Brief Communications

Accelerated Aβ Deposition in APPswe/PS1ΔE9 Mice with Hemizygous Deletions of *TTR* (Transthyretin)

Se Hoon Choi,[1] Susan N. Leight,[3] Virginia M.-Y. Lee,[3] Tong Li,[4] Philip C. Wong,[4] Jeffrey A. Johnson,[5] Maria J. Saraiva,[6] and Sangram S. Sisodia[2]

[1]Committee on Neurobiology and [2]Department of Neurobiology, University of Chicago, Chicago, Illinois 60637, [3]Department of Pathology and Laboratory Medicine, Center for Neurodegenerative Disease Research, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, [4]Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, [5]Neuroscience Training Program, University of Wisconsin, Madison, Wisconsin 53705, and [6]Institute of Molecular and Cellular Biology, Porto, Portugal

A cardinal pathological lesion of Alzheimer's disease (AD) is the deposition of amyloid β (Aβ) in the brain. We previously reported that exposing transgenic mice harboring *APPswe/PS1ΔE9* transgenes to an enriched environment resulted in reduced levels of Aβ peptides and deposition, findings that were correlated with an increase in the expression of *TTR*, encoding transthyretin (TTR). TTR is expressed at high levels in the choroid plexus and known to bind Aβ peptides and modulate their aggregation *in vitro* and *in vivo*. To explore the impact of TTR expression on Aβ levels and deposition *in vivo*, we crossed *ceAPPswe/PS1ΔE9* transgenic mice to mice with genetic ablations of *TTR*. We now report that the levels of detergent-soluble and formic acid-soluble levels of Aβ and deposition are elevated in the brains of *ceAPPswe/PS1ΔE9/TTR+/−* mice compared with age-matched *ceAPPswe/PS1ΔE9/TTR+/+* mice. Moreover, Aβ deposition is significantly accelerated in the hippocampus and cortex of *ceAPPswe/PS1ΔE9/TTR+/−* mice. Our results strongly suggest that TTR plays a critical role in modulating Aβ deposition *in vivo*.

Key words: **transthyretin; presenilin 1; transgenic mice; Aβ peptide; Alzheimer's disease; amyloid**

Introduction

Alzheimer's disease (AD) is associated with progressive memory loss and severe cognitive decline. These clinical features are associated with deposition of 40–42 amino acid β-amyloid (Aβ) peptides in the cerebral cortex and hippocampal formation. Aβ peptides are liberated from amyloid precursor proteins (APP), by the concerted action of BACE 1 (Vassar et al., 1999; Yan et al., 1999) and "γ"-secretase (Sisodia and St George-Hyslop, 2002; De Strooper, 2003). Early onset, familial forms of the disease (FAD) are caused by expression of mutant variants of APP, presenilin 1 (PS1), or presenilin 2 (PS2) (Price and Sisodia, 1998).

We previously reported that *APPswe/PS1ΔE9* transgenic mice exposed to an "enriched" environment exhibited reduced Aβ in the cortex and hippocampus compared with *APPswe/PS1ΔE9* mice maintained in standard housing conditions (Lazarov et al., 2005). Extending these findings, our high density DNA microarray profiling studies revealed that expression of *TTR*, a gene encoding transthyretin (TTR), was significantly upregulated in the brains of the enriched *APPswe/PS1ΔE9* transgenic mice. TTR, a homoteramеric protein of 127 amino acid subunits, is synthesized in the liver and by epithelial cells of the choroid plexus (CSF). Serum TTR is involved in the transport of thyroxine (Schreiber et al., 1990; Chanoine et al., 1992) and plasma retinol-binding protein complexed to vitamin A (Monaco, 2000). A series of biochemical and *in vivo* studies have revealed that TTR may also play a role in modulating Aβ aggregation both *in vitro* and *in vivo*. For example, Aβ forms stable complexes with TTR *in vitro* and prevents aggregation/amyloid formation (Schwarzman et al., 1994), whereas expression of human TTR in *Caenorhabditis elegans* rescues the morphological and behavioral alterations in worms expressing human Aβ peptides in the muscle (Link, 1995). Finally, microarray studies of hippocampi from 6-month-old *Tg2576* transgenic mice (Stein and Johnson, 2002), or cortical tissue from Tg2576/PS1$^{P264L/P264L}$ mice analyzed well before the onset of Aβ deposition (Wu et al., 2006), have revealed markedly elevated levels of *TTR* transcripts. These studies suggested that *TTR* gene expression was induced in response to overproduction of Aβ peptides (Stein and Johnson, 2002) and that overexpressed TTR would sequester Aβ species and thus preclude their subsequent aggregation and deposition.

To explore the impact of TTR expression on Aβ levels and deposition *in vivo*, we crossed mice that harbor FAD-linked *APPswe* and *PS1ΔE9* transgenes (Jankowsky et al., 2001) to mice with homozygous deletions of *TTR*. Brain Aβ levels and amyloid deposition in *ceAPPswe/PS1ΔE9/TTR+/+* or *ceAPPswe/PS1ΔE9/TTR+/−* mice were examined as a function of age. We now report that amyloid deposition is accelerated and Aβ levels are

Received April 27, 2007; accepted May 18, 2007.

This work was supported by National Institutes of Health Grants AG021494 and AG027854 (S.S.S.) and by the Edward H. Levi Fund, the Adler Foundation, and Cure Alzheimer's Fund. S.S.S. discloses that he is a paid Consultant of Neuropharma, Torrey Pines Therapeutics, and Eisai Research Labs but is not a shareholder in any company that is a maker or owner of an FDA-regulated drug or device. We thank John Robinson and Yi Huang for excellent technical assistance.

Correspondence should be addressed to Sangram S. Sisodia, Department of Neurobiology, The University of Chicago, 947 East 58th Street, AB 308, Chicago, IL 60637. E-mail: ssisodia@bsd.uchicago.edu.

DOI:10.1523/JNEUROSCI.1919-07.2007

 0270-6474/07/277006-05$15.00/0

significantly elevated in the brains of *ceAPPswe/PS1ΔE9/TTR+/−* compared with *ceAPPswe/PS1ΔE9/TTR+/+* mice at all ages examined. Our results strongly suggest that TTR plays a critical role in modulating Aβ deposition *in vivo*.

Materials and Methods

Transgenic mice. The *ceAPPswe/PS1*ΔE9 transgenic mouse line #57 (Jankowsky et al., 2001) was obtained from The Jackson Laboratory (Bar Harbor, ME). Mice with a targeted insertion into exon 2 of the *TTR* gene (Episkopou et al., 1993) were obtained from Dr. William Blaner (Columbia University, New York, NY).

Tissue processing. The mice were deeply anesthetized with a mixture of ketamine and xylazine and then decapitated. Isolated brains were bisected longitudinally, and hemispheres were separated and frozen on dry ice. The left hemisphere was used for examination of cerebral Aβ quantification by ELISA or protein expression by Western blot analysis. The right hemisphere was kept intact for immunohistochemistry (see below).

Sandwich ELISA analysis, immunocytochemistry, and Western blot analysis. The levels of cerebral Aβ were detected using ELISA protocols described previously (Suzuki et al., 1994; Turner et al., 1996). Immunostaining of sections, confocal imaging, and quantification of amyloid deposition were performed as described by Lazarov and et al. (2005). For Western blot analysis, brains were homogenized in detergent-containing buffer and subject to Western blot analysis as described previously (Lazarov et al., 2005). APP and APP-C-terminal fragments (CTFs) were detected using Ab369, raised against the APP C terminus (Xu et al., 1997). Full-length and soluble APP derivatives were detected with Ab22C11, a monoclonal antibody raised against the extracellular domain of APP (Hilbich et al., 1993), whereas the soluble derivative derived from APPswe was detected with Ab192swe (Haass et al., 1995). TTR was detected with rabbit anti-mouse TTR antibody (Sousa et al., 2007). Mouse anti-α-tubulin was used to normalize for protein loading.

Statistical analysis. Data are expressed as mean values ± SEM. Student's *t* test and ANOVA tests were applied to study the relationship between the different variables. Values of $p < 0.05$ were considered to be significant.

Results

Increased levels of cerebral Aβ levels in brains of hemizygous *TTR*-deficient mice harboring *ceAPPswe/PS1ΔE9* transgenes

The *ceAPPswe/PS1*ΔE9 transgenic mouse line #57 (Jankowsky et al., 2001) harbors cointegrated *APPswe* and *PS1ΔE9* transgenes are driven by the mouse prion promoter (PrP). The *APPswe* transgene encodes a chimeric mouse-human APP695 harboring a human Aβ domain and mutations (K595N, M596L) linked to Swedish FAD pedigrees (*APPswe*), and the human *PS1*ΔE9 transgene is linked to familial AD (Borchelt et al., 1996, 1997; Lee et al., 1997). The bigenic *ceAPPswe/PS1ΔE9* were crossed with *TTR-deficient* mice (Episkopou et al., 1993) to generate *ceAPPswe/PS1ΔE9/TTR+/−* or *ceAPPswe/PS1ΔE9/TTR+/+* mice. Although we obtained a number of *ceAPPswe/PS1ΔE9/TTR−/−* in the course of breeding, we chose not to evaluate Aβ levels and deposition in these animals to avoid indirect effects of *TTR-deficiency* that could confound our interpretations. For example, *TTR-deficient* mice exhibit significantly elevated expression of mRNA encoding peptidylglycine α-amidating monooxygenase, the rate-limiting enzyme in neuropeptide maturation, and hence, leads to elevated levels of neuropeptide Y (NPY) (Nunes et al., 2006). NPY acts on energy homeostasis by increasing white adipose tissue lipoprotein lipase and decreasing thermogenesis. Indeed, *TTR-deficient* mice exhibit decreased body temperature, increased carbohydrate consumption, and preference (Nunes et al., 2006), and we observed that both *TTR−/−* and *ceAPPswe/PS1ΔE9/TTR−/−* mice are lethargic and exhibit increased body weight relative to their hemizygous *TTR* (or *ceAPPswe/PS1ΔE9/TTR+/−*) or *TTR+/+* (or *ceAPPswe/PS1ΔE9/TTR+/+*) littermates (S.H.C. and S.S.S., personal observations). In addition, the limbic forebrain of *TTR-deficient* mice exhibits significantly elevated levels of noradrenaline (Sousa et al., 2004), a catecholamine neurotransmitter that has been shown to modulate Aβ burden in a transgenic mouse model of AD (Kalinin et al., 2007). Finally, because retinol and thyroid hormones are essential for normal mammalian brain physiology and are particularly critical during development (Porterfield and Hendrich, 1993) and because TTR is the only thyroid hormone-binding protein found at a substantial level in the CSF (Herbert et al., 1986), it is possible that TTR reduction could cause developmental abnormalities.

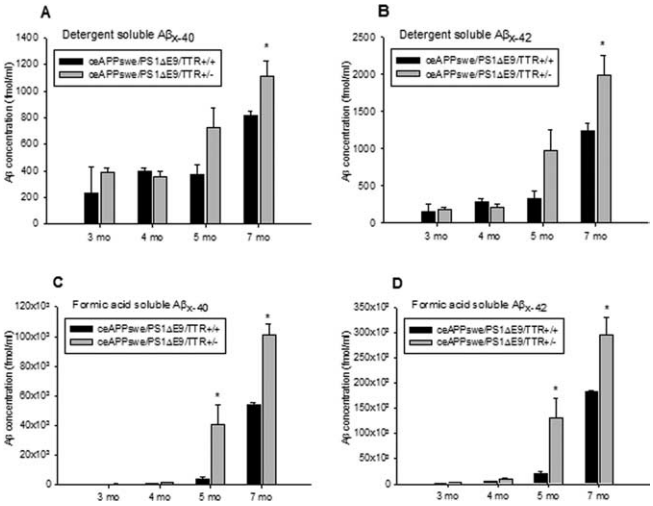

Figure 1. Genetic reduction of *TTR* elevates steady-state levels of cerebral Aβ in the brains of *ceAPPswe/PS1ΔE9* mice. ***A***, ***B***, Levels of detergent-soluble $A\beta_{X\text{-}40}$ and $A\beta_{X\text{-}42}$ in brain extracts of *ceAPPswe/PS1ΔE9/TTR+/−* and *ceAPPswe/PS1ΔE9/TTR+/+* mice at 7 month time point. ***C***, ***D***, Levels of formic acid-soluble $A\beta_{X\text{-}40}$ and $A\beta_{X\text{-}42}$ in brain extracts of *ceAPPswe/PS1ΔE9/TTR+/−* and *ceAPPswe/PS1ΔE9/TTR+/+* mice at 5 and 7 month time points. The asterisk indicates a significant difference from *ceAPPswe/PS1ΔE9/TTR+/+* at $p < 0.05$ (3–4 animals/group). Error bars represent SE.

To examine the influence of TTR expression on cerebral steady-state levels of Aβ, we generated mice that harbored *ceAPPswe/PS1ΔE9* transgenes on a *TTR+/−* background. Cohorts of these animals were aged for either 3, 4, 5, or 7 months. Aβ levels in detergent and formic acid extracts of hemibrains of either *ceAPPswe/PS1ΔE9/TTR+/+* or *ceAPPswe/PS1ΔE9/TTR+/−* mice were quantified using sandwich ELISA analysis. We show that 5-month-old *ceAPPswe/PS1ΔE9/TTR+/−* mice exhibit elevated steady-state levels of detergent soluble $A\beta_{X\text{-}40}$ and $A\beta_{X\text{-}42}$ compared with 5-month-old *ceAPPswe/PS1ΔE9/TTR+/+* mice (Fig. 1*A* and *B*, respectively), but the differences failed to reach significance. In contrast, the levels of detergent soluble $A\beta_{X\text{-}40}$ and $A\beta_{X\text{-}42}$ peptides were significantly elevated in the brains of 7-month-old *ceAPPswe/PS1ΔE9/TTR+/−* compared with *ceAPPswe/PS1ΔE9/TTR+/+* mice (Fig. 1*A*, *B*). These studies suggested that a fraction of Aβ peptides that would otherwise aggregate and deposit remain in a detergent extractable state when *TTR* gene dosage is reduced. Most notably, the levels of formic acid-soluble $A\beta_{X\text{-}40}$ (Fig. 1*C*) and $A\beta_{X\text{-}42}$ (Fig. 1*D*) peptides were significantly elevated in the brains of the *ceAPPswe/PS1ΔE9/TTR+/−* compared with *ceAPPswe/PS1ΔE9/TTR+/+* mice at both the 5 and 7 month time points, findings that would argue in support for a role of TTR in preventing Aβ aggregation and subsequent deposition.

Genetic reduction of *TTR* leads to accelerated Aβ deposition in *ceAPPswe/PS1ΔE9* mice

To examine the influence on reduced expression of *TTR* on amyloid deposition, brain sections from *ceAPPswe/PS1ΔE9/TTR+/−* and *ceAPPswe/PS1ΔE9/TTR+/+* mice were probed with Aβ-specific 3D6 antibodies (Kim et al., 2001), and bound antibodies were detected by fluorescently labeled secondary antibodies. Confocal images of amyloid burden were quantified by morphometric methods.

Although we failed to detect any significant difference in the levels of Aβ deposition between genotypes at the 3 and 4 month time points, we observed a dramatic and significant elevation in amyloid deposition in the cortex of *ceAPPswe/PS1ΔE9/TTR+/−* at 5 and 7 months compared with *ceAPPswe/PS1ΔE9/TTR+/+* mice (Fig. 2*A*, compare *f* and *g* with *b* and *c*, respectively). Similarly, amyloid deposition in the hippocampus of 7-month-old *ceAPPswe/PS1ΔE9/TTR+/−* was clearly elevated compared with *ceAPPswe/PS1ΔE9/TTR+/+* mice (Fig. 2*A*, compare *h* and *d*, respectively). Morphometric analysis confirmed that *ceAPPswe/PS1ΔE9/TTR+/−* exhibited significantly higher amyloid burden in the cortex (Fig. 2*Ba*) and hippocampus (Fig. 2*Bb*) compared with *ceAPPswe/PS1ΔE9/TTR+/+* mice at the 5- and 7-month-old time points. We then costained brain sections from 5-month-old mice with thioflavin S and 3D6 antibodies (Fig. 2*C*). These studies revealed that thioflavin S staining in *ceAPPswe/PS1ΔE9/TTR+/−* was elevated compared with the *ceAPPswe/PS1ΔE9/TTR+/+* mice, and that this staining was at the core of the amyloid deposits in both cases.

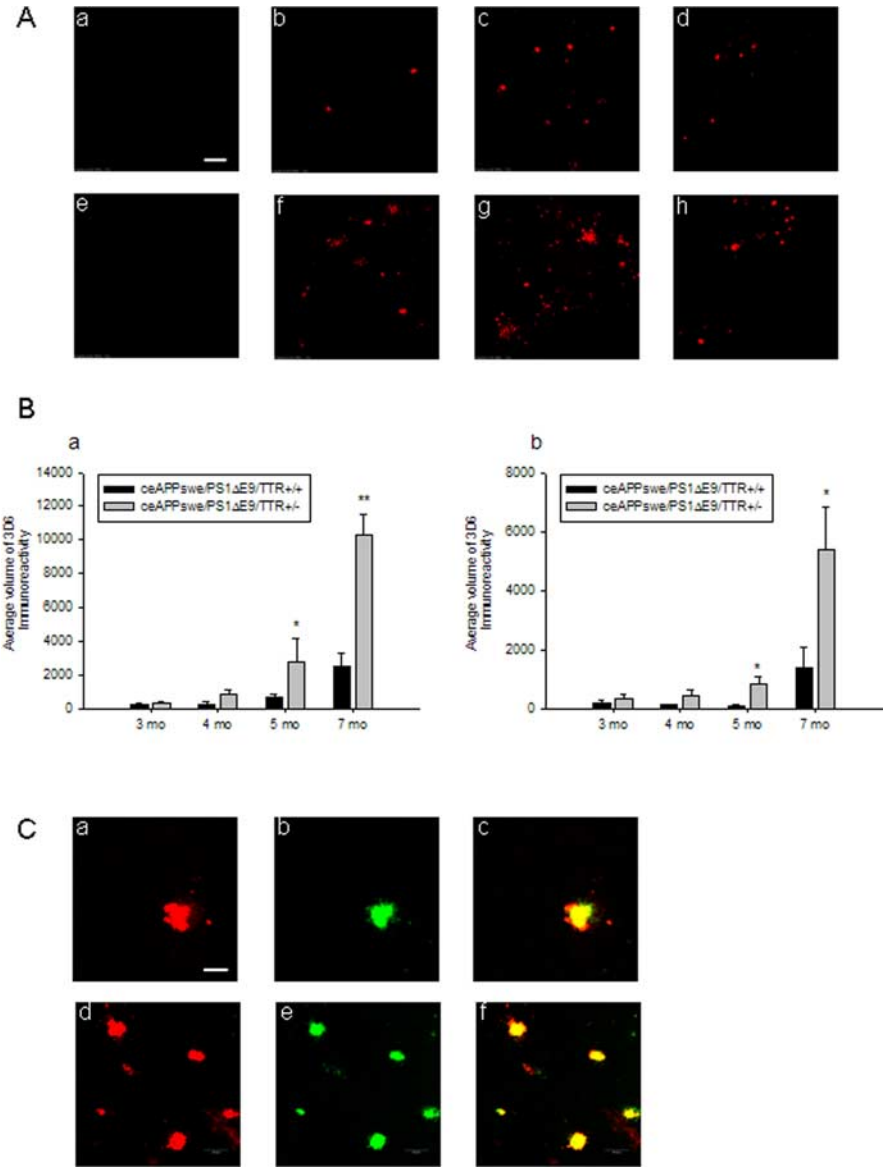

Figure 2. Increased amyloid deposition in the cortex and hippocampus of *ceAPPswe/PS1ΔE9/TTR+/−*. ***A***, Immunohistochemical analysis of brain sections of *APPswe/PS1ΔE9/TTR+/+* (***a–c***, cortex, 4, 5, and 7 month; ***d***, hippocampus, 7 month) and *ceAPPswe/PS1ΔE9/TTR+/−* (***e***, ***f***, cortex, 4, 5, and 7 month; ***h***, hippocampus, 7 month) mice immunolabeled with anti-Aβ 3D6 antibodies. Scale bar, 200 μm. ***B***, Quantitative analysis of volume of amyloid burden in the cortex (***a***) and the hippocampus (***b***) of *APPswe/PS1ΔE9/TTR+/−* versus *APPswe/PS1ΔE9/TTR+/+* mice. Volume is in arbitrary units (mean voxel count ± SE). The asterisk indicates a significant difference from *ceAPPswe/PS1ΔE9/TTR+/+* at $*p < 0.05$; $**p < 0.01$ (4 animals/group). Error bars represent SE. ***C***, Thioflavine S-stained amyloid deposits in the cortex of 5-month-old *ceAPPswe/PS1ΔE9/TTR+/−* versus 5-month-old *ceAPPswe/PS1ΔE9/TTR+/+* mice. Costaining of brain sections with 3D6 antibodies (***a***, ***d***) and thioflavine S (***b***, ***e***) and overlap (***c***, ***f***). Scale bar, 50 μm.

Genetic reduction of *TTR* does not alter APP processing in *ceAPPswePS1ΔE9* mice

To assess whether alterations in APP processing might account for the observed elevations of Aβ levels in *ceAPPswe/PS1ΔE9/TTR+/−*, we prepared detergent-soluble extracts from these animals and *ceAPPswe/PS1ΔE9/TTR+/+* mice and subjected these preparations to Western blot analysis. To confirm the TTR genotype, extracts were probed with an anti-TTR antibody. We demonstrate that an ~14 kDa TTR antibody-immunoreactive polypeptide is present in extracts from brains of *ceAPPswe/PS1ΔE9/TTR+/−*, and this species is absent in extracts from *ceAPPswe/PS1ΔE9/TTR−/−* mice (Fig. 3*A*, lanes 1 and 2, respectively). The ~14 kDa immunoreactive species is also present in extracts from brains of *ceAPPswe/PS1ΔE9/TTR+/+* mice at all ages examined (Fig. 3, lanes 3, 5, 7), and the levels are clearly lower in brains of *ceAPPswe/PS1ΔE9/TTR+/−* mice at all ages (Fig. 3, lanes 4, 6, 8). Analysis of Western blots using Ab369 failed to disclose any differences in the levels of full-length APP (APP-FL) or membrane-tethered APP C-terminal derivates (APP-CTFs) in extracts prepared either from *ceAPPswe/PS1ΔE9/TTR+/+* (Fig. 3*B*, lanes 3, 5, 7) or *ceAPPswe/PS1ΔE9/TTR+/−* (Fig. 3*B*, lanes 1, 4, 6, 8) mice. Furthermore, we failed to observe any differences in total levels of full-length APP and soluble derivatives using Ab22C11, raised against the extracellular domain of APP, or soluble Swedish βAPPs detected by Ab192swe between *ceAPPswe/PS1ΔE9/TTR+/+* and *ceAPPswe/PS1ΔE9/TTR+/−* mice at all time points. These results suggest that genetic reduction of *TTR* in *ceAPPswe/PS1ΔE9* mice does not result in a discernable impact on APP processing at steady state.

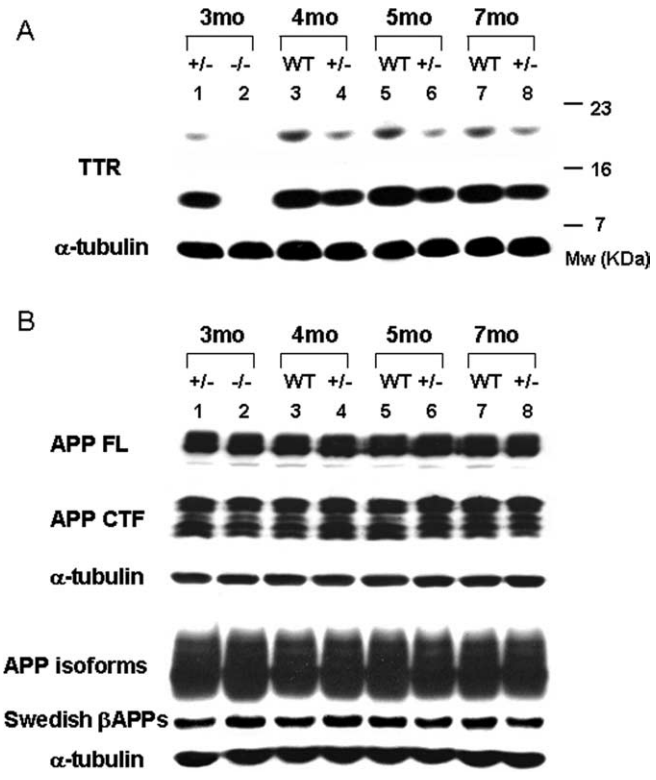

Figure 3. Western blot analysis of protein extracts of brains of *APPswe/PS1ΔE9/TTR+/+* (WT), *APPswe/PS1ΔE9/TTR+/−* (+/−), or *APPswe/PS1ΔE9/TTR−/−* (−/−) mice. ***A***, Western blot analysis using anti-TTR antibody. ***B***, APP processing. Lane 1, APP-FL; Lane 2, APP-CTFs; Lane 3, α-tubulin; Lane 4, full-length and soluble APP; Lane 5, soluble Swedish βAPPs.

Discussion

A series of preceding biochemical and transgenic mouse studies have provided compelling evidence that TTR plays a role in modulating Aβ aggregation and deposition. For example, Aβ forms stable complexes with TTR and inhibits aggregation *in vitro* (Schwarzman et al., 1994), and expression of human TTR rescues the morphological and behavioral alterations in *C. elegans* that express human Aβ (Link, 1995). Indeed, studies have revealed that the levels of brain TTR were significantly lower in human AD patients compared with age-matched controls and negatively correlated with the abundance of amyloid plaques (Serot et al., 1997; Merched et al., 1998). Supporting these lines of evidence, DNA microarray studies in transgenic mice have revealed that expression of *TTR* mRNA is markedly elevated well before the onset of amyloid deposition (Stein and Johnson, 2002; Costa et al., 2006), suggesting that upregulation of TTR is a physiological response to elevated Aβ levels that in turn blocks their aggregation. To these studies, we demonstrated that relative to *APPswe/PS1ΔE9* mice maintained in standard housing conditions, *APPswe/PS1ΔE9* transgenic mice exposed to an "enriched" environment exhibited reduced Aβ deposition in the cortex and hippocampus (Lazarov et al., 2005), a setting in which steady-state levels of *TTR* transcripts were elevated in the brain. Collectively, these latter data provided support for the notion that TTR plays an important role in regulating Aβ deposition *in vivo*.

In the present study, we tested the hypothesis that lowering brain levels of TTR, a protein that can sequester Aβ peptides and prevent fibril formation, would accelerate amyloid deposition in *APPswe/PS1*ΔE9 mice, and we now offer several important insights. First, we demonstrate that the levels of detergent-soluble Aβ peptides are elevated in the brains of *ceAPPswe/PS1ΔE9/TTR+/−* compared with *ceAPP/PS1ΔE9/TTR+/+* mice at all time points tested. Although these studies would suggest that lowering *TTR* levels might affect APP processing, we have not observed any alterations in APP metabolism in steady-state Western blot studies. Our interpretation of the finding of elevated soluble Aβ levels in *APPswe/PS1ΔE9/TTR+/−* mice is that these species are either oligomeric assemblies that are not deposited or represent the amorphous nonfibrillar assemblies that are present in the "penumbra" of the thioflavin-positive deposits. In any event, our confocal immunofluorescence and morphometric studies reveal that amyloid burden both in the cortex and hippocampus of *ceAPPswe/PS1ΔE9/TTR+/−* was dramatically increased compared with *ceAPPswe/PS1ΔE9/+/+* mice from the 5 month time point onwards. These morphological studies were validated by sandwich ELISA analyses in which we observed that the levels of formic acid-soluble $A\beta_{X\text{-}40}$ and $A\beta_{X\text{-}42}$ peptides are markedly elevated in the brains of the *ceAPPswe/PS1ΔE9/TTR+/−* mice at all time points. Collectively, our immunohistochemical and biochemical studies convincingly demonstrate that genetic reduction of *TTR* elevates Aβ deposition in the brains of *ceAPPswe/PS1ΔE9/TTR+/−* mice.

The nature of the interaction(s) between TTR and Aβ and the mechanism(s) by which TTR alters the aggregation of Aβ *in vivo* are not fully understood. Liu and Murphy (2006) reported that TTR significantly decreased the rate of aggregation in a strong concentration-dependent manner. Moreover, the region near tryptophan 41 of TTR is involved in binding to Aβ aggregates by Trp fluorescence quenching experiments (Liu and Murphy, 2006), a finding consistent with studies showing that peptide fragments containing Trp41 of TTR bind to Aβ (Schwarzman et al., 2005). Although the domain(s) within Aβ that bind to TTR are not known, future efforts to obtain high-resolution information pertaining to the nature of Aβ-TTR interactions would be of considerable interest.

Finally, the sites within the brain where Aβ binds to TTR have not been fully resolved. Recent studies using laser dissection microscopy and PCR studies have clearly demonstrated that *TTR* transcripts are excluded from the brain parenchyma but restricted to choroid plexus (Sousa et al., 2007). These findings would argue that $A\beta_{40/42}$, produced in the brain parenchyma, is subject to efflux into the CSF (Seubert et al., 1992; Shoji et al., 1992) where the peptides encounter TTR that is secreted from the choroid plexus and subsequently sequestered. Although this latter notion is attractive, it is important to note that TTR is not the only protein that binds Aβ in CSF. Indeed, evidence has accumulated that α-1-antichymotrypsin, apolipoprotein J, and apolipoprotein E, proteins present in CSF, can also bind Aβ *in vitro* and, in certain cases, *in vivo* (Abraham et al., 1988; Ghiso et al., 1993; Strittmatter et al., 1993), and the respective contributions of each of these proteins to Aβ clearance remains to be established. Notwithstanding the importance of these latter species to Aβ metabolism *in vivo*, our data supporting a role for TTR in modulating Aβ deposition suggest that approaches aimed at enhancing Aβ sequestration and clearance with TTR as a template would be of significant therapeutic value.

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