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Chronic Mild Stress Accelerates the Onset and Progression of the Alzheimer's Disease Phenotype in Tg2576 Mice

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Abstract. The etiology of the more common (sporadic) forms of Alzheimer's disease (AD) remains unknown, although age is the most important risk factor. Nevertheless, interactions between environmental risk factors and genetic background may also influence the onset and progression of sporadic AD. Chronic stress, associated with altered memory and other neurological processes, is thought to influence the pathogenesis of AD. Hence, we evaluated the effect of unpredictable and consecutive chronic mild stressors on the onset of an AD-related pathology in the Tg2576 mouse line that overexpresses the human amyloid-β protein precursor with the Swedish mutation (hAβPP^{Swe}). Two months after exposure to chronic mild stress, 4 month-old animals that normally display no pathological features of AD, not only expressed pathological markers but also experienced cognitive dysfunction in the Morris water maze test. These findings suggest that chronic mild stress accelerates the onset of cognitive impairment and produces an increase in hippocampal amyloid-β and phospho-tau levels on a background of AD susceptibility.

Keywords: Amyloid-β, cognitive impairment, GSK3β, stress, tau

Supplementary data available online: http://www.j-alz.com/issues/28/vol28-3.html#supplementarydata03

1. INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder pathologically characterized by the formation of extracellular neuritic plaques composed primarily of fibrillar amyloid-β (Aβ) peptide, and of intracellular neurofibrillary tangles (NFTs) containing hyperphosphorylated tau [1]. While the accumulation of Aβ and hyperphosphorylated tau appear to contribute to the onset and progression of AD, it remains unclear how these two factors interact during the development of AD. Familial forms of AD involve mutations in several genes, such as AβPP and presenilins (PS1 and PS2), which leads to an increase in Aβ production. However, in the majority of AD cases, there is no obvious genetic factor involved but rather, age is the most important risk factor. It is therefore likely that complex interactions between environmental risk factors and genetic background lead to the increased risk of developing AD [2].

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Given the well-documented changes induced by chronic stress in the hippocampus [3–5], stress may influence the pathogenesis of AD [6] and indeed, there is epidemiological evidence that it is a risk factor for AD [7]. Moreover, clinical studies have reported increases in plasma cortisol levels in AD patients and in individuals with mild cognitive impairment [6, 8, 9]. In agreement with these findings, increased Aβ and enhanced tau-pathology have been reported in triple-transgenic (3XTg-AD) mice following glucocorticoid administration [10]. A relationship between behavioral stress and the exacerbation of the AD phenotype has also been described in other murine models of AD using chronic immobilization stress, considered to be a strong stressor [11–13]. The aim of the present study was to analyze the effect of chronic mild stress (CMS) on the development of AD. CMS is considered more similar to the forms of stress experienced by humans in everyday life and it is routinely used in models of depression [14]. Accordingly, we applied CMS to 4 month-old transgenic AD mice (Tg2576) with no signs of cognitive impairment to determine the consequences of CMS in combination with genetic risk factors for the development of AD.

2. MATERIALS AND METHODS

2.1. Animals

4 month-old female Tg2576 mice were divided in two groups ($n = 10$), the "chronic-mild-stress" (CMS, stressed) group and the control (non-stressed) group. Tg2576 transgenic mice express the human 695-aa isoform of AβPP containing the Swedish double mutation (hAβPP^{Swe}: (AβPP695) Lys670-Asn, Met671-Leu) driven by the hamster prion promoter. Brain Aβ-peptide content increases exponentially between 6 and 12 months of age in these mice, and memory impairment in the Morris water maze (MWM) is evident by 12–15 months [15–17]. At the age these mice were analyzed (4 months), they did not display any features of AD. The mice were housed in individual cages and they were habituated to their environment for 2 weeks before the experiments began. Food and water were available *ad libitum* for the duration of the experiments unless otherwise specified. The animals were maintained in a temperature and humidity-controlled room with a 12 h light-dark cycle. All procedures were carried out in accordance with European and Spanish regulations (86/609/CEE; RD1201/2005) and the study was approved by the Ethical Committee of the University of Navarra (no. 018/05).

2.2. Chronic mild stress procedure

The CMS protocol involved the exposure of mice to a variety of mild and unpredictable stressors applied in a random order for several weeks [18–20]. Unpredictable repeated mild stressors were applied for 6 weeks following the protocol described previously with minor modifications [21]. Of the following stressors, 2-3 were applied in any 24 h period: Low intensity stroboscopic illumination (in darkness, 8 h), intermittent bell ringing (10 db, 1/10 s), or white noise (an un-tuned radio, 4 h), 45° cage tilt (8 h), damp bedding (200 ml of water per cage, 6 h), rat odor (saw dust from rat cages, 8 h), darkness during the day (3 h), transfer of cages to another room (4 h), placement of a novel object in the cage (3 h), overnight water and food deprivation, illumination and removal of nesting material (12 h), and swimming in cold water (18°C, 5 min). For the complete protocol, See Table 1 in Supplemental Data (available online: http://www.j-alz.com/issues/28/vol28-3.html#supplementarydata03). During the final week of CMS, animals from both groups were subjected to the MWM task. To determine long-lasting biochemical changes, the animals were sacrificed two months after completing the CMS procedure. The time course of the experimental procedure is shown in Fig. 1.

2.3. Morris Water Maze test

The MWM test was used to evaluate working and reference memory as described previously [22]. Briefly, the test was carried out in a circular pool (1.2 m in diameter) filled with water (20°C) that was opaque due to the addition of non-toxic white paint. Mice were trained with a visible-platform above the surface of the water on 3 consecutive days (eight trials per day). This was followed by 8 consecutive days (four trials per day) of hidden-platform training in the presence of all the visible cues, during which the mice were trained to locate a platform in the opposite quadrant submerged 1 cm beneath the surface. In both visible- and hidden-platform training sessions, mice were pseudo-randomly placed at specific locations facing the wall of the pool to eliminate the potentially confounding influence of extra-maze spatial cues. Each trial was terminated when the mouse reached the platform or after 60 s, whichever occurred first. Mice that failed to reach the platform were finally guided onto

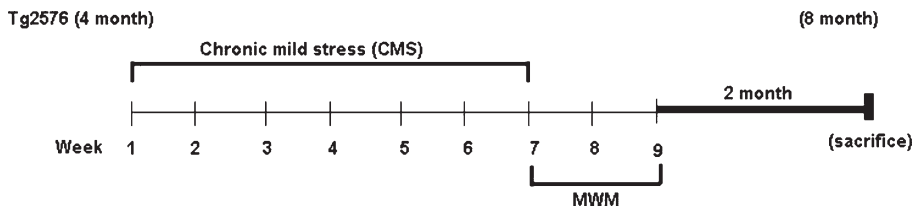

Fig. 1. Time course of the CMS procedure and behavioral test. 4-month-old Tg2576 mice were exposed to CMS (chronic mild stress procedure) which lasts 6 weeks. Immediately after the CMS completion, the Morris water maze test was conducted and 2 month later the animals were sacrificed.

it and after each hidden platform trial, the mice were left on the platform for 20 s. Twenty hours after the 12th, 24th, and 32nd trials, all the mice were subjected to a probe trial in which they swam for 60 s in the pool with no platform. Mice were monitored with a ceiling-mounted camera directly above the pool, and the all trials were recorded using an HVS water maze program and subsequently analyzed with SMARTLD software (PanLab S.L., Barcelona, Spain) to determine the latency to reach the platform, swimming speed, path length, and the time spent in each quadrant of the pool during the probe trials. All experimental procedures were performed by a researcher blind to the experimental groups. Two months after the MWM task the animals were sacrificed by cervical dislocation or transcardial perfusion.

2.4. Corticosterone measurement

Blood samples were collected after completion of the CMS procedure, at the beginning of the light period (8 a.m.). The blood samples were centrifuged and the plasma was collected and immediately stored at –80°C. Plasma corticosterone levels were measured using a commercially available enzyme immunoassay kit (Assay Designs Inc., Ann Arbor, MI) with a 6.25 μl aliquot of each sample diluted (1 : 20) in buffer. Optical densities were read at 405 nm using the Multiskan Ex (Thermolab systems) microplate reader.

2.5. Determination of Aβ levels

Cortical $A\beta_{40}$ and $A\beta_{42}$ levels were measured using a sensitive sandwich ELISA kit from Biosource (Camarillo, CA). Briefly, the tissue was weighed and homogenized in ice-cold guanidine buffer (5 M guanidine HCl/50 mM Tris-HCl pH 8.0). The homogenates were mixed for 4 h at room temperature and diluted 1 : 50 in Dulbecco's phosphate buffered saline containing 5% BSA and 0.03% Tween-20 (DPBS-BSAT) supplemented with protease inhibitor cocktail (CompleteTM Protease Inhibitor Cocktail, Roche Diagnostics, Mannheim, Germany). After centrifugation at 16,000 g for 20 min at 4°C, the supernatant was diluted and loaded onto ELISA plates in duplicate. The assays were performed according to the manufacturer's instructions and the Aβ standards were prepared in a buffer with the same composition as the final tissue samples.

2.6. Production of protein extracts

Mice were sacrificed by cervical dislocation and the hippocampus and prefrontal cortex were quickly dissected from the brains. To determine the AβPP carboxy-terminal fragments, the prefrontal cortex of one hemisphere was homogenized in a buffer containing 2% SDS, 10 mM Tris-HCl [pH 7.4], protease inhibitors (Complete™ Protease Inhibitor Cocktail, Roche) and phosphatase inhibitors (0.1 mM Na_3VO_4, 1 mM NaF). The homogenates were sonicated for 2 min and centrifuged at 100,000 g for 1 h. The protein concentrations determined by the Bradford method using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) and aliquots of the supernatant were frozen at -80°C. To assay other proteins, total tissue homogenates were obtained by homogenizing the hippocampus in ice-cold RIPA buffer (50 mM Tris-HCl [pH 7.4], 0.25% DOC, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 1 mM Na_3VO_4, and 1 mM NaF) and they were centrifuged at 14,000 g 4°C for 20 min. The protein concentration of the supernatant was determined (Bradford protein assay, BioRad Laboratories, Hercules, CA) and aliquots were stored at –80°C.

2.7. Immunoblotting

To analyze AβPP fragments in western blots, aliquots of protein extracts were mixed with XT sample buffer™ plus XT reducing agent™ or Tricine sample buffer™ (Bio-Rad) and boiled for 5 min. The proteins

were separated in a Criterion™ precast Bis-Tris 4–12% (Bio-Rad) and transferred to nitrocellulose membranes. The membranes were blocked with 5% fat-free milk, 0.05% Tween-20 in TBS followed by overnight incubation with the 6E10 mouse monoclonal antibody (amino acids 1–17 of Aβ peptide, 1 : 1000, Millipore) or a rabbit polyclonal antiserum raised against the C-terminal of AβPP (amino acids 676–695 of hAβPP, 1 : 2000, Sigma-Aldrich, St. Louis, MO, USA). To determine other proteins, samples were mixed with an equal volume of 2X Laemmli sample buffer, resolved on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% fat-free milk, 0.05% Tween-20 in PBS or TBS and incubated overnight with the following primary antibodies: Mouse monoclonal anti-phospho tau AT8 (Ser-202, Thr-205; 1 : 1000, Pierce Biotechnology Inc., Rockford), mouse monoclonal PHF-1 (Ser-396, Ser-404; 1 : 100, from Peter Davies at Albert Einstein University), mouse monoclonal anti-tau (1 : 1000 clone Tau46, Sigma-Aldrich), rabbit polyclonal anti-pGSK3β Ser9 (1 : 000, Cell Signalling Technology, Beverly, MA), rabbit polyclonal anti-GSK-3β (1 : 000, Santa Cruz Biotechnology, Santa Cruz, CA). After two washes in PBS or TBS/Tween-20 and one wash in PBS or TBS alone, the proteins were detected with HRP-conjugated anti-rabbit or anti-mouse antibody (1 : 5000, Santa Cruz), and they were visualized by enhanced chemiluminiscence (ECL, GE Healthcare Bioscience) and autoradiographic exposure to Hyperfilm ECL (GE Healthcare Bioscience). Signals were quantified using the Quantity One™ software v.4.6.3 (Bio-Rad).

2.8. Immunohistochemistry

Under xylazine/ketamine anesthesia, animals from both groups ($n = 4$) were perfused transcardially with saline and 4% paraformaldehyde in phosphate buffer (PB). After perfusion, the animal's brain was removed, post-fixed in the same fixative solution for 1 h at room temperature (RT) and cryoprotected in 30% sucrose solution in PB overnight at 4°C. Coronal microtome sections (30 μm thick) were collected free-floating and stored in 30% ethylene glycol, 30% glycerol, and 0.1 M PB at –20°C, before floating tissue sections containing the hippocampal formation were processed for immunohistochemistry. Brain sections were rinsed with 0.125 M PB [pH 7.4] three times each for 10 min. Endogenous peroxidases were inactivated with 0.3% hydrogen peroxide in methanol for 45 min and after washing the section in 0.125 M PB, they were incubated in a blocking solution for 2 h at RT (PB containing 0.5% Triton X-100, 0.1% BSA and 2% normal goat serum). The sections were then incubated overnight with one of the following primary antibodies in blocking solution: Mouse monoclonal 6E10 (amino acids 1-17 of Aβ peptide, 1 : 1000, Millipore), mouse monoclonal 4G8 (amino acids 17–24 of Aβ peptide, 1 : 500, Millipore), mouse monoclonal anti-phospho-tau (Ser202/Thr205) AT8 (1 : 50, Pierce Biotechnology Inc., Rockford). For 6E10 immunostaining, sections were treated with 70% formic acid for 10 min to expose the epitope prior to incubating with the antibody. After washing in PB, the sections were incubated for 2 h at RT with the appropriate biotinylated secondary antibodies (1 : 200, Vector Laboratories, Burlingame, CA, USA) diluted in blocking solution and for 1 h with avidin-biotin complex. Finally, antibody binding was visualized with the DAB-kit (diaminobenzidine from Vector). To ensure comparable immunostaining, sections were processed together under identical conditions. To assess the specificity of staining, sections from each experimental group were incubated in the absence of the primary antibodies and in such cases no immunostaining was observed. Non-specific secondary immunostaining was also evaluated by incubating sections with the primary and a non-specific secondary antibody, and again no immunostaining was observed.

2.9. Statistical analysis

Statistical analysis was performed using SPSS 15.0 for Windows. Unless otherwise indicated, the results are presented as the mean ± the standard error of the mean (SEM, error bars). In the MWM test ($n = 10$ per group), escape latencies were analyzed with the Student's t test and the non-parametric Friedman test was used to analyze the differences between groups. Each biochemical assay was repeated 3 times ($n = 6$ per group) and the data analyzed using the Mann Whitney test. In all cases the level of significance was set at 0.05 ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

3. RESULTS

3.1. Chronic mild stress accelerates cognitive impairment

To investigate the relationship between CMS and cognitive deficits, learning and spatial memory was assessed using the MWM task. No significant differences were observed between stressed and

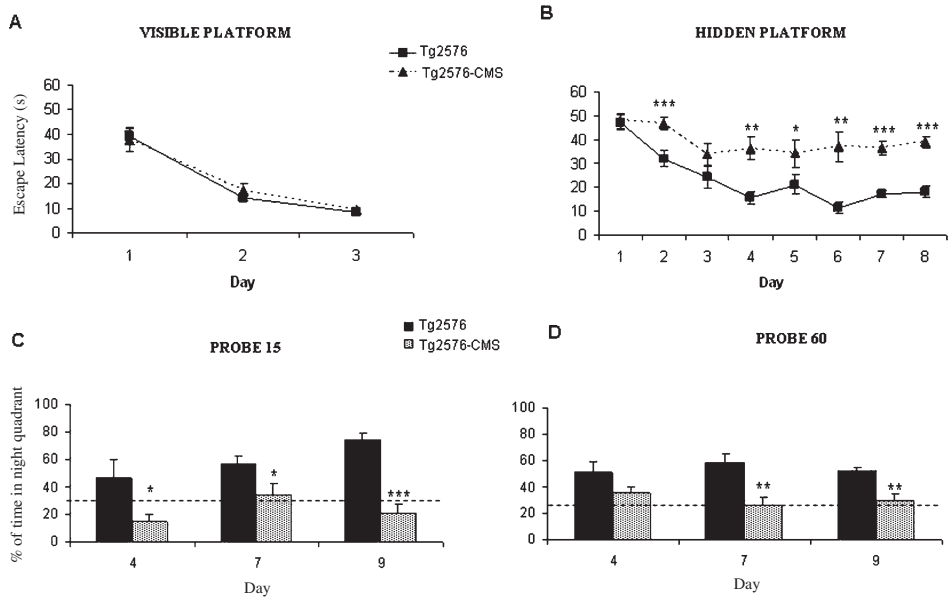

Fig. 2. CMS impairs learning and memory in Tg2576 mice. Latency of stressed (Tg2576-CMS) and unstressed mice (Tg2576) in reaching the visible (A) or hidden platform (B) in the water maze test, presented as the mean ± S.E.M. ($n = 10$ per group). CMS-Tg2576 mice showed significantly greater latencies to detect the invisible platform than unstressed Tg2576 littermates (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, ANOVA with Scheffe's post-hoc test). C, D) Percentage time spent searching for the target (trained) quadrant in the probe test expressed as the mean ± S.E.M. ($n = 10$ per group). Stressed CMS-Tg2576 mice performed significantly worse than unstressed littermates in both the 15 s (C) and 60 (D) s probe trials (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, ANOVA with Scheffe's post-hoc test).

non-stressed mice during visible-platform training (Fig. 2A). However, the spatial memory component of the test (invisible platform) revealed significant differences in latency to reach the platform between these two groups of mice ($p < 0.01$), with stressed Tg2576 mice performing significantly poorer than their non-stressed counterparts. Indeed, exposure to CMS increased escape latencies on several days of the invisible platform test ($p < 0.01$, Fig. 2B). The variance of the intra-group latencies in the hidden-platform training (over trials) was analyzed with the non-parametric Friedman test (see Methods) and while the mean latency to reach the platform decreased in unstressed mice as the training sessions progressed (days 3–8; $x^2 r = 15.08$ $p < 0.05$), no such change was observed in stressed mice ($x^2 r = 3.31$, $p = 0.7$). These results indicated that exposure to CMS impaired the capacity of Tg2576 mice to learn in this component of the test.

To study memory retention, the mice were subjected to a probe trial in which the platform was removed 24 h after the training session on days 3, 6, and 8. The performance of the mice in each trial was measured at 15 and 60 s, as the sensitivity of the MWM test may be increased in short trials [23]. Indeed, one-way ANOVA revealed significant differences between the two groups of mice at both time points (Fig. 2C, D; $p < 0.01$) and the mice subjected to CMS spent significantly less time in the target quadrant than the corresponding unstressed age-matched controls (Fig. 2C and D). While the swim speed did not differ significantly between these two groups of mice in any of the parts of the test, the distance travelled followed a similar pattern as the escape latency and it differed significantly between stressed and unstressed animals (data not shown). These results indicated that CMS significantly affected the behavior of Tg2576 mice in the MWM test.

As CMS is reported to increase corticosterone levels in adult animals [24–26], this hormone was measured in blood samples collected from animals sacrificed 2 months after the MWM assays. In this case, there was significantly less corticosterone in the plasma of stressed than in unstressed Tg2576 mice (Fig. 3).

3.2. *Intraneuronal Aβ and amyloid plaques are augmented in CMS-Tg2576 mice*

Aβ ($A\beta_{40}$ and $A\beta_{42}$) formation is mediated by the sequential cleavage of AβPP by the β- and

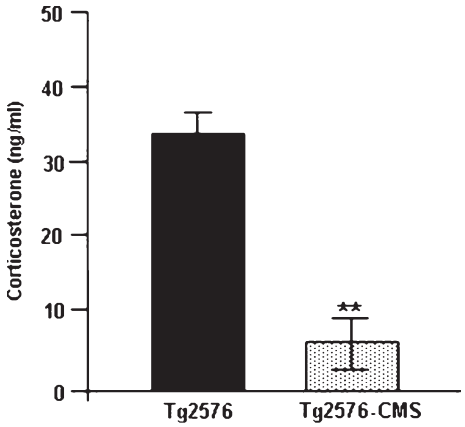

Fig. 3. Effects of CMS on corticosterone levels in Tg2576 mice. ELISA was used to measure corticosterone levels in blood samples taken two months after completion of the CMS procedure. The data are presented as means ± SEM $^{**}p < 0.01$ ($n = 6$ per group).

γ-secretases, a process promoted in Tg2576 mice by the presence of the Swedish mutation. To determine whether CMS affected hAβPP processing in 4 month-old Tg2576 mice, the hAβPP-derived $A\beta_{40}$ and $A\beta_{42}$ (Fig. 4A, B) and the hAβPP-CTFs C83 and C99 (Fig. 4C, D) were quantified in the cerebral cortex of stressed versus unstressed Tg2576 mice. ELISA assays revealed that there was significantly more $A\beta_{42}$ in the cerebral cortex of stressed Tg2576 mice than in that of unstressed controls (Fig. 4B; $p < 0.05$). Similarly, $A\beta_{40}$ levels augmented in the cortex of stressed but not unstressed Tg2576 mice (Fig. 4A; $p < 0.05$). The amount of the Aβ precursor, C99 (Fig. 4C), but that not of C83 (data not shown), also increased significantly in stressed (196 ± 9%) versus unstressed (100 ± 5%) Tg2576 mice ($p < 0.05$). Consequently, the ratio of C99/C83 was significantly higher in stressed versus non-stressed Tg2576 mice (Fig. 4D; $p < 0.05$).

The extracellular amyloid plaque load and intraneuronal Aβ levels were analyzed by immunohistochemistry using the 6E10 antibody (Fig. 5C), which specifically recognizes the 1–17 amino acid sequence of human Aβ. Intraneuronal Aβ immunoreactivity and AβPP-CTFs increased markedly in the hippocampus of stressed but not unstressed Tg2576 mice (Fig. 5). Tg2576 mice subjected to CMS also exhibited enhanced plaque deposition and there were abundant Aβ plaques in the hippocampus and entorhinal cortex of stressed Tg2576 mice, but these were rarely observed in similar tissue from age-matched unstressed mice (Fig. 5).

3.3. CMS increases hippocampal tau phosphorylation

As tau hyperphosphorylation is an early event in the development of AD in both patients and animal models of the disease, the tau phosphorylation was analyzed in the hippocampus of Tg2576 mice. We analyzed two sites in tau that are phosphorylated by glycogen synthase kinase 3β (GSK-3β): Ser396/404, which is recognized by the PHF-1 antibody; and Ser202, which is recognized by AT8 antibody. When the results were normalized against the total tau content, detected with the T46 monoclonal antibody, the phosphorylated tau detected with the AT8 antibody was 2.5-fold higher in the hippocampus of stressed Tg2576 mice versus unstressed mice ($p < 0.05$; Fig. 6A, left panel). Indeed, tau phosphorylation at the PHF1 epitope was also significantly enhanced in the stressed versus non-stressed Tg2576 mice ($p < 0.05$; Fig. 6A, right panel), even though the total amount of tau normalized to the actin content was similar in both groups (data not shown). Furthermore, the presence of tau phosphorylated at the AT8 epitope could be detected in the hippocampus of stressed animals by immunocytochemistry, primarily in the CA1 and the hilus of the dentate gyrus (DG: Fig. 6C). Phosphorylated tau was not detected in unstressed animals, and cell loss could not be seen in either stressed or unstressed animals by Nissl staining (data not shown).

Dystrophic neurites (DNs) are abnormal neuronal processes associated with amyloid deposits (neuritic plaques) and they represent an important pathological feature of AD closely related to synaptic defects. While AT8-phospho-tau-positive DNs are normally found in Tg2576 mice from 12 months of age [27], we observed several AT8-positive DNs surrounding some amyloid plaques (Fig. 6D) in the entorhinal cortex of stressed Tg2576 mice (8 months old), suggesting that CMS accelerated the AD pathology.

Tau phosphorylation is regulated by various protein kinases and phosphatases. However, since GSK-3β mediates the phosphorylation of tau protein at Ser202 and Ser396/404 (AT8 and PHF1 immunoreactivity) [28–30], we determined the proportion of the total GSK3β that was inactive (pGSK-3β-Ser9). There was significantly less pGSK-3β-Ser9 in the hippocampus of stressed than in unstressed Tg2576 mice (Figure 6B; $p < 0.01$, Student's t-test), although the total GSK-3β normalized to actin content was similar in both these groups of mice (data not shown). Taken together these data suggested that CMS enhanced the tau pathology in Tg2576 mice and that this accumulation of tau may

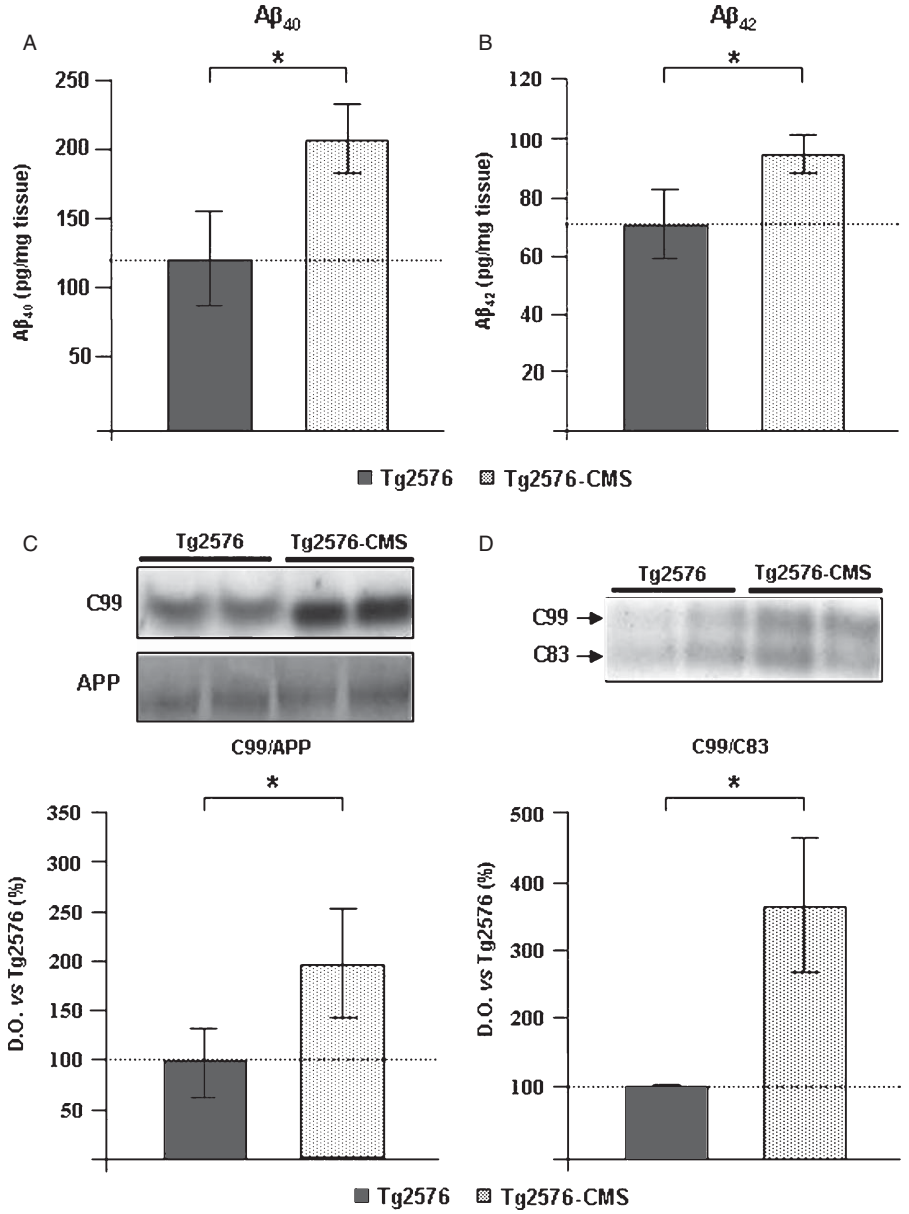

Fig. 4. CMS exposure increases Aβ and CTF (C99) levels in Tg2576 mice. $A\beta_{40}$ (A) and $A\beta_{42}$ (B) in cortical samples from stressed and unstressed Tg2576 mice measured by ELISA and expressed as the mean ± S.E.M. ($n = 6$ per group, $*p < 0.05$ Student's test). C, D) Analysis of full-length AβPP and the AβPP-derived CTFs, C99 and C83, in 8 month old stressed and unstressed Tg2576 mice. Representative western blots of prefrontal cortex samples, from which the AβPP, C99 and C83 immunoreactive bands were quantified and presented in histograms as the C99/AβPP (C) and C99/C83 (D) ratios. The data is expressed as the mean ± S.E.M. ($n = 4$-5 per group, $*p < 0.05$ Student's test).

contribute to the memory impairment evident in the MWM task.

4. DISCUSSION

The findings presented here indicate that CMS accelerates the onset of cognitive impairment in the Tg2576 transgenic mouse model of AD. This impairment was accompanied by an increase in the accumulation of Aβ and phospho-tau in the hippocampus of stressed Tg2576 mice two months after experiencing CMS. While several studies have demonstrated that chronic stress may accelerate the onset and progression of an AD-like phenotype in different murine models of AD [11–13], most of these studies involved the use of strong stressors. Here, we employed a CMS protocol

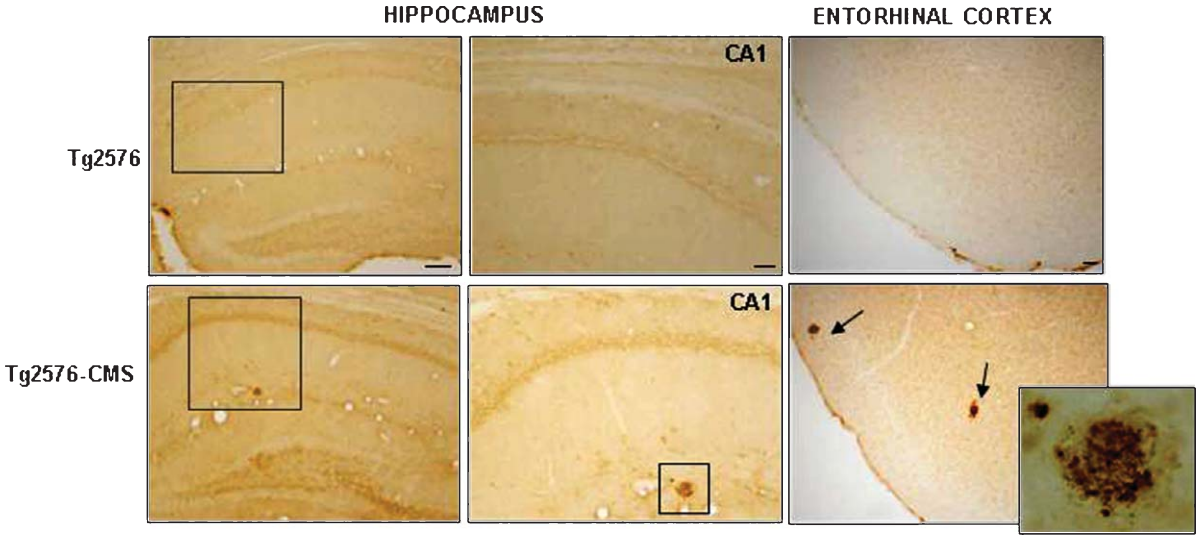

Fig. 5. CMS exposure increases intraneuronal Aβ and amyloid deposits in Tg2576 mice. Representative images showing immunostaining with the 6E10 antibody. Intraneuronal Aβ immunoreactivity increased markedly in the CA1 area of the hippocampus in stressed (CMS-Tg2576) (A) but not in unstressed (Tg2576) mice. B) Extracellular deposits stained with the 6E10 antiserum were mainly detected in stressed Tg2576 mice but they were rarely observed in age-matched unstressed controls. Representative brain sections (hippocampus and entorhinal cortex) of stressed (C) and unstressed (D) Tg2576 mice are shown.

in which several mild stressors were presented in a random order. This approach is thought to represent a good model of everyday stress in humans better suited to study the progress and/or pathogenesis of AD.

Stress is classically associated with the hypersecretion of cortisol, which may contribute to impaired hippocampal function [31]. Increases in plasma corticosterone levels have been associated with CMS [24, 25] and cognitive impairment [21, 32]. However, we observed a decrease in corticosterone levels in stressed mice 2 months after terminating the CMS protocol. Indeed, corticosterone levels decreased two weeks after CMS in a model of depression [26] and in animal models of post-traumatic stress disorder [33]. Decreases in corticosterone levels have also been reported in patients with different stress-related disorders such as post-traumatic stress disorder, chronic fatigue syndrome or atypical depression [34]. Thus, it would appear likely that the elevated corticosterone levels observed in response to stress return to baseline levels or even decrease over time. Indeed, a rebound effect has been proposed following prolonged hyperactivity of the hypothalamic-pituitary-adrenal axis due to chronic stress [35].

4.1. Cognitive function

CMS had a dramatic influence on spatial memory in transgenic mice, witnessed by the cognitive impairment in the last invisible platform training trial (learning phase) and in the probe trials (indicative of impaired memory consolidation). Tg2576 mice overexpress human AβPP695 with the "Swedish" mutation, and they develop memory deficits and plaques with age [15]. Aβ levels increase rapidly at 6 months and amyloid plaques appear at 10–12 months [36]. The 4 month-old Tg2576 mice used here do not display cognitive deficits or amyloid plaques. However, CMS markedly accelerates the onset of cognitive deficits in these mice in the MWM task, suggesting that it contributes to the progression of memory impairment in AD. Exposure to CMS has been previously reported to impair cognitive performance in wild type mice, although stressed wild type animals can still learn at the end of the training sessions in the invisible platform phase [37]. Here, we found that stressed Tg2576 mice were unable to learn during the invisible platform training sessions, suggesting that the combination of stress with a genetic risk factor (overexpression of $hA\beta PP^{Swe}$) markedly exacerbates the cognitive deficit.

4.2. Amyloid Pathology

Hippocampal Aβ levels and amyloid deposits increase significantly in stressed Tg2576 mice two months after CMS. It is important to note that stress-induced increases in glucocorticoid levels enhance β-secretase transcription, contributing to enhanced

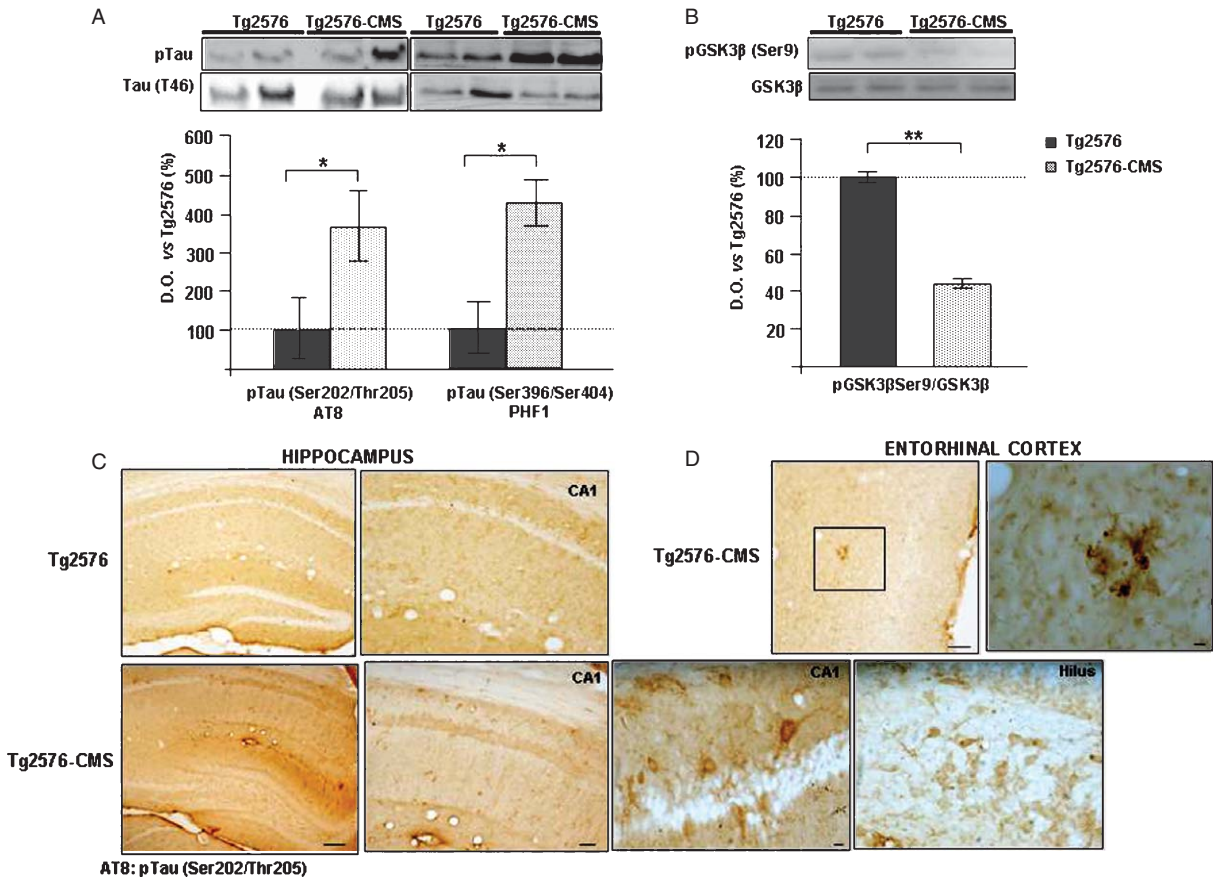

Fig. 6. CMS regulates tau phosphorylation via the pGSK3β pathway in Tg2576 transgenic mice. A) Probing western blots with the phospho-specific AT8 (left panel) or PHF1 (right panel) antibodies, normalized to total tau (T46), revealed tau hyperphosphorylation in the hippocampus of 8 month-old Tg2576 mice exposed to CMS when compared with the unstressed controls ($n = 4$-5 per group, $*p < 0.05$ Student's test). B) The levels of pGSK-3β-Ser9 normalized to total GSK-3β protein were significantly lower in the stressed Tg2576 mice ($*p < 0.05$, $**p < 0.01$ Student's test, $n = 4$-5 per group). The data are expressed as the mean percentage (±S.E.M.) with respect to unstressed Tg2576 mice (100%). C) Representative images of different brain sections stained with the AT8 antibody. Hyperphosphorylated tau at the AT8 epitopes was rarely observed in the hippocampus of stressed Tg2576 mice. Representative brain sections of stressed and unstressed Tg2576 mice. D) AT8-positive staining is detected around an amyloid plaque in the entorhinal cortex of the stressed Tg2576 mice.

Aβ production [10]. However, these changes were observed long after the CMS protocol was completed, when corticosteroid secretion had decreased. It is possible that the elevated corticosterone levels that are evident at the time of stress induction [24, 25] induce long-lasting changes that affect Aβ-processing. However, the effects of some strong chronic stressors on brain Aβ (in Tg2576 mice) are independent of corticosterone levels [38]. Thus, it is also possible that the elevated corticosterone *per se* does not directly affect the increase in Aβ in CMS-Tg2576 mice, and that other components of the hypothalamic axis that are deregulated in response to CMS are involved in this effect.

We observed an increase in intraneuronal Aβ in the CA1 hippocampal layer in stressed Tg2576 mice but not in the age-matched Tg2576 controls. Intraneuronal Aβ accumulation has been correlated with deficits in long-term synaptic plasticity and with the downregulation of the Akt survival pathway in different transgenic AD mouse models [39–41]. The downregulation of the Akt survival pathway may have given rise to the decrease in the inactive pGSK-3β-Ser9 form of GSK-3β observed here (see below). Aβ plaques begin to appear in Tg2576 mice at 10–12 months of age [15] and they are not detected in younger animals. Consistent with the effects of harsh stress protocols in different mouse models of AD [11, 12], Aβ plaques were readily detected in the hippocampus and entorhinal cortex of stressed 8 month old Tg2576 mice. Thus, like stronger stressors, exposure to CMS also appears to enhance AβPP-processing and increase the

intraneuronal Aβ and extracellular plaques. This enhancement is primarily observed in the hippocampus, a particularly vulnerable region of the brain that is specifically targeted by stress hormones (glucocorticoids) [5, 42]. Exposure of wild-type mice to CMS has previously been reported to increase AβPP processing but via the non-amylodogenic pathway [37]. Together, these findings suggest that CMS affects AβPP processing in mice in different ways, depending on the presence or absence of AβPP mutations that provide the amyloidogenic pathway.

4.3. Tau pathology

We show that tau phosphorylation increases in the hippocampus of stressed mice and the decrease in the inactive form of GSK-3β observed suggests that this effect may result from enhanced GSK-3β activity. Acute environmental insults, such as ether exposure [43], starvation [44], or cold water stress [45, 46], activate kinases that induce hippocampal tau phosphorylation at several serine and threonine sites equivalent to those phosphorylated in AD brains. While tau phosphorylation in response to acute stress returns to basal levels once the stressor is removed [43–46], we found that tau phosphorylation was altered two months after termination of the CMS procedure. Hence, chronic stress would appear to produce permanent or more long-standing alterations in tau phosphorylation and thus, CMS may be a risk factor contributing to AD-like neuropathology in a tau-dependent manner. As increases in phospho-tau have been described in wild type animals exposed to CMS [37], this probably reflects a neural response to stress that occurs irrespective of alterations to AβPP or Aβ burden.

GSK-3β is one of the most important kinases involved in the phosphorylation of tau associated with AD [47, 48]. Several authors have demonstrated that GSK-3 over-activity accounts for three key hallmarks of AD: Memory impairment, tau hyperphosphorylation, and increased Aβ production (see [49] for review). There is less GSK-3β in the hippocampus of Tg2576 mice exposed to CMS, suggesting that tau phosphorylation in these mice is at least partially modulated by the upregulation of this kinase. The Aβ peptide has been proposed to promote GSK-3β activation, in turn leading to tau phosphorylation [50, 51], suggesting that the increase in AβPP processing via the amyloidogenic pathway in CMS-Tg2576 mice may underlie this effect. This hypothesis is in line with previous reports of altered CDK5 activity in wild type animals exposed to CMS. Thus, it appears that increases in phospho-tau in non-transgenic animals are primarily mediated by CDK5 [37], while in Tg2576 mice GSK-3β is the main kinase involved in tau phosphorylation. Indeed, the CDK5 activity in stressed Tg2576 mice was similar to that in unstressed animals when determined by the p25/p35 ratio (data not shown). The higher levels of active GSK-3β may also result from the increases in Aβ, which has been reported to activate GSK-3β [52].

Taken together the present findings suggest that CMS induces cognitive impairment, increases intraneuronal and extracellular Aβ levels, and enhances GSK-3β-dependent tau phosphorylation in the Tg2576 mouse model of AD. These results suggest that CMS may influence the onset and development of AD by interacting with genetic risk factors.

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