Early cognitive stimulation compensates for memory and pathological changes in Tg2576 mice

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A B S T R A C T

Education and cognitive occupations are commonly associated to reduce risk of Alzheimer’s disease (AD) or dementia. Animal studies have demonstrated that cognitive stimulation (CS) achieved by social/physical activities and/or enriched environments compensates for memory decline. We have elaborated a novel paradigm of CS that is devoid of physical/social activity and enriched environments. 4 month-old Tg2576 mice were cognitively trained for 8 weeks and, after a break of 8 months, long-lasting effects of CS on cognitive abilities and AD-like pathology were measured. Morris Water Maze (MWM) and Novel Object Recognition (NOR) tests showed that deficits in spatial and recognition memories were compensated by CS. These outcomes were accompanied by increased levels of hippocampal post-synaptic markers (PSD95 and NR1) and proteins involved in synaptic formation (Arc, β-catenin). CS softened amyloid pathology in terms of reduced levels of Aβ42 and the dodecameric assembly, referred as Aβ56. CS appeared to affect the APP processing since differences in levels of ADAM17, BACE1 and C99/C83 ratio were found. Tau hyper-phosphorylation and high activities of tau kinases were also reduced by CS. In contrast, CS did not induce any of these molecular changes in wild-type mice. The present findings suggest beneficial and long-lasting effects of CS early in life on cognitive decline and AD-like pathology.

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1. Introduction

Dementia is a syndrome defined by clinical abnormalities in such cognitive abilities as memory, attention and problem solving. The most common form of dementia is Alzheimer’s disease (AD). Aetiology of sporadic AD (99% of cases) may be the result of the combination of several genetic and environmental factors. Although the pathological traits of AD are present long before patients manifest clinical symptoms, an associative link between them does not have to be necessarily implied. Accordingly, numerous neuroimaging and autopsy observations have brought to light the disparity about clinical and pathological correlates. This lack of association is conceptualised by the hypothesis of “cognitive reserve”, and is likely to be regarded as life environmental factor-induced mechanisms that help in maintaining the cognitive ability in the face of deleterious AD pathology [1]. Epidemiological studies have determined education [2], cognitively complex occupations [3,4], leisure activities [5], and to a lesser extent, physical activity [6], as the most important protective factors that can delay or prevent the onset of clinical symptoms. Although little improvement of cognitive function has been observed in humans after “brain training” sessions, further empirical evidences from animal studies are required [7].

Murine models of AD and other neurodegenerative diseases have been tested in enriched environment (EE) paradigms that are initially aimed at stimulating cognitive activities. Exposure of EE animal models of AD has reported both prevention of cognitive dysfunction [8–10] or little or no effect on memory [11,12]. Some controversies about the effects on amyloid pathology have also been reported [13–15]. A key question lies on the interpretation of what the EE is modelling. For example, EE may be modelling a normal daily lifestyle relative to standard housing as a deprivation state instead [16]. Another point refers to the complexity of EE protocol. It usually requires the exposition to physical activity, social interaction or enriched environments, therefore making hard the identification of the relative contribution of each factor to the observed cognitive benefits. In this sense, isolated social or physical enrichment was reported to occur with either insufficient or poor cognitive benefits [9,17,18], suggesting that enhanced cognitive stimulation is also required. A paradigm for cognitive enrichment evading other types of sensory-motor stimulations might be developed in order to provide support to the hypothesis formulated by epidemiological data that intellectual occupations contribute to cognitive reserve and, eventually, compensation for AD pathology.
In the present work, we have elaborated and validated a novel protocol for cognitive stimulation (CS) that comprises a long training on problem-solving tasks. The transgenic mice Tg2576 and their wild-type littermates were used to study the long-term effects of cognitive stimulation on AD pathology and cognitive functioning.

2. Material and methods

2.1. Animals

Sixteen female transgenic mice (Tg2576) over-expressing human amyloid precursor protein (hAPP) carrying the Swedish familial mutation (K670N/M671L) [19] under the genetic mixed hybrid background C57BL/6SJ and sixteen wild-type littermates (WT) with the same genetic background were used. At the age of 3 months, the mice were housed in standard laboratory cages (43 × 27 × 15 cm) in groups of 4. All mice were kept in the same room under controlled temperature (21 ± 1 °C) and humidity (55 ± 5%) on a 12-h light–dark cycle. Cage bottoms, water bottles and sipper tubes were cleaned twice per week, wire lids were cleaned monthly, and food/water were provided ad libitum. All the animals were handled under the same conditions and by the same experimenters throughout the duration of experiments in order to avoid potential influence on cognition.

As described later in the text, following the assessment of memory function through a short version of Morris Water Maze, the mice were assigned to “cognitive stimulation” (CS) or “naïve” groups in order to create uniform groups in terms of basal cognitive function.

Experimental procedures were conducted in accordance with the European and Spanish regulations (2003/65/EC; 1201/2005) and approved by the Ethical Committee of University of Navarra (068-11).

2.2. Cognitive stimulation paradigm

Cognitive stimulation paradigm was aimed at keeping the mice cognitively active for a period of 8 weeks. For this purpose, the mice were allowed to find and learn the pathway to reach a goal through a Lashley-type maze. The goal consisted of a food stimulus, in particular a piece of wheaten “digestive” biscuit. This paradigm intends to generate a response learning, where mice choose the correct sequence of right–left responses depending on which choice led them to the food stimulus in previous trials. The food stimulus reinforces the correct choices. No extra-maze cues were allowed in order to avoid place learning. Cognitive stimulation was induced by exposing mice to solve 8 different problems, one per week. 8 different Lashley-type mazes were manufactured in our lab, having every maze (made of black wooden walls: 27 × 54 × 18 cm) a unique design so that there is only one pathway, different to each other maze, to reach the goal. A schematic illustration of each maze can be seen in Fig. 1A. The animals were trained for 4 days (Monday, Tuesday, Wednesday and Friday) and 2 trials per day in each of the 8 mazes for 8 weeks. Number of entry errors and latency to reach the goal was measured as the main outcomes. Three criteria were applied in the discrimination index (percentage of time exploring the novel object to the total time of object exploration).

Prior to the training in the CS paradigm, cognitive behaviour of 4-month-old mice was analysed by a short version of MWM. 3 days prior to the hidden platform phase, a visible platform version was carried out for 3 consecutive days and 4 trials per day with an inter-trial interval of 2 h. Animals that showed to be floating, thigmotactic, wandering and other behaviours that could affect learning were discarded from the study, and the rest were next trained in the hidden platform version for 3 consecutive days and 4 trials per day with an inter-trial interval of 2 h. The retention phase (no platform) was carried out on the 4th day. Mice were allowed to swim for 60 s and time in the target quadrant was measured. Besides checking the cognitive status of WT and Tg2576 mice before the beginning of the training paradigm, the short version of MWM allowed an equally distribution of animals between CS and naïve groups in terms of cognitive abilities.

Just after the 8-week training paradigm, 15-month-old mice were trained for 6 consecutive days and 4 trials per day with an inter-trial interval of 2 h in the hidden platform version. To ensure that memory differences were not due to lack of task learning, mice that did not reach the platform were led to the platform and allowed to stay for 15 s. The visible platform version was avoided since it was performed at the beginning of the study. The retention phase (no platform) was carried out at the 4th and 7th days. The mice were allowed to swim for 60 s and time in the target quadrant was measured.

2.4. Biochemical measurements

2.4.1. Protein extraction

15-month-old fasting mice were killed by decapitation. For immune-blotting of most of the proteins shown in the present work, proteins were extracted from hippocampi after homogenization in HEPES buffer saline (0.2 M NaCl, 2 mM Na₂HPO₄, 0.1 M HEPES, 10% glycerol, 200 mM, 5 mM EDTA, 1 mM EGTA, 2 mM DTT) containing protease and phosphatase inhibitors (NaF, 0.5 mM PMSF, 1 mM Na₂VO₄, 1 mM benzamidine,
10 mg/ml leupeptin, 400 U/ml aprotinin) and 1% NP-40. Homogenates were centrifuged at 14,000 g for 20 min at 4 °C and supernatant stored at −80 °C.

For immune-blotting of APP-derived fragments, proteins were extracted from hippocampi after homogenization in a buffer containing Tris–HCl (10 mM, pH 7.4), SDS 2% and protease and phosphatase inhibitors. Homogenates were sonicated and centrifuged at 100,000 g for 1 h. Supernatant was stored at −80 °C. Protein concentration was determined by Bradford (Bio-Rad, Hercules, CA, USA).

For ELISA determination of Aβ1–42 and Aβ1–40 peptides from soluble and insoluble fractions, protein were extracted as follows: hippocampi were homogenized in 4 volumes of ice-cold guanidine buffer (5 M guanidine–HCl, 10 mM, pH 8.0) diluted 1:20. After over-night mixing at room temperature in DPBS buffer containing 5% BSA and 0.03% Tween-20, sample was centrifuged at 20,000 g for 20 min at 4 °C. Supernatant contained the "soluble fraction" and pellet contained the "insoluble fraction". To prepare the insoluble fraction, pellet was dissolved in 4 volumes of ice-cold guanidine buffer (5 M guanidine–HCl, 50 mM Tris–HCl, pH 8.0) diluted 1:20. After over-night mixing at room temperature sample was centrifuged at 20,000 g for 20 min at 4 °C. Aβ1–42 and Aβ1–40 peptides from Tg2576 samples were measured in duplicate by a sandwich nitrocellulose membranes (Pall Corporation, Port Washington, NY, USA). Gel conditions and primary antibodies are summarized in Table 1. Secondary antibodies conjugated to IRDye 800CW or IRDye 680CW (LI-COR Biosciences, Lincoln, NE, USA) were diluted to 1/1,500 in TBS with 5% BSA. Bands were visualized using Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). β-Actin was used as loading control. Results were expressed as O.D. values relative to WT-naive mice. For APP-derived fragments, samples were mixed with XT sample buffer plus XT reducing agent (Bio-Rad, Hercules, CA, USA) and boiled for 5 min. Proteins were transferred to PVDF membranes (Amersham Biosciences, Buckinghamshire, UK). HRP-conjugated secondary antibodies were diluted to 1:5,000 (Dako, Glostrup, Denmark). Bands were detected by enhanced chemiluminescence system (ECL, Amersham Biosciences, Buckinghamshire, UK), and autoradiographic exposure to Hyperfilm ECL (Amersham Biosciences), Signal was quantified by using Quantity One v4.6.3 software (Bio-Rad, Hercules, CA, USA).

Note that some proteins bands were obtained as a result of stripping (stripping buffer, Pierce, IL, USA) and re-probing the membrane. However, membranes were never reused for more than 4 times (Table 1).

2.4.2. Immuno-blotting

Samples were mixed with equal volume of loading buffer (0.16 M Tris–HCl pH 6.8, 4% SDS, 20% glycerol, 0.01% bromphenol blue, 0.1 M DTT) and ran in SDS-PAGE. Proteins were transferred to BioTrace™ Nitrocellulose membranes (Pall Corporation, Port Washington, NY, USA). Gel conditions and primary antibodies are summarized in Table 1. Secondary antibodies conjugated to IRDye 800CW or IRDye 680CW (LI-COR Biosciences, Lincoln, NE, USA) were diluted to 1/1,500 in TBS with 5% BSA. Bands were visualized using Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). β-Actin was used as loading control. Results were expressed as O.D. values relative to WT-naive mice. For APP-derived fragments, samples were mixed with XT sample buffer plus XT reducing agent (Bio-Rad, Hercules, CA, USA) and boiled for 5 min. Proteins were transferred to PVDF membranes (Amersham Biosciences, Buckinghamshire, UK). HRP-conjugated secondary antibodies were diluted to 1:5,000 (Dako, Glostrup, Denmark). Bands were detected by enhanced chemiluminescence system (ECL, Amersham Biosciences, Buckinghamshire, UK), and autoradiographic exposure to Hyperfilm ECL (Amersham Biosciences), Signal was quantified by using Quantity One v4.6.3 software (Bio-Rad, Hercules, CA, USA).

Note that some proteins bands were obtained as a result of stripping (stripping buffer, Pierce, IL, USA) and re-probing the membrane. However, membranes were never reused for more than 4 times (Table 1).
**Table 1**

Antibodies and conditions used in immunoblotting experiments.

<table>
<thead>
<tr>
<th>Protein</th>
<th>% Polyclayamide</th>
<th>Primary antibody (dilution)</th>
<th>T</th>
<th>Lysis buffer</th>
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<tr>
<td>Arc</td>
<td>10%</td>
<td>ARCl (1:1000)</td>
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<td>HBS</td>
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<tr>
<td>β-Catenin</td>
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<td>β-catenin (1:1000)</td>
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<tr>
<td>PSD95</td>
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<td>PSD95 (1:1000)</td>
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<td>2 HBS</td>
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<tr>
<td>Synaptophysin</td>
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<td>Synaptophysin (1:500)</td>
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<td>3 HBS</td>
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<tr>
<td>APP</td>
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<td>APP 676–695 (1:2000)</td>
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<td>Tris–HCl</td>
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<tr>
<td>Aβ1–56</td>
<td>Gradient (4–12)%</td>
<td>Aβ-amyloid-1–16 (SE10) (1:1000)</td>
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<tr>
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<td>ADAM17 (1:1000)</td>
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<tr>
<td>BACE1</td>
<td>Gradient (4–12)%</td>
<td>BACE1 (1:1000)</td>
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<td>1 HBS</td>
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<tr>
<td>Phospho-tau</td>
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<td>0 HBS</td>
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<td>Total tau</td>
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<tr>
<td>NR1</td>
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<td>NR1 (1:1500)</td>
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<tr>
<td>NR2A</td>
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<td>NR2A (1:1000)</td>
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<tr>
<td>NR2B</td>
<td>7.5%</td>
<td>NR2B (1:1000)</td>
<td></td>
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<tr>
<td>VGLUT1</td>
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<tr>
<td>MAP2A</td>
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</tr>
<tr>
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<td>p35/p25</td>
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<tr>
<td>Phospho-GSK-3β Ser9</td>
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<td>Phospho-GSK-3β S9 (1:1000)</td>
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<tr>
<td>Phospho-GSK-3β Thr207</td>
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<td>Phospho-GSK-3β Thr207 (1:1000)</td>
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<td>Total GSK-3β</td>
<td>7.5%</td>
<td>GSK-3β (1:1000)</td>
<td></td>
<td>2 HBS</td>
</tr>
</tbody>
</table>

* T refers to the number of times that membranes were stripped before probing with the corresponding antibody.
HBS, HEPES buffer saline containing protease and phosphatase inhibitors, and NP-40.
Tris–HCl, Tris–HCl buffer containing protease and phosphatase inhibitors, and SDS 2%.
* a Santa Cruz Biotechnology, Santa Cruz, CA, USA.
* b Cell Signalling Technology, Danvers, MA, USA.
* c Abcam, Cambridge, UK.
* d Sigma-Aldrich, St. Louis, MO, USA.
* e Covance, Princeton, NJ, USA.
* f Pierce, Rockford, IL, USA.
* g Millipore, Billerica, MA, USA.
* h Bio-Rad, Hercules, CA, USA.

**2.4.4. Amyloid plaque staining**

Left hemispheres from 4 mice per group were fixed by immersion in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 24 h followed by 10% sucrose solution. Brains were cut into series of 20 μm slides. Free-floating sections were incubated in 70% formic acid for 10 min, then blocked with 2% of normal goat serum in PBS with 0.3% Triton X-100 for 30 min prior to incubation with 6E10 primary antibody overnight at 4 °C. Then, sections were incubated with secondary antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h in blocking solution, followed by incubation in ABC-Elite HRP (Vector Laboratories, Burlingame, UK). Reactions were visualized by developing the sections in DAB (Vector Laboratories, Burlingame, UK). Sections were rinsed in PBS, dehydrated and mounted in DPX (BDH, Poole, UK). For control staining the primary antibody was omitted. 8 sections per hippocampus were processed and captured into images with Nikon Eclipse E800 microscope and Nikon FXD35 camera. Area covered by amyloid plaques was analysed by ImageJ v. 1.43u software (NIH Image).

**2.5. Statistical analysis**

All results are expressed as mean ± standard error of the mean (SEM). The outcomes measured in the cognitive stimulation paradigm were analysed by one-way ANOVA (genotype (WT/Tg2576)) with and additional factor (days) as repeated measurement for each week. As no significant interaction between genotype and days was found, main effect of “days” was assessed by one-way ANOVA followed by Tukey’s test. Biochemical data were evaluated by two-way ANOVA (genotype (WT/Tg2576), training (naive/CS)). When interaction between factors was significant, single effects were analysed by one-way ANOVA followed by post hoc Tukey’s test. The value from F of interaction together with the probability associated to the single comparison was indicated. When no significant interaction between factors was found, main effects were analysed. Statistical analysis of MWM acquisition was performed by two-way ANOVA with an additional factor (days) as repeated measurement. When interaction between factors was found, intra-group differences were checked by one-way ANOVA followed by Tukey’s test. In those cases where only two groups were compared, data were evaluated by Student’s t test. Number of animals per group ranged from 6 to 8. The level of significance was set at P < 0.05. All analyses were performed using SPSS 15.0 packages of Windows (IL, USA).

**3. Results**

**3.1. Effects of cognitive stimulation on memory and synaptic markers**

Prior to training, cognitive function of mice was tested in a short version of the MWM (3 days of acquisition in the hidden platform version plus a probe trial in the next day). Normal cognitive abilities were confirmed as both WT and Tg2576 showed better escape latencies on the 3rd day of the hidden platform (main effect of “Day”; F(2,45) = 5.66, P < 0.05 vs 1st day, WT group; F(2,45) = 3.92, P < 0.05 vs 1st day, Tg2576 group) as well as on the visible platform version (main effect of “Day”; F(2,45) = 14.04, P < 0.01 vs 1st day, WT group; F(2,45) = 18.72, P < 0.01 vs 1st day, Tg2576 group) (Fig. S1A–C).

The mice were considered to have achieved a successful performance when latency to reach the goal was significantly reduced from the 1st day to the last (4th). No improvement would have indicated a lack of learning, and therefore, a fail in the purpose of inducing cognitive stimulation. Notably, the mice showed improved escape latencies and decreased number or entry errors to find the goal over days (main effect of “day”; Week 1 as a representative example: F(1,28) = 8.89, P < 0.01 for escape latency; F(1,28) = 12.33, P < 0.01 for number of entry errors; see Fig. 1C and D). A video recorded the 4th day of week 7 shows a Tg2576 mouse that was able to reach the goal with no entry errors (Supplementary material). As it can be observed, transgenic animals showed a better performance, which was particularly evident at weeks 4 and 5. A possible beneficial effect of β-amyloid on learning at a young age, when levels are not high enough to cause neurodegeneration [22], cannot be ruled out.

After the training sessions the mice were kept in standard-housing conditions for 8 months until the age of 15 months-old. Then, effects of CS on memory were assessed by two different behavioural tests: MWM and NORT. These tasks are mainly dependent on spatial and recognition memory, respectively, which are two types of memory largely affected in AD and Tg2576 mice as well [19,23]. This long lag between the end of CS paradigm and the beginning of cognitive characterization is due to the onset of cognitive decline in Tg2576 mice. Despite some discrepancies about the onset of spatial memory impairment in Tg2576 mice [19,24,25], the age of 14–15 months seems to be proper for detecting decline in both types of memories [21,26]. As expected, Tg2576-naïve mice displayed impaired retention memories in the probe trials carried out at day 4 (F(1,28) = 8.02, P < 0.05 vs WT-naïve; Fig. 2A) and 7 (F(1,28) = 9.74, P < 0.01 vs WT-naïve; Fig. 2A).
Tg2576-naïve mice, which showed similar swimming speed than WT (data not shown), did not achieve significant longer escape latencies during the acquisition phase (Fig. 2B). It is important to note that Tg2576-naïve mice did not show lower escape latencies over days, which means that they were not able to learn the platform location ($F_{(3,35)} = 1.51, P = 0.21$), while Tg2576-CS mice did shorten escape latency to platform over days ($F_{(3,30)} = 2.63, P < 0.05$; Fig. 2B). No significant differences on escape latencies between groups were found in the visible platform version as well (data not shown). Regarding memory retention, transgenic animals subjected to CS presented better performance in both probe trials, reaching similar retention scores than WT animals (Fig. 2A). In contrast, WT animals subjected to CS did not perform better retention than WT-naïve (Fig. 2A). In the same manner, Tg2576-naïve mice showed impaired object recognition memory as they did not preferentially discriminate in favour of the novel object ($F_{(1,26)} = 8.94, P < 0.01$ vs WT-naïve, 1 h; $F_{(1,26)} = 6.22, P < 0.01$ vs WT-naïve, 24 h) but, interestingly, Tg2576-CS achieved similar discrimination indexes than WT-naïve mice at both retention times (Fig. 2C).

It is well accepted that hippocampal synaptic plasticity is essential for mediating the formation of new memories [27]. The observed improvement of memory abilities in cognitive-stimulated Tg2576 mice might be thus attributable to different expression levels of long-term activity-associated synaptic proteins. To test this hypothesis, we then assessed the state of several synaptic markers. Pre-synaptic markers were found either unaltered, as the case of synaptophysin, or decreased, as the case of VGLUT-1 ($F_{(1,21)} = 7.74, P < 0.05$), but unaffected by CS (Fig. 3A). Interestingly, some proteins recruited into the post-synaptic density as PSD95 and its partner the NR1 subunit of NMDA receptors (Fig. 3B), as well as such proteins involved in synaptic consolidation and formation as Arc and β-catenin (Fig. 3C), were found decreased in Tg2576 mice (PSD95, $F_{(1,21)} = 10.85, P < 0.05$; NR1, $F_{(1,23)} = 30.96, P < 0.01$; Arc, $F_{(1,23)} = 5.425, P < 0.01$; β-catenin, $F_{(1,23)} = 6.26, P < 0.05$), but preserved by CS.

### 3.2. Decreased soluble Aβ species but constant plaque burden in Tg2576 mice subjected to cognitive stimulation

Accumulating evidence supports soluble Aβ oligomers, rather than Aβ fibrils, as fundamental triggers for the pathological and cognitive symptoms observed in AD [28]. We found that SDS-soluble Aβ1–42 and Aβ1–40 levels in cognitive-stimulated Tg2576 mice were reduced by 40% and 20%, respectively, when compared to the naïve group ($t_{(1,12)} = 5.86, P < 0.01$ for Aβ1–42; $t_{(1,12)} = 2.46, P < 0.05$ for Aβ1–40; Fig. 4A). Tg2576-CS mice presented unchanged levels of insoluble GuHCl-extracted Aβ1–40 and a slight but significant decrease in Aβ1–42 ($t_{(1,12)} = 5.72, P < 0.01$) (Fig. 4A). In contrast, CS did not appear to affect the levels of murine Aβ1–42 in either the soluble or insoluble fraction of WT brains (Fig. 4A).

The total burden of 6E10-immunoreactive amyloid plaques remained similar in both groups as well (Fig. 4B). These results indicate that CS is likely to induce major changes in the levels of soluble Aβ1; however, it fails to provide real information about which Aβ

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**Fig. 2.** Cognitive stimulation delays the memory deficits observed in 16-month-old Tg2576 mice, as studied by MWM (A–B) and NOR (C) tests. (A) Percentage of time spent in the target quadrant over a total duration of 60 s. At both the 4th and 7th day after the beginning of training Tg2576 mice showed impaired retention, which was reverted by cognitive stimulation. (B) In the hidden platform version of acquisition, no significant differences on escape latencies were found between groups. However, while Tg2576-naïve mice did not manage to lower escape latency to find the platform over days, Tg2576-CS as well as WT-naïve and WT-CS mice did. $P < 0.05$, day 6 vs day 1. (C) Tg2576 mice showed impaired recognition memory, as shown by decreased discrimination index to a novel object, both 1 and 24 h after training with 2 similar objects. Tg2576-CS mice presented a recovered recognition memory, which only reached the level of significance at 24 h post-training. $P < 0.05$, $P < 0.01$ vs WT-naïve; $P < 0.05$, $P < 0.01$ vs Tg2576-naïve. Data are expressed as mean ± S.E.M. WT-naïve, $n = 7$; WT-CS, $n = 8$; Tg2576-naïve, $n = 8$; Tg2576-CS, $n = 7$. 
oligomeric assemblies are involved in this process. To solve this drawback, electrophoresis of extracellularly-enriched SDS homogenates was run. Notably, a band around 56-kDa was found in Tg2576 samples but was absent in WT samples when immuno-blotting the membranes with 6E10 antibody (4C). This band, which could presumably correspond to the amyloid dodecamer (also referred as Aβ*56) was found dramatically reduced by 45% in cognitive-stimulated Tg2576 mice (t(1,10) = 6.19, P < 0.01; Fig. 4C). To discard misinterpretations caused by artefacts, samples were pre-treated with 10% hexafluoroisopropanol (HFIP), an organic solvent that breaks up Aβ oligomers, and immunoblotted again with 6E10 antibody, resulting in the dissolution of the 56-kDa band (Fig. 4C).

Remarkably, levels of soluble Aβ1–42 as well as Aβ*56 showed a strong and inverse correlation to both NORT (Pearson’s r = −0.90, P < 0.01; Pearson’s r = −0.70, P < 0.01, respectively) and MWM retention scores (Pearson’s r = −0.78, P < 0.01; Pearson’s r = −0.58, P < 0.01, respectively), suggesting an important link between decreased levels of soluble Aβ species and the CS-induced reversion of cognitive deficits (Fig. 4D).

3.3. Cognitive stimulation compensates for tau pathology in Tg2576 mice

As hyper-phosphorylation of tau is a common feature of AD and other neurodegenerative disorders, it was next examined whether CS might be beneficial on this pathological event. According to reported data, tau pathology in Tg2576 mice in terms of altered phosphorylation state of tau and its related kinases was observed [29]. Immuno-blotting with AT-8 antibody revealed that phosphorylation at Ser202/Thr205 residues was markedly increased in Tg2576-naïve when compared to WT animals (F(1,123) = 5.30, P < 0.05; Fig. 6A and E). Notably,
cognitive-stimulated Tg2576 mice showed similar levels of phospho-tau than WT mice (Fig. 6A and D). No differences in total tau levels between experimental groups were found (Fig. 6A). The activity of several kinases involved in abnormal tau phosphorylation was next assessed by calculating the ratio between the phosphorylated form and the total level of each kinase. Active GSK-3β (phospho-Tyr216) was found increased (F(1,23) = 8.48, P < 0.05; Fig. 6B and F) and inactive form (phospho-Ser9) decreased in Tg2576-naïve mice (F(1,23) = 4.87, P < 0.05; Fig. 6B and E), CS returned GSK-3β activity to WT levels (Fig. 6B and E). In addition, despite no differences in Cdk5 levels were detected (Fig. 6B and E), a two-fold increase of the ratio p25/p35 was found in Tg2576-naïve mice (F(1,16) = 10.17, P < 0.01; Fig. 6B and E), suggesting a higher constitutive activation of Cdk5 in these animals [30]. Yet again, Tg2576-CS mice showed similar levels than WT (Fig. 6B and E).

Tau’s main function is to modulate the stability of axonal microtubules. Other microtubule-associated proteins (MAPs), such as MAP2A, are able to stabilize microtubules in dendrites as well, and its lost has

Fig. 4. Tg2576 mice exposed to cognitive stimulation shows reduced levels of soluble Aβ species at the age of 16 months. (A) Soluble Aβ1–40 and, particularly, Aβ1–42 assessed by ELISA were found significantly decreased in hippocampus of Tg2576-CS mice (left panel). However, only levels of the insoluble species Aβ1–42 presented a little and significative change (right panel). Levels of both soluble and insoluble murine Aβ1–42 were found unaltered in WT CS when compared to WT-naive mice. WT-naive, n = 7; WT-CS, n = 7; Tg2576-naive, n = 8; Tg2576-CS, n = 6. (B) Immunostaining with 6E10 antibody did not show any particular change in the amyloid plaque burden between naïve and cognitive-stimulated Tg2576 animals. Scale bar, 100 μm. Tg2576-naive, n = 3; Tg2576-CS, n = 3. (C) Immunoblotting with 6E10 antibody revealed a band around 56 KDa, which was present in Tg2576 mice but absent in WT mice (left panel). The fact that this band is not present in WT mice and is dissolves by pre-treating samples with HFIP, makes this band likely attributable to the amyloid dodecamer Aβ*56. Aβ*56 was strikingly decreased by CS (middle and right panel). Tg2576-naive, n = 5; Tg2576-CS, n = 6. (D) Soluble levels of Aβ1–40 and Aβ1–42 strongly correlated with memory retention scores at both NOR (Discrimination index; left panel) and MWM tests (Time in target zone; right panel). Pearson’s correlation coefficient “r” is shown. Western-blot quantifications are normalized to β-actin levels. *P < 0.05, **P < 0.01 vs Tg2576-naive. Data are expressed as mean ± S.E.M. OD refers to optical density.
been associated with dendrite degeneration. Immuno-blotting revealed that MAP2A levels were indeed decreased in Tg2576-naïve when compared to WT mice ($F(1,19) = 4.51, P<0.01$) but recovered in Tg2576-CS group (Fig. 6D and H).

Together these data suggest that CS is able to delay the tauopathy inherent to Tg2576 mouse at the age of 15 months. It is worthy of notice that decreased tau hyper-phosphorylation is strongly correlated with reduced Aβ*56 and Aβ1–42 (Pearson’s $r = 0.683, P<0.01$; Pearson’s $r = 0.763, P<0.01$, respectively; data not shown), however, whether cognitive stimulation prevents tau pathology directly or secondary to modulation of amyloid pathology cannot be addressed in the present work.

4. Discussion

In the present study, a novel training paradigm for CS, devoid of social, physical or enriched environments, has been elaborated and validated. AD transgenic mice exposed to this training throughout early-life manifested normal cognitive function, reduced synaptic deficit and decreased amyloid/tau pathology at an older age. One of the main findings is the simple and striking fact that a transient CS delays the onset of spatial and object recognition memory decline in Tg2576 mice after a lag of 8 months. The reason by which this CS induces strong long-lasting effects on cognitive function might lie in the time frame where CS was applied, since early-life stimulatory events appear to build cognitive reserve more effectively and, therefore, make the brain less susceptible to pathological changes. An in vivo study has added evidence on this assumption by showing that exposure to a transient stimulation with an enriched environment in early-life reduced the AD-related cognitive deficits more efficiently than exposure later in life [31]. In contrast to the beneficial effects of CS observed in Tg2576 mice, trained WT mice did not show improved cognitive abilities in any of the tests performed. These observations may indicate that CS fails to provide any supplement against the physiological cognitive decline associated to ageing [32], but may instead compensate for the memory loss associated to AD pathogenic processes. A previous study carried out in eleven thousand cognitively healthy humans subjected to “brain training” that failed to report benefits in untrained tasks might support this assumption [7].
Given the genotypic nature of Tg2576 mouse, APP should be a major player in the memory dysfunction developed by this transgenic mouse. Although effects of amyloid load on memory may be nonlinear and exhibit saturation [25], data from correlational and experimental studies might suggest a causal role in memory disruption [33,34]. We have found that cognitive training led to lowered soluble amyloid load while leaving amyloid plaque burden intact. Among the different amyloid assemblies that are present in the soluble fraction, the dodecamer referred to as Aβ*56 was found notably reduced in CS transgenic animals. Previous studies that identified this oligomeric form as being most closely related to memory loss in younger Tg2576 mice [35,36] imply a potential rationale by which CS might be compensating for the memory decline. Likewise, repeated training in a MWM over a period of 10 months reported similar benefits on the levels of Aβ*56 and MWM-related memory in the 3xTg-AD transgenic mice [37]. In contrast to this study, the present work evaluated memory abilities in untrained tasks. Additionally, it was found a strong correlation between levels of Aβ*56 and retention scores in MWM and NOR tests, which, although far from providing a causal association, confirms the close connection of decreased Aβ*56 to CS-induced delay of cognitive decline. It is hypothesized that oligomeric Aβ*56 may rise prior to the formation of amyloid plaques, and therefore act as seeds for plaques [35,38]. Speculatively, CS might be reducing Aβ*56 levels by 1) decreasing the production of monomers; 2) inducing its clearance; or 3) promoting its aggregation into plaques. The fact that training did not alter plaque burden and that Aβ*56 showed correlation to soluble but not to insoluble levels seems to discard the latter point. In contrast, the fact that CS showed reduced levels of C99 and BACE1, as well as increased the levels of C83 and ADAM17 indicates that the production of monomer might be decreased, thus leaving to reduced formation of the dodecamer. However, the idea that CS may lower Aβ*56 load by favouring its clearance cannot be ruled out. It is important to note that CS likely induced a shift towards a more non-amyloidogenic processing of APP but remained the amyloid plaque burden intact. Although soluble amyloid species were found indeed decreased, the production of those amyloid species that are more prone to aggregate might have not been affected by CS. Also noteworthy in the present study is that CS compensated for the abnormal tau phosphorylation and high activities of GSK-3β and cdk5 found in Tg2576 mice. Although transgenic mice do not suffer from real tau pathology in terms of neurofibrillar tangles, these animals manifest age-related hyperphosphorylated tau-containing neurites and higher activities of various tau kinases [21,39,40]. High inter-correlation between levels of Aβ*56/Aβ1–42 and phospho-tau and a lack of CS effect on tau phosphorylation in WT mice was also observed. These data, together with the increasing evidence of tau abnormalities as a secondary event to amyloid pathology in the pathogenic continuum of AD [40–42], invite to speculate that prevention of tau pathology by CS is likely to be a consequence of reduced amyloid load.

Fig. 6. Cognitive stimulation compensates for the tau pathology inherent to 16 month-old Tg2576 mice. (A, D) Immuno-blot with monoclonal antibody AT8 revealed that tau phosphorylation at residues S202 and T205 were increased in Tg2576-naïve but unchanged in Tg2576-CS mice. (B, E) Immuno-blots and quantification of tau kinases that showed altered state of activity. Levels of inactive p-S9-GSK3β were found decreased and active p-Y207-GSK3β increased in Tg2576-naïve animals, while unchanged in Tg2576-CS mice. The ratio p25/p35, and indicator of cdk5 activity, was also increased in Tg2576-naïve mice but unaltered in Tg2576-CS. (C, F) Levels of the microtubule-associated protein MAP2A were found decreased in Tg2576-naïve animals but recovered in cognitive-stimulated transgenic mice. Quantifications of phospho-proteins are normalized to both total protein and β-actin levels. *P < 0.05, **P < 0.01 vs WT-naïve; ^P < 0.05, ^^P < 0.01 vs Tg2576-naïve. Data are expressed as mean ± S.E.M. WT-naïve, n = 6; WT-CS, n = 6; Tg2576-naïve, n=6–8; Tg2576-CS, n = 6. OD refers to optical density.
The strong differences at the molecular level between transgenic mice exposed to CS and naive conditions after a lag of 9 months are quite striking. Based on previous animal studies, which have suggested that EE can impact a variety of processes involved with amyloid processing [10,14,18], it is possible to speculate that CS may have reduced the production of toxic Aβ species to further induce changes on the development of other AD-related pathological events or cognitive impairments. However, biochemical characterization at different time points after the CS paradigm should be carried out in order to accurately assess how big the delay caused by CS intervention could be. It would also be of great interest to understand why CS effects are specific for Tg2576 mice. The observation that CS did not affect levels of murine Aβ1–42 in WT mice implies that CS shows more benefits under particular pathological conditions, such as amyloid pathology, than under normal ageing. However, the molecular mechanisms underlying the lowering effects of CS on amyloid need to be elucidated.

EE has been used for several decades to study neuroanatomical and behavioural changes associated with cognitive stimulation. The application of EE to AD animal models has shown beneficial effects on cognitive and pathological symptoms. 5–6 months of EE in young APP/PS1 mice improved performance in radial water maze and in the classical and reversal version of MWM [8,10], and an EE of 6 months in old APP23 mice also improved MWM performance [9]. However, discrepancies have risen since not all the EE paradigms have been able to reduce memory impairments. For example, shorter durations of EE (2–4 months) failed to improve Y-maze or Barnes maze performances in either TgCRND8 or APP/PS1 mice [11,12]. Varying outcomes on amyloid pathology [9,12–14,17] have also been reported. Most studies have shown increased or stable amyloid plaque load after an EE ranging from 4 to 6 months [9,12,13], but some studies with an EE ranging from 5 to 6 months have found decreased deposition of amyloid [14,17]. It is important to note that the effect on amyloid pathology does not necessarily correlate with cognitive function in many of these studies. EE protocols generally consist of a set of different stimuli as running wheels, coloured tunnels, visual stimulating toys, ladders and group housing [43], therefore providing a complex interaction between social abilities, physical skills and anti-stress environments. A novel approach to induce CS in WT and Alzheimer’s mice while avoiding enriched environments and social/physical activities has been used and validated. The present findings would thus provide empirical evidence on the relative beneficial and long-lasting contribution of an early-life CS paradigm on cognitive decline associated to AD and, in particular, on some pathological processes, such as synaptic, amyloid and tau pathologies.

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