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Gender-Dependent Transthyretin Modulation of Brain Amyloid-β Levels: Evidence from a Mouse Model of Alzheimer's Disease

Sandra Marisa Oliveira[a], Carlos A. Ribeiro[a], Isabel Cardoso[a,b] and Maria João Saraiva[a,c,*]
[a]*Molecular Neurobiology, IBMC- Instituto de Biologia Molecular e Celular, Porto, Portugal*
[b]*Escola Superior de Tecnologia da Saúde do Porto, Instituto Politécnico do Porto, Porto, Portugal*
[c]*ICBAS- Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal*

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Abstract. Alzheimer's disease (AD) is a neurodegenerative disorder affecting tens of millions of people worldwide, with women being at greater risk of developing the disease. A growing body of evidence suggests transthyretin (TTR) as an important modulator of AD pathogenesis. Aiming at providing further insight into the potential neuroprotective role of TTR and gender differences in AD, we crossed transgenic AβPPswe/PS1A246E mice with TTR-null mice and investigated both male and female AβPPswe/PS1A246E/TTR$^{+/+}$, AβPPswe/PS1A246E/TTR$^{+/-}$, and AβPPswe/PS1A246E/TTR$^{-/-}$ animals for brain amyloid-β (Aβ) levels and deposition. The levels of circulating TTR between non-transgenic and AD mice were evaluated. Decreased levels of circulating TTR in AD mice as compared to non-transgenic littermates were observed in early stages of AD-like neuropathology, but not at later stages where an opposite relationship was found. Elevated brain levels of $A\beta_{42}$ were observed in AβPPswe/PS1A246E/TTR$^{+/-}$ female mice as compared to AβPPswe/PS1A246E/TTR$^{+/+}$ female littermates; no significant differences were found among males of different TTR genotypes. We subsequently quantified the brain levels of testosterone and 17β-estradiol in these animals and verified that AβPPswe/PS1A246E/TTR$^{+/-}$ female mice present reduced brain levels of both hormones as compared to AβPPswe/PS1A246E/TTR$^{+/+}$ females; no significant differences were detected among males of different TTR genotypes. Our results provide evidence for a gender-associated modulation of brain Aβ levels and brain sex steroid hormones by TTR, and suggest that reduced levels of brain testosterone and 17β-estradiol in female mice with TTR genetic reduction might underlie their increased AD-like neuropathology.

Keywords: Alzheimer's disease, amyloid-β peptide, 17β-estradiol, testosterone, transgenic mouse, transthyretin

INTRODUCTION

Alzheimer's disease (AD), the most common cause of dementia worldwide, is a devastating incurable neurodegenerative disorder. Clinically, patients initially manifest memory decline and, as the disease progresses, other cognitive domains become severely affected. The disease is pathognomically characterized by the occurrence of extraneuronal amyloid plaques, consisting of aggregates of amyloid-β (Aβ) peptide, and intraneuronal neurofibrillary tangles composed of aggregates of abnormally hyperphosphorylated tau protein. Further pathological changes include cellular inflammatory responses and other cellular disturbances resulting from calcium dyshomeostasis, defective proteolysis, and oxidative stress [1–3]. It is noteworthy that epidemiological and neuropathological studies have provided evidence for gender differences in AD,

*Correspondence to: Maria João Saraiva, Molecular Neurobiology, IBMC, Rua do Campo Alegre, 823, 4150-180, Porto, Portugal. Tel.: 00351 22 6074900; Fax: 00351 22 6099157; E-mail: mjsaraiv@ibmc.up.pt.

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with women being more susceptible to developing the disease than men [4].

It is crucial to understand the molecular pathways that lead to AD pathology. Although being the subject of very intensive research, the exact cascade(s) of mechanisms is still unknown. Several lines of evidence suggest that transthyretin (TTR), a plasma and cerebrospinal fluid (CSF)-circulating 55 kDa homotetrameric protein, has an important role in AD. TTR and other few CSF proteins (such as apolipoproteins E, J, and A1) have been shown to bind soluble Aβ *in vitro* and to prevent Aβ from forming amyloid fibers [5]. By mediating its solubility and transport, these Aβ sequesters constitute potential contributors to its clearance from the brain. Further, *in vitro* studies performed in our laboratory have shown that TTR is able to proteolytically cleave Aβ, with the resulting peptides being less amyloidogenic [6]. Interestingly, decreased levels of TTR have been found in the CSF of elderly AD patients [7]. Further, the levels of TTR in the CSF of AD patients have been shown to be inversely correlated with Aβ plaque burden [8]. Over the last decade, data from transgenic mouse models provided further *in vivo* evidence for a role of TTR in AD, although the results have not always been consistent. Lazarov and colleagues [9] reported that AβPPswe/PS1ΔE9 mice exposed to an enriched environment presented reduced Aβ levels and amyloid deposits, this being associated with altered expression of several genes including upregulation of TTR. The work by Choi et al. [10] has shown that the levels and deposition of brain Aβ were elevated in AβPPswe/PS1ΔE9/TTR$^{+/-}$ mice compared with their AβPPswe/PS1ΔE9/TTR$^{+/+}$ counterparts. Subsequently, Buxbaum and coworkers [11] have also shown evidence for a protective role of TTR since AβPP23/TTR$^{-/-}$ mice presented increased Aβ deposition as compared to AβPP23/TTR$^{+/+}$ animals. Moreover, AβPP23 mice overexpressing human TTR had improved cognition and diminished levels and deposition of brain Aβ. On the other hand, the works by Wati et al. [12] and Doggui et al. [13] reached different conclusions. Wati and colleagues [12] reported reduced total and vascular Aβ burdens in Tg2576/TTR$^{-/-}$ mice as compared to Tg2576/TTR$^{+/-}$, and Doggui et al. [13] have shown reduced Aβ plaque burden in the hippocampus of TgCRND8/TTR$^{+/-}$ mice as compared to TgCRND8/TTR$^{+/+}$.

While compelling evidence suggests TTR as a potentially important modulator of AD pathogenesis, the precise mechanisms behind TTR action are not fully understood. Furthermore, the role of TTR in AD in the context of the disease's inherent gender differences has not yet been explored. Aiming at addressing this issue, we crossed transgenic AβPPswe/PS1A246E mice [14], in which gender differences in hippocampal Aβ levels and deposition have been described [15], with TTR-null mice [16] and investigated brain Aβ levels and deposition in both male and female AβPPswe/PS1A246E/TTR$^{+/+}$, AβPPswe/PS1A246E/TTR$^{+/-}$, and AβPPswe/PS1A246E/TTR$^{-/-}$ littermates.

MATERIALS AND METHODS

Animals

AβPPswe/PS1A246E transgenic mice [14] were purchased from The Jackson Laboratory. These transgenic mice coexpress a chimeric mouse-human amyloid-β protein precursor (AβPP) bearing a human Aβ domain with mutations (K595 N and M596 L) linked to Swedish (Swe) familial AD (FAD) pedigrees and human presenilin 1 (PS1) bearing a mutation (A246E) which also causes FAD. Expression of both transgenes is under the control of the mouse prion protein promoter (PrP).

AβPPswe/PS1A246E/TTR mouse model was generated in our animal house by crossing AβPPswe/PS1A246E mice (B6/C3 H background) with TTR-null mice (TTR$^{-/-}$) (SV129 background) [16]. F1 animals AβPPswe/TTR$^{+/-}$ and PS1A246E/TTR$^{+/-}$ were crossed to obtain AβPPswe/PS1A246E/TTR$^{+/+}$, AβPPswe/PS1A246E/TTR$^{+/-}$, AβPPswe/PS1A246E/TTR$^{-/-}$, and non-transgenic controls TTR$^{+/+}$, TTR$^{+/-}$, and TTR$^{-/-}$. The colony was maintained on a B6/C3 H/SV129 genetic background. Hereafter, the AβPPswe/PS1A246E/TTR colony will be referred to as AD/TTR, and the different genotypes AβPPswe/PS1A246E/TTR$^{+/+}$, AβPPswe/PS1A246E/TTR$^{+/-}$, and AβPPswe/PS1A246E/TTR$^{-/-}$ referred to as AD/TTR$^{+/+}$, AD/TTR$^{+/-}$, and AD/TTR$^{-/-}$, respectively.

Littermate cohorts of male and female animals were used in this study. For each gender, six groups of animals were studied (AD/TTR$^{+/+}$, AD/TTR$^{+/-}$, AD/TTR$^{-/-}$, TTR$^{+/+}$, TTR$^{+/-}$, and TTR$^{-/-}$) at three ages (3, 6, and 10 months). Animals were housed in a controlled environment (12-h light/dark cycle; temperature, 22 ± 2°C; humidity, 45–65%), with food and water freely available. All procedures involving animals were carried out in accordance with the European Communities Council Directive.

Tissue processing

Mice were deeply anesthetized with a mixture of ketamine and medetomidine. Blood was collected from the inferior vena cava in syringes containing EDTA as anticoagulant, followed by centrifugation at $1000 \times g$ for 15 min at room temperature (RT), and plasma samples collected and stored at $^{-}80^{\circ}$C. Brains were removed from the skull and bisected longitudinally: each half was either immediately frozen for biochemical analyses, or dissected to isolate the hippocampus also for biochemical analyses, or fixed for 24 h at 4°C in 10% neutral buffered formalin and then transferred to a 30% sucrose solution for cryoprotection before cryostat sectioning for immunohistochemical analyses.

Plasma TTR levels determination

Plasma TTR levels were quantified using Mouse Prealbumin ELISA Kit (MyBioSource) according to the manufacturer's instructions. Data were expressed in mg/mL.

Aβ immunohistochemistry

Free-floating immunohistochemistry of 30 μm-thick cryostat coronal brain sections using a monoclonal biotinylated $A\beta_{1-16}$ antibody (6E10) (Covance Research Products, Inc.) was used to determine Aβ plaque burden.

Briefly, free-floating brain sections were washed twice in phosphate-buffered saline (PBS), and once in distilled water (dH_2O). In order to partially denature amyloid, sections were then immersed in 70% formic acid (FA) for 15 min at room temperature (RT), with gentle agitation. After washing in dH_2O and then PBS, endogenous peroxidase was blocked by incubation in PBS containing 1% H_2O_2 for 20 min at RT. Following PBS washes, nonspecific staining was blocked with PBS containing 10% fetal bovine serum (FBS) and 0.5% Triton X-100 for 1 h at RT. Sections were then incubated with biotinylated 6E10 primary antibody (diluted 1/750 in blocking buffer) overnight (O/N) at 4°C, with gentle agitation. After PBS washing, sections were incubated in Vectastain® Elite ABC Reagent (Vector Laboratories, Inc.) according to the manufacturer's instructions. Sections were once more washed in PBS, followed by development with diaminobenzidine (Sigma-Aldrich, Inc.). The sections were then mounted on 0.1% gelatin-coated slides and were left to dry O/N at RT in dust-free conditions. After dehydration, slides were coverslipped under Entellan® (Merck & Co., Inc.). Sections were examined with an Olympus BX50 light microscope.

Aβ plaque burden was estimated by analysing the 6E10-immunostained area fraction in the hippocampus and cortex (expressed as percentage of analyzed area). Three sections per animal were analyzed using digitized images (Olympus DP71 microscope digital camera) and the Image-Pro Plus programme.

Brain $A\beta_{40}$ and $A\beta_{42}$ levels determination

Sandwich ELISA analysis was used to determine the levels of Aβ in brain extracts: detergent-soluble and FA-soluble Aβ.

Each half brain was homogenized in 1 mL of 0.1% Triton X-100 and 2 mM EDTA in 50 mM Tris-buffered saline (TBS) (pH 7.4) with protease inhibitors (Amersham Biosciences), and centrifuged at $21500 \times g$ for 15 min at 4°C. The supernatant was collected, aliquoted, and frozen at −80°C for the analysis of the detergent-soluble fraction of brain Aβ. The pellet was then homogenized with 1 mL 70% FA in dH_2O and centrifuged as before. The supernatant was collected, neutralized with 1 M Tris (pH 11.0) (1/20 dilution), aliquoted and frozen at $^{-}80^{\circ}$C for the analysis of the FA-soluble fraction of brain Aβ. Sandwich ELISA analyses of $A\beta_{40}$ and $A\beta_{42}$ in the obtained fractions were performed using Human $A\beta_{40}$ and Human $A\beta_{42}$ ELISA Kits (Invitrogen) according to the manufacturer's instructions. Data were expressed in pmol/g wet tissue.

Brain testosterone and 17β-estradiol levels determination

Brain testosterone and 17β-estradiol levels were quantified in TBS/Triton X-100 brain extracts (prepared as described in the above section) using Testosterone EIA Kit and Estradiol EIA Kit (Cayman), respectively, according to the manufacturer's instructions. Data were expressed in ng/g wet tissue. Before starting these analyses, we undertook a series of tests to evaluate the levels of both testosterone and 17β-estradiol in TBS/Triton X-100 brain extracts before and after diethyl ether extraction, and verified that the levels were similar in both conditions.

Statistical analyses

Data are expressed as mean values ± standard error of the mean (SEM). One-way ANOVA with Bonfer-

roni's post test was performed using GraphPad Prism version 5.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Values of $p < 0.05$ were considered to be significant.

RESULTS

Plasma TTR levels in AβPPswe/PS1A246E/TTR mice

The AD/TTR mouse colony was successfully established in our laboratory, with both male and female animals being viable and fertile.

To obtain a better knowledge of this newly established mouse colony, we decided to quantify the levels of circulating TTR by performing sandwich ELISA analysis of plasma samples. We analyzed male and female littermate cohorts of both non-transgenic ($TTR^{+/+}$ and $TTR^{+/-}$) and transgenic ($AD/TTR^{+/+}$ and $AD/TTR^{+/-}$) mice, aged 3, 6, and 10 months. For all three age groups analyzed, we confirmed that the genetic reduction of TTR in both non-transgenic $TTR^{+/-}$ and $AD/TTR^{+/-}$ heterozygous mice translates in phenotypically reduced levels of circulating TTR (data not shown). Notably, we verified that, at 3 and 6 months of age, both male and female $AD/TTR^{+/+}$ mice present reduced levels of circulating TTR as compared to non-transgenic $TTR^{+/+}$ littermates, the reduction being statistically significant for male mice aged 3 months and for female mice aged 6 months (Fig. 1A, B). Strikingly, we obtained different results for the age of 10 months, as both male and female $AD/TTR^{+/+}$ mice present increased levels of circulating TTR as compared to non-transgenic $TTR^{+/+}$ littermates, the difference being significant for female animals (Fig. 1C). While comparing the levels of circulating TTR between male and female mice, our results revealed increased levels in male animals (both $TTR^{+/+}$ and $AD/TTR^{+/+}$) as compared to their female littermates (Fig. 1A–C). With regard to heterozygous mice, we also observed elevated levels of circulating TTR in male mice as compared to female littermates (data not shown). However, differences in circulating TTR levels between non-transgenic and AD mice were not observed (data not shown). The results of this study provide evidence for: i) altered circulating TTR levels in $AD/TTR^{+/+}$ mice as compared to their non-transgenic $TTR^{+/+}$ littermates; ii) gender differences in the levels of circulating TTR.

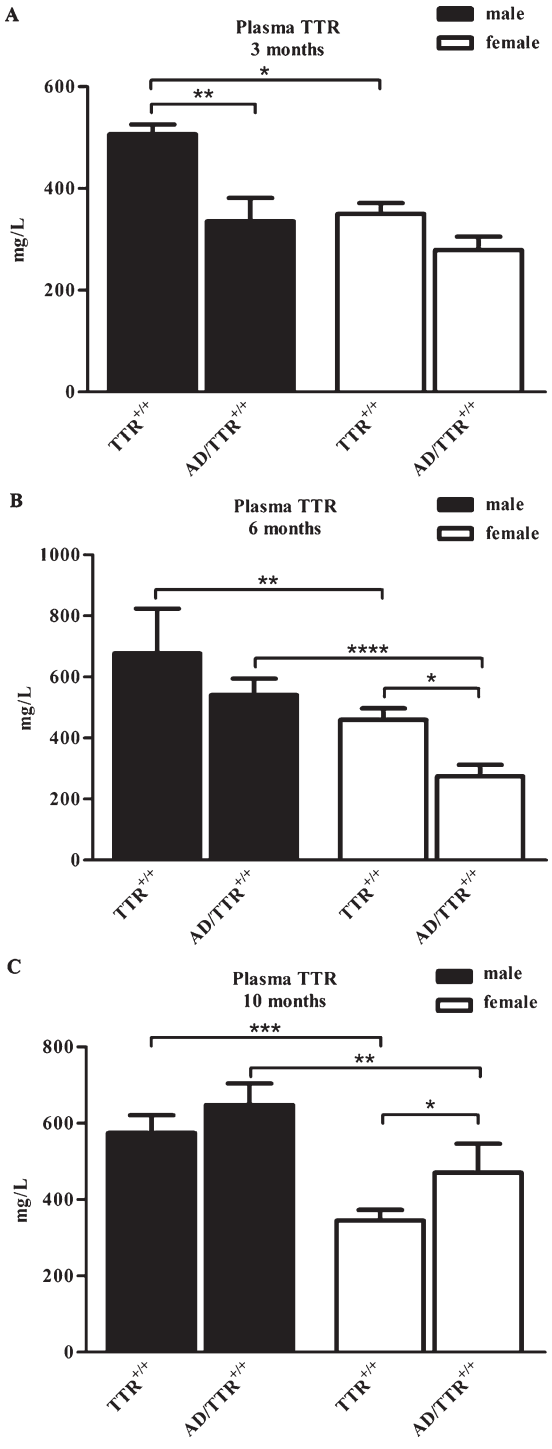

Fig. 1. Plasma TTR levels in male and female non-transgenic $TTR^{+/+}$ and transgenic $AD/TTR^{+/+}$ littermate mice. Plots show quantification of circulating TTR levels by ELISA for the ages of 3 (A), 6 (B), and 10 months (C). Error bars represent SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. ($n = 6$–10 animals/group).

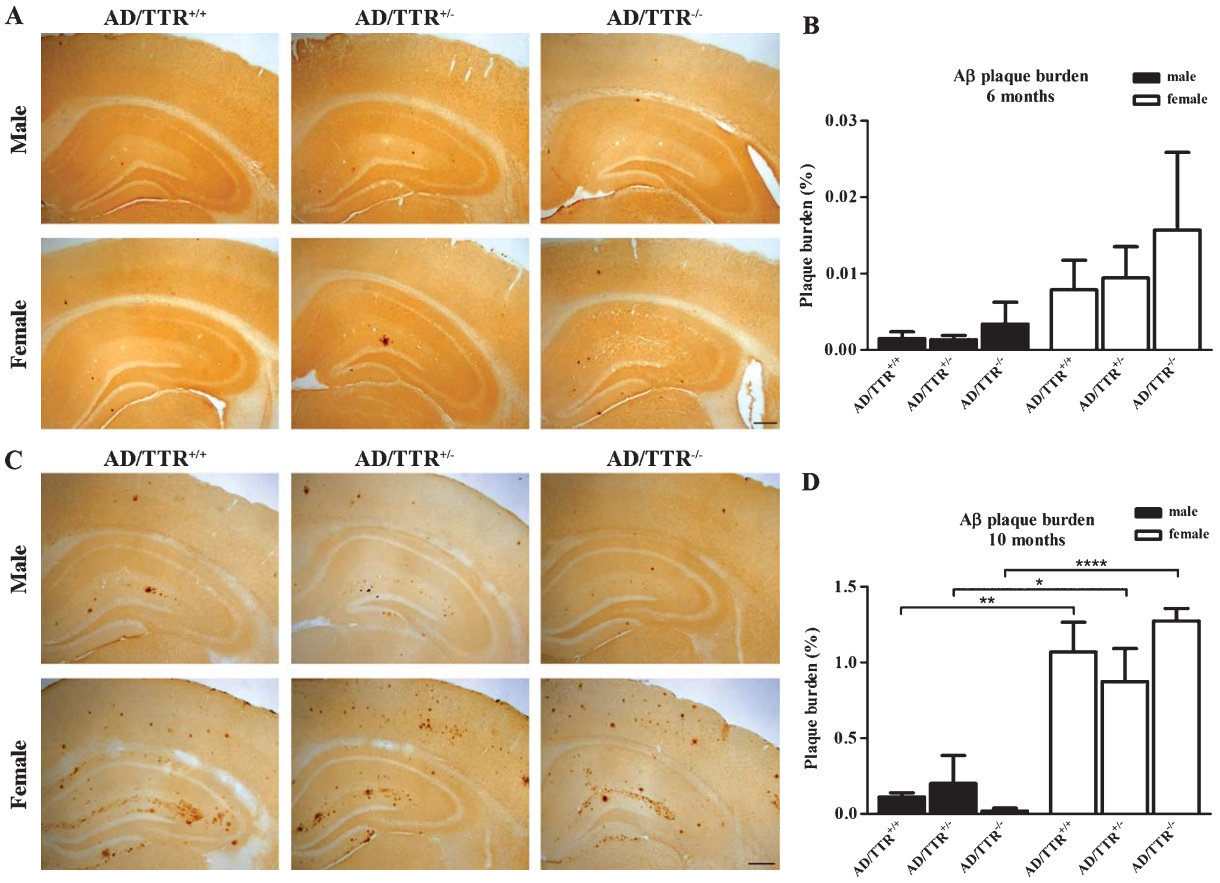

Fig. 2. Aβ plaque burden in male and female transgenic AD/TTR$^{+/+}$, AD/TTR$^{+/-}$ and AD/TTR$^{-/-}$ littermate mice. Photomicrographs illustrate immunohistochemical analysis of brain Aβ plaques using the 6E10 antibody, and plots show plaque burden estimation for the ages of 6 (A, B), and 10 months (C, D). Scale bar, 250 μm. Error bars represent SEM. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$. ($n = 4$ animals/group).

Aβ plaque burden in AβPPswe/PS1A246E/TTR mice

To evaluate the onset of Aβ deposition in the AD/TTR colony, we analyzed male and female littermate cohorts of AD/TTR mice aged 3, 6, and 10 months by performing immunohistochemical analyses using the 6E10 antibody. We verified that both male and female AD/TTR mice bear Aβ plaques from as early as 6 months of age (Fig. 2A, C).

In order to investigate the influence of TTR on Aβ deposition, Aβ plaque burden was estimated at the ages of 6 and 10 months in male and female littermate cohorts of AD/TTR$^{+/+}$, AD/TTR$^{+/-}$, and AD/TTR$^{-/-}$ animals. As it can be seen in Fig. 2, the plaque burden increases with age in all groups of animals analyzed, with females presenting the most dramatic changes. At 6 months of age (at the beginning of deposition) (Fig. 2A, B), a trend for increased Aβ plaque burden in female animals compared to their male counterparts is observed, and at 10 months (Fig. 2C, D) this gender difference becomes significant. No significant differences were found in Aβ plaque burden among the male and female different TTR genotypes (Fig. 2B, D). Taken together, the results show clear gender differences in Aβ plaque burden in this mouse model, with female mice being particularly affected, and suggest that TTR does not exert significant influence on Aβ plaque burden in this mouse model.

Brain Aβ levels in AβPPswe/PS1A246E/TTR mice

In order to assess the effect of TTR on brain Aβ levels, male and female littermate cohorts of 6- and 10-month-old AD/TTR$^{+/+}$, AD/TTR$^{+/-}$, and

AD/TTR$^{-/-}$ animals were used. Aβ levels in detergent and FA extracts of hemibrains of these animals were quantified using sandwich ELISA analysis. As shown in Fig. 3, the levels of $A\beta_{40}$ and $A\beta_{42}$ in both detergent (Fig. 3A, B, E, F) and FA (Fig. 3C, D, G, H) extracts clearly increase with age in all groups of animals examined, with FA extracts presenting the most dramatic Aβ levels elevations. Our data also noticeably show that, at both 6 and 10 months of age, female mice, independently of their TTR genotype, present elevated levels of both detergent- and FA-soluble $A\beta_{40}$ and $A\beta_{42}$ as compared to their male counterparts (Fig. 3A–H). No significant differences among the different TTR genotypes were observed in male mice of the different ages in brain levels of both detergent- and FA-soluble $A\beta_{40}$ and $A\beta_{42}$. On the contrary, female mice heterozygous for TTR (AD/TTR$^{+/-}$) present elevated brain levels of detergent- and FA-soluble $A\beta_{42}$ as compared to AD/TTR$^{+/+}$ female littermates at the ages of 6 (Fig. 3B) and 10 months (Fig. 3H), respectively. Taken together, these results clearly show gender differences in Aβ levels, and suggest that TTR influences brain Aβ levels (in particular $A\beta_{42}$) in a gender-dependent manner, with female mice being more affected by TTR genetic reduction.

Brain testosterone and 17β-estradiol levels in AβPPswe/PS1A246E/TTR mice

Given our evidence that female mice are particularly affected by TTR genetic reduction with regard to increased brain $A\beta_{42}$ levels, we decided to focus on the age of 10 months (the analyzed time point with more pronounced AD-like neuropathology) and evaluate whether this gender-specific alteration could be related to altered brain levels of sex steroid hormones. We addressed this issue by quantifying the levels of testosterone and 17β-estradiol in brain extracts of 10-month-old male and female AD/TTR$^{+/+}$, AD/TTR$^{+/-}$, and AD/TTR$^{-/-}$ mice. We also quantified the levels of these sex steroid hormones in brain extracts of non-transgenic TTR$^{+/+}$, TTR$^{+/-}$, and TTR$^{-/-}$ littermate mice. As it can be seen in Fig. 4, mice in a full TTR genetic background do not exhibit differences in brain levels of testosterone and 17β-estradiol, regardless of gender, or being non-transgenic or AD. It is clear that TTR reduction and/or abolition influences both hormone levels, with differences being found not only among the male and female different TTR genotypes, but also between male and female mice of the same genotype. Thus, TTR reduction/abolition positively correlates with lower brain hormone levels, in particular, in female mice. Consequently, lower brain hormone levels are generally found in female mice relative to males of the same genotype. The main exceptions in this positive correlation between reduced/absent TTR levels and reduced brain hormone levels are found among AD animals. One of the exceptions concerns AD male mice which do not present significant differences in the levels of brain testosterone (Fig. 4C) and 17β-estradiol (Fig. 4D) among the different TTR genotypes. The other exception involves AD/TTR$^{-/-}$ females which, in the absence of TTR, do not exhibit decreased brain 17β-estradiol levels. Taken together, these results suggest that TTR modulates brain sex steroid hormones in a gender-dependent manner, with female mice being more affected by the genetic reduction of TTR.

DISCUSSION

This work presents, for the first time, evidence for a gender-dependent modulation of AD pathogenesis by TTR. Further, it brings to light the importance of analyzing questions concerning AD considering the disease's inherent gender differences [4, 17].

There are several epidemiological [18–29] and neuropathological [30-33] studies which have been providing compelling evidence that women are at greater risk of developing AD and a more severe pathology than men. It has been suggested that this increased vulnerability to AD in women is most likely associated with the abrupt loss of estrogens and progesterone at menopause, in contrast with the much less dramatic age-related fall in testosterone that occurs in men [4, 17].

Although transgenic mice represent a powerful and widely used research tool in AD [34], only a few studies have documented gender differences in AD transgenic mouse models. Three different mouse models have been described as exhibiting gender differences in the degree of AD-like neuropathology [15, 35, 36]. Female mice from Tg2576 [35], and 3×Tg-AD [36] strains present increased levels of brain Aβ and increased Aβ plaque burden as compared to age-matched male mice, as well as female mice from the strain (albeit in a different genetic background) used in the current studies, the AβPPswe/PS1A246E mice [15].

Prior to the studies on the role of TTR in this AD mouse model which was crossed with TTR-null mice [16] (AD/TTR), we evaluated the levels of circulating TTR in both non-transgenic and transgenic littermates of the different TTR genotypes. Our results

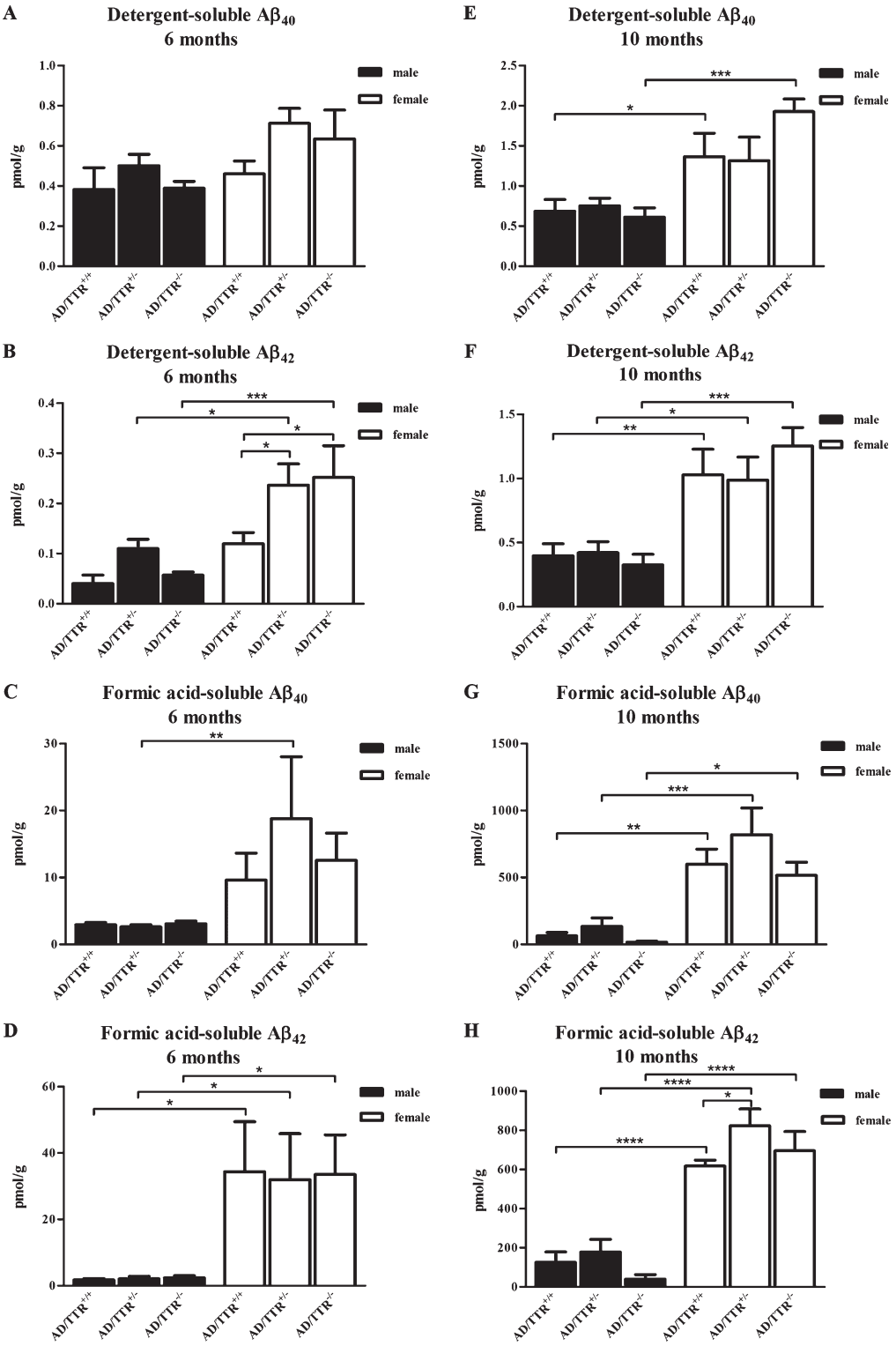

Fig. 3. Brain Aβ levels in male and female transgenic AD/TTR$^{+/+}$, AD/TTR$^{+/-}$ and AD/TTR$^{-/-}$ littermate mice. Plots show quantification of brain Aβ levels by ELISA for the ages of 6 (A–D), and 10 months (E–H): A, E, Levels of detergent-soluble Aβ$_{40}$; B, F, Levels of detergent-soluble Aβ$_{42}$; C, G, Levels of FA-soluble Aβ$_{40}$; D, H, Levels of FA-soluble Aβ$_{42}$. Error bars represent SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. ($n = 5$, 6 animals/group).

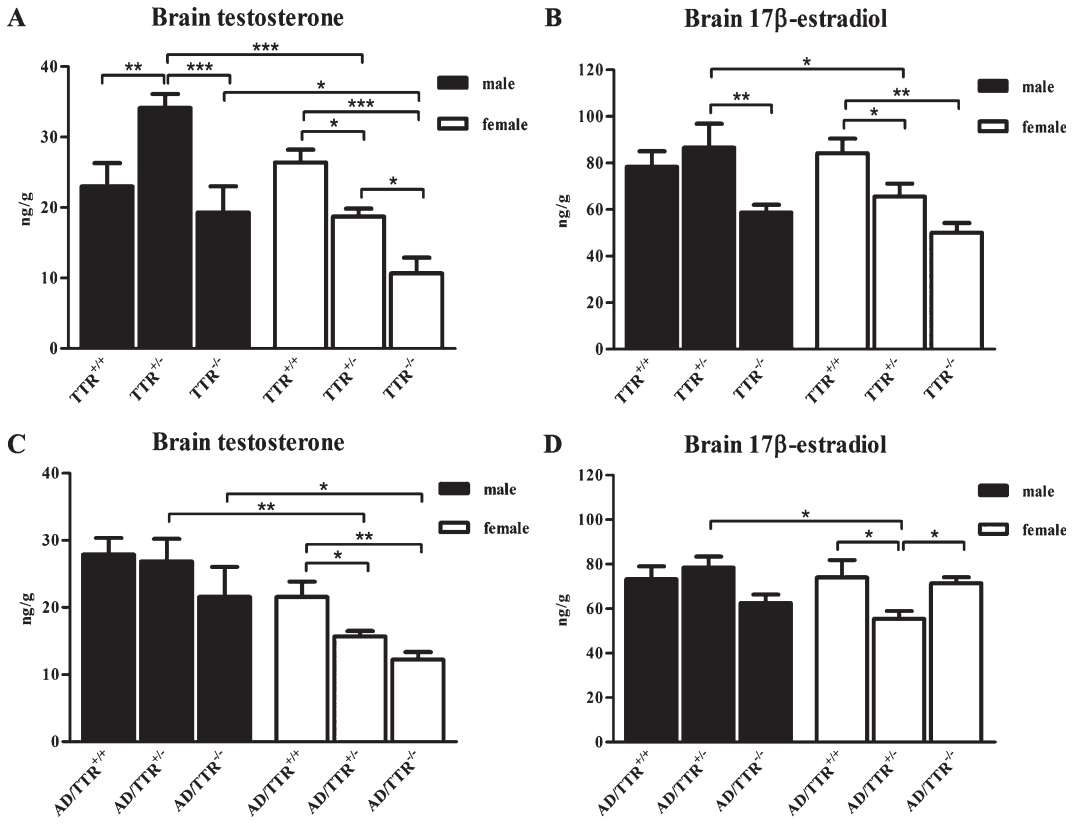

Fig. 4. Brain testosterone and 17β-estradiol levels in male and female non-transgenic $TTR^{+/+}$, $TTR^{+/-}$ and $TTR^{-/-}$, and transgenic $AD/TTR^{+/+}$, $AD/TTR^{+/-}$ and $AD/TTR^{-/-}$ littermate mice aged 10 months. Plots show quantification of brain testosterone and 17β-estradiol levels by EIA: A) Levels of brain testosterone in non-transgenic mice; B) Levels of 17β-estradiol in non-transgenic mice; C) Levels of testosterone in transgenic mice; D) Levels of 17β-estradiol in transgenic mice. Error bars represent SEM. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. ($n = 4, 5$ animals/group).

revealed a striking reduction of circulating TTR levels in both male and female $AD/TTR^{+/+}$ aged 3 and 6 months mice as compared to non-transgenic $TTR^{+/+}$ animals, whereas $AD/TTR^{+/+}$ animals aged 10 months presented increased levels of circulating TTR as compared to their non-transgenic $TTR^{+/+}$ littermates. These results suggest a relationship between decreased circulating TTR levels and early stages of AD-like neuropathology. The inverse relationship observed at 10 months of age suggests the existence of a potential compensatory mechanism. Further, this study revealed increased levels of circulating TTR in both non-transgenic and AD male mice as compared to their female littermates. We suggest that increased levels of circulating TTR in AD male mice might confer further protection against AD-like neuropathology when compared to AD females. The altered circulating TTR levels in $AD/TTR^{+/+}$ mice versus non-transgenic $TTR^{+/+}$, and in male mice versus female animals might be the result of altered regulation of TTR gene expression and/or TTR protein degradation. Evaluation of TTR levels in the CSF of these animals, and studies such as transcriptomics/proteomics in liver and choroid plexus (which are the main sources of TTR in the plasma and CSF, respectively) are critical. These studies will generate important clues regarding the mechanisms associated with altered TTR levels between genders and between non-transgenic and AD animals.

By analyzing brain Aβ plaques, we verified that the onset of Aβ deposition in the AD/TTR colony established in our laboratory is at around 6 months of age in both male and female mice, which is earlier than the age of 9 months previously described for this model [14, 15]. This discrepancy is most probably due to differences in the genetic background of the colonies. While our colony has been maintained on a B6/C3 H/SV129 background, the colonies investigated by Borchelt et al. [14] and Wang et al. [15] were maintained on B6/C3 H and B6 backgrounds, respectively. It is also important

to mention that even standard housing conditions in different laboratories can lead to phenotypic variations. Other studies have also used transgenic mouse models on mixed backgrounds to evaluate the role of genetic manipulations of the TTR gene in AD-like neuropathology [10, 13].

Our study singles out from the previous ones [10–13] as it is the first to address the role of TTR in AD analyzing male and female mice separately. Although Aβ plaque burden estimation (through semi-quantitative immunohistochemical analysis) suggests that, in this mouse model, TTR does not exert significant influence in the levels of Aβ deposition, the results of the quantification of brain Aβ levels (through ELISA analysis) clearly revealed significant alterations in the levels of $A\beta_{42}$—a highly neurotoxic Aβ species [37, 38]—in female animals. Thus, we found elevated levels of detergent-soluble $A\beta_{42}$ in 6-month-old AD/TTR$^{+/-}$ and AD/TTR$^{-/-}$ female mice and of FA-soluble $A\beta_{42}$ in 10-month-old AD/TTR$^{+/-}$ female animals as compared to age-matched AD/TTR$^{+/+}$ female littermates, which argues in favor of a neuroprotective role of TTR in AD. Since no significant differences in brain Aβ levels were found among male mice of the different genotypes, we suggest that TTR influences brain Aβ levels but in a gender-associated manner.

With the aim of gaining insight into the possible mechanisms underlying the observed gender-dependent modulation of brain Aβ levels by TTR, we quantified the levels of testosterone and 17β-estradiol in brain extracts of both non-transgenic and transgenic 10-month-old male and female littermates of the different TTR genotypes. It is noteworthy that mice in a full TTR genetic background do not present differences in brain levels of both hormones, regardless of gender, or being non-transgenic or AD. A recent study done in rat also showed no gender differences in the levels of sex steroid hormones measured in different brain areas of adult animals [39]. Our results clearly show that TTR reduction/abolition influences brain levels of testosterone and 17β-estradiol, and that this influence is gender-dependent. This is the first study providing evidence for a role of TTR as a potential modulator of brain sex steroid hormone levels. Other studies have shown that sex steroid hormones themselves can regulate de expression levels of TTR, and suggest a positive correlation between sex steroid hormone levels and TTR levels [40–42]. Our work also provides evidence for this positive correlation, in particular, in female mice. We suggest that CSF-circulating TTR (synthesized and secreted by the CP) might be able to modulate brain sex steroid hormone levels in different brain areas through receptor(s)-binding and/or internalization [43–47], followed by activation of yet unknown signaling pathways.

An increasing number of studies have been providing evidence for neuroprotective actions of sex steroid hormones in AD. Both estrogen and testosterone neuroprotective actions consist in conferring increased neuronal resistance against AD-related brain insults, as well as directly preventing accumulation of Aβ. Testosterone neuroprotection can be achieved both directly by activation of androgen pathways and indirectly by aromatization to estradiol and triggering of protective estrogen pathways [4]. We observed that AD/TTR$^{+/-}$ female mice present reduced levels of both brain testosterone and 17β-estradiol as compared to AD/TTR$^{+/+}$ females, whereas AD male mice of the different TTR genotypes do not exhibit altered levels of these hormones. Given the well-recognized role of sex steroid hormones in AD neuroprotection, our data suggest that the increased AD-like neuropathology (i.e., increased brain levels of FA-soluble $A\beta_{42}$) observed in female mice as a result of genetic reduction of TTR might, in part, be explained by the reduced levels of brain testosterone and 17β-estradiol.

The detrimental effects of the genetic reduction of TTR found especially in AD/TTR$^{+/-}$ female mice were not always observed in AD/TTR$^{-/-}$ animals. In fact, AD/TTR$^{-/-}$ female mice exhibit neither increased levels of brain FA-soluble $A\beta_{42}$, nor decreased levels of brain 17β-estradiol. One can hypothesize that AD mice bearing homozygous deletion of the TTR gene might undergo transcriptional remodeling through which compensatory mechanisms may occur, overcoming the deleterious effects of the total lack of TTR in the pathogenesis of AD. For instance, studies in non-transgenic TTR-null mice have described a few alterations resulting from the lack of TTR such as elevated levels of noradrenaline in the limbic forebrain [48], and elevated levels of neuropeptide Y (NPY) in the peripheral and central nervous systems [49]. Overall, the reason for AD/TTR$^{-/-}$ female mice not to present increased brain levels of FA-soluble $A\beta_{42}$ might lie on compensatory mechanisms including neuroprotection by non-decreased brain 17β-estradiol levels.

In the course of preparing this manuscript, a study was published showing a negative correlation between serum TTR levels and AD, as human serum TTR levels were shown to be decreased in AD patients as compared to non-demented controls. Further, non-demented men were shown to exhibit elevated levels

of circulating TTR when compared to non-demented women. This difference, however, was not observed in the AD case [50]. Our studies in a transgenic mouse model of AD have reached similar results, except that we have also found elevated levels of circulating TTR in AD male mice as compared to AD female littermates. It is essential to further investigate the role of TTR in AD using larger male and female cohorts of non-demented and AD patients, and also mild cognitive impairment patients to address the early stages of this disease.

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