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Gender dependent APP processing in a transgenic mouse model of Alzheimer's disease

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Summary Epidemiological studies have reported a higher prevalence and incidence of Alzheimer's disease (AD) in women. The biochemical basis for this gender-disparate susceptibility is unknown. A gender effect on AD-typical plaque pathology has been shown in APP transgenic mouse models of AD. Female mice elicit higher plaque load than male mice. In an effort to analyze gender-dependent APP processing during postnatal development, we examined APP transgenic mice at time points prior to plaque deposition. At 14 weeks of age there was a significant elevation of C99 and Aβ in female mice compared to males. Furthermore we observed a slight decrease of BACE-activity in male mice as well as higher cerebral manganese levels in females. Although the decline in estrogen levels due to menopause in female patients is still discussed to be a risk factor for AD our results implicates that additional factors like modified BACE-activity or metal levels may contribute to the higher prevalence and incidence of AD in females.

Keywords: Gender, APP processing, BACE, sex difference, transgenic mice

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder of the brain and represents the most common form of dementia among the elderly. The disease is neuropathologically characterized by abundant neuritic plaques, as well as neurofibrillary tangles. While hyperphosphorylated tau protein is present in neurofibrillary tangles, the so-called ''senile'' plaques consist mainly of a small 40- to 42-amino acid peptide (Aβ), which is derived by proteolytical cleavage from the larger amyloid precursor protein (APP). Cleavage by β-secretase releases a truncated APP ectodomain and leads to the generation of a C-terminal fragment named C99. Subsequent cleavage by γ-secretase generates Aβ peptides. During the alternative α-secretase pathway, the APP holoprotein is cleaved within the Aβ domain, preventing the generation of Aβ peptides. This cleavage step results in the release of a secreted form of APP (sAPPα), which possesses neurotrophic activities (for review see Bayer et al., 2001).

Epidemiological studies have shown gender differences in the incidence and prevalence of Alzheimer's disease with females being at higher risk (Fratiglioni et al., 1997, 2000; Andersen et al., 1999; Jorm and Jolley, 1998). The biochemical basis for this gender-based predisposition remains still unclear, although the decline in estrogen, following menopause, is being discussed to be a contributing factor. However, hormone replacement therapy (HRT) did not fulfill the expectations. Whereas some clinical studies were able to demonstrate a decreased incidence as well as a delay in the onset of AD due to the supplemental estrogen (Asthana et al., 2001; Tang et al., 1996), other studies failed (Mulnard et al., 2000; Wang et al., 2000). Furthermore, there is growing evidence that elevated levels of gonadotropins, particularly luteinizing hormone, are more likely to drive the pathogenesis of AD than does the decline of estrogen (reviewed in Webber et al., 2004). Gender differences in AD are also obvious by a slight increase in plaque load, a significantly different distribution of plaques in cortical areas of female AD patients (Kraszpulski et al., 2001) and gender-disparate oxidative stress parameters in AD affected brains (Schuessel et al., 2004). Interestingly, gender-dependent elevated plaque formation has been reported in

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several APP transgenic mouse models: APP23 (Sturchler-Pierrat and Staufenbiel, 2000; Bayer et al., 2003), Tg2576 (Callahan et al., 2001) and APP/Tau double transgenic mice (Lewis et al., 2001). Like in AD patients, this gender effect has been attributed to the decline of the estrogen status.

Recent evidence indicates that metal homeostasis in AD brain is disturbed. Therefore increased Fe and Mn levels (Perry et al., 2002; Loeffler et al., 1995) or decreased Cu levels (Deibel et al., 1996) were detected. Cu deficiency in the CNS seems to enhance Aβ accumulation and plaque formation (Bayer et al., 2003; Phinney et al., 2003). Synaptic zinc levels, which were shown to be modulated by estrogen (Lee et al., 2004) were linked to greater plaque pathology in female Tg2576 mice (Lee et al., 2002).

In the present study we examined young (up to 14 week old) APP transgenic mice (Thy1-APP751^{SL}) in order to analyze gender-dependent APP processing during postnatal development. The Thy1-APP751^{SL} mouse model develops plaques at 24 weeks of age (Blanchard et al., 2003). Therefore, we studied gender-dependent production of C99 and Aβ prior to extracellular Aβ deposition into plaques and age-dependent decline of estrogen levels due to menopause.

Materials and methods

Transgenic mice

Thy1-APP751^{SL} transgenic mice were a generous gift of Dr. Laurent Pradier (Sanofi-Aventis, Paris). Generation and initial characterization of these APP transgenic mice bearing two mutations Swedish (KM670/671NL) and London (V717I) under the control of the Thy1-promotor has been previously described (Blanchard et al., 2003). In brief, the modified APP cDNA (Sal I fragment) was cloned into the murine Thy1-expression construct. To optimize the translation initiation site of APP, an optimized Kozak consensus sequence was introduced using PCR-directed mutagenesis. Transgene status of the mice was determined by PCR of tail genomic DNA. All animals were handled according to French and German guidelines for animal care. The mice were anaesthetised by inhalation of Forene and sacrificed by cervical dislocation. The head was dissected by a cut down the atlantoaxial connection. The brain was removed and separated down the midline into two hemispheres. The olfactory bulb, cerebellum, pons and medulla were removed from the hemispheres. The remaining cerebra were homogenized (1/10; w.w./vol) in 0.01 M PBS pH 7.4 (Sigma, suprapure) by 10 strokes in a glass-teflon homogenizer at 650 rpm. The homogenate was divided in several aliquots depending on further application (ELISA, Western blot, ICP-MS, activity assay). In order to determine metal levels by ICP-MS, aliquots were snap frozen in liquid nitrogen. The appropriate amount of complete protease inhibitor (Roche, Germany) was added to the remainder of the homogenate.

Western Blotting

For isolating the SDS-soluble protein fraction 10% SDS (final concentration: 2%) was added to the brain homogenate followed by a centrifugation step of 16,000 rpm for 30 min at 4°C. Protein extracts were fractionated onto a 4–12% NuPage SDS-polyacrylamide gels (Invitrogen, Karlsruhe, Germany) and transferred to Hybond nitrocellulose membranes (Amersham Biosciences, Little Chalfont, UK). Western blots were probed with antibody W0-2 (1:2000), the polyclonal antiserum 40090 against APP (1:500), the monoclonal antiserum BSC-1 against BACE1 ((Schmechel et al., 2004); 1:2000) and an antiserum against β-actin (1:5000, Sigma, Taufkirchen, Germany) to ensure equal loading. For Aβ-detection the blotted membrane was heated in boiling PBS for 5 min to enhance the signal and blocked with 10% non-fat dry milk in PBS containing 0.05% Tween-20 for 1 h. After washing the membrane, the primary antibody was added and incubated overnight at 4°C. The bound antibodies were detected by horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (Amersham) followed by ECL detection system (Amersham) according to the manufacturer's instructions. The analysis of band intensity was performed using an imaging densitometer (BioRad, Model GS-700, Germany) and the Quantity-One-software (BioRad, Quantity One, Version 4.3, Germany).

Determination of cerebral BACE1-activity

The activity of β-secretase (BACE1) in brain lysates of 4, 8 and 14 week-old mice was determined using the commercially available fluorogenic β-secretase activity assay (Calbiochem, Germany). For preparation of brain (cerebra) homogenates, 0.01 M PBS (suprapure) was used. The lysates were centrifuged for 1 min at 10,000 *g*, the supernatant was transferred to a fresh tube and 100 µg of total protein were applied for the assay. The measurement was performed in microplates following the instructions of the manufacturer in a fluorescence plate reader (Wallac Victor2^{TM}, 1420 Multilabel counter, Perkin Elmer) set to an excitation wavelength of 355 nm and an emission wavelength of 510 nm.

ELISA of Aβ40 peptides

For isolating the PBS-soluble protein fraction brain homogenate was incubated on ice for 30 min and occasionally vortexed. After a centrifugation step of 30 min at 16,000 rpm and 4°C the supernatant, including the desired fraction, was collected for analysis. For the quantification of PBS-soluble Aβ40 in the 4, 8 and 14 week-old Thy1-APP751^{SL} mice a microplate enzyme immunoassay was used (The Genetics company, TKHS-set, Switzerland) following the instructions of the manufacturer. Dependent on age and genotype of the analyzed mice, different dilutions of the samples were prepared (1:5 and 1:10). Aβ42 peptide concentration was below the detection limit using ELISA in young animals.

Determination of metal levels

For analysis of metal concentration, brain samples were prepared by HNO_3 closed-vessel microwave digestion and diluted in Milli-Q water to a final concentration of 6.5% HNO_3 for analysis by ICP-MS as described elsewhere (Bayer et al., 2003).

Protein content

The Pierce BCA Protein Assay was used for total protein quantification. The assay is based on the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium (biuret reaction) and on the colorimetric detection of the cuprous cation by bicinchoninic acid (BCA). The measurement was performed in microplates according to the protocol of the supplier. The absorbance was determined in a microplate reader (Biotek-Instruments, µQuant, USA) set to 562 nm.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 (SPSS Inc., Illinois, USA) and GraphPad Prism 4.02 (Graphpad Software Inc., San Diego

California, USA). Outliers were defined as values differing more than three times the interquartile range from the third or first quartile in the boxplot figure of the explorative data analysis in SPSS. If not otherwise indicated outliers were excluded and the number from each experimental group is mentioned in the legend of the figure. Student's two-tailed *t*-tests were calculated for differences between elemental groups without confounding variables. All data were expressed as the mean ± SEM. $P < 0.05$ was considered to be significant.

Results

Age- and gender-dependent processing of APP

To study postnatal APP expression levels in Thy1-APP751^{SL} mice, brain lysates of transgenic mice ranging from postnatal day 3 (P3) to postnatal day 20 (P20) were examined by Western blotting. Transgenic human APP expression was already detectable at P3 and increased up to full expression at P20 (Fig. 1a). At later time points the expression of human full-length APP remained constant between the age groups as shown in accordance with Schuessel et al. (2005).

We further investigated gender-dependent differences in APP processing in 4, 8 and 14 week-old Thy1-APP751^{SL} mice by means of Western blotting, subsequent densitometric evaluation and Aβ40 ELISA. In contrast to the constant APP expression levels, the level of the β-secretase cleavage product C99 changed in an age and gender dependent manner. The ratio of band intensity of C99 to APP

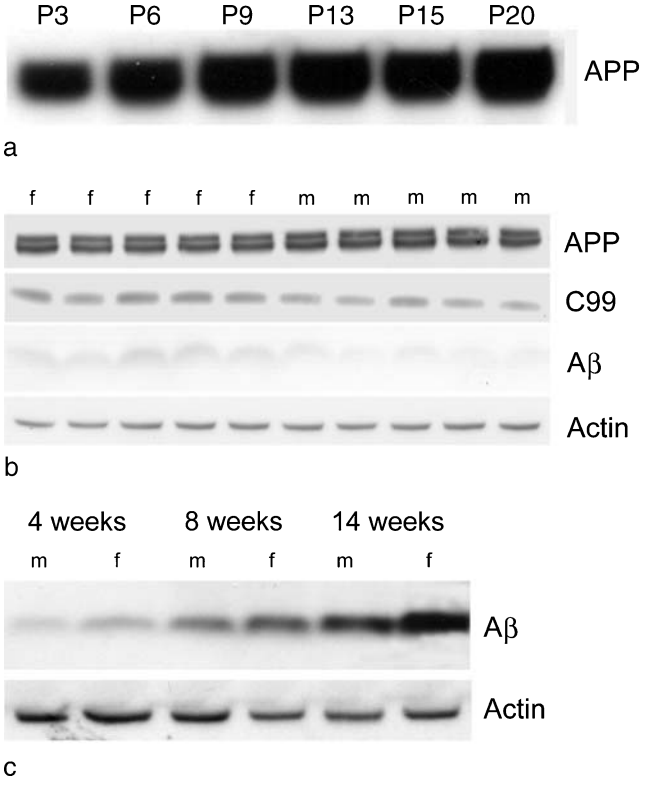

Fig. 1. **a** Western-blot analysis of Thy1-driven transgenic human APP-expression revealed an age-dependent increase in APP expression up to full expression at P20. The gel represents 3 independent experiments with 4 mice at each time point. **b** Western Blot analysis of SDS-soluble brain extracts from male and female Thy1-APP751^{SL} with the age of 14 weeks. Human full-length APP, C99 and Aβ are detected with the monoclonal antiserum W0-2 and equal loading is assured by reprobing the membrane with an antibody against actin. Each lane represents an individual animal. **c** Aβ and actin bands in Western blots from SDS-soluble brain extracts from female and male Thy1-APP751^{SL} transgenic mice of varying age, each lane representing one individual animal. *f* Female; *m* male

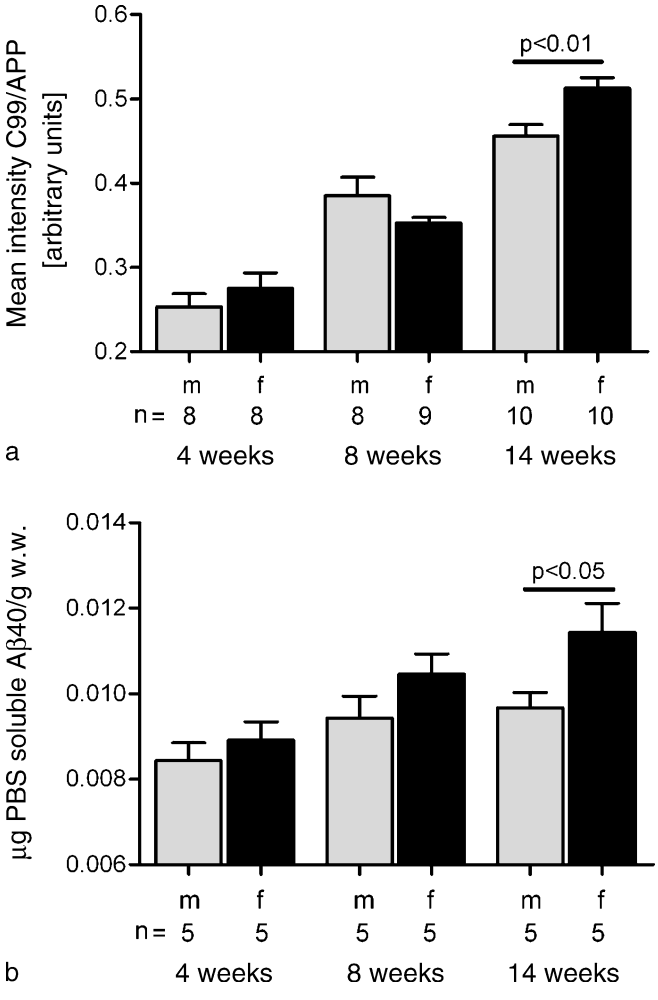

Fig. 2. **a** Densitometric evaluation of SDS-soluble C99 and APP-bands revealed a significant increase in the C99/APP ratio with aging (ANOVA, $p < 0.0001$). With the age of 14 weeks female Thy1-APP751^{SL} mice showed a significantly higher C99/APP ratio compared to age-matched males (*t*-test, $p = 0.006$). One outlier (male, eight weeks) was excluded from the analysis. **b** Mean values ± SEM for PBS soluble Aβ40 levels in Thy1-APP751^{SL} mice of varying age determined by ELISA. The Aβ40 levels increased in an age-dependent manner (ANOVA, $p = 0.002$) and showed a gender effect (ANOVA, $p = 0.01$). Statistical tests revealed a significant increase of PBS soluble Aβ40 in female Thy1-APP751^{SL} mice compared to male animals with the age of 14 weeks (*t*-test, $p < 0.05$). *f* Female; *m* male; *n* number of individual mice

Table 1. *Percentage of the alteration between four to14 week-old Thy1-APP751^{SL} mice and differences between 14 week-old female and male Thy1-APP751^{SL} mice regarding C99/APP ratio and Aβ40-level as well as BACE1 activity*

		Age effect 4–14 weeks (%)	*p* (*t*-test)	Gender effect at 14 weeks female versus male (%)	*p* (*t*-test)
C99/APP	f	+86	<0.001	+7	<0.05
	m	+80	<0.001		
Aβ40	f	+29	<0.05	+19	<0.05
	m	+14	<0.05		
BACE1 activity	f	+0.2	ns	+11	<0.05
	m	−5	ns		

f Female; *m* male; *ns* not significant; *p* significance.

was calculated, which enabled the comparison of band density between individual animals. ANOVA revealed a significant increase of the C99/APP ratio during aging ($p < 0.0001$) (Fig. 2a). Despite of an unchanged expression level of full-length APP, a difference in C99/APP ratio between males and females was obvious and showed a statistical significance at 14 weeks of age (*t*-test, $p = 0.006$). In agreement with a former study (Schuessel et al., 2005), female mice (0.51 ± 0.04 C99/APP ratio) had a significantly higher C99/APP ratio than age-matched male animals (0.46 ± 0.04 C99/APP ratio). Total Aβ levels in the Thy1-APP751SL animals also increased during aging. The monoclonal antibody W0-2 (epitope Aβ5–8) detected a faint band in the four-week old animals in the Western blot, which increased steadily up to 14 weeks of age (Fig. 1c). To further validate the Western blot data concerning Aβ levels, we performed an Aβ40 ELISA. As expected, an age dependent increase in Aβ40 levels was found (ANOVA, $p = 0.002$) (Fig. 2b). Furthermore, a significant influence of gender on Aβ40 levels was detected across all age groups (ANOVA, $p = 0.01$), with female Thy1-APP751SL mice having significantly higher levels than male animals. Specifically, the mean Aβ40 levels in females were elevated in 14 week-old mice by 19% compared to age-matched male animals (Table 1).

Aβ42 values were not determined in young animals, due to the low abundance close to the detection limit (Blanchard et al., 2003). For similar reasons and the lack of plaque pathology in 14 week-old Thy1-APP751SL mice, most Aβ was found in the PBS soluble fraction.

Influence of BACE1

The most obvious difference between 4 and 14 weeks of age was the dramatic increase in C99 levels (+86% in

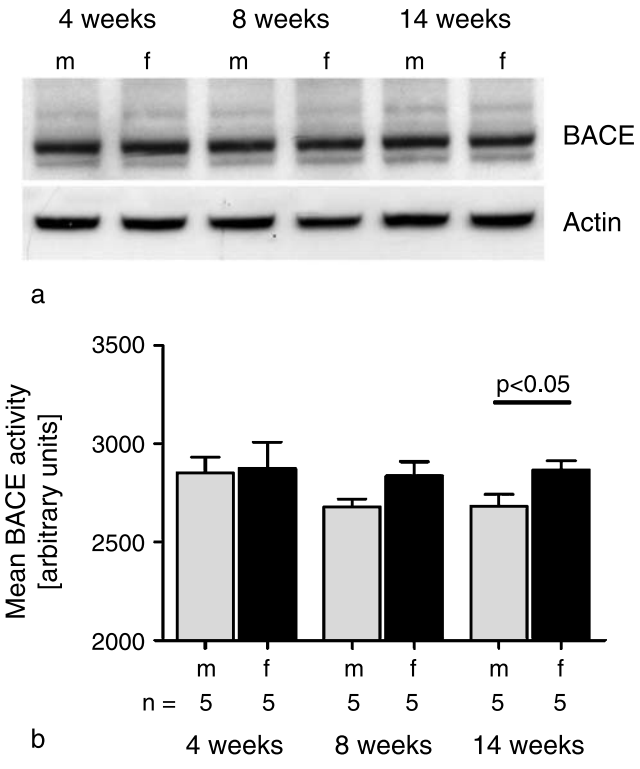

Fig. 3. **a** Western blot analysis of murine BACE1 expression in SDS brain homogenates of Thy1-APP751^{SL} using the monoclonal antiserum BSC-1. The expression of BACE1 does not differ in relation with age or gender. **b** Mean BACE1-activity ± SEM for mean BACE1-activity in PBS brain homogenates of Thy1-APP751^{SL}. At the age of 14 weeks male mice show a significantly decreased activity of BACE1 in comparison to females (*t*-test, $p = 0.039$). *f* Female; *m* male; *n* number of mice

females and +80% in males; Table 1), the β-cleavage product of APP. Therefore we investigated the possibility that BACE1 protein levels or activity might be responsible for an increase in APP cleavage. We performed quantitative Western blots using the monoclonal antiserum BSC-1, which recognizes the N-terminus of BACE1 (Schmechel et al., 2004). Equal loading was ensured by re-probing the blot with an antibody against actin. No difference in BACE1 expression was observed between males and females in 4, 8 and 14 week-old animals (Fig. 3a). Additionally, no change in BACE1 expression level was detected with increasing age. Regarding all examined age groups, ANOVA revealed a tendency for decreased BACE1 activity in male Thy1-APP751^{SL} transgenic mice (ANOVA, $p = 0.069$) (Fig. 3b). During aging BACE1 activity remained stable in female Thy1-APP751^{SL} mice. Interestingly, eight-week-old Thy1-APP751^{SL} male mice exhibited a tendency towards reduced levels (*t*-test, $p = 0.095$) compared to age-matched female mice. At 14 weeks of age, the effect became significant in comparison with female mice (*t*-test, $p = 0.039$). Thus, 14-week-old male Thy1-APP751^{SL}

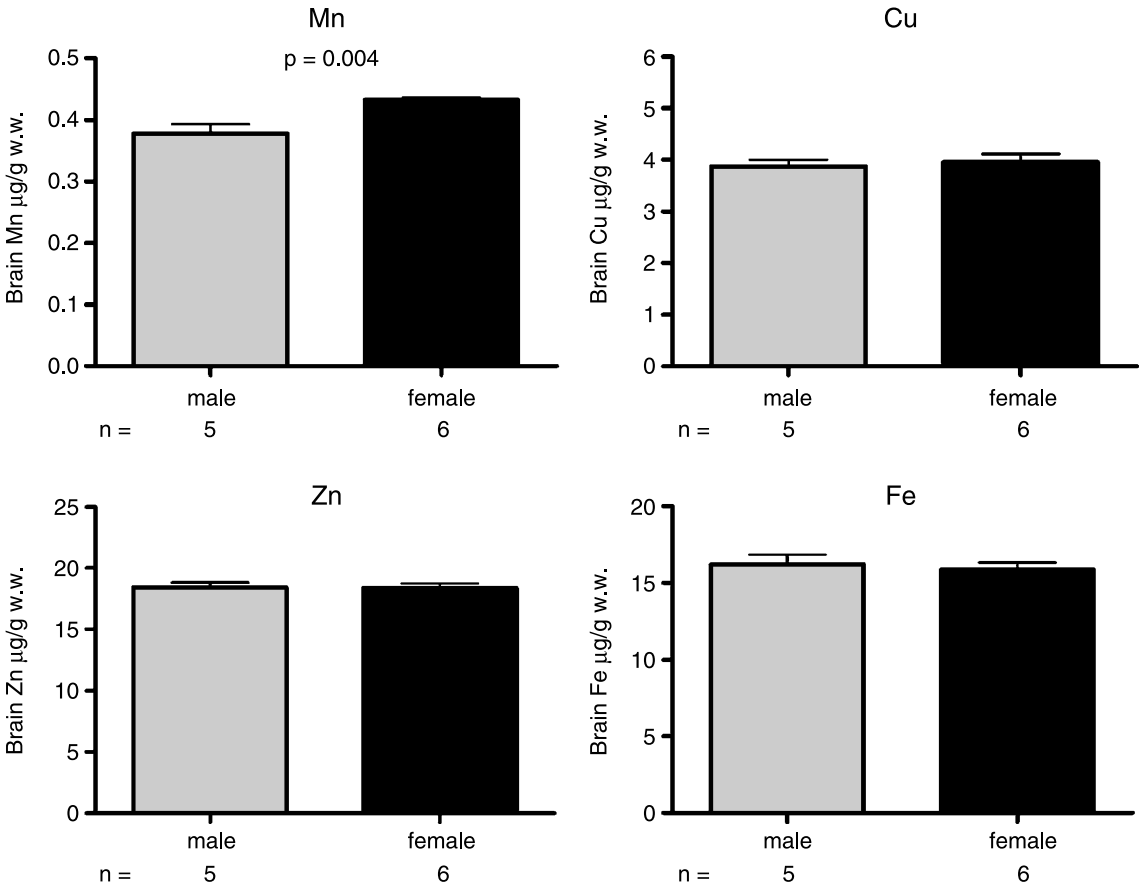

Fig. 4. Mean cerebral Mn levels in 14 week-old Thy1-APP751^{SL} mice determined by ICP-MS. Female mice exhibited significantly elevated metal levels compared to age-matched male animals (*t*-test, $p = 0.004$)

mice showed a modest but significant decrease in BACE1 activity of approximately 7% compared to female mice. However, BACE1 activity did not correlate with the abundant increase in C99 and Aβ levels between 4 and 14 weeks of age.

Metal levels in 14 week-old mice

The cerebral levels of Mn, Fe, Cu and Zn were compared between sexes in the Thy1-APP751^{SL} mice at an age of 14 weeks. Whereas Fe, Cu and Zn showed no significant sex difference, a significant effect of sex was found on the Mn levels in brain. Female Thy1-APP751^{SL} mice displayed significantly increased levels of Mn (13%; $p < 0.01$) compared to male APP transgenic mice (Fig. 4).

Discussion

We examined a transgenic mouse model overexpressing human APP751, with the Swedish and London mutations under the control of the murine Thy1-promotor (Thy1-APP751^{SL}), in order to analyze the potential gender effect regarding APP metabolism in the brain of young mice. First, we examined early expression of transgenic APP expression by means of Western blotting. Thy1-driven human APP expression was detected already at postnatal day three. This is in accordance with earlier studies reporting an onset of expression at P6 – 10 (Caroni, 1997). There were no detectable age- or gender-dependent differences in human APP expression in mice older than 20 days. However, we found a significant age and gender dependent increase in C99/APP ratio and Aβ levels, which did not result from higher expression of human mutant APP (mean values and statistical significance are summarized in Table 1). It is already known that female APP transgenic mice show a more pronounced accumulation of extracellular Aβ compared to age-matched male animals. This observation has been reported in a variety of APP transgenic mouse models: in Tg2576 (Callahan et al., 2001; Lee et al., 2002), APP23 (Sturchler-Pierrat and Staufenbiel, 2000; Bayer et al., 2003) APP/PS1 (Wang et al., 2003), as well as in APP/Tau transgenic mice (Lewis et al., 2001).

Regarding the incidence of Alzheimer's disease (Andersen et al., 1999; Fratiglioni et al., 1997, 2000; Jorm and Jolley, 1998) and a human gender-effect (Kraszpulski et al., 2001; Schuessel et al., 2004), a decrease in endogenous estrogen level due to menopause has been proposed as a trigger for the development of AD in females (Paganini-Hill and Henderson, 1994; Tang et al., 1996). However, clinical trials concerning estrogen replacement therapy (ERT) have resulted in inconclusive data (Asthana et al., 2001; Tang et al., 1996; Wang et al., 2000; Mulnard et al., 2000; Henderson et al., 2000; Shumaker et al., 2003).

The average age at the onset of reproductive decline in C57Bl6 mice may range from 11 to 16 months (Felicio et al., 1984). In order to study contributing factors to the gender effect in addition to the decrease in estrogen levels we examined only young mice up to an age of 14 weeks. Since Thy1-APP751^{SL} mice were maintained on a C57Bl6 background and considering that the age of mice used in this study is well below the onset of reproductive decline, a decline of estrogen levels in the present study is unlikely.

To our knowledge only two studies exist, dealing with a gender effect in APP transgenic mice prior to plaque load. We examined in a former study three and 12 months old Thy1-APP751^{SL} mice and detected that at the age of only three months differences in both C99 and Aβ levels between female and male mice are observed using Western blotting (Schuessel et al., 2005). Wang et al. (2003) detected also higher levels of Aβ40, Aβ42 and a higher Aβ42/Aβ40 ratio in female APP/PS1 mice already at 4 months of age. The authors suggested, in agreement with our assumption, that estrogen is not the most likely candidate for the gender difference.

Recently, disturbed metal homeostasis in brain is associated with AD (reviewed in Maynard et al., 2005). Some studies implicated changes in cerebral metals levels and the gender effect regarding Aβ generation. Lee et al. (2002) noted a female-restricted increase of synaptic zinc with age, which could be correlated with higher levels of insoluble Aβ and a higher plaque load in female Tg2576 mice. This gender effect was completely absent in APP-transgenic animals lacking the zinc-transporter (ZnT3$^{-/-}$). The results of the recent study of Lee et al. (2004) indicate that brain levels of synaptic vesicle zinc are affected by changes in the levels of estrogen. Thus, ovariectomy increased the levels of synaptic zinc in the brain, whereas estrogen replacement reduced the levels. Therefore, changes in zinc levels of aged animals could contribute to the gender effect, but could not explain the differences already obvious in young animals in the present study. In addition measurement of cerebral metal levels of Thy1-APP751^{SL} mice by the means of ICP-MS showed no statistically significant sex difference in Cu, Zn and Fe levels. However, a sex difference in Mn levels with increased levels in female compared to male animals was detected. Our results are in good agreement with the study of Maynard et al. (2006). They also determined cerebral metal levels in various APP transgenic and wildtype mice. At the age of 2.8–3 months none of the examined mouse lines displayed a significant sex difference regarding Cu, Fe, Zn or Co levels. In contrast, cerebral Mn levels were significantly elevated in females independent of mouse line as early as 2.8 months of age. In a former study Maynard et al. (2002) showed that overexpression of APP resulted in significantly increased Mn levels in brains of transgenic mouse model of AD. Abnormally high concentrations of Mn in the brain are associated with a neurological syndrome (manganism) with symptoms similar to Parkinson's disease (Takeda, 2003). The exact mechanism how Mn can damage the CNS is still unclear (Dieter et al., 2005). However, if excess levels of Mn contribute to the pathogenic alterations observed in AD, the elevated Mn levels in female APP transgenic animals may accelerate this process.

Until now a gender effect regarding APP processing has only been described in APP transgenic mouse models harboring the Swedish mutation alone or in combination with the London mutation. It is known that the Swedish mutation, due to its localization near the BACE1 cleavage site, results in increased concentrations of β-cleaved C-terminal fragments and Aβ40 (Bodendorf et al., 2002; Moechars et al., 1999). This mutation could possibly contribute to the dramatic accumulation of C99 with increasing age but not to the observed gender differences. To study the influence of β-secretase (BACE1) on the generation of C99, both the expression level and activity of BACE1 were determined. According to the findings of Fukumoto et al. (2004) we replicated the expression of BACE1 protein as being independent of genotype and age.

Fukumoto et al. (2004) examined in their study young (4 months) as well as old (14–18 months) mice and reported an increase in BACE1 activity with aging. We observed in our young mice (up to 3.5 months) a stable activity over all examined time points in female Thy1-APP751^{SL} mice and a modest but significant decrease of BACE1 activity in male Thy1-APP751^{SL} mice compared to age-matched female animals (14 weeks of age). There is growing evidence from studies in mice overexpressing human BACE that in vivo, the rate-limiting cleavage in the generation of Aβ is regulated by BACE rather than by the processing event mediated by γ-secretase (Bodendorf et al., 2002). The fact that male Thy1-APP751^{SL} mice exhibited a slight

diminished β-secretase activity in comparison to age-matched female animals in our study might explain the reduced C99 and consequently lower Aβ levels in male compared to female Thy1-APP751^{SL} mice. Since we used young mice in the present study, we cannot rule out that there is an age-dependent increase in BACE1 activity in very old Thy1-APP751^{SL} mice. However, we found no significant age-dependent difference in BACE1 activity and protein level at the analyzed time points.

It is feasible that an age-dependent loss in degradation of APP proteolytic products exists. Several proteases have been implicated in the degradation of Aβ peptides. For example, Leissring et al. (2003) have shown that transgenic overexpression of insulin-degrading enzyme (IDE) or neprilysin (NEP) in neurons significantly reduces brain Aβ levels, leading to retardation or complete prevention of amyloid plaque formation in APP transgenic mice. While no gender-dependent differences have been reported for NEP, a gender effect has been observed in association studies using polymorphisms in the insulin-degrading enzyme gene associated with type 2 diabetes (Karamohamed et al., 2003). However, the molecular mechanisms responsible for the significant gender effect in AD need to be examined in more detail. At present it seems unlikely that the decline in estrogen levels represents a main contributing factor to the gender effect observed in APP transgenic mice.

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