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Title:

Melatonin plus physical exercise are highly neuroprotective in the 3xTg-AD mouse

Authors:

Yoelvis García-Mesa[a], Lydia Giménez-Llort[b], Luis C López[c], Carmen Venegas[c], Rosa Cristòfol[a], Germain Escames[c], Darío Acuña-Castroviejo[c], Coral Sanfeliu[a,*]

Affiliations:

a. Institute of Biomedical Research of Barcelona (IIBB), CSIC, IDIBAPS, E-08036 Barcelona, Spain.

b. Institute of Neuroscience and Medical Psychology Unit, Department of Psychiatry and Forensic Medicine, Autonomous University of Barcelona, E-08193 Bellaterra, Barcelona, Spain.

c. Instituto de Biotecnología, Departamento de Fisiología, Centro de Investigación Biomédica, Parque Tecnológico de Ciencias de la Salud, Universidad de Granada, E-18100 Armilla, Granada, Spain.

Corresponding author:

Coral Sanfeliu, PhD.

IIBB-CSIC, IDIBAPS,

Rosselló 161, 6th floor,

08036 Barcelona, Spain.

E-mail: coral.sanfeliu@iibb.csic.es

Tel: +34 93 3638338

FAX: +34 93 3638301

Abstract

Alzheimer's disease (AD) is a devastating age-related neurodegenerative disease with no specific treatment at present. Several healthy lifestyle options and over-the-counter drugs that it has been suggested delay the onset of the disease are in an experimental phase, but it is unclear whether they will have any therapeutic value against AD. We assayed physical exercise and melatonin in 3xTg-AD male mice aged from 6 to 12 months, therefore from moderate to advanced phases of AD pathology. Analysis of behavior and brain tissue at termination showed differential patterns of neuroprotection for the two treatments. Both treatments decreased soluble amyloid β oligomers, whereas only melatonin decreased hyperphosphorylated tau. Melatonin was effective against the immunosenescence that 3xTg-AD mice present. Voluntary physical exercise protected against behavioral and psychological symptoms of dementia such as anxiety, a lack of exploration and emotionality. Both treatments protected against cognitive impairment, brain oxidative stress and a decrease in mtDNA. Interestingly, only the combined treatment of physical exercise plus melatonin was effective against the decrease of mitochondrial complexes. Therefore, melatonin plus physical exercise may exert complementary, additive or even synergistic effects against a range of disturbances present in AD.

Keywords: Alzheimer's disease; mitochondrial aging; 3xTg-AD mice; melatonin; physical exercise.

Introduction

Healthy lifestyle options such as physical exercise, caloric restriction and a diet rich in antioxidant food increase physical reserve and delay symptoms of aging and hence they may protect against age-associated diseases (Joseph et al., 2009; Smith et al., 2010; Middleton and Yaffe, 2010; Daffner et al., 2010). The health-promoting lifestyle intervention known as the most effective to date is aerobic exercise (Warburton et al., 2006). According to several studies in people, chronic physical exercise helps maintain cognitive function in older age and reduces the risk of cognitive decline and Alzheimer's disease (AD) (Angevaren et al., 2008, Lautenschlager et a., 2008; Taaffe et al., 2008; Barnes et al., 2008; Geda et al., 2010). In individuals with established cognitive impairment, a physical exercise program induces positive mood and behavior, but the amelioration of cognitive function is modest (Heyn et al., 2004; Deslandes et al., 2009). More studies are required to establish conditions for overcoming physiological brain deterioration in AD patients. Physical exercise has been tested against AD pathology in several AD mouse models and has resulted in beneficial effects at moderate stages of pathology (Adlar et al., 2005; Pietropaolo et al., 2008; Um et al., 2008; Yuede et al., 2009; Giménez-Llort et al., 2010). At advanced AD stages, promising results of higher cognitive performance after running wheel exercise have been reported in the aged Tg2576 mouse (Nichol et al., 2007; Parachikova et al., 2008) but not in the aged APP23 mouse (Wolf et al., 2006). In a previous study with the 3xTg-AD mouse model (Oddo et al., 2003) we demonstrated that chronic running wheel starting at 1 month of age greatly improves behavior and cognition tested at 7 months of age (García-Mesa et al., 2011). Therefore, 6 months of voluntary physical exercise significantly ameliorated nerve cell and brain tissue functionality throughout early and moderate stages of AD pathology. One of the observed benefits was an amelioration of the cerebral oxidative stress of 3xTg-AD mice (Resende et al., 2008; Giménez-Llort et al., 2010; García-Mesa et al., 2011). However, exercise induction of antioxidant defense (Radak et al., 2008) may not be enough to protect highly oxidized brain tissue at advanced AD stages. Similarly, specific neuroprotective mechanisms triggered by physical exercise such as secretion of neurotrophic factors and strengthening of synaptic function (Cotman and Berthold, 2002; Cotman et al., 2007; García-Mesa et al., 2011) may not be enough to recover from advanced brain damage.

Treatment with the hormone melatonin has also been reported to be neuroprotective in AD mouse models (Matsubara et al., 2003; Feng et al., 2004; Olcese et al., 2009; Spuch et al., 2010). Melatonin is a pleiotropic molecule, believed to have anti-aging activity because of its powerful effects as an antioxidant, anti-inflammatory and enhancer of mitochondrial activity (Carretero et al., 2009; Acuña-Castroviejo et al., 2011; Hardeland et al., 2011). In humans, melatonin levels decrease with normal aging and even more so in the presence of neurodegenerative diseases (Kunz

et al., 1999; Liu et al., 1999; Zhou et al., 2003). Treatment of AD patients with melatonin to regulate their circadian rhythm has suggested a possible therapeutic action beyond wakefulness and sleep quality (Srivasan et al., 2005; Furio et al., 2007; Cardinale et al., 2010).

Combined healthy lifestyle options have an additive effect in the decrease of human mortality rates and induce a commensurate decrease of biological age (Khaw et al., 2008). Therefore, two anti-aging therapies with promising effects against AD risk, such as physical exercise and melatonin, may be effective against AD-related neurodegeneration on simultaneous administration. Thus, the aim of this study was to test for additive effects of melatonin with physical exercise against advanced AD neurodegeneration, with emphasis on mitochondrial status. We chronically treated 3xTg-AD mice with either melatonin, voluntary exercise or both from 6 to 12 months of age. The study was initiated at a stage of already overt AD pathology and cognitive loss (Oddo et al., 2003; Giménez-Llort et al., 2007; García-Mesa et al., 2011) that progressed to advanced brain damage in the untreated 3xTg-AD mice at the termination stage (Oddo et al., 2003; Giménez-Llort et al., 2007).

1. Materials and Methods

1.1. *Animals*

The 3xTg-AD mouse strain harboring familial AD mutations PS1/M146V, APPSwe and tauP301L, was genetically engineered at the University of California Irvine (Oddo et al., 2003). Male 3xTg-AD mice from the Spanish colony of homozygous mice, established in the Medical Psychology Unit, Autonomous University of Barcelona (Giménez-Llort et al., 2006), were used in the present study. The non-transgenic (NTg) mouse colony had the same genetic background hybrid (129 x C57BL6) as 3xTg-AD. Genotypes were confirmed by PCR analysis of DNA obtained from tail biopsies. Animals were maintained in Macrolon cages under standard laboratory conditions of food and water *ad libitum*, 22 ± 2 ºC and 12 h light / 12 h dark cycle (lights were turned on at 9:00 h. and turned off at 21:00 h. local time).

The exercise and melatonin treatments, general behavioral studies and necropsies were performed at the facilities of the animal unit of the University of Barcelona, under approval from the local animal experimentation ethics committee (CEEA, UB). All the studies were performed in accordance with Spanish legislation concerning the protection of animals used for experimental and other scientific purposes and the European Communities Council Directive (86/609/EEC) on this subject.

1.2. Treatments

Six-month old 3xTg-AD mice were divided into four different treatment groups (n = 8-10 per group), as follows: (i) Tg, 3xTg-AD mice control group; (ii) Tg-M, 3xTg-AD mice that received melatonin in their drinking water; (iii) Tg-E, 3xTg-AD mice subjected to physical exercise; and (iv) Tg-ME, 3xTg-AD mice that both received melatonin and were subjected to exercise. A fith group of 6-month old NTg mice was added as an NTg control. The mice were housed 4-5 animals per cage, 2 cages per group.

Melatonin (Sigma-Aldrich, Madrid, Spain) was dissolved in a minimum volume of absolute ethanol in bottles protected from light and added to the drinking water at a concentration that would yield a daily dose of 10 mg/kg b.w. (average per mouse for each cage). The initial concentration of melatonin was established in a preliminary study of water consumption per mouse. Thereafter, bottles were changed twice a week. The mice were weighed, and their weight and the amount of water consumed in each cage were recorded. These date were used to adjust the concentration of melatonin whenever necessary. The concentration of ethanol in the final solution was 0.066%. The control groups received this vehicle in the drinking water. Physical exercise was implemented by free access to one running wheel present in the housing cage (Activity Wheel Cage System for Mice, Techniplast, Buguggiate, Italy), as described previously (García-Mesa, et al., 2011). The average running distance (Km) per mouse for each cage was calculated from the total counts for each wheel per week.

Treatments were administrated chronically for 6 months. At treatment termination, the mice were 12 months old and were evaluated for physical condition, behavior and cognition, and killed for tissue analysis two weeks later.

1.3. *Sensorimotor function*

The body weight of the mice was measured at the beginning and end of the treatments period.

Their sensorimotor responses were evaluated by a standard task battery as previously described (García-Mesa, et al., 2011). Briefly, reflexes (visual reflex and posterior legs extension reflex tests) were measured as the limb extension response after holding the animal by its tail and slowly lowering it towards a black surface. Motor coordination and equilibrium were assessed by means of distance covered and the latency to fall off a horizontal wooden rod and metal wire. Prehensility and motor coordination were measured as the distance covered on the wire hang test. Muscle strength was measured as the time taken to fall off the wire. All the apparatus was suspended 80 cm above a padded table.

1.4. Behavioral and cognition profile

Behavioral and cognitive responses were evaluated by a variety of widely used tests, as previously described (García-Mesa et al, 2011). Briefly, the *corner test* was used to assess neophobia to a new home-cage by measuring the number of corners visited and rearings during a period of 30 s, and the latency of the first rearing. The *open field test* was utilized to assess the horizontal and vertical locomotor activity by counting crossings and rearings, respectively. The latency of initial movement, the self-grooming behavior and the number of time the mice urinated and defecated were also registered to assess response to a relatively low stress environment. The *dark and light box test* was used to measure anxiousness of behavior. The mice were introduced into the black compartment and observed for 5 min. The latency of enter into the lit compartment, the time spent in the lit compartment and the horizontal (crossings) and vertical (rearings) activity that took place once there were recorded. The numbers of times the mice urinated and defecated were also recorded. *Boissier's four hole-board test* was used to asses exploratory behavior by measuring the number of head-dips and time spent head-dipping at each of the four holes. The latencies of movement, first dipping and four hole dipping, and the number of times the mice urinated and defecated were also recorded. The *Morris water maze (MWM) test* was used to test spatial learning and memory; it consisted of one day of cue learning of a visual platform and six days of place learning for spatial reference memory (four trial sessions per day). On day 7, after one trial of place learning, the platform was removed from the maze and the mice performed a probe trial. A computerized tracking system (SMART, Panlab S.A., Barcelona, Spain) allowed the escape latency during the learning tasks to be measured, along with the time spent in each quadrant of the pool after the removal of the platform in the probe trial. All behavioral testing was performed during the first hours of the light period, from 9:00 h. to 14:00 h. local time).

1.5. *Tissue samples*

After completion of all behavioral and cognition tests, animals were decapitated and immediately the brain was dissected on ice to obtain the cerebral cortex and hippocampus. Brain tissue samples were stored at −80ºC for further analysis. Animals were killed on two successive days between 10:00 h. and 13:00 h. local time (half the number of each group per day).

The weight of intraabdominal white adipose tissue (WAT), brown adipose tissue (BAT) and thymus was recorded and relative weight were calculated as the percentage of total body weight of each dissected tissue.

1.6. Lipid peroxidation, glutathione peroxidase, and superoxide dismutase assays

For lipid peroxidation and enzymatic assays, 100 mg of cerebral cortex tissue was sonicated for 30 s in 1 ml of ice-cold 50 mM potassium phosphate buffer containing 1 mM EDTA

pH 7.4 and then centrifuged at 12,000 x g for 30 min at 4 ºC. The supernatants were collected and stored at -80 ºC until assayed. Enzyme activities and lipid peroxidation were determined as described previously (Sebastià et al., 2004). Lipid peroxidation was measured using a Lipid Peroxidation Assay Kit from Calbiochem (EMD Biosciences Inc., Darmstadt, Germany). Glutathione peroxidase (GPx) activity was determined by spectrophotometrically measuring the rate of β-nicotinamide adenine dinucleotide phosphate (NADPH) oxidation in the presence of hydrogen peroxide. Total superoxide dismutase (SOD) activity was measured using the Ransod SOD assay kit (Randox Laboratories Ltd, Crumlin, UK). After total SOD (Cu/Zn SOD and Mn SOD) was determined, the samples were again analyzed in the presence of 500 µM KCN to inhibit CuZn SOD and to determine Mn SOD activity. Proteins were measured in 10 µl of supernatants following the Bradford method.

1.7. Reduced glutathione and oxidized glutathione assays

For reduced glutathione (GSH) and oxidized glutathione disulfide (GSSG) assays, 200 mg of cerebral cortical tissue was sonicated in 1 ml of 3.3% sulfosalicylic acid. Acid homogenates were centrifuged at 12,000 g for 30 min at 4 ºC and supernatant fractions were collected and stored at -80 ºC until assayed. The levels of GSH and GSSG were determined as described previously (García-Mesa et al., 2011) using an enzymatic assay that is essentially a modification of Tietze's recycling method. Briefly, samples for GSSG determination were first incubated at room temperature with 2-vinyl pyridine in order to conjugate any GSH present in the sample, so that only GSSG was recycled to GSH. For total glutathione determination, each sample was mixed with phosphate buffer containing 1 mM dithiobisnitrobenzoate, 20 U/ml glutathione reductase, and 1 mM NADPH. The kinetics of the formation of 5-thio-2-nitrobenzoic acid was immediately recorded at 30ºC and 405 nm, every 15 s over a 5-min period.

1.8. Mitochondrial DNA quantification

Mouse mitochondrial DNA (mtDNA) was quantified in hippocampus samples by real-time PCR using a Stratagene Mx3005P^{TM} Real-Time PCR system (Agilent Technologies, Inc., CA, USA) as described previously (Spinazzola et al., 2006; López et al., 2009), using primers and probes for murine COXI gene (mtDNA) and mouse glyceraldehyde-3-phosphate dehydrogenase (nuclear DNA, nDNA) (Spinazzola et al., 2006; López et al., 2009). The values of mtDNA levels were normalized by nDNA, and the data are expressed in terms of percentage relative to NTg mice.

1.9. Western blotting

Protein extracts from hippocampi were obtained in 50 mM Tris/HCl (pH 7.6), 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 1 mM DTT and 10 μg/ml aprotinin. Fifteen μg of the hippocampus protein extracts were electrophorezed in a PhastGel™ Homogeneous12.5 using a PhastSystem instrument (GE Healthcare Europe GmbH, Spain). The proteins were transferred to an Amersham Hybond™ ECL™ nitrocellulose membrane (GE Healthcare Europe GmbH, Spain) and probed with a Rodent Total OXPHOS Complexes Detection Kit cocktail of antibodies supplemented with extra complex II (CII) subunit 30 kDa monoclonal antibody (MitoSciences, Eugene, OR, USA) (López et al., 2009). Protein–antibody interactions were detected with peroxidase-conjugated horse anti-mouse IgG antibody (BD Biosciences, Spain), using Western Lightning™ *Plus*-ECL detection kits (PerkinElmer, Spain). Bands quantification was carried out using an Image Station 2000R (Kodak, Spain) and Kodak 1D 3.6 software. Quantitative values of the bands corresponding to ATP synthase subunit α (CVα, complex V), ubiquinol-cytochrome-c reductase complex core protein 2 (Core2, complex III), cytochrome c oxidase I (COXI, complex IV) and NADH-ubiquinone oxidoreductase chain 6 (ND6, complex I) were normalized by complex II subunit 30 kDa (CII), and the data are expressed in terms of percentage relative to wild-type mice.

Protein extracts from cerebral cortices were similarly processed using PVDF membranes (Immobilon-P, Millipore, Billerica, MA). Membranes with soluble protein were probed with anti-amyloid-β (1:1,000, clone 6E10, 4 kDa, Signet, Emerville, CA) and anti-phospho-tau (1:1,000, clone AT8 (Ser202), 55 kDa, Pierce, Rockford, IL). These cerebral cortical blots were normalized to those stained with anti-pan actin (1:10,000, 42 kDa, Sigma) and anti-GAPDH (1:2,000, 36 kDa, Assay Designs, Ann Arbor, MI), respectively.

1.10. Quantification of coenzyme Q_9 and coenzyme Q_{10} levels

Coenzyme Q_9 (CoQ_9) and coenzyme Q_{10} (CoQ_{10}) from the hippocampus were extracted by mixing tissue extracts with 1-propanol. After 2 min vortexing, the solution was centrifuged at 13,000 rpm for 5 min. The resultant supernatant was injected into an HPLC system (Gilson, WI, USA) and the lipid components were separated in a reverse-phase Symmetry C18 3.5 μm, 4.6 x 150 mm column (Waters, Spain), using a mobile phase consisting of methanol, ethanol, 2-propanol, acetic acid (500:500:15:15) and 50 mM sodium acetate at a flow rate of 0.9 ml/min. The electrochemical detector consisted of an ESA Coulochem III with the following settings: Guard cell (upstream of the injector) at +900 mV; and conditioning cell at –600 mV (downstream of the column) followed by the analytical cell at +350 mV. CoQ_9 and CoQ_{10} concentrations were estimated by comparison of the peak areas with those of standard solutions of known concentrations (López et al., 2010). The results are expressed in ng CoQ/mg protein.

1.11. Determination of melatonin brain levels

Steady-state brain melatonin levels were measured in two additional groups of 3xTg-AD mice that were treated with melatonin or vehicle. Mice were housed in the same conditions as those in the main study and given melatonin at a daily dose of 10 mg/kg b.w. (average per mouse for each cage) or the corresponding vehicle. Mice were treated for 3 months and then killed immediately (without behavior testing). The hippocampi (11-17 mg each one) were processed individually. Tissue was sonicated in 400 µl of 0.01 mM phosphate buffer containing 0.15 M NaCl, pH 7.4, and centrifuged at 3,000 g for 10 min at 4ºC. An aliquot of the supernatant was frozen at -80 ºC for protein determination. Another supernatant aliquot (320 µl) was mixed with 1 ml chloroform, shaken for 20 min and centrifuged at 9,000 g for 10 min at 4ºC. The organic phase was washed twice with 0.05 M carbonate buffer, pH 10.25. Five hundred µl of this mixture was evaporated to dryness in a SPD2010 SpeedVac System (Thermo Scientific, Asheville, NC, USA). The residue was dissolved in 100 µl of an HPLC mobile phase for processing.

The melatonin content of the extracted samples was measured by HPLC (Shimadzu, Shimadzu Corporation, Duisburg, Germany) with a 4.6 x 150 mm reverse-phase C18 Sunfire Column (Waters Corporation, Milford, MA, USA). After stabilizing the column with the mobile phase, samples (40 µl) were injected onto the HPLC system. The mobile phase consisted in 0.1 M sodium phosphate, 0.1 mM EDTA, and 25% acetonitrile, pH 5.2, at a flow rate of 1 ml/min. A standard curve for melatonin was constructed with 17.9, 35.9, 71.9, 143.7, and 287.5 pg/ml. 5-fluorotryptamine was used as the internal standard. The fluorescence of the samples was measured with a fluorescence detector (Shimadzu, RF-10A XL, Shimadzu Corporation, Duisburg, Germany), with excitation/emission wavelengths of 285/345 nm, respectively. Melatonin concentration is expressed in pg/mg protein.

1.12. *Statistics*

Results are expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism 4 software. All quantitative results showing a single factor (treatment) were analyzed by one-way ANOVA followed by Newman-Keuls multiple comparison test. This *post hoc* test was used to compare the means of all groups to the NTg mice, those of the Tg-M, Tg-E and Tg-ME groups to the Tg mice, and the Tg-M, Tg-E and Tg-ME groups. Whenever two factors were present (treatment and either: week of running, day of latency acquisition, MWM quadrant, mitochondrial complex or CoQ type) we used two-way ANOVA followed by Bonferroni *post hoc* tests to compare the means. Non-parametric data (reflex test) were compared using the chi-square

and Fisher's exact tests. Student's *t*-test was used where indicated to confirm the reliability of some results (removal test in the Tg-E group and phospho-tau levels in Tg and Tg-E groups).

2. Results

2.1. Melatonin treatment did not change mouse running behavior

There was no decrease of basal melatonin levels in the 3xTg-AD mice (10.59 ± 0.45 pg/mg protein) as compared to the NTg mice (9.51 ± 0.73 pg/mg protein) at 12 months of age. In the middle of the 6-month treatment period (9-month-old mice), the level of melatonin in the hippocampus of the mice dosed with melatonin was 66.09 ± 2.24 pg/mg protein and in the undosed mice it was 10.65 ± 0.65 pg/mg protein. At the time of the final tissue collection (two weeks after the end of the 6-month treatment period) brain melatonin was back to untreated animal levels and therefore it did not interfere with the biochemical tissue analyses.

Melatonin treatment of the Tg-ME mouse group did not change the steady-state level of running activity from that of the undosed Tg-E group (Fig. 1). There was only a slower progression of the Tg-ME group during the first week of training until the plateau was reached as compared to Tg-E group [two-way ANOVA, effect of week factor $F_{(23,432)} = 4.577$, $p < 0.0001$, and effect of week x treatment interaction $F_{(23,432)} = 1.813$, $p = 0.0126$].

2.2. Melatonin and physical exercise differentially improved body fitness and immunoendocrine status

Melatonin, exercise, and the combination of melatonin plus exercise did not change the body weight of the 3xTg-AD mice, as there were no significant differences between any experimental groups (Fig. 2A). However, all three treatments significantly reduced the WAT weight of the 3xTg-AD mice (Fig. 2B) [one-way ANOVA $F_{(4,42)} = 5.644$, $p = 0.001$]. Melatonin treatment was the most effective, with a reduction of WAT weight to values lower than those of the NTg mice. BAT and thymus weight in the Tg group were significantly lower than in the NTg group, while melatonin treatment (Tg-M) protected against this effect (Fig. 2C, D) [$F_{(4,42)} = 3.354$, $p = 0.018$, for BAT weight; $F_{(4,42)} = 4.355$, $p = 0.005$ for thymus weight].

2.3. *Melatonin and physical exercise ameliorated sensorimotor reflexes*

In the sensorimotor characterization (Table 1), the untreated 3xTg-AD mice showed poorer reflex responses than the NTg mice, whereas the reflex responses of the treated groups (Tg-M, Tg-E and Tg-ME) were similar to those of the NTg group [$X^2_4 = 11.051$, $p = 0.026$]. The 3xTg-AD

mice showed better responses in the wooden rod and metal wire tests than the NTg mice, as previously reported for this mouse colony (Giménez-Llort et al., 2010; García-Mesa et al., 2011). Coordination was further improved by exercise.

2.4. *Melatonin and physical exercise differentially improved behavioral exploratory responses*

All the treatments ameliorated the neophobia of the 3xTg-AD mice (Fig. 3A, B) [$F_{(4,54)}$ = 4.683, p = 0.0026, for the number of corners, and $F_{(4,54)}$ = 5.410, p = 0.001 for latency of vertical activity]. The 3xTg-AD mice showed greater latency of movement and lower activity in the open field than the NTg mice (Fig. 3C-F). The treatments did not ameliorate total activity but reduced latency of movement [$F_{(4,54)}$ = 3.032, p = 0.025, for horizontal movement latency; $F_{(4,54)}$ = 4.931, p = 0.0018, for vertical movement latency]. The self-grooming time was significantly increased in the 3xTg-AD mice as compared to the NTg mice, and it was ameliorated by all treatments (Fig. 3G) [$F_{(4,54)}$ = 3.044, p = 0.025]. Also, the high number of urine spots in the Tg group was reduced to the NTg group values by exercise in the Tg-E group and exercise plus melatonin in the Tg-ME group (Fig. 3H) [$F_{(4,54)}$ = 6.564, p = 0.0003].

The anxious behavior of the 3xTg-AD mice in the dark-light box was shown by an increased latency of entry to the lit area (Fig. 4A). This behavior was reduced by exercise, as shown by the decreased latency of the Tg-E and Tg-ME groups down to values similar to those of the NTg group [$F_{(4,54)}$ = 4.405, p = 0.0037]. However, the positive changes in the time of permanence in the lit area were not significant (Fig. 4B).

The results in the hole-board test confirmed the reduced exploratory behavior of the 3xTg-AD mice (Fig. 4C, D). Mice in the Tg group waited longer before entering to the first hole and dipped less number of times their heads than their counterparts of the NTg group. These behavioral changes were improved to the NTg group values by voluntary exercise and exercise plus melatonin treatments [$F_{(5,54)}$ 5.050, p = 0.0016, and $F_{(5,54)}$ 6.211, p = 0.0003, respectively].

In both the later tests, the number of urine spots (as indicative of emotional changes) increased in the Tg group and was reduced to the NTg group levels in the Tg-E and Tg-ME groups (not shown), as also happened in the open field test. No changes in the number of times the mice defecated were registered across the treatment groups (not shown).

2.5. *Melatonin and physical exercise differentially improved learning and memory loss*

The results obtained in the cue learning and the acquisition and retention of spatial learning in the MWM are shown in Fig. 3E,F. All animals were able to reach the platform during the cue learning. However, there were differences between the groups in the distance covered (Fig. 3E, results indicated as CL) [one-way ANOVA, $F_{(4,58)}$ = 8.675, p < 0.0001]. Mice of the Tg-M group

followed shorter pathways than those of the NTg, Tg and Tg-E groups. There were differences between the groups in the 6-day task acquisition curves (Fig. 4E) [two-way ANOVA, effect of day factor $F_{(5,324)} = 30.31$, $p < 0.0001$, treatment factor $F_{(4,324)} = 5.360$, $p < 0.0003$, and day x treatment interaction $F_{(20,324)} = 1.541$, $p < 0.0660$]. The Tg-ME group showed a better acquisition than the groups subjected to either treatment alone did. Final acquisition of the exercised mice in the Tg-E and Tg-M groups was also significantly better than that of the Tg group. In the removal test, the untreated 3xTg-AD mice were not able to locate the right platform quadrant whereas all the treatments improved the spatial retention of learning (Fig. 4F). Melatonin was the best treatment as the Tg-M group response was similar to that of the NTg group [two-way ANOVA, effect of quadrant factor $F_{(1,94)} = 89.33$, $p < 0.0001$, and quadrant x treatment interaction $F_{(4,94)} = 9.094$, $p< 0.0001$], whereas the improvement due to exercise alone only reached statistical significance by Student's *t*-test [Tg-E group, Platform vs. Opposed quadrant, $p = 0.01$].

2.6. *Melatonin and physical exercise differentially reduced brain pathology*

Levels of soluble amyloid-β oligomers and phospho-tau in the cerebral cortex tissue of 3xTg-AD mice indicated significant amyloid and tau pathologies (Fig. 5A, B). All the treatments reduced the levels of a distinct oligomer band of approximately 24 kDa which might correspond to hexameric assemblies of amyloid-β [$F_{(4,16)} = 4.150$, $p < 0.0245$] (Fig. 5A). Levels of tau with abnormal phosphorylation at Ser202 were reduced by some of the treatments [$F_{(4,27)} = 2.906$, $p < 0.0384$] (Fig. 5B). Melatonin and melatonin plus exercise induced a clear tendency to decrease the levels of phospho-tau, but the multiple comparison *post-hoc* test did not reveal statistical significance, possibly due to the dispersion of the data. Student's *t*-test showed a significant increase of phospho-tau only in the untreated 3xTg-AD mice and those submitted to physical exercise alone [Tg and Tg-E groups vs. NTg group, $p = 0.01$].

2.7. *Melatonin and physical exercise reduced brain oxidative stress*

Brain oxidative stress in the 3xTg-AD mice was demonstrated by the increased lipoperoxidation levels of their cerebral cortex as compared to their NTg counterparts (Fig. 6A). All the treatments reduced the elevated LPO levels of the Tg group [$F_{(4,31)} = 29.18$, $p < 0.0001$]. Also, reduced glutathione (GSH) levels in the Tg group were significantly lower than in the NTg group, and they were increased by all the treatments assayed (Fig. 6B) [$F_{(4,31)} = 16.59$, $p < 0.0001$]. However, no differences in oxidized glutathione (GSSG) levels were detected across the mouse groups (Fig. 6C). The GSH/GSSG ratio showed that the Tg-M group had the best glutathione cycle status (Fig. 6D) [$F_{(4,31)} = 115.2$, $p = 0.0026$].

Activities of the antioxidant enzymes GPx, GR, CuZnSOD and MnSOD were significantly lower in the Tg group than in the NTg group (Fig. 6E-H). The GPx activity of the Tg group was significantly increased by melatonin (Tg-M) and exercise (Tg-E) and partially recovered under the combined treatment (Tg-ME) (Fig. 6E) [$F_{(4,31)}$ = 6.91, p = 0.0004]. Although not statistically significant, all treatments partially ameliorated GR activity (Fig. 6F). CuZnSOD activity was improved by melatonin (TgM) and exercise (Tg-E) (Fig. 6G) [$F_{(4,31)}$ = 4.49, p = 0.0056]. All the treatments assayed were effective in increasing MnSOD activity (Fig. 6H) [$F_{(4,31)}$ = 5.646, p = 0.0016].

2.8. *Melatonin and physical exercise ameliorated mitochondrial DNA*

Analysis of mitochondria showed a depletion of mtDNA in the hippocampus of the 3xTg-AD mice as compared to the NTg mice, measured as a 20% depletion of the mtDNA-encoded protein COXI (Fig. 7). All the treatments protected against such mtDNA reduction, and the Tg-M, Tg-E and Tg-ME groups all showed mtDNA content similar to that of the NTg group [$F_{(4,15)}$ = 7.298, p = 0.0018].

2.9. *Melatonin and physical exercise induced a synergistic increase of mitochondrial functional markers*

Impairment of mitochondrial oxidative phosphorylation in the Tg group was demonstrated by a deficiency of the markers assayed for the different mitochondrial complexes: complex I, ND6; complex III, Core2; complex IV subunit 1, COXI; and complex V, CVα (Fig. 8A). The Tg-ME mice (combined melatonin and physical exercise treatment) showed values indistinguishable from those of NTg mice for all the oxidative phosphorylation mitochondrial markers, whereas only a slight improvement resulted for treatments alone (Tg-M and Tg-E) [two-way ANOVA showed an effect of the treatment factor $F_{(4,60)}$ = 17.68, p < 0.0001, but no effect of the mitochondrial complex factor].

CoQ_{10}, another component of the electron transporter chain that is essential for ATP generation in mitochondria, did not show any significant changes across groups (Fig. 8B). However, its precursor, CoQ_9, was significantly enhanced by melatonin plus exercise treatment (Tg-ME) (Fig. 8C) [two-way ANOVA showed an effect of the treatment factor $F_{(4,56)}$ = 3.334, p < 0.05, and the coenzyme $F_{(1,56)}$ = 56.57, p < 0.0001].

Discussion

This study examined in 3xTg-AD male mice whether the combined treatment of melatonin plus voluntary exercise afforded higher neuroprotection than each treatment alone. Several mice were housed in each cage throughout the study. Therefore, we anticipated some slight differences in the amount of melatonin received from the drinking bottle, or the time of exercise performed in the running wheel, between animals in the same cage. However, the reliability of the results obtained with mice housed in social groups, where they freely interacted and where the treatments were administrated with minimum disturbance, overcame these inconveniences. Six-month chronic treatments began at a moderate pathology phase, when animals already present cognitive loss and brain pathology. Overall, both melatonin and exercise groups showed a remarkable amelioration of cognitive and brain redox states up to NTg mouse levels. Differential neuroprotection was obtained against other alterations. Namely, behavioral and psychological symptoms of dementia were protected by exercise and senescence parameters by melatonin. Interestingly, the combined treatment induced neuroprotective effects against brain mitochondria deterioration.

Twelve-month-old 3xTg-AD mice show greater behavioral and cognitive deterioration than 7-month olds, as evaluated in a previous study with voluntary physical exercise (García-Mesa et al., 2011). This is in agreement with an advanced stage of AD at the age of 12 months (Oddo et al., 2003). The 12-month-old mice also showed bodily deterioration that confirms the reported neuroimmunoendocrine impairment of male 3xTg-AD mice (Giménez-Llort et al., 2008). Nevertheless, the amount of voluntary running activity from 6 to 12 months of age was similar to that from 1 to 7 months of age (García-Mesa et al., 2011). Twelve-month-old NTg mice were behaviorally and cognitively healthy, as we saw from their good results for all tests, as compared with previous results at younger ages (García-Mesa et al., 2011).

Melatonin induced a large decrease of WAT and an increase of BAT and thymus weights in the 3xTg-AD mice, in keeping with its proposed modulatory and invigorating action on the immunoendocrine function (Carrillo-Vico et al., 2005; Tan et al., 2011; Hardeland et al., 2011). The decrease of thymus involution brought about by the melatonin treatment indicates an improvement in the immunosenescence of these mice (Giménez-Llort et al., 2008). Regulation of both WAT and BAT are important for maintaining the correct balance between energy intake and expenditure, and it is associated with healthy immunoendocrine function. Age- or diet-related increases of WAT in rats have been reduced by melatonin, through mechanisms associated with leptin and insulin regulation (Rasmussen et al., 1999; Ríos-Lugo et al., 2010). An increase in thermogenic BAT is known to be induced by melatonin through activation of brown adipocytes (Tan et al., 2011). Running induced a decrease in WAT in the 3xTg-AD mice in agreement with the reported beneficial effects of exercise against abdominal obesity (Slentz et al., 2009).

Unpredictably, the combined melatonin plus exercise treatment was less effective in ameliorating the peripheral immunoendocrine markers than melatonin alone. Exercise itself has been reported to counteract immunosenescence in older humans (Senchina et al., 2007). It is assumed that physical exercise enhances immune surveillance and vigilance through an increase in and modulation of circulating immune cells (Kruger and Mooren, 2007; Maltseva et al., 2011). We speculate that the low level of stress induced by chronic physical exercise interferes with the immunoendocrine system rescue status induced by melatonin in highly impaired 3xTg-AD mice.

Physical exercise induced a positive response in a range of behavior tested in the 12-month-old 3xTg-AD mice, in keeping with previous results reported at the younger age of 7 months (García-Mesa et al., 2011). Neuroprotection induced by physical exercise was similarly efficacious at both ages, despite the higher amyloid and tau pathology of the older mice (Oddo et al., 2003; Mastrangelo et al., 2008). Physical exercise improved 3xTg-AD mouse fitness as shown by a decrease in their abnormally high WAT levels to NTg mouse levels, as discussed above, and an improvement in their motor coordination and equilibrium. It also ameliorated the impaired reflexes tested. Physical exercise decreased the latency of activity and the level of emotionality in all the behavioral tests (corner, open field, dark and light, and hole-board tests), which became similar to NTg mouse levels. Physical exercise also increased exploratory activity (hole-board test) and reduced anxiety (dark and light test). These results are in agreement with the anxiolytic and mood improvement effect of physical exercise shown in AD patients (Teri et al., 2003; Williams et al., 2007; Williams and Tappen, 2008). Physical exercise is known to activate multiple molecular pathways that enhance brain activity and upregulate the expression of growth factors that regulate synaptic plasticity, neurogenesis and angiogenesis, and can exert a direct effect on neural function (Cotman et al., 2006; Cotman et al., 2007). Hence, through these mechanisms, physical exercise may normalize behavioral and psychological symptoms of dementia associated with AD. In contrast, melatonin only weekly improved 3xTg-AD mouse behavior, but in the combined treatment it did not offset the effects of physical exercise and it also ameliorated reflex responses. Similarly, non-cognitive effects were not induced by chronic melatonin treatment of APP+PS1 double-transgenic mice, as tested in somatosensorial and anxiety tests (Olcese et al., 2009). Indeed, no antidepressant action has been reported for melatonin in humans, but it could help mood disorders through restoring the circadian rhythm (Quera-Salva et al., 2011). Sleep disturbances and insomnia in AD patients are at least partially caused by greatly decreased melatonin levels (Liu et al., 1999; Zhou et al., 2003). Twelve-month-old 3xTg-AD mice did not show decreased brain melatonin levels even though some circadian changes have been reported in these mice (Sterniczuk et al., 2010).

Twelve-month-old 3xTg-AD mice were not capable of spatial learning. Their curve of latency acquisition was almost flat and they were not able to later locate the platform position in the MWM tests. This indicates a high degree of functional disturbance of the hippocampus, one of the main brain regions affected in AD (Rossler et al., 2002). Learning and memory appeared to be improved by either physical exercise or melatonin, and also the combined treatment; however, the combined treatment facilitated acquisition and melatonin alone produced the best outcome in the retention of learning of the platform position. Melatonin also induced the best outcome in the previous assay of cue learning. Therefore, melatonin might facilitate visuospatial attention and motivation. In the retention of spatial learning, physical exercise was more effective in 7-month-old 3xTg-AD male mice whose treatment starting at the age of 1 month (García-Mesa et al., 2011) than in the present 12-month-old mice whose treatment started at 6 months of age. Therefore, the exercise treatment was less neuroprotective against cognitive loss in 3xTg-AD mice with advanced AD pathology. Their response is in between the amelioration of aged Tg2576 mice (Nichol et al., 2007; Parachikova et al., 2008) and the poor response to exercise of aged APP23 mice (Wolf et al., 2006). Differential responses are probably derived from the degree of AD pathology of the different mouse models and its interplay with the neuroprotective mechanisms triggered by physical exercise (see above). Physical exercise has been shown to yield poor results with regard to amyloid-β and phospho-tau pathology in the hippocampus of 7-month-old 3xTg-AD (García-Mesa et al., 2011). Physical exercise reduced the levels of soluble amyloid-β oligomers but did not decrease phospho-tau in the cerebral cortex of 12-month-old 3xTg-AD mice. Previous mixed results regarding insoluble or soluble amyloid-β decrease after exercise treatment in other AD transgenic mice in the presence of a cognitive improvement (Parachikova et al., 2008; Yuede et al., 2009; Mirochnic et al., 2009) suggest that clearance of amyloid is not the main neuroprotective mechanism of physical exercise, but rather a gain in cognitive reserve is. Nevertheless, even a modest therapeutic effect of physical exercise once cognitive impairment is established represents a huge benefit for AD patients (Heyn et al., 2004; Lautenschlager et al., 2008). The combined treatment with exercise and melatonin induced a more greatly improved cognitive response than exercise alone. Melatonin itself showed a significant neuroprotective effect against cognitive loss. Two previous studies of chronic melatonin treatment of young AD mice of the strains APP695 (Feng et al., 2004) and APP+PS1 double-transgenic (Olcese et al., 2009), also reported amelioration of learning and memory deficits in several tests. Nonetheless, the latter study reported no improvement in spatial memory in the MWM test. Melatonin may act directly against amyloid-β aggregation (Pappolla et al, 1998; Olcese et al., 2009) and tau hyperphosphorylation (Wang et al., 2007) through its extraordinary antioxidant potential (see below). Accordingly, melatonin reduced both amyloid-β oligomers and phospho-tau in the

cerebral cortex of 12-month-old 3xTg-AD mice. These effects and protection of cholinergic activity in the hippocampus and cortex (Feng et al., 2004) may be the basis of its cognitive benefits in AD. However, melatonin treatment does not protect against advanced brain amyloid pathology in old Tg2576 mice (Quin et al., 2005). Few studies of melatonin treatment have been performed on mild cognitively impaired or AD patients, but some cognitive amelioration has been reported at the early phases of cognitive loss (Srivasan et al., 2005; Furio et al., 2007; Cardinale et al., 2010). The decrease in melatonin levels correlates with the severity of dementia and appears to be a consequence rather than a cause of the disease (Magri et al., 1997). Restoration of melatonin levels is likely to induce a noticeable benefit in AD patients whenever melatonin receptors are functional (Hardeland et al., 2011).

Cerebral cortex tissue from 12-month-old 3xTg-AD mice presented higher levels of oxidative stress markers than that from younger mice (Resende et al., 2008; García-Mesa et al., 2011), demonstrating a progression of oxidative damage in advanced stages of AD. In those reports, 3xTg-AD mouse tissue showed a depletion of GSH together with induction of SOD enzymes and GSH cycle enzymes, indicative of a functional antioxidant defense. In the present study, the depletion of both GSH and antioxidant enzymes showed exhaustion of the brain antioxidant capacity. All the treatments (melatonin, exercise and melatonin plus exercise) protected against glutathione cycle impairment and the decrease in antioxidant enzymes. Consequently, oxidative damage was reduced to NTg mouse levels. It is known that regular physical exercise can attenuate oxidative damage in the brain by reducing the production of free radicals and stimulating the antioxidant systems (Radak et al., 2005) and this would therefore protect from AD-related oxidative damage (Radak et al., 2010). Melatonin further increased GSH levels over the control values, in agreement with its considerable antioxidant capacity. Melatonin is a potent scavenger of destructive hydroxyl and many other free radicals as well as other oxidizing radicals (Hardeland et al., 1993; Tan et al., 2002). Moreover, it has the capacity to up-regulate antioxidant enzymes, as we found in 3xTg-AD mice; mainly GPx (Hardeland 2005). The remarkable antioxidant capacity of melatonin is considered the principal mechanism by which it protects cells (Reiter et al., 2001; Hardeland et al., 2011). Melatonin protects against oxidative damage in the mouse models Tg2576 (Matsubara et al., 2003), APP695 (Feng et al, 2006) and APP+PS1 double-transgenic (Olcese et al., 2009), and also in rats injected with amyloid-β in the hippocampus (Rosales-Corral et al., 2003). However, melatonin treatment at old age in Tg2576 mice did not afford protection against oxidative damage (Quinn et al. 2005). Oxidative damage is a biochemical hallmark of AD and a possible link between aging and AD (Castellani et al., 2008; García-Matas et al., 2010). Oxidative-stress-related derangements of neurotransmission and

general brain function in aging and AD could be prevented or delayed by physical exercise and/or melatonin or other potent antioxidants and enhancers of antioxidant cell defenses.

Untreated 12-month-old 3xTg-AD mice had impaired mitochondrial machinery, as shown by partial depletion of mtDNA and decreased markers for the respiratory chain complexes in the hippocampal tissue. mtDNA is an extra-chromosomal genetic element and therefore it is highly vulnerable to oxidative damage. Oxidative lesions in the mtDNA lead to its depletion (Oka et al., 2008). In AD, amyloid-β may be the agent that triggers or facilitates the mitochondrial deleterious cycle of oxidative stress and respiratory impairment (Casley et al., 2002; Rhein et al., 2009). Several defects of the mitochondrial electron transport chain enzymes have been reported in AD, but the most consistent is deficiency in cytochrome c oxidase (COXI, complex IV) (Maurer et al., 2002). In 3xTg-AD mice, this complex was shown to be heavily damaged, as was ATP synthase (CVα, complex Vα), whereas decreases in complex I and complex III were not statistically significant. mtDNA levels were preserved completely in the 3xTg-AD mice treated with melatonin or physical exercise. Both treatments had antioxidant potential against the increased oxidative stress of the brain tissue, as discussed above, that would mitigate mtDNA damage and thus avoid its depletion. The amelioration of respiratory mitochondrial complexes was partial in the mice treated with either melatonin or physical exercise; however, the combined treatment (melatonin plus physical exercise) induced levels of mitochondrial complex markers indistinguishable from those of NTg mice. Interestingly, this combined treatment increased the hippocampal levels of CoQ9, the precursor of CoQ10 (ubiquinone), indicating a protective response against oxidative stress. CoQ10 is an essential factor in the respiratory chain and acts both as an antioxidant and electron acceptor for complexes I and II (Ernest and Dallner, 1995). Its deficiency is considered a culprit in age-related mitochondrial disturbances (Ochoa et al., 2011). Therefore, melatonin and physical exercise exerted a synergistic effect in the protection of the mitochondrial functionality. Melatonin is highly effective in preserving of mitochondria respiration processes because it improves the electron transport chain while it reduces oxidative damage (Acuña-Castroviejo et al., 2001). Exercise, particularly aerobic exercise, has been reported to increase mitochondrial biogenesis in experimental animals through activation of PGC1α (Viña et al., 2009). Activation of PGC1α by melatonin has been demonstrated in aged cultured neurons (Tajes et al., 2009). Therefore, this and other pathways probably contributed to the healthy status of mitochondria in the 3xTg-AD mice treated with the combination of melatonin and exercise.

In conclusion, anti-aging therapies and healthy life-style options such as melatonin and physical exercise showed a noticeable potential to increase cognitive reserve and bodily resistance to AD-related changes. In 12-month-old 3xTg-AD mice, they induced partially different survival

and neuroprotection pathways that resulted in some additive or synergistic neuroprotective effects when the two treatments were combined. The study was initiated at 6 months of age when 3xTg-AD mice are in a stage of moderate pathology, and therefore both melatonin and exercise showed therapeutic effects by resversing many analyzed parameters to NTg levels. However, more studies should be done to conclude a possible therapeutic effect of restoration of function linked to a decrease in amyloid-β and tau pathology by melatonin, and to a lesser extend by physical exercise. Similarly to the EPIC-Norfolk prospective population study, where the combination of physical exercise plus another three healthy life-style options predicted a 4-fold difference in total mortality (Khaw et al., 2008), these combined treatments may significantly alleviate AD incidence in the population.

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Figure Legends

Fig. 1. Wheel-running activity of 3xTg-AD (Tg) mice. Mice were subjected either to a treatment of voluntary physical exercise with free access to a running wheel (Tg-E) or a combination of exercise and melatonin (Tg-ME) from 6 months to 12 months of age. Melatonin at a dose of 10 mg/kg/day was administrated via drinking water. Melatonin treatment did not affect the running activity of the mice after the first two weeks of training. The distance (kilometers) run per mouse was calculated as the average of the distance covered by the 4-5 animals in each cage in a week. Values are mean ± SEM, n = 7-13. Statistics: *p < 0.05 compared to Tg-ME.

Fig. 2. Differential effects of melatonin, voluntary exercise and melatonin plus exercise treatments on immunoendocrine function in 3xTg-AD (Tg) mice. (A) There were no significant differences in total body weight between non-transgenic (NTg) mice, Tg mice and Tg mice subjected to melatonin (Tg-M), exercise (Tg-E) or melatonin plus exercise (Tg-ME). Weights of: (B) white adipose tissue (WAT); (C) brown adipose tissue (BAT); and (D) thymus, are expressed as a percentage of total body weight. Differences in these values between Tg mice and NTg mice were offset by physical exercise and melatonin plus exercise, whereas melatonin alone caused a great reduction in WAT. Values are mean ± SEM, n = 7-15. Statistics: * p < 0.05, ** p < 0.01 compared to NTg; # p < 0.05, ## p < 0.01 compared to Tg.

Fig. 3. Differential effects of melatonin, voluntary exercise and melatonin plus exercise treatments on the neophobic behavior (A, B) and the open field behavior (C-H) of 3xTg-AD (Tg) mice. In the corner test, alterations of corner number (A) and latency of vertical activity (B) in Tg mice were ameliorated by melatonin (Tg-M) and to a greater extent by physical exercise (Tg-E). In the open field test, changes in horizontal (C, D) and vertical (E, F) activity, self-grooming (G) and emotionality (H) of Tg mice were reduced by exercise or melatonin and exercise combined (Tg-ME). Values are mean ± SEM, n = 7-15. Statistics: * p < 0.05, ** p < 0.01, *** p < 0.001 compared to non-transgenic (NTg) mice; # p < 0.05, ## p < 0.01 compared to Tg; & p < 0.05, && p < 0.01 compared to Tg-M.

Fig. 4. Voluntary physical exercise reduced anxiety (A, B) and increased exploratory behavior (C, D) in 3xTg-AD (Tg) mice, while melatonin treatment was more effective than exercise on the Morris water maze behavior (E, F). Increased anxiety and reduced exploration of Tg mice in the dark and light box test (A, B) and Boissier's 4 hole-board test (C, D) respectively, were reduced by exercise (Tg-E) and melatonin plus exercise (Tg-ME), but not by melatonin (Tg-M). Melatonin, physical exercise and melatonin plus exercise treatments ameliorated the spatial

memory acquisition and retention of 3xTg-AD (Tg) mice assessed in the Morris water maze test (E, F). (E) Distance covered to reach the visible platform during the cue learning day (CL) was reduced by melatonin treatment (Tg-M). Distance covered to reach the platform location during the 6 days of training in Tg mice were ameliorated by exercise and melatonin plus exercise. (F) Time spent swimming in the platform quadrant of the pool (Platform Q) and the opposite quadrant (Opposed Q) when the platform was removed to test the retention of learning was improved by all treatments. Melatonin was the best treatment for improving spatial learning retention of Tg mice. Values are mean ± SEM, n = 7-15. Statistics A-E: * $p < 0.05$, *** $p < 0.001$ compared to nontransgenic (NTg) mice; # $p < 0.05$, ,## $p < 0.01$, ### $p < 0.001$ compared to Tg; & $p < 0.05$, && $p < 0.01$, &&& $p < 0.001$ compared to Tg-M. Statistics F: * $p < 0.05$, *** $p < 0.001$ compared to the platform quadrant; Tg-E platform location was significant according to Student's *t*-test (see text).

Fig. 5. Representative immunoblots and densitometry analyses of (A) amyloid-β and (B) phospho-tau in the cerebral cortex of non-transgenic mice (NTg) mice, 3xTg-AD (Tg) mice, and Tg mice treated with melatonin (Tg-M), physical exercise (Tg-E), and melatonin plus exercise (Tg-ME). (A) Immunoblot probed with 6E10 antibody showed a distinc band of approximately 24 kDa corresponding to hexameric aggregates (6-mer) of amyloid-β. The immunoreactivity of this band was normalized to that of pan actin. Levels of soluble oligomers of amyloid-β in Tg mice were reduced by all the treatments. (B) Tau protein abnormally phosphorylated at Ser202 was labeled with AT8 antibody and the blot densities were normalized to that of GAPDH. Phospho-tau levels were increased in Tg mice and reduced by melatonin and melatonin plus exercise treatments. Values are mean ± SEM, n = 5 - 6. Statistics: ** $p < 0.01$ compared to NTg; ## $p < 0.01$ compared to Tg. Levels of Tg and Tg-E phospho-tau in (B) were significanty different from NTg according to Student's *t*-test (see text).

Fig. 6. Melatonin, physical exercise and melatonin plus exercise treatments ameliorated cerebral cortical redox status of 3xTg-AD (Tg) mice. Tg mice showed increased lipoperoxidation (LPO) (A), lower levels of reduced glutathione (GSH) (B), but no significant changes of oxidized glutathione (GSSG) (C) or of the GSH/GSSG (D) as compared to non-transgenic (NTg) mice. Oxidative stress changes were ameliorated to the corresponding NTg levels by all treatments. Tg mice had reduced activity levels of the antioxidant enzymes glutathione peroxidase (GPx) (E), glutathione reductase (GR) (F), CuZn superoxide dismutase (CuZn-SOD) (G) and Mn-SOD (H). The loss of enzymatic defense was mitigated by melatonin, exercise and/or melatonin plus exercise. Values are mean ± SEM, n = 5-8. Statistics: * $p < 0.05$, *** $p < 0.001$ compared to NTg,

# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to Tg, & $p < 0.05$ compared to Tg-M, $ $p < 0.05$ compared to Tg-E.

Fig. 7. Melatonin, physical exercise and melatonin plus exercise treatments countered the depletion of mtDNA in the hippocampus of 3xTg-AD (Tg) mice. Tg mice had diminished levels of mtDNA. All treatments induced levels of mtDNA similar to non-transgenic (NTg) mouse levels. Results are normalized to those of nDNA. Values are mean ± SEM, n = 4. Statistics: *** $p < 0.001$ compared to NTg; ## $p < 0.01$, ### $p < 0.001$ compared to Tg.

Fig. 8. Levels of mitochondrial proteins in the hippocampus of nontransgenic (NTg) mice, 3xTg-AD (Tg) mice, and Tg mice subjected to melatonin (Tg-M), physical exercise (Tg-E), and melatonin plus exercise (Tg-ME). (A) Representative immunoblot and densitometry analysis of proteins from the respiratory chain complexes. The immunoreactivity of each band is normalized to that of complex II (CII) protein. Reduced levels of phosphorylative oxidation markers cytochrome c oxidase I (COXI, complex IV) and ATP synthase subunit α (CVα, complex V) in Tg mice were slightly ameliorated by all the treatments but only recovered to NTg levels with the combined treatment of melatonin plus exercise. NADH-ubiquinone oxidoreductase chain 6 (ND6, complex I) and ubiquinol- cytochrome-c reductase complex core protein 2 (Core2, complex III) were also increased to NTg levels in the Tg-ME group. (B, C) A tendency to decrease both coenzyme Q_{10} (CoQ_{10}) (B) and its precursor coenzyme Q9 (CoQ_9) (C) in Tg mice was offset by melatonin and physical exercise. The combined treatment of melatonin plus exercise induced an increase of CoQ9 to above the values of NTg. Values are mean ± SEM, n = 4-7. Statistics: * $p < 0.05$ compared to NTg; # $p < 0.05$ and ## $p < 0.01$ compared to Tg.

Table 1

Effects of melatonin, voluntary physical exercise and both combined treatments on the sensorimotor function of 3xTg-AD mice.

	NTg (*n*=15)	Tg (*n*=13)	Tg-M (*n*=10)	Tg-E (*n*=14)	Tg-ME (*n*=7)
Reflex test					
Incidence of both reflexes	15/15	8/13*	9/10	14/14$^{\#}$	7/7
Wooden rod test					
Equilibrium (mean latency to fall, s)	10.0± 1.4	18.2± 1.2***	18.6± 0.9***	19.9± 0.1***	20.0± 0.0***
Coordination (mean distance, cm)	0.2± 0.1	1.1± 0.1	1.2± 0.5	8.2±1.3***, ###, &&&	9.9± 1.8***, ###, &&&
Wire rod test					
Equilibrium (mean latency to fall, s)	2.0± 0.7	2.7± 0.5	4.1± 0.6	6.1± 1.2**, #	5.7± 1.5
Coordination (mean distance, cm)	0± 0	0± 0	0± 0	1.1± 0.5	0.9± 0.5
Wire hang test (2 trials 5 s)					
Strenght (mean latency to fall, s)	1.1± 0.3	2.7± 0.4**	3.1± 0.5**	2.7± 0.3**	2.6± 0.4*
Coordination (mean distance, cm)	0.03± 0.03	0.04± 0.04	0.10± 0.07	0.14± 0.11	0.07± 0.07
Elements of support (*n*)	1.1± 0.04	1.7± 0.14***	1.9± 0.16***	2.1± 0.16***	2.1± 0.13***
Wire hang test (1 trial 60 s)					
Strength (mean latency to fall, s)	1.8± 0.5	11.8± 4.8	9.8± 5.7	3.5± 0.5	8.5± 4.7
Coordination (mean distance, cm)	0± 0	1.1± 0.7	0.7± 0.5	0.2± 0.1	0.9± 0.7
Elements of support (*n*)	1.0± 0	2.0± 0.2***	1.9± 0.2***	2.4± 0.2***	2.4± 0.3***

Note: NTg and Tg, non transgenic and 3xTg-AD mice housed in standard conditions; Tg-M and Tg-E, Tg mice submitted to a 6-month melatonin and voluntary exercise treatment, respectively; Tg-ME, Tg mice submitted to both tratments. Results are the mean ± SEM. Statistics: *p<0.05, **p<0.01 and ***p<0.001 compared to NTg; $^{\#}$p<0.05 and $^{\#\#\#}$p<0.001 compared to Tg; $^{\&\&\&}$p<0.001 compared to Tg-M.

Fig. 1

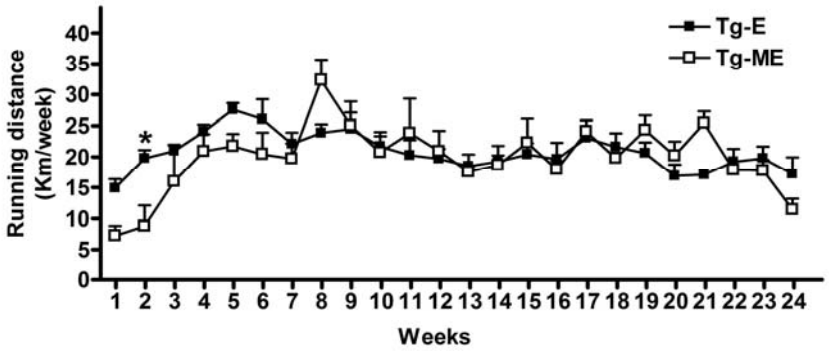
Tg-E
Tg-ME
Running distance (Km/week)
Weeks
*

Fig. 2

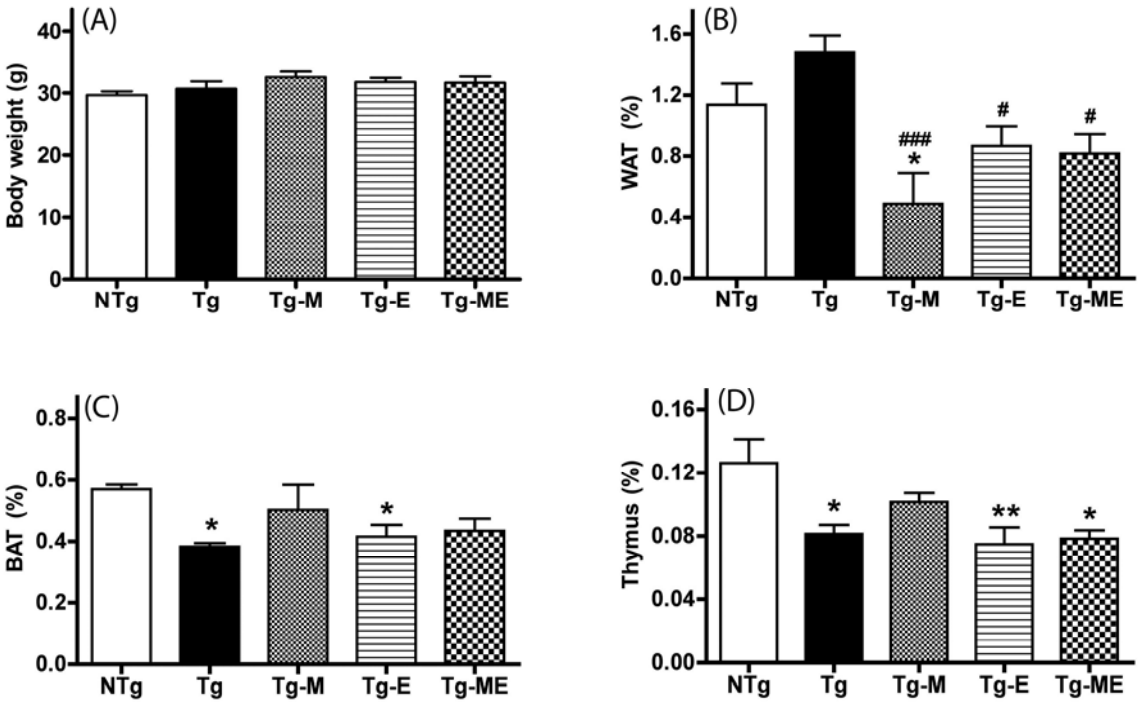
(A)
Body weight (g)
NTg
Tg
Tg-M
Tg-E
Tg-ME
(B)
WAT (%)
###
*
#
#
(C)
BAT (%)
*
*
(D)
Thymus (%)
*
**
*

Fig. 3

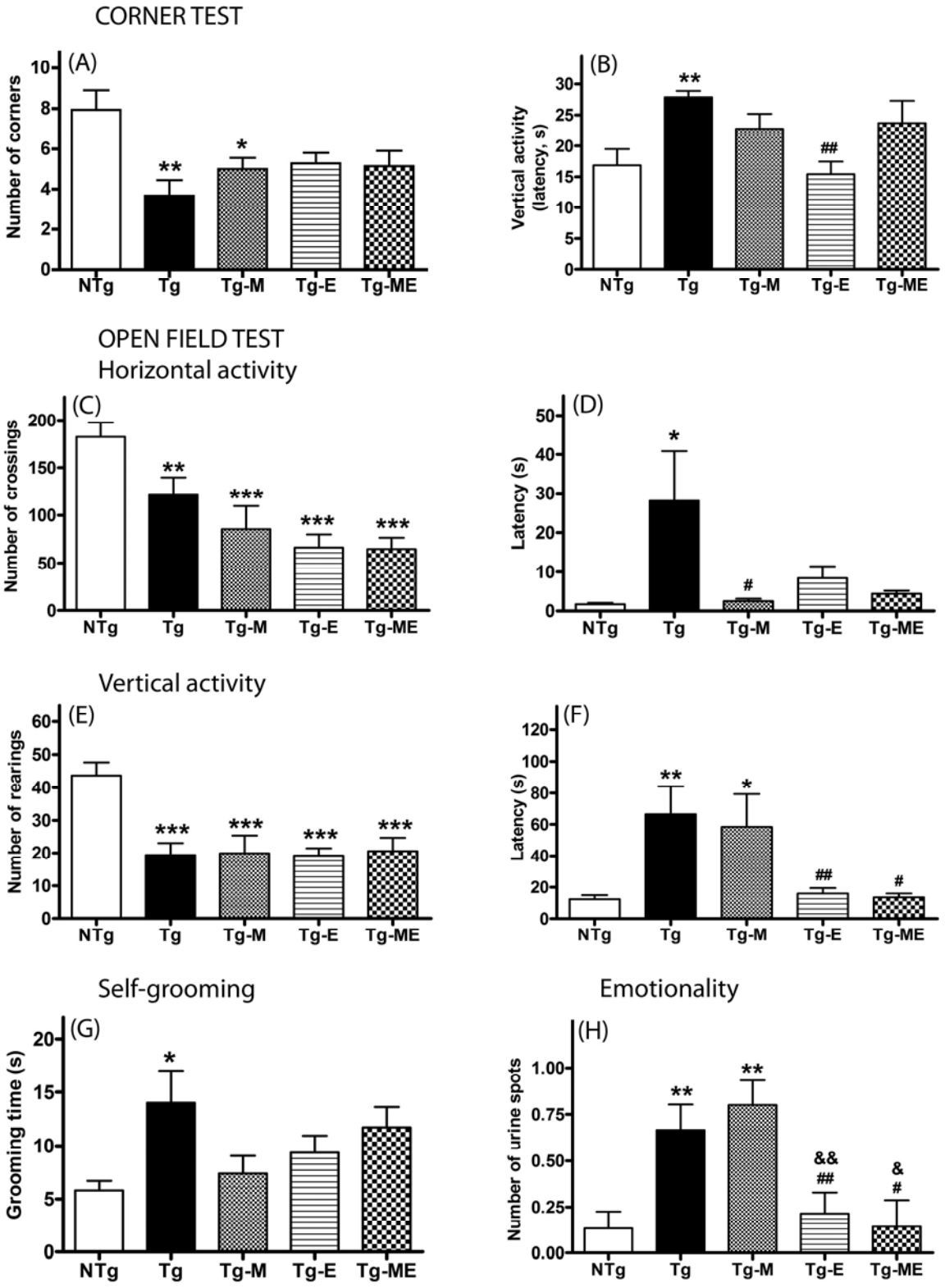
CORNER TEST
(A)
Number of corners
(B)
Vertical activity (latency, s)
OPEN FIELD TEST
Horizontal activity
(C)
Number of crossings
(D)
Latency (s)
Vertical activity
(E)
Number of rearings
(F)
Latency (s)
Self-grooming
(G)
Grooming time (s)
Emotionality
(H)
Number of urine spots
NTg
Tg
Tg-M
Tg-E
Tg-ME

Fig. 4

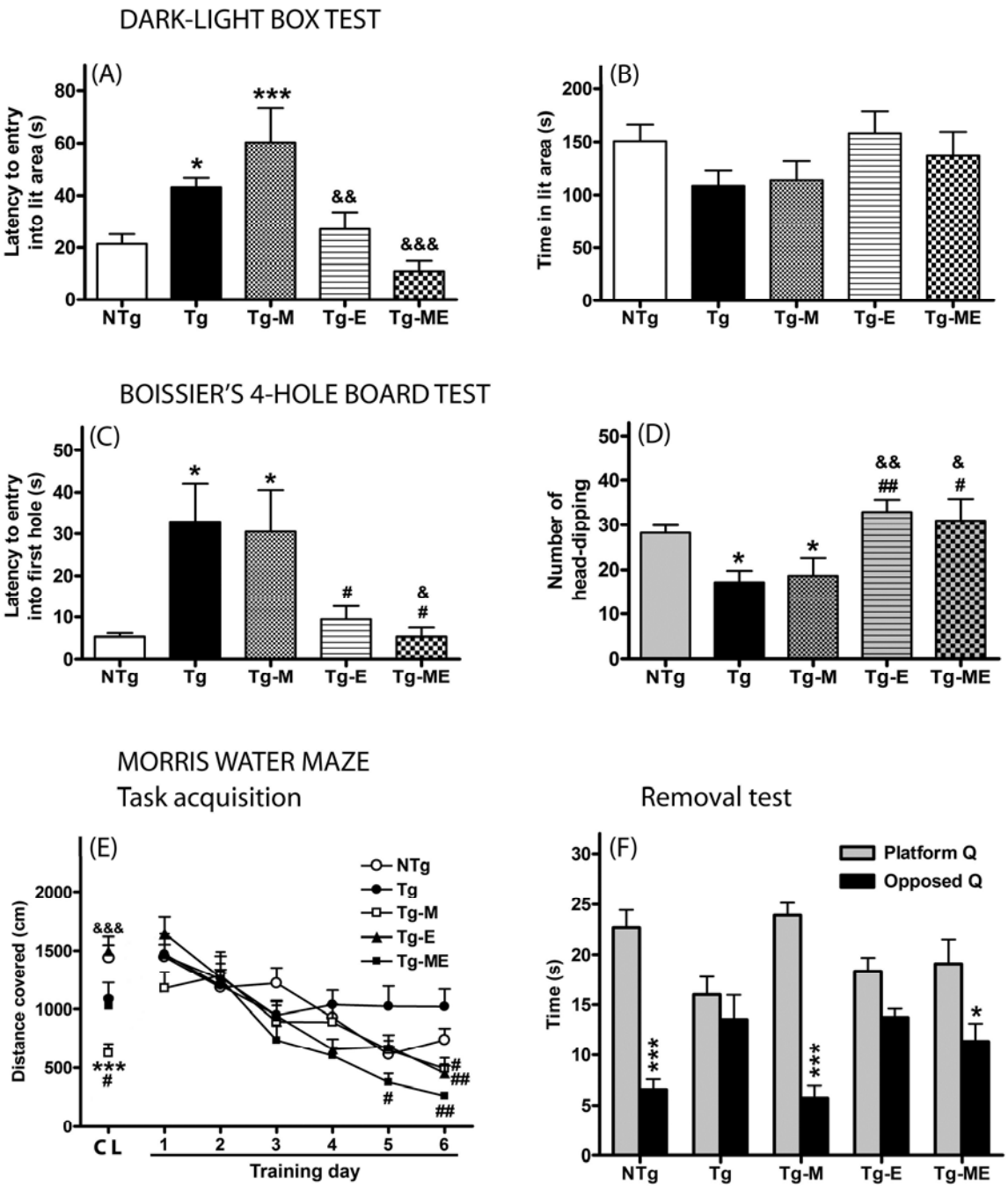
DARK-LIGHT BOX TEST
(A)
Latency to entry into lit area (s)
NTg
Tg
Tg-M
Tg-E
Tg-ME
(B)
Time in lit area (s)
BOISSIER'S 4-HOLE BOARD TEST
(C)
Latency to entry into first hole (s)
(D)
Number of head-dipping
MORRIS WATER MAZE
Task acquisition
Removal test
(E)
Distance covered (cm)
CL
Training day
(F)
Platform Q
Opposed Q
Time (s)

Fig. 5

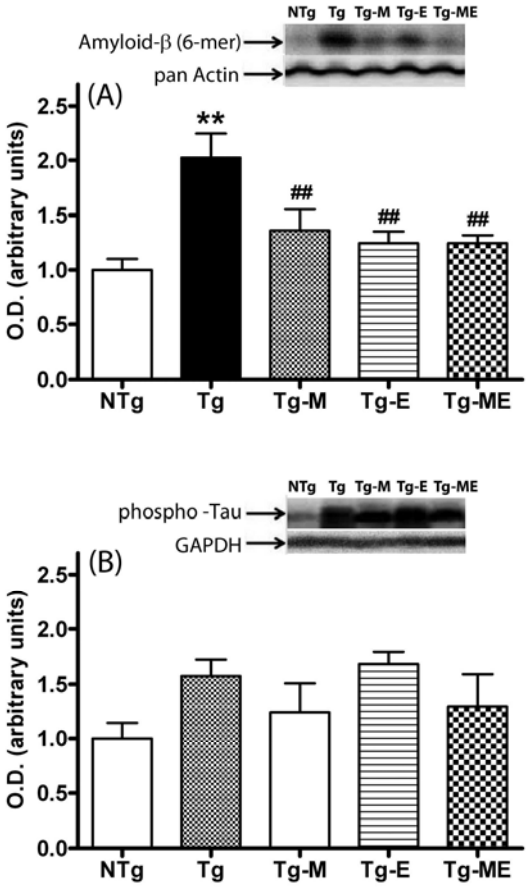
NTg Tg Tg-M Tg-E Tg-ME
Amyloid-β (6-mer)
pan Actin
(A)
O.D. (arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
**
##
##
##
NTg
Tg
Tg-M
Tg-E
Tg-ME
NTg Tg Tg-M Tg-E Tg-ME
phospho -Tau
GAPDH
(B)
O.D. (arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
NTg
Tg
Tg-M
Tg-E
Tg-ME

Fig. 6

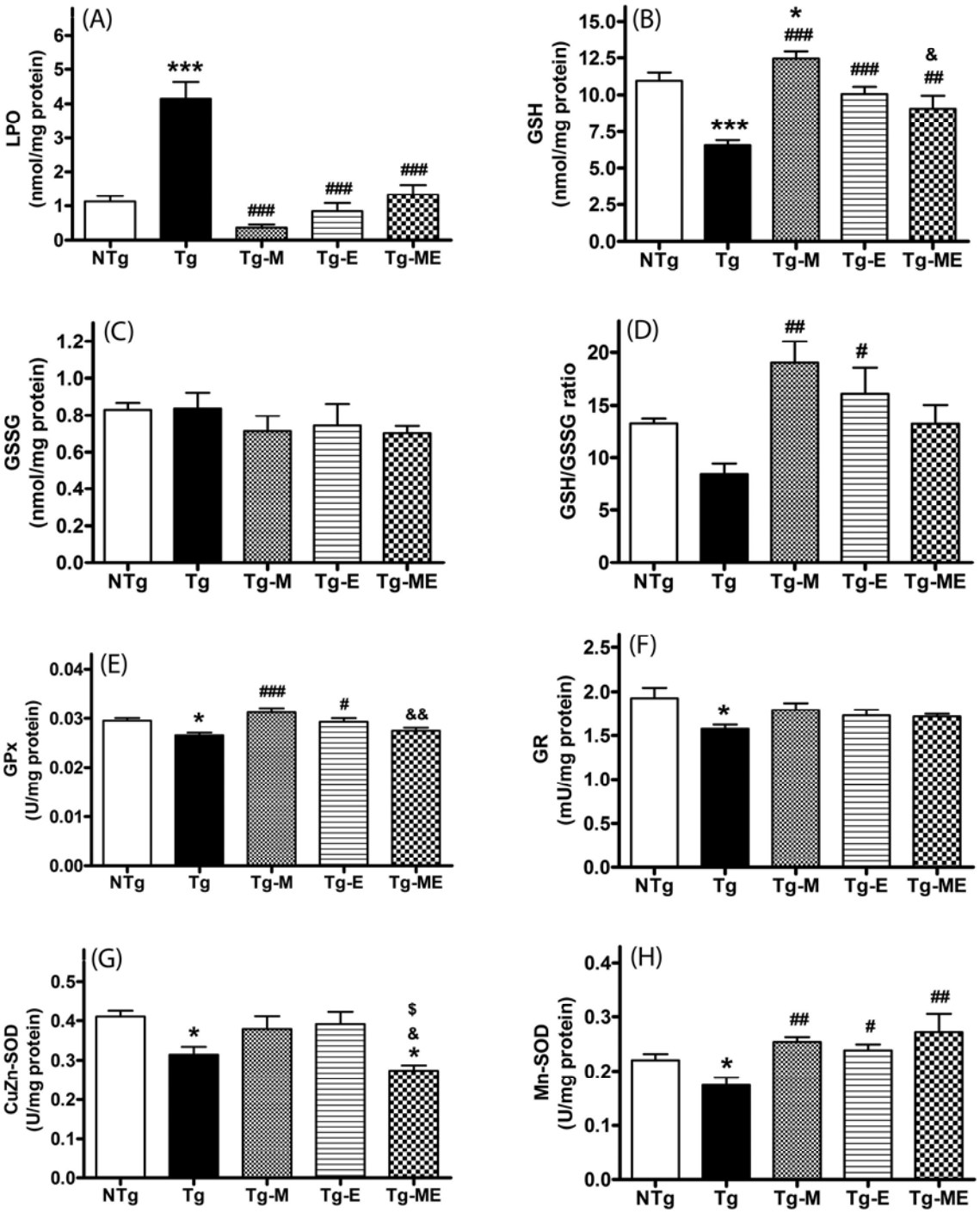
(A)
LPO (nmol/mg protein)
***
###
###
###
NTg
Tg
Tg-M
Tg-E
Tg-ME
(B)
GSH (nmol/mg protein)
*
###
***
###
&
##
(C)
GSSG (nmol/mg protein)
(D)
GSH/GSSG ratio
##
#
(E)
GPx (U/mg protein)
*
###
#
&&
(F)
GR (mU/mg protein)
*
(G)
CuZn-SOD (U/mg protein)
*
$
&
*
(H)
Mn-SOD (U/mg protein)
*
##
#
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Fig. 7

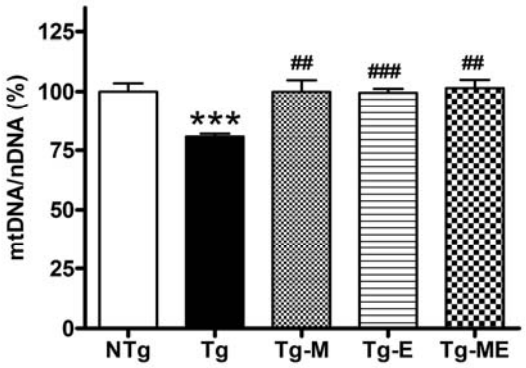
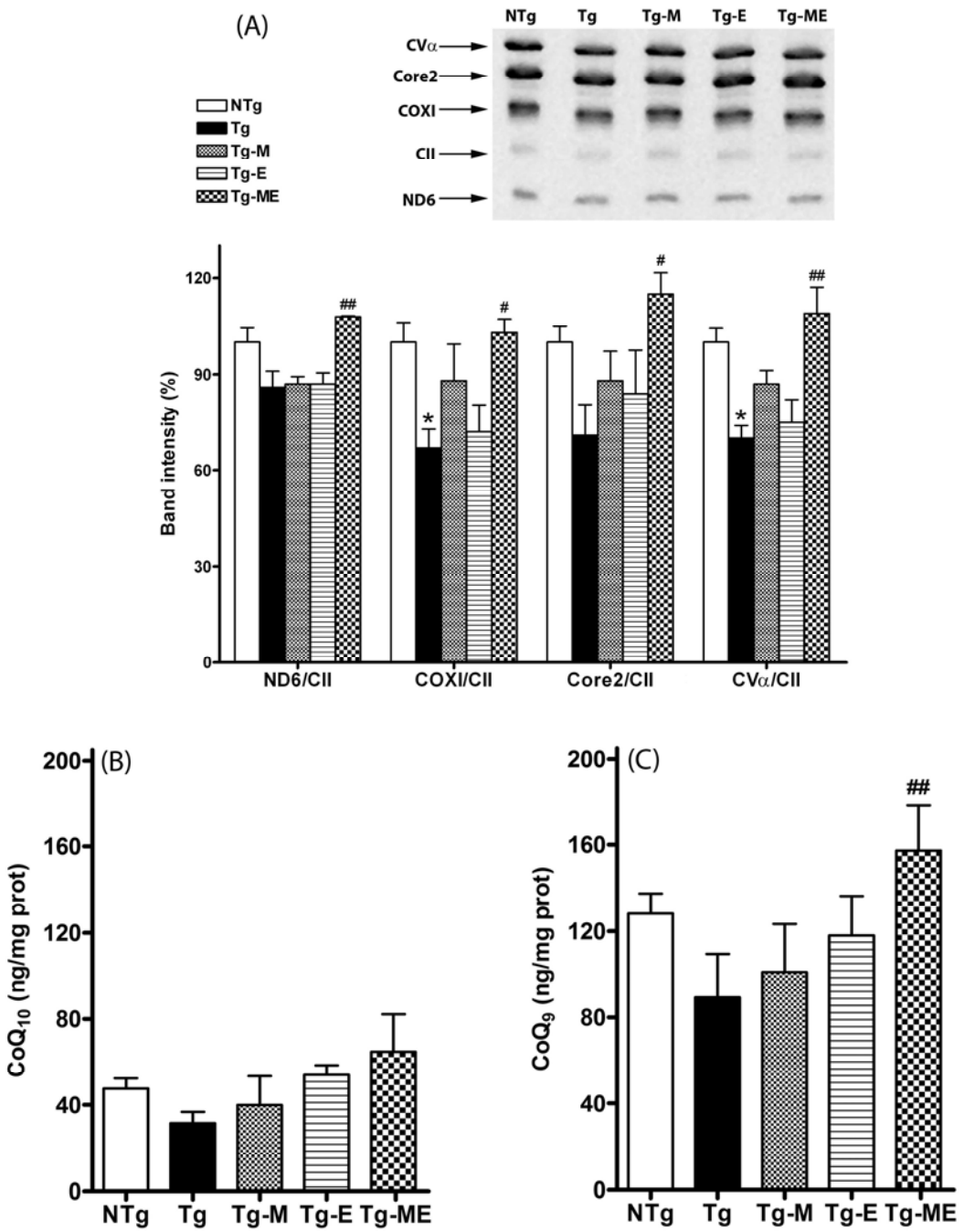


Fig. 8


(A)
NTg
Tg
Tg-M
Tg-E
Tg-ME
CVα
Core2
COXI
CII
ND6
Band intensity (%)
ND6/CII
COXI/CII
Core2/CII
CVα/CII
(B)
CoQ10 (ng/mg prot)
(C)
CoQ9 (ng/mg prot)