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# Physical exercise improves synaptic dysfunction and recovers the loss of survival factors in 3xTg-AD mouse brain

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ABSTRACT

Physical exercise has become a potentially beneficial therapy for reducing neurodegeneration symptoms in Alzheimer's disease. Previous studies have shown that cognitive deterioration, anxiety and the startle response observed in 7-month-old 3xTg-AD mice were ameliorated after 6 months of free access to a running wheel. Also, alterations in synaptic response to paired-pulse stimulation were improved. The present study further investigated some molecular mechanisms underlying the beneficial effects of 6 months of voluntary exercise on synaptic plasticity in 7-month-old 3xTg-AD mice. Changes in binding parameters of [$^3$H]-flunitrazepam to $GABA_A$ receptor and of [$^3$H]-MK-801 to NMDA receptor in cerebral cortex of 3xTgAD mice were restored by voluntary exercise. In addition, reduced expression levels of NMDA receptor NR2B subunit were reestablished. The synaptic proteins synaptophysin and PSD-95 and the neuroprotective proteins GDNF and SIRT1 were downregulated in 3xTgAD mice and were recovered by exercise treatment. Overall, in this paper we highlight the fact that different interrelated mechanisms are involved in the beneficial effects of exercise on synaptic plasticity alterations in the 3xTg-AD mouse model.



## 1. Introduction

AD is a progressive neurodegenerative disease eventually leading to cognitive impairment of the brain and consequently memory and short-term attention loss. However, apart from the cognitive deficiencies, most affected individuals also present non-cognitive alterations known as Behavioral and Neuropsychiatric Symptoms of Dementia (BPSD), including apathy, depression, emotional disorders, psychosis and anxiety (Giménez-Llort et al., 2007). Recent studies performed in aged animals and in animal models of AD, have revealed that deterioration in cognitive performances is associated with significant changes in synaptic plasticity (Balietti et al., 2012).

It is suggested that changes in our lifestyle might retard either the onset or the progression of AD. In fact, regular physical exercise enhances brain functionality by improving neuroplasticity, neurotransmission, learning, memory and cognition (Molteni et al., 2002; Van Praag et al., 2005; Pang and Hannan, 2013). Moreover, running exercise has been shown to improve memory task performance in several transgenic strains of mice, including Tg2576 (Yuede et al., 2009) and 3xTg-AD (García-Mesa et al., 2011, 2012). Importantly, the results of many studies confirm that the enhancement of LTP and synaptic plasticity occurs in response to physical exercise (Cotman and Berchtold, 2002).

One of the functions of the excitatory receptor N-methyl-D-aspartate (NMDA) is to maintain synaptic plasticity. It has been shown that mRNA and protein expression levels of NR2A/2B subunits of the NMDA receptor are significantly reduced in susceptible regions of the AD brain as pathology progresses (Mishizen-Eberz et al., 2004; Hynd et al., 2004). In parallel with the declines in the glutamatergic receptor system, there is also evidence revealing age-related changes in the γ-aminobutyric acid (GABA) system (Rissman and Mobley, 2011). Rissman et al. (2007) have reported that $GABA_A$ receptors appear to be the least affected class of receptor in the AD brain hippocampus. Biochemical investigations have demonstrated moderate reductions (13.5%) of α5 subunit with

*Abbreviations:* AD, Alzheimer's disease; APP, amyloid precursor protein; BPSD, Behavioral and Neuropsychiatric Symptoms of Dementia; ANOVA, analysis of variance; BDNF, brain-derived neurotrophic factor; EXE, exercise; GABA, γ-aminobutyric acid; GDNF, glial-derived neurotrophic factor; NMDA, N-methyl-D-aspartate; NTg, non-3xTg-AD mouse; LTP, long-term potentiation; SED, sedentary; SEM, standard error of the mean; Tg, 3xTg-AD mouse.

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increasing AD neuropathology (Rissman et al., 2003). Ultimately, Hill et al. (2010) have demonstrated that voluntary exercise induced changes in $GABA_A$ receptor subunits in the forebrain of rats. Indeed, previous studies using the 3xTg-AD mice approach, have demonstrated that forced exercise reversed the flunitrazepam binding affinity to control values, although it barely ameliorated the behaviors associated with anxiety and depression (Giménez-Llort et al., 2010).

There is emerging evidence that synaptic plasticity and cognitive function can be strongly affected by shifts in the relative abundance of the various synaptic scaffolding such as PSD95 (Ehrlich and Malinow, 2004). With respect to pre-synaptic systems, synaptophysin has been demonstrated to be closely related to cognitive processes and has been shown to develop a relevant role in synaptic plasticity (Schmitt et al., 2009). Several authors have reported changes of synaptophysin and PSD95 protein expression in brain areas of AD patients (Leuba et al., 2008; Proctor et al., 2010). On the other hand, several authors have reported that synaptophysin and PSD95 levels increased in the cerebral cortex and hippocampus of rats after physical activity (Vaynman et al., 2006; Hu et al., 2009; Quirié et al., 2012) supporting the hypothesis that exercise enhances learning and memory processes.

In contrast to the brain-derived neurotrophic factor (BDNF) that is already known to be critically involved in plasticity, memory and other cognitive processes, the role of glial-derived neurotrophic factor (GDNF) in such functions is less studied. A recent report indicated that GDNF concentrations are significantly up-regulated in cerebrospinal fluid and down-regulated in serum in patients with early AD (Straten et al., 2009). Given its involvement in synaptogenesis, GDNF could potentially play a further role in synaptic plasticity so as to face the alterations in learning and memory characteristic of AD. It is well recognized that BDNF plays a role in exercise-mediated neuroprotective effects; however it is not known if GDNF plays a similar role. Recently, McCullough et al. (2013) have reported that short-term exercise increases GDNF protein in the lumbar spinal cord of 6-month-old rats.

SIRT1 regulates chromatin remodeling and histone acetylation (Vaquero et al., 2004). Their beneficial roles are promoting longevity in lower organisms and also improve health in rodents (Baur, 2010). SIRT1 promotes benefits in age-dependent neurodegenerative disorders (Herskovits and Guarente, 2013), and also promotes plasticity and memory in a direct manner through a mechanism distinct from its established neuroprotective activity (Gao et al., 2010; Michán et al., 2010). A few studies have addressed the role of SIRT1 in the brain effects of exercise in rats (Gomez-Pinilla and Ying, 2010; Bayod et al., 2011).

Previous results have demonstrated that physical activity not only barely ameliorates Aβ and tau pathologies (García-Mesa et al., 2011), but also, enhances mitochondrial biogenesis (García-Mesa et al., 2012), and cognitive function (Giménez-Llort et al., 2010). This study therefore aims to elucidate the neurochemical mechanisms of voluntary exercise in the 3xTg-AD mice mouse model. To achieve this goal, an evaluation will be carried out on the beneficial effects of long-term voluntary exercise therapy on $GABA_A$ and NMDA receptors in terms of functionality whilst addressing the changes in the expression levels of their respective α5 and NR 2A and 2B subunits in the 3xTg-AD mouse model. Alterations in synaptic plasticity will also be analyzed in order to confirm the benefits of voluntary physical activity against neurodegenerative disorders such as AD.

## 2. Material and methods

### 2.1. Animals

The 3xTg-AD (Tg) mice were derived by co-microinjecting two independent transgenes encoding human APPSwe and the human tauP301L (both under control of the mouse Thy1.2 regulatory element) into single-cell embryos harvested from homozygous mutant PS1M146V knockin (PS1-KI) mice. These mice were genetically engineered at the University of California, Irvine (Oddo et al., 2003). For these studies, 7 months Tg and age-matched non-transgenic (NTg) mice from the Spanish colony of homozygous 3xTg-AD mice established in the Medical Psychology Unit, Autonomous University of Barcelona (Giménez-Llort et al., 2007) were subjected to different physical exercise therapies (see below). All mice were maintained (Makrolon, 35 × 35 × 25 cm) under standard laboratory conditions (food and water ad libitum, 22 ± 2 °C, 12 h light: dark starting at 08:00 a.m.).

### 2.2. Administration of voluntary exercise on the running wheel

Animals were randomly assigned to one of two treatment groups (exercise, EXE; sedentary, SED). NTg and Tg groups submitted to voluntary exercise (NTgEXE and TgEXE) had access to a running wheel for 6 months, and were evaluated when they were 7 months old, at a moderate pathological stage of AD. Groups of healthy mice (NTgSED) and of transgenic mice (TgSED) with no access to exercise were used as controls respectively. Exercising animals were placed in standard cages equipped with one running wheel of free access in each cage (Activity Wheel Cage System for Mice; Techniplast, Buguggiate, Italy), as previously described (García-Mesa et al., 2011). Sedentary animals were left undisturbed in their respective homecages. Wheel revolutions were recorded by computer using Vital Viewer software (Mini Mitter Company, Inc., OR, USA). Brain was dissected to obtain hippocampus (used for western-blotting) and cerebral cortex (used in radioligand experiments). Tissue samples were stored at −80 °C until analysis (see below).

### 2.3. Flunitrazepam and MK-801 binding assays

Synaptic membrane preparations were obtained from mice cerebral cortices. Brain samples were thawed and homogenized in 50 volumes (w/v) of Tris Citrate NaCl buffer (50 mM, pH 7.4) or Tris–HCl buffer (50 mM, pH 7.4) for flunitrazepam or MK-801 binding experiments, respectively. Then, samples were centrifuged at 20,000 × *g*, for 20 min 4 °C. Supernatants were discarded and the pellet was resuspended in 1.5 mL of the same buffer and again centrifuged at 20,000 × *g*, at 4 °C for 20 min. This procedure was repeated five times after which, pellets were resuspended in 20 volumes of 50 mM Tris Citrate NaCl or Tris–HCl buffer. The last suspension was frozen at −80 °C until immediately before the binding assay, when they were thawed on ice and the tissue was dispersed by pulse homogenization and centrifuged at 16,000 × *g*, for 20 min at 4 °C. The membranes were then resuspended in 1 mL of 50 mM Tris–HCl buffer/200 mM NaCl. Proteins were measured in 200 μL by the Bradford method. For the flunitrazepam binding assays an aliquot of each membrane sample containing 30 μg of protein was combined with 0.3–8 nM [$^3$H]-flunitrazepam, (specific activity 87 Ci/mmol, NEN Life Science Products, USA), 50 mM Tris–HC1 (pH 7.4), 20 μM diazepam (Sigma Chemical, Co, St Louis, MI, USA) and 100 μM GABA (for specific binding) in a final reaction volume of 1 mL for 30 min at 25 °C. Binding obtained in the presence of 20 μM diazepam was considered to be non-specific. For the MK-801 binding assays, an aliquot of each membrane sample containing 200 μg of protein was combined with 2.5 nM [$^3$H]-MK-801 (120 Ci/mmol, Perkin Elmer, MA) and different concentrations (0 nM, 1 nM, 3 nM, 10 nM, 30 nM, 100 nM) of unlabeled MK-801 (Sigma Chemical, Co, St Louis, MI, USA) for 30 min at 25 °C. Binding obtained in the presence of 100 μmol/L of unlabeled MK-801 was considered to be non-specific. Incubations were terminated by rapid filtration through 0.05% polyethyleneimine-treated Whatman GF/C filters (BDH, Lulterworth, UK) using a Brandel equipment (Gaithersburg, MD, USA) and rinsed with cold Tris buffer for 10 times. Just in MK-801 experiments, filters were previously incubated for 10 min in Tris–HCl buffer 50 μM containing 0.2% of polyethyleneimine (Sigma Chemical, Co, St Louis, MI, USA) to avoid the inespecific binding of the radioligand to the filter. The amount of bound radioactivity retained on the filters was determined following the addition of 2 mL of OptiPhase "Hisafe" scintillation cocktail in a Wallac 1414 Winspectral liquid scintillation counter (Perkin Elmer, Waltham, MA). Specific binding was calculated as the difference between total and non-specific binding. Radioligand binding data were subjected to analysis in order to obtain the variables for binding site density ($B_{max}$, in pmol/mg protein) and binding affinity ($K_d$, in nM).

### 2.4. Immunoblotting

Hippocampus was suspended in 15 volumes of ice-cold RIPA buffer (1% IGEPAL, 0.5% sodium salt of the desoxicolic acid, 0.1% SDS in PBS) supplemented with 1 mM orthovanadate, 5 mM sodium fluoride, 2% SDS and 1 tablet Complete Protease Inhibitor Cocktail/50 mL solution (Roche, Manheim, Germany). Then, samples were sonicated and, after centrifuging (12,000 × *g*, 20 min, 4 °C), supernatants were collected in order to determine the total protein concentration by the Bradford method using bovine serum albumin as standard. Sixty μg of protein were charged in a polyacrylamide gel, and separated by electrophoresis. Finally, the gel was transferred to a 0.45 μm PVDF membrane (Immobilon-P, Millipore). The membranes were blocked for 1 h at room temperature in different solutions depending on the proteins analyzed: for phosphorylated proteins, we used TBS-T buffer (0.02 M Tris base pH 7.5, 0.15 M NaCl (TBS), 0.1% Tween 20) with 5% BSA; to analyze the protein APP, Aβ peptide and C-terminal fragments 99 and 83, we used TBS buffer with 10% skim milk and 0.1% BSA; for other proteins, we used TBS-T buffer with 5% skim milk.