a tone was presented without any shocks. On the third day, each mouse was placed in the same visual context as the first day without any tones or shocks for 5 min. CFC was evaluated by quantifying the freezing time. During sessions 2 and 3, freezing time provided a measure of the association between the unconditioned stimuli (shock) and the conditioned stimuli (tone in session 2 and context in session 3, respectively).

2.5. Immunohistochemistry

2.5.1. Tissue preparation. The animals were anesthetized and perfused, and their hippocampi were removed and processed for histology and cell counting, as previously described [26]. The hippocampi were coronally sliced in a cryostat (50-μm-thick sections), and 1 random section out of every 9 was used for the immunohistochemistry protocol.

2.5.2. Nissl staining. To calculate the total area of the subgranular zone (SGZ) of each mouse , a randomly chosen series was used to perform Nissl staining. The total SGZ extension was measured by the standard Cavalieri method as previously described [30], using a semiautomatic system (ImageJ v.1.33, NIH, USA, http://rsb.info.nih.gov/ij/).

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2.5.3. Cell proliferation (Ki67 immunofluorescence). Ki67 immunohistochemistry in a series of one-in-nine slices was performed as described by Corrales et al. [26]. Briefly, free-floating slices were incubated with primary antibodies (rabbit anti-Ki67 (1:750; Neo Markers, UK)) diluted in phosphate buffer with 0.5% Triton X-100 and 0.1% BSA (PBTBSA) for two days at 4°C. Then, the slices were incubated overnight at 4°C with secondary antibody (donkey anti-rabbit-Alexa Fluor 488 (1:1,000, Molecular Probes, Eugene, OR, USA)). The sections were counterstained with 4'6-diamidino-2-phenylindole (DAPI, Calbiochem, 1:1,000) for 10 min in 0.1 M phosphate buffer (PB) and mounted on gelatin-covered slides. The total number of Ki67-positive cells was counted in the selected sections using an optical fluorescence microscope (Zeiss Axioskop 2 plus, 40x objective) via the optical dissector method [31] and divided by the total area of the SGZ.

2.5.4. Mature granule cells. Mature granule cells in the hippocampal granule cell layer (GCL) were counted in the series of one-in-nine sections stained with DAPI, using a previously described physical dissector system coupled with confocal microscopy [30]. Random numbers were generated to select the points at which to locate the dissectors. In each series, 6 dissectors, in which the frame was a square situated randomly inside the GCL, were photographed and analyzed using the confocal microscope (Leica SPE) and ImageJ software, respectively. The cells were counted with the NIH ImageJ Cell Counter in the series of confocal images. The number of cells was then divided by the reference volume of the dissector to obtain the number of cells per volume unit (cell density).

2.6. Oxidative stress assays

2.6.1. Sample preparation. Cortical and hippocampal samples were homogenized and processed following the protocol of Parisotto et al. [25]. The supernatant was used to perform all the biochemical determinations, which were carried out in triplicate.

2.6.2 Antioxidant enzyme assays. All the enzymatic activities were measured as described by Parisotto et al. [25]. Briefly, catalase (CAT) activity was analyzed at 240 nm to quantify the decrease in the level of H_2O_2 (expressed in mmol/min/g) in a 10 mM H_2O_2 fresh solution. SOD activity (expressed in USOD/g) was quantified at 480 nm, monitoring the oxidation of epinephrine (pH 2.0 to pH 10.2), which produces superoxide anions and pink chromophores. Glutathione peroxidase (GPx, expressed in μmol/min/g) activity was determined by measuring the oxidation of NADPH at 340 nm. Glutathione reductase activity (GR, expressed in μmol/min/g) was analyzed by quantifying the oxidation of NADPH at 340 nm due to the formation of reduced glutathione (GSH) from its oxidized form (GSSG) via the GR present in the assay solution. Glutathione S-

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transferase (GST) activity (expressed in µmol/min/g) was measured at 340 nm using 1-chloro-2,4-dinitrobenzene as the substrate and 0.1 M GSH.

2.6.3. Lipid peroxidation assessment. Lipid oxidation was determined spectrophotometrically at 535 nm via the quantification of thiobarbituric acid-reactive substances (TBARS, expressed in nmol/g) as described by Parisotto et al. [25].

2.6.4. Protein carbonyls (PC). Oxidative damage caused by protein carbonylation was determined by measuring carbonyl absorbance at 360 nm as previously described [25] and expressed in nmol/mg.

2.7. Statistical analysis

All analyses were performed in SPSS (version 22.0, Chicago, IL, USA) for Windows. The MWM data from the acquisition period and rotarod latencies to fall at constant speeds were analyzed by MANOVAs (multiple analyses of variance) with repeated measures (RM) (`session x genotype x treatment' or 'trial x genotype x treatment´), respectively. The total home cage count activity performed in the actimetry test was analyzed by RM-MANOVA (`hour x genotype x treatment´). The rest of the behavioral, neuromorphological and OS data were analyzed using two-way ('genotype x treatment') MANOVAs. For post-hoc group comparisons, Bonferroni tests were performed when all groups were compared, and Student's t-tests were performed when two individual groups were compared.

3. RESULTS

Table 1 shows the levels of plasma melatonin in the pups of the different groups at post-natal day 17 during both phases of the daily cycle. MANOVA revealed that all groups of animals treated with or without melatonin showed elevated levels of melatonin during the dark period of the daily cycle regardless of the experimental group to which they belonged (MANOVA `light/dark cycle´ $F_{(1,21)}$= 17.51, $p \leq 0.001$). Vehicle-treated TS and CO pups had undetectable plasma melatonin levels two hours after the beginning of the light phase. However, during the dark phase of the cycle, the levels were significantly increased in mice of both genotypes. As expected, the exogenous administration of melatonin to TS and CO mice increased the level of circulating melatonin in comparison to those groups that did not receive the treatment. TS and CO pups

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treated with melatonin or vehicle did not differ in the amount of melatonin found in the plasma during the light or the dark phase of the cycle (Table 1).

3.1. Melatonin treatment during pre- and post-natal stages did not affect spatial memory in the MWM or contextual fear conditioning in the CFC test of TS mice

In the MWM, the four groups of mice exhibited reduced latencies to reach the platform across the acquisition sessions, indicating that all groups progressively learned to escape from the tank (RM MANOVA `session´: $F_{(7,49)}=20.01$, $p<0.001$). TS mice exhibited impairment in spatial reference memory because they had more difficulties reaching the escape platform than CO mice under the same treatment condition during these sessions (MANOVA `genotype´: $F_{(1,49)}=135.6$, $p<0.001$). Pre- and post-natal melatonin treatment did not reduce the latency to reach the platform in TS or CO mice during these sessions (MANOVA `treatment´: $F_{(1,49)}=1.71$, $p=0.19$), and it therefore did not improve spatial memory in these animals (Fig. 1A).

During the cued sessions, in which the platform was visible, all groups of mice showed similar latencies to reach the platform (MANOVA `genotype´: $F_{(1,49)}=3.2$, $p=0.086$; `treatment´: $F_{(1,49)}=3.34$, $p=0.075$; Fig. 1B). Additionally, the four groups of mice did not differ in their swimming speeds during the entire test (data not shown).

In the CFC test, the TS mice exhibited a reduced percentage of freezing time, and they therefore showed impaired cued- (MANOVA 'genotype': $F_{(1,49)}=11.60$; $p\leq0.001$) and context- fear conditioning (MANOVA 'genotype': $F_{(1,49)}=4.75$; $p\leq0.05$; Fig. 2A). TS mice under both treatments also exhibited increased freezing latencies during the context conditioning test session (i.e., the time spent from the start of the test until the first freezing episode; MANOVA 'genotype': $F_{(1,49)}=5.73$; $p\leq0.05$; Fig. 2B).

Chronic melatonin treatment did not exert any significant effect on the performance of TS or CO mice in either of these tests. Melatonin-treated TS mice behaved similarly to TS animals treated with vehicle, as they tended to spend less time freezing during the cued- (MANOVA 'treatment': $F_{(1,49)}=2.28$; $p=0.13$) and context-conditioning sessions ($F_{(1,49)}=0.06$; $p=0.80$), and in this last session, they also exhibited higher freezing latencies ($F_{(1,49)}=0.38$; $p=0.54$) than CO mice under the same treatment (Fig. 2A and B).

3.2. Melatonin did not affect sensorimotor abilities, motor coordination or spontaneous activity but reduced the exploratory activity of TS and CO mice

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Table 2 shows the scores obtained for the different sensorimotor abilities tested in TS and CO mice after vehicle or melatonin treatment during the pre- and post-natal periods. No differences in vision, response to an auditory stimulus, grip strength, prehensile reflex, traction capacity and equilibrium on the wooden or aluminum rod were found between TS and CO mice. In addition, chronic melatonin treatment from conception did not affect any of the sensorimotor abilities and reflexes tested in TS or CO mice.

To evaluate motor coordination in more demanding conditions, the rotarod test was used. Mice from the different genotypes and treatments did not differ in their latency to fall from the rotarod at different constant speeds (MANOVA 'genotype': $F_{(1,49)}=3.16$; $p=0.081$; 'treatment': $F_{(1,49)}=0.096$; $p=0.75$) or during the acceleration cycle (MANOVA 'genotype': $F_{(1,49)}=0.61$; $p=0.43$; 'treatment': $F_{(1,49)}=0.02$; $p=0.88$). Therefore, neither the genotype nor treatment affected the motor coordination of these animals (Fig. 3).

To evaluate the effect of early melatonin administration on spontaneous locomotor activity, the movement of the animals was recorded in their home cages during a complete dark-light cycle of 24 h. No differences were found between the activities displayed by the mice of both genotypes (MANOVA 'genotype': light period: $F_{(1,49)}=0.45$; $p=0.50$; dark period: $F_{(1,49)}=3.08$; $p=0.09$), indicating a normal daily rhythm. This activity was highest during the period of darkness, as befits nocturnal animals. Melatonin treatment during the pre- and post-natal stages did not alter the spontaneous locomotor activity during the dark (MANOVA 'treatment': $F_{(1,49)}=.451$; $p=0.24$) or the light phase (MANOVA 'treatment': $F_{(1,49)}=0.25$; $p=0.61$) in either TS or CO mice (Fig. 4).

On the hole board test, which evaluates exploratory activity, no significant effect of genotype or treatment was found in horizontal or vertical activity (distance travelled and rearings, respectively). TS mice showed greater exploratory activity than CO animals because they performed a larger number of explorations. Melatonin treatment significantly decreased the number of explorations and the time that mice spent exploring holes in both genotypes, but this effect was higher in TS mice, which indicates that melatonin partially reduced the hyperactivity found in vehicle-treated mice. Furthermore, melatonin treatment decreased the ABA index (number of explorations of recently explored holes), suggesting an improvement in attention. However, this decrease in the number of repetitions was partially due to the reduction in exploratory behavior induced by melatonin, because when this index was corrected by relating it to the number of explorations (ABA/number of head dippings), the effect of melatonin on the number of repetitions (i.e., attention) did not reach statistical significance (Table 3).

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3.3. Melatonin did not induce anxiety in the open field or plus maze tests in TS and CO mice

To evaluate possible effects of pre- and post-natal melatonin treatment on general activity and anxiety, we used the open field (Fig. 5) and elevated plus maze (Fig. 6) tests. Table 4 shows the results of the multivariance analysis of each variable tested in both experimental paradigms.

In the open field test, TS mice treated with either melatonin or vehicle were hyperactive compared to CO animals. TS mice exhibited greater horizontal activity, as they travelled a higher distance in the center and in the periphery of the apparatus, although no significant differences between mice of both genotypes were found in the number of rearings (vertical activity) performed in the apparatus. However, unlike the finding in the hole board test, melatonin treatment did not rescue the hyperactivity of TS mice and did not affect the vertical activity of either TS or CO mice (Fig. 5A and B).

In the elevated plus maze test, no differences were found in the motor components of anxiety (number of arm entries, time spent in the open arms of the maze and the time spent freezing at the start of the test), which were also similar for both genotypes (Fig. 6A, B and C). In addition, the cognitive components of anxiety were similar between TS and CO mice, as indicated by the lack of differences in the number of risk-associated behaviors (i.e., HDs and SAPs) performed by mice of both genotypes (Fig. 6D). Pre- and post-natal treatment with melatonin did not affect the cognitive (i.e., the number of risk assessment behaviors) or motor components of anxiety (number of arm entries, time spent in the open arms and freezing time). All these results indicate that the levels of anxiety of the TS and CO mice under both treatments did not differ in this test.

3.4. Melatonin treatment during pre- and post-natal stages did not improve the decreased cell proliferation or hypocellularity found in the hippocampus of TS mice

Figure 7A shows representative images of the immunocytochemical detection of mature granule neurons (DAPI+ cells) in the GCL and of proliferating cells (ki67+ cells) in the SGZ of the DG of TS and CO mice treated with either melatonin or vehicle. The quantitative analysis of both markers showed that TS mice had a significantly lower density of Ki67+ and DAPI+ cells than CO animals (MANOVA `genotype´: Ki67: $F_{(1,25)}=25.13$, $p\leq0.001$; DAPI: $F_{(1,25)}=40.19$, $p\leq0.001$). Melatonin treatment during pre- and post-natal periods did not modify the density of either population of cells (MANOVA `treatment´: Ki67: $F_{(1,25)}=2.97$, $p=0.10$; DAPI: $F_{(1,25)}=0.07$, $p=0.78$; Fig. 7B and C).

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3.5. Melatonin administered at early stages partially regulated the activity of several antioxidant enzymes and decreased the levels of lipid peroxidation in the brains of TS mice

Table 5 shows the results of the multivariance analysis of each oxidative stress marker.

To evaluate the effects of melatonin treatment on the levels of brain oxidative damage, we measured the levels of PC (a marker of protein damage induced by reactive oxygen species) and TBARS (a marker of lipid peroxidation) in the hippocampus and cortex of the four groups of mice. MANOVA revealed no significant differences due to genotype in the levels of oxidized proteins (PC) in both brain structures of young animals. However, TS mice exhibited higher hippocampal levels of TBARS than CO animals, while the levels of TBARS were similar in the cortices of the different groups of mice. Melatonin administration did not significantly affect the levels of protein damage or lipid peroxidation in the brains of TS mice. However, after melatonin treatment, the levels of TBARS in the hippocampus of TS mice were similar to those of CO animals ($p=0.60$) (Fig. 8A and B).

As expected, the activity of SOD1 was higher in the cortex and in the hippocampus of TS mice compared to CO mice. Pre- and post-natal melatonin treatment did not modify the activity of this enzyme in the hippocampus but normalized its levels in the cortex of TS mice (Fig. 9A).

Because SOD action must be coordinated with CAT and GPx activity to metabolize the H_2O_2 derived from SOD activity into water and oxygen, we also analyzed the activity of these antioxidant enzymes. CAT activity was increased in the hippocampus of TS mice treated with vehicle, probably to compensate for the increase the SOD1 activity. However, in this group of mice, the increase in SOD activity was not adequately compensated for by CAT activity in the cortex, because TS mice presented similar levels of CAT activity as those of CO animals. Pre- and post-natal treatment with melatonin decreased the levels of CAT activity in the cortices of mice of both genotypes and in the hippocampus of TS animals (Fig. 9B). Regarding GPx, the activity of this enzyme was similar in the cortex and hippocampus of the four experimental groups (Fig. 9C).

GR is a central player in the conversion of GSSH to its reduced form (GSH), acting as the major non-enzymatic endogenous antioxidant. MANOVA showed that GR activity was decreased in the hippocampus but not in the cortex of TS mice compared to that of CO mice. However, post hoc comparisons also showed a decrease in the levels of the activity of this enzyme in the cortex of

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vehicle-treated TS mice compared to the levels in CO mice under the same treatment. Melatonin treatment did not affect the activity of this enzyme in any of the studied brain structures (Fig. 9D).

GST activity, an enzyme that participates in the detoxification of the endogenous hydroperoxides continuously generated through cellular lipoperoxidation processes, exhibited no significant differences in the cortex or hippocampus of the different groups of mice (Fig. 9E).

4. DISCUSSION

We previously reported that administration of melatonin to adult TS mice improves spatial learning and memory, hippocampal LTP, some neuromorphological alterations in the hippocampus (increases in glutamatergic transmission and density of immature neuroblasts and mature granular cells) and reduces neurodegeneration (decreased cholinergic neuron degeneration, hippocampal oxidative stress and the density of senescent cells in the hippocampus). In this study, we evaluated whether melatonin treatment initiated at the pre-natal stages is an effective neuroprotective therapy that could prevent some of these neuromorphological alterations that appear early in DS (increased oxidative stress, hypocellularity and impaired neurogenesis) and drive the cognitive deficits in adult TS mice.

To assess the effectiveness of oral melatonin administration on early life stages in TS mice, we measured melatonin concentrations in the plasma of pups from the four groups. Exogenous melatonin administration at pre- and post-natal stages led to an increase in the levels of melatonin in both phases of the daily cycle and in the pups of both genotypes, demonstrating that the addition of melatonin to the drinking water of the dams (presumably reaching the pups through lactation) effectively increased the circulating levels of this indoleamine. Our previous study, performed in adult animals [24], confirmed that no differences in diurnal or nocturnal melatonin levels were observed between TS and CO pups. However, in the DS population, conflicting results have been described in studies analyzing endogenous melatonin concentrations at different ages. Whereas Uberos et al. [32] found lower plasma levels of melatonin in children with DS than in healthy subjects, Reiter et al. [33] reported that the circadian production of melatonin is preserved in individuals with DS between the ages of 3 and 55 years.

The effects of early melatonin administration on the cognitive deficits of TS mice have been studied using two experimental paradigms, the MWM and the CFC test. Consistent with previous findings [29,34,35], TS mice exhibited poorer spatial memory in the MWM test and acoustic

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(independent of the hippocampus) and contextual memory (hippocampus-dependent) associated with fear in the CFC test. These deficits are likely due to the numerous morphological and functional alterations in the hippocampus. In addition, TS animals also presented deficits during the cued-conditioning session, a cognitive process that has been traditionally attributed to the amygdala, a structure that seems to be unaltered in TS mice [36]. However, numerous studies have demonstrated that this type of conditioning also involves the integrity of the perirhinal, entorhinal and postrhinal cortices and the thalamus, which are also compromised in this model of DS [37,38].

Early treatment with melatonin did not produce the same beneficial effects on spatial learning and memory in the MWM test in TS mice as those that were observed when melatonin was administered during adulthood [24]. This lack of beneficial effects after early melatonin treatment does not seem to be due to changes in motor function, because no alterations were found in swimming speed or in their performance during the cued sessions among the different groups of mice. Furthermore, melatonin did not improve acoustic or contextual memory in the TS or CO mice, which is consistent with the findings of Yang et al. [39] in rats.

The lack of pro-cognitive effects following treatment with melatonin during the early stages in TS mice was not due to changes in daily activity or sensorimotor alterations, as suggested by the results obtained in the battery of motor tests, including the actimetry and rotarod tests, in which TS and CO animals under both treatments did not exhibit any impairment. Consistent with the results shown here, our research group and others have described that TS mice do not show impaired sensorimotor abilities [29, 35, 40, 41] or impairments in spontaneous activity [24, 34]. However, other studies show severe deficits in balance and motor coordination as well as abnormal daily behavioral rhythmicity [24, 34, 42. 43].

Other behavioral disturbances such as attention deficits [35, 44-46] or hyperactivity in settings that provoke caution and lack of movement in normal animals, such as in the open-field, hole board and plus-maze tests, are found in the Ts65Dn mouse at different ages [24, 29, 34, 35, 41, 43, 47] However, some studies have not found attention deficits in this model [24, 34]. In addition, within the same study, TS mice can be hyperactive in some experimental paradigms but not in others [24, 34]. This fact is mainly due to the between-subjects variability in the penetrance of these symptoms in Ts65Dn mice, similar to what is seen in the DS population. Another reason for these behavioral differences observed between studies could be the different apparatus or protocols used to measure a specific behavioral phenotype. In this study, TS mice did not show

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attention deficits in the hole board and were hyperactive in both the hole board and open-field tests but not in the plus maze test. Chronic treatment with melatonin reduced the exploratory behavior of TS mice in the hole board but not in the open field test. Pierrefiche et al. [48] also found that melatonin reduced the exploratory behavior of animals in the hole board test.

Thus, our results suggest the ineffectiveness of melatonin administered to TS mice during fetal and post-natal stages in preventing the cognitive deficits characteristic of these animals. Exogenous melatonin has been demonstrated to be effective in improving spatial memory during the later stages in many rodent models of various neuropathologies [17-19, 21]. However, its effect on spatial learning and memory after administration during the early stages in young animals is unclear. Consistent with our results, Baydas et al. [20] reported that the impact of melatonin in the prevention of learning and memory deficits is higher in aged animals than in young animals. In addition, Cao et al. [49] found deficits in spatial learning and memory in the MWM test after administration of melatonin to newborn rats and rats exposed to lead from weaning until the third month of life. Furthermore, prenatal administration of melatonin also failed to have any effect on the impaired performance in the MWM induced after chronic intrauterine hypoxia in rats [50].

Spatial memory dysfunction is quantitatively related to hippocampal neurogenesis [51]. TS mice show a positive correlation between performance in the MWM and the number of newly generated cells during adulthood in the DG [1,52]. Melatonin administered to middle aged TS mice improved both spatial memory [24] and hippocampal neurogenesis [26]. In this study, we did not find a pro-cognitive effect or an enhancement of hippocampal neurogenesis after pre- and post-natal melatonin treatment in TS mice. Thus, the failure of this treatment to improve cognition in young TS animals may be due to its inability to rescue these neuromorphological deficits.

The differential effects induced by melatonin on neurogenesis in TS mice depending on the time of administration could be explained by the many intrinsic and extrinsic factors that regulate adult neurogenesis [53], which could interfere with or influence the effects observed in older animals. In addition, melatonin also ameliorated some altered processes (cholinergic degeneration, increases in oxidative stress-induced damage and cellular senescence) that appear and progress as TS mice age, contributing to their neurodegeneration. Therefore, it is possible that the pro-cognitive effects induced by melatonin in the old TS mice could be mainly due to the prevention of neurodegeneration rather than to increases in neurogenesis. In this regard, some of these alterations, such as cholinergic degeneration, start at 6 months of age in TS mice and progress

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as the animals age. Thus, melatonin treatment in young mice may not be as effective as when administered to older animals because the TS animals used in the present study may be too young to exhibit these alterations and, in the case that they present these changes, these alterations may not be very severe at this stage.

Because OS plays an early role in cognitive dysfunction in DS, we also examined the effects of pre- and post-natal melatonin treatment on the brain OS status.

Due to an excess of gene expression, the activity of SOD was increased in the cortex and in the hippocampus of TS mice at 5 months of age, which may result in the accumulation of H_2O_2 that should also induce an increase in the activities of CAT and GPx. In this regard, CAT activity is upregulated in DS children to counteract, at least in part, the chronic enhanced H_2O_2 levels generated by the excess SOD present in cells [54,55]. CAT is a second pseudo-order enzyme specific for metabolizing H_2O_2 and maintaining the relatively low cellular generation of the hydroxyl radical ($HO^{\bullet}$) via the Haber-Weiss-Fenton reactions [56]. Accordingly, we observed an increase in CAT activity in the hippocampus but not in the cortex of TS mice. The fact that CAT activity was not enhanced in the cortex could result in insufficient removal of H_2O_2, favoring the generation of $HO^{\bullet}$ and thereby promoting persistent OS in this structure. In contrast to the previously observed enhancement in GPx activity in the cortex and hippocampus of 12-month-old TS mice [25], the activity of this enzyme was not increased at 5 months of age. Conflicting results have been found regarding the cellular responses involving GPx activity in the erythrocytes of individuals with DS. While Garlet et al. [54] revealed no significant differences between children with DS and normal controls, Pastor et al. [57] found enhanced GPx in individuals with DS between the ages of 1 and 50 years. However, in the plasma of subjects with DS and in the brains of rats, GPx activity seems to increase with age due to the formation of peroxides [58-60]. Thus, it is possible that in the brains of TS mice, GPx activity is not increased while the animals are young, but its activity is probably induced by increases in SOD levels and therefore by the accumulation of H_2O_2 as the animal ages.

The levels of GSH, the most important endogenous antioxidant, are decreased in subjects with DS [54,61]. Although its levels were not measured in the present study, the activity of the GST enzyme, which acts in xenobiotic detoxification by catalyzing the conjugation of GSH to chemical toxins and in the detoxification of hydroperoxides derived from lipoperoxidation [61], was unchanged in the cortex or the hippocampus of TS mice. However, the low activity of GR found in the hippocampus and cortex of TS mice compared to CO mice in the present study may impair

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the efficient replacement of the levels of GSH, which may be an additional factor contributing to the exacerbation of oxidative damage in this structure. In addition to these enzymes, the redox system involving GSH also includes the activity of γ-glutamylcysteine synthase and glucose-6-phosphate dehydrogenase [62], which were not measured in our study.

An imbalance between OS and the antioxidant system produces cell damage by oxidation of DNA, lipids and proteins. At 12 months of age, TS animals display higher levels of TBARS and PC, markers of lipid peroxidation and protein damage, respectively, in the hippocampus compared to the levels observed in CO animals [25]. In agreement with our previous results in older animals and with the levels of other markers of lipid peroxidation (the t8isoPGF2α) found in the cortex and hippocampus of TS mice at 4 months of age [63], in the present study TS animals also showed increased levels of TBARS in the hippocampus but not increased levels of PC in either of the two brain structures. The failure to find increased PC levels in the brain may be because the damage to proteins requires a more extended period to develop. In fact, Tramutola et al. [64] found that the total levels of protein oxidation start to rise at 6 months but become significantly increased at 12 months of age in the TS mouse, and Zitnanová et al. [65] did not find altered levels of PC in the plasma of DS children.

Melatonin decreases OS, acting as a free radical scavenger and/or regulating the endogenous antioxidant enzyme activities [23]. In the brains of adult TS mice, melatonin does not exert its antioxidant effects by regulating the antioxidant defense system [25]. At 5 months of age, pre- and post-natal melatonin administration partially regulated the antioxidant enzymes in the brains of TS mice because it diminished the activity of SOD in the cortex and the activity of CAT in both brain structures. However, it did not regulate the altered activity of SOD or GR in the hippocampus of TS animals. These differences may be due to the fact that melatonin differentially regulates the antioxidant enzymes depending on the basal or elevated OS levels [62]. In old TS mice, the main antioxidant action of this indoleamine was due to its effects as a free radical scavenger, normalizing the levels of protein damage and lipid peroxidation in the hippocampus of TS mice [25]. However, in young TS mice, it only exerted partial effects as a ROS scavenger, because it slightly decreased the levels of TBARS in the hippocampus. Consistent with these results, melatonin appeared to have different effects to prevent brain oxidative damage in young and in old animals and was more effective in old animals [66,67]. Melatonin administration resulted in differential restoration of lipid peroxidation and antioxidant enzymes in the livers of 3-, 12- and 24-month-old rats [68]. Exogenous melatonin treatment decreased aged-induced lipid peroxidation (malondialdehyde) in the brain of rats and was more effective at 20 than at 4 months

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of age [69]. Furthermore, melatonin administration reduces the age-related accumulation of TBARS in the plasma and homogenates of spleen and bone marrow, and its effects were also stronger in old than in young hamsters [70]. Therefore, melatonin may be a more efficient ROS scavenger as lipid peroxidation increases, which occurs in the hippocampus of TS mice as demonstrated by the higher levels of TBARS at 12 [25] than at 5 months of age. The fact that pre- and post- natal melatonin administration did not decrease SOD activity and did not completely restore the levels of lipid peroxidation in the hippocampus of TS animals may be partially responsible for the spatial learning and memory deficits that are still present in adult TS mice after treatment with this indoleamine.

The discrepancies found between the effects of melatonin when it was administered during adulthood or during pre- and early post-natal stages to TS mice could be due to different mechanisms. A possible explanation might be the dose of melatonin used in each case. We administered the same dose of melatonin in the present study (pre- and post-natal stages) as that used in our previous study (adult stages: 5 to 12 months of age, [24,26]), but it may have been necessary to administer a different dose of melatonin in young mice to obtain the same benefits found in adult mice. The pharmacokinetics of melatonin may be different in young than in adult TS animals, affecting its final concentration in the brain and reducing its beneficial effects on neurogenesis and cognitive deficits. Future studies should explore the dose-range and pharmacokinetic profile to define the optimal concentration at which the neuroprotective actions of melatonin at pre- and post-natal stages in TS mice are most effective.

The potential response of fetal brains to maternal melatonin signals have only been detected on the 17th or 18th day of gestation in rats [71], and changes in maternal melatonin could influence the expression of melatonin receptors in different brain areas of the offspring [72,73], thus compromising the response to the indoleamine in the first gestational days. Recently, Vilches et al. [74] demonstrated that the induction of chronodisruption in pregnant rats alters the transplacental melatonin signals responsible for the entrainment of daily rhythms of fetal developing tissues, including the hippocampus, and exerts negative effects on long-term cognitive functions in the offspring when assessed as adults. In our experiment, maternal exogenous melatonin administration during the gestational period could be in conflict with endogenous signals, thus inducing alterations in the development of plasticity of several brain areas modulated by maternal melatonin. Furthermore, oral melatonin administration to pregnant rats retards the secretion of postnatal testosterone and some neurotransmitters, such as substance P and neurokinin A, in various brain structures in the offspring [75]. Therefore, it is

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possible that melatonin administered during the early life stages in TS animals exerts some of these adverse effects, preventing the pro-cognitive effects induced by this indoleamine in later life-stages.

Although most studies have not found side effects after long-term treatment with melatonin in children [76-78] or after maternal or early post-natal life treatment in animals [79-81], some studies revealed negative effects of melatonin in developing organisms, such as reductions in litter size, pup growth and weight, increased mortality, and alterations in sexual maturation, fertility and levels of luteinizing hormone [50, 82-85].

Other pharmacotherapies have been shown to be effective when administered pre-natally or during the early post-natal stages to TS mice. For example, the neuroprotective peptides, NAP and SAL, and fluoxetine and choline have been shown to improve cognitive dysfunction and prevent neurogenesis defects and hypocellularity [52, 86-88]. On the other hand, conflicting results have been found regarding the use of antioxidants at pre- and post-natal stages in TS mice. While the administration of SGS-111, a neural antioxidant similar to piracetam, from conception to adulthood did not improve memory and spatial learning in TS animals [28], early intervention with α-tocopherol ameliorated oxidative stress and induced cognitive improvements in this mouse model [63]. In children and teenagers with DS, early antioxidant intervention with antioxidants, such as vitamins E and C, attenuated systemic oxidative damage [55,89,90]. However, there is no clinical evidence regarding the benefits of antioxidant supplementation on cognitive function in children and young adults with DS [91-93].

Conclusions

In the present work, pre- and post-natal melatonin treatment partially regulated OS in the brains of young TS mice. However, this pattern of administration did not attenuate the reduced hippocampal cell proliferation and hypocellularity or the cognitive deficits of these animals. These results show the inability of melatonin to prevent the cognitive impairment of TS mice when it is administered at pre- and post-natal stages and suggest that to produce pro-cognitive effects in TS mice during the early stages of development, in addition to attenuating OS, therapies should be targeted toward the improvement of other altered processes, such as hippocampal neurogenesis and/or hypocellularity.

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6. REFERENCES

1. Rueda N, Flórez J, Martínez-Cué C (2012), Mouse models of Down syndrome as a tool to unravel the causes of mental disabilities. Neural Plast 2012:584071.

2. Bianchi P, Ciani E, Guidi S, Trazzi S, Felice D, Grossi G, Fernandez M, Giuliani A, Calzà L, Bartesaghi R (2010), Early pharmacotherapy restores neurogenesis and cognitive performance in the Ts65Dn mouse model for Down syndrome. J Neurosci 30:8769-8779.

3. Bartesaghi R, Guidi S, Ciani E (2011), Is it possible to improve neurodevelopmental abnormalities in Down syndrome? Rev Neurosci 22:419-455.

4. Haydar TF, Reeves RH (2012), Trisomy 21 and early brain development. Trends Neurosci 35:81-91.

5. Lott IT (2012), Antioxidants in Down syndrome. Biochim Biophys Acta 1822:657-663.

6. Perluigi M, Di Domenico F, Buttterfield DA (2014), Unraveling the complexity of neurodegeneration in brains of subjects with Down syndrome: insights from proteomics. Proteomics Clin Appl 8:73-85.

7. Perluigi M, di Domenico F, Fiorini A, Cocciolo A, Giorgi A, Foppoli C, Butterfield DA, Giorlandino M, Giorlandino C, Schininà ME, Coccia R (2011), Oxidative stress occurs early in Down syndrome pregnancy: A redox proteomics analysis of amniotic fluid. Proteom Clin Appl 5:167-178.

8. Perluigi M, Butterfield DA (2012), Oxidative Stress and Down Syndrome: A Route toward Alzheimer-Like Dementia. Curr Gerontol Geriatr Res 2012:724904.

9. Uysal N, Ozdemir D, Dayi A, Yalaz G, Baltaci AK, Bediz CS (2005), Effects of maternal deprivation on melatonin production and cognition in adolescent male and female rats. Neuro Endocrinol Lett 26:555-560.

10. Guzman-Marin R, Suntsova N, Methippara M, Greiffenstein R, Szymusiak R, McGinty D (2005), Sleep deprivation suppresses neurogenesis in the adult hippocampus of rats. Eur J Neurosci 22:2111-2116.

11. Alers NO, Jenkin G, Miller SL, Wallace EM (2013), Antenatal melatonin as an antioxidant in human pregnancies complicated by fetal growth restriction--a phase I pilot clinical trial: study protocol. BMJ Open 3:e004141.

12. Morceli G, Honorio-França AC, Fagundes DL, Calderon IM, França EL (2013), Antioxidant effect of melatonin on the functional activity of colostral phagocytes in diabetic women. PLoS One 8:e56915.

13. Serón-Ferré M, Mendez N, Abarzua-Catalan L, Vilches N, Valenzuela FJ, Reynolds H., Llanos AJ, Rojas A, Valenzuela GJ, Torres-Farfan C (2012), Circadian rhythms in the fetus: Mol Cell Endocrinol 349:68-75.

14. Rawashdeh O, Maronde E (2012), The hormonal Zeitgeber melatonin: role as a circadian modulator in memory processing. Front Mol Neurosci 5: 27.

15. Spasojevic N, Stefanovic B, Jovanovic P, Dronjak S (2016), Anxiety and hyperlocomotion induced by chronic unpredictable mild stress can be moderated with melatonin treatment. Folia Biol 62:250-257.

ACCEPTED MANUSCRIPT

16. Iggena D, Winter Y, Steiner B (2017), Melatonin restores hippocampal neural precursor cell proliferation and prevents cognitive deficits induced by jet lag simulation in adult mice. J Pineal Res. 2017 May;62(4). doi: 10.1111/jpi.12397.

17. Yoo DY, Kim W, Lee CH, Shin BN, Nam SM, Choi JH, Won MH, Yoon YS, Hwang IK (2012), Melatonin improves D-galactose-induced aging effects on behavior, neurogenesis, and lipid peroxidation in the mouse dentate gyrus via increasing pCREB expression: J Pineal Res 52:21-28.

18. Liu XJ, Yuan L, Yang D. Han WN, Li QS, Yang W, Liu QS, Qi JS (2013), Melatonin protects against amyloid-β-induced impairments of hippocampal LTP and spatial learning in rats: Synapse 67:626-636.

19. Peng C, Hong X, Chen W, Zhang H, Tan L, Wang X, Ding Y, He J (2017), Melatonin ameliorates amygdala-dependent emotional memory deficits in Tg2576 mice by up-regulating the CREB/c-Fos pathway. Neurosci Lett 638:76-82.

20. Baydas G, Yasar A, Tuzcu M (2005), Comparison of the impact of melatonin on chronic ethanol-induced learning and memory impairment between young and aged rats. J Pineal Res 39:346-352.

21. Olcese JM, Cao C, Mori T, Mamcarz MB, Maxwell A, Runfeldt MJ, Wang L, Zhang C, Lin X, Zhang G, Arendash GW (2009), Protection against cognitive deficits and markers of neurodegeneration by long-term oral administration of melatonin in a transgenic model of Alzheimer disease: J Pineal Res 47:82-96.

22. Bertaina-Anglade V, Drieu-La-Rochelle C, Mocaër E, Seguin L (2011), Memory facilitating effects of agomelatine in the novel object recognition memory paradigm in the rat. Pharmacol Biochem Behav 98:511-517.

23. Tan DX, Manchester LC, Qin L, Reiter RJ (2016) Melatonin: A Mitochondrial Targeting Molecule Involving Mitochondrial Protection and Dynamics. Int J Mol Sci 17(12):2124.

24. Corrales A, Martínez P, García S, Vidal V, García E, Flórez J, Sanchez-Barceló EJ, Martínez-Cué C, Rueda N (2013), Long-term oral administration of melatonin improves spatial learning and memory and protects against cholinergic degeneration in middle-aged Ts65Dn mice, a model of Down syndrome. J Pineal Res 54:346-358.

25. Parisotto EB, Vidal V, García-Cerro S, Lantigua S, Wilhelm Filho D, Sanchez-Barceló EJ, Martínez-Cué C, Rueda N (2016), Chronic Melatonin Administration Reduced Oxidative Damage and Cellular Senescence in the Hippocampus of a Mouse Model of Down Syndrome. Neurochem Res 41:2904-2913.

26. Corrales A, Vidal R, García S, et al. (2014), Chronic melatonin treatment rescues electrophysiological and neuromorphological deficits in a mouse model of Down syndrome. J Pineal Res 56:51-61.

27. O'Neal-Moffitt G, Delic V, Bradshaw PC, Olcese J (2015), Prophylactic melatonin significantly reduces Alzheimer's neuropathology and associated cognitive deficits independent of antioxidant pathways in AβPP(swe)/PS1 mice. Mol Neurodegener 10:27.

28. Rueda N, Florez J, Martinez-Cue C (2008), Effects of chronic administration of SGS-111 during adulthood and during the pre- and post-natal periods on the cognitive deficits of Ts65Dn mice, a model of Down syndrome. Behav Brain Res 188:355-367.

ACCEPTED MANUSCRIPT

29. García-Cerro S, Martínez P, Vidal V, Corrales A, Flórez J, Vidal R, Rueda N, Arbonés ML, Martínez-Cué C (2014), Overexpression of Dyrk1A is implicated in several cognitive, electrophysiological and neuromorphological alterations found in a mouse model of Down syndrome. PLoS One 9:e106572.

30. Llorens-Martín M,Torres-Alemán I, Trejo JL (2006), Pronounced individual variation in the response to the stimulatory action of exercise on immature hippocampal neurons. Hippocampus 16:480-490.

31. Llorens-Martín MV, Rueda N, Tejeda GS, et al. (2010), Effects of voluntary physical exercise on adult hippocampal neurogenesis and behavior of Ts65Dn mice, a model of Down syndrome. Neuroscience 171:1228-1240.

32. Uberos J, Romero J, Molina-Carballo A, et al. (2010), Melatonin and elimination of kynurenines in children with Down's syndrome. J Pediatr Endocrinol Metab 23:277-282.

33. Reiter RJ, Barlow-Walden L, Poeggeler B, et al (1996), Twenty-four hour urinary excretion of 6-hydroxymelatonin sulfate in Down syndrome subjects. J Pineal Res 20:45-50.

34. Vidal V, García S, Martínez P, Corrales A, Flórez J, Rueda N, Sharma A, Martínez-Cué C (2012), Lack of behavioral and cognitive effects of chronic ethosuximide and gabapentin treatment in the Ts65Dn mouse model of Down syndrome. Neuroscience 220:158-168.

35. Martínez-Cué C, Martínez P, Rueda N, Vidal R, García S, Vidal V, Corrales A, Montero JA, Pazos Á, Flórez J, Gasser R, Thomas AW, Honer M, Knoflach F, Trejo JL, Wettstein JG, Hernández MC (2013), Reducing GABAA a5 receptor-mediated inhibition rescues functional and neuromorphological deficits in a mouse model of Down syndrome. J Neurosci 33:3953-3966.

36. Kleschevnikov AM, Belichenko PV, Faizi M, Jacobs LF, Htun K, Shamloo M, Mobley WC (2012), Deficits in cognition and synaptic plasticity in a mouse model of Down syndrome ameliorated by GABAB receptor antagonists.J Neurosci 32:9217-9227.

37. Goosens KA, Maren S (2001), Contextual and auditory fear conditioning are mediated by the lateral, basal, and central amygdaloid nuclei in rats. Learn Mem 8:148–155.

38. Kholodar-Smith D B, Allen TA, Brown TH (2008), Fear conditioning to discontinuous auditory cues requires perirhinal cortical function. Behav Neurosci 122:1178-1185.

39. Yang Z, Li C, Huang F (2013), Melatonin impaired acquisition but not expression of contextual fear in rats. Neurosci Lett 552:10-14.

40. Whitney KN, Wenger GR (2013), Impulsivity and motor activity in aged, male Ts65Dn mice. Exp Clin Psychopharmacol 21:345-354.

41. Escorihuela RM, Fernández-Teruel A, Vallina IF, Baamonde C, Lumbreras MA, Dierssen M, Tobeña A, Flórez J (1995), A behavioral assessment of Ts65Dn mice: a putative Down syndrome model. Neurosci Lett 199:143-146.

42. Costa ACS, Walsh K, Davisson MT (1999), Motor dysfunction in a mouse model for Down syndrome. Physiol Behav; 68:211-220.

43. Stewart LS, Persinger MA,. Cortez MA, Snead OC (2007), Chronobiometry of behavioral activity in the Ts65Dnmodel of Down syndrome,Behavior Genetics, 37:388-398.

44. Martínez-Cué C, Rueda N, García E, Flórez J (2006), Anxiety and panic responses to a predator in male and female Ts65Dn mice, a model of Down syndrome. Gen Brain Behav 5:413-422.

ACCEPTED MANUSCRIPT

45. Escorihuela RM, Vallina IF, Martínez-Cué C, Baamonde C, Dierssen M, Tobeña A, Flórez J, Fernández-Teruel A (1998), Impaired short- and long-term memory in Ts65Dn mice, a model for Down syndrome.Neurosci Lett 247:171-174.

46. Driscoll LL, Carroll JC, Moon J, Crnic LS, Levitsky DA, Strupp BJ (2004), Impaired sustained attention and error-induced stereotypy in the aged Ts65Dn mouse: a mouse model of Down syndrome and Alzheimer's disease. Behav Neurosci 118:1196-1205.

47. Coussons-Read ME, Crnic LS (1996), Behavioral assessment of the Ts65Dn mouse, a model for Down syndrome: altered behavior in the elevated plus maze and open field. Behav Genet 26:7-13.

48. Pierrefiche G, Zerbib R, Laborit H (1993), Anxiolytic activity of melatonin in mice: involvement of benzodiazepine receptors. Res Commun Chem Pathol Pharmacol 82:131-142.

49. Cao XJ, Wang M, Chen WH, Zhu DM, She JQ, Ruan DY (2009), Effects of chronic administration of melatonin on spatial learning ability and long-term potentiation in lead-exposed and control rats. Biomed Environ Sci 22:70-75.

50. Dubovický M, Ujházy E, Kovacovský P, Navarová J, Juráni M, Soltés L (2004), Effect of melatonin on neurobehavioral dysfunctions induced by intrauterine hypoxia in rats. Cent Eur J Public Health 12:23-25.

51. Drapeau E, Mayo W, Aurousseau C, Le Moal M, Piazza PV, Abrous DN (2003). Spatial memory performances of aged rats in the water maze predict levels of hippocampal neurogenesis. Proc Natl Acad Sci 100:14385-90.

52. Velazquez R, Ash JA, Powers BE, Kelley CM, Strawderman M, Luscher ZI, Ginsberg SD, Mufson EJ, Strupp BJ (2013) Maternal choline supplementation improves spatial learning and adult hippocampal neurogenesis in the Ts65Dn mouse model of Down syndrome. Neurobiol Dis 58:92-101.

53. Balu DT, Lucki I (2009). Adult hippocampal neurogenesis: regulation, functional implications, and contribution to disease pathology. Neurosci Biobehav 33:232-252.

54. Garlet TR, Parisotto EB, de Medeiros G, et al (2013), Systemic oxidative stress in children and teenagers with Down syndrome. Life Sci 93:558-563.

55. Parisotto EB, Garlet TR, Cavalli VL, Zamoner A, da Rosa JS, Bastos J, Micke GA, Fröde TS, Pedrosa RC, Wilhelm Filho D (2014), Antioxidant intervention attenuates oxidative stress in children and teenagers with Down syndrome. Res Dev Disabil 35:1228-1236.

56. Halliwell, B and Gutteridge JMC (Eds) (2007). Free radicals in biology and medicine (4th ed). Oxford: Carendon press.

57. Pastor MC, Sierra C, Doladé M, Navarro E, Brandi N, Cabré E, Mira A, Serés A (1998), Antioxidant enzymes and fatty acid status in erythrocytes of Down's syndrome patients. Clin Chem 44:924-929.

58. Mattei JF, Baeteman MA, Baret A, Ardissone JP, Rebuffel P, Giraud F (1982), Erythrocyte superoxide dismutase and redox enzymes in trisomy 21. Acta Paediatr Scand 71:589-591.

59. Annerén G, Gebre-Medhin M, Gustavson KH, Plantin LO (1985), Selenium in plasma and erythrocytes in patients with Down's syndrome and healthy controls. Variation in relation to age, sex and glutathione peroxidase activity in erythrocytes. Acta Paediatr Scand 74:508-514.

60. Öztürk G, Akbulut KG, Güney Ş, Acuna-Castroviejo D (2012), Age-related changes in the rat brain mitochondrial antioxidative enzyme ratios: modulation by melatonin. Exp Gerontol 47:706-711.

ACCEPTED MANUSCRIPT

61. Hamed RR, Maharem TM, Abdel-Meguid N, Sabry GM, Abdalla AM, Guneidy RA (2011), Purification and biochemical characterization of glutathione S-transferase from Down syndrome and normal children erythrocytes: a comparative study. Res Dev Disabil 32:1470-1482.

62. Rodriguez C, Mayo JC, Sainz RM, et al. (2004), Regulation of antioxidant enzymes: a significant role for melatonin. J Pineal Res 36:1-9.

63. Shichiri M, Yoshida Y, Ishida N, et al. (2011), Alpha-Tocopherol suppreses lipid peroxiation and behavioural and cognitive impairments in the Ts65Dn mouse model of Down syndrome. Free Radic Biol Med 15:1801-1811.

64. Tramutola A, Lanzillotta C, Arena A, Barone E, Perluigi M, Di DomenicoF (2016), Increased Mammalian Target of Rapamycin Signaling Contributes to the Accumulation of Protein Oxidative Damage in a Mouse Model of Down’s Syndrome. Neurodegener Dis 16:62-68.

65. Zitnanová I, Korytár P, Sobotová H, Horáková L, Sustrová M, Pueschel S, Duracková Z (2006), Markers of oxidative stress in children with Down syndrome. Clin Chem Lab Med 44:306-310.

66. Manda K, Bhatia AL (2003), Melatonin-induced reduction in age-related accumulation of oxidative damage in mice. Biogerontology 4:133-139.

67. Campbell A, Sharman E, Bondy SC (2014), Age-related differences in the response of the brain to dietary melatonin. AGE 36:49-55.

68. Manikonda PK, Jagota A (2012), Melatonin administration differentially affects age-induced alterations in daily rhythms of lipid peroxidation and antioxidant enzymes in male rat liver. Biogerontology 13:511-524.

69. Akbulut KG, Gonül B, Akbulut H (2008), Exogenous melatonin decreases age-induced lipid peroxidation in the brain. Brain Res 1238:31-35.

70. Vishwas DK, Mukherjee A, Haldar C, Dash D, Nayak MK (2013), Improvement of oxidative stress and immunity by melatonin: an age dependent study in golden hamster. Exp Gerontol 48:168-182.

71. Williams LM, Martinoli MG, Titchener LT, Pelletier G (1991), The ontogeny of central melatonin binding sites in the rat. Endocrinology 128:2083-2090.

72. Zitouni M, Masson-Pévet M, Gauer F, Pévet P (1995), Influence of maternal melatonin on melatonin receptors in rat offspring. J Neural Transm Gen Sect 100:111-122.

73. Zitouni M, Pévet P, Masson-Pévet M (1996), Brain and pituitary melatonin receptors in male rat during post-natal and pubertal development and the effect of pinealectomy and testosterone manipulation. J Neuroendocrinol 8:571-577.

74. Vilches N, Spichiger C, Mendez N, Abarzua-Catalan L, Galdames HA, Hazlerigg DG, Richter HG, Torres-Farfan C (2014), Gestational chronodisruption impairs hippocampal expression of NMDA receptor subunits Grin1b/Grin3a and spatial memory in the adult offspring. PLoS One 9(3):e91313.

75. Díaz Rodríguez E, Díaz López B, Debeljuk L, Esquifino Parras AI, Arce Fraguas A, Marín Fernández B (1999), Developmental changes of hypothalamic, pituitary and striatal tachykinins in response to testosterone: influence of prenatal melatonin. Peptides 20:501-508.

76. Palm L, Blennow G, Wetterberg L (1997), Long-term melatonin treatment in blind children and young adults with circadian sleep-wake disturbances. Dev Med Child Neurol 39:319-325.

ACCEPTED MANUSCRIPT

77. Jan J, Wasdell MB, Freeman RD, Bax M (2007), Evidence supporting the use of melatonin in short gestation infants. J Pineal Res 42:22-27.

78. Gitto E, Aversa S, Salpietro CD, Barberi I, Arrigo T, Trimarchi G, Reiter RJ, Pellegrino S (2012), Pain in neonatal intensive care: role of melatonin as an analgesic antioxidant. J Pineal Res 52:291-295.

79. Jahnke G, Marr M, Myers C, Wilson R, Travlos G, Price C (1999), Maternal and developmental toxicity evaluation of melatonin administered orally to pregnant Sprague-Dawley rats. Toxicol Sci 50:271-279.

80. Uyanikgil Y, Baka M, Ateş U, Turgut M, Yavaşoğlu A, Ulker S, Sözmen EY, Sezer E, Elmas C, Yurtseven ME (2007), Neuroprotective effects of melatonin upon the offspring cerebellar cortex in the rat model of BCNU-induced cortical dysplasia. Brain Res 1160:134-144.

81. Watanabe K, Hamada F, Wakatsuki A, Nagai R, Shinohara K, Hayashi Y, Imamura R, Fukaya T (2012), Prophylactic administration of melatonin to the mother throughout pregnancy can protect against oxidative cerebral damage in neonatal rats. J Matern Fetal Neonatal Med 25:1254-1259.

82. Colmenero MD, Díaz B, Miguel JL, González ML, Esquifino A, Marín B (1991), Melatonin administration during pregnancy retards sexual maturation of female offspring in the rat. J Pineal Res 11:23-27.

83. Edmonds KE (2013), Melatonin, But not Auxin, Affects Postnatal Reproductive Development in the Marsh Rice Rat (Oryzomys palustris). Zoolog Sci 30:439-345.

84. Singh HJ, Saleh HI, Gupalo S, Omar E (2013), Effect of melatonin supplementation on pregnancy outcome in Wistar-Kyoto and Sprague-Dawley rats. Acta Physiolog Sinica 65:149-157.

85. González-Candia A, Veliz M, Araya C, Quezada S, Ebensperger G, Serón-Ferré M, Reyes RV, Llanos AJ, Herrera EA (2016), Potential adverse effects of antenatal melatonin as a treatment for intrauterine growth restriction: findings in pregnant sheep. Am J Obstet Gynecol. 215:245.e1-7.

86. Vink J, Incerti M, Toso L, Roberson R, Abebe D, Spong CY (2009), Prenatal NAP+SAL prevents developmental delay in a mouse model of Down syndrome through effects on N-methyl-D-aspartic acid and gamma-aminobutyric acid receptors. Am J Obstet Gynecol 200:524.e1-4.

87. Moon J, Chen M, Gandhy SU, Strawderman M, Levitsky DA, Maclean KN, Strupp BJ (2010), Perinatal choline supplementation improves cognitive functioning and emotion regulation in the Ts65Dn mouse model of Down syndrome. Behav Neurosci 124:346-361.

88. Guidi S, Stagni F, Bianchi P, Ciani E, Giacomini A, De Franceschi M, Moldrich R, Kurniawan N, Mardon K, Giuliani A, Calzà L, Bartesaghi R (2014), Prenatal pharmacotherapy rescues brain development in a Down's syndrome mouse model. Brain 137:380-401.

89. Parisotto EB, Giaretta AG, Zamoner A, Moreira EA, Fröde TS, Pedrosa RC, Filho DW (2015), Persistence of the benefit of an antioxidant therapy in children and teenagers with Down syndrome. Res Dev Disabil 45:14-20.

90. Mustafa Nachvak S, Reza Neyestani T, Ali Mahboob S, Sabour S, Ali Keshawarz S, Speakman JR (2014), α-Tocopherol supplementation reduces biomarkers of oxidative stress in children with Down syndrome: a randomized controlled trial. Eur J Clin Nutr 68:1119-1123.

91. Ani C, Grantham-McGregor S, Muller D (2000), Nutritional supplementation in Down syndrome: theoretical considerations and current status. Dev Med Child Neurol 42:207-213.

ACCEPTED MANUSCRIPT

92. Bennett FC, McClelland S, Kriegsmann EA, Andrus LB, Sells CJ (1983) Vitamin and mineral supplementation in Down's syndrome. Pediatrics 72:707-713.

93. Ellis JM, Tan HK, Gilbert RE, Muller DP, Henley W, Moy R, Pumphrey R, Ani C, Davies S, Edwards V, Green H, Salt A, Logan S (2008), Supplementation with antioxidants and folinic acid for children with Down's syndrome: randomised controlled trial. BMJ 336:594-597.

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7. Figure legends

Fig. 1 Latency (in seconds) to reach the platform during the 8 acquisition sessions (A) and during the 4 cued sessions (B) in the MWM after chronic administration of melatonin or vehicle during the pre- and post-natal period in TS and CO mice. Data are expressed as the means ± SEM. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ TS vs. CO; ##: $p<0.01$ vehicle-treated vs. melatonin-treated mice. Bonferroni tests were performed after significant MANOVAs.

Fig. 2 Means ± S.E.M. of the percentage of time spent freezing (A) and of the freezing latency (B, time spent from the start of the test until the first freezing episode) during the second (cued conditioning) and third (context conditioning) sessions in the CFC test for melatonin and vehicle treated-TS and CO mice. ** $p<0.01$; TS vs. CO. Bonferroni tests were performed after significant MANOVAs.

Fig. 3. Fall latency (in seconds) on the rotarod test (A) at different constant speeds (5, 20 and 40 r.p.m.) and during the acceleration cycle for CO and TS mice treated with either vehicle or melatonin. Data are expressed as the means ± SEM.

Fig. 4. Spontaneous locomotor activity. Means ± SEM of the total home cage count activity performed over a 24-h period by CO and TS mice treated with vehicle or melatonin.

Fig. 5. Number of crossings (A, horizontal activity) and rearings (B, vertical activity) performed by CO and TS mice treated with vehicle or melatonin during the pre- and post-natal periods in the open field test. Data shown are the means ± SEM. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ TS vs. CO. Bonferroni tests were performed after significant MANOVAs.

Fig. 6. Means ± SEM of the scores obtained for the CO and TS mice treated with vehicle or melatonin during the pre- and post-natal period in the plus maze test. (A) Number of arm entries, (B) the time spent in the open arms of the apparatus, (C) freezing behavior (in seconds) and (D) number of risk-associated behaviors (SAP: stretch attend posture; HD: head dipping). *$p<0.05$ TS vs. CO. Bonferroni tests were performed after significant MANOVAs.

Fig. 7. (A) Representative images of DAPI (upper row), Ki67 (second row) and co-immunostaining of Ki67 and DAPI (third row) in the DG of TS and CO mice treated with vehicle or melatonin during the pre- and post-natal periods. (B) Means ± S.E.M. of the density of Ki67+ cells in the SGZ and (C) of mature granule cells in the GCL of the vehicle- or melatonin- treated TS

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and CO mice. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ TS vs. CO. Bonferroni tests were performed after significant MANOVAs.

Fig. 8. Means ± S.E.M. of the levels of PC (A) and TBARS (B) in the cortex and hippocampus of TS and CO mice treated with melatonin or vehicle. *$p<0.05$, TS vs. CO. Bonferroni tests were performed after significant MANOVAs.

Fig. 9. Means ± S.E.M. of the activity levels of different antioxidant enzymes in the hippocampus and cortex of TS and CO mice treated with melatonin or vehicle. Superoxide dismutase (SOD, A), catalase (CAT, B), glutathione peroxidase (GPx, C), glutathione reductase (GR, D) and glutathione-S-transferase (GST). Mean ± SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$ TS vs. CO; #: $p<0.05$ vehicle-treated vs. melatonin-treated mice. Bonferroni tests were performed after significant MANOVAs.

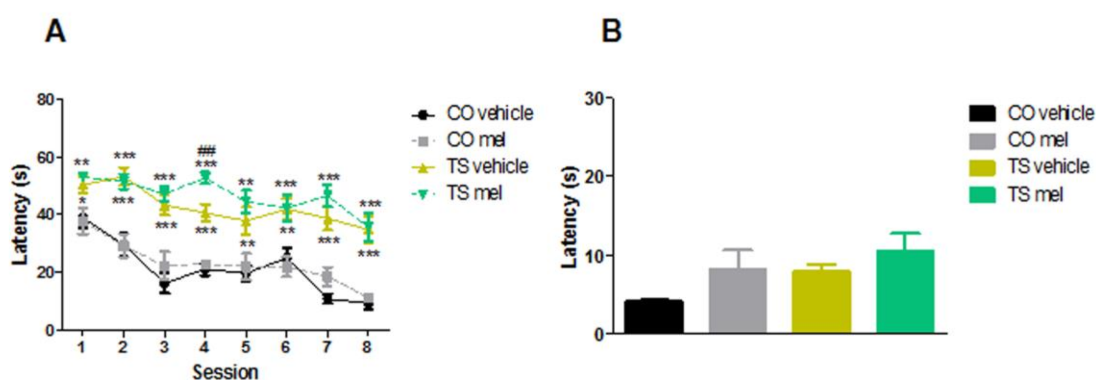

Figure 1

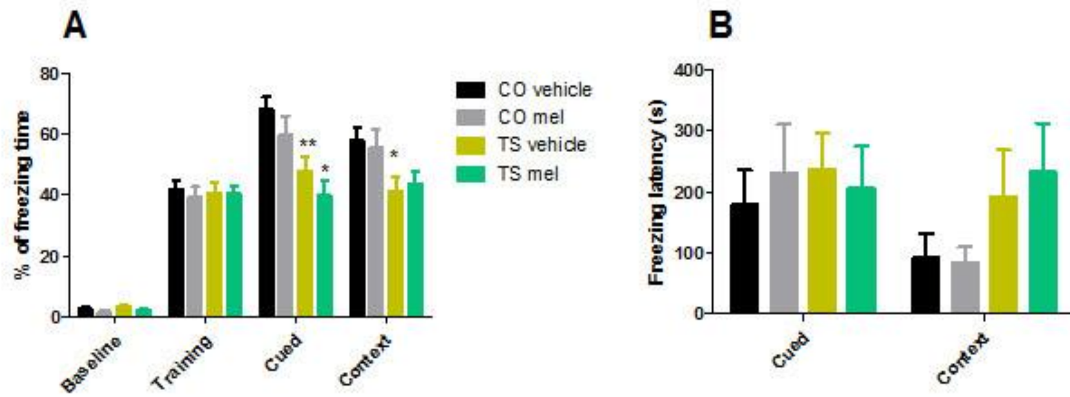

Figure 2

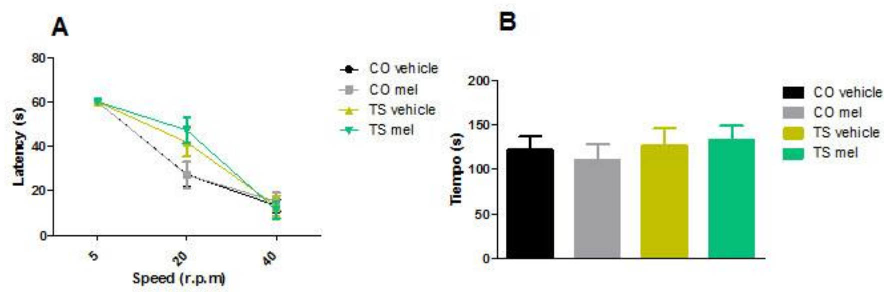

Figure 3

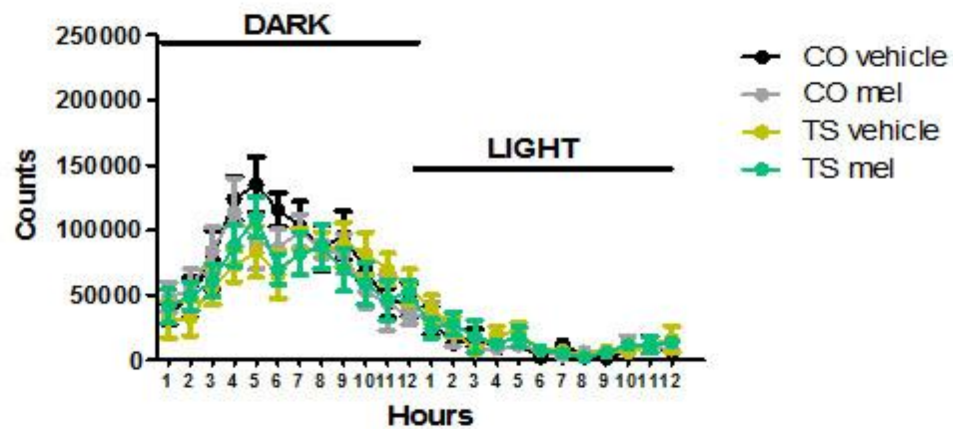

Figure 4

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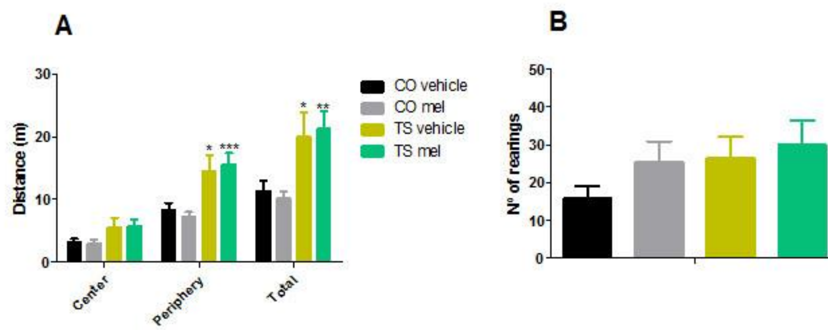

Figure 5

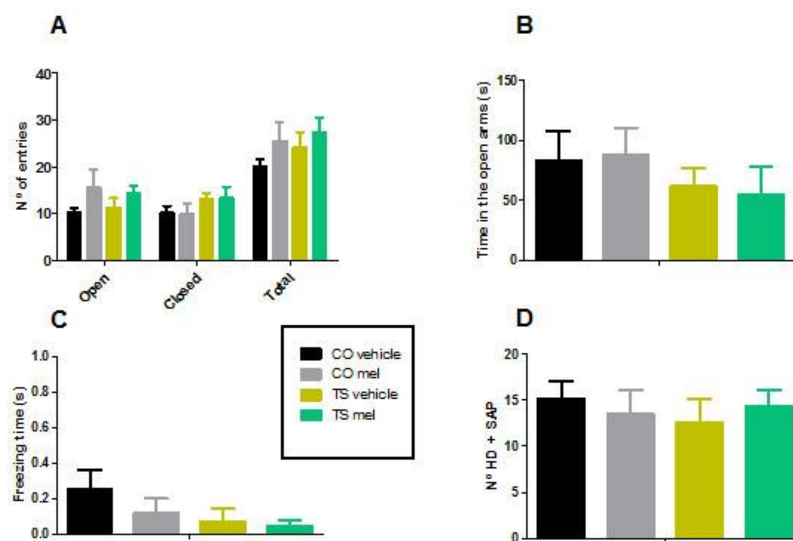

Figure 6

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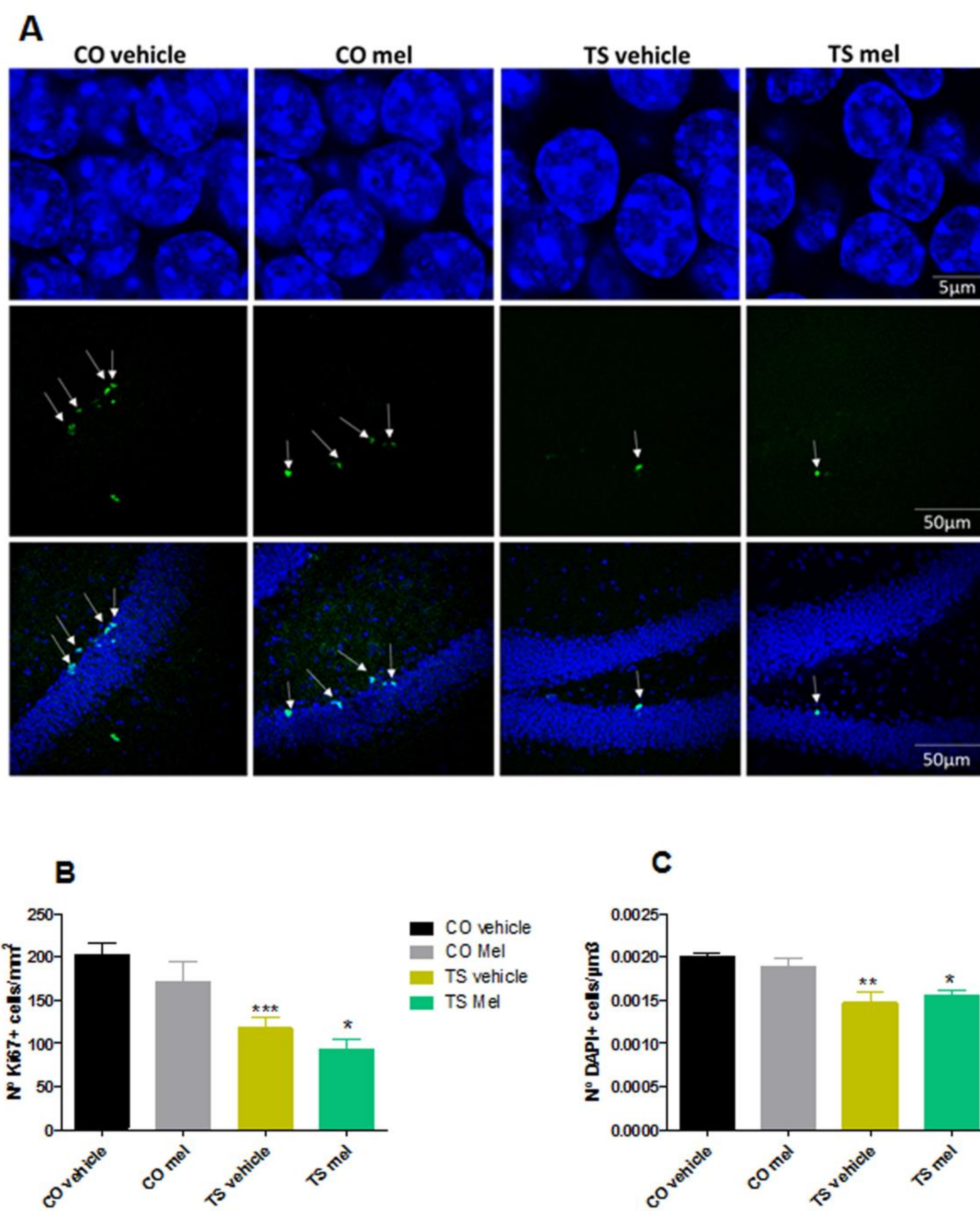

Figure 7

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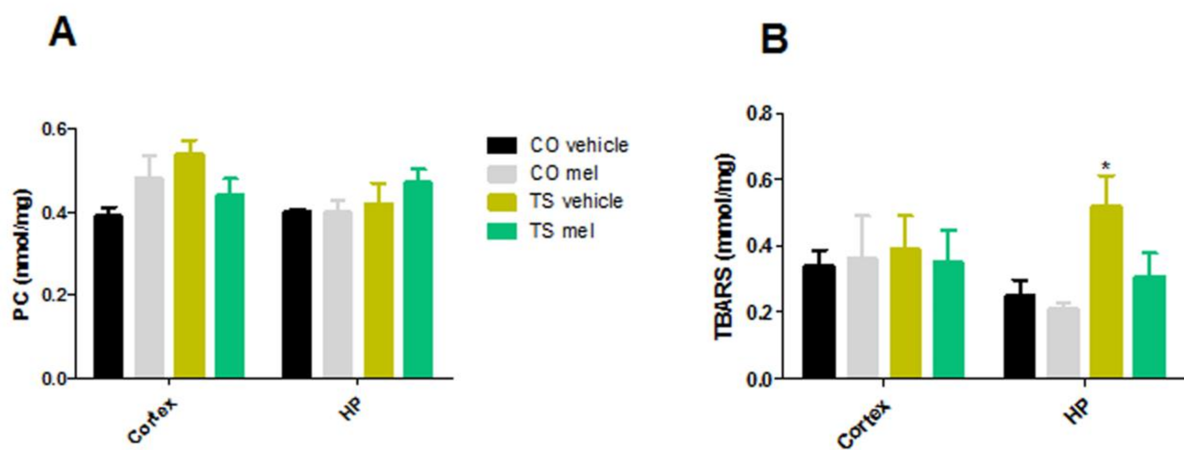

Figure 8

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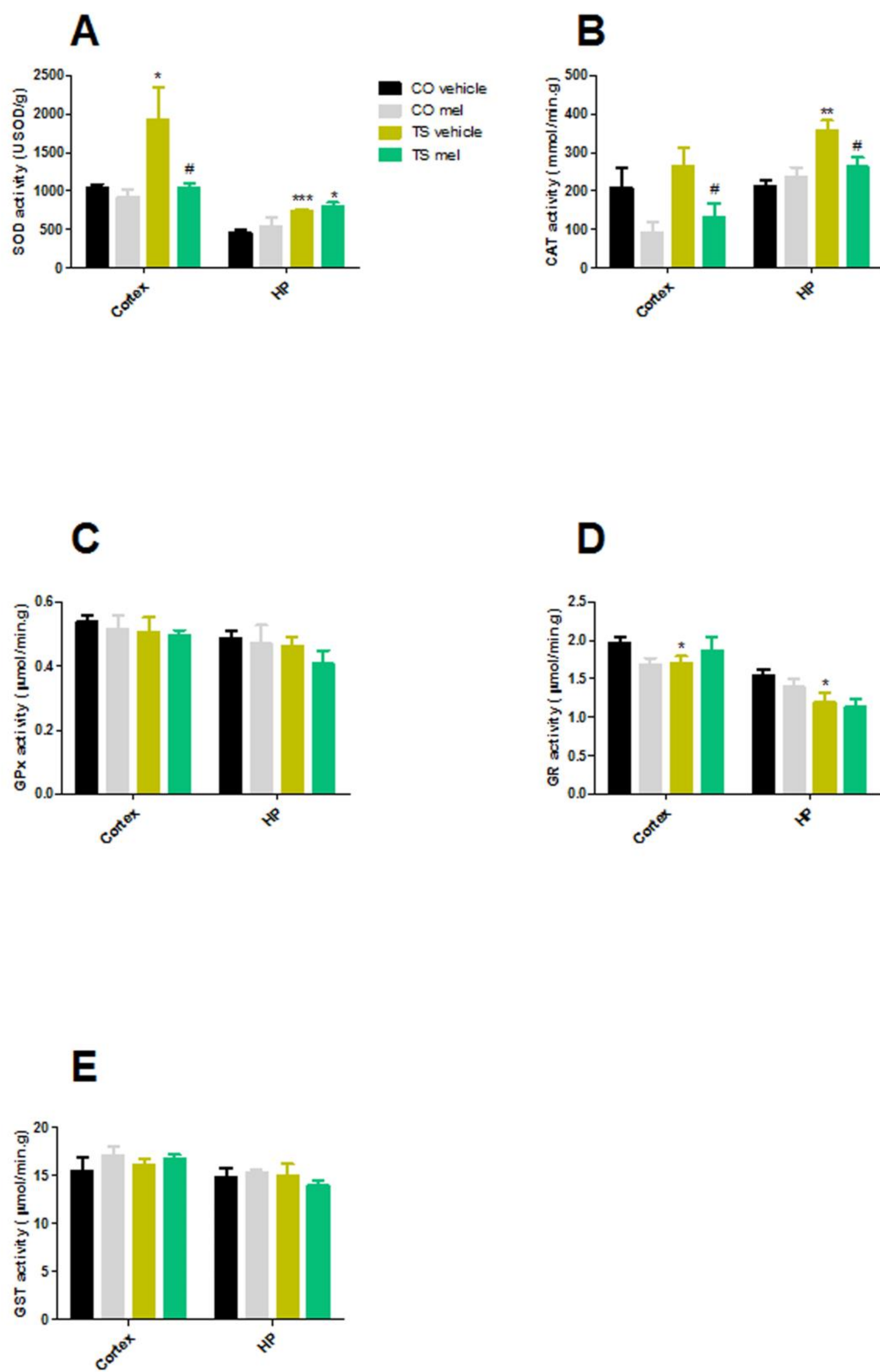

Figure 9

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Table 1: Plasma melatonin concentration in TS and CO pups treated with either melatonin or vehicle. Data are expressed as the means ± S.E.M. ##p<0.01; ###p<0.001 vehicle-treated vs. melatonin-treated mice. Bonferroni tests were performed after significant MANOVAs.

	TREATMENT				**STATISTICS**		
	VEHICLE		**MELATONIN**		**MANOVA $F_{(1,21)}$**		
Plasma melatonin	**CO**	**TS**	**CO**	**TS**	**Genotype**	**Treatment**	**Genotype x treatment**
pg/ml (light)	Under kit detection range (<12 pg/ml)	Under kit detection range (<12 pg/ml)	87.69 ± 16.12	105.04 ± 16.68	-	**48.26, p<0.001**	1.38, p=0.25
pg/ml (dark)	26.82 ± 8.10	31.83 ± 9.19	130.96## ± 26.70	143.47### ± 12.87	0.31, p=0.58	**47.51, p<0.001**	0.05, p=0.81

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Table 2. Sensorimotor tests. Scores are presented as the means ± SEM obtained from TS and CO mice after vehicle or melatonin treatment during pre- and post-natal periods.

		TREATMENT				STATISTICS		
		VEHICLE		MELATONIN		MANOVA $F_{(1,49)}$		
		CO	TS	CO	TS	Genotype	Treatment	Genotype x treatment
Vision		2.42 ± 0.17	2.50 ± 0.19	2.50 ± 0.20	2.27 ± 0.19	F=0.163 p=0.68	F=0.16, p=0.68	F=0.59, p=0.44
Auditory startle		1.28 ± 0.16	1.08 ± 0.08	1.07 ± 0.07	1.36 ± 0.20	F=0.10, p=0.74	F=0.05, p=0.81	F=3.22, p=0.07
Vibrissa placing reflex		0.00 ± 0.00	0.00 ±0.00	0.07 ± 0.07	0.00 ± 0.00	F=0.81, p=0.37	F=0,81, p=0.37	F=0.81, p=0.37
Righting reflex		3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00	3.00 ± 0.00			
Grip strength		3.00 ± 0.14	2.75 ± 0.13	3.00 ± 0.10	3.00 ± 0.19	F=0.75, p=0.38	F=0.75, p=0.38	F=0,75, p=0.38
Equilibrium	**Wooden rod**	2.07 ± 0.16	2.16 ± 0.16	2.57 ± 0.25	2.09 ± 0.09	F=1.05 p=0.31	F=1.27, p=0.26	F=2.34, p=0.13
	Aluminum rod	1.14 ± 0.49	0.50 ± 0.23	1.64 ± 0.58	0.90 ± 0.54	F=1,91, p=0.17	F=0.83, p=0.36	F=0.08, p=0.92
Latency to fall wooden rod		19.60± 0.39	20.00± 0.00	20.00± 0.00	20.00 ± 0.00	F=0.81, p=0.37	F=0.81, p=0.37	F=0.81, p=0.37
Latency to fall aluminum rod		7.42 ± 1.77	6.58 ± 1.69	11.39 ± 1.86	8.04 ±2.21	F=1.22, p=0.27	F=2.04, p=0.15	F=0.43, p=0.51
Prehensile reflex		2.28 ± 0.28	2.50 ± 0.23	2.85 ± 0.09	2,36 ± 0.27	F=0.36, p=0.55	F=0,87, p=0.35	F=2.31, p=0.13
Traction capacity		2.92 ± 0.70	2.50 ± 0.66	1.21 ± 0.45	2.451 ± 0.74	F=0.39 p=0.53	F=1.84, p=0.18	F=1.65, p=0.20
Latency to fall coat hanger		42.28 ± 6.81	43.75 ± 6.2	37.14 ± 5.96	37.18 ± 7.51	F=0.01, p=0.91	F=0.77, p=0.38	F=0.01, p=0.91
Nº crossings coat hanger		4.28 ± 0.89	3.83 ± 0.57	4.50 ± 1.03	3.45 ± 0.81	F=0.73, p=0.39	F=0.009, p=0.92	F=0.11, p=0.73
Latency of arrival coat hanger		35,71 ± 5.46	32.41 ± 6.05	30.85 ± 5.79	40.90 ± 6.09	F=0.33, p=0.56	F=0.096, p=0.75	F=1.29, p=0.26

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Table 3. Mean scores ± SEM of melatonin- and vehicle-treated TS and CO mice during the pre- and post-natal periods in the hole board test. #: p<0.05 vehicle-treated vs. melatonin-treated mice. Bonferroni tests were performed after significant MANOVAs.

	TREATMENT				**STATISTICS**		
	Vehicle		**Melatonin**		**MANOVA $F_{(1,49)}$**		
	TS	**CO**	**TS**	**CO**	**Genotype**	**Treatment**	**Genotype x treatment**
Distance	5.88 ± 0.57	7.46 ± 0.92	8.16 ± 1.02	8.36 ± 1.20	F=0.64, p=0.42	F=2.85, p=0.09	F=0.36, p=0.54
Rearings	8.00 ± 2.54	9.85 ± 2.15	11.5 ± 2.84	14.71 ± 2.91	F=0.91, p=0.34	F=2.47, p=0.12	F=0.06, p=0.79
Number of explorations	29.25 ± 3.77	20.14 ± 1.80	18.40# ± 3.09	15.21 ± 1.48	**F=5.76, p≤0.05**	**F=9.49, p≤0.01**	F=1.33, p=0.25
Time exploring holes	32.43 ± 6.36	33.21 ± 5.70	14.40 ± 3.09	24.05 ± 4.35	F=0.98, p=0.32	**F=6.69, p≤0.01**	F=0.71, p=0.40
ABA index	7.08 ± 0.94	5.42 ± 0.64	3.90# ± 0.83	3.28 ± 0.64	F=2.18, p=0.14	**F=12.04, p≤0.001**	F=0.46, p=0.50
ABA/number of explorations	0.26 ± 0.02	0.28 ± 0.03	0.19 ± 0.02	0.21 ± 0.04	F=0.33, p=0.56	F=3.54, p=0.06	F=0.01, p=0.89

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Table 4. *F* values of MANOVA (genotype x treatment) of each variable tested in the open field and plus maze tests.

	MANOVA $F_{(1,49)}$		
	Genotype	**Treatment**	**Genotype x treatment**
Open field			
Distance center	**F= 5.54; p≤ 0.05**	F= 0.004; p=0.95	F= 0.038; p= 0.84
Distance periphery	**F=17.29; p≤0.001**	F=0.016; p=0.90	F=0.023; p=0.88
Total distance	**F=13.71; p≤0.001**	F= 0.012; p=0.91	F=0.001; p=0.97
Rearings	F= 1.55; p=0.21	F= 1.51; p= 0.22	F= 1.88; p=0.17
Plus maze			
Number of entries			
Open	F = 0.00; p= 0.99	F= 2.72; p= 0.10	F= 0.26; p= 0.99
Closed	F= 2.75; p= 0.10	F= 0.00; p= 0.12	F= 0.002; p= 0.96
Total entries	F= 1.06; p= 0.30	F= 1.85; p= 0.18	F= 0.15; p= 0.69
Time open arms	F= 1.99; p= 0.16	F= 0.004; p= 0.95	F= 0.096; p= 0.75
Freezing time	F= 1.81; p= 0.18	F= 0.79; p= 0.37	F= 0.22; p= 0.63
HD + SAP	F= 0.07; p= 0,79	F= 0.06; p= 0,93	F= 0.41; p= 0.52

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Table 5. *F* values of MANOVA (genotype x treatment) for oxidative stress markers and antioxidant enzymatic activities in the cortex and hippocampus of the four groups of animals.

	MANOVA $F_{(1,21)}$					
	CORTEX			***HIPPOCAMPUS***		
	Genotype	***Treatment***	***Genotype x Treatment***	***Genotype***	***Treatment***	***Genotype x Treatment***
PC level	F=1.74, p=0.20	F=0.00, p=0.98	**F=4.72, p≤0.05**	F=1.41, p=0.24	F=0.42, p=0.52	F=0.38, p=0.54
TBARS level	F=0.13, p=0.71	F=0.001, p=0.97	F=0.18, p=0.67	**F=6.12, p≤0.05**	F=2.93, p=0.10	F=1.43, p=0.24
SOD activity	**F=6.79, p≤0.05**	**F=6.33, p≤0.05**	F=3.60, p=0.99	**F=23.26, p≤0.001**	F=2.14, p=0.16	F=0.04, p=0.82
CAT activity	F=0.77, p=0.39	**F=6.98, p≤0.05**	F=0.00, p=0.67	**F=12.16, p≤0.01**	F=1.64, p=0.21	**F=4.66, p≤0.05**
GPx activity	F=0.56, p=0.46	F=0.23, p=0.63	F=0.04, p=0.82	F=0.84, p=0.37	F=0.52, p=0.47	F=0.33, p=0.56
GR activity	F=0.11, p=0.74	F=0.20, p=0.65	F=3.04, p=0.09	**F=6.76, p≤0.05**	F=0.81, p=0.37	F=0.07, p=0.79
GST activity	F=0.008, p=0.93	F=1.79, p=0.19	F=0.27, p=0.61	F=1.17, p=0.29	F=0.12, p=0.72	F=1.72, p=0.20