

*Regular Article*

**Ageing and Amyloid-beta peptide deposition contribute to an impaired brain tissue plasminogen activator activity by different mechanisms**

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## **ABSTRACT**

Alzheimer's disease (AD) is the most common form of neurodegenerative disorder in the ageing population. It is characterized by the cerebral accumulation of toxic amyloid-beta peptide assemblies (A $\beta$ ). The serine protease plasmin, which is generated from the inactive zymogen plasminogen through its proteolytic cleavage by tissue- (tPA) or urokinase-type plasminogen activator, has been implicated in the catabolism of A $\beta$  peptides. In this report, we studied the regulation of tPA activity *in vivo* during ageing in normal mice and in a mouse model of AD characterized by an exacerbated endogenous A $\beta$  accumulation. We observed that cerebral tPA activity was decreased during ageing in normal mice and that this effect was worsened in mice overproducing A $\beta$  peptides. These phenomena result, respectively, from a decrease in tPA expression and from an increase in the production of one of the tPA inhibitors, the plasminogen activator inhibitor type 1 (PAI-1). A similar study in sporadic AD and age-matched control brain tissues revealed that the tPA proteolytic activity was negatively correlated to A $\beta$  peptides levels supporting the data observed in mice. Altogether, our data support a model in which amyloid deposition induces a decrease in tPA activity through the overproduction of PAI-1 by activated glial cells.

**Keywords :** tissue plasminogen activator; ageing; amyloid-beta peptides; transgenic mice; Alzheimer's disease; PAI-1

## INTRODUCTION

Amyloid-beta peptide ( $A\beta$ ) is a central element in Alzheimer's disease (AD) pathogenesis (Hardy and Selkoe, 2002; Walsh and Selkoe, 2004). Although  $A\beta$  overproduction has been proposed as the main cause of rare familial AD cases, it seems that a defect in  $A\beta$  clearance could be more relevant for common sporadic forms of AD (Tanzi et al., 2004). In this report, we focused on the secreted serine proteinase tissue plasminogen activator (tPA), which has been involved in  $A\beta$  catabolism.

tPA is the main plasminogen activator in the brain (Sappino et al., 1993). It is mostly expressed by neurons (Salles and Strickland, 2002) and is implicated in several physiological and pathological processes (Melchor and Strickland, 2005). For instance, it is involved in extracellular matrix remodelling during brain ontogenesis (Krystosek and Seeds, 1981; Hawkins and Seeds, 1986; Friedman and Seeds, 1995); and in synaptic plasticity and glutamatergic signalling in the adult brain (Qian et al., 1993; Huang et al., 1996; Madani et al., 1999; Nicole et al., 2001; Pang et al., 2004).

Although tPA mediates most of its effects through the activation of plasminogen into plasmin, another serine protease, recent studies report that it has also a plasminogen-independent activity, through proteolytic (Nicole et al., 2001; Fernandez-Monreal et al., 2004a) or non-proteolytic mechanisms (Zhuo et al., 2000; Siao and Tsirka, 2002). To control this multimodal activity, tPA is regulated at several levels. Its translation and release in the extracellular space are coupled to neuronal activity (Gualandris et al., 1996; Fernandez-Monreal et al., 2004b; Shin et al., 2004). Once released, tPA proteolytic activity is regulated through its interaction with several serine protease inhibitors, belonging to the SERPIN family (Silverman et al., 2001), and through its uptake by astrocytes (Fernandez-Monreal et al., 2004b).

In AD, the tPA/plasmin system has been reported to have a beneficial role. Indeed, plasmin promotes  $A\beta$  peptides clearance both *in vitro* and *in vivo* (Tucker et al., 2000a; Exley and Korchazhkina, 2001; Melchor et al., 2003). In turn,  $A\beta$  peptides seem to modulate the tPA/plasmin system. Indeed,  $A\beta$  fibrils enhance the proteolytic activity of tPA by acting like a cofactor (Kingston et al., 1995; Wnendt et al., 1997; Kranenburg et al., 2002; Kruithof and Schleuning, 2004), or induce its expression in neuronal cell cultures and in transgenic mice overproducing  $A\beta$  (Tucker et al., 2000b). Altogether, these data suggest that brain  $A\beta$  levels are

controlled by a positive feedback loop mechanism involving tPA and plasmin. However, a recent work has challenged this view. This study showed, in contrast, that the proteolytic activity of tPA is decreased in the brain of another transgenic mouse model overproducing A $\beta$  (Ledesma et al., 2003; Melchor et al., 2003). It is difficult to reconcile the view that A $\beta$  accumulation leads on one hand to an increase in tPA expression (Tucker et al., 2000b) and on the other hand to a decrease in its activity.

The main purpose of this study was to investigate the regulation of tPA in the brain both at its expression level and at its activity level. We chose to examine the regulation of this protease both in normal mice and in mice overproducing A $\beta$  peptide. In an attempt to better understand how tPA could be modulated in the brain, we also monitored the expression of the main inhibitors of tPA in the brain of both animals. Finally, we questioned whether tPA proteolytic activity could be related to AD pathogenesis by monitoring its activity in a population of sporadic AD cases.

## **MATERIALS AND METHODS**

*Animals.* Experiments were performed in accordance with French (act no. 87-848; Ministère de l'Agriculture et de la Forêt) and European Communities Council (Directives of November 24, 1986, 86/609/EEC) guidelines for the care and use of laboratory animals, agreement number: C 14 118 001. Transgenic mice were generated as described (Blanchard et al., 2003) and provided by Sanofi-Aventis. Animals (referred to as APPsl) overexpress the human 751 isoform of the amyloid precursor protein, carrying both the double Swedish mutation (KM595/596NL) and the London mutation (V642I). The transgene is driven by the neuronal specific promoter Thy 1.2. All transgenic animals included in the present study were heterozygote for the transgene, as checked by PCR genotyping. Moreover, the transgene expression was confirmed by qPCR for human APP. All animals studied were males. The light/dark cycle condition was 12:12 with lights on from 8:00 PM to 20:00 AM. Animals were sacrificed between 10:00 and 11:00PM.

*Brain processing.* At the age of analysis, animals were anaesthetized by intraperitoneal injection of chloral hydrate (500mg/kg) and transcardially perfused with cold 0.9% NaCl. Then, brains were carefully removed and hemispheres were separated for biochemical and mRNA analyses or for immunohistochemistry.

*Immunohistochemistry.* Brain hemispheres were fixed with 1% paraformaldehyde in 0.1M phosphate buffer pH 7.4 for 6 days at 4°C. Then, hemispheres were cryoprotected in 3 baths of 0.1M phosphate buffer containing 20% sucrose. Hemispheres were next embedded in O.C.T. compound (VWR international<sup>®</sup>, France) and quickly frozen in liquid nitrogen. Sagittal cryosections of 25µm were fixed on SuperFrost<sup>®</sup> Plus slides (VWR international<sup>®</sup>, France). Sections were incubated with the primary antibodies FCA35-42 (1/1000) or FCA33-40 (1/1000) (both from Calbiochem) and subsequently with a biotinylated anti-rabbit antibody (1/500) before staining with the ABC kit from Vector labs. Congo red staining was performed according to the method of Puchtler.

*Protein and RNA extractions.* Briefly, brain cortices were homogenized in ice-cold TNT buffer (50mM Tris-HCl pH 7.4; 150mM NaCl; 0.5% Triton X-100) at 1mg/mL. Debris were removed by centrifugation (13.000g, 4°C, 30min). Supernatants were stored at -80°C until activity analysis. Protein quantification was performed according to the BCA method (Pierce, France). RNA extraction was performed by using Nucleospin RNA II column (Macherey-Nagel, France).

*tPA activity measurement.* Three techniques were used to evaluate the proteolytic activity of tPA. 1) Zymography assays were performed according to previously published methods (Sappino et al., 1993). Briefly, 5µg of protein extracts were resolved on SDS-PAGE (12%) containing casein (1mg/mL) and plasminogen (4.5µg/mL). After migration, the gel was washed in 2.5% Triton X-100 solution and incubated for 2h in a buffer containing glycine (100mM) and EDTA (10mM), pH 8.3. Finally, clear bands indicating proteolytic activity were visualized by Coomassie staining. 2) ELISA for free catalytic site tPA (Molecular Innovation<sup>®</sup>, USA) was performed on 100µg of protein extract. Briefly, biotinylated human PAI-1 was adsorbed on a streptavidin coated plate and used to capture “free catalytic site tPA”, corresponding to active tPA. A polyclonal antibody for mouse tPA was used to detect the captured tPA (Molecular Innovation<sup>®</sup>, USA). 3) tPA activity was measured by using a commercial fluorogenic substrate for human tPA (American Diagnostica<sup>®</sup>, France). This substrate is a modified peptide corresponding to the cleavage site of tPA (methylsulfonyl-D-phenyl-glycyl-arginine-7-amino-4-methylcoumarin acetate). Briefly, 200µg of protein extract were mixed with equal volume of tPA substrate (final

concentration: 5 $\mu$ M). Fluorescence (excitation: 360, emission: 440 nm) was monitored during 30 min and activity was next estimated by calculating the initial slope of the reaction. Results were expressed in arbitrary units (AU). Substrate was prepared according to manufacturer's instructions. The specificity of the substrate for human tPA was assessed by performing competition experiments with a monoclonal antibody (H27B6) directed to the protease domain of tPA (Abcam®, France) (Supplementary data, Figure 1).

*Quantitative Real-Time RT-PCR.* Total RNAs (1  $\mu$ g) from each sample were reverse-transcribed using the Promega RT system (Promega, Charbonnières, France). Two primers were designed for each gene using the Beacon Designer software (Bio-Rad, Marnes-la-Coquette, France). The following primer sequences were used: tPA forward (F) primer: CTC CGA CCC ATG CTC AGA A; and reverse (R) primer: TTG TAC CAG GCC GCT GTT G; neuroserpin forward (F) primer: CGC CAT TCA ATG GGA TAT G; and reverse (R) primer: CAA AGA GCG AAT TGG CAA G. PAI-1 forward (F) primer: CGC TGC ACC CTT TGA GAA AG; and reverse (R) primer: GGG CAG CCT GGT CAT GTT. Protease nexin-1 (PN-1) forward (F) primer: TGT GTT TCA GTG TGA AGT GCA GAA; and reverse (R) primer: CGA TCA GAT TTG GGG AAA GCA GAT; human APP forward (F) primer: AGC CAC AGA GAG AAC CAC CAG; and reverse (R) primer: TCA AAG TAC CAG CGG GAG ATC A; GFAP forward (F) primer: GAG GAG TGG TAT CGG TCT AAG TTT G; and reverse (R) primer: GCC GCT CTA GGG ACT CGT T; CD11b forward (F) primer: GAC ATG GAC GCT GAT GGC AAT AC ; and reverse: CGA GGC AAG GGA CAC ACT GAC ; NMDA NR1 subunit forward (F) primer: CTC TAG CCA GGT CTA CGC TAT CC; and reverse (R) primer: GAC GGG GAT TCT GTA GAA GCC A. Sequence alignments were performed with the BLAST database in order to ensure the specificity of primers. PCR solutions were prepared with RNase-free water containing primers and IQ SYBR Green Supermix (BioRad, Marnes-la-Coquette, France). For PCR amplification, 20 $\mu$ L of mix were added to 5 $\mu$ L of reverse transcription reaction previously diluted (1:20). Two negative controls were performed during each qPCR experiment. We use samples without reverse transcriptase in order to control RNA contamination by genomic DNA, and samples without cDNA to check the absence of primer dimers. Assays were run in triplicate on the iCycler iQ real-time PCR detection system (BioRad). The amplification conditions were as follows: Hot Goldstar enzyme activation, 95°C for 3 minutes; 50 cycles of PCR (Denaturation: 95°C, 15

seconds; Hybridation/Extension: 60°C, 1 minute). Several house-keeping genes were tested for normalization (beta-actine; RNA polymerase 2; GAPDH, NMDA receptor subunit NR1) and NR1 was considered as the best gene since its expression was the most stable both with age and with the genotype. Data were expressed in arbitrary units using the  $2^{-\Delta C_t}$  method and relative to NR1 by the  $2^{-\Delta\Delta C_t}$  method. Both representations have been used in figures.

*Alzheimer's disease and age-matched control brain samples.* All frontal cortex tissue samples were recovered from a human brain bank constituted between 1998 and 2003 at INSERM U422 (current U815). All patients came from Lille University Hospital where autopsies were performed. Brains were recovered in the Department of Neuropathology. Brains were processed as follow: The right hemisphere was deep-frozen for biochemical and molecular analyses and the left hemisphere was formalin-fixed for histopathological examination. All dissections were done by the same investigator. The diagnosis of AD was established according to the criteria of the National Institute on Aging and the Reagan Institute working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease. All patients had been previously diagnosed at the Lille Memory Clinic, according to the criteria of the NINCDS-ADRDA (McKhann et al., 1984). Tau pathology was investigated as already described (Delacourte et al., 1999). Amyloid-beta aggregates were quantified both by dot-blot and Western blots according to the procedure described by Delacourte et al., 2002. Briefly, 100mg of brain tissue were homogenized in 1mL of pure formic acid in order to extract A $\beta$  aggregates. Two  $\mu$ l of brain homogenate were directly loaded on a polyvinylidene difluoride (PVDF) membrane for dot-blot immunoassay; 50 $\mu$ l were evaporated under nitrogen, solubilized in 50 $\mu$ L of denaturing loading buffer, boiled and submitted to SDS-PAGE. Membranes were blotted with a home-made rabbit polyclonal antiserum, named ADA42, generated against the seven last C-terminal amino acids of A $\beta$ <sub>42</sub>. The quantification was performed by running parallel experiments with serial dilutions of A $\beta$ <sub>42</sub> synthetic peptides as standards. Both techniques gave similar results. tPA activity measurement was performed on TNT buffer brain extracts by using human specific fluorogenic substrate for tPA as described in the tPA activity measurement section. All clinical and biochemical data are summarized in Table 1 (supplementary data).

*Statistical analyses.* Statistical analyses were performed using Statview v4.57. Non-parametric test were used for animals studies: Mann-Whitney U-test was used for two-group comparison (genotype and age comparisons). Kruskal-Wallis multi-parametric test was used for three-group analyses (age comparison), followed by a *post-hoc* two-group comparison using the Mann-Whitney U-test. For human studies, normal distribution of each parameter was assessed by the test of Kolmogorov-Smirnov, and Student t-test was used for two-group comparison. For correlation analysis, Pearson's correlation coefficient and Z test of correlation were used.

## RESULTS

### tPA activity is decreased by age

The first part of this study was conducted to monitor tPA activity in the mouse brain during ageing. For this purpose, brains of C57BL6 mice were harvested at 3, 11 and 14 months old. To ensure that the tPA activity measured was independent of vascular tPA activity, brains were collected after intracardiac perfusion with cold saline (see Materials and Methods). tPA activity was monitored by using a modified enzyme-linked immunosorbant assay (ELISA), using recombinant PAI-1 as a capture protein, trapping only tPA which is not bound to a SERPIN (Molecular Innovation<sup>®</sup>, USA). By using this approach, we observed a significant decrease in tPA activity with age (Fig. 1A; 3m.:  $38 \pm 1.6$  ng/g; 11m.:  $30.7 \pm 1.7$  ng/g; 14m.:  $27.5 \pm 1.9$  ng/g,  $p=0.018$ ).

We next measured the total tPA protein levels. For this, we used an ELISA directed against both free active tPA and tPA/SERPIN complexes (Molecular Innovation<sup>®</sup>, USA). We observed a decrease in total tPA protein levels between 3 and 14 months old (Fig. 1B; 3m.:  $35.4 \pm 3.6$  ng/g, 14m.:  $23.6 \pm 3.2$  ng/g,  $p=0.059$ ).

We also measured tPA mRNA levels by using semi-quantitative real-time PCR. In agreement with the data obtained at the protein level, we observed a significant decrease in tPA mRNA levels at 14 months when compared to 3 months old (Fig. 1C ; 3m.:  $1.01 \pm 0.09$ , 14m.:  $0.70 \pm 0.02$ ,  $p=0.021$ ).

Altogether these data show that tPA activity is decreased in the brain parenchyma of mice with ageing, a phenomenon that is due to a reduced transcriptional activity of the tPA gene.

### Neuroserpin, an inhibitor of tPA, is increased by age

In order to further investigate the decrease of tPA activity observed during ageing in the brain, we wondered whether an increase in SERPIN expression could be also involved in this phenomenon. Thus, we performed semi-quantitative RT-PCR to follow the expression of the main tPA inhibitors in the brain of these mice, *i.e.* neuroserpin (NSP), protease-nexin-1 (PN-1) and PAI-1. We observed a significant increase in NSP mRNA levels at 14 months when compared to 3 months old (Fig. 2A; 3m.:  $1.01 \pm 0.08$ , 14m.:  $1.33 \pm 0.10$ ,  $p=0.043$ ). Although not significant, we also observed a decrease in PN-1 mRNA levels (Fig. 2B; 3m.:  $1.00 \pm 0.03$ , 14 m.:  $0.83 \pm 0.06$ ,  $p=0.059$ ), and no modification of PAI-1 mRNA levels with age (Fig. 2C; 3m.:  $1.01 \pm 0.08$ , 14m.:  $1.13 \pm 0.17$ ,  $p=0.248$ ). Thus, it is possible that an increase in neuroserpin expression could also contribute to the decrease in tPA activity observed in the brain of mice during ageing.

### tPA activity is decreased in a mouse model of Alzheimer's disease

We next measured tPA activity in a mouse model of AD, overexpressing the amyloid precursor protein carrying the *London* and the double *Swedish* mutations (APP<sup>sl</sup> mice). In order to characterize this model, we performed immunohistochemistry against A $\beta$  peptide in these mice at 3 and 14 months old (Fig. 3A). We observed a robust A $\beta$  deposition in the brain of APP<sup>sl</sup> at 14 months old, in contrast to 3 months old where no A $\beta$  deposit was detected. Next, tPA activity was monitored by free catalytic site tPA ELISA as previously done in wild type mice. Unfortunately, the sensitivity of this method was too low to be applied to this model, especially in transgenic animals (data not shown). So, we changed to a more sensitive technique using the tPA zymography assays (Sappino et al., 1993). We compared APP<sup>sl</sup> mice and WT littermates at 3, 11 and 14 months of age and observed a decrease in tPA activity at 11 (Fig. 3B,C; WT:  $100 \pm 10.7\%$ , APP<sup>sl</sup>:  $52 \pm 4.9\%$ ,  $p=0.049$ ) and 14 months (Fig. 3B,C; WT:  $100 \pm 14.5\%$ , APP<sup>sl</sup>:  $28.2 \pm 5.8\%$ ,  $p=0.049$ ).

We next compared total tPA protein levels and tPA mRNA levels by total tPA ELISA and semi-quantitative real time PCR, respectively. Interestingly, and in contrast to what was observed in the aged mice, we evidenced that neither the total tPA protein levels (Fig. 4A; 3m.-WT:  $40.5 \pm 4.2$  ng/g, 3m.-APP<sup>sl</sup>:  $29 \pm 4.3$  ng/g,  $p=0.083$ ; 14m.-WT:  $28.5 \pm 3.8$  ng/g, 14m.-APP<sup>sl</sup>:  $25.2 \pm 2.1$

ng/g,  $p=0.564$ ), nor the tPA mRNA levels were decreased in APPsl as compared to WT littermates (Fig. 4B; 3m.-WT:  $1.00 \pm 0.04$ , 3m.-APPsl:  $1.17 \pm 0.17$ ,  $p=0.24$ ; 14m.-WT:  $1.03 \pm 0.16$ , 14m.-APPsl:  $1.13 \pm 0.10$ ,  $p=0.47$ ).

Finally, we wondered whether ageing could change tPA mRNA levels in the brain of APPsl mice like in normal mice. We compared the level of tPA mRNA at 3 and 14 months by semi-quantitative PCR and we observed a similar decrease in tPA mRNA levels as in normal mice (Fig. 4C; 3m.:  $1.00 \pm 0.05$ , 14m.:  $0.49 \pm 0.05$ ;  $p=0.021$ ).

Altogether, these data show that tPA activity is lowered in APPsl brain as compared to wild type mice, but that this phenomenon is not due to a decrease in tPA expression. This view is reinforced by the fact that APPsl mice present a similar magnitude of decrease in tPA expression with ageing as the normal mice (Fig. 1C).

### **PAI-1 is over-expressed in APPsl mice**

In order to understand the molecular mechanisms by which tPA activity was decreased in APPsl mice, we next investigated the expression of the main tPA inhibitors in the brain of these mice at 3 and 14 months old. We observed that neither **neuroserpin** mRNA levels (Fig. 5A; 3m.-WT:  $1.01 \pm 0.08$ , 3m.-APPsl:  $1.06 \pm 0.14$ ,  $p=0.773$ ; 14m.-WT:  $1.01 \pm 0.08$ , 14m.-APPsl:  $0.88 \pm 0.06$ ,  $p=0.149$ ), nor **PN-1** mRNA levels (Fig. 5B; 3m.-WT:  $1.01 \pm 0.09$ , 3m.-APPsl:  $1.16 \pm 0.08$ ,  $p=0.189$ ; 14m.-WT:  $1.01 \pm 0.07$ , 14m.-APPsl:  $1.10 \pm 0.05$ ,  $p=0.386$ ) were significantly modulated in APPsl mice at both ages.

However, even though PAI-1 mRNA levels were unchanged in the brain of 3 months old mice as compared to WT littermates, they were increased by two fold at 14 months old (Fig. 5C; 3m.-WT:  $1.00 \pm 0.08$ , 3m.-APPsl:  $1.08 \pm 0.04$ ,  $p=0.564$ ; 14m.-WT:  $1.04 \pm 0.16$ , 14m.-APPsl:  $2.12 \pm 0.16$ ,  $p=0.021$ ). As glial cells are a source of PAI-1 in the brain, we next wondered whether PAI-1 production could be linked to gliosis associated with amyloid deposition. Thus, we evaluated the degree of microglial activation and astrogliosis by measuring the level of CD11b and GFAP mRNA, respectively. At 3 months old, an age where no amyloid deposits had been detected (Fig. 3A), we observed no variation of CD11b mRNA levels (Fig. 6A; WT:  $1.02 \pm 0.11$ , APPsl:  $0.95 \pm 0.09$ ,  $p=0.771$ ), nor of GFAP mRNA levels (Fig. 6B; WT:  $1.02 \pm 0.10$ , APPsl:

0.96 ± 0.08,  $p=0.773$ ). However, at 14 months old, when numerous A $\beta$  deposits were detected, we observed a two fold increase in CD11b mRNA levels (Fig. 6A; WT: 1.00 ± 0.05, APPsl: 2.00 ± 0.12,  $p=0.021$ ) and a six fold increase in GFAP mRNA levels in APPsl mice as compared to WT animals (Fig. 6B; WT: 1.00 ± 0.07, APPsl: 6.50 ± 0.63,  $p=0.021$ ).

Altogether, these data suggest that PAI-1 overexpression might be linked to gliosis associated with A $\beta$  deposition.

### **tPA activity in Alzheimer's disease brains**

To determine whether tPA could be involved in AD evolution as proposed by experiments performed in rodents, we measured tPA activity in AD brain samples and examined whether it could be linked to biochemical or clinical markers of AD progression, especially brain A $\beta$  levels. For this purpose, we used a fluorogenic substrate specific for human tPA and measured tPA activity in 16 clinically and neuropathologically confirmed sporadic AD and in 9 age-matched control brain samples. Table 1 (supplementary data) summarizes clinical and biochemical data of all samples.

We observed that, even though tPA activity was comparable in AD and non AD samples (mean ± SD: AD: 4.2 ± 2.3 AU, Non AD: 3.3 ± 1.9 AU,  $p=0.32$ , Student t-test), tPA activity was negatively correlated to the A $\beta_{(x-42)}$  levels (Fig. 7A;  $R^2=0.354$ , Z-test,  $p=0.015$ ). As APOE allele E4 polymorphism has been previously associated with a decreased in plasmin activity in AD brain (Lesdesma et al., 2003), we also wondered whether tPA activity could be influenced by this polymorphism. Thus, all patients were regrouped in two categories depending on the absence (APO  $\epsilon 4^-$ ) or the presence of at least one APO E4 allele (APO  $\epsilon 4^+$ ). We did not observe any difference between the two groups (Fig. 7B; APO  $\epsilon 4^-$ : 4.4 ± 2.1 AU; APO  $\epsilon 4^+$ : 3.5 ± 2.2 AU,  $p=0.27$ ), suggesting that tPA activity is not related to APO $\epsilon$  polymorphism.

## **DISCUSSION**

In this study, we provide evidence that the proteolytic activity of tPA is decreased in the brain of mice displaying an Alzheimer's disease phenotype (APPsl). Interestingly, this phenomenon

results, on the one hand, from the overexpression of the tPA inhibitor: PAI-1, associated with gliosis, and on the other hand, from an age-dependent reduction of tPA transcription (Fig. 8). We also observed that the proteolytic activity of tPA was negatively correlated to A $\beta$  levels in AD brains. Our findings underscore the importance that the tPA/plasmin system may have in AD.

In physiological conditions, tPA displays pleiotropic functions in the brain. It is involved in matrix degradation, NMDA signalling, long term potentiation and long term depression, through both plasmin-dependent and -independent processes (Melchor and Strickland, 2005). In this study, we showed that tPA proteolytic activity is decreased in the brain of mice during ageing. Such decrease in tPA activity has been previously reported in the blood of rodent (Emeis et al., 1992) but to our knowledge, this is the first time that this phenomenon is described in the brain. Although, at this time, it is not clear whether blood and brain tPA levels are related, our study supports the view that tPA activity could be decreased in the whole body during ageing. In the brain, such a decrease would lead to an impairment of the tPA biological function with ageing. For instance, a decrease in tPA expression and activity may have detrimental effects on synaptic plasticity by impairing memory and learning processes. However, this view needs to be further investigated, since this study focused on the cerebral cortex. It is possible that this phenomenon might be different in other brain structures expressing tPA, like the hippocampus, the cerebellum or the spinal cord.

In the context of Alzheimer's disease, initials studies have proposed that the tPA/plasmin system may have a beneficial effect on the disease evolution by regulating brain A $\beta$  peptides levels (Tucker et al., 2000b). They suggest that brain A $\beta$  levels might be regulated through a feedback loop mechanism, A $\beta$  peptides inducing the tPA/plasmin system which in turn degrades A $\beta$  peptides. Here we observed that tPA activity is decreased with age. As most sporadic AD starts at around 60-80 years old, we suggest that tPA activity could be already low at the age of AD onset and that the beneficial effect of the feedback loop may be less effective. In our study, we also observed that A $\beta$  accumulation worsens the decrease in tPA activity associated with ageing by inducing the expression of the tPA inhibitor: PAI-1. As the overexpression of PAI-1 is observed only when astroglia is developed and because astrocytes are a major source of PAI-1 (Buisson et al., 1998), we suggest that activated astrocytes associated with A $\beta$  deposits could be the main source of PAI-1. This view is in accordance with a previous study (Melchor et al.,

2003), in which PAI-1 protein levels were shown to be increased in astrocytes of a transgenic mice overproducing A $\beta$ . However, we can not rule out that microglia also contribute to this phenomenon since it is increased when amyloid deposition occurs in the AD mouse model.

However, in contrast to the study of Tucker et al., (2000b), we have not observed an increase in tPA mRNA levels in the brain of APPsl mice. This might be explained by the different ages analysed in these two studies. Indeed, Tucker et al. (Tucker et al., 2000b) analysed mice at 22 months old, whereas our study has been performed in mice at 3 and 14 months old. Maybe the observed increase appears at a later stage, when A $\beta$  deposition is more pronounced. However, as the present study shows that both tPA activity and tPA expression decrease with age, this seems less probable.

Furthermore, the study of tPA activity in the brain of AD patient bring some new informations supporting a role of tPA in AD progression. Indeed, we observed that brain tPA activity is similar in AD and non AD patients. These data would suggest that tPA is not implicated in AD pathogenesis, that is to say that a deficit in tPA activity *per se* may not be enough to induce AD. However, the negative correlation observed between tPA activity and A $\beta$  levels in the AD population suggests a link between the metabolism of these two proteins. On the one hand, patients with a high content of amyloid may present a decrease in tPA activity, as suggested by the study performed in AD transgenic mice. On the other hand, the correlation between tPA activity and A $\beta$  levels may also suggest that having a low tPA activity increases the rate of A $\beta$  accumulation by decreasing the rate of A $\beta$  clearance from the brain. This idea is supported by other reports since tPA/plasmin system had been shown to degrade A $\beta$  both *in vivo* and *in vitro* (Tucker et al., 2000a; Exley and Korchazhkina, 2001; Melchor et al., 2003). Both hypotheses are not incompatible, since there might be a negative feedback loop mechanism between tPA activity and A $\beta$  deposition which might accelerate AD progression by accelerating amyloid accumulation.

Altogether, our findings underscore the role that the tPA/plasmin system may play in Alzheimer's disease. They support the model by which tPA/plasmin system favours the amyloid clearance from the brain; and that an impairment of the tPA proteolytic activity may decrease the efficiency of A $\beta$  clearance. Moreover, they add new insight on the tPA regulation *in vivo* by showing that the expression and the proteolytic activity of tPA is impaired with ageing. Thus, we

would propose that increasing the endogenous tPA activity, for instance by down-regulating the expression of its brain inhibitors or delivering compounds that block the activity of these inhibitors may have a beneficial role on AD evolution.

## **ACKNOWLEDGMENTS**

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## FIGURE LEGENDS

### **Fig. 1. Both tPA activity and tPA expression are decreased in the mouse brain by age**

An ELISA raised against active tPA (A) or total tPA (B) was performed on proteins extracted from the cerebral cortex of C57BL6 mice at 3, 11 and 14 months old. Data are represented in ng of tPA / g of protein. C, qPCR for tPA was performed on mRNAs extracted from the cerebral cortex of the same mice. Data are represented relative to 3 months old mice with the  $2^{-\Delta Ct}$  method (open columns) or with the  $2^{-\Delta\Delta Ct}$  method (relative to NR1 mRNA levels) (hatched columns). Mean +/- SEM (n=4 per group). Kruskal Wallis test was used for multi-group comparison (A) and Mann-Whitney U-test for two-group comparison (A, B, C) (\*, p<0.05).

### **Fig. 2. Brain SERPINs mRNA levels during ageing**

qPCR for NSP (A), PN-1 (B), PAI-1 (C) were performed on mRNAs extracted from the cerebral cortex of 3 and 14 months old mice. Data are represented relative to 3 months old mice with the  $2^{-\Delta Ct}$  method (open columns) or with the  $2^{-\Delta\Delta Ct}$  method (relative to NR1 mRNA levels) (hatched columns). Mean +/- SEM (n=4 per group). Mann-Whitney U-test (\*, p<0.05).

### **Fig. 3. tPA activity is decreased in the brain of APPs1 mice**

A, Sections from APPs1 at 3 months (a, b, c) and 14 months old (d, e, f) were stained with Congo red (a, d) and immunostained for A $\beta$ (x-40) (b, e) and A $\beta$ (x-42) (c, f) (scale bar: 400 $\mu$ m). B, Zymography analysis of tPA proteolytic activity was performed on proteins extracted from the cerebral cortex of WT and APPs1 mice at 3, 11, and 14 months old. C, Densitometric analysis of the corresponding zymograms. Data are represented as mean +/- SEM (n=3 per group) relative to WT mice of the same age. Mann-Whitney U-test (\*, p<0.05).

### **Fig. 4. tPA expression is stable in the brain of APPs1 mice**

A, An ELISA raised against total tPA was performed on proteins extracted from the cerebral cortex of WT and APPs1 mice at 3 and 14 months old. Data are represented in ng of tPA / g of protein. B, qPCR for tPA was performed on mRNAs extracted from the cerebral cortex of the same mice. Data are represented relative to WT mice with the  $2^{-\Delta Ct}$  method (open columns) or with the  $2^{-\Delta\Delta Ct}$  method (relative to NR1 mRNA levels) (hatched columns). C, tPA mRNA levels

were compared at 3 and 14 months old in APPsl brain. Data are represented relative to 3 months old mice. Mean +/- SEM (n=4 per group). Mann-Whitney U-test (\*, p<0.05).

**Fig. 5. Brain SERPINs mRNA levels in the brain of APPsl mice**

qPCR for neuroserpin (A), Protease-nexin-1 (B) and PAI-1 (C) were performed on mRNAs extracted from the cerebral cortex of control (light grey bars) and APPsl mice (grey bars) at 3 and 14 months of age. Data are represented relative to WT mice with the  $2^{-\Delta C_t}$  method (open columns) or with the  $2^{-\Delta\Delta C_t}$  method (relative to NR1 mRNA levels) (hatched columns). Mean+/-SEM (n=4 per group). Mann-Whitney U-test (\*, p<0.05).

**Fig. 6. Amyloid deposition is associated with an increase in both microglial and astroglial markers in the brain of APPsl mice**

qPCR for CD11b (A), a biological marker for microglia, and GFAP (B), a biological marker for astrocytes, were performed on mRNAs extracted from the cerebral cortex of control (light grey bars) and APPsl mice (grey bars) at 3 and 14 months of age. Data are represented relative to WT mice with the  $2^{-\Delta C_t}$  method (open columns) or with the  $2^{-\Delta\Delta C_t}$  method (relative to NR1 mRNA levels) (hatched columns). Mean+/-SEM (n=4 per group). Mann-Whitney U-test (\*, p<0.05).

**Fig. 7. Brain tPA activity in Alzheimer's disease**

tPA activity was measured in 16 clinically and neuropathologically confirmed sporadic AD and in 9 age-matched frontal brain protein samples by using a fluorogenic substrate specific for human tPA. Points represent the tPA activity measured as a function of brain  $A\beta_{(x-42)}$  levels (A). B, All samples were pooled in two groups, one presenting no APOE4 allele (APO  $\epsilon 4^-$ ) and the other one having at least one APOE4 allele (APO  $\epsilon 4^+$ ). Data are represented in two ways. Points indicate the tPA activity measured in each brain sample. Box charts represent the distribution characteristics, by indicating the median with 5<sup>th</sup>, 25<sup>th</sup>, 75<sup>th</sup> and 95<sup>th</sup> percentiles. Mean is represented by empty squares. No significant differences were measured between the two groups (Student t-test; p=0.27).

**Fig. 8. Ageing and PAI-1 decrease tPA activity**

Ageing leads to the decrease in tPA activity in the normal mouse brain through a decrease in tPA expression. This phenomenon is worsened in AD transgenic mice through an increased in PAI-1 transcription probably related to gliosis.

## SUPPLEMENTARY MATERIALS

### **Table 1: Summary of clinical and biochemical data collected for each patient**

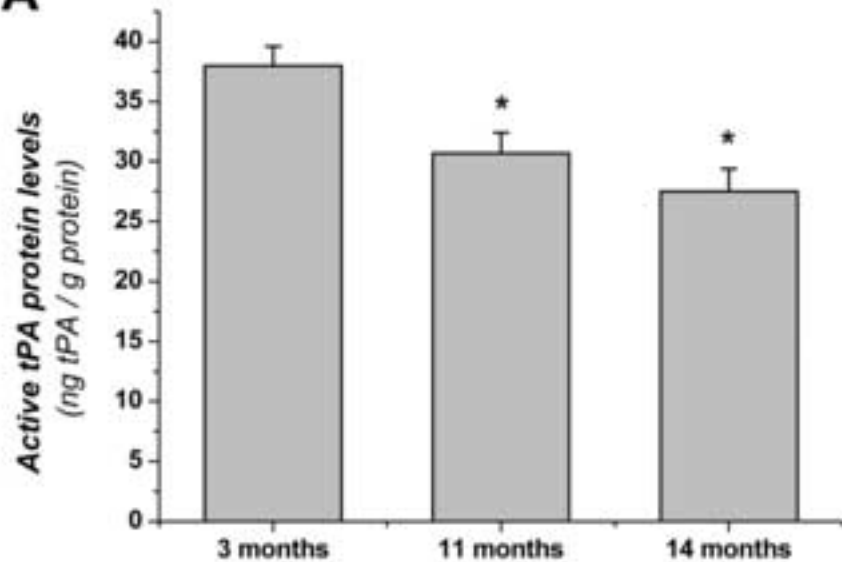
Age is expressed in years. Tau stages are expressed according to the biochemical classification of Delacourte et al. (1999). This scale categorizes Tau pathology progression in 10 stages according to the brain region affected: transentorhinal cortex (S1), entorhinal (S2), hippocampus (S3), anterior temporal cortex (S4), inferior temporal cortex (S5), medium temporal cortex (S6), polymodal association areas (prefrontal, parietal inferior, temporal superior) (S7), unimodal areas (S8), primary motor (S9a) or sensory (S9b, S9c), and all cortical areas (S10). A $\beta$  (x-42) stands for the level amyloid-beta 42 aggregates and is expressed in  $\mu\text{g/g}$  of tissue. tPA proteolytic activity is expressed in arbitrary units (AU). AD: Alzheimer's disease; ND: Not determined.

### **Fig. 1. tPA activity is decreased both *in vitro* and *in vivo* by an antibody directed to the protease domain of tPA**

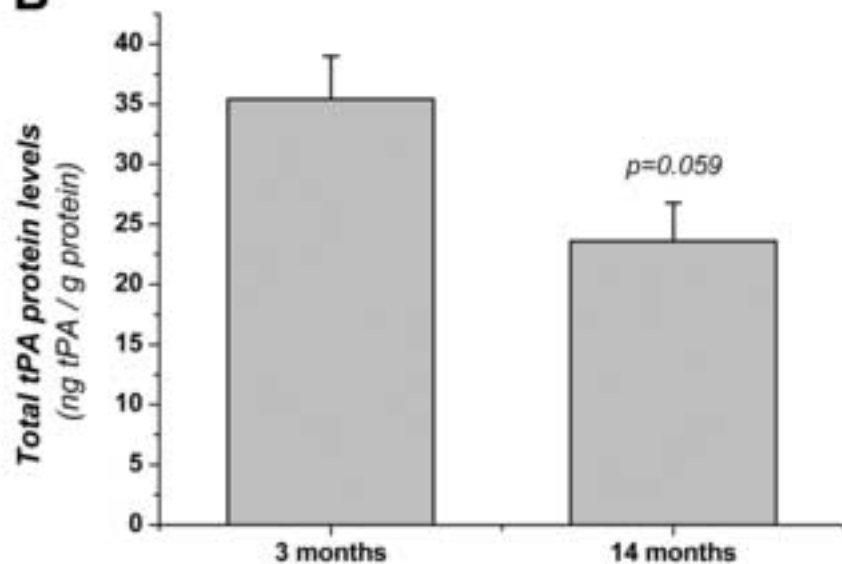
The monoclonal antibody H27B6 raised against the protease domain of tPA was used to assess the specificity of the fluorogenic substrate used in the enzymatic assay. A, 1,5  $\mu\text{g}$  of recombinant tPA (Actilyse®, Boehringer Ingelheim, Germany) was incubated with an excess of H27B6 antibody and activity was monitored as described in the Materials and Methods. B, tPA proteolytic activity was monitored on 200 $\mu\text{g}$  of protein extracted from an AD brain, with or without an excess of the H27B6 antibody or an excess of an anti-CDK5 antibody (C-8) (Santa Cruz, Germany) used as a control.

## Figure 1

**A**



**B**



**C**

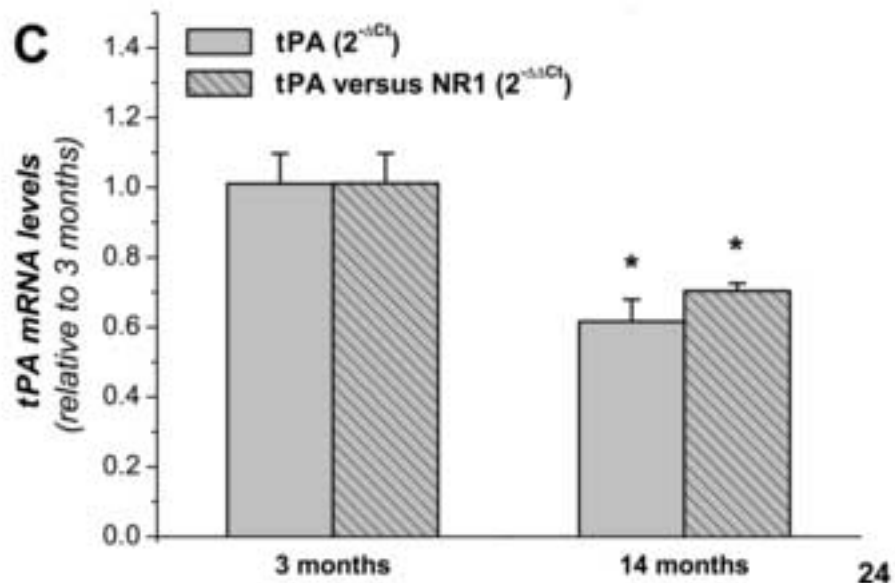
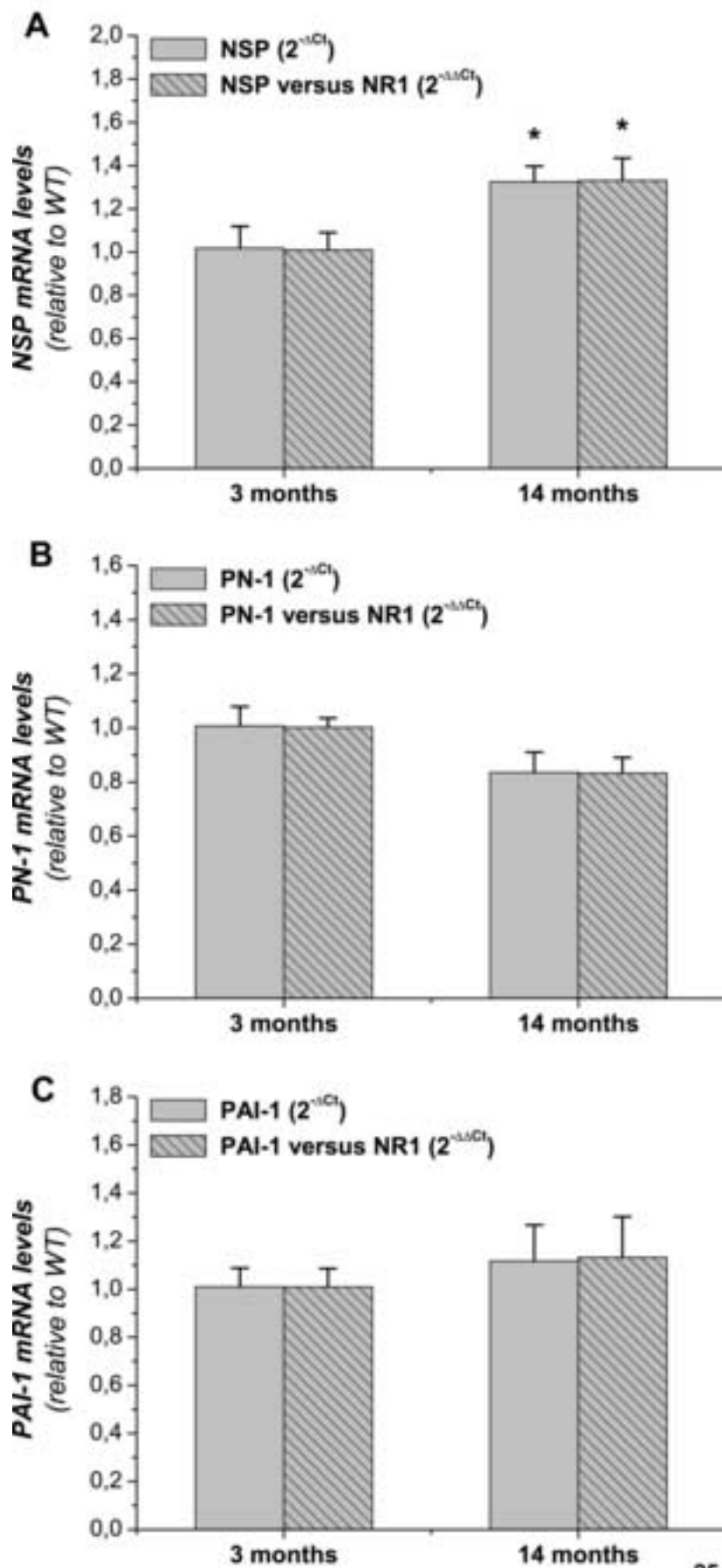
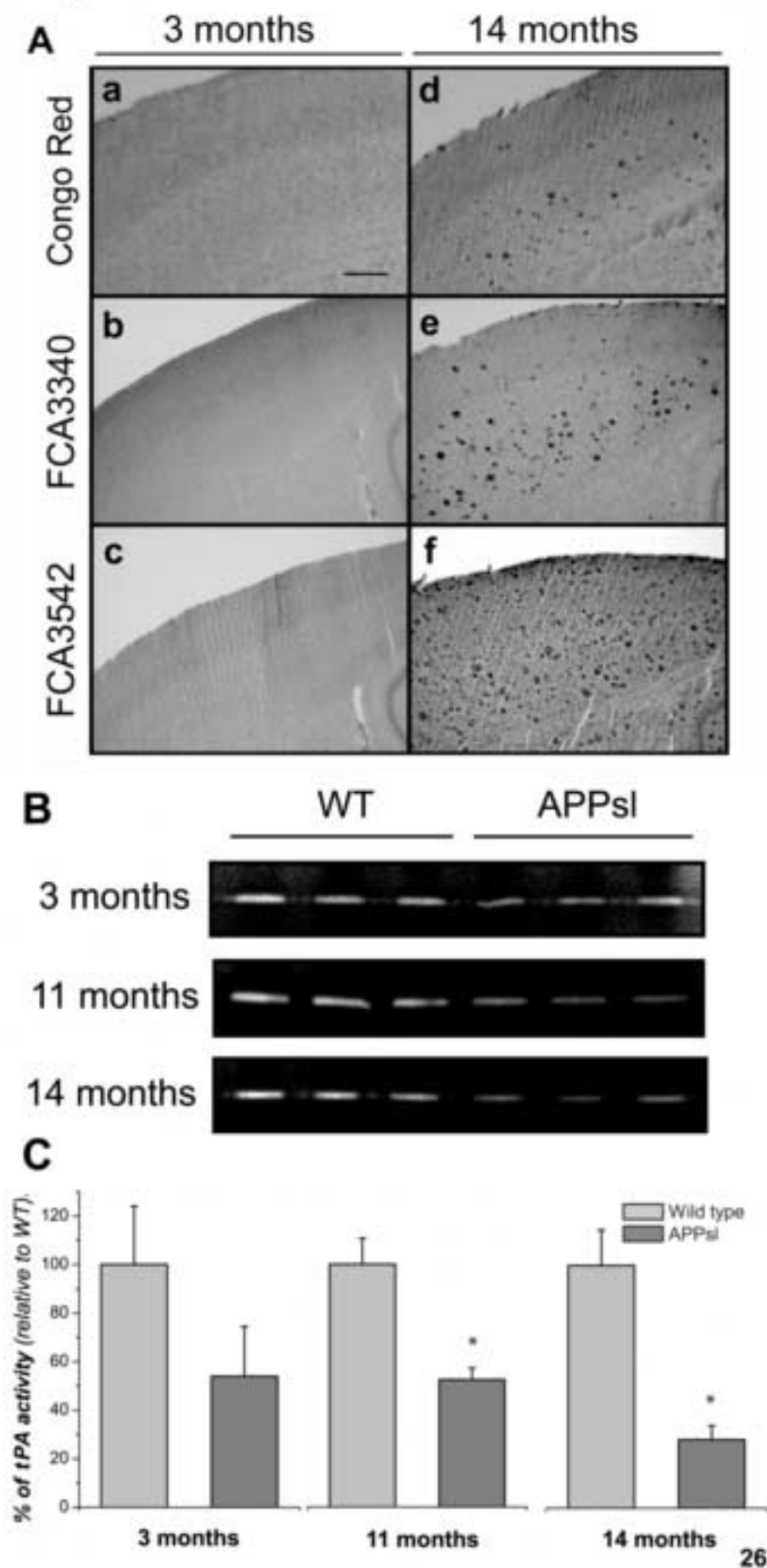


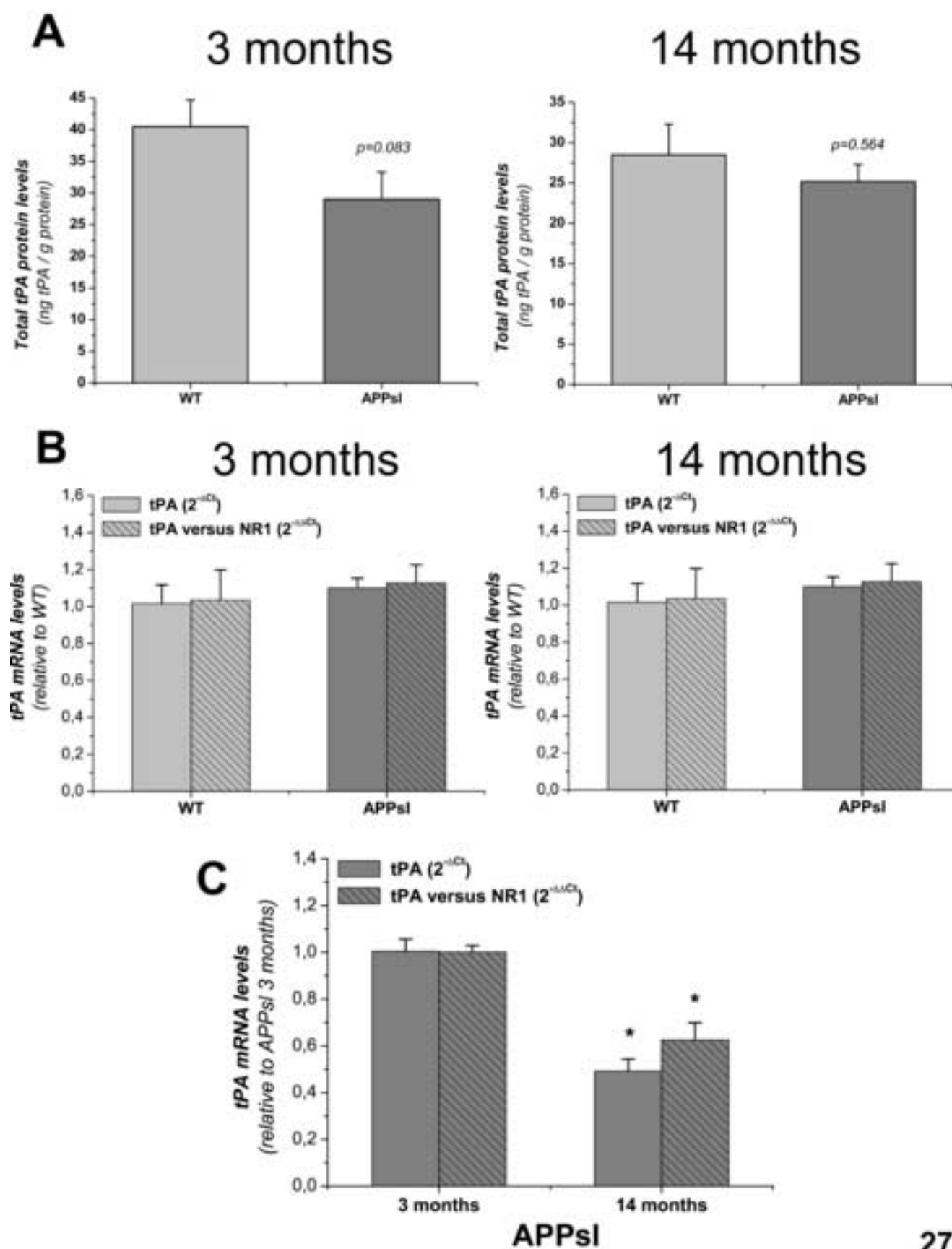
Figure 2



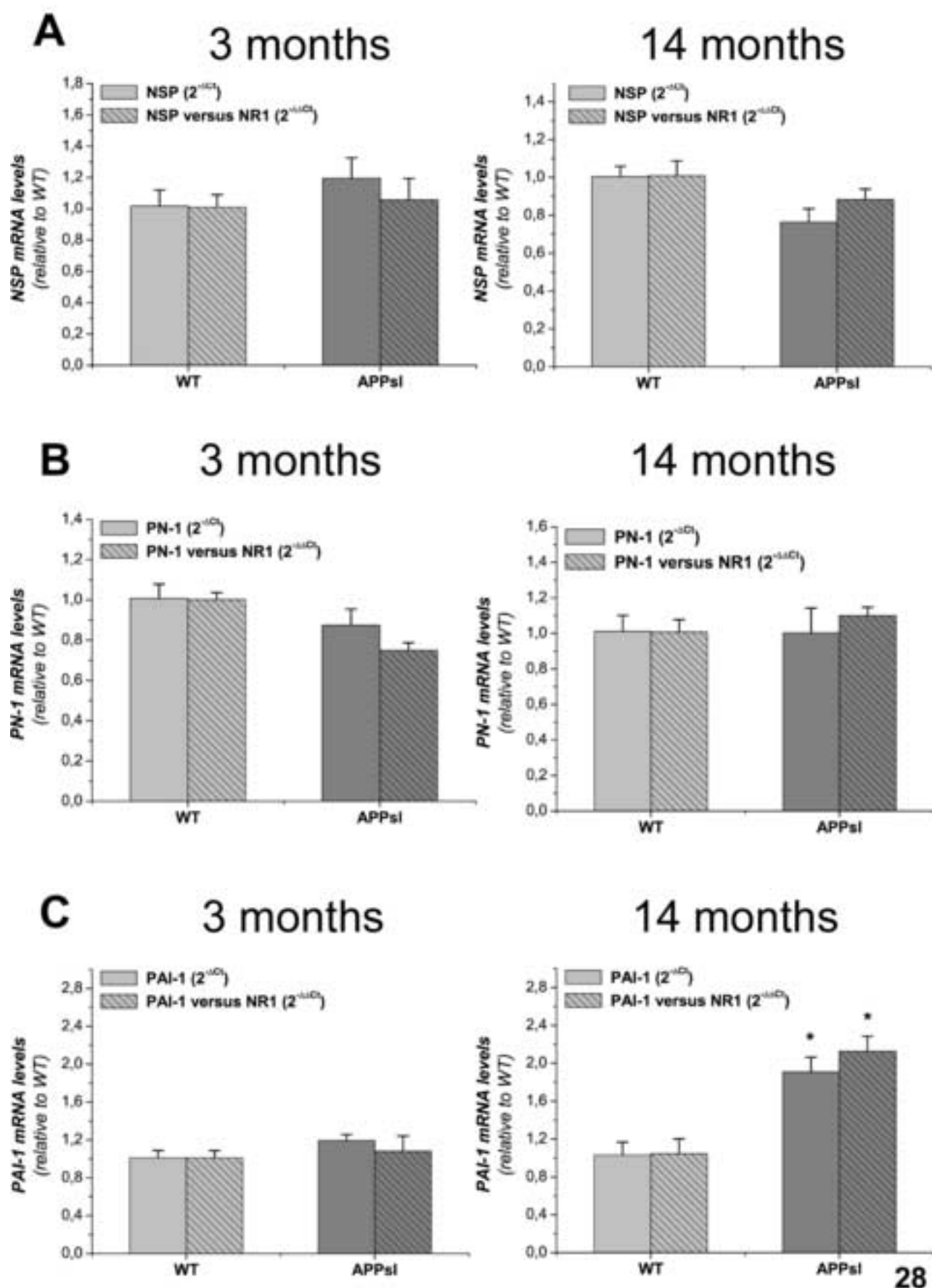
## Figure 3



# Figure 4



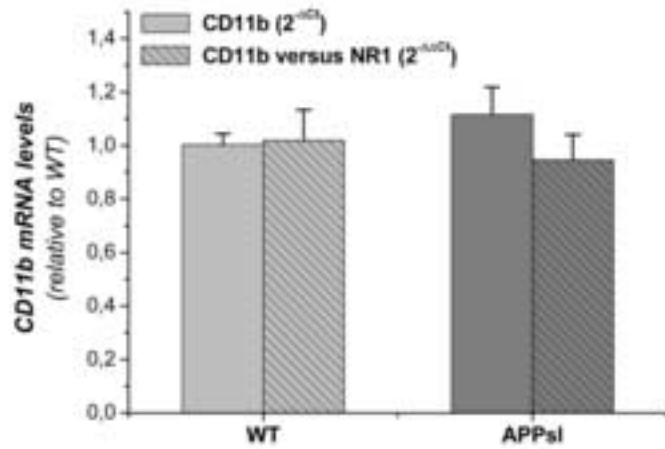
# Figure 5



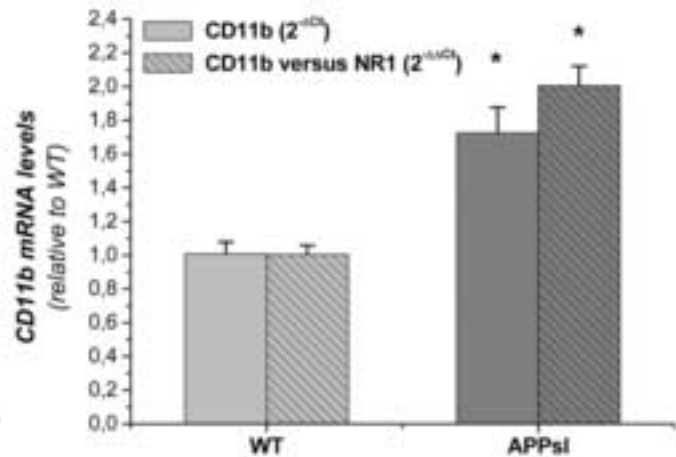
# Figure 6

**A**

3 months

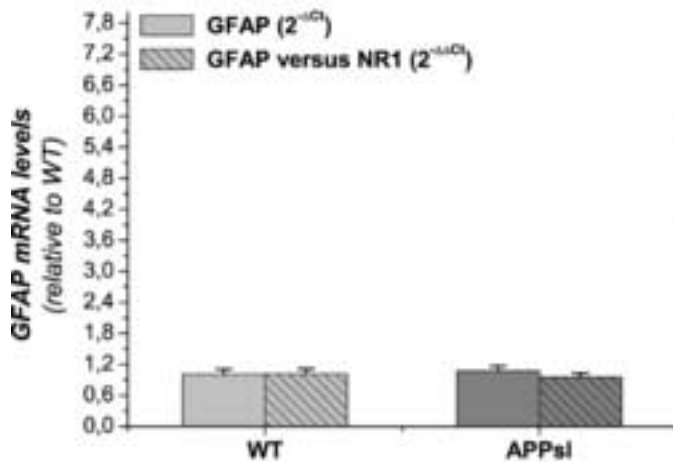


14 months

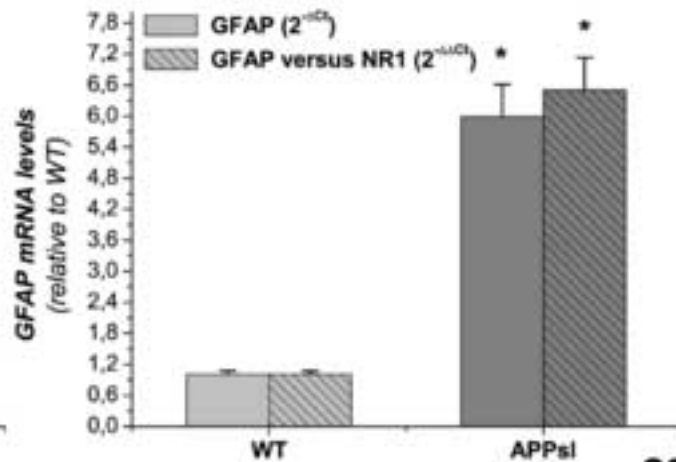


**B**

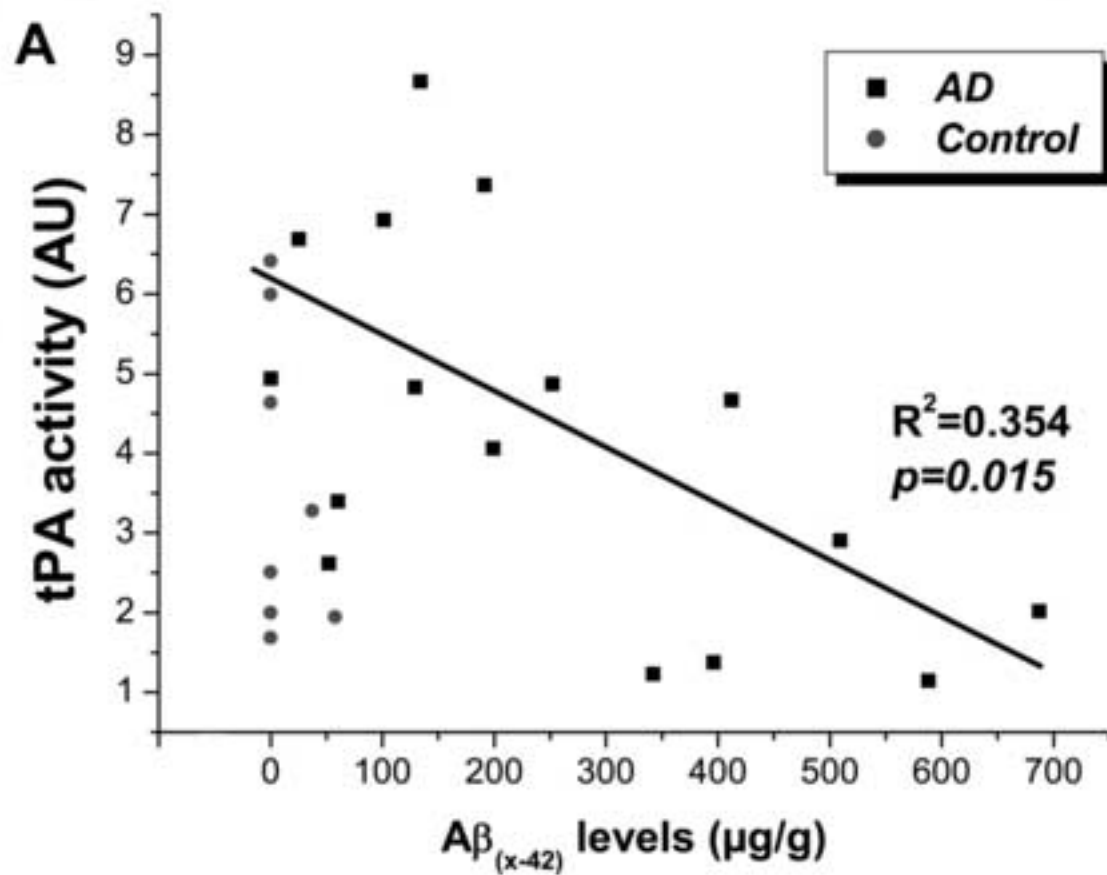
3 months



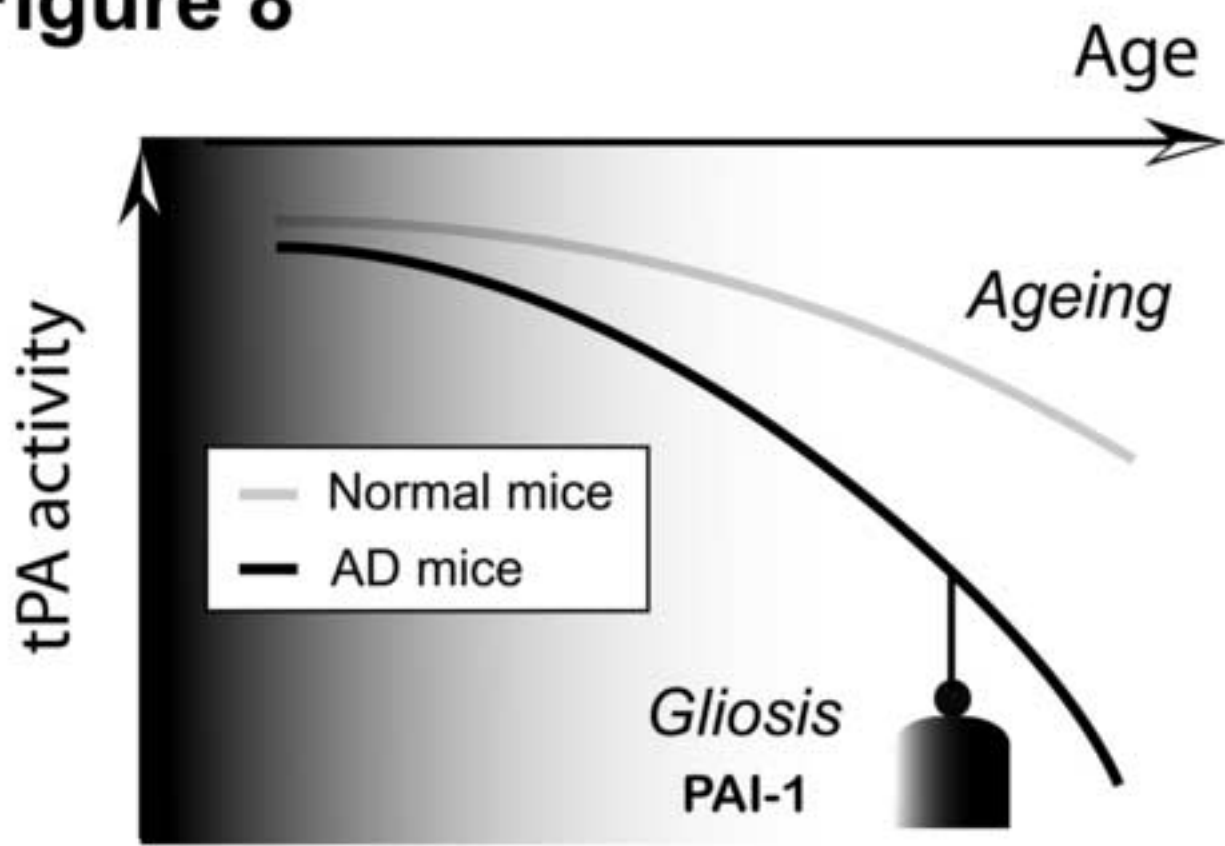
14 months



# Figure 7



**Figure 8**



**Table 1**

[Click here to download 6-Supplementary Material: S-Table 1.tif](#)

**Figure 1**

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