Title:

Physical exercise neuroprotects ovariectomized 3xTg-AD mice through BDNF mechanisms

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Running title: Wheel-running in ovariectomized 3xTg-AD

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Summary:

Postmenopausal women may be more vulnerable to cognitive loss and Alzheimer's disease (AD) than premenopausal women because of their deficiency in estrogens, in addition to their usually older age. Aerobic physical exercise has been proposed as a therapeutic approach for maintaining health and well-being in postmenopausal women, and for improving brain health and plasticity in populations at high risk for AD. To study the neuroprotective mechanisms of physical exercise in a postmenopausal animal model, we submitted previously ovariectomized, six-month old non-transgenic and 3xTg-AD mice to three months of voluntary exercise in a running wheel. At nine months of age, we observed lower grip strength and some exacerbation of the behavioral and psychological symptoms of dementia (BPSD)-like involving active exploratory activities. A similar major cognitive impairment was observed of ovariectomized 3xTg-AD mice in comparison with sham-operated 3xTg-AD mice. A reduction of bodily fitness and lack of retention of memory were observed in the ovariectomized non-transgenic mice. Physical exercise protected against all deleterious behaviors and normalized learning and memory. It also protected against body frailty, as expected. Analyses of hippocampal key markers of antioxidant and neuroplasticity signaling pathways, showed that ovariectomy impairs the activation of CREB through physical exercise. Furthermore, molecular and behavioral correlates suggested a central role of BDNF in the neuroprotection mediated by physical exercise therapy against apathy and memory loss induced by ovariectomy and the AD-genotype.

Keywords: Alzheimer's disease; Ovariectomy; 3xTg-AD mice; Physical exercise; Frailty; Behavioral tests; Cognition; Catalase; p-CREB; BDNF

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the elderly and the estimated number for AD patients worldwide in years to come is staggering. As women have a higher life expectancy than men, the higher proportion of female patients could be due to the higher proportion of women in the upper age bracket. Despite the suggested higher risk of AD at menopause, several cohort studies have demonstrated no gender effect in the risk of developing AD nor in the incidence rates (Rocca et al., 1998; Hebert et al., 2001; Barnes et al., 2003). However, the fact that there are many more women than men suffering from AD has led to the research into gender-specific therapies. Hormone reposition therapy, diet and physical exercise have been recommended as strategies to prevent and reverse the bodily effects of menopause in women. While more effective treatments are being developed, these general strategies may lead to better health and higher resistance to AD development in post-menopausal women. Treatment with estrogens has been shown to be neuroprotective in many experimental systems (Viña et al., 2011). However, clinical trials of hormone therapy yielded inconsistent results, including some negative outcomes in cognition and dementia, thus warranting further research before hormone replacement could be recommended as a treatment against AD (for review see: Maki and Henderson, 2012). Some nutrients, mainly those with antioxidant properties, yielded positive results in preclinical studies, although clinical trials held so far have not produced evidence in support of such cognitive benefits for AD patients (Kamat et al., 2008; Aisen et al., 2012). Aerobic exercise was demonstrated as improving cognitive processes in animal models of AD (Intlekofer and Cotman, 2013) and in studies involving upper-aged human subjects with or without a known cognitive impairment (Heyn et al., 2004; Angevaren et al., 2008). Physical exercise also impeded the cognitive deficits induced in ovariectomized rodents (Ben et al., 2010), an animal model of menopause. Therefore, regular physical exercise could be a preventive and/or protective strategy to decrease the AD burden in women and more research into its benefits and mechanisms will help to improve the lives of elderly females at risk of frailty and AD.

In this study we analyzed the presence of neuroprotection as a result of voluntary running exercise, on the effects induced by ovariectomy in the triple transgenic mouse model of AD (3xTg-AD) (Oddo et al., 2003) and in non-transgenic mice. In this mouse model, ovariectomy has been reported to induce an increase of AD-like pathology (Carroll et al., 2010) including exacerbated mitochondrial dysfunction (Yao et al., 2012). Physical exercise has been proved to be neuroprotective at different stages of pathological severity in the 3xTg-AD mice (García-Mesa et al., 2011, 2012), although it has not been assayed in a context mimicking ovarian

hormone deficiency in AD Tg models. We aimed to investigate the benefits and mechanisms of a physical exercise therapy along a hypothesized progressive neurodegeneration from ovariectomized wild type mice, to control AD mice and to ovariectomized AD mice. For this purpose, we studied the mechanisms underlying physical exercise-mediated neuroprotection in ovariectomized and sham-operated wild type mice and AD mice. This was done by way of characterizing behavioral and cognitive changes, brain pathology, and the levels of selected markers of neuroprotection and plasticity in the hippocampus. Namely, we analyzed the antioxidant enzymes catalase, glutathione peroxidase (GPx) and Mn superoxide dismutase (SOD); the modulator of mitochondrial function and biogenesis, peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α); the mediators of synaptic plasticity and long-term memory, phosphorylated cAMP response element-binding protein (p-CREB) and brain derived neurotrophic factor (BDNF); and the receptor tyrosine kinase B (TrkB) that binds BDNF with high affinity.

2. Materials and Methods

2.1. Animals and experimental design

For this study we used 3xTg-AD (Tg) mice, which were genetically engineered at the University of California Irvine to express familial AD mutations of amyloid precursor protein (APPSwe), presenilin-1 (PS1M146V), and tau (tauP301L) (Oddo et al., 2003). These mice presented a progressive AD-like brain pathology and deterioration in multiple aspects of brain physiology and behavior (Oddo et al., 2003; Giménez-Llort et al., 2007; García-Mesa et al., 2011), suggesting close adherence to AD disease progression in patients. We used 3xTg-AD female mice and non-transgenic (NTg) mice with the same genetic background hybrid (129 x C57BL/6) as the 3xTg-AD mice. Both strains were bred in homozygosis from progenitors obtained from Dr Frank M LaFerla (University of California Irvine, CA). The animals were maintained in Macrolon cages under standard laboratory conditions of food and water *ad libitum*, 22 ± 2 °C and 12 h light:dark. The genotypes were confirmed by PCR analysis of DNA obtained from tail biopsies. Animal handling and procedures were approved by the local animal ethics committee (Ref: DMAH-5150, CEEA, UB), in accordance with the EU Directive 2010/63/EU for animal experiments.

Mice were submitted to bilateral ovariectomy or sham operation at 4 months of age. Two months later, at 6 months of age, half of the animals were subjected to a chronic treatment of voluntary physical exercise. Four to five animals from different litters were housed together to

avoid social isolation stress, grouped by genotype, surgery type and exercise treatment. For those submitted to exercise, a readily-available running wheel connected to a computerized system (Activity Wheel Cage System for mice, Techniplast, Buguggiate, Italy) was added to each of the corresponding cages, as described previously (García-Mesa, et al., 2011). The system allowed a continuous recording of the wheel turn number and therefore calculation of the average mouse running activity per cage. The study was terminated at 9 months of age, after 3 months of physical exercise. Subsequently, grip strength, behavior and cognitive patterns were evaluated along 13 days. Three days later, the animals were euthanized. Mice submitted to physical exercise treatment had access to the running wheel until sacrificed. All animal procedures were performed between 9:00 h and 15:00 h in a balanced manner between mice of the different experimental groups.

The experimental groups (n=8-9 per group) were as follows: (i) NTg, sham-operated NTg mice; (ii) NTg+Ex, sham-operated NTg mice subjected to exercise; (iii) NTgOv, ovariectomized NTg mice; (iv) NTgOv+Ex, ovariectomized NTg mice subjected to exercise; (v) Tg, sham-operated Tg mice; (vi) Tg+Ex, sham-operated Tg mice subjected to exercise; (vii) TgOv, ovariectomized Tg mice; and (viii) TgOv+Ex, ovariectomized Tg mice subjected to exercise.

2.2. Fitness, and behavioral and cognitive tests

Body weight of the mice was recorded at the beginning and end of the study. The running activity of those mice submitted to exercise was recorded throughout the study. The average distance covered (Km) per mouse was calculated in a weekly basis for each cage.

Fore limb muscle strength was measured by a *grip strength test*. The mouse was held at the base of the tail and allowed to place its forepaws on the pull bar connected to the force gauge of an electronic grip strength meter (Harvard Apparatus, Panlab, Cornellà, Barcelona, Spain). The animal was slowly pulled away from the pull bar at a rate of 2 to 3 cm/sec until it released the bar. The force gauge indicated the peak tension exerted by the animal. Each mouse was tested in three sequential trials and the highest grip strength reading was recorded as the total peak force (gram force).

Non-cognitive behavioral responses were evaluated as previously described (García-Mesa et al., 2011). Each mouse was submitted to sequential testing of the following four tests, one test per day. The *corner test* was executed to evaluate neophobia. Mice were placed into a home cage with fresh bedding and examined for 30 sec for signs of adaptation to the new environment. Latency of rearing, number of rearings and number of corners were recorded. The *open field test* was used to evaluate vertical and horizontal locomotor activity for a single trial period of 5 min.

The mouse was positioned in the center of the apparatus (home-made, wooden, white, 55 x 55 x 25 cm high) and measurements were taken of latency of rearing, number of rearings, grooming activity, latencies and number of defecation and urine spots. Distance covered was measured as the number of crossings along a virtual grid on the floor of the apparatus. The *dark-light box test* was used to evaluate anxiety-like behavior for 5 min. The dark-light box consisted of two compartments (black, 27 × 18 × 27cm; white, 27 × 27 × 27 cm; lit with a 20W white bulb) connected by an opening (7 × 7 cm) (Panlab, Barcelona, Spain). In this test the mouse was placed in the dark compartment of the apparatus and measures were taken for latency of entry into the illuminated compartment as well as the number of entries and the time spent in the illuminated compartment once there. *Boissier's 4 hole-board test* was used to assess exploratory behavior by measuring the number of head-dips, latency and the time spent head-dipping at each of the four holes during 5 min (3 cm diameter holes in a home-made 32 x 32 cm, white, wooden board, with walls 32 cm high).

Cognitive testing was then initiated on the fifth day of behavior evaluation. Spatial learning and memory were measured using the *Morris water maze (MWM) test* as previously described (García-Mesa *et al.*, 2011). The test consisted of a preliminary day for the cue learning of a visual platform and six days of place task learning for spatial reference memory (days 1 to 6, four trial sessions per day). Mice were trained to locate a platform (7 cm diameter, 1.5 cm below the water surface) in a circular pool (91 cm diameter, 40 cm height, 25°C opaque water) surrounded by black curtains, by relying on visual cues. Within the black enclosure, four different landmarks hung from a false ceiling at 90° to each other. The platform was placed between two of these landmarks. 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) enabled swimming distance to be measured during the learning tasks, in addition to the time spent in each quadrant of the pool following the removal of the platform in the probe trial.

2.3. Tissue analyses.

The animals were decapitated under a light CO₂ anesthesia. Each mouse brain was immediately dissected on ice to isolate the hippocampus and cerebral cortex. The samples were stored at -80 °C for further analysis. The weight of the uterus and the intra-abdominal white adipose tissue (WAT) was obtained upon termination and measured as a percentage of body weight. WAT was measured as indicative of obesity and fitness related changes. The uterus was measured to check the efficacy of ovariectomy surgery.

RNA expression of antioxidant enzymes in the hippocampus was determined by real-time quantitative reverse transcriptase-polymerase chain reaction (qPCR). Total RNA was isolated from hippocampal tissue using RNeasy® Mini Kit (Qiagen Distributors, Valencia, CA) in accordance with the manufacturer's instructions. TaqMan FAM labeled specific probes (Applied Biosystems, Foster City, CA) were used for PCR quantitation of catalase (Mm00437992_m1), GPx (Mm00656767_g1) and MnSOD (Mm01313000_m1) mRNA in an iCycler iQ detection system (Bio-Rad, Hercules, CA). Gene expression was normalized to that of glyceraldehyde-3P-dehydrogenase (GAPDH, Mm99999915_g1) and 18S (Mm03928990_g1) gene expression.

Protein levels of BDNF were quantified in the hippocampus by ELISA (CYT306 Millipore, Bedford, MA), as stipulated by the manufacturer.

Western immunoblotting studies of cerebral cortical and hippocampal tissues were performed according to standard procedures. The following primary antibodies were used: anti-CREB (1:800, Cell Signaling, Danvers, MA), anti-p-CREB (1:800, Cell Signaling), anti-TrkB (1:1000, Cell Signaling), anti-PGC-1 α (1:1000, Cayman, Ann Arbor, MI), anti-C-terminus of the β -amyloid precursor protein (APP-CT) (1:1000, Covance, Princeton, NJ), anti-p-tau clone AT8, specific for double phosphorylated tau at Ser202/Thr205 (Pierce, Rockford, IL), anti-pan-actin (1:2000, Sigma-Aldrich, St. Louis, MO), and anti- α -tubulin (1:10000 Cell Signaling). The secondary antibody was a horseradish peroxidase-conjugated antibody (BD Amersham, Arlington Heights, IL).

2.9. Statistics

Data are expressed as the mean $\pm$ SEM. Normal distribution was checked with Kolmogorov-Smirnov test whereas the Shapiro-Wilks test was used for small samples (C99/APP and p-tau protein levels). Skewed variables (i.e.: latency to enter into the lit area in the dark-light box test; and latency to enter into the four holes in the Boissier's 4 hole-board test) were analyzed with Kruskal-Wallis H test. Post-hoc, non-parametric comparisons were performed with Dunn's test. Normally distributed variables were analyzed for variance equality between groups with Levene's test. Those variables that did not meet this assumption (i.e.: uterus weight, open field test parameters, and p-tau levels) were transformed to their natural logarithm. Next, all data were submitted to ANOVA. Fisher's LSD post-hoc tests were performed where factor interactions were detected. Bonferroni test was used where indicated. Correlation between variables was analyzed with Pearson's test. Descriptive statistics and analyses were performed using GraphPad Prism 4.02 and IBM SPSS Statistics 21.0 software.

3. Results

3.1. Physical exercise protected ovariectomy-increased body frailty

Sham-operated NTg and Tg mice showed a pattern of running activity similar to that seen in previous studies with younger female mice of the same strains (García-Mesa et al., 2011), with Tg mice being more active than NTg ones (Fig. 1A). Ovariectomy induced a general decrease in running activity that was greater in NTg than Tg mice, with respective decreases of 63% and 28% compared to the activity of corresponding sham-operated group. There were statistical effects of the genotype [$F_{1,32}=88.24$, $p<0.001$] and ovariectomy [$F_{1,32}=142.2$, $p<0.001$] factors, and an interaction genotype x ovariectomy [$F_{1,32}=11.89$, $p=0.002$] on the running distance.

The measure of grip strength of NTg mice was 44.0 ± 4.6 gram force. Tg mice showed 113.2 ± 7.8 gram force, in agreement with a higher muscular strength reported for this Tg mouse colony compared to NTg (García-Mesa et al., 2011). Results of grip strength of the diverse treatment groups are expressed as the percentage of their corresponding NTg or Tg values in Fig. 1B. Ovariectomy reduced grip strength in sedentary Tg mice but not in NTg mice, whereas physical exercise generally improved grip strength. There were effects of the genotype [$F_{1,67}=51.217$, $p<0.001$], ovariectomy [$F_{1,67}=4.295$, $p=0.030$] and exercise [$F_{1,67}=16.546$, $p<0.001$] factors, and an interaction genotype x ovariectomy [$F_{1,67}=8.623$, $p=0.005$].

All experimental groups had similar body weights at the beginning of the study, with group averages of 20-22 g. Body weights at termination and WAT values are shown in Fig. 1C and D, respectively. Ovariectomy induced a general decrease in body weight [ovariectomy factor, $F_{1,67}=9.956$, $p=0.003$]. WAT values were increased in ovariectomized NTg mice, whereas physical exercise induced a general decrease of WAT. There were effects of ovariectomy [$F_{1,67}=11.424$, $p=0.001$] and exercise [$F_{1,67}=9.837$, $p=0.003$] and an interaction genotype x ovariectomy [$F_{1,67}=4.159$, $p=0.046$] on WAT. Mouse uterus weight values are shown in Fig. 1E. Uterus weight decreased significantly in all ovariectomized mice [$F_{1,67}=5.732$, $p<0.001$], a clear indication of the efficacy of ovariectomy and consequent lack of ovarian hormones.

3.2. Physical exercise protected behavioral and cognitive responses

The behavioral tests confirmed the phenotype of neophobia, anxiety and reduced exploratory activity of the 3xTg-AD mice and their protection by way of a therapy of physical exercise (García-Mesa et al., 2011, 2012). Selected results are shown in Figs. 2 and 3. Tg mice showed higher latencies of rearing (Fig. 2A), decreased number of rearings (Fig. 2B), and reduced number of corners (Fig. 2C) in the corned test [genotype factor, all F 's ≥ 8.620 , $p \leq 0.007$].

Ovariectomy did not induce significant changes in neophobia behavior. However, it induced a marginal decrease on the number of rearings ($p=0.065$) and the number of corners ($p=0.053$). Physical exercise improved the latency of rearing and the number of corners of the Tg mouse groups [exercise factor, $F's \geq 4.706$, $p \leq 0.034$].

In the open field test, Tg mouse groups showed higher latency of rearing (Fig. 2D), a decreased number of rearings (Fig. 2E) and a decreased number of crossings (Fig. 2F) [genotype factor, all $F's \geq 6.580$, $p \leq 0.013$]. Ovariectomized Tg mice showed more significant changes than sham-operated Tg mice (Fig. 2D), but ovariectomy factor did not reach significance. Physical exercise improved the response of Tg groups in the latency of rearing and the number of rearings [interaction genotype x exercise for both parameters, $F's \geq 6.950$, $p \leq 0.011$; effect of exercise on the number of rearings $F_{1,67}=6.756$, $p=0.012$]. Other changes such as those of grooming activity and emotionality (number of urine spots and defecation boli) did not reach a level of significance (not shown).

In the dark-light box test, the non parametric analysis of the latencies to entry into the lit area (Fig. 3A) showed statistically significant differences between the experimental groups [$H_7=17.891$, $p=0.012$]. Post-hoc comparisons indicated higher latencies in the dark-light box for the Tg group and the ovariectomized NTg and Tg, as well as protection through physical exercise. Tg mice showed a declining trend in the number of entrances (Fig. 3B) and the time spent in the lit area (Fig. 3C), but the genotype factor not reaching significance. Physical exercise induced an increase in both parameters [all $F's \geq 5.740$, $p \leq 0.020$], although effects on the time spent in the lit area were higher in Tg mice than the NTg [interaction genotype x exercise, $F_{1,67}=11.258$, $p=0.001$],

Ovariectomized Tg mice showed lower performance than sham-operated Tg mice in the hole-board test. Seven out of 8 mice of the former group did not explore the four different holes during the 5 min test, reaching the highest latency of the the different experimental groups (Fig. 3D) [$H_7=28.919$, $p<0.001$]. Ovariectomy also had a marginal effect on the number on head-dips ($p=0.051$). Physical exercise totally protected from this apathetic behavior. Genotype factor did not reach significance with respect to the number of head-dips (Fig. 3E) or the dipping time (Fig. 3F), whereas exercise increased both parameters [all $F's \geq 39.549$, $p<0.001$]. Furthermore, the time of dipping was increased more notably by exercise in the ovariectomized than in the sham-operated mice [interaction ovariectomy x exercise, $F_{1,67}=4.645$, $p=0.035$].

The MWM test confirmed the known cognitive deficits of these Tg mice. Results are shown in Fig. 4. In the acquisition of learning there were differences of performance between the groups (Fig. 4A). All but ovariectomized and sham-operated Tg animal groups, showed a

progressive decrease in swimming distance to the platform along the 6 days of training [ANOVA repeated measures within each group: Tg and TgOv not significant; NTgOv $F_{5,53}=4.218$, $p=0.004$; all other groups $F \geq 5.099$, $p \leq 0.001$]. Also, a contrast multivariate analysis showed a significance in day factor ($p < 0.001$), and interactions day x genotype (0.08), day x ovariectomy ($p=0.007$), day x genotype x exercise ($p=0.033$), and day x genotype x ovariectomy x exercise ($p=0.004$). However, Tg and TgOv groups of mice exhibited rather short swimming distances from the beginning of the acquisition assay, which adding a level of uncertainty to their degree of acquisition deficit. In the analysis of the retention of learning, ovariectomized NTg and Tg mice, and sham-operated Tg showed a lack of preference for the platform quadrant as compared to the opposed quadrant (Fig. 4B). There was an effect of genotype [$F_{1,62}=3.932$, $p=0.052$], an interaction ovariectomy x exercise [$F_{1,62}=6.512$, $p=0.014$], and an interaction between the three factors genotype x exercise x ovariectomy [$F_{1,62}=4.755$, $p=0.034$], on the ratio between the searching time in the platform quadrant and that of searching in the opposed quadrant. When analyzed by two-way ANOVA as in Fig. 4B, there was an effect of the quadrant ($p < 0.001$) and an interaction of the quadrant with the mouse group ($p < 0.001$). Ovariectomized NTg and Tg mice, and sham-operated Tg mice showed overall reduced skills overall in the MWM which, in addition, were more evident in the retrieval of memory than in the acquisition of learning. Contrarily, all animals submitted to exercise performed at a level near to that of NTg mice.

3.3. Physical exercise and ovariectomy barely modified amyloid and tau pathology

Amyloid and tau pathology markers were analyzed in homogenates of cerebral cortical tissue, including neocortex and amygdala, with both areas bearing AD type pathology in this mouse model. Levels of C99 and p-tau are shown in Fig. 5. We analyzed the protein levels of C99 because this C terminal fragment of the APP is reported as a key contributor of 3xTg-AD mouse pathology (Lauritzen et al., 2012). The Western blotting analysis of C99 showed an increase of C99 in Tg mice, no further increase in ovariectomized Tg mice and a modest protective effect of physical [one-way ANOVA $F_{4,19}=4.459$, $p=0.014$]. Levels of tau analyzed by clone AT8 showed the presence of p-tau (Ser202/Thr205) in Tg mice, but the high variability obtained in the cerebral cortex of the different mouse groups did not allow significant results to be obtained.

3.4. Physical exercise activated antioxidant and signaling pathways

Levels of neuroprotection and plasticity markers are shown in Fig. 6. The Tg group had an increased gene expression of catalase compared to NonTg, showing its tendency to be

abolished in the sedentary ovariectomized Tg mice, but not in those submitted to exercise (Fig. 6A). ANOVA showed an effect of genotype on catalase RNA levels [$F_{1,47}=26.441$, $p<0.001$], but interaction within the diverse factors did not reach significance.

Analysis of the RNA levels of MnSOD indicated a lack of effect of ovariectomy (Fig. 6B). However, there was an interaction genotype x exercise [$F_{1,49}=6.052$, $p=0.018$], that indicated a significant decrease of this enzyme in ovariectomized Tg mice but not in those submitted to exercise (Fig. 6B). Levels of GPX RNA showed a similar response to those of MnSOD, although the interaction genotype x exercise ($p=0.037$) did not lead to any further differences of significance between groups (not shown).

Physical exercise induced an increase of PGC-1 α protein in NTg mice (Fig. 6C). Accordingly, there was an interaction of genotype x exercise [$F_{1,48}=4.722$, $p=0.036$] on PGC-1 α .

Exercise also increased p-CREB in all sham-operated, but not in ovariectomized mouse groups (Fig. 6D). ANOVA showed an effect of ovariectomy [$F_{1,40}=9.283$, $p=0.005$], exercise [$F_{1,40}=9.880$, $p=0.004$], and an interaction between both factors [$F_{1,40}=8.095$, $p=0.008$] in p-CREB protein levels.

BDNF levels were generally increased by exercise, including Tg and all ovariectomized mice (Fig. 6E) [exercise factor, $F_{1,51}=11.867$, $p=0.001$]. Interaction genotype x exercise did not reach significance ($p=0.064$), although sham-operated NTg mice showed an unexpected lack of response. As regards to BDNF receptor, the protein levels of TrkB were generally decreased in Tg mice [genotype factor, $F_{1,51}=5.547$, $p=0.023$], without changes induced through exercise or ovariectomy (Fig. 6F).

Correlation studies showed that PGC-1 α , p-CREB and TrkB were inter-correlated (all Pearson correlations: $r\geq 0.549$, $p\leq 0.001$). Levels of BDNF showed a positive correlation with the time in the illuminated area in the dark-light box test (Pearson correlation: $r=0.325$, $p=0.019$, $n=52$), and with the total dipping time in the Boissier's 4 hole-board test (Pearson correlation: $r=0.291$, $p=0.037$, $n=52$). BDNF levels correlated negatively with the distance covered in the place task acquisition test (Pearson correlation: $r=-0.324$, $p=0.020$, $n=51$).

4. Discussion

In this study we analyzed the protective effects of three months of physical exercise by way of free access to a running wheel in ovariectomized AD mice of the strain 3xTg-AD. As expected, ovariectomy induced changes that indicate a lower fitness level of the animals in the control NTg group. Fewer changes were induced in the ovariectomized Tg animals than in the NTg group, due to the fact that AD transgenes were already inducing a low-fitness phenotype.

The combination of increased weakness (grip strength), decreased physical activity (running) and weight loss fulfilled the criteria for frailty in the ovariectomized mice, and the frailty status is associated with dementia (Gray et al., 2013). However, physical exercise maintained a moderate level of body fitness and prevented the mice from entering into a state of frailty induced by ovariectomy surgery and AD genes. A decrease in peripheral risk factors through exercise contributes to maintaining brain health (Cotman et al., 2007).

Ovariectomy did not induce major effects on the behaviors of neophobia and anxiety in the Tg mice or induced a deterioration in NTg mice. Effects of ovariectomy for both strains were only detected as some marginal trends of decreased performance in the corner test, open field test and dark-light box test. However, ovariectomy-induced impairments were detected in a test requiring active exploration such as the hole-board test. The main effect was an increase of the latency to explore the four holes in ovariectomized Tg mice. Interestingly, exercise training impeded most signs of neophobia, anxiety, decreased activity and apathetic behavior in ovariectomized Tg mice similarly to the protection afforded to control Tg mice, as previously reported this AD mouse model (García-Mesa et al., 2011, 2012). Moreover, physical exercise improved the behavior of NTg mice; whether sham-operated or ovariectomized, as showed by their improved performance in the corner test, dark-light box test and hole-board test. These results confirm the general mood improvement effect of physical exercise, including the effects reported in postmenopausal women (Villaverde Gutiérrez et al., 2012) and AD patients (Knöchel et al., 2012).

Ovariectomy induced a trend towards a decreased capacity of learning acquisition in the NTg mice, whereas ovariectomized Tg mice showed a similar acquisition curve as sham-operated Tg mice. Retention of learning was impaired in ovariectomized NTg and Tg and sham-operated Tg mice. Females of the 3xTg-AD strain had shown a consistent lack of learning and memory in the MWM test from early ages (García-Mesa et al., 2011). Ovariectomy induces a premature aging of the nervous system (Baeza et al., 2010), and it decreases cognitive abilities in rodents (Ben et al., 2010). However, 3xTg-AD mice already show traits of neurosenescence (Giménez-Llort et al., 2008). In humans it has long been known that sex steroids influence behavior and cognition, including visual-spatial abilities (Hampson, 1995). Menopause induces a rapid loss of estrogens in the brain, that will pave the way to the deterioration of cognitive functions (for review see: Pompili et al., 2012).

Physical exercise protected Tg mice and both groups of ovariectomized mice from cognitive loss. These results support those of previous studies on the cognitive improvement seen in this strain and other strains of AD mice following a treatment of physical exercise (García-

Mesa et al., 2011; Intlekofer and Cotman, 2013) and demonstrate the protective effect of exercise on spatial learning and memory in the ovariectomized AD Tg mice. A variety of patterns of aerobic physical exercise have been shown to induce cognitive benefits in older women. Only long-term strenuous activity prior to menopause was reported to decrease cognitive outcome in later stages of life (Tierney et al., 2010). Aerobic exercise improves executive performance, and decreases cognitive decline and AD risk in elder women (Yaffe et al., 2001; Bixby et al., 2007). Furthermore, some meta-analyses have suggested that exercise interventions improve cognition in AD patients (Heyn et al., 2004).

We did not find an exacerbation of the amyloid and p-tau parameters by ovariectomy, as has been reported by other authors in this mouse model at the younger age of 6 months (Carroll et al., 2010; Yao et al., 2012). This pathology was not significantly protected by physical exercise in either sham-operated or ovariectomized Tg mice. Nonetheless, running exercise protected all Tg mice against BPSDs and learning and memory loss as discussed above. Therefore, the protective effects of exercise in both groups of Tg mice would be mainly triggered by prosurvival pathways involving antioxidant signaling and neurotrophic factors.

Brain oxidative stress increases with older age and it is a proposed link between aging and AD (Viña et al., 2004). Ovarian hormone loss induces mitochondrial dysfunction and oxidative stress in ovariectomized mice (Yao et al., 2012), in line with the above mentioned induction of premature aging. Oxidative imbalance of 3xTg-AD female mice increases with the mouse age and AD-like pathology severity, with progressively increased levels of lipoperoxides and oxidized glutathione (Resende et al., 2008; Yao et al., 2009, 2012; García-Mesa et al., 2011, 2012). One of the functional mechanisms of physical exercise is an increase in the antioxidant response that restores redox homeostasis in the brain (Radák et al., 2001). In the present study at 9 months of age, an increase in gene expression of catalase in the hippocampus of the sedentary and exercised Tg groups would indicate an active defense against oxidative stress. Furthermore, the low level of changes of GPx and MnSOD would be in accordance with an intermediate stage of oxidative stress at this age.

It is known that physical exercise enhances brain plasticity and improves neuronal survival through the triggering of signaling cascades and growth factor induction (Cotman and Berchtold, 2002; Cotman et al., 2007). Tg and ovariectomized mice showed reduced plasticity responses compared with NTg mice. PGC-1 α was activated by physical exercise in NTg mice but not in ovariectomized mice or Tg mice. Transcription dysregulation of PGC-1 α will contribute to mitochondrial dysfunction in ovariectomized and Tg mice. Indeed, the 3xTg-AD mice have shown mitochondrial complex deficiencies (García-Mesa et al., 2012) and decreased

respiration (Yao et al., 2009), in addition to the above discussed oxidative stress. A novel role recently attributed to PGC-1 α is and the formation and maintenance of dendritic spines and maturation of synapses in hippocampal neurons (Cheng et al., 2012). As regards CREB, impaired CREB signaling has been implicated in memory deficits associated with aging and AD (Saura and Valero, 2011). As expected, chronic wheel-running activity increased p-CREB in the sham-operated NTg mice. Furthermore, Tg mice showed a similar response of CREB activation. Nonetheless we did not observe basal changes of p-CREB, whereas other authors have reported either a decrease (Caccamo et al., 2010) or an early increase in this mouse model (Müller et al., 2011). The impairment of this plasticity pathway following ovariectomy supported previously reported decreases of hippocampal p-CREB in ovariectomized rats (Sharma et al., 2007). Thus, deficient signaling of this transduction factor is probably implicated in the cognitive deficits of Tg and ovariectomized mice.

In this study the exercise-induced increase of BDNF strongly correlated with behavioral responses of decreased anxiety, increased active exploration, and improved learning. Therefore, hippocampal levels of BDNF increased coordinately with the behavioral and cognitive improvement in ovariectomized and Tg groups, thus reinforcing the mediation of BDNF in the neuroprotective changes induced by physical exercise (Cotman and Bertchold 2002; Adlar et al., 2005; Cotman et al., 2007). In humans, increasing BDNF through aerobic exercise was reported to ameliorate age-related hippocampal atrophy and memory dysfunction and reduce depression (Erickson et al., 2012). We observed a decrease of TrkB expression in the hippocampus of the Tg mice, which would contribute to the derangements of BDNF signaling in this mouse model. However we did not find a significant decrease of BDNF in the hippocampus of this mouse model, as previously reported (Caccamo et al., 2010). Activation of TrkB receptors by BDNF induces several signalling pathways (Santos et al., 2010) that would converge in the synaptic plasticity and neurotransmission improvements required for the mood-related and memory improvements discussed previously. An effect of increased levels of BDNF by physical exercise was noticeable in Tg mice and ovariectomized NTg and Tg mice, but not in sham-operated NTg. Therefore, it appears that BDNF was increased through exercise when the hippocampal signaling was disturbed. Alternatively, middle-aged NTg mice submitted to chronic exercise training exhibited a lesser degree of BDNF increase than expected for young mice (Adlard et al., 2005). Furthermore, differential induction of BDNF could be influenced by the complex interactions that occur between ovarian steroids and BDNF in the hippocampus (Franklin and Perrot-Sinal, 2006; Scharfman and MacLusky, 2014). Studies in humans have found either positive or

negative correlations between physical exercise and BDNF levels in blood (Vega et al., 2011; Lee et al., 2014), thus highlighting the complexity of BDNF regulation.

In summary, ovariectomy induced a deterioration of fitness and cognition in NTg mice and a worsening of apathy responses in the Tg mice. However, ovariectomy did not induce a significant exacerbation of AD pathology. Nonetheless, three months of physical exercise in a freely available running wheel protected 9-month old Tg and NTg female mice submitted to ovariectomy (at two month before starting the exercise training) against brain alterations and loss of brain plasticity. Physical exercise induced a recovery of BPSD-like changes in Tg groups, and cognitive loss as seen in Tg mice and in ovariectomized Tg and NTg mice. Neuroprotection in Tg mice did not appear mediated through a reduction of amyloid or tau pathology, but rather through the enhancement of mechanisms of neuroprotection and plasticity, such as those involving catalase, p-CREB and BDNF. Remarkably, there was a positive correlation between BDNF levels and amelioration of behavior and cognition. These results confirm the value of moderate aerobic exercise training as a lifestyle that effectively induces neuroprotection in postmenopausal women and reinforce the role of BDNF as a molecular effector of physical exercise neuroprotection.

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Figures

Figure 1 Effects of ovariectomy on running activity (A) and ovariectomy and physical exercise on frailty status (B-D) in 3xTg-AD (Tg) and non-transgenic (NTg) mice. A) Tg mice submitted to physical exercise (Ex) through free access to a running wheel were more active than NTg mice, whereas ovariectomized (Ov) mice showed lower running activity than those of the same strain in sedentary conditions. B) Physical exercise increased grip strength in NTg mice, both

control and ovariectomized mice. Exercise protected against a decrease in grip strength seen in TgOv mice. C) Ovariectomized mice showed decreased body weight. D) White abdominal fat (WAT) was higher in ovariectomized NTg mice, whereas exercise showed a tendency to decrease WAT in most groups of mice. C) Uterus weight is included to monitor the efficacy of ovariectomy. Values are the mean $\pm$ SEM, n=8-9. $\Delta\Delta p < 0.01$, $\Delta\Delta\Delta p < 0.001$ overall effect of ovariectomy in (C, E). $**p < 0.01$, $***p < 0.001$ compared to NTg+Ex group, and $####p < 0.001$ compared to Tg+Ex group in (A). $**p < 0.01$ compared to NTg group, $##p < 0.01$ compared to Tg group, $\&\&p < 0.01$ compared to the ovariectomized NTg group, $\$p < 0.05$ compared to the ovariectomized Tg group in (B, D) (Fisher's LSD post-hoc test).

Figure 2 Effects of ovariectomy and physical exercise on neophobia and open field test behavior in 3xTg-AD (Tg) and non-transgenic (NTg) mice. A-C). In the corner test, Tg mice showed a higher latency of rearing (A) and a reduced number of rearings (B) and corners (C). These effects were ameliorated by physical exercise (Ex). Mouse neophobia was not modified by ovariectomy (Ov). D-F) In the open field test, Tg mice showed an increased latency of rearing

(D) and reduced number of rearings (E) and crossings (F). Physical exercise ameliorated these behaviors. Ovariectomy slightly impaired the open field behavior. Values are the mean $\pm$ SEM, n=8-9. ⁺⁺p<0.01, ⁺⁺⁺p<0.001 overall effect of genotype. ^sp<0.05, ^{sss}p<0.001 overall effect of physical exercise. *p<0.05, **p<0.01, ***p<0.001 compared to NTg group; #p<0.05, ##p<0.01 compared to Tg group; &&&p<0.001 compared to the ovariectomized NTg group; \$p<0.01, \$\$\$p<0.01 compared to the ovariectomized Tg group (Fisher's LSD).

Figure 3 Effects of ovariectomy and physical exercise on anxiety and exploratory behavior in 3xTg-AD (Tg) and non-transgenic (NTg) mice. A-C) In the dark-light box test, Tg mice showed higher latency to enter into the illuminated area (A), a lower number of entrances (B) and a lower time of permanence in the illuminated area (C). These anxiety behaviors were barely increased in the ovariectomized (Ov) Tg mice, whereas physical exercise showed an effective protection. D-F) The Boissier's hole-board test showed lower exploratory behavior in the ovariectomized Tg

mice than in the sham-operated mice, as can be seen in the latency of the first entry into the four holes (D), number of head-dips (E) and total dipping time (F). All parameters were effectively ameliorated by physical exercise. Values are the mean $\pm$ SEM, n=8-9. $^{\S}p<0.05$, $^{\S\S\S}p<0.001$ overall effect of physical exercise. $^{*}p<0.05$, $^{**}p<0.01$ compared to NTg group; $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ compared to Tg group; $^{\&}p<0.05$, $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$ compared to the ovariectomized NTg group; $^{\$}p<0.01$, $^{\$ \$ \$}p<0.001$ compared to the ovariectomized Tg group (Dunn's test in (A, D), Fisher's LSD in (B, C, E, F)).

Morris Water Maze

Figure 4 Effects of ovariectomy and physical exercise on spatial memory acquisition and retention in 3xTg-AD (Tg) and non-transgenic (NTg) mice measured by the Morris water maze test. A) Swimming distance to reach the platform location. B) Time spent swimming in the platform quadrant of the pool and in the opposite quadrant after removal of the platform to test the retention of learning. Ovariectomy partially worsened place task acquisition and impaired memory retention in NTg mice. Exercise ameliorated learning and memory in the ovariectomized NTg mice and all Tg mice. Values are the mean $\pm$ SEM, $n=8-9$. See text for statistics in (A); * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to the platform quadrant for each experimental group in (B) (Bonferroni).

Figure 5 Effects of ovariectomy and physical exercise on the amyloid β and tau pathology in cerebral cortical tissue of 3xTg-AD (Tg) mice. A) Representative immunoblot and densitometric analysis of the APP carboxy-terminal fragment C99 relative to APP levels (C99/APP). B) Representative immunoblots and densitometric analyses of p-tau protein detected with clone AT8. Data were normalized to pan actin levels. Ovariectomy did not induce a worsening of amyloid and tau pathology in cerebral cortex. Physical exercise induced a marginal reduction of C99/APP levels. Values are the mean $\pm$ SEM, $n=4$. * $p < 0.05$ compared to NonTg (Bonferroni).

Figure 6 Effects of ovariectomy and physical exercise on the levels of antioxidant and neuroplasticity signaling molecules in the hippocampus of 3xTg-AD (Tg) and non-transgenic

(NTg) mice. A) Catalase, RNA levels. B) MnSOD, RNA levels. C) PGC-1 α , and D) p-CREB/CREB, protein levels by Western blot (representative immunoblots and densitometric analysis; data were normalized to α -tubulin levels). E) BDNF, protein levels by ELISA. F) TrkB, protein levels by Western blot. Catalase was increased in Tg mice. Ovariectomy inhibited the induction of p-CREB through physical exercise but did not significantly interact with the induction of PGC-1 α and BDNF. Values are the mean $\pm$ SEM, n=5-8. ⁺p<0.05 overall effect of genotype; ^{§§§}p<0.001 overall effect of physical exercise. *p<0.05, **p<0.01 compared to NTg group; ##p<0.01 compared to Tg group (Fisher's LSD).