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Biochemical and Morphological Characterization of the A β PP/PS/Tau Triple Transgenic Mouse Model and its Relevance to Sporadic Alzheimer's Disease

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Abstract

Transgenic (Tg) mouse models of Alzheimer's disease (AD) have been genetically altered with human familial AD genes driven by powerful promoters. However, a Tg model must accurately mirror the pathogenesis of the human disease, not merely the signature amyloid and/or tau pathology, as such hallmarks can arise via multiple convergent or even by pathogenic mechanisms unrelated to human sporadic AD. The 3 $\times$ Tg-AD mouse simultaneously expresses 3 rare familial mutant genes that in humans independently produce devastating amyloid- β protein precursor (A β PP), presenilin-1, and frontotemporal dementias; hence, technically speaking, these mice are not a model of sporadic AD, but are informative in assessing co-evolving amyloid and tau pathologies. While end-stage amyloid and tau pathologies in 3 $\times$ Tg-AD mice are similar to those observed in sporadic AD, the pathophysiological mechanisms leading to these lesions are quite different. Comprehensive biochemical and morphological characterizations are important to gauge the predictive value of Tg mice. Investigation of A β PP, amyloid- β (A β), and tau in the 3 $\times$ Tg-AD model demonstrates AD-like pathology with some key differences compared to human sporadic AD. The biochemical dissection of A β PP reveals different cleavage patterns of the C-terminus of A β PP when compared to human AD, suggesting divergent pathogenic mechanisms. Human tau is concomitantly expressed with A β PP/A β from an early age while abundant extracellular amyloid plaques and paired helical filaments are manifested from 18 months on. Understanding the strengths and limitations of Tg mouse AD models through rigorous biochemical, pathological, and functional analyses will facilitate the derivation of models that better approximate human sporadic AD.

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Keywords

Alzheimer's disease; amyloid- β precursor protein; presenilin; tau; transgenic mice

INTRODUCTION

The neuropathological landmarks of Alzheimer's disease (AD) are the presence of abundant extracellular amyloid- β (A β) deposits in the brain gray matter and vasculature, and neurofibrillary tangles (NFT) mainly composed of polymerized tau. AD has been classified into two major categories: 1) familial; attributed to specific mutations in the amyloid- β protein precursor (A β PP) and presenilin (PS) genes representing 3% of all AD cases (reviewed in [1]), and 2) sporadic; accounting for 97% of all AD cases and with no clearly defined genetic basis. However, the likelihood of developing sporadic AD is strongly linked to the apolipoprotein E ϵ 4 genotype [2]. The 3 $\times$ Tg-AD mouse expresses three separate human genes each with mutations that cause familial AD in humans [3]. These mice reproduce a hierarchical sequence of pathological events thought to unfold in sporadic AD in which initial synaptic dysfunction, due to increased intracellular A β , is followed by amyloid plaque deposition and subsequent NFT accumulation [3, 4].

The 3 $\times$ Tg-AD model, carrying the A β PP^{KM670-671NL}₇₇₀, the PS1^{M146V}, and the tau^{P301 L} mutations, has been very informative in terms of molecular kinetics and magnitude of intra- and extracellular A β and tau/phosphorylated-tau pathological lesions, reaction to immunotherapy, cognitive outcomes, inflammatory responses, and neurochemical and neuroanatomical changes. Aged 3 $\times$ Tg-AD mice have been actively and passively immunized against A β as well as subjected to direct intracerebral injection of anti-A β antibodies. Active immunization did not significantly reduce thioflavine-positive plaques, while peripheral antibody perfusion and direct injection did [5]. Immunotherapy did not reduce NFT, but caused a decrease in the levels of both soluble tau and A β that correlated with cognitive improvements [5, 6]. However, despite the promising results in Tg mice, a clear demonstration of disease-modifying capability has yet to be replicated in clinical trials [7–13]. This failure may reflect significant anatomical and physiological species differences, the artificial nature of the model and the multifactorial complexity of AD [10].

We performed longitudinal histological and biochemical evaluations of the A β and tau species in 3 $\times$ Tg-AD mouse brains. In this investigation we compare: 1) 3 $\times$ Tg-AD mice to human sporadic AD to assess how closely this model, based on massive transcriptional overexpression of mutant familial AD genes, conforms to the sporadic AD pattern, and 2) the longitudinal changes in A β PP/A β and tau in three different age groups of 3 $\times$ Tg-AD mice.

METHODS

Mouse and human tissues

The 3 $\times$ Tg-AD mice were provided by Frank LaFerla (University of California, Irvine). Several lines of 3 $\times$ Tg-AD mice were generated by Oddo et al., including the B1 line used in this study [3]. Homozygous 3 $\times$ Tg-AD mice were monogamously mated to produce offspring, which were housed until sacrifice at the designated age. All genotypes were confirmed by PCR. Control wild-type (wt) mice were age-matched 129/C57BL/6. All mice were housed and bred in accordance with University of Rochester or Barrow Neurological Institute, St. Joseph's Hospital and Medical Center requirements for animal welfare and care. For comparative studies, frontal cerebral cortex was acquired from 3 AD (2 females

and 1 male, average age = 81 years) and 3 non-demented control (NDC) cases (2 females and 1 male, average age, 80 years) were included. Human gray matter was obtained in the immediate postmortem from the Brain and Body Donation Bank at Banner Sun Health Research Institute in Sun City, AZ. A battery of histochemical diagnostic stains, that included Campbell-Switzer silver, thioflavine-S and Gallyas stains as well as apolipoprotein E genotyping, was performed for each AD and NDC case used in this study [14]. The Braak Stage was assessed for the 3 NDC (I, I, II) and 3 AD (IV, V, V) cases as well as the plaque density: moderate in 1 case and frequent in 2 cases of the AD cohort, and zero in 2 cases and sparse in 1 case of the NDC group. In addition, the diagnosis of AD was assessed following the Consortium to Establish a Registry for Alzheimer's disease [15] and the National Institute on Aging and Regan Institute [16].

Immunohistochemistry

Mouse brains were processed for immunohistochemistry (IHC) as described previously [17, 18], and imaged using an Olympus AX-70 microscope with a motorized stage (Olympus, Center Valley, PA). The following antibodies were used at the designated working dilutions: anti-amyloid- β protein precursor A4, corresponding to the NPXY motif of human A β PP, (Clone Y188; AbCam, Cambridge, MA, 1 : 750); anti-A β_{1-42} clone 12F4 reactive to the C-terminus of A β and specific for the isoform ending at amino acid 42 (Covance, Berkeley, CA, 1 : 1000); anti-A β_{1-42} polyclonal antibody for intracellular A β staining (Invitrogen, Carlsbad, CA, 1 : 1000); anti-human tau HT7, reactive to human tau residues 159 to 163 (Pierce, Rockford, IL; 1 : 200); anti-human phosphorylated tau PHF-1 (gift from Dr. Peter Davies, Albert Einstein College of Medicine; 1 : 30).

Congo red stain

Brain sections were processed for Congo red histochemistry as described previously [19], and imaged using an Olympus AX-70 microscope with polarized light filters.

Quantification of soluble and insoluble human A β by ELISA

All steps were performed at 4°C. One hundred mg of mouse cerebrum were gently homogenized with a Teflon tissue grinder in 6 volumes of 20 mM Tris-HCl, 5 mM EDTA, pH 7.8 with a protease inhibitor cocktail (PIC, Roche, Mannheim, Germany) and centrifuged at $430,000 \times g$ for 20 min in a Beckman TLA 120.2 rotor (Fullerton, CA). The supernatant, containing the soluble A β , was collected and total protein measured with a Micro BCA protein assay (Pierce). The pellets were reconstituted in 500 μ l of 5 M guanidine hydrochloride (GHC), 50 mM Tris-HCl, pH 8.0 with an electric grinder (Omni International) and shaken for 4 h. The above centrifugation was repeated, the supernatant was collected and total protein was also determined by the Pierce Micro BCA protein assay. Both Tris-soluble and GHC-soluble human A β_{40} and A β_{42} were measured by ELISAs from Invitrogen and Innogenetics (Gent, Belgium), respectively, and according to the manufacturers' instructions.

Quantification of human total tau and human phosphorylated tau (p-tau) by ELISA

All steps were performed at 4°C. Each mouse cerebrum was homogenized in 8 volumes of 5 M GHC, 50 mM Tris-HCl, pH 8.0 with an electric grinder (Omni International) and shaken for 4 h. The GHC homogenates were then diluted 10 $\times$ in phosphate buffered saline plus PIC and centrifuged at $16,000 \times g$ for 20 min. The supernatant was collected and total protein determined with a Micro BCA protein assay (Pierce). Human total tau and human p-tau (S396) were measured with ELISA kits obtained from Invitrogen, following the manufacturers' instructions.

Western blot analysis

A detailed account of the protocol for Western blots (WB) is described elsewhere [20, 21]. Briefly, brain tissue was homogenized in RIPA buffer (Sigma), containing a PIC (Roche). The proteins were separated by SDS electrophoresis and then transferred onto nitrocellulose membranes. The following primary antibodies were used: 22C11 (recognizes amino acids 66–81 of human and mouse A β PP; Millipore, Billerica, MA), CT9APP (recognizes the last 9 amino acids of human and mouse A β PP; Millipore) and anti-tau HT7 (recognizes amino acids 159–163 of human tau; Pierce). After detection, all membranes were stripped and re-probed with rabbit or mouse anti-actin (Abcam, Cambridge, MA) for normalization of total protein. A GS-800 calibrated densitometer (Bio-Rad, Hercules, CA) and Quantity One software (Bio-Rad) were used for scanning and quantitative analysis.

In addition, WB were performed on high performance liquid chromatography (HPLC) fractions as described above and elsewhere [20, 21] with anti-A β ₄₀ and anti-A β ₄₂ (Invitrogen) and CT9APP (Millipore), and anti-tau (Pierce) as the primary antibodies.

Fast protein liquid chromatography (FPLC)

The cerebellum and brainstem of mouse brains were removed and the remaining cerebrum pooled from multiple 3 $\times$ Tg-AD mice of similar age. Brain tissue was solubilized in 90% glass-distilled formic acid (GDFA) and the acid supernatant submitted to size-exclusion FPLC using a Superose 12 column, as previously described [8, 21]. Three different molecular weight fractions were collected (1 = 300–70 kDa; 2 = 70–10 kDa; 3 = 10–2 kDa) and the volume reduced by vacuum centrifugation (SpeedVac, Savant Instruments Inc., Farmingdale NY).

High performance liquid chromatography (HPLC)

The FPLC fractions were further purified by HPLC using a reverse-phase C8 column (4.6 $\times$ 250 mm, Zorbax SB, Mac Mod) maintained at 80°C. For further technical details, see [8]. A total of 9 fractions were collected. To eliminate the trifluoroacetic acid (TFA) and acetonitrile solvent, the fractions were washed with water (200 μ l each) and the volume reduced by vacuum centrifugation (SpeedVac) a total of three times. Each fraction was then submitted to WB (see above).

RESULTS

Histological assessment

Immunohistochemistry was employed to document the temporal and regional evolution of A β PP/PS/tau transgene expression related to amyloid deposition, intracellular A β , tau and p-tau within the hippocampi of 6, 12, and 18 month-old male 3 $\times$ Tg-AD mice. As reported previously [17], we confirm here that 6 month-old 3 $\times$ Tg-AD mice exhibit robust hA β PP^{SWE} expression (Figs. 1A and D) and show evidence of intracellular A β ₄₂ accumulation (Figs. 1C and F). Neither extracellular A β ₄₂ deposition (Figs. 1B and C) nor Congo Red-stained deposits (Figs. 1G, J and K) were observed in these young mice. Similarly, while evidence of human tau^{P301 L} mutant transgene expression can be readily observed (Figs. 1H and L), pathogenic paired helical filaments (PHF) were not detectable in 6 month-old 3 $\times$ Tg-AD mice (Figs. 1I and M). Twelve month-old 3 $\times$ Tg-AD mice continue to show immunohistochemical evidence of hA β PP^{SWE} and tau^{P301 L} transgene expression (Figs. 2A and D, and H and L, respectively). While intracellular A β ₄₂ staining in male 3 $\times$ Tg-AD mice is significant at this age (Figs. 2C and F), extracellular A β ₄₂ deposits (Figs. 2B and E), congophilic plaques (Figs. 2G, J and K) and intracellular PHF pathologies remain noticeably absent (Figs. 2I and M). By 18 months of age, hA β PP^{SWE} and tau^{P301 L} transgene expression continues (Figs. 3A, D, H and L), while extracellular A β ₄₂ burden is severe with

large dense plaques apparent in the caudal hippocampus at the area of the subiculum/CA1 interchange (Figs. 3B and E). To further assess the status of insoluble A β , Congo red staining was used. A characteristic yellow-green birefringence is observed upon visualizing Congo red-stained amyloid plaques in the brains of 18 month-old 3 $\times$ Tg-AD mice under polarized light (Figs. 3G, J and K). Paired helical filament pathology, as detected by PHF-1 IHC, is also quite apparent in the pyramidal neurons of the 3 $\times$ Tg-AD mouse hippocampus at 18 months of age (Figs. 3I and M). These immunohistochemical and histological observations regarding the age-related progression of hallmark AD pathologies in 3 $\times$ Tg-AD mice, while temporally delayed in relation to what was initially described for this mouse model [3, 4], are largely consistent with subsequent reports [17, 18, 22].

Human A β_{40} and A β_{42} ELISAs

Immunoassay analyses revealed a wide range of variability in the levels of A β_{40} and A β_{42} peptides among the 3 $\times$ Tg-AD mice at 7, 12–16, and 18 months of age in the Tris-soluble and GHCl-soluble fractions (Fig. 4). In agreement with other studies in which sexual dimorphism plays an important role in determining the A β levels and neuropathology severity [23, 24], the female rodents contained higher values of A β peptides. In the three age groups studied, the total A β_{40} detected in the Tris-soluble fractions, demonstrated a wide distribution of values among the 7, 12–16, and 18 month-old 3 $\times$ Tg-AD mice (Fig. 4A). Similar patterns were also seen in the A β_{42} Tris-soluble fraction (Fig. 4B). There was a large difference between 12 and 16 months of age in A β production as there is a large increase during these 4 months (Figs. 4A and B). More extreme fluctuations in A β_{40} and A β_{42} levels were observed in the GHCl-soluble fractions (Figs. 4C and 4D), respectively. Interestingly, there were two specific animals (12 and 18 months) in which the values for A β_{42} were below the levels of the immunoassay detection and hence are reported as zero. The wt mice did not harbor detectable levels of human A β peptides in our immunoassays.

Human total tau and p-tau (Ser396) ELISAs

GHCl-soluble total tau levels decreased with age with an average of 508, 481 and 354 ng/mg total protein for the 7 months, 13–16 months, and 18 months 3 $\times$ Tg-AD mice (Fig. 4E). A trend of decreased total tau levels, as the 3 $\times$ Tg-AD mice age, was also observed in the WB (see below). Likewise, the p-tau ELISAs revealed a decrease in levels with age (Fig. 4F). Figures 4E and F also revealed that the female mice had more tau and p-tau levels than the males, which was also the case for the A β ELISA results (Figs. 4A–D), however, we recognize that the sample set is too small to reach conclusions related to the gender of the Tg animals. There was minimal cross-reactivity in endogenous wt mouse tau and p-tau human ELISA kits being, on average 0.853 ng/mg total protein and 2.791 ng/mg total protein, respectively.

Western blots of cerebral cortex homogenates

The 22C11 antibody reacts with the N-terminal domain of human and mouse A β PP and revealed a heavy band corresponding to the complete A β PP (~110 kDa) which was on the average slightly more prominent in the 7 and 12–16 months 3 $\times$ Tg-AD mice compared to the wt mice of the same age (Fig. 5A). This was probably due to a small elevation in the expression of the human A β PP in 3 $\times$ Tg-AD mice over the endogenous mouse A β PP seen in the wt mice. The amount of A β PP at 18 months appeared to be very similar in the 3 $\times$ Tg-AD and wt mice, suggesting that the expression of the A β PP transgene may also be reduced with time or increasingly degraded. A single band of ~25 kDa was visible in all the human specimens, but appeared as a doublet in the 3 $\times$ Tg-AD and wt mice (Fig. 5A) being less apparent in the latter group. In addition, the 3 $\times$ Tg-AD and wt mice harbored a faint species at ~20 kDa that was completely absent in the human homogenates (Fig. 5A). Interestingly, a very different pattern of C-terminal A β PP (CT-A β PP) processing emerged between the

3×Tg-AD mouse and human samples (Fig. 5B), as detected by an antibody against the last 9 amino acids of AβPP (CT9AβPP). The AβPP CT99 or CT83 fragments resulting from the β- and α-secretase cleavage, corresponding to a ~13 kDa band in AD and NDC specimens, was conspicuously absent or very faint in 3×Tg-AD and wt mice of all ages. In contrast, the 12–16 month-old 3×Tg-AD and wt mice showed bands at ~35 and ~24 kDa that were much less abundant in human specimens, suggesting a different sequence of AβPP degradation in mice. These bands were less intense or absent in younger and older 3×Tg-AD mice, suggesting an alteration in the temporal regulation of AβPP cleavage or an alternative activation of other AβPP proteases. Bands at ~110, ~80, and ~40 kDa did not differ dramatically amongst 3×Tg-AD, wt and human samples, but the CT40 kDa band was more abundant in the 18 month-old 3×Tg-AD mice relative to wt mice (Fig. 5B).

The 3×Tg-AD mouse WB patterns demonstrated that the expression of the human tau transgene, represented by a ~55 kDa band, decreases as the mice aged (Fig. 5C) in agreement with the tau values quantitatively determined by ELISA. The ~55 kDa peptide was abundant in humans as well as in 3×Tg-AD mice, but substantially reduced in the wt mice. AD and NDC cases exhibited significantly higher levels of ~55 kDa tau, than those expressed in the 3×Tg-AD mice, with multiple lower molecular weight forms that were not evident in the mice. A higher molecular mass tau oligomer, probably corresponding to dimeric tau, was apparent in one AD case. The HT7 monoclonal antibody (generated against human tau residues 159–163 and detects all tau isoforms) in the 3×Tg-AD and wt mice showed a double band corresponding to ~80 and 70 kDa peptides that were not visible in the AD or NDC cases (Fig. 5C). The 3×Tg-AD and wt mice also had a strong band at ~28 kDa which was lighter in humans. The single ~55 kDa band in 3×Tg-AD mice is likely due to the mouse transgene being expressed from a cDNA with no splicing capability resulting in a single tau isoform.

Sequential chromatographic separations and western blot analyses

The GDFA-homogenized 3×Tg-AD cerebral cortex supernatants were separated by FPLC and collected in 3 fractions corresponding to molecular weights of 300–70 kDa, 70–10 kDa, and 10–2 kDa proteins and designated as fractions 1, 2 and 3, respectively. Fraction 3, more clearly separated from the heavier M_r fractions, contained the monomeric and dimeric Aβ peptides. These 3 fractions were further separated by C8 reverse-phase HPLC and the resulting 8 peaks from each chromatography were submitted to WB analysis using antibodies raised against Aβ₄₀, Aβ₄₂, CT-AβPP and tau (Figs. 6–8).

The monomeric form of the Aβ₄₀ was detected in FPLC fraction 3 at 12–16 and 18 months of age in the 3×Tg-AD mice (Figs. 7A and 8A) while none was seen at 7 months (Fig. 6A). Other higher molecular weight molecules were also detected with this antibody in the 12–16 month-old female mice (Fig. 7A) and to a much lesser degree in the 18 month-old mice (Fig. 8A). The Aβ₄₂ peptide was well represented at 7, 12–16, and 18 months of age (Figs. 6B, 7B and 8B) in monomeric, and in some gels is manifested as hexameric Aβ ~25kDa and dodecameric Aβ ~56 kDa forms. In our experience with humans and several strains of Tg mice, these two oligomeric Aβ₄₂ aggregates may be an artifact generated during the purification procedure especially when the Aβ peptides are concentrated by vacuum centrifugation. Several protein bands reacted with the CT9AβPP antibody in the 7, 12–16, and 18 month-old 3×Tg-AD mice (Figs. 6C, 7C, and 8C). Multiple peptides potentially containing the CT-AβPP fragment were observed at ~110, ~80, ~60, ~40, ~35, ~24 and ~10 kDa. The ~35 and ~40 are of great interest because they may represent an earlier fragment of CT-AβPP in the absence of the human CT99, which in our system appears as a ~13 kDa band, that results from the β-secretase cleavage of AβPP. The peptide at ~10 kDa likely results from the action of the mutant γ-secretase and may represent the AβPP intracellular domain (AICD).

The tau dominant bands in the 3×Tg-AD mice were observed at ~55 and ~28 kDa that may correspond to the human tau transgene and to a degradation product, respectively (Figs. 6D, 7D and 8D). Additional tau positive bands with ~80 and ~70 kDa are detected at 7 months becoming more prominent as the mice age. In addition, higher molecular weight aggregates, probably corresponding to dimeric tau, as well as an increase in degradation products with molecular weights of 40, 25 and 20 kDa become more prominent as the mice age.

Histological and biochemical correlations and summary

6–7 month-old 3×Tg-AD mice—At this age, the 3×Tg-AD mice demonstrated a robust AβPP expression in both IHC (Figs. 1A and 1D) and WB analyses (Figs. 5A, 5B and 6C). Intracellular Aβ was detected in brain IHC sections (Figs. 1C and 1F), however, no extracellular deposits were revealed by Congo red staining (Fig. 1G, 1J and 1K). Aβ levels were at their lowest levels as measured by ELISA (Figs. 4A–D) and visualized by WB (Figs. 6A and 6B). Tau immunoreactivity was positive in brain sections (Figs. 1H and 1L), but negative for the PHF-1 antibody (Figs. 1I and 1M). Tau was detected by WB (Figs. 5C and 6D) and ELISA of brain homogenates with high values of tau (Fig. 4E) and p-tau (Fig. 4F).

12–16 month-old 3×Tg-AD mice—AβPP continued to be expressed as seen in the IHC sections (Figs. 2A and 2D) and confirmed by WB (Figs. 5A, 5B and 6C). There was a higher expression of intracellular Aβ (Figs. 2C and 2F) as detected by IHC. ELISA Aβ quantification is at its highest for this age group, being more prominent in female mice (Figs. 4A–D). Western blots also showed a wider Aβ peptide diversity (Figs. 7A and 7B) than at 6–7 months. There was no Congo red evidence of extracellular Aβ (Figs. 2G, 2J and 2K). Tau is still expressed (Figs. 2H and 2L), but without evidence of PHF-1 (Figs. 2I and 2M). ELISA (Figs. 4E and 4F) and WB (Fig. 5C) levels of tau and p-tau are similar to those seen at 7 months, but with a more HPLC diversified pattern on tau WB (Fig. 7D).

18 month-old 3×Tg-AD mice—AβPP (Fig. 3A and 3D) and intracellular Aβ (Fig. 3C and 3F) continue to be expressed as detected by IHC. AβPP continued to be detected by WB as well (Figs. 5A, 5B and 6C). Compared to 12–16 month mice, ELISA levels drop off slightly (Fig. 4A–D), which may be due to gender differences or conversion to insoluble amyloid that escapes detection in brain homogenates. Separation by HPLC and analysis by WB also revealed a similar reduction (Fig. 8A and 8B). Importantly, in addition to intracellular Aβ (Fig. 3C and 3F), there is a severe deposition of extracellular amyloid deposits (Fig. 3B and 3E) with very apparent PHF-1 pathology (Fig. 3I and 3M) that is detectable for the first time. ELISA tau (Fig. 4E) and p-tau (Fig. 4F) are at lower levels at this age. In addition, HPLC WB demonstrated a variety of tau fragments (Fig. 8D).

DISCUSSION

The 3×Tg-AD mouse model is a sophisticated reproduction of key AD histopathological features achieved through the combined overexpression of genetically determined AβPP, PS, and tau mutations. Although producing a pathological phenotype grossly similar to that of sporadic AD, they do not replicate many aspects of this neurodegenerative disorder. This in part may be explained by the observation that mice have substantially more proteolytic enzymes, despite a genome 14% smaller than the human [25, 26]. Moreover, humans and mice have evolutionary diverged in regards to the age-related expression pattern of neuronal genes, being repressed in humans and rhesus macaques [27].

The differences between human and Tg mice could also be due to alternative AβPP degradation pathways elicited by an overwhelming expression of mutant foreign transgenes. Familial PS and AβPP mutations produce early clinical onset, a rapid disease evolution and

premature death. Presenilin mutations yield an impaired γ -secretase activity that ultimately not only affects A β PP processing, but impacts at least 25 additional substrates, among them Notch-1, N-cadherin, Erb-B4, and low density lipoprotein receptor-related protein that participate in a large number of vital cellular functions [28, 29]. Analysis of homogenates from 3 $\times$ Tg-AD mice by WB demonstrated that the CT99 and CT83 protein bands resulting from A β PP cleavage by the β - and α -secretases respectively, which are prominent in humans, were barely detectable in this animal model. In the 3 $\times$ Tg-AD mice, a novel peptide of 10 kDa was evident in the WB of the HPLC fraction 8 from the 12–16 and 18 months old mice. It is possible that this band corresponds to the 49-amino acid long AICD, resulting from γ -secretase hydrolysis at the peptide bond between amino acid residues 645-646 (ϵ -site) of A β PP₆₉₅. This band could also represent alternative CT-A β PP AICD peptide resulting from the γ -secretase hydrolysis that generates the A β ₄₀/A β ₄₂ peptides. A direct consequence of an increased synthesis and degradation of A β PP in the 3 $\times$ Tg-AD mice will be a concurrent elevation of AICD peptides which may have deleterious consequences for neuronal homeostasis and promote deficits in working memory [30].

Other prominent CT-A β PP fragments are represented by the 40 and 35 kDa bands which theoretically correspond to peptides of ~360 and ~320 amino acids. These longer CT-A β PP fragments are also present in the WB of human and 3 $\times$ Tg-AD mouse brain homogenates, suggesting that prior to the β -secretase cleavage, other hydrolysis sites along the A β PP molecule yield extended CT fragments and potentially generate reciprocal A β PP N-terminal peptides that may serve as ligands for the death receptor-6 [31]. In addition, if the generation of the 40 kDa peptide precedes the β - and γ -secretase cleavage, the production of this larger CT-A β PP peptide may be a potential limiting factor for the generation of A β peptides and hence an alternative target for therapeutic intervention.

Transgenic mice have demonstrated a strain-specific heterogeneity in the proteolytic processing of A β PP [32]. Van Nostrand and colleagues [33] used the C57BL/6 mouse background to create the TgSwDI mouse and, as we previously established [21], the pattern of A β PP degradation is very similar to that exhibited by 3 $\times$ Tg-AD mice. Hence, the observed differences between humans and 3 $\times$ Tg-AD mice in CT-A β PP processing could reflect the use of the C57BL/6 mouse strain. Intriguingly, when using the CT9A β PP antibody in WB of the TgCRND8 mice which uses the C3 H/B6 background, also revealed lower molecular weight bands [34] similar to those observed in the 3 $\times$ Tg-AD and TgSwDI Tg mice. Mass spectrometric analysis and immunoprecipitation of these peptides in the TgCRND8 mouse using the monoclonal antibody 369, raised against the 50 CT amino acids of A β PP, suggest correspondence between the CT-A β PP peptides produced by γ -secretase hydrolysis at the γ - and ϵ -sites [34]. Given the apparent differences in CT-A β PP processing between Tg mice and humans, whether some strains of A β PP/A β /PS Tg mice are faithful and reliable models for the testing of drugs intended to inhibit β - and γ -secretase activities in AD is questionable.

In the 3 $\times$ Tg-AD mice, the noticeably decreased levels of total tau as these mice age maybe due to diminished net production of tau caused by an increased rate of degradation or decreased solubility as it is being converted to PHF. Similar results were previously observed in the 3 $\times$ Tg-AD mice with IHC staining (HT7 antibody) where tau was detectable at 2–3 months, followed by a decrease starting at 12 months, and thereafter remaining constant as the mice aged [17]. Likewise, the decrease in p-tau may be due to alterations in kinase and phosphatase activity ratios. In our experience, the amount of soluble tau recovered from enriched human NFT after extraction with either SDS or GHCl or GDFA, represents only a small fraction of the total tau present in the AD brain. This is probably due to extreme insolubility, lipid complexation and molecular cross-linkage conditions that hinder isolation. Furthermore, AD electron microscopy has revealed that PHF are apparently

derived from stacks of coalescing organelles, such as degenerating mitochondria and endoplasmic reticulum [35]. It is possible that the pathophysiological mechanisms behind the production of PHF in sporadic AD and the 3×Tg-AD mouse model are fundamentally different, since in humans, PHF result from a long standing disease process while in Tg mice, pathology results from the forced introduction of a mutant human Tau^{P301L} gene product specifically responsible for frontotemporal dementia P-17. In humans, this tau mutation does not produce Aβ plaques and has a distinctly different clinical presentation than either sporadic or familial AD. Histological observations show that as the 3×Tg-AD mice age, they develop plaques and PHF that are detectable with the same histochemical techniques used on human tissue, and are therefore similar on a gross histochemical basis. However, the apparent similarities exhibited by the 3×Tg-AD mice require further investigation in terms of expression patterns, cleavage products, and degradation pathways to determine whether the mechanistic underpinnings of these signatures are consistent with what is known for sporadic AD.

The differences between mouse and human suggest that Tg animals reflect neither native protein product stoichiometry nor the consequential pathological conditions prevailing in sporadic AD. Although 3×Tg-AD mice, as well as other engineered AD mice, are effective models for studying mutant familial AD transgene overexpression, they cannot be expected to completely reproduce the conditions or full spectrum of pathology of sporadic AD, which is not the consequence of mutations or significantly upregulated gene expression. Furthermore, there is no reason to doubt that the mutated AβPP, PS and tau transgenes present in the 3×Tg-AD mice are in temporal terms co-expressed, a condition that may not be encountered in sporadic AD. In addition, plaques are dynamic [36] but more permanent in humans than in Tg mice, perhaps due to the numerous post-translational modifications and complex association with glycoproteins and glycolipids [37–40]. Once amyloid plaques are formed in humans they may not be remediable by any means other than by specific antibodies.

Nearly every therapeutic intervention intended to slow the progression of sporadic AD has some basis in the amyloid cascade hypothesis and extrapolates efficacy data from mouse models of familial AD directly to human sporadic AD. Heavy reliance on a single and controversial hypothesis, which is linked to a biomarker that does not correlate well with clinical dementia [41–43], and the observations derived from incomplete models of human disease may be factors contributing to the disappointing outcomes of late-stage clinical trials. The field of AD has been focused on a dogmatic hypothesis that advances the presence of amyloid plaques and tangles as the ultimate target to defeat rather than inquiring about the numerous possible reasons underlying their appearance. So far, Aβ immunotherapy clinical trial data have suggested that amyloid plaques, although pathophysiologically important in AD, are not the primary dementia culprit. Furthermore, the existence of non-demented individuals with high number of pathological lesions that equate those observed in AD [41, 44–46] also detracts from the possibility of amyloid plaques as the ultimate perpetrator for the AD dementia. The ultimate conquest of AD dementia may demand meticulous scrutiny of the entire constellation of assumptions regarding fundamental aspects of dementia causality.

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Fig. 1.

Histological assessment of 6 month-old 3xTg-AD mice. Coronal mouse brain sections (30 μ m) were prepared from 6 month-old male 3xTg-AD mice ($n = 4$). Histochemistry/IHC was performed to detect the following: human A β PP A4 using the Y188 monoclonal antibody (A, D), extracellular A β ₁₋₄₂ accumulation using the 12F4 monoclonal antibody (B, E), intracellular A β ₁₋₄₂ accumulation by microwave and buffer retrieval using polyclonal anti A β ₁₋₄₂ antibody (C, F), amyloid fibrils using Congo red staining (G, K) and polarized light for birefringence (J), human tau^{P301L} transgene using the HT7 monoclonal antibody (H, L), and paired helical filaments using the PHF-1 monoclonal antibody (I, M). Photomicrographs in panels A, B, C, G, H, and I were obtained at 10 $\times$, where scale bar in I represents 200 μ m. Photomicrographs in panels D, E, F, J, K, L, and M were obtained at 100 $\times$, where scale bar in M represents 10 μ m.

Fig. 2.

Histological assessment of 12 month-old 3×Tg-AD mice. Coronal mouse brain sections (30 μm) were prepared from 12 month-old male 3×Tg-AD mice ($n = 4$). Histochemistry/IHC was performed to detect the following: human AβPP A4 using the Y188 monoclonal antibody (A, D), extracellular Aβ₁₋₄₂ accumulation using the 12F4 monoclonal antibody (B, E), intracellular Aβ₁₋₄₂ accumulation by microwave and buffer retrieval using polyclonal anti Aβ₁₋₄₂ antibody (C, F), amyloid fibrils using Congo red staining (G, K) and polarized light for birefringence (J), human tau^{P301L} transgene using the HT7 monoclonal antibody (H, L), and paired helical filaments using the PHF-1 monoclonal antibody (I, M). Photomicrographs in panels A, B, C, G, H, and I were obtained at 10×, where scale bar in I represents 200 μm. Photomicrographs in panels D, E, F, J, K, L, and M were obtained at 100×, where scale bar in M represents 10 μm.

Fig. 3.

Histological assessment of 18 month-old 3×Tg-AD mice. Coronal mouse brain sections (30 μm) were prepared from 18 month-old male 3×Tg-AD mice ($n = 4$). Histochemistry/IHC was performed to detect the following: human AβPP A4 using the Y188 monoclonal antibody (A, D), extracellular Aβ₁₋₄₂ accumulation using the 12F4 monoclonal antibody (B, E), intracellular Aβ₁₋₄₂ accumulation by microwave and buffer retrieval using polyclonal anti Aβ₁₋₄₂ antibody (C, F), amyloid fibrils using Congo red staining (G, K) and polarized light for birefringence (J), human tau^{P301L} transgene using the HT7 monoclonal antibody (H, L), and paired helical filaments using the PHF-1 monoclonal antibody (I, M). Photomicrographs in panels A, B, C, G, H, and I were obtained at 10 ×, where scale bar in I represents 200 μm. Photomicrographs in panels D, E, F, J, K, L, and M were obtained at 100 ×, where scale bar in M represents 10 μm.

Fig. 4. Scatter plots of A β , tau, and p-tau ELISAs in 3×Tg-AD mice. Levels of A β ₄₀ (A) and A β ₄₂ (B) extracted with Tris-buffer. Quantification of A β ₄₀ (C) and A β ₄₂ (D) after extraction with GHCl. Total human tau (E) and p-tau (F) were determined by ELISA as well. Each data point corresponds to one mouse. mo = months; GHCl = guanidine hydrochloride; M = male; F = female p-tau (S396) = phosphorylated tau at serine 396.

Fig. 5.

Western blot analysis of 3xTg-AD mice at 7, 12–16, and 18 months, 3 human AD and 3 human NDC cases using RIPA homogenates of gray matter. All blots were re-probed for β -actin as a loading control. A) 22C11 is an antibody against amino acids 66–81 of A β PP. 2) CT9A β PP detects the last 9 amino acids of A β PP. C) Tau (HT7 clone) recognized amino acids 159–163 of human tau. mo = months; Tg = transgenic; wt = wild-type; AD = Alzheimer's disease; NDC = non-demented control.

Fig. 6. HPLC and Western blots of 7 month-old 3xTg-AD mice. Fractions 1, 2, and 3 from the FPLC were each run on a C8 column developed with 0–60% water/acetonitrile containing 0.1% TFA. A total of 8 fractions were collected and submitted to WB analysis using antibodies against Aβ₄₀ (A), Aβ₄₂ (B), CT9AβPP (C) and tau (D). Lanes 9 in the Aβ₄₀ (A) and Aβ₄₂ (B) WB contain a purified peptide as a standard. HPLC = high performance liquid chromatography; FPLC = fast protein liquid chromatography; min = minutes.

Fig. 7. HPLC and Western blots of 12 to 16 month-old 3xTg-AD mice. Fractions 1, 2, and 3 from the FPLC were each run on a C8 column developed with 0–60% water/acetonitrile containing 0.1% TFA. A total of 8 fractions were collected and submitted to WB analysis using antibodies against Aβ₄₀ (A), Aβ₄₂ (B), CT9AβPP (C), and tau (D). Lanes 9 in the Aβ₄₀ (A) and Aβ₄₂ (B) WB contain a purified peptide as a standard. HPLC = high performance liquid chromatography; FPLC = fast protein liquid chromatography; min = minutes.

Fig. 8. HPLC and Western blots of 18 month-old 3 $\times$ Tg-AD mice. Fractions 1, 2, and 3 from the FPLC were each run on a C8 column developed with 0–60% water/acetonitrile containing 0.1% TFA. A total of 8 fractions were collected and submitted to WB analysis using antibodies against A β ₄₀ (A), A β ₄₂ (B), CT9A β PP (C), and tau (D). Lanes 9 in the A β ₄₀ (A) and A β ₄₂ (B) WB contain a purified peptide as a standard. HPLC = high performance liquid chromatography; FPLC = fast protein liquid chromatography; min = minutes.