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Long-term Phenylbutyrate administration prevents memory deficits in Tg2576 mice by decreasing Aβ

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1. ABSTRACT

Aberrations in protein folding, processing, and/or degradation are common features of neurodegenerative diseases, such as Alzheimer's disease (AD). Sodium 4-phenylbutyrate (PBA) is a well-known histone deacetylase inhibitor, which increases gene transcription of a number of genes, and also exerts neuroprotective effects. PBA acts as a chemical chaperone reducing the load of mutant or unfolded proteins during cellular stress. Previously, we reported that 5-week administration of PBA reinstated memory loss and dendritic spine densities in the Tg2576 mouse model of AD. In this study we reported that chronic administration of PBA, starting before the onset of disease symptoms (6 month-old) prevents age-related memory deficits in Tg2576 mice. The amelioration of the memory impairment is associated to a decrease in amyloid beta pathology and the glial fibrillary acidic protein (GFAP), suggesting that inflammation was reduced in PBA-treated animals. Together, the beneficial effects of PBA make it a promising agent for the prevention of AD.

2. INTRODUCTION

Alzheimer´s disease (AD) is a neurodegenerative disorder clinically characterized by a progressive cognitive decline. The hallmark pathological features of AD involve misfolding and aggregation of proteins including the accumulation of amyloid β (Aβ) protein in plaques (neuritic plaques), and the aggregation of hyperphosphorylated Tau protein in intraneuronal filaments that originate the neurofibrillary tangles (1). Aβ is processed from the amyloid precursor protein (APP), a transmembrane protein folded and modified in the endoplasmic reticulum (ER) and transported through the Golgi complex to the outer membrane (2, 3). The accumulation of proteins in the ER may both, disturb its function and induce ER stress, a cellular response that restores cellular homeostasis. However, if the provoking insult is prolonged, ER stress may trigger apoptotic cell death (4). According to the amyloid hypothesis, the accumulation of Aβ in the brain is the fundamental cause of the disease (5), and could be, at least in part, the responsible of the ER stress that different authors have demonstrated in AD brains (6, 7).

ER stress might activate the expression of glycogen synthase kinase 3β (GSK-3β), a well-known Tau kinase, and in that way, immunohistochemical studies have revealed a close connection between the ER stress and Tau pathology in neurons (8). Moreover, recent studies have indicated that ER stress may also trigger inflammatory responses to defend brain tissue against necrotic injuries (9). Importantly, a chronic inflammatory response is associated to the Aβ pathology and it is known that activated microglia and astrocytes closely associate to amyloid plaques in AD brains (10, 11). Overall, ER stress is clearly involved in the pathogenesis of AD. On one hand the pathological characteristics of AD (oxidative stress, impaired Ca^{2+} homeostasis, intracellular deposition of Tau proteins and amyloid-β peptides) may be caused by ER stress in neurons. On the other hand, these pathological features may also trigger ER stress and thus aggravate AD pathology (2, 9, 12).

Recently, we have demonstrated that the administration of the histone deacetylase (HDAC) inhibitor, 4-phenylbutyrate (PBA) for 5 weeks reversed the memory impairments in the AD mouse model Tg2576 mice at the age of 15 month (13). PBA is a short chain acid fat (SCAF), acts as a chemical chaperone and, furthermore, exerts anti inflammatory properties (14, 15), thus, may be a potential pharmacological therapy to revert AD-like symptoms. We have shown that the effect of PBA in Tg2576 mice was associated with a rescue of the synaptic dendritic markers and dendritic spine loss related to the memory failure (16). Tg2576 mice overexpress the human Swedish mutation of the APP (APPK670N, M671L) (17) and by 15 months of age they have considerable amyloid pathology, quantifiable inflammatory response (18) and serious learning impairment in the traditional spatial reference memory water maze (19, 20).

Although the 5-week treatment was significantly effective in the amelioration of memory deficits in aged mice, no changes were found in total Aβ cortical levels measured by ELISA (13). However, in further studies focused in the mechanism involved in PBA-induced memory restoration, a decrease in the intracellular accumulation of Aβ in CA1 pyramidal cells was revealed in transgenic mice receiving PBA treatment (16). These data suggested that PBA may facilitate the clearance of Aβ and this effect could be related to its chaperone-like activity and ability to mitigate ER stress (21). It has been postulated that PBA could have an anti inflammatory effect through inhibition of ER stress (14, 15, 22). The present study was aimed to determine whether a chronic treatment with PBA, starting at the onset of AD symptoms, would prevent the progressive amyloid pathology (and histopathological changes linked to the disease) as well as the associated immunological response. For this purpose, 6-month old Tg2576 mice were treated until 12 months of age, when behavioural and biochemical assays were carried out to asses the potential preventive effect of PBA in AD.

3. MATERIAL AND METHODS

3.1. Mouse model and treatment

In this study, Tg2576 AD transgenic mice, which express the human 695-aa isoform of APP containing the Swedish double mutation (APPswe) [(APP695)Lys670→Asn, Met671→Leu] driven by a hamster prion promoter, were used. In the Tg2576 AD mouse model, Aβ peptide content in the brain accumulates exponentially between 7 and 12 months of age and mice show impaired memory in the water maze test at the age of 12 - 15 months (17, 23).

The aim of this study was to investigate whether PBA could prevent Alzheimer phenotype, thus, 6 month old Tg2576 mice (before the onset of symptoms) were administered daily with PBA (200 mg/kg, i.p.) or vehicle for 6 months. PBA solution was prepared by titrating equimolecular amounts of 4-phenylbutyric acid (Sigma, Madrid, Spain) and sodium hydroxide to pH 7.4. As a control, a group of strain- and age-matched non-transgenic mice was used. All procedures were carried out in accordance with European and Spanish regulations (86/609/CEE; RD1201/2005). This study was approved by the Ethical Committee of the University of Navarra (no. 018/05).

3.2. Morris Water Maze Test (MWM)

The MWM test was used to evaluate spatial memory function in response to the treatment as previously described (13, 17, 23). Briefly, mice were trained for three consecutive days (8 trials/day) swimming to a platform above the water (visible-platform). The same platform location was used for all visible-platform sessions and was changed for the invisible-platform training, conducted over the next 8 consecutive days (4 trials/day) and during which, the time required to reach the hidden platform was recorded. To test the retention, three probe trials were performed at the beginning of 4th, 7th, and 9th. In the probe trials the platform was removed from the pool and the percent of time spent in the quadrant where previously was the platform was recorded. All trials were monitored by a camera above the center of the pool connected to a SMART-LD program (Panlab S.L., Barcelona, Spain). All experimental procedures were performed blind to groups.

On day 10th, the platform was moved diagonally across its initial position in the invisible platform, without changing any distal visual cues, and relearning was assessed. Each animal performed the test as described above with the new platform location. Two probe trials were performed on days 12^{th} and 14^{th}. The time spent into the quadrant of the previous platform location and time spent in the new location quadrant, indicated learning of the new condition, was measured. In all cases, animals were killed 24 h after the last trial. The brains were removed and immediately frozen on dry ice before dissection. Some animals were perfused transcardially with paraformaldehyde for immunohistochemistry.

3.3. Determination of Aβ levels

For the analysis of total $A\beta_{42}$ burden, mice frontal cortex were homogenized in a buffer containing 5 M guanidine HCl and 50 mM Tris HCl, pH 8, protease inhibitors (CompleteTM Protease Inhibitor Cocktail, Roche, Barcelona, Spain) and phosphatase inhibitors (0.1 mM Na_3VO_4, 1 mM NaF). $A\beta_{42}$ levels were measured using a sensitive sandwich ELISA kit from Biosource

(Camarillo, Ca, USA) following the manufacturer's instructions.

3.4. Immunohistochemistry

Floating tissue sections comprising hippocampal and cortex formation were processed for immunohistochemistry. Brain sections were rinsed (3 x 10 min) with 0.125 M phosphate buffer (pH 7.4). The endogenous peroxidase activity was eliminated by pre-treatment with 0.3% hydrogen peroxide in methanol for 45 min and washed in 0.125 M phosphate buffer solutions (PBS). The sections were then incubated in a blocking solution (PBS containing 0.5% Triton X-100, 0.1% BSA and 2% normal goat serum) for 2 h at room temperature and incubated overnight with primary antibodies in blocking solution. Subsequently, the sections were washed in PBS and incubated in a biotinilated secondary antibodies that matched the primary antibodies (1:200, Vector Laboratories, Burlingame, CA, USA) diluted in blocking solution for 2 h and then avidin-biotin complex for 1 h at room temperature. The reaction was developed using the diaminobenzidine (DAB) kit (Vector Laboratories). For 3D6 immunostaining, sections were incubated in 70% formic acid for 10 min to expose the epitope. The primary antibodies used were as follows: mouse monoclonal 3D6 (amino acids 1–5 of Aβ peptide, 1:4000, Chemicon, Temecula, CA), mouse monoclonal anti phospho-Tau (Ser202/Thr205) AT8 (1:50, Pierce Biotechnology Inc., Rockford), rabbit polyclonal anti-GFAP (1:1000, Sigma-Aldrich, St Luis, MO). For the double immunostaining, the secondary antibodies used were Alexa Fluor 488 goat anti-mouse, highly cross-absorbed, and Alexa Fluor 546 goat anti-rabbit, highly cross-absorbed (both 1:200, Molecular Probes, Eugene, Oregon, USA). Sections were mounted on super frost plus slides, air dried for 24 h, rinsed in toluene (2 x 5 min), and cover slip with DPX mounting medium. To ensure comparable immunostaining, sections were processed together under identical conditions. For the assessment of nonspecific primary immunostaining, some sections from each experimental group were incubated without the primary antibodies; in this case no immunostaining was observed. Nonspecific secondary immunostaining was also evaluated by incubating sections with primary and its nonspecific secondary antibodies; again, no immunostaining was observed.

3.5. Production of protein extracts

Mice were sacrificed by cervical dislocation and hippocampi quickly dissected from the brains. Total tissue homogenates were obtained by homogenizing the hippocampus in a cold lysis buffer with protease inhibitors (0.2 M NaCl, 0.1 M HEPES, 10% glycerol, 200 mM NaF, 2 mM $Na_4P_2O_7$, 5 mM EDTA, 1 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 1 mM Na_3VO_4, 1 mM benzamidine, 10 μg/ml leupeptin, 400 U/ml aprotinin), centrifuged at 14,000 x *g* 4ºC for 20 min and the supernatant was aliquoted and stored at -80ºC. Total protein concentrations were determined using the BioRad Bradford protein assay (BioRad Laboratories).

For APP carboxy-terminal fragments determination, the hippocampus was homogenized in a buffer containing SDS 2%, Tris–HCl (10 mM, pH 7.4), protease inhibitors (Complete™ Protease Inhibitor Cocktail, Roche) and phosphatase inhibitors (0.1 mM Na3VO4, 1 mM NaF). The homogenates were sonicated for 2 min and centrifuged at 100,000 g for 1 h. Aliquots of the supernatant were frozen at −80 °C and protein concentration was determined by the Bradford method using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA).

3.6. Immunoblotting

Protein samples were mixed with an equal volume of 2 x Laemmli sample buffer, resolved onto SDS-polyacrylamide gels and transferred to nitrocellulose membrane. The membranes were blocked with 5% milk, 0.05% Tween-20 in PBS or TBS followed by overnight incubation with the following primary antibodies: mouse monoclonal anti-p-Tau AT8 (1:1000, Pierce Biotechnology), mouse monoclonal anti Tau (clone Tau46, 1:1000, Sigma-Aldrich), rabbit polyclonal anti-pGSK3-Ser9 (1:1000, Cell Signalling Technology, Beverly, MA), rabbit polyclonal anti-pAkt-Ser473 (1:1000, Cell Signalling Technology), rabbit polyclonal anti-GSK3 (1:1000), rabbit polyclonal anti-GFAP (1:100, Sigma-Aldrich) and mouse monoclonal anti-actin (1:4000, Santa Cruz Biotechnology, Santa Cruz, CA) in the corresponding buffer. Following two washes in PBS/Tween-20 or TBS/Tween20 and one PBS or TBS alone, immunolabeled protein bands were detected by using HRP-conjugated anti-rabbit or anti-mouse antibody (Santa Cruz; dilution 1:5000) following an enhanced chemiluminescence system (ECL, GE Healthcare Bioscience, Buckinghamshire, UK), and autoradiographic exposure to Hyperfilm^{TM}ECL (GE Healthcare Bioscience). Signals quantification was performed using Scion Image software (Scion Corporation).

For western blot analysis of APP-derived fragments, aliquots of the protein extracts were mixed with XT sample buffer™plus XT reducing agent™ or Tricine sample buffer™ (Bio-Rad) and boiled for 5 min. Proteins were separated in a Criterion™ precast Bis–Tris 4–12% gradient precast gel (Bio-Rad) and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk, 0.05% Tween-20 in TBS followed by overnight incubation with the following primary antibodies: mouse monoclonal 3D6 (amino acids 1–5 of Aβ peptide, 1:4000, Chemicon), rabbit polyclonal anti-APP C-terminal (amino acids 676–695) (1:2000, Sigma-Aldrich).

3.7. Data analysis and statistical procedures

The results were processed for statistical analysis using SPSS package for Windows, version 15.0 (SPSS, Chicago, IL, USA). Unless otherwise indicated, results are presented as mean±SEM. In the MWM and fear conditioning test, escape latencies during training were analyzed using one-way analysis of variance (ANOVA) followed by Scheffé post hoc test. In the MWM, Friedman´s test was performed to determine the intra group comparisons over trials. Biochemical data were analyzed using one-way ANOVA followed by Scheffé post hoc test.

4. RESULTS

4.1. Morris Water Maze test (MWM)

To test whether PBA prevents memory deficits in AD transgenic mice, we assessed learning/memory

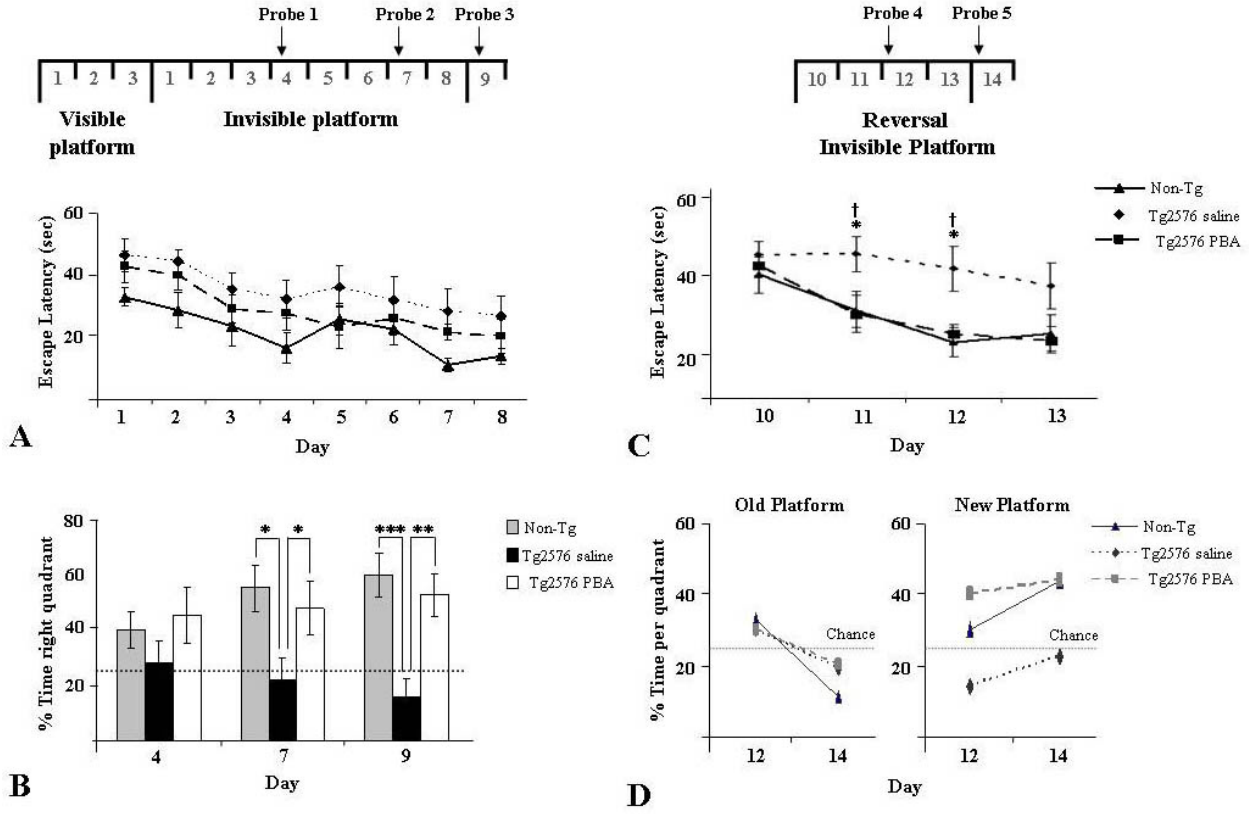

Figure 1. A chronic treatment with PBA improves memory deficits in 12-month-old Tg2576 mice. A, Escape latency in the invisible platform training. No significant differences were detected among groups during any of the 8 days of invisible platform training. B, Percentage time spent in the training quadrant during 60 s probe trials. Saline-treated Tg2576 mice performed significantly worse than PBA-treated or non-transgenic littermate (Non-Tg) controls in 7th and 9th probe trials (*p< 0.05, **p< 0.01, ***p< 0.001, ANOVA with Scheffe's post-hoc tests). C, Escape latency during the reversal phase of learning, that is, 4 days (days 10 to 14) of acquisition training in which the platform was in the opposite quadrant. Saline-treated Tg2576 mice showed significantly longer escape latencies when compared to Non-Tg control group (†p< 0.05 ANOVA with Scheffe's post-hoc test) and to PBA-treated group (*p< 0.05, ANOVA with Scheffe's post-hoc test). D, Percentage time spent in the training quadrants (former and new platform location) during 60 s probe trials on days 12th and 14th. In the former platform location (old platform), a gradual reduction in percent time between days 12 and 14 can be seen for all the groups. In the new platform location (new platform) a gradual increase in percent time between days 12 and 14 was observed in Non-Tg mice and transgenic mice treated with PBA, presenting more than 25% time since day 12, whereas Tg2576-saline mice were not able to learn the new platform location, showing a percent time below 25% at the end of the task (day 14). Twenty-five percent (dashed line) would equal chance performance.

performance at 12 months of age after six months of treatment with PBA or vehicle. At the end of the treatment no significant differences were found between the groups in their performance in both the visible and the invisible platform training. Escape latencies from the hidden platform were analyzed using the Friedman´s repeated measure nonparametric test for the intra-group comparisons. The mean latencies spent to reach the platform decreased significantly over the training sessions for all the groups: non-transgenic (Non-Tg; $\chi 2r=19.67$, $p<0.01$), Tg2576-saline ($\chi 2r=17.67$, $p<0.01$) and Tg2576-PBA ($\chi 2r=14.61$, $p<0.01$) mice. Specifically, intra-group comparisons of escape latencies showed a significant effect of the training in all the groups, reflecting its ability to learn the platform location.

After the 12 th, 24th and 32 th trial, all mice were subjected to a probe trial in which they swam in the pool for 60 s with the platform removed. Memory retention was measured as the percentage of time (within 60 s) that mice spent in the target quadrant (held the platform during training). The probe on days 7th and 9th showed that transgenic mice treated with saline spent significantly less time in the target quadrant than non transgenic mice. Transgenic mice that underwent PBA treatment spent a percent of time in the target quadrant that not differ from that of age-matched controls (Figure 1B). These data suggest that PBA, administered chronically to Tg2576 mice, prevents the deficits in the water maze memory retention assay.

One day after the probe in day 9, the platform was moved to the opposite quadrant location and mice were trained for 4 days (four trials per day, days 10th to 13th) in this reversed setting (reversal learning). Tg2576 saline mice

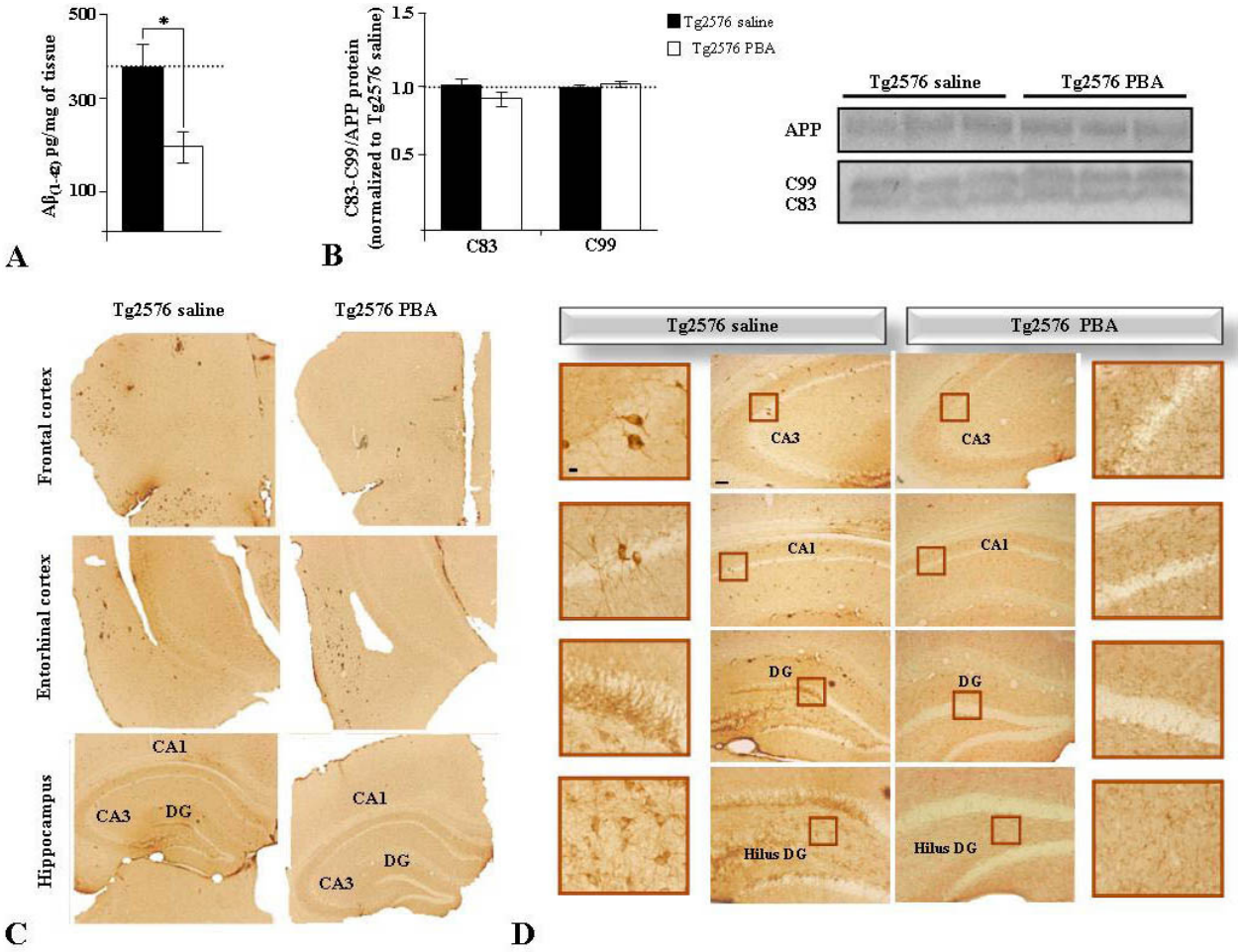

Figure 2. Chronic PBA treatment decreases Aß burden in 12-month-old Tg2576 transgenic mice. A, Total $Aß_{42}$ levels determined by ELISA were decreased (50%) in the frontal cortex in Tg2576 mice treated with PBA compared to that of Tg2576 mice receiving saline (mean ± S.E.M. of 5 individual determinations). B, No change on full-length APP, and on APP C-terminal fragments, C99 and C83 were detected in transgenic mice after chronic administration of PBA. Representative bands of western blot made from cerebral tissues of mice are shown. The histogram shows the quantification of the immunochemically reactive bands in the western blot of APP, C99 and C83. Results are expressed as mean ± SEM n= 5-6 in each group. C, Multiple extracellular deposits stained with 3D6 antiserum were detected in frontal and entorhinal cortex, and hippocampus in Tg2576-saline mice. Tg2576-PBA mice showed a decrease in Aβ deposits mainly in the hippocampus and frontal cortex. D, Tg2576 mice receiving PBA displayed a marked decrease in intraneuronal Aβ immunoreactivity in CA1, CA3, dentate gyrus (DG) and hilus when compared to saline-treated transgenic mice. Representative brain sections of saline- and PBA-treated Tg2576 mice are shown.

showed significantly longer escape times over the training blocks on days 11 and 12, whereas the non-transgenic and PBA-treated mice more rapidly reached the platform in the new location (Figure 1C). These results demonstrate that a persistent search in the previous target zone resulted in a significant delay for Tg2576 saline mice to find the new target location. The probe test, in which the platform was again removed, revealed significant differences in performance between groups (Figure 1D). In the quadrant that had previously contained the platform (old platform), a gradual reduction in percent time can be seen for all the groups. A shift to the 'new' goal position (new platform) was observed on day 12 for PBA-treated mice, whereas non-transgenic mice reached similar memory retention in the reversed setting (new location) two days later (14^{th} probe day). Transgenic-saline treated mice spent less than 25% of time (random learning) in the new location at the end of the task, indicative of memory retention impairment (Figure 1D).

Thus, at the age of 12 month, Tg2576 mice had clear deficits in spatial memory consolidation and in the reversal task. Reversion of these deficits by chronic treatment with PBA supports a role of PBA in plasticity remodelling and memory enhancement in the AD transgenic mice.

4.2. Aβ pathology

The effect of PBA treatment on the Aβ levels in Tg2576 mice brain was determined in the cerebral cortex

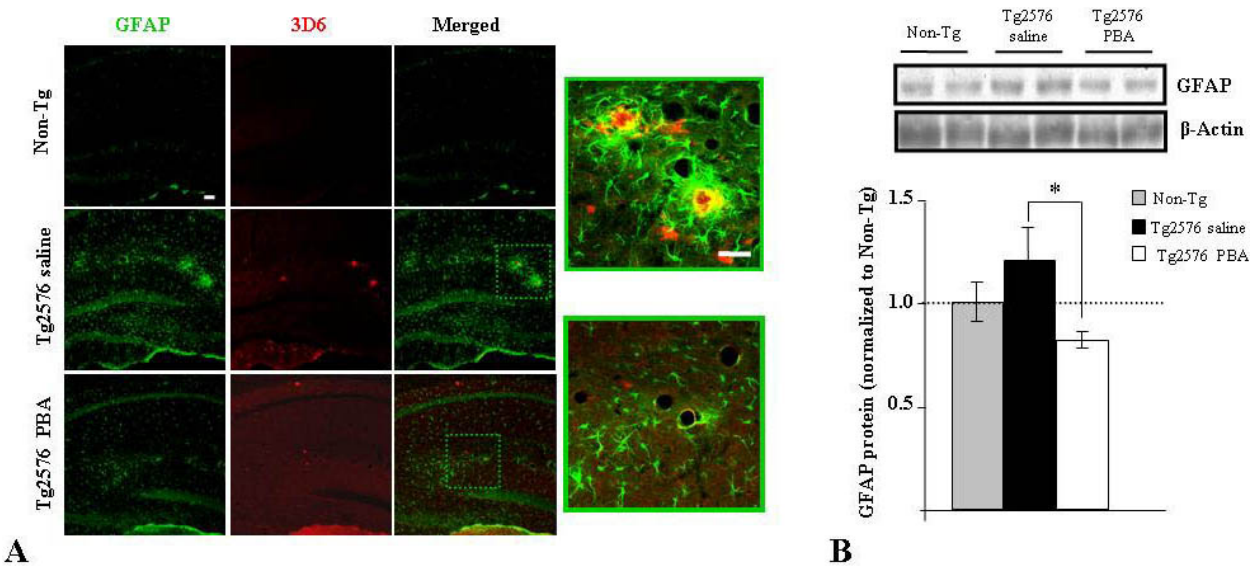

Figure 3. The activation of plaque-associated astrocytes in the hippocampus of Tg2576 mice was decreased after 6 month treatment with PBA. A, Double immunostaining in which Aβ deposits are labelled with 3D6 antibody and astrocytes are labelled with anti-GFAP antibody. Reactive astrocytes colocalize with extracellular Aβ deposits in the hippocampus of Tg2576 mice whereas they were almost absent in PBA-treated mice, where hippocampus is devoid of visible Aß deposits. B, Steady-state levels of the astrocyte marker GFAP was measured in hippocampal extracts and blots were normalized to β-Actin. GFAP levels were increased in Tg2576 mice compared to non transgenic mice (Non-Tg, * $p < 0.05$) and reversed after PBA treatment. Representative bands of western blot made from cerebral tissues of mice are shown. The histogram shows the quantification of the immunochemically reactive bands in the western blot. Results are expressed as mean ± SEM n= 5-6 in each group.

by sandwich ELISA. As shown in Figure 2A, a significant difference (50% reduction, $p < 0.05$, Student´s t test) was seen in $A\beta_{42}$ levels in Tg2576 mice treated with PBA compared to saline-treated transgenic mice. No Aβ was detected in wild type littermates (data not shown). The levels of the carboxyl terminal fragments of APP, C83 and C99, were analyzed using Tris/tricine PAGE 16.5% gels for a better resolution. No differences in intensity of any of the fragments were found between the two groups of transgenic mice, suggesting that PBA does not affect APP processing (Figure 2B).

Aβ immunoreactivity was assayed using 3D6 antibody to stain intraneuronal Aβ and extracellular deposits. Fewer senile plaques were detected in the frontal cortex and hippocampus of mice receiving chronic PBA treatment compared to Tg2576-saline treated mice (Figure 2C). Interestingly, 3D6 immnuoreactivity displayed largely reduced levels of intraneuronal Aβ in the pyramidal cells of CA3 and CA1 and in the granular layer and the hilus of dentate gyrus (GD) (Figure 2D) of mice treated with PBA compared to saline-treated animals.

Inflammatory processes are intimately involved in several crucial events in the pathological cascade, in particular with dense-core Aβ plaques. Previous studies had shown that PBA have anti inflammatory effect (14). Analysis of astroglial activation by double staining for Aβ42 (using 3D6 antibody) and the astrocytic marker molecule, glial fibrillary acidic protein (GFAP), were carried out in mice hippocampus. Immunostaining revealed that GFAP-positive cells were mostly located around amyloid plaques in the brain of Tg2576 mice (Figure 3A). Remarkably, amyloid plaques and activated astrocytic cells were decreased in the brain of Tg2576 mice receiving PBA for 6 months. In the brain of non-transgenic mice, neither 3D6 nor GFAP immunostaining was detectable (data not shown).

In order to confirm these immunohistochemical findings, western blotting of hippocampal homogenates was carried out. Statistical comparison of GFAP band optic density normalized with β-actin showed significant differences among the different groups (Figure 3B). Expression levels of GFAP in Tg2576-PBA treated mice (82 %) were significantly decreased ($p < 0.05$) when compared to that of untreated Tg2576 mice (121 %), suggesting that PBA prevents the astrocytosis observed in the untreated Tg2576 animals.

4.3. Tau pathology

Hiperphosphorylation of Tau is another pathological mark that contributes to the development of AD. An enhancement in Tau phosphorylation in transgenic animals was observed by immunocytochemistry using the AT8 antibody, which recognizes aberrantly hyperphosphorylated epitopes. In fact, positive neurons for AT8 staining were easily found in the CA3 region and the DG of the hippocampus in these animals but were not presented in the samples from their respective non-transgenic age-matched controls or in the transgenic mice receiving PBA (Fig 4A). The levels of Tau phosphorylation

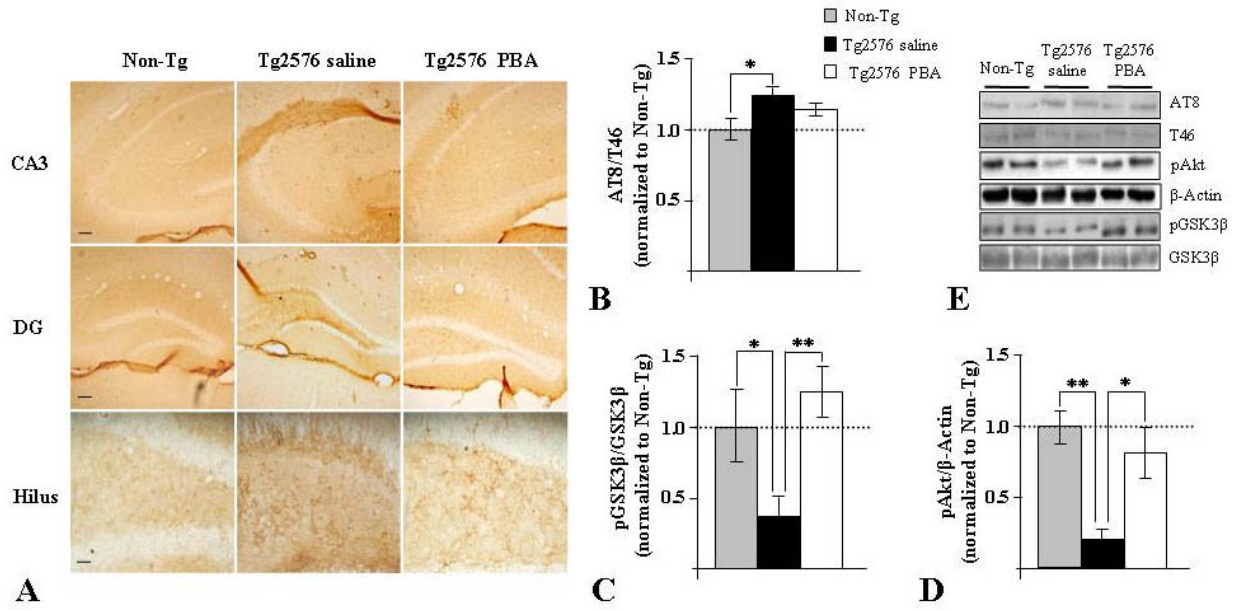

Figure 4. PBA regulates Tau phosphorylation through the pGSK3β pathway in Tg2576 transgenic mice. A, Tg2576 mice receiving PBA display a marked decrease in phosphospecific antibody AT8 immunostaining in CA3, dentate gyrus (DG) and hilus, when compared to saline-treated mice. AT8 immunostaining was absent in age-matched control mice. B, Western blot analysis using AT8 antibody normalized to total Tau (T46) showed that Tau was hyperphosphorylated in the hippocampus of 12-month-old saline-treated Tg2576 mice compared to non-transgenic group (Non-Tg) (*p< 0.05) and partially reverse with PBA treatment. The histogram shows the quantification of the immunochemically reactive bands in the western blot. Results are expressed as mean ± SEM n= 5-6 in each group. C, pGSK3β–Ser9 levels normalized to GSK3β total protein were significantly decreased in saline-treated Tg2576 mice compared with Non-Tg mice (*p< 0.05; **p< 0.01). This decrease was inverted after PBA treatment. The histogram shows the quantification of the immunochemically reactive bands in the western blot. Results are expressed as mean ± SEM n= 5-6 in each group. D, pAkt levels normalized to actin were significantly decreased in saline-treated Tg2576 mice compared with Non-Tg mice (*p< 0.05; **p< 0.01). This decrease was inverted after PBA treatment. The histogram shows the quantification of the immunochemically reactive bands in the western blot. Results are expressed as mean ± SEM n= 5-6 in each group.

in the mice hippocampus were measured by western blot analysis using the same antibody (AT8) and normalized to total Tau (T46). As depicted in Figure 4B, a one-way ANOVA showed a significant group effect (p< 0.05). Scheffe's multiple-comparison test revealed that phosphorylation of Tau was significantly increased in the hippocampus of 12-month-old Tg2576 (124 %, p < 0.05) compared to non-transgenic mice (100 %) and was only partially reversed in transgenic mice treated with PBA (114 %) (Figure 4B). Western blot analysis showed that PBA moderately reduced Tau phosphorylation in transgenic mice, whereas the effect observed using immunohistochemistry, seems more consistent. This could be due to the differences between the two techniques, and, although the immnuhistochemistry is not quantitative, allows it to detect changes in specific areas (*i.e.*: CA3 and DG) which can be undetectable if the whole hippocampus is analyzed by western blot.

Tau hyperphosphorylation may be the result of dysregulation of different pathways, including the pGSK3/pAkt pathway. The levels of inactive GSK3β, phosphorylated at Ser 9 (pGSK3β-Ser9), normalized to GSK3β were measured. A one-way ANOVA followed by Scheffe's multiple-comparison test revealed that pGSK3β-Ser9 levels were markedly decreased in the hippocampus of transgenic mice treated with vehicle (36 %) compared to non-transgenic mice (100 %). No significant differences in pGSK3ß-Ser9 levels were found in transgenic mice treated with PBA (125 %) compared to non-transgenic mice (Figure 4C). In the same extracts the level of pAkt, which phosphorylates GSK3β at Ser 9, were analyzed. The expression of pAkt in transgenic mice that received PBA was no significantly different from that of non-transgenic mice (80 % vs non-Tg: 100 %); in contrast, the saline-treated Tg2576 mice showed a marked reduction in pAkt (20 %; p< 0.01 vs non-Tg and p< 0.05 vs Tg2576-PBA) (Figure 4D). These data suggest that PBA induces a reduction in pTau levels through the modulation of the pAkt/GSK3 pathway.

5. DISCUSSION

Few studies have tested whether or not a treatment at the pre-plaque stage, that would parallel the MCI stage of AD, would prevent/delay the progressive cognitive decline in AD models. Our previous studies demonstrated that 5-week treatment with PBA restored the memory impairments in aged Tg2576 mice without decreasing total brain Aβ burden (13, 16). Here, we

investigated whether PBA would be effective in preventing Aβ pathology and the associated memory deterioration in an AD mouse model when the treatment starts before the onset of the disease's symptoms. For this aim, 6 month-old Tg2576 mice were administered daily with PBA (200 mg/kg, i.p.) or vehicle for 6 months. At the end of the treatment, memory function in mice was tested using the Morris water maze test with a reversal phase, in which animals have to extinguish their initial learning of the platform's position and acquire a direct path to a new goal position (24). A platform reversal appears to be a suitable test to detect subtle changes in synaptic plasticity leading to a cognitive deficit that may be eventually prevented by PBA treatment.

In this study, and corroborated by others (23, 25), 12 month Tg2576 mice were able to acquire and use a spatial map in the standard version of the Morris water maze task, whereas they were impaired in probe trials (memory consolidation). PBA treatment prevented their impairment in memory retention, showing a similar performance to non transgenic mice. Analysis of reversal trial performance showed that, whereas untreated transgenic mice did not learn the new platform location, both, the non-transgenic and transgenic PBA-treated mice showed decreasing latencies escape in the new platform position with repeated reversal trials. Pompl *et al.*, (26) also demonstrated that, at the age of 9 month, Tg2576 performed similar to non-transgenic mice in the spatial learning phase (invisible platform) of MWM but performed worse in the reversal learning phase. Our data demonstrated that transgenic mice receiving chronic PBA treatment performed in the reversal learning phase similar to non transgenic mice, suggestive of an improvement of synaptic plasticity.

Eventhough 5 week-treatment with PBA did not modified Aβ burden in aged mice with extensive amyloid pathology (13), in the present study we show that when the PBA treatment starts before the onset of amyloid pathology, a decrease in total Aβ levels detected in mice brain might contribute to the memory restoration. Other therapeutic approaches that decrease the level of soluble Aβ in animal models, even in the absence of detectable changes in plaque load, ameliorate cognitive deficits (27, 28). These results demonstrate that PBA, administered before the onset of the disease symptoms, may prevent spatial learning and memory decline of Tg2576 mice by decreasing $A\beta_{42}$ accumulation in the brain. This could be due to the PBA cytoprotective chaperone effect that facilitates the clearance of $A\beta_{42}$ without affecting its production, since APP processing was not affected.

When cells are subjected to protein misfolding in the ER, a stress cascade with pathological consequences, including inflammation and cell death, is triggered. In a recent study we demonstrated that PBA clear intraneuronal Aβ staining in CA1 and affects the levels of ER stress markers (16). On analyzing inflammation-associated transcripts in 12-month Tg2576 mice, an up-regulation of GFAP, which reflects astro- and micro- gliosis occurs (18, 29) (30). GFAP-positive astrocytes are located in the vicinity of the Aβ plaques (31) and may promote Aβ plaque formation and maturation (19). Noteworthy, here we report a reduction in astrocyte activation (GFAP expression) in the hippocampus in PBA-treated mice compared to untreated transgenic mice, which correlates with a reduction in Aβ burden. Thus, it may be hypothesized that a reduction in intracellular Aβ, regulates ER stress markers and mediate events that reduce GFAP immunoreactivity.

ER stress activates GSK3β through dephosphorylation of Ser9 (32). Previously, we showed that PBA inhibited GSK3β in Tg2576 mice hippocampus after a 5 week-treatment (13). These results suggest that PBA protects neurons from ER stress and leads to GSK3β inhibition, which in turns prevents tau phosphorylation and contributes to restore neuronal plasticity. Here, using immunoshistochemitry and western blot analysis, we corroborated that PBA is able to decreased pTau in the hippocampus of Tg2576 mice, which could be mediated by the GSK3β inactivation, as deduced by the increase in GSK3β-Ser 9 levels detected in the hippocampal extracts.

Overall, there are two possible mechanisms in the preventive and beneficial effect of PBA in Tg2576 after 6 month-treatment: (1) PBA decreases the inflammatory reaction engendered by amyloid deposits and/or (2) PBA modulates the clearance of Aβ and amyloid deposition, and thereby reduces ER stress and inflammatory reaction.

6. ACKNOWLEDGMENTS

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Abbreviations: AD: Alzheimer´s disease, APP: amyloid precursor protein, Aβ: amyloid β protein, ER: endoplasmic reticulum, PBA: 4-Phenylbutyrate, GFAP: glial fibrillary acid protein

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