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BRAIN RESEARCH

Research Report

Females exhibit more extensive amyloid, but not tau, pathology in an Alzheimer transgenic model

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ABSTRACT

Epidemiological studies indicate that women have a higher risk of Alzheimer's disease (AD) even after adjustment for age. Though transgenic mouse models of AD develop AD-related amyloid beta (Abeta) and/or tau pathology, gender differences have not been well documented in these models. In this study, we found that female 3xTg-AD transgenic mice expressing mutant APP, presenilin-1 and tau have significantly more aggressive Abeta pathology. We also found an increase in beta-secretase activity and a reduction of neprilysin in female mice compared to males; this suggests that a combination of increased Abeta production and decreased Abeta degradation may contribute to higher risk of AD in females. In contrast to significantly more aggressive Abeta pathology in females, gender did not affect the levels of phosphorylated tau in 3xTg-AD mice. These results point to the involvement of Abeta pathways in the higher risk of AD in women. In addition to comparison of pathology between genders at 9, 16 and 23 months of age, we examined the progression of Abeta pathology at additional age points; i.e., brain Abeta load, intraneuronal oligomeric Abeta distribution and plaque load, in male 3xTg-AD mice at 3, 6, 9, 12, 16, 20 and 23 months of age.

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These findings confirm progressive Abeta pathology in 3xTg-AD transgenic mice, and provide guidance for their use in therapeutic research.

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1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that is the most common form of dementia. Only a small portion (< 1%) of cases have known genetic causes, while the majority of AD cases are sporadic (Cummings, 2004). The most significant non-genetic risk factor for AD is age; the risk of AD doubles between the age groups of 65–70 and 70–74. In addition to age, various factors, such as medical history (head injury, stroke, hypertension, hypercholesterolemia, stress, etc.), life style (diet, lack of exercise, alcohol consumption, smoking, etc) and education, may be associated with AD (McDowell, 2001). Epidemiological studies also indicate that women have a higher risk of AD (Brookmeyer et al., 1998) even after adjusting for age (Hy and Keller, 2000). The precise cause of the higher risk of AD in women is unknown.

The pathological hallmarks of AD are amyloid plaques in the extracellular space and intraneuronal neurofibrillary tangles (Hyman, 1997). Amyloid beta (Abeta), the primary component of amyloid plaques, is generated from the amyloid precursor protein (APP) by sequential proteolytic cleavage at the beta and gamma sites (Hardy and Selkoe, 2002). Freshly generated Abeta forms oligomers on and within neurons (Walsh et al., 2000) and compromises hippocampal long-term potentiation in vivo (Walsh et al., 2002). Neurofibrillary tangles are composed of hyperphosphorylated tau, a neuronal microtubule-associated protein. Phosphorylation of tau regulates its ability to promote microtubule assembly (Lindwall and Cole, 1984); hyperphosphorylation interferes with the normal biological functions of tau by reducing its ability to bind to and stabilize microtubules (Trojanowski and Lee, 1995). All genetic mutations that cause familial AD are linked to the Abeta cascade, while tau mutations are associated with AD and several other forms of dementia including frontotemporal dementia and progressive supranuclear palsy (Hardy and Selkoe, 2002). The clinical progression of AD is closely related to tau pathology (Braak and Braak, 1995). In animal models, modulation of Abeta or tau cascades alter pathology and influence cognitive function (Barten et al., 2005; SantaCruz et al., 2005).

To investigate the mechanisms of these pathological events and explore therapeutic strategies, various lines of transgenic mice have been created (McGowan et al., 2006). Overexpression of mutant APP elevates Abeta production leading to plaque formation (Games et al., 1995; Hsiao et al., 1996). Overexpression of mutant presenilin (PS)-1 elevates the level of endogenous Abeta slightly, but does not induce Abeta plaque deposition (Duff et al., 1996). Mice obtained by crossing APP and PS-1 transgenic mice show dramatically accelerated Abeta pathology (Holcomb et al., 1998) associated with oxidative stress and neuroinflammation (Matsuoka et al., 2001a,b). Overexpression of mutant tau causes hyperphosphorylation and tangle formation (Lewis et al., 2000). In many tau transgenic mice, tauopathy is more evident in the brainstem and spinal cord and is associated with motor dysfunction. Some tau transgenic mice, including the one used in this study, show tau pathology in the brain along with cognitive impairment (Ramsden et al., 2005).

Currently available AD mouse models are well-characterized, but the effects of gender on Abeta and tau pathology in animal models have not been carefully examined. Previously, gender differences have been studied in Tg2576 APP transgenic mice, which develop Abeta, but not tau pathology. In that study, female mice showed more histologically-determined Abeta plaques at 15 and 19 months of age and ELISA-quantified Abeta 1–40 (but not Abeta 1–42) load at 15 months of age (Callahan et al., 2001). Mice at these two time points bear Abeta plaques; the effect of gender at the pre-plaque stage was not evaluated. Abeta and tau pathology influence each other, but gender influences on the co-occurrence of these two aspects of AD pathology have not been examined. In this study, we examined the progression of AD-related pathology in male and female mice during the course of their lifespan using transgenic mice which develop both Abeta and tau pathology. In addition, we also investigated the progression of Abeta pathology in male mice at additional age points.

2. Results

2.1. *Soluble and total Abeta 1–40 and 1–42 are increased with aging after plaque formation*

Our Abeta ELISAs were composed of N and C-terminus end-specific antibodies to quantify full-length Abeta 1–40 and 1–42 (Horikoshi et al., 2004). Both the Abeta 1–40 and 1–42 ELISAs provide linear (r^2>0.99) standard curves over the range of 1–800 fmol/ml (pM). In this study, results from multiple plates needed to be combined; therefore, to assure quality control, we used a higher cut-off, 10 fmol/ml, which is equivalent to 220 and 6500 fmol/g tissue in diethylamine (DEA) and formic acid (FA) fractions, respectively (this cut-off level is indicated by dashed line in Fig. 1). Samples were analyzed using multiple ELISA plates. The variance of the internal reference, pooled human plasma, was within 5% among plates; thus we used values obtained by ELISA assays without adjustment.

DEA-extracted soluble Abeta 1–40 was detectable from 3 months of age, the youngest age we examined in this study, and the level was stable until 12 months of age in male 3xTg-AD mice (Fig. 1A squares). The soluble Abeta 1–40 level began to rise between 12 and 16 months of age in male 3xTg-AD mice, and continued to increase with aging. Abeta plaques were first detected around 14 months of age in male 3xTg-AD mice (Fig. 4E), which is close to when ELISA-detected Abeta 1–42 began to rise. Soluble Abeta 1–42 was below the quality control cut-off until 12 months of age and barely detectable at 16 months of age in male 3xTg-AD mice (Fig. 1A circles). Soluble Abeta 1–40 was much more abundant, >10 fold,

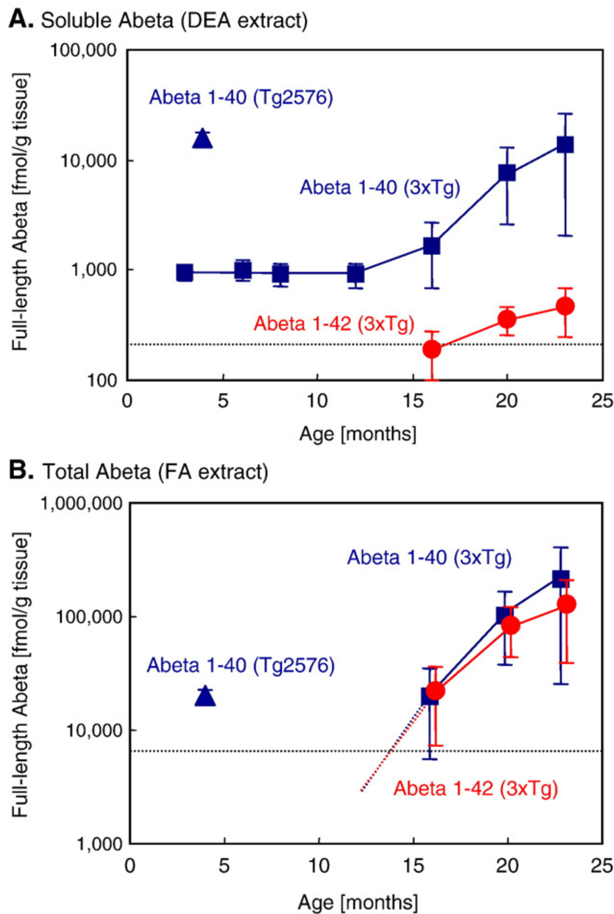


Fig. 1 – Levels of full-length Abeta 1–40 and 1–42 are increased with aging in male 3xTg-AD mice. Levels of DEA-extracted soluble (A) and FA-extracted total (B) Abeta 1–40 and 1–42 were determined in male 3xTg-AD mice (n=6 each) and male Tg2576 mice (n=5 each). Dashed lines at 220 and 6500 fmol/g tissue in DEA and FA fractions, respectively, indicate the limit of fully-reproducible and reliable Abeta detection (10 fmol Abeta/ml extract, note: the detection limit is 1 fmol/ml extract). FA extraction requires 1:20 neutralization during the sample preparation, and the signals were diluted in young mice. Please note that Y-axis is in log scale.

compared to soluble Abeta 1–42 (Fig. 1A squares vs circles). The commonly used APP single transgenic Tg2576 mice (male) had much higher soluble Abeta 1–40 at 4 months of age (Fig. 1A triangle), but soluble Abeta 1–42 was not detectable. 3xTg-AD mice with a similar level of Abeta 1–40 (>20 months of age) bear Abeta plaques, while Tg2576 mice at 4 months of age are free of any plaque pathology. This suggests that the absolute level of Abeta 1–40 is not a critical factor in Abeta plaque formation.

FA-extracted total Abeta 1–40 and 1–42 were not detectable until 16 months of age in male 3xTg-AD mice, while total Abeta 1–40 was detectable in Tg2576 mice from 3 months of age, the youngest age we examined in this study (Fig. 1B triangle). FA extraction required 1:20 neutralization prior to ELISA analysis, and the neutralization dilutes the signals; thus FA-extracted total Abeta levels were below the cut-off at younger ages. Levels of total Abeta 1–40 and 1–42 were similar and gradually increased with aging (Fig. 1B squares and circles, respectively).

2.2. *Abeta plaques and intraneuronal Abeta increase with aging*

We examined Abeta and APP pathology using two antibodies: 82E1, specific to the N-terminus end of Abeta (epitope: 1–5 amino acids of cleaved Abeta) and not cross-reactive to uncleaved APP, and 6E10, fully cross-reactive to uncleaved APP, soluble APP cleaved at alpha site (sAPPalpha), and Abeta (epitope: 3–8). 82E1-immunostaining was detectable in neurons in the frontal cerebral cortex starting at 6 months of age, and extended to the middle of the brain by 9 months of age (Fig. 2A). Abeta plaques were first observed in the subiculum and then gradually extended to the hippocampus (Fig. 2C). In the hippocampal formation, 82E1 detected plaques as well as some pyramidal neurons weakly (Fig. 2G). Intraneuronal staining was much more intense in the cerebral cortex (Figs. 2G vs H). APP-cross-reactive 6E10 stained neurons in the cerebral cortex and hippocampal CA1 prior to the age at which plaques became detectable (Figs. 2D, E). In addition to intraneuronal staining, 6E10 stained extracellular plaques (Figs. 2F, I). Both 82E1 and 6E10 resulted in similar intraneuronal staining in the cerebral cortex (Figs. 2H, J). In contrast to the cerebral cortex, 82E1 and 6E10 staining in the hippocampal formation were distinct. While uncleaved APP-cross-reactive 6E10 stained pyramidal neurons strongly (Fig. 2I), cleaved Abeta-specific 82E1 detected only a few neurons weakly (Fig. 2G). These results suggest that 6E10 immunostaining in the hippocampal pyramidal neurons is primarily due to its cross-reactivity to uncleaved APP and sAPPalpha.

2.3. *Oligomeric Abeta accumulates inside of neurons in 3xTg-AD mice*

Oligomeric Abeta was examined using an anti-Abeta-derived diffusible ligands (ADDL) antibody, clone NU-1 (Lambert et al., 2007). In 3xTg-AD mice, intraneuronal staining in the cortex of the frontal region was detectable from 6 months of age, and became more evident at 9 months of age (Fig. 3C). Neuronal staining in the cortex in the middle region was less evident compared to the frontal brain (Figs. 3A vs B). A conventional Abeta antibody, 82E1, also detected intraneuronal staining in the cortex (Figs. 2A–C). In addition to the staining in the cortex, NU-1-staining was seen in the intraneuronal space in the subiculum where Abeta plaques first became detectable at 14 months of age (Figs. 3B, D). At older ages, intraneuronal staining became more evident and plaques were also stained by NU-1 antibody (Figs. 3E–I). Some cells surrounding the plaques were also immunostained with NU-1 (Fig. 3H).

We also examined oligomeric Abeta in a different AD transgenic mouse model, PS/APP (Duff et al., 1996). APP transgene expression is under the control of the prion promoter, which drives transgene expression mainly in neuronal cells in PS/APP mice, while it is under the control of neuron-specific Thy-1 promoter in 3xTg-AD mice. In PS/APP mice, Abeta plaques were detectable from 2 months of age (Fig. 3J). At 2 months of age, cells surrounding plaques were not immunopositive with NU-1 (Fig. 3M); with aging, more plaques appeared and cells

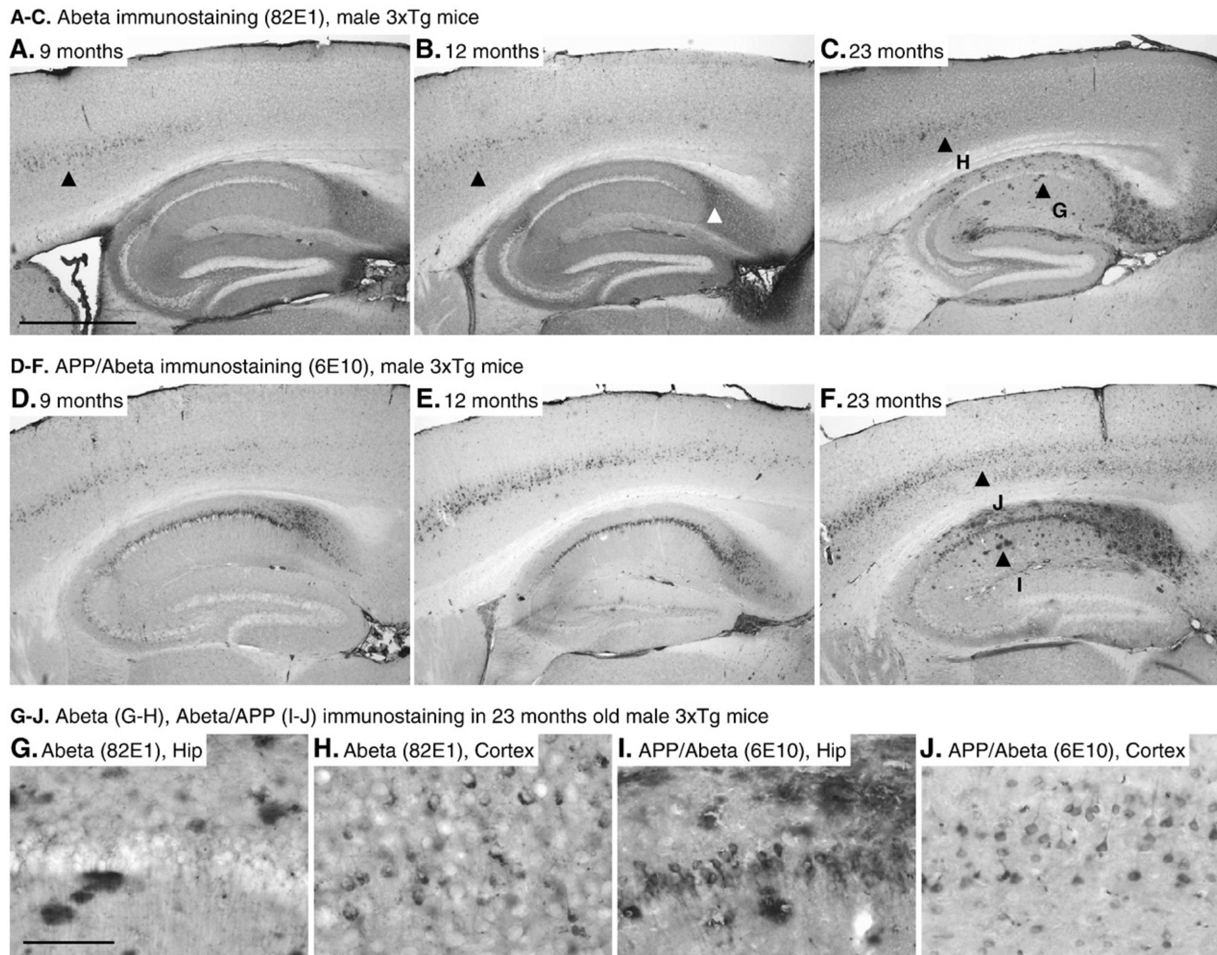


Fig. 2 – Abeta and APP pathology increase with aging. Sagittal sections from male 3xTg-AD mice at 9, 12, 14 and 23 months of age were immunostained with 82E1 antibody (A–C, G, H) or 6E10 antibody (D–F, I, J). 82E1, not cross-reactive with uncleaved APP, detected intraneuronal Abeta in the cerebral cortex of the frontal brain region from 6 months of age, and expanded toward the middle of the brain at 9 and 12 months of age (A, B, indicated by closed arrow heads). At 12 months of age, 82E1 detected intraneuronal staining in the subiculum (B, indicated by open arrow heads). Uncleaved APP and sAPPalpha cross-reactive antibody, 6E10, detected strong intraneuronal staining in wider area; cortex and hippocampal pyramidal neurons (D–F, I. J). Bar = 200 μm in A for A–F; 20 μm in G for G–J.

surrounding Abeta plaques became NU-1 immunopositive (Figs. 3N–P). However, intraneuronal Abeta staining was hard to detect in PS/APP mice under the experimental conditions we used. We also used another ADDL antibody, NU-2 (Lambert et al., 2007), for 3xTg-AD and PS/APP mice and obtained similar staining patterns (data not shown).

2.4. *Female mice show more aggressive Abeta pathology than male mice after plaque formation*

We examined gender differences in Abeta load using age-matched male and female mice. Tg2576 mice at 4 months of age and 3xTg-AD mice at 9 months of age are free of Abeta plaques. Levels of soluble Abeta 1–40 were similar between genders (Fig. 4A). FA-extracted total Abeta 1–40 was detectable in Tg2576 mice, and there was no difference between genders (Fig. 4C).

In contrast to the plaque-free stage, there was a significant gender difference in plaque-bearing mice. Levels of soluble Abeta 1–40 and 1–42 in female mice were higher (Abeta 1–40: 461%, $P = 0.058$; Abeta 1–42: 175%, $P = 0.065$) at 16 months of age (Figs. 4A, B), and the difference became clearer at 23 months (Abeta 1–40: 407%, $P = 0.006$; Abeta 1–42: 190%, $P = 0.003$). FA-extracted total Abeta levels were significantly higher in female 3xTg-AD mice at 16 (Abeta 1–40: 648%, $P = 0.017$; Abeta 1–42: 330%, $P = 0.047$) and 23 months of age (Abeta 1–40: 161%, $P = 0.003$; Abeta 1–42: 208%, $P = 0.011$) (Figs. 4C, D). Histology revealed similar gender differences (see below).

As suggested by the ELISA results, Abeta plaque pathology was more evident in female mice compared to age-matched male mice. At 14 months of age, Abeta plaques were barely detectable in male mice, while several plaques were seen in the subiculum in the female mice (Figs. 4E/F vs G/H).

2.5. *Female mice have higher beta-secretase activity and less efficient Abeta degradation*

It has been suggested that Abeta production and degradation are altered with aging (Hyman, 1997; Iwata et al., 2005). Changes

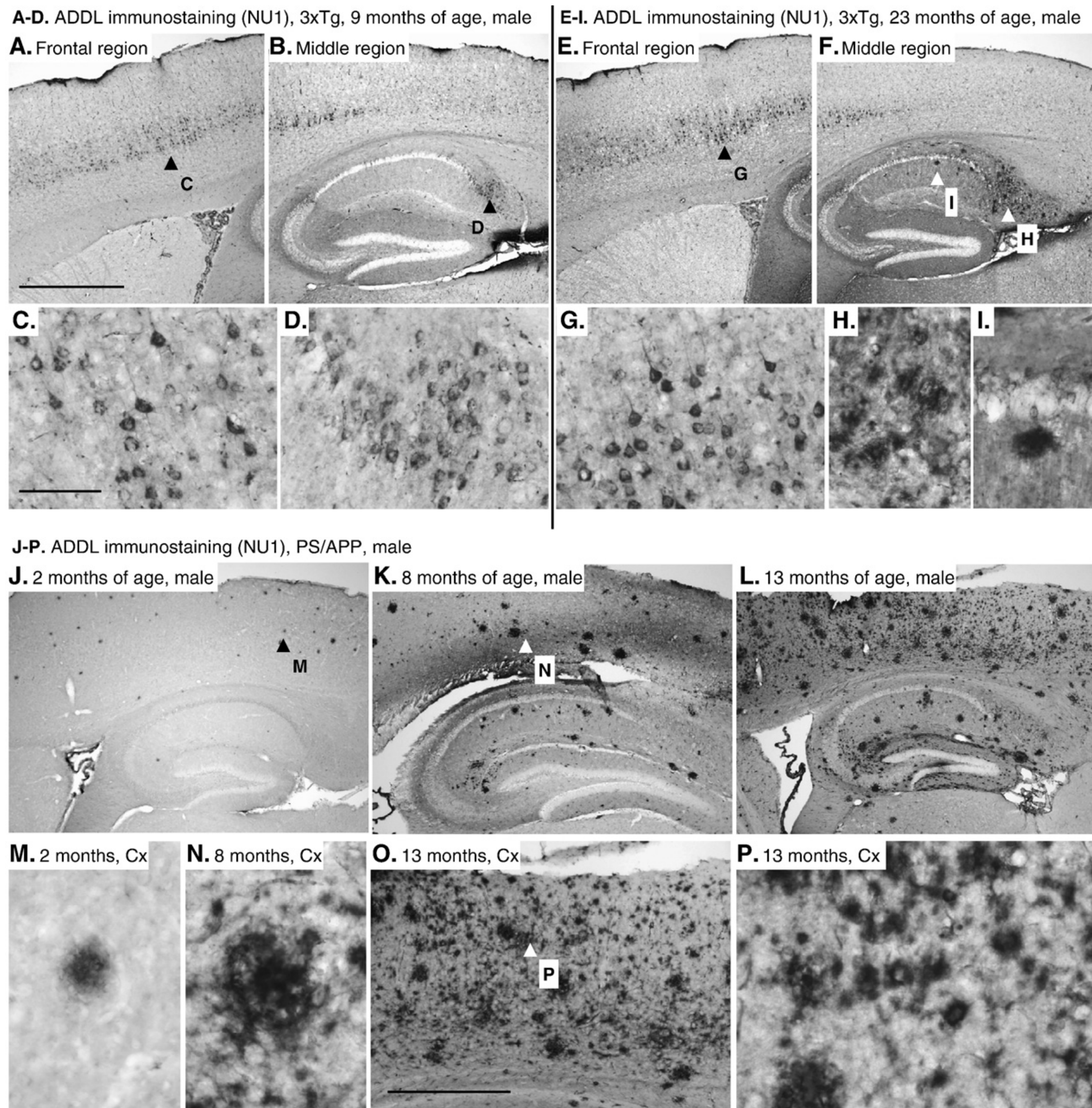


Fig. 3 – Intraneuronal Abeta in 3xTg-AD mice. Sagittal sections from male 3xTg-AD (A–I) and PS/APP (Holcomb et al., 1998) (J–P) mice were immunostained using an anti-ADDL antibody NU-1 (Lambert et al., 2007). NU-1 detected intraneuronal staining in the cortical neurons from 6 months of age in the frontal brain region, and expanded toward the middle of the brain at 9 months of age (A–C). In addition, the intraneuronal, but not the extracellular plaque, staining was detected in the subiculum at 9 months of age (D). At older ages, intraneuronal staining became stronger and the extracellular plaques were also detected (H, I). In PS/APP mice, there is no obvious intraneuronal staining with NU-1 (J–L). At 2 months of age, plaques were stained with NU-1 but there is no cellular staining surrounding the plaques (J, M). At older age, cells surrounding plaques were also ADDL-immunopositive at 8 and 13 months of age (N–P). Bar = 200 μm in A for A, B, E, F, J–L; 20 μm in C for C, D, G–I, M, N, P; 100 μm in O for O.

in Abeta production and degradation in 3xTg-AD mice were examined using levels of soluble APP cleaved at the beta site (sAPPbeta) and neprilysin as indicators. A rabbit polyclonal antibody we previously developed (Nishitomi et al., 2006) is specific to wild-type sAPPbeta and not cross-reactive with Swedish mutant sAPPbeta. For the present study, we developed a mouse monoclonal antibody against Swedish-type sAPPbeta, clone 6A1 (Fig. 5B); this antibody did not cross-react with uncleaved Swedish APP or wild-type sAPPbeta (Fig. 5A). Immunocytochemical staining of cells transfected with either wild-type or Swedish mutant APP confirmed the specificity of these antibodies (data not shown).

The level of sAPPbeta was significantly higher in female 3xTg-AD mice compared to male 3xTg-AD mice at 9 months of

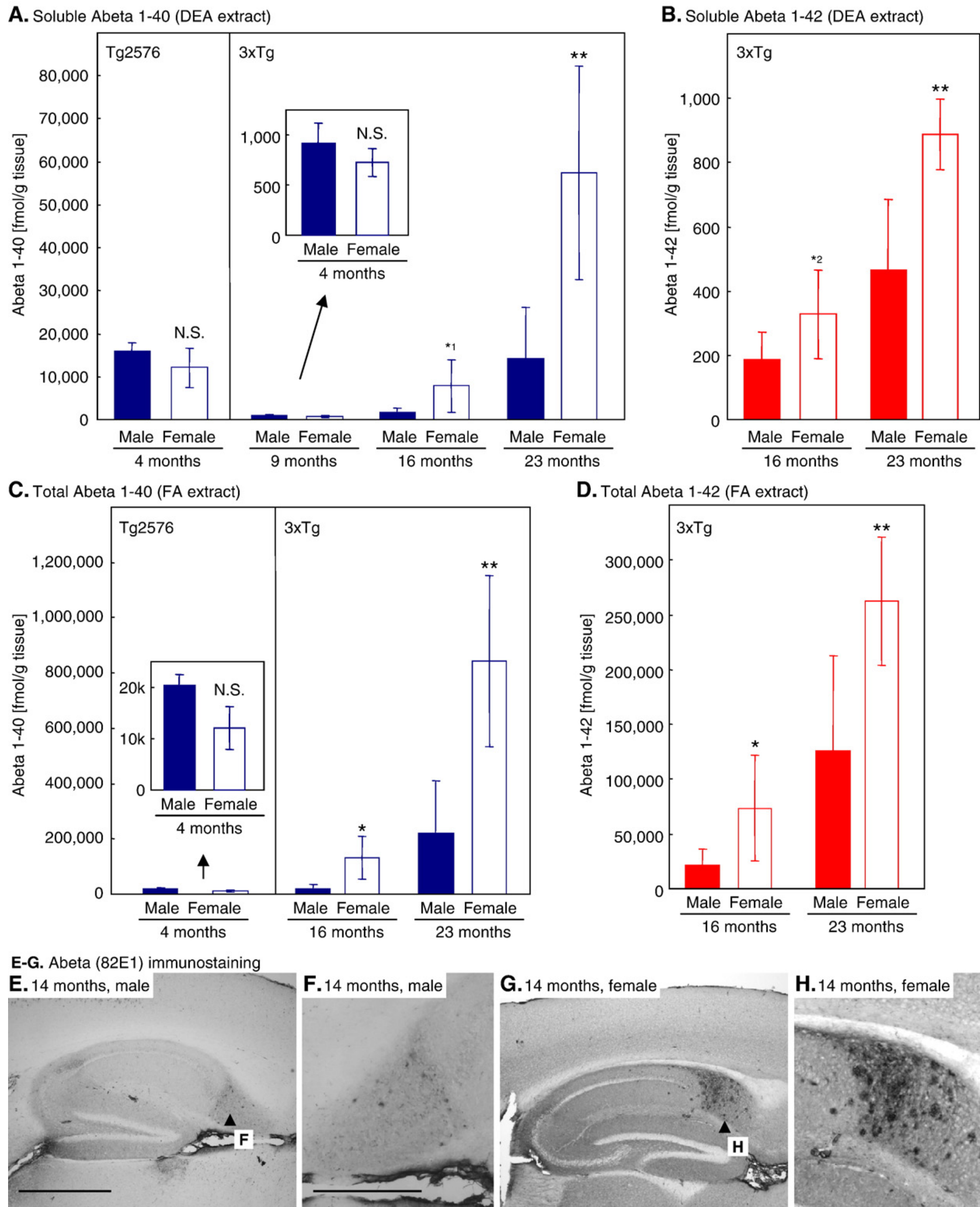
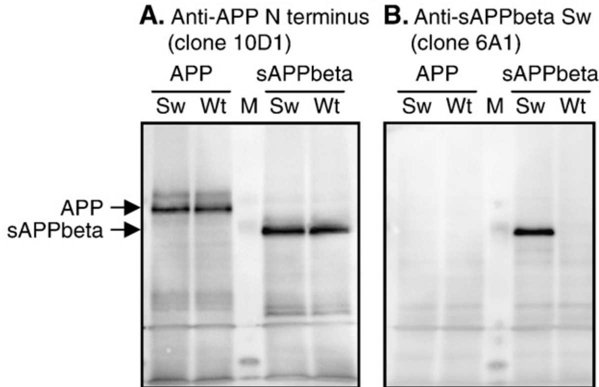
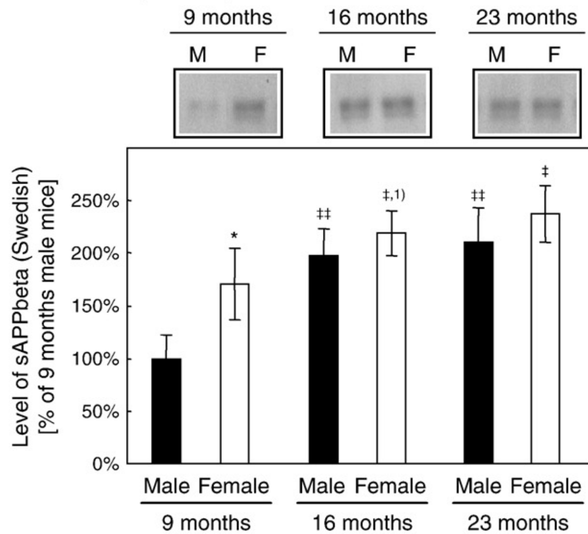
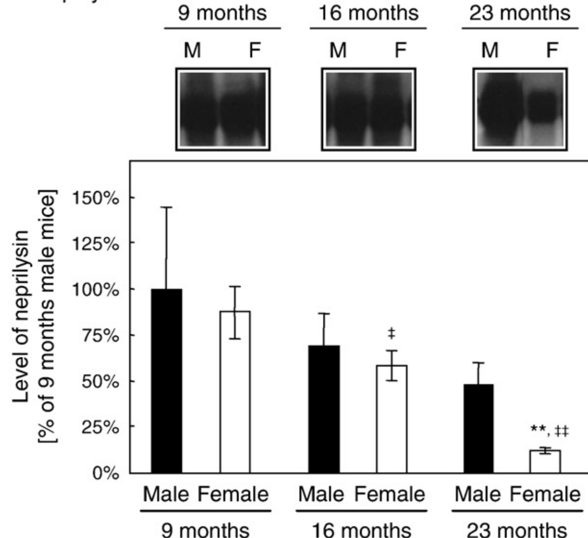


Fig. 4 – Female mice have higher level of soluble and total Abeta in 3xTg-AD. Levels of DEA-extracted soluble and FA-extracted total Abeta 1–40 (A, DEA; C, FA) and 1–42 (B, DEA; D, FA) in each gender were determined in 3xTg-AD mice and Tg2576 mice. 3xTg-AD at 9 months and Tg2576 mice at 4 months of age are free from any plaque pathology. *$P<0.05$ and **$P<0.01$ compared with age-matched male mice using *t*-test. DEA-extracted soluble Abeta 1–40 (A) and 1–42 (B) in mice at 16 months of age showed marginal significance: $^{*1}P=0.058$ and $^{*2}P=0.065$. Gender difference was also seen in Abeta plaque pathology. Female mice at 14 months of age have plaques in the subiculum (G, H), while plaques are not evident in male mice (E, F). Bar = 200 μm in E for E, G; 100 μm in F for F, H.

age (Fig. 5C, 170% of age-matched male mice). However, no statistical difference was detected between genders at 16 and 23 months of age: 197% vs 219% and 209% vs 236% in male vs female mice, respectively. sAPPbeta levels were significantly increased with aging compared to mice at 9 months of age: 100%→197%→209% in male mice, 170%→219%→236% in female mice at 9, 16 and 23 months of age, respectively.







Neprilysin levels were not different between genders at 9 and 16 months of age; 100% vs 87%, 68% vs 58% in male and female mice at 9 and 16 months of age, respectively (Fig. 5D). At 23 months of age, neprilysin levels were significantly reduced in female mice compared to age-matched male mice (47% vs 12%, respectively). Compared to gender-matched 9 month old mice, female mice showed greater reduction with aging than males; 100%→68%→47% in male mice, 87%→58%→12% in female mice at 9, 16 and 23 months of age, respectively.

2.6. *Tau pathology was not influenced by gender*

To determine whether gender difference affected tau phosphorylation as it did Abeta, we examined levels of phosphorylated tau in male and female mice at 9, 16 and 23 months of age. Levels of phosphorylated tau were markedly increased with aging (Matsuoka et al., unpublished observation). Levels of phosphorylated tau in female mice at a given age were compared with age-matched male mice. No statistically significant gender difference was detected at any of these ages (Fig. 6).

3. Discussion

Epidemiological studies indicate that women have a higher risk of AD even after adjustment for age (Brookmeyer et al., 1998), the most important risk factor for AD (Hy and Keller, 2000). Transgenic mice expressing pathogenic genes of AD develop Abeta and/or tau pathology (McGowan et al., 2006); however, previous studies of AD transgenic mouse models have not necessarily reflected a consistent gender difference. In Tg2576 mice, the Abeta 1–40 level has been reported to be higher in females than males (~115% in soluble Abeta 1–40 and ~180% in total Abeta 1–40 in females compared to males), but

Fig. 5 – Female mice have higher beta-secretase activity and lower neprilysin levels. Antibodies specific to APP N-terminus and Swedish-type sAPPbeta, clones 10D1 and 6A1, were characterized (A and B, respectively). Levels of sAPPbeta (C) and neprilysin (D) were compared and densitometrically quantified between genders at 9, 16 and 23 months of age. A–B: COS1 cells transfected with wild-type- (Wt) or Swedish mutant (Sw) type-full-length APP or sAPPbeta were run on a gel and probed with antibodies against APP N-terminus and Swedish sAPPbeta, clones 10D1 and 6A1, respectively. C: sAPPbeta level was significantly higher in female compared to male 3xTg-AD mice at 9 months of age. However, there is no significant difference between genders at older ages, 16 and 23 months of age. sAPPbeta levels were significantly elevated at 16 and 23 months of age compared to gender-matched mice at 9 months of age. D: Neprilysin levels decrease with advancing age in both male and female 3xTg-AD mice, with the magnitude of the decrease being greater in female mice. [1)]$P=0.062$, *$P<0.05$, **$P<0.01$ compared with age-matched male mice using *t*-test. ‡$P<0.05$, ‡‡$P<0.01$ compared with gender-matched mice at 9 months of age using ANOVA.

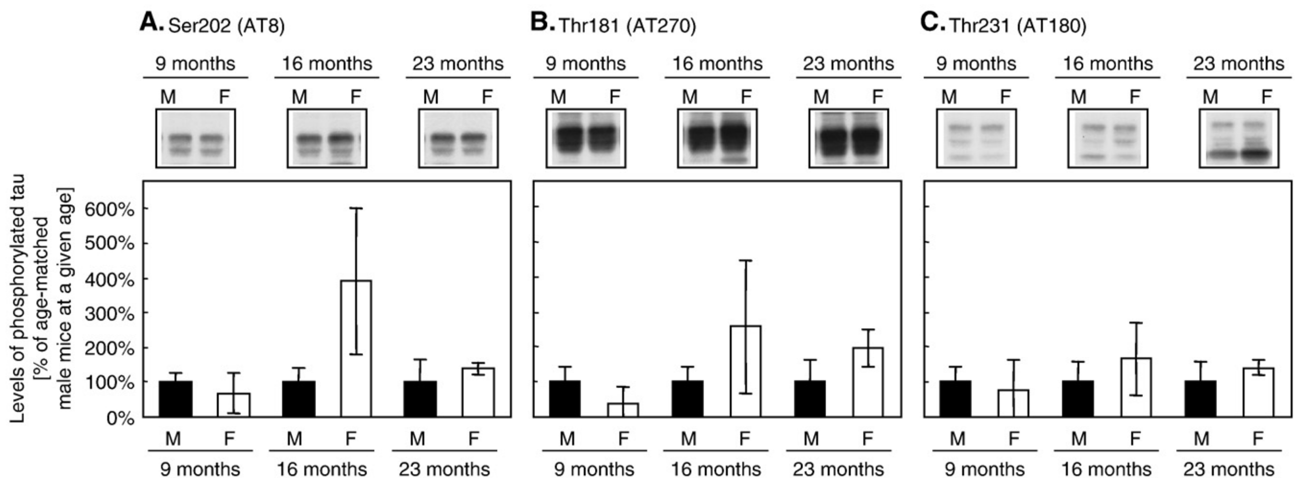


Fig. 6 – Gender difference had no effect on tau phosphorylation status. Levels of phosphorylated tau in unfractionated total tau were compared between age-matched female and male mice at 9, 16 and 23 months of age; i.e. 100% at each age point represents the level of phosphorylated tau in male mice at a given age. Since levels of phosphorylated tau were drastically increased with aging, the immunoblots from 9 and 23 months of age were outside the linear dynamic range in a single gel of immunoblotting. We therefore compared male and female mice brain homogenate side-by-side at a given age to examine gender differences. No statistically significant difference was detected.

levels of Abeta 1–42 were not different at 15 months of age (Abeta levels were not determined by ELISA at other ages) (Callahan et al., 2001). In our study, we compared Abeta levels at pre- and post-plaque pathological stages, and found that the gender difference in Abeta level became significant only after Abeta plaques were formed. Interestingly, the magnitude of the gender difference was much more significant in 3xTg-AD mice (161–648% depending on Abeta species and ages of mice) compared to Tg2576 mice. We also found that Abeta production and metabolism also affected by gender difference in 3xTg-AD mice. These findings suggest that 3xTg-AD mice may be useful as tools to investigate causes of gender differences in AD.

This study suggests possible causes of the higher risk of AD in women. The Abeta degrading enzyme neprilysin (Iwata et al., 2005) was significantly reduced with aging as previously reported in wild-type (non-transgenic) mice (Iwata et al., 2002) and AD mouse models (Caccamo et al., 2005; Lazarov et al., 2005). The decrease of neprilysin level found in 3xTg-AD mice was more exacerbated than that found in wild-type mice (Iwata et al., 2002), and notably, the reduction of neprilysin level was more prominent in female mice. Ovariectomy significantly reduces brain neprilysin activity and estrogen replacement restored the reduced neprilysin activity to the level in sham-operated mice (Huang et al., 2004), suggesting that expression or activity of neprilysin is in part regulated by estrogen in females, and supporting out finding. We found that Abeta level was not different between genders when mice were free from plaque pathology, though the sAPPbeta level, which reflects beta-secretase activity, was significantly higher in female mice. With aging, beta-secretase activity was increased as found in AD postmortem cases (Fukumoto et al., 2002; Holsinger et al., 2002; Yang et al., 2003; Zhao et al., 2007). These findings suggest that both up-regulation of Abeta production and down-regulation of Abeta degradation may contribute to the higher risk of AD in women.

Loss of ovarian steroids, particularly estrogens, at menopause may increase the susceptibility of the aging brain to AD neurodegeneration (Gandy and Duff, 2000), and estrogen is central to the current hypothesis on the gender difference in risk of AD. However, the effects of estrogen reduction through ovariectomy and hormone replacement therapy in transgenic models of AD are not completely consistent; ovariectomy increases the amount of Abeta and estrogen replacement therapy reduces Abeta in Tg2576 and PS/APP mice (Zheng et al., 2002), but not in PDAPP mice (Green et al., 2005). Although the majority of retrospective and prospective epidemiological studies suggest a beneficial effect of estrogen (Tang et al., 1996; Kawas et al., 1997; Waring et al., 1999; Zandi et al., 2002), clinical trials have shown conflicting results. In humans, three double-blind placebo-controlled clinical trials failed to detect beneficial effects of estrogen replacement therapy (Henderson et al., 2000; Mulnard et al., 2000; Wang et al., 2000). Estrogen has a variety of roles in addition to classic sex hormone function, including effects on neurotransmission (Mukai et al., 2006), which may complicate the overall outcomes in animal studies. Further studies investigating CNS estrogen-related signaling are required to elucidate the role of estrogen in AD pathology.

In the amyloid hypothesis, tau abnormalities are considered to be downstream of Abeta pathology (Hardy and Selkoe, 2002), and evidence supports this hypothesis. Abeta advances tau phosphorylation and neurofibrillary tangle formation in vivo (Lewis et al., 2001; Gotz et al., 2001), and reduction of endogenous tau ameliorates Abeta-induced effects in vivo (Roberson et al., 2007). However, it is not completely clear whether Abeta pathology is the major determinant of tau pathology and synaptic dysfunction. Abeta reduction ameliorated tau pathology at earlier, but not at later, pathological stages in 3xTg-AD mice (Oddo et al., 2004). Treatment with lithium chloride, a glycogen synthase kinase 3 inhibitor, reduced tau phosphorylation, but did not reduce Abeta load or improve cognitive performance in 3xTg-AD mice (Caccamo

et al., 2007). A neuronal tubulin-interacting peptide, NAPVSIPQ, reduced tau phosphorylation and insoluble tau, and also improved cognitive impairment without reducing Abeta load in 3xTg-AD mice (Matsuoka et al., 2008). Taken together, although evidence supports the Abeta hypothesis overall, the relationship between Abeta and tau pathologies is not straightforward. While there are some reports suggesting that estrogen-related signaling may be also involved in tau phosphorylation in cultured cells in vitro (varez-de-la-Rosa et al., 2005), our in vivo study revealed that female mice show more aggressive Abeta pathology, although levels of phosphorylated tau were similar between genders. This suggests that the Abeta pathway, rather than the tau pathway, is dominantly involved in the higher risk of AD in women. Further studies are necessary to address the role of tau pathology in the higher risk of AD in women.

This transgenic mouse line was originally created by a group at the University of California, Irvine (Oddo et al., 2003). In the process of investigating gender differences, we also examined the progression of Abeta pathology at additional age points using strictly-defined groups with small age variance, specific gender and larger sample size. Although there are some discrepancies in the finding of intraneuronal Abeta, other key pathological features, such as Abeta plaques, hyperphosphorylated tau-bearing neurons, and thioflavin T-positive mature neurofibrillary tangles were found in 3xTg-AD mice housed at Georgetown University as originally reported (Oddo et al., 2003). However, the progression of pathology in our colony is slower than that reported in the original report (Oddo et al., 2003), and other investigators found similar slow progression of pathology at their institute (Drs. Donna M. Barten and Margi Goldstein at Bristol-Myers Squibb, and Dr. Mary Ann Ottinger at University of Maryland, personal communication). We suspect that some genetic and/or environmental factors altered this line after distribution by the University of California, Irvine. These findings provide guidance for the use of 3xTg-AD mice in therapeutic research.

Overall, this study demonstrated that 3xTg-AD mice show gender differences that may be relevant to human AD. Our findings suggest that both an increase in Abeta production and a decrease in Abeta degradation may contribute to the higher risk of AD in women.

4. Experimental procedures

4.1. *Animals and sampling*

We used 3xTg-AD mice (Oddo et al., 2003) generated by co-microinjection of mutant APP (K670M/N671L) and tau (P301L) transgenes under the control of Thy 1.2 promoter into mutant PS-1 (M146V) knock-in mice (Guo et al., 1999). 3xTg-AD mice were created by a group at the University California, Irvine, in collaboration with a group at the National Institute on Aging. The colony was established at Georgetown University using breeding pairs provided by the National Institute on Aging after homozygosity was confirmed by crossing with non-transgenic mice. All 3xTg-AD mice used in this study were bred and housed at the Georgetown University animal facility. Two other transgenic mouse lines were used for comparison purposes: Tg2576 APP transgenic mice (Taconic, Hudson, NY), the most widely used APP single transgenic mouse model, which expresses mutant APP (K670M/N671L) under the control of prion promoter (Hsiao et al., 1996), and PS/APP double transgenic mice (Holcomb et al., 1998) obtained by crossing Tg2576 with PS-1 transgenic mice (Duff et al., 1996). PS/APP mice were housed and the brains were collected at the Nathan Kline Institute for Psychiatric Research (Orangeburg, NY). This study has been reviewed and approved by the Georgetown University Animal Care and Use Committee.

For quantitative biochemical studies, we used male 3xTg-AD mice at 3 (3.3±0.0), 6 (6.0±0.2), 9 (8.7±0.1), 12 (12.2±0.1), 16 (16.1±0.1), 20 (20.2±0.3) and 23 (23±0.0) months of age (n=6 at each age), and female 3xTg-AD mice at 9 (9.1±0.4), 16 (16.1±0.1) and 23 (23.0±0.0) months of age (n=6 each). These mice were selected from at least two litters from different parents to avoid possible litter effects. Tg2576 APP transgenic male and female mice (n=5 each) were used at 4 (3.7±0.1) months of age. For histochemical analysis, we used additional mice: 3xTg-AD mice at 9 (9.1±0.2), 12 (11.7±0.1), 14 (14±0.2), 23 (22.7±0.3) and 28 (28±0.5) months of age (n=5 each), and PS/APP mice at 2 (2.5±0.0), 8 (7.6±0.0) and 13 (12.9±0.0) months of age (n=3, 2 and 2, respectively).

Mice were sacrificed by cervical dislocation to eliminate anesthesia-mediated tau phosphorylation (Planel et al., 2007), and the brains were quickly isolated. After the olfactory bulb and cerebellum were discarded, the hemi brain was snap-frozen in dry ice or immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for biochemical and histochemical analyses, respectively.

4.2. *Biochemical analysis of Abeta pathology*

Brains were homogenized in 10 volumes of 50 mM Tris–HCl buffer, pH 7.6, containing 250 mM sucrose and protease inhibitor cocktail (Sigma, St. Louis, MO). Soluble and total Abeta were extracted in 0.4% DEA and 70% FA, respectively, as previously described (Nishitomi et al., 2006; Takata et al., 2007; Sakaguchi et al., in press). Levels of full-length Abeta 1–40 and 1–42 were quantified using ELISAs developed by our group as previously described (Horikoshi et al., 2004; Matsuoka et al., 2008). Statistical significance was tested using t-tests or ANOVA followed by Bonferroni post-hoc tests (SPSS, Chicago, IL).

The DEA fraction and unfractionated crude brain homogenates were used to determine the levels of sAPPbeta and neprilysin, respectively. Samples were mixed with Laemmli buffer, run on a 4–15% gradient SDS-PAGE gel (BioRad, Hercules, CA) and the proteins were transferred to PVDF membrane (Millipore, Bedford, MA). The membrane was probed with mouse monoclonal antibodies against Swedish mutant sAPPbeta (clone 6A1, see below, 0.2 μg IgG/ml) or mouse neprilysin (0.2 μg IgG/ml, R&D Systems, Minneapolis, MN). The sAPPbeta Swedish antibody does not react with uncleaved APP or wild-type sAPPbeta (see below for sAPPbeta antibody characterization). The primary antibodies were detected by HRP-coupled anti-mouse IgG antibody (Jackson Immuno Research, West Grove, PA), and visualized with a chemiluminescence kit (Pierce). The protein bands were densitometrically analyzed (Quantity One, BioRad). Statistical significance was tested using t-tests or ANOVA (SPSS).

4.3. Biochemical analysis of tau pathology

Crude tau fraction was prepared as previously described (Matsuoka et al., 2008). Samples were run on a 4–15% gradient SDS-PAGE gel and the proteins were transferred to a PVDF membrane. The membrane was probed with a primary antibody: phosphorylated tau at Ser202 (clone AT8) (Biernat et al., 1992), Thr181 (clone AT270) (Greenberg and Davies, 1990) and Thr231 (clone AT180) (Greenberg and Davies, 1990) (all phosphorylated tau antibodies were used at 1 μg IgG/ml, Pierce Biotechnology, Rockford, IL). The primary antibodies were detected and quantified as described above.

4.4. Histochemical analysis of Abeta pathology

Brains were immersion fixed and sections were prepared as previously described (Matsuoka et al., 2001b). Sections were incubated with the primary antibodies in 100 mM phosphate buffered saline consisting of 0.3% Triton X-100 overnight. Sections were incubated with primary antibodies against Abeta (clone 82E1 (Horikoshi et al., 2004), Immuno-Biological Laboratories), APP/Abeta (clone 6E10 (Kosik et al., 1988), Signet Laboratories/Covance, Berkley, CA), and oligomeric Abeta (ADDL) (clone NU-1 (Lambert et al., 2007)). We used all primary antibodies at 1 μg IgG/ml. Primary antibodies were detected by a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) and then visualized using the ABC method (Vectastain, Vector Laboratories).

4.5. Development and characterization of an antibody against Swedish-type sAPPbeta

Mice (Balb/c, Charles River, Japan) were immunized with thyroglobulin-conjugated synthetic peptide corresponding to the C-terminus of Swedish mutant sAPPbeta (ISEVNL) or recombinant human APP N-terminal portion. After a series of immunizations, the spleen was isolated and spleen cells were fused with X63-Ag8 myeloma cells. We determined selectivity and sensitivity of these antibodies by microplate assay using the antigen and other recombinant proteins.

The full-length cDNA of human APP695 was amplified from human brain cDNA (Clontech, Mountain View, CA) using specific primers synthesized based on the published human APP cDNA sequence. To introduce the Swedish mutation, we used the following primers: Swe-sense: 5′-TCTGAAGTGAACTTGGATGCAGAA-3′, and Swe-antisense: 5′-TTCTGCATCCAAGTTCACTTCAGA-3′. The amplified products were digested with SalI and NotI, ligated into pGEX-6P-1 vector (Amersham Bioscience/GE Healthcare, Piscataway, NJ) and transformed into *Escherichia coli* JM109 cells. The sequence of the cloned wild-type and Swedish mutant APP cDNAs was confirmed. Wild-type sAPPbeta cDNA was amplified from APP695 cDNA by using following primers: Sal-APP: 5′-CGGTCGACTCGCGATGCTGCCCGGTTTGGC-3′ and sAPPbeta: 5′-GCGCGGCCGCCTACATCTTCACTTCAGAGAT-3′. Swedish sAPPbeta cDNA was also amplified from APP695 cDNA by using following primers: Sal-APP: 5′-CGGTCGACTCGCGATGCTGCCCGGTTTGGC-3′ and Swe-sAPPbeta-antisense: 5′-GCGCGGCCGCCTACAAGTTCACTTCAGAGATCTCCTCCG-3′. The amplified products were digested with SalI and NotI, ligated into pGEX-6P-1 vector and used to transform *E. coli* JM109 cells. The sequence of the cloned wild-type sAPPbeta and Swedish sAPPbeta cDNAs was confirmed.

The wild-type APP, wild-type sAPPbeta, Swedish APP and Swedish sAPPbeta cDNA in pGEX-6P-1 vector were transferred into pcDNA3.1(+) (Invitrogen, Carlsbad, CA). Then, APP and sAPPbeta cDNAs in pcDNA3.1(+) were transfected into COS-1cells by using FuGENE6 (Roche Diagnostics, Basel, Switzerland). Two days after transfection, cells were harvested by scraping with ice-cold 10 mM Tris buffer, pH 8.0, consisting of 1% NP-40, 150 mM NaCl and 1 mM EDTA. Cell lysates were run on a 10% SDS-PAGE gel and then transferred as described above. The membrane was probed with primary antibodies against the APP N-terminus or sAPPbeta (clone 10D1 or 6A1 at 5 or 1 μg IgG/ml, respectively).

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Disclosure statement: Northwestern University owns intellectual property concerning the use of globular Abeta oligomers (ADDLs) for Alzheimer's therapeutics and diagnostics which has been licensed to Acumen Pharmaceuticals. Dr. Klein is a co-founder of Acumen and a member of its Scientific Advisory Board.

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