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AβPP induces cdk5-dependent tau hyperphosphorylation in transgenic mice Tg2576

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Abstract. Previous studies of Aβ-induced neuronal damage of hippocampal cells in culture have provided strong evidence that deregulation of the Cdk5/p35 kinase system is involved in the neurodegeneration pathway. Cdk5 inhibitors and antisense probes neuroprotected hippocampal cells against the neurotoxic action of Aβ. To further investigate the mechanisms underlying the participation of Cdk5 in neuronal degeneration, the transgenic mouse containing the Swedish mutations, Tg2576, was used as an animal model. Immunocytochemical studies using anti-A$\beta_{(1-17)}$ antibody evidenced the presence of labeled small-clustered core plaques in the hippocampus and cortex of 18-month-old transgenic mice brains. The loss of granular cells without a compressed appearance was detected in the vicinity of the cores in the dentate gyrus of the hippocampus. Immunostaining of Tg2576 brain sections with antibodies AT8, PHF1 and GFAP labeled punctuate dystrophic neurites in and around the amyloid core. Reactive astrogliosis around the plaques in the hippocampus was also observed. Studies at the molecular level showed differences in the expression of the truncated Cdk5 activator p25 in the transgenic animal, as compared with wild type controls. However no differences in Cdk5 levels were detected, thus corroborating previous cellular findings. Interestingly, hyperphosphorylated tau epitopes were substantially increased as assessed with the AT8 and PHF1 antibodies, in agreement with the observation of a p25 increase in the transgenic animal. These observations strongly suggest that the increased exposure of Alzheimer's type tau phosphoepitopes in the transgenic mice correlated with deregulation of Cdk5 leading to an increase in p25 levels. These studies also provide further evidence on the links between extraneuronal amyloid deposition and tau pathology.

Keywords: Alzheimer's disease, neurodegeneration, transgenic mouse Tg2576, amyloid precursor protein, Cdk5, regulatory aspects

1. Introduction

Alzheimer's disease (AD) is one of the most common types of dementia affecting the elderly, characterized by the formation of senile plaques [39,40] and neurofibrillary tangles [23,24]. These aggregates are involved in the process leading to neuronal loss of function and progressive neuronal death. Senile plaques are mainly composed of fibrils of amyloid-β (Aβ), a peptide derived from the proteolytic processing of the amyloid β-protein precursor (AβPP) [34,39]. Tau protein is the major component of PHFs, filaments that form the network termed neurofibrillary tangles [23]. In normal brain, the equilibrium between tau phosphorylations/dephosphorylations modulates the stability of the cytoskeleton and therefore axonal morphol-

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ogy [24]. Consequently, the breakdown of this equilibrium under pathological conditions such as those of neurodegenerative disorders, results in damage to the mature neurons [23,24,26]. In AD, it has been suggested that amyloid deposition is one of the processes that trigger the cascade of molecular events leading to neurofibrillary degeneration. However, the presence of senile plaques is not the only factor responsible for neurodegeneration. Alvarez et al., [3] showed an unusual activation of Cdk5 of hippocampal cells treated with Aβ. This deregulation of Cdk5 was concomitant with tau hyperphosphorylation and gradual neuronal death of hippocampal neurons, but pretreatment of cells with specific inhibitors of Cdk5 such as butyrolactone I or by using anti-sense probes for Cdk5, protected cells against tau hyperphosphorylation and cell death. Neuroprotection of cells against the neurotoxic effects of fibrillary Aβ strongly suggest that alterations in the regulatory patterns of this enzyme play a key role in the sequence of molecular events involved in neurofibrillary degeneration in hippocampal cells [2]. Other authors showed that cleavage of p35 to p25, with the subsequent release of p25 activator, seems to be responsible for the activation o f this protein kinase during fibrillary degeneration and suggested that the proteolytic product p25 concentrates in patients with AD [1,19,32]. In the context of the preceding information, it seems reasonable to think that the reactions leading to neuronal death in this disease are part of an integrated process involving extra- and intracellular changes in the normal neuronal machinery, in which Aβ deposition appears to be one of the promoting factors (for a review [23, 25]). Despite these findings, no conclusive evidence has been reached in order to identify the fine molecular changes underlying Cdk5 deregulation and the precise involvement of p35 and p25 in Cdk5 activation leading to tau hyperphosphorylation.

To further clarify the role of Cdk5 in AD, it is interesting to examine the expression of this enzyme in an in vivo model overexpressing the Swedish double mutation of human AβPP. This mutant progressively develops typical Aβ plaques including gliosis and astrocytosis, diminished glucose utilization, and increased oxidative stress in the cerebral cortex [29]. Tg2576 transgenic mice expressing human AβPP with the Swedish familial AD double mutation K670N-M671L (hAPPSw) under control of the hamster prion protein promoter recapitulate several clinical and pathological features of human AD. It has been observed that Tg2576 mice develop normally but, by 16 months of age, they exhibit progressive Aβ deposits in cortical and limbic regions associated with major inflammatory changes, age-related cognitive deficits and impairment in hippocampal long-term potentiation [6,11,15]. Here we analyze the relationships between the overexpression of mutated AβPP and deregulation of the Cdk5/p35 complex in an in vivo model. These studies indicate that besides amyloid deposition in senile plaques of brains in the transgenic mice, clear evidence for changes in the Cdk/p25 system and tau hyperphosphorylation patterns was found.

2. Materials and methods

2.1. Biological model

Tg2576 mice were bred from germinal lines produced and described previously [11]. The genetic background was contributed by C57B6 and SJL strains. Four heterozygote transgenic animals and four non-transgenic mice were studied at 18 months of age. The animals were sacrificed by anesthesia overdose and after dissection their brains were frozen in cold isopentane and further processed for immunocytochemistry.

2.2. Immunocytochemistry

For immunocytochemistry, 40 μm serial coronal sections were cut in a cryostat and then fixed in 4% paraformaldehyde for 6 h and maintained in 90% glycerol at -20°C. Sections were then used for immunocytochemical analyses using the free floating approach. Briefly, sections were post-fixed in 4% paraformaldehyde, and endogenous peroxidase was inactivated by incubation of the sections in 0.3% H_2O_2 in methanol during 10 minutes. After removal of endogenous peroxidase the sections were blocked with 5% BSA in PBS containing 0.2% Triton X-100 for 1 hour at room temperature and then incubated with primary antibodies in 1% BSA buffer, in a humid chamber at 4°C overnight. Primary antibodies used in immunocytochemistry experiments were from Santa Cruz: anti-Cdk5 antibody (C-8), anti-p35 (C-19), anti-phospho-(Tyr^{15})Cdk5, anti-phospho-GSK3 (α/β), anti-MAPK, anti-phospho-MAPK, while an anti-GSK-3β antibody was from Affinity, anti-GAFP was from Sigma, and anti-Bcl2, anti-caspase-3 active and anti-p85-fragment of PARP were from Promega. Antibodies were used at a dilution of 1:100 in PBS containing 1% BSA and 0.3% Triton X-100. The antibodies to Tau5 and PHF1 were used at dilutions of 1:1000 and 1:100 respectively.

The sections were then washed and incubated with horseradish peroxidase secondary antibodies (1:100, Amersham) for 2.5 hours at room temperature. Immunostaining was developed using 0.05% diaminobenzidine and 0.03% H_2O_2 as peroxidase substrate. Controls without the primary antibody were included in all the experiments.

2.3. Western blots

Brain protein extracts were prepared in 20 mM Tris/HCl pH 7.2 homogenization buffer containing 150 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 0.5% NP-40 and protease inhibitors and then centrifuged for 30 min at 5000 x g. The high speed supernatants were separated on SDS-PAGE and transferred onto PVDF membranes [48]. After blocking with 5% non-fat dry milk the membranes were incubated overnight with primary antibodies in a 1% BSA containing buffer at $4°$C in a humid chamber. Primary antibodies used in this study were rabbit anti-Cdk5 (C-8) and anti-p35 (C-19) from Santa Cruz; and the monoclonal phosphorylation-dependent tau antibodies AT8 and PHF1 against Alzheimer's type tau epitopes and the Tau-5 monoclonal antibody. After washing, the membranes were incubated with horseradish peroxidase secondary antibodies diluted in 1% BSA containing buffer during 1 hour at room temperature. Finally the reaction was developed using enhanced chemiluminiscence with the ECL Western blotting analysis system (Bio-Rad).

2.4. Immunoprecipitation and Cdk5 activity assays

200–500 μg of total protein extract was used for immunoprecipitation with anti-Cdk5 antibody (C8) or with anti-p35 (C19) from Santa Cruz at a final dilution of 1:50. For the in vitro kinase assay, the immunoprecipitates were rinsed three times with 20 mM Tris/HCl pH 7.2 containing 150 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 0.5% NP-40 and protease inhibitors and once with kinase buffer (50 mM HEPES, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT) plus 1 μM cold ATP. Protein A beads were then incubated with kinase buffer containing 2.5 μg of mouse brain purified tau plus 5 μCi [^{32}P]ATP in a final volume of 50 μl for 30 min at $24°$C. After incubation, 50 μl of 2X Laemmli sample buffer was added to each sample and the samples were analyzed by SDS-PAGE.

3. Results

In the first set of experiments we decided to analyze the expression pattern of Cdk5 and p35 in brain sections of transgenic and control animals. All transgenic sections showed deposits of Aβ as recognized by an antibody directed against the first 17 aminoacids of the protein (Fig. 1A). These Aβ deposits morphologically resemble those observed in senile plaques in AD. In contrast, control littermates did not show Aβ deposits (Fig. 1B). The transgenic animals contained numerous Aβ plaques that were stained with Congo red dye in the cortical and limbic structures [15]. Thus, the presence of Aβ deposits in Tg2576 animals is due to the overexpression of the transgene. Moreover, Congo-red staining was verified in sections of the transgenic line, confirming the results obtained in the immunocytochemistry assay using an anti-Aβ antibody (i.e., compare Figs 2A, B with 2C, D and E).

Brain sections derived from Tg2576 and control littermates were then analyzed for Cdk5-induced tau phosphorylation. Tau hyperphosphorylation has been related to the formation of paired helical filaments (PHFs), the main structure involved in the appearance of neurofibrillary tangles (NFT). For this purpose, we selected antibodies that specifically recognize phosphoepitopes of tau. The phosphoepitopes recognized by AT8 and PHF1 antibodies are dependent on the activity of proline-directed protein kinases (PDPKs). There was an increase in the immunostaining with both AT8 and PHF1 antibodies in sections derived from transgenic animals (compare Fig. 2A and B with 2C and D). Interestingly, the increase in staining was mainly verified in areas surrounding the Congo-red stained structures. A higher magnification of the Congo-red stained structures revealed the presence of significant dystrophic neurites immunostained with the AT8 antibody (Fig. 2E, arrowheads). These changes in the quantity and distribution of hyperphosphorylated tau in the area surrounding the Congo-red stain was not indeed due to an increase in the expression of tau protein, as indicated by immunocytochemistry using Tau5, a phosphorylation independent antibody (Figs 3A and D). This effect was also found to be specific for tau protein, since MAP2 staining revealed no variation in the staining levels of transgenic and control mice brain sections (Figs 3B and E). Additionally, immunostaining of brain sections with an antibody against glial fibrillary acidic protein (anti-GFAP) revealed that in the Tg2576 line there was an increase in reactive gliosis, especially in the area surrounding plaques (Figs 3C and

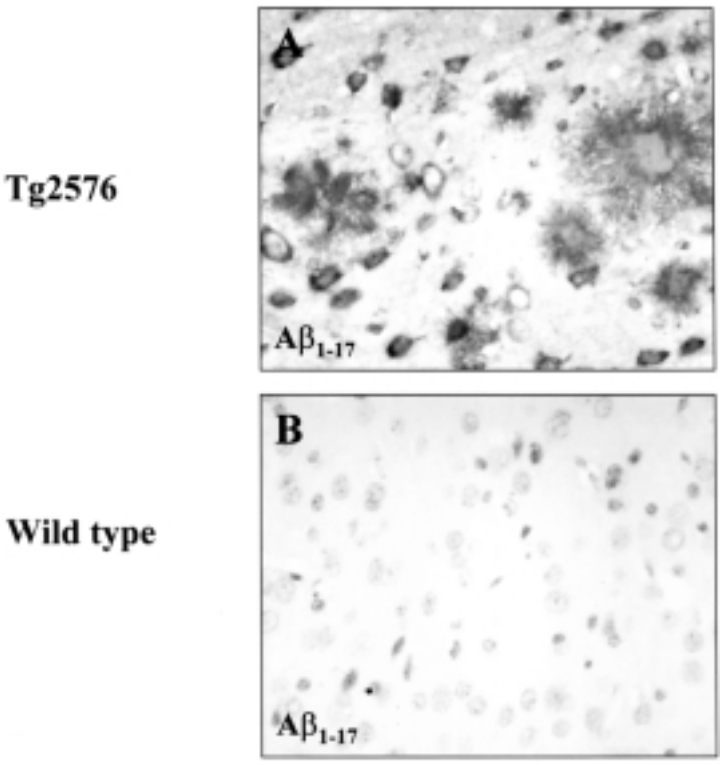

Fig. 1. Immunocytochemistry of Aβ plaques in the Tg2576 brain. (A) The anti-Aβ_{1-17} antibody labeled clustered core neuritic plaques in the hippocampus and cerebral cortex of the brain of a transgenic 18-month-old mouse. (B) Control of a wild type mouse of the same age. Immunocytochemistry was developed using the DAB staining procedure, and the nuclei were revealed by using hematoxilin staining. Other details are as described in Methods. Magnification, 100X.

F, arrows in C). The increase in reactive gliosis has been shown to occur in other transgenic models that recapitulate neurodegenerative diseases such as AD [22] and Huntington's disease [51].

In order to further evaluate the histochemical observations we decided to perform Western blot analyses. Brain extracts of Tg2576 showed increased immunoreactivity with AT8 antibody, specifically in hyperphosphorylated tau isoforms (Fig. 4A). An increase in the reactive Alzheimer's type phosphoepitopes was also evidenced with the PHF1 antibody, since brains from the Tg2576 mice exhibited a significantly higher reactivity than the wild type brains (Fig. 4B). Changes in the tau phosphoepitope patterns were not due to an increase in total tau levels, as was determined with the phosphoindependent antibody Tau5 (Fig. 4C). Densitometric analysis confirmed the visual inspection data (Fig. 4D).

The fact that phosphoepitopes of tau were increased with no variations in the overall total protein levels, suggests that an in vivo increase of the Aβ peptide might deregulate a kinase system responsible for tau hyperphosphorylation. For such reason the next issue to address was to find which protein kinase was deregulated in response to Aβ overexpression in the transgenic mice Tg2576. Previous results obtained in hippocampal cell cultures had suggested to us that the Cdk5/p35 system shown to be involved in tau phosphorylation is overactivated by Aβ [2,3]. Therefore, we carried out Western blot studies to ascertain the contribution of Cdk5 to the tau hyperphosphorylation induced by overexpression of AβPP. In this context, we analyzed the expression levels of activators p35 and p25 in brain protein extracts derived from Tg2576 and control mice. Even though Fig. 5 shows that there is no variation in the levels of total Cdk5 in both transgenic and control mice (Fig. 5A) as revealed in the Western blots, there is a significant increase in p25 in protein extracts derived from Tg2576 as compared with controls (Fig. 5B, lower band indicated by arrow). The amount of p35 is unchanged (Figure 5B, upper band), confirming previous results showing no changes in the mRNA of both proteins [2,25]. Densitometry analyses from five determinations confirmed the marked increase in the amount of

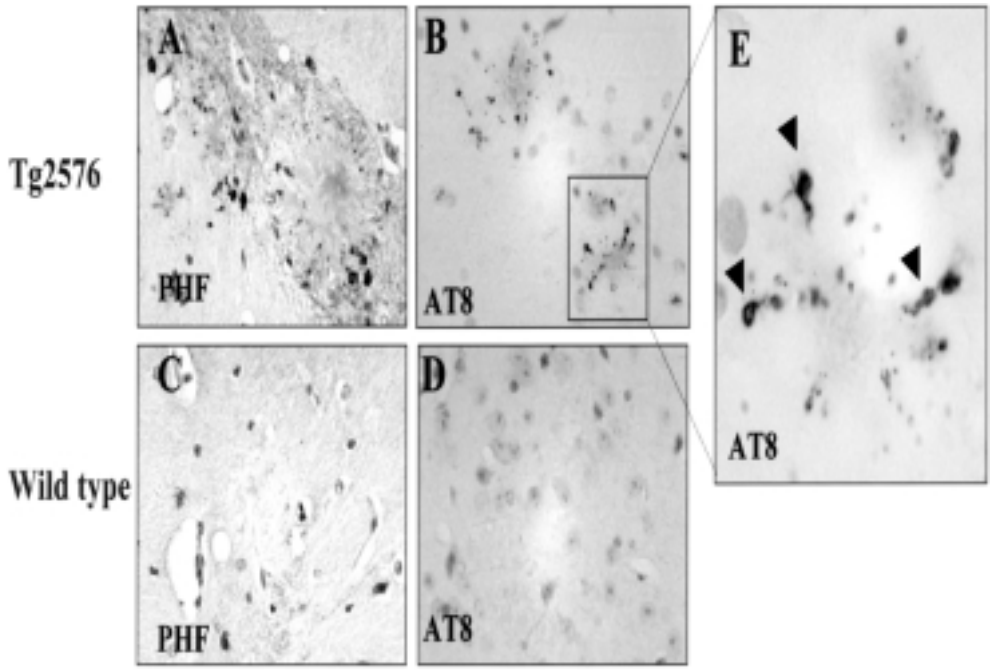

Fig. 2. Immunocytochemistry of cytoskeletal proteins in the Tg2576 mouse. Cytochemical experiments were carried out by using antibodies against tau protein of the Alzheimer type (PHF-1) (panels A and C) and AT8 (B, D and E). Panels A, B and E correspond to the transgenic mice and panels C and D the wild type control animal. Figure in panel 2E (arrowheads) shows a magnification of the plaque zone indicated in B, and indicates dystrophic neurites staining with AT8 antibody around amyloid-β plaques. The figure shows dystrophic neurites immunolabeled around the senile plaque cores stained with Congo red. Magnification, 100X in A, B, C and D. 250X in E.

the soluble p25 fragment ($p \leqslant 0.005$), with no significant alterations in the levels of the membrane-anchored p35 protein (Fig. 5C). Thus, the increase of p25 most likely occurs due to an enhanced activity of the protease responsible for such processing rather than an overexpression of the p35 protein.

In line with these observations, we analyzed the distribution of Cdk5 in brain sections derived from transgenic and control mice. As can be seen in Fig. 6 no major differences were found in the staining patterns, except for the observation that in Tg2576 brains Cdk5 staining surrounded the Congo red stained core of neuritic plaques. Staining is prominent in transgenic as well as in the wild type control brain sections. The pattern of Cdk5 staining was widely distributed in the brain of transgenic mice, including hippocampus and cortex. A comparison of the levels of Cdk5 showed that there is no noticeable increase in the staining of Cdk5 in transgenic mice sections (Figs 6A and D). Staining of the neurospecific activator p35 was also important in the areas surrounding the Aβ plaques stained with Congo red in the cortex and hippocampus of transgenic mice (Figs 6B and E). In contrast with the results obtained for Cdk5 staining, transgenic sections were more immunoreactive with the anti-p35 antibody (Figs 6B and E) indicating either an up-regulation of p35 expression or an increase in the production of the antibody-reactive soluble p25 fragment (Fig. 6B, arrowheads). To address this question we then performed immunocytochemical analyses using an antibody that specifically recognizes the activated form of Cdk5 [2]. Phospho-Cdk5 was also increased in the AβPP transgenic mice as compared with control littermates (compare Figs 6C and F). Cdk5 and p35 from control and transgenic brain extracts were then immunoprecipitated with anti-Cdk5 antibody and assayed for kinase activity. We measured the activity of the kinase through phosphorylation of in vitro purified tau and the activities obtained were then corrected against total and activated-Cdk5 (phospho-Cdk5) levels. Tg2576 brain extracts showed higher Cdk5 activity as compared with control brains in immunoprecipitated Cdk5 dependent tau phosphorylation assays (Fig. 7A). Besides the levels of phospho-Cdk5, the active form of the kinase was also significantly increased in the Tg2576 model (Fig. 7B). Then, we analyzed the significance of changes of protein levels and kinase activity in transgenic and control mice. Normalized values for densitometry analyses indicate an increase in the activity of the kinase (Figs 7C and D).

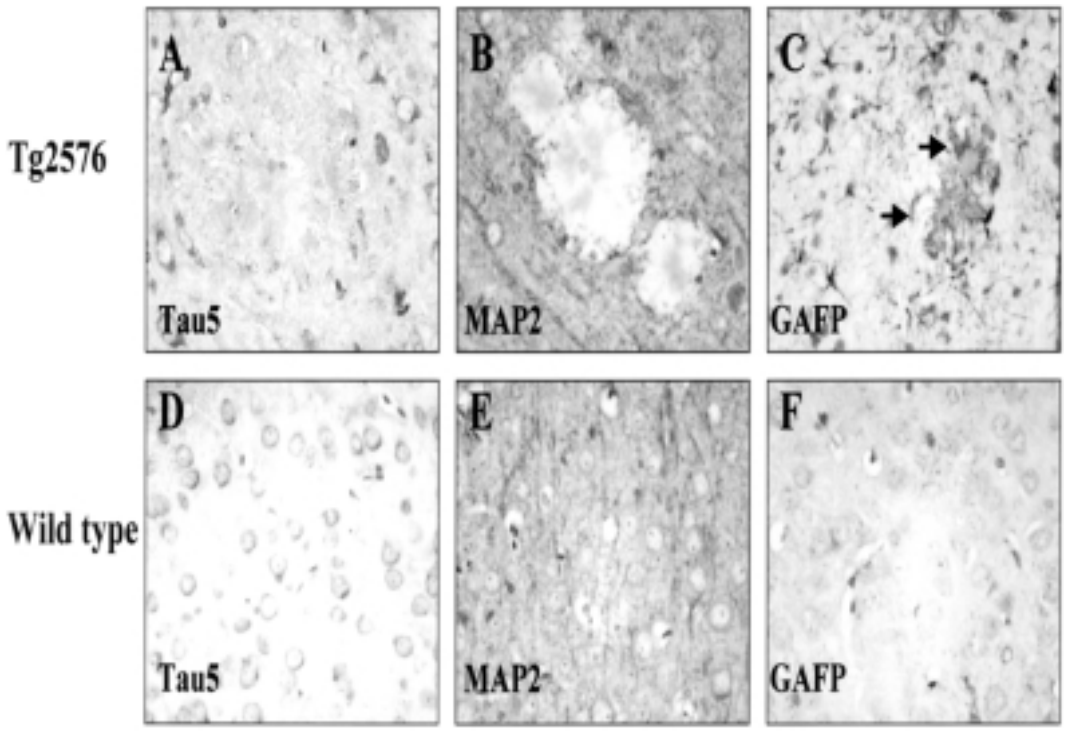

Fig. 3. Immunocytochemistry of cytoskeletal proteins in the Tg2576 mouse. Cytochemical experiments were carried out by using antibodies against phosphorylation independent tau proteinl, Tau5 (panels A and D); MAP2 (B and E) and GFAP (C and F). Panels A, B and C correspond to the transgenic mice and panels D, E and F to the wild type control animal. The figure shows that there were no variations in the immunodected tau or MAP2 between transgenic and control groups. It also shows activated astrocytes around plaques in the cerebral cortex of Tg2576 mouse brain (C, arrows). Magnification, 100X.

Since other different kinases from the Cdk5/p35 complex could be involved in Aβ-mediated tau hyperphosphorylation we decided to analyze the distribution and expression levels of inactive and active forms of glycogen synthase kinase 3 (GSK3) and MAPK. Figure 8 shows the immunocytochemistry of brain sections from transgenic and control mice with antibodies that recognize the activated forms of kinases GSK3β and MAPK. We did not find any increase in the amount of phospho-GSK3 (Figs 8B and F) or phospho-MAPK (Figs 8D and H) in the transgenic mice (i.e., compare Figs 8B and F, and 8D and H). Moreover, we did not detect any up-regulation in the levels of those kinases in transgenic mice (compare Figs 8A and C with 8E and G). Taken together, these results with previous findings [2,3], that suggest Aβ-induced abnormal hyperphosphorylation specifically involves the Cdk5/p35 complex with a lesser role of other kinase systems such as those of GSK3β and MAPK. Considering these observations it was important to assess apoptotic markers in the transgenic mice. Immunocytochemical studies (Fig. 9) revealed no upregulation of pro-apoptotic or anti-apoptotic proteins occurs in Tg2576 as compared with the control animals. We did not find immunoreactivity with antibodies to active Caspase 3 (Figs 9A and E) or anti-p85 fragment PARP (Figs 9B and F). The anti-apoptotic protein Bcl2 was distributed in the periphery of the Congo red stained plaques in the cortex (Figs 9C and G). Finally we analyzed the effect of Aβ overexpression in the generation of oxidative stress phenomena. Thus, brain sections derived from control and transgenic mice were immunostained with an antibody that recognizes HNE, a marker of oxidative stress [27]. HNE is an aldehydic product of lipid peroxidation that is found in neurons suffering oxidative stress [27]. Figure 10 shows that the overexpression of Aβ induces an increase in the staining pattern of HNE in transgenic brain sections (comp are Figs 10A and B).

4. Discussion

In this paper we have analyzed the changes in the function of the Cdk5/p35 system in response to an in-

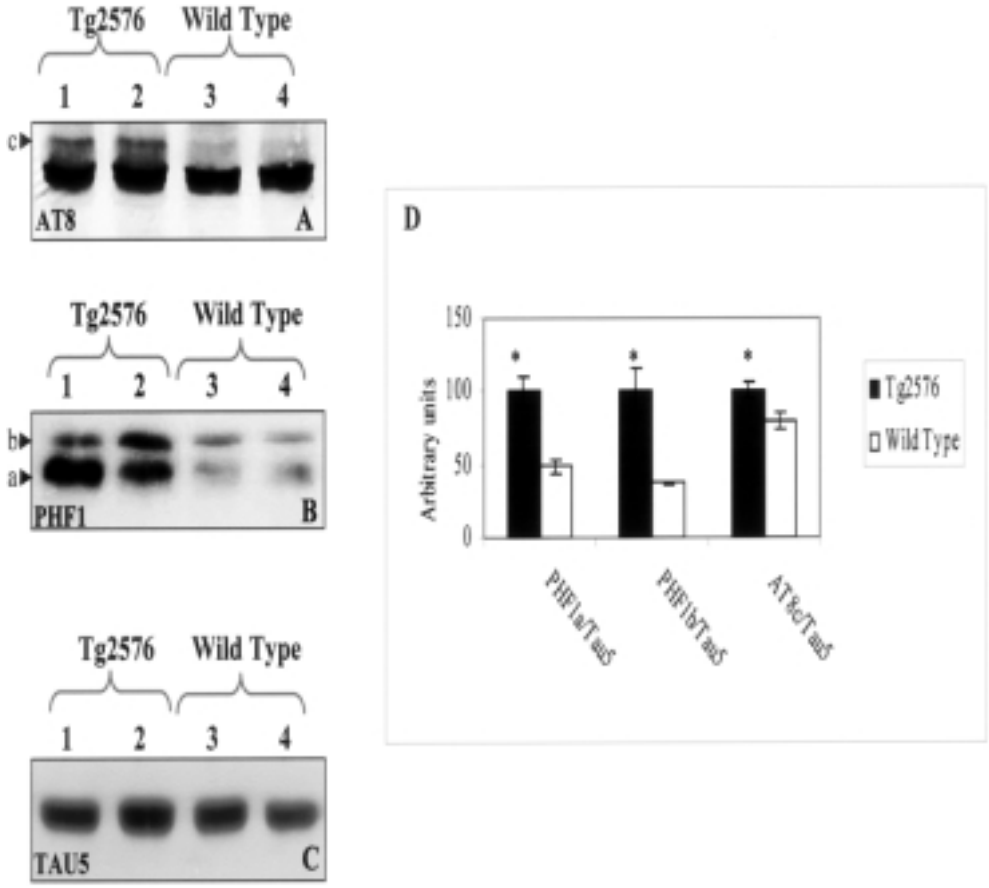

Fig. 4. Immunodetection of tau protein in both transgenic Tg2576 and control mice. A. Detection of hyperphosphorylated tau with AT8 antibody in a Tg2576 mouse extract (lanes 1 and 2), and wild type mouse control (lanes 3 and 4). Lanes 1 and 3 correspond to cerebral cortex and lanes 2 and 4 to hippocampus. B. Immunodetection of hyperhosphorylated tau using PHF1 antibody of Tg2576 extracts (lanes 1 and 2) and controls lanes (3 and 4). C. Immunodetection of tau by using Tau5 antibody that recognizes conformational tau epitopes in the Tg2576 mouse (lanes 1 and 2) and control mouse (lanes 3 and 4). D. Quantitation from Western blot data of hyperphosphorylated tau protein in transgenic and control groups, indicating a significant increase in the amount of Alzheimer's type tau in transgenic mice ($p < 0.05$; $n = 5$). The ratios between the tau phosphoepitopes with either PHF1 (epitopes a and b) and AT8 (epitope c) and Tau5 epitopes for both the transgenic brains (Tg2576) and controls are shown. Bars indicate standard deviations.

crease in the production of endogenous Aβ peptide in an animal model. Transgenic mice Tg2675 have more Aβ deposits as shown by immunocytochemistry and Congo-red dye staining. Their brains also showed an increase of reactive astrogliosis. In the present report, we show that Tg2576 mouse brains present tau hyperphosphorylation as revealed by immunocytochemistry, immunodetection and kinase activity assays. In this animal model tau hyperphosphorylation is produced by an increase in the activity of the Cdk5/p35 complex, with minor contribution of other kinases such as GSK3β and MAPK. These observations are in agreement with previous findings on the involvement of the Cdk5 system in Aβ-induced neurodegeneration in rat brain hippocampal neurons [1–3,19,25,32].

An early modification found in AD brains is tau hyperphosphorylation by the action of protein kinases and phosphatase systems which lead to structural changes in tau, thus affecting its binding with tubulin and its ability to promote microtubule assembly [4,26, 38]. The most relevant protein kinases involved in tau modifications in neurofibrillary degeneration are GSK3β [13,14,44] and Cdk5. The former kinase has been implicated in tau hyperphosphorylation in transgenic models that overexpress AβPP [47] and in a transgenic conditional model [22].

Cdk5 forms an active complex with p35 and with its cleavage product p25 [2,3,30,49]. Different phosphorylated sites have been detected in AD PHFs [23, 42]. Cdk5 has been characterized as a proline-directed

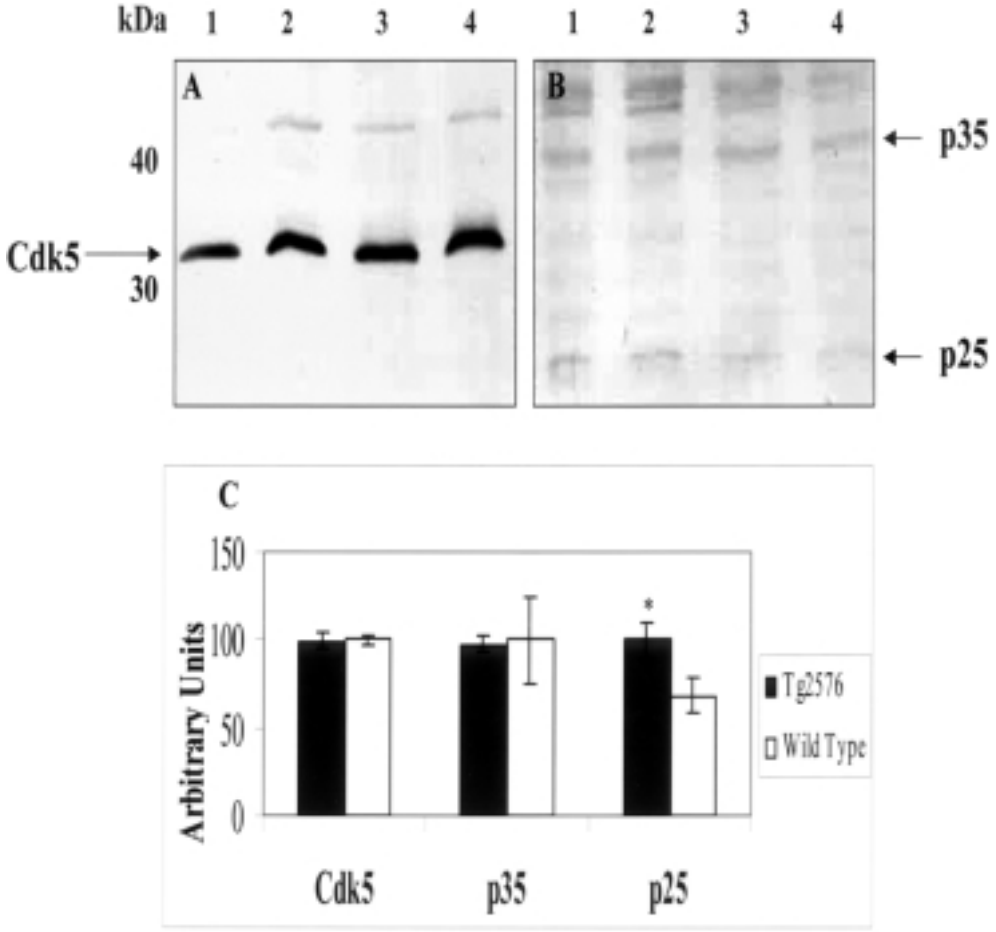

Fig. 5. Immunodetection of Cdk5 and p35 proteins. A. Immunodetection of Cdk5 protein with antibody anti-Cdk5 (H291) in brain extracts of Tg2576 (lanes 1 and 2) and control mouse (lanes 3 and 4). Lanes 1 and 3 correspond to cortex and hippocampus and lanes 2 and 4 to the rest of the brain. B. Immunodetection of p35 with anti-p35 antibody (C19) in brain extracts of Tg2576 (lanes 1 and 2) and control mouse (lanes 3 and 4). C. Densitometry scans of immunoreactive Cdk5, p35 and p25 in transgenic and wild type animals based on five different determinations ($n = 5$). Standard deviations are shown in each bar of the histograms ($p < 0.05$).

Ser/Thr protein kinase, which contributes to phosphorylation of human tau on Ser-202, Thr-205, Ser-235 and Ser-404 [2,25,41]. Cdk5 and its neuron specific regulator p35 are essential for neuronal migration in the cerebral cortex [31,45]. Pioneering experiments reported by Busciglio and coworkers succeeded in demonstrating that incubation of hippocampal cells in primary cultures with Aβ activated tau phosphorylation [5]. Moreover, previous findings in our laboratory have described that a stabilization of the Cdk5/p35 complex, mediated by a site-specific Cdk5 phosphorylation [2], is involved in tau hyperphosphorylation [3,23]. Genetic analysis supports the finding that tau pathology is preceded by Aβ accumulation [12]. Tomidokoro et al., [46] analyzed how Aβ deposits induce tauopathy. The presence of phosphorylated tau, glycogen synthase kinase 3α (GSK3α), GSK3β, cyclin-dependent kinase 5 (Cdk5), mitogen-activated protein kinase (MAPK) and fyn were examined in Tg2576 brains. Substantial brain Aβ amyloidosis and behavioral abnormalities were also observed. Tau phosphorylated at Ser199, Thr231/Ser235, Ser396 and Ser413 accumulated in the dystrophic neurites of senile plaques [46,47]. This study merely compared the histochemical staining of these kinases in the dystrophic neurites in and around the senile plaque cores of the Tg2576 brains without further biochemical or kinase activity analyses.

In this report, we have extended previous reports that did not identify the kinase responsible for tau hyperphosphorylation induced by the overexpression of Aβ. Thus, the increased p35 in transgenic sections supports a key role for the kinase in the phosphorylation of tau.

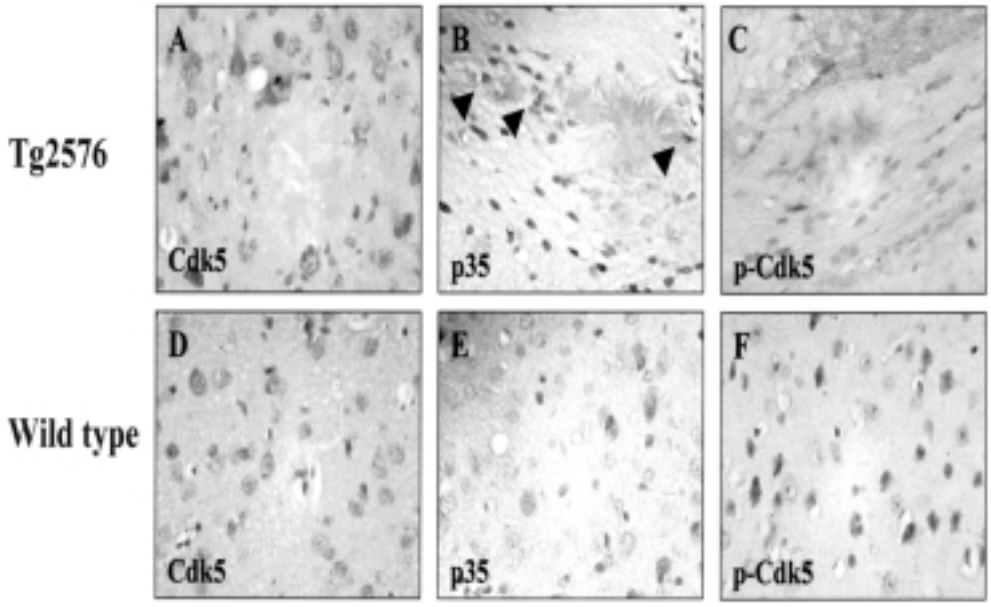

Fig. 6. Immunocytochemistry of Cdk5 and p35. Immunostaining of Cdk5 with a polyclonal anti-Cdk5 antibody (H291) of the transgenic mouse brain (A), or Cdk5 staining of the wild type control (D). Staining using an antibody against the activator p35 (anti-p35 antibody C19) in the transgenic animal (B) and in the wild type control (E). Staining using the antibody against the phosphorylated form of Cdk5 in the Tg2576 animal (B) and wild type control (E) is also shown. Note the increase of p-Cdk5 in the area surrounding the plaque of transgenic mice (Fig. 6B, arrowhead). Magnification, 100X.

Since the antibody used in the immunocytochemistry analyses was directed against the C-terminal of p35, it could recognize both the membrane anchored p35 and the soluble p25 fragment. Previous studies with hippocampal cells treated with Aβ suggested the synthesis of Cdk5 or p35 is not upregulated [2]. A possible mechanism for the increased phosphorylation of tau in transgenic is increased proteolytic processing of p35 to give rise to the soluble p25 fragment. The p25 fragment as been proposed as the in vivo cellular activator of Cdk5. This fragment has been shown to increase in AD brain [32]. Moreover, a line of transgenic mice overexpressing p25 protein showed tau hyperphosphorylation [1]. Even though in the hippocampal cell cultures the involvement of p25 release is not clear [2], the present data indicating an increased level of this activator in the Tg2576 mice favors the idea of calpain proteolysis involvement in Aβ-promoted neurodegeneration. Alternatively, AβPP overexpression could trigger Cdk5 phosphorylation through protein kinase systems.

The results obtained here using an in vivo model support previous findings based on hippocampal neurons in culture that implicate Aβ in the regulation of the Cdk5/p35 complex [3]. Other in vivo reports based on the genetic engineering of double AβPP and tau transgenic models support our observations. Hence, Gotz et al., [10] have shown that injection of Aβ fibrils in the brain of tau transgenic mice cause a fivefold increase in the production of NFT. On the other hand, Lewis et al., [20] demonstrated an increase in the amount of NFT in a double mutant model overexpressing both Aβ peptide and a mutated tau, especially in the limbic and olfactory cortex. Moreover, by using tau deficient mice it has been shown that the presence of tau protein is essential for Aβ induced neurotoxicity [35]. Our findings here of Cdk5 deregulation and tau pathology in mice that carry a transgene coding for a mutant form of the 695-amino acid isoform of human Alzheimer's AβPP support the link between tau and AβPP that leads to an increase in formation of neurofibrillary tangles. In this way a deregulated Cdk5/p35 complex should be responsible for tau hyperphosphorylation and the subsequent production of neurofibrillary tangles. It is worth mentioning that having both Aβ production and tau hyperphosphorylation in the same in vivo model should facilitate development of new pharmacological strategies to be developed that consider not only Aβ deposition but also NFT formation and neuronal loss that really recapitulates the ethiopathology of AD.

Along these lines, it was of interest to ascertain the contribution of apoptosis and oxidative stress to neurodegeneration in this animal model. Evidence exists

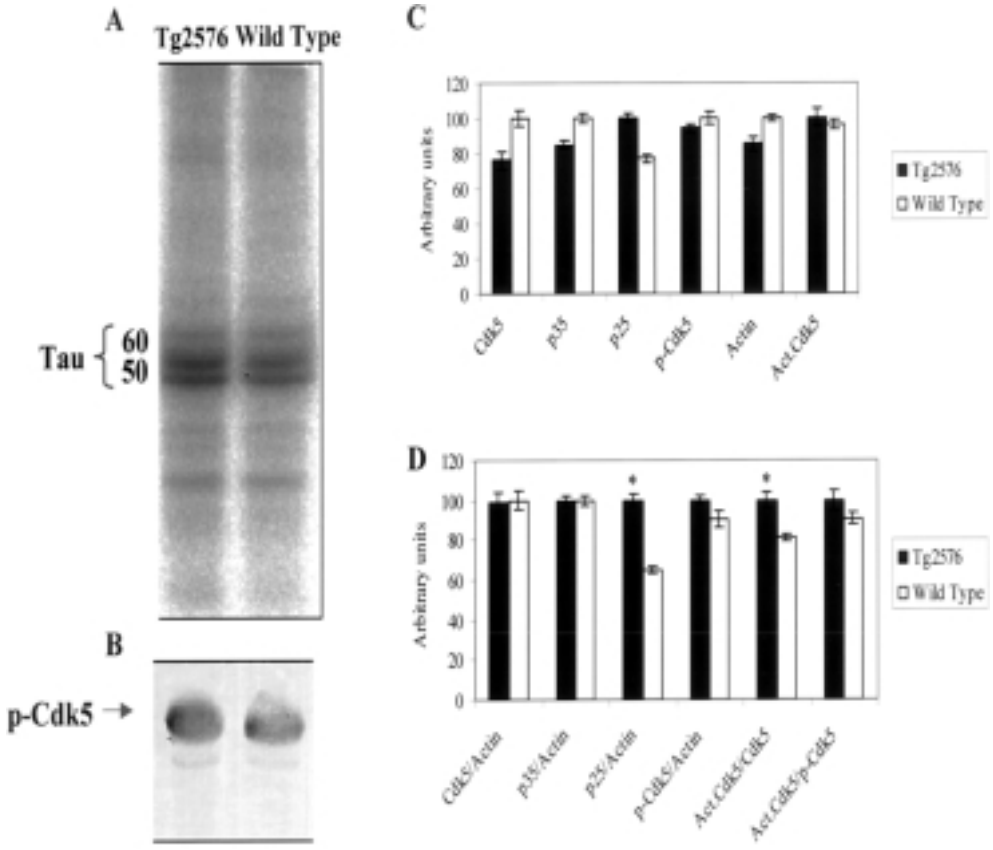

Fig. 7. Increased activity of Cdk5 in Tg2576 animals A. Immunoprecipitated Cdk5 from Tg2576 and control animal brains was assayed for tau phosphorylation activity. Note a mild increase in tau phosphorylation. B. Active Cdk5 (phospho-Cdk5) was immunoprecipitated from Tg2576 and control mice and a higher level of activity detected in Tg2576. C. Relative levels of p35, p25, Cdk5 and p-Cdk5 proteins in brain extracts from Tg2576 and control animals as analyzed by western blots. The mean and standard deviation values were from three independent experiments. D. Corrected levels of the same brain proteins shown in C., derived from Tg2576 and control animals were normalized against actin, Cdk5 and phospho-Cdk5. These levels show a significant increase in p25 levels and Cdk5 activity. As analyzed by the procedure in A ($p < 0.05$). The analysis in D followed the procedure of Lee et al., (1999) [18].

that certain neuronal cells die by activation of apoptotic mechanisms in AD, even though it is not known how neuronal apoptosis is linked with the effects of fibrillary Aβ or tau phosphorylation, and the precise sequence of molecular events leading to neuronal death. Although Aβ neurotoxicity has been demonstrated in vitro [7, 17,21,28,33], an in vivo demonstration has been difficult [8,43]. Moreover, the amount of Aβ deposited in AD brain does not correlate with the amount of neuronal loss [9]. Thus a transgenic mouse that exhibits age-related Aβ deposition [11] has allowed us to test the in vivo effects of Aβ deposits. With regard to the contribution of apoptosis to neurodegeneration, the present studies indicate that no dramatic changes in apoptotic markers are evidenced in the Tg2576 transgenic model, at least based on immunocytochemical analysis of active caspase 3 levels and distribution, as well as the anti-apoptotic protein Bcl2. No changes were observed either in proapoptotic Bax and p85 fragment of PARP in Tg2576 as compared with controls. On the other hand, the overexpression of AβPP in transgenic mice induced HNE adducts, suggesting that Aβ production triggered the oxidative stress response in the brain of transgenic mice. It is worth noting that some authors have indicated that the oxidative stress response might be the earliest neuronal change in AD [16,37]. Moreover, it has recently been suggested that AβPP overexpression could induce lipid peroxidation [50]. Our findings suggest that Aβ overexpression indeed produces an oxidative stress response and deregulation of the Cdk5/p35 kinase complex. The molecular mechanisms linking these two phenomena are currently being explored.

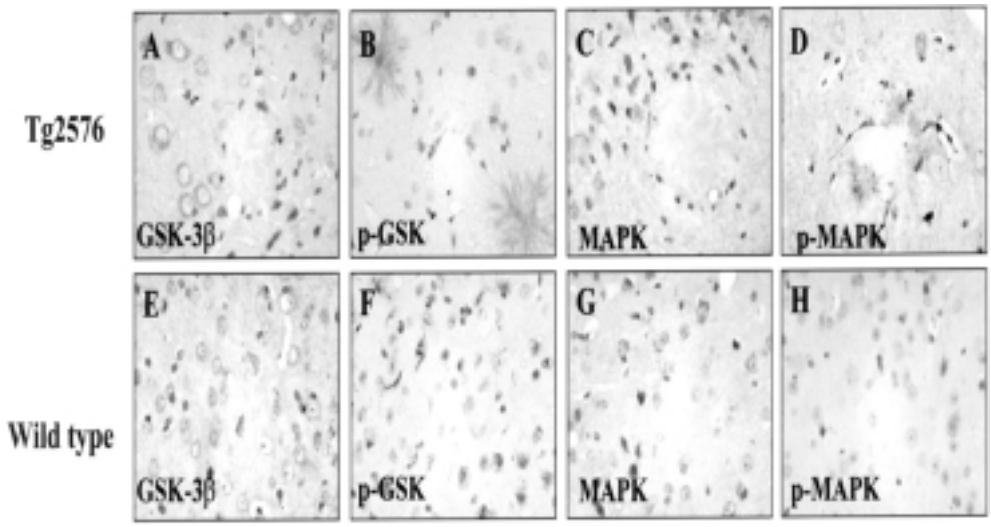

Fig. 8. Immunocytochemistry of GSK-3β and MAPK. Hippocampal brain sections of the transgenic Tg2576 (A to D) and control animals (E to H) were immunostained with antibodies to GSK-3β (A and E), phosphorylated GSK3 (p-GSK3) (B and F), MAPK (C and G) and phosphorylated MAPK (p-MAPK) (D and H). The figure shows punctate dystrophic neurites immunolabeled around the senile plaque cores in hippocampus and cortex stained with Congo red. Magnification 100X.

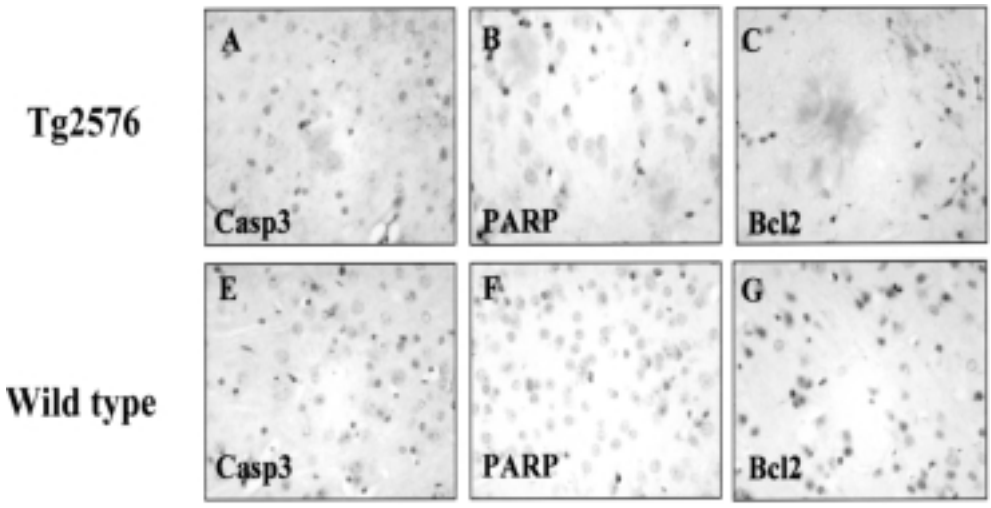

Fig. 9. Immunocytochemistry of apoptotic proteins. Cortex brain sections of the transgenic Tg2576 (A to D) and control (E to H) animals were immunostained with antibodies against the apoptosis enzymes caspase-3 (active forms) (panels A and C); PARP (B and F); Bcl2 (panels C and G) and Bax (D and H). No reactivity for caspase 3 or the p85-fragment of PARP was revealed. No increase in anti-apoptotic Bcl2 was found. Magnification 100X.

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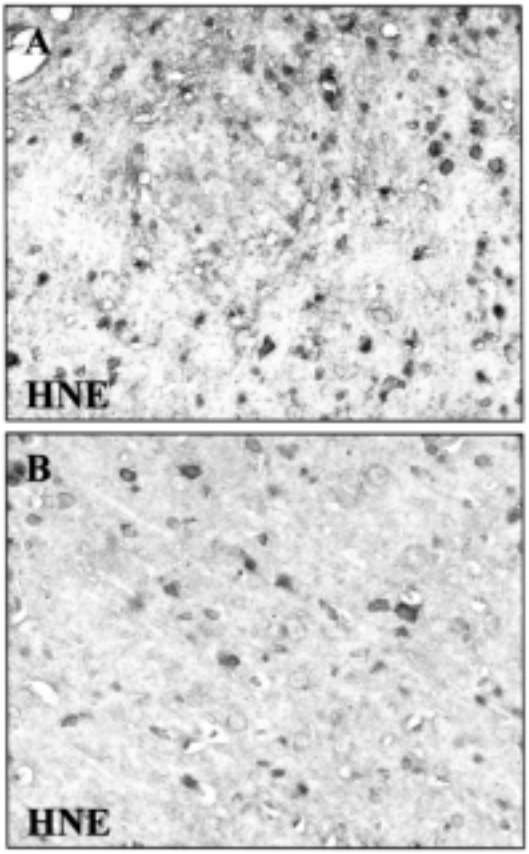

Fig. 10. Immunocytochemistry of oxidative stress marker Brain cortex sections of the transgenic Tg2576 (A) and control (B) animals were immunostained with anti-HNE antibody. Note the increased immunoreaction with anti-HNE in transgenic sections as compared with control littermates. Magnification 100X.

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