Amyloid Activates GSK-3 β to Aggravate Neuronal Tauopathy in Bigenic Mice

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The hypothesis that amyloid pathology precedes and induces the tau pathology of Alzheimer's disease is experimentally supported here through the identification of GSK-3 isozymes as a major link in the signaling pathway from amyloid to tau pathology. This study compares two novel bigenic mouse models: APP-V717I $\times$ Tau-P301L mice with combined amyloid and tau pathology and GSK-3 β $\times$ Tau-P301L mice with tauopathy only. Extensive and remarkable parallels were observed between these strains including 1) aggravation of tauopathy with highly fibrillar tangles in the hippocampus and cortex; 2) prolonged survival correlated to alleviated brainstem tauopathy; 3) development of severe cognitive and behavioral defects in young adults before the onset of amyloid deposition or tauopathy; and 4) presence of pathological phospho-epitopes of tau, including the characteristic GSK-3 β motif at S396/S404. Both GSK-3 isozymes were activated in the brain of parental APP-V717I amyloid mice, even at a young age when cognitive and behavioral defects are evident but before amyloid deposition. The data indicate that amyloid induces tauopathy through activation of GSK-3 and suggest a role for the kinase in maintaining the functional integrity of adult neurons. (Am J Pathol 2008, 172:786–798; DOI: 10.2353/ajpath.2008.070904)

In Alzheimer's disease (AD), the extracellular amyloid plaques and intracellular neurofibrillary tangles are inseparable as definitive postmortem diagnosis, but exact molecular relations to each other and to the synaptic defects and neurodegeneration remain primarily unknown. Amyloid peptides, excised from the amyloid precursor protein (APP) are considered the primary pathological agents in AD, but their earliest modes of action, in what physical form and at which cellular sites they act, all remain controversial.^{1–3} Unresolved are the activation pathways and molecular changes triggered by amyloid

peptides that drive the phosphorylation of protein tau into its eventual aggregation into tangles.

GSK-3 β has a long history in AD as tau kinase-I,⁴ based on experiments with isolated recombinant proteins and cellular models.^{4–13} The pathological evidence is circumstantial and limited to co-localization of GSK-3 β with disease-related structures in AD brain.¹³ Whether GSK-3 β is needed and sufficient to cause aggregation of protein tau in mammalian brain is unclear. In GSK-3 β transgenic mice, the endogenous mouse tau becomes phosphorylated without producing authentic tauopathy.^{14–17}

Conversely, increased GSK-3 β activity did rescue the axonopathy of human Tau-4R transgenic mice.¹⁵ We proposed that GSK-3 β phosphorylated tau at S396/S404, as observed, thereby reducing binding of tau to microtubuli to restore normal axonal transport.¹⁵ Although this hypothesis was supported in cellular models¹⁸ it does not explain the role of tau and its only known physiological function as microtubule-associated protein in amyloid peptide generation and pathology in AD.^{19,20} Implication of GSK-3 β in downstream actions of amyloid has been inferred from cellular models^{21–24} but conclusive evidence is lacking *in vivo*.^{23,25–28} In double²⁵ and triple transgenic mice^{26,27} increased GSK-3 β activity was observed but the relation to or from amyloid and tau pathology was not resolved.

Previously, we modeled amyloid pathology in mutant APP-V717I mice, which develop the entire complement of

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amyloid defects, beginning with intracellular amyloid at young age, evolving into extracellular diffuse and senile plaques, and finally angiopathy at old age.^{29–32} Because tauopathy remains conspicuously absent in the brain of amyloid mouse models^{31,33–35} incorporation of mutant human tau into the model is needed to obtain complete AD pathology in mouse brain.^{36,37}

Here, we generated and analyzed APP-V717I $\times$ Tau-P301L bigenic mice with combined amyloid and tau-pathology, and compared them to another novel model, ie, GSK-3 β $\times$ Tau-P301L bigenic mice with dramatic tauopathy in forebrain. We report the remarkable parallel in both bigenic strains with respect to a wide set of criteria, ie, brain pathology, biochemistry, survival, behavior, and cognition. Combined with data from the parental single transgenic mice, the novel bigenic models firmly position GSK-3 β , and even GSK-3 α , as major signaling link between amyloid and tau pathology in AD.

Materials and Methods

Transgenic Mice

APP-V717I, GSK-3 β , and Tau-P301L transgenic mice expressing the human genes under control of the mouse thy1 gene promoter were generated and characterized.^{15,29,38} APP-V717I and GSK-3 β heterozygous mice

were crossed with Tau-P301L homozygous transgenic mice and bigenic offspring were crossed with homozygous Tau-P301L mice to obtain offspring APP-V717I $\times$ Tau-P301L or GSK-3 β $\times$ Tau-P301L mice. The bigenic mice described and characterized in the current study were heterozygous for either APP-V717I or GSK-3 β and were all homozygous for Tau-P301L. Genotyping was performed on tail-biopsy DNA by polymerase chain reaction-specific sets of primers as described.^{15,29,38} Heterozygosity and homozygosity for Tau-P301L was demonstrated by backcrossing with nontransgenic mice and genotyping for Mendelian inheritance of the transgenes in the offspring of at least two large litters.

Immunohistochemistry (IHC)

Anesthetized mice were transcardiacally perfused with ice-cold saline and the brain was rapidly excised. For IHC one hemisphere was immersion-fixed overnight in 10 vol of 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C. Free-floating vibratome sections (40 μ m) were cut and stored in PBS containing 0.1% azide at 4°C. IHC was performed as described with the antibodies listed in Table 1. Briefly, free-floating sections were rinsed in PBS, and treated with 1.5% H₂O₂ and 50% methanol in PBS before washing in 0.1% Triton X-100 in PBS (PBST). Nonspecific binding of antibodies was blocked by treat-

Table 1. Overview of Antibodies Used

Antibody	Type	Epitope	Host	Dilution or concentration (μ g/ml)	Source
Protein tau					
Tau-5	mAb	Mouse tau phospho-independent	Mouse	IHC: 0.1; WB: 0.5	Pharmingen
HT-7	mAb	Human tau phospho-independent	Mouse	IHC: 0.02; WB: 0.02	Innogenetics
AT-8	mAb	P-S202/P-T205	Mouse	IHC: 0.2; IF: 0.4; WB: 0.2	Innogenetics
AD2	mAb	P-S396/P-S404	Mouse	IHC: 0.02; WB: 0.1	Bio-Rad
AT-270	mAb	P-T181	Mouse	IHC: 0.04; WB: 0.4	Innogenetics
CP13	mAb	P-S199/P-S202	Mouse	IHC: 1/500; WB: 1/250	P. Davies
PHF1	mAb	P-S396/P-S404	Mouse	IHC: 1/500; WB: 1/250	P. Davies
AT-100	mAb	P-T212/P-S214	Mouse	IHC: 0.2; IF: 0.2; WB: 0.07	Pierce
PG5	mAb	P-S409	Mouse	IHC: 1/1000; WB: 1/50	P. Davies
AP422	Poly	P-S422	Rabbit	IHC: 1/1000	A. Delacourte
MC1	mAb	Conformational	Mouse	IHC: 1/5000; IF: 1/500	P. Davies
PY18	Poly	P-tyrosine 18	Rabbit	IHC: 1/1000	G. Lee
Amyloid/APP					
B10.4	Poly	APP 20 C-terminal a.a.	Rabbit	IHC: 1/1000; WB: 1/5000	Homemade
22C11	mAb	APP ectodomain	Mouse	IHC: 1/5000	Chemicon
T668	Poly	P-T668	Rabbit	IHC: 1/50000; WB: 1/5000	A. Delacourte
3D6bio	mAb	Human Ab 1-5	Mouse	IHC: 0.15; IF: 1.5	Innogenetics
PanAb	Poly	Human Ab 15-30	Rabbit	IHC: 0.02; IF: 0.04	Oncogene
Other/markers					
NeuN	mAb	Neuronal nuclei	Mouse	IHC: 0.01; WB: 1.0	Chemicon
CD45	mAb	Activated microglia	Rat	IHC: 0.01	Pharmingen
MHCII	mAb	Activated microglia	Rat	IHC: 0.05	Pharmingen
GFAP	Poly	Astrocytes	Rabbit	IHC: 0.11; IF: 1.1	DAKO
JNK-p	Poly	P-T183/Y-185	Rabbit	IHC: 1/250	Santa Cruz
CREB-p	Poly	P-S133	Rabbit	IHC: 1/1000	Cell Signalling
ERK1	Poly	ERK1/ERK2	Goat	IHC: 1/1000	Santa Cruz
C19	Poly	p35/p25	Rabbit	IHC: 0.5; IF: 2.0; WB: 0.5	Santa Cruz
GSK-3ab	mAb	GSK-3 isozymes	Mouse	IHC: 1; WB: 0.005	Biosource
GSK-3ab	Poly	P-aY279/P-bY216	Rabbit	WB: 0.64	Biosource
phospho					
GSK-3b (pS9)	Poly	P-S9	Rabbit	IHC: 1/500; WB: 1/1000	Cell Signalling
Caspase-3	Poly	Activated caspase-3	Rabbit	IHC: 1/500; WB: 1/1000	Cell Signalling

IHC, immunohistochemistry; IF, immunofluorescence; WB, Western blotting; a.a., amino acid.

ment with 10% fetal calf serum in PBST (blocking buffer). The sections were incubated with primary antibody diluted as indicated (Table 1) in blocking buffer at 4°C overnight. Sections were rinsed with PBST and incubated for 1 hour with goat anti-mouse or goat-anti-rabbit IgG, labeled with horseradish peroxidase (two-step procedure) or biotin (three-step procedure). In the two-step procedure the sections were washed with PBS and incubated with 3,3'-diaminobenzidine, 0.3% H₂O₂ in 50 mmol/L Tris-HCl (pH 7.6) for 4 minutes. In the three-step procedure, sections were washed with PBST and incubated with avidin-biotinylated peroxidase complex (Vectastain Elite ABC; Vector Laboratories, Burlingame, CA) diluted in 1:800 in blocking buffer for 30 minutes at ambient temperature. The rest of the procedure was identical as in the two-step procedure. Finally, sections were rinsed in PBS, fixed on siliconized object glasses and air-dried at 50°C. Sections were counterstained with hematoxylin, dehydrated by passage through a graded series of alcohol and xylene, and mounted.^{30,38} For immunofluorescence, the secondary antibodies were goat anti-mouse or goat anti-rabbit IgG labeled with Alexa 488 or Alexa 594 (Molecular Probes, Leiden, The Netherlands).

Histology

X34 staining³⁹ was performed on free-floating sections (40 μ m) that were washed twice with PBS and incubated for 10 minutes with 10 μ mol/L X34 in 40% ethanol in 50 mmol/L Tris-HCl (pH 9.5). After rinsing with water, sections were incubated in 50 mmol/L NaOH in 80% ethanol in water for 2 minutes at room temperature. Sections were washed twice for 5 minutes in tap water, mounted on siliconized object glasses and dried at 50°C. After two washes in xylene, sections were mounted (Depex; BDH, Poole, UK). ThioflavinS staining of free-floating sections was performed by incubation for 30 minutes in 0.01% ThioflavinS in 50% ethanol. After washing with 50% ethanol and tap water, sections were mounted on siliconized object glasses, air-dried, and washed twice in xylene before being mounted (Depex). Stained sections were examined microscopically with epifluorescence illumination optics and a 3CCD camera (Leica, Wetzlar, Germany). Images were captured and analyzed using dedicated software (IM500, Leica). Alternatively, sections were analyzed by confocal imaging (LSM 510; Zeiss, Göttingen, Germany).

Western Blotting

Anesthetized mice were transcardiacally perfused with ice-cold saline and the brain was rapidly excised and tissue samples snap-frozen in liquid N₂ and stored at -70°C. Hemispheres, hippocampus, brainstem, and spinal cord were homogenized in 6 vol of 25 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 5 mmol/L sodium pyrophosphate, 30 mmol/L sodium fluoride, 2 mmol/L sodium vanadate, 1 μ mol/L okadaic acid, 1 mmol/L phenylmethyl sulfonyl fluoride, 5 μ g/ml leupeptin, 5 μ g/ml pepstatin, and 5 μ g/ml soybean trypsin inhibitor in a Potter-Elvehjem homogenizer (20

strokes, 700 rpm; VWR, Leuven, Belgium). Extracts were diluted appropriately in sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 8% polyacrylamide gels (Novex, San Diego, CA). Proteins were transferred to nitrocellulose filters (Hybond-ECL; GE Healthcare Life Sciences, Buckinghamshire, UK) at 70 mA overnight, in 25 mmol/L Tris-HCl (pH 8.6), 190 mmol/L glycine, and 20% methanol. Filters were stained with Ponceau red to visualize proteins as a first loading control, and washed three times in 50 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, and 0.1% Tween (TBS-Tween) for 10 minutes. To prevent nonspecific binding of antibody the blots were blocked by incubation in 5% nonfatty milk in TBS-Tween for 1 hour. The blots were incubated at 4°C for 2 hours with the primary antibody diluted in blocking buffer as indicated (Table 1). Primary antibody was removed by washing four times with TBS-Tween and filters were incubated for 1 hour with appropriate secondary antibody and immune reactions visualized by chemiluminescence (ECL; Amersham Biosciences, Buckinghamshire, UK).

Western blots for specified proteins or phospho-epitopes on protein tau in extracts of hippocampus and brainstem were quantified by densitometric scanning and image analysis as described.^{38,40} The data were analyzed by two-way analysis of variance with brain region and genotype of the mice as independent variables and as dependent variable the ratio of a specified phospho-epitope defined by blotting with a specific antibody, to the total level of protein tau defined by blotting with antibody Tau-5.³⁸ Differences for separate independent variables were assessed by posthoc Student-Newman-Keuls analysis.

Electron Microscopy and Immunogold Labeling of Tangles

Mice were anesthetized and transcardiacally perfused with 0.9% NaCl for 2 minutes followed by 4% paraformaldehyde in PBS for 10 minutes. Brains were removed and fixed in the same fixative overnight at 4°C. Sagittal vibratome sections (40 μ m) were stored in PBS with 0.1% NaN₃. After rinsing in 0.1 mol/L phosphate buffer (pH 7.4) sections were placed in 0.1% sodium borohydride to reduce residual aldehyde groups and subsequently treated with 0.05% Triton X-100 in PBS to improve reagent penetration. To prevent nonspecific binding of antibody, sections were incubated in blocking solution (Aurion, Wageningen, The Netherlands). After washing in PBS containing 0.2% bovine serum albumin (BSA) sections were incubated overnight with mAb AT8 (1/200) in 0.2% BSA/PBS. After six washes in 0.2% BSA/PBS, sections were incubated overnight with the secondary antibody, ie, gold-conjugated F(ab')₂ fragment of goat anti-mouse IgG (Aurion), diluted 1/100 in 0.2% BSA/PBS. Sections were thoroughly washed with 0.2% BSA/PBS and finally with PBS. Before silver enhancement sections were fixed in 2.5% glutaraldehyde in PBS. After washing in PBS, sections were incubated for enhancing the signal in R-gent SE-EM (Aurion) for 90 minutes. Enhancement was stopped by washing the sections in distilled water, before fixation with 0.5% osmium tetroxide, dehydration,

and embedding in Agar100 resin between sheets of Aclar film (Agar Scientific, Stansted Essex, UK). The resin was allowed to polymerize by incubation at 60°C for 72 hours. Ultra-thin sections (80 nm) were cut and stained with uranyl acetate and lead citrate. For ultrastructural evaluation, 300- μ m vibratome sections were postfixed for 60 minutes in 1% osmium tetroxide, dehydrated and impregnated with Agar100 resin. Specific regions of the brain sections were dissected and flat-embedded in Agar100 as above. Ultrathin sections (80 nm) were cut and stained with uranyl acetate and lead citrate. Digital images were made with a transmission electron microscope (JEM-2100; Jeol, Tokyo, Japan) at 160kV.

Behavior and Cognition

The different groups of mice analyzed for behavioral and cognitive defects were 4 to 6 months old, and kept under an inverted 12-hour light/dark cycle. Food and water was available *ad libitum* unless indicated otherwise. One week before the tests, mice were transferred to the vivarium of the behavior lab. All experimental procedures were performed in accordance with regulations of, and authorized by, the Ethical Commission for Animal Experimentation of the K.U. Leuven. Statistical analysis was by analysis of variance single factor as described.^{29,38,41,42}

The open-field test analyzes spontaneous locomotor activity, exploratory behavior, and anxiety. Mice were placed individually in a corner of a box (52 $\times$ 52 $\times$ 40 cm) with black walls and a translucent floor, dimly illuminated from underneath. They were allowed to explore the field for 5 minutes under continuous video observation. The arena was divided into an outer zone (10 cm from the walls) and corner zones (10 $\times$ 10 cm in each corner) leaving the remaining as center zone. Paths were tracked digitally (EthoVision; Noldus, Wageningen, The Netherlands) at a frequency of 4.2 Hz and a spatial resolution of 256 $\times$ 256 pixels. The parameters calculated using dedicated software (EthoVision) included total distance traveled, velocity, time, and distance in the different zones (center, corners, outer).

The light/dark exploration test was performed by placing mice in a box (52 $\times$ 52 $\times$ 40 cm) divided in two compartments, one dark and one brightly illuminated from above. The test was started by placing a mouse in the dark compartment and recording the time spent in each compartment and the number of transitions between both compartments during a 5-minute observation period.

The passive inhibitory avoidance was performed by placing the mice into the lit compartment of a chamber with a grid floor connected to a current shocker (Medical Associates, St. Albans, VT). After 1 second the door of the dark compartment opened to allow the mice to enter and the time needed was recorded as training latency (L_{training}) which was averaged for all mice in each group and genotype. Once the mouse entered the dark compartment, the door closed automatically and a mild foot-shock (0.5 mA for 2 seconds) was administered. Ten seconds after the shock the mouse was removed from the dark compartment and returned to the home cage. Twenty-four hours after the training, the mice were again

analyzed by placing them into the lit compartment and the latency taken to enter the dark compartment was measured as test latency (L_{test}), limited to maximum 300 seconds. The avoidance index was calculated as (L_{test})/(L_{training}) as a measure of learning and memory engaging amygdala and hippocampus.

Object recognition test was performed as described.^{41,42} Briefly, mice were individually habituated for 10 minutes to the Plexi test-box and the next day in the same box submitted to a 10-minute acquisition trial, whereby mice were confronted with two identical objects at two positions (A and B). The time spent exploring object in position A was recorded with the criterion that the snout of the mouse was directed toward the object and at a distance of less than 1 cm. During a 10-minute retention trial performed 3.5 hours later, a novel object was placed in position B, leaving a familiar object in position A, and the exploration time was recorded for each object (t_A and t_B). The recognition index was defined as the ratio of the time spent exploring the novel object over the time spent exploring both objects ($t_B/t_A + t_B$) as a measure of nonspatial memory engaging hippocampus.^{41,43}

A conditioned taste aversion test was performed with mice that were deprived from drinking water *ad libitum*, but were trained to drink during two daily sessions of 30 minutes each with a 4-hour interval for 4 days. Water was presented in two identical 15-ml vessels, that were weighted before and after the test as measure of water intake. During the morning session on the conditioning day (day 5) mice were allowed to drink a 0.5% saccharin solution as conditioning stimulus, and 30 minutes later mice were injected intraperitoneally with LiCl (4.5 mmol/kg) in saline as the nausea-inducing agent as unconditioned stimulus. In the next session after a 2-hour interval, mice were allowed to drink water before being returned to their home cage. During the 30-minute test session, 24 hours later, the mice were presented with two identical vessels with either water or 0.5% saccharin-water solution and the volume of consumed fluid was measured for each, to calculate the aversion index as $V_{\text{water}}/V_{\text{water}} + V_{\text{saccharin}}$.

Results

To test the GSK-3 β hypothesis *in vivo* we generated and compared two novel strains of bigenic mice by crossing APP-V717I mice²⁹ or human GSK-3 β mice¹⁵ each with the same mutant Tau-P301L mice.³⁸ In all transgenic constructs the mouse thy1-gene promoter was used to warrant neuron-specific, postnatal expression.^{14,15,29,32} For clarity, the two bigenic mouse strains are further denoted as biAT and biGT, respectively.

At this point, it is important to recall briefly the phenotypic and pathological characteristics of the parental Tau-P301L mice³⁸ that are incorporated in both bigenic models, because they are affected remarkably similar, as detailed in the following sections. In Tau-P301L mice, the clinical symptoms set in at age 6 to 7 months, and aging Tau-P301L mice become progressively lethargic and eventually motor impaired. In terminal stages they lose weight and die sud-

Figure 1. Combined amyloid and tau pathology in old biAT mice. **A:** Low power of sagittal brain section (**a**) and higher power of details in cortex (**b**), subiculum and CA1 (**c**), piriform cortex (**d**), and superior colliculus (**e**). Double IHC with Pan-A β (brown) and AT100 (dark blue) reveals amyloid and tau pathology, respectively. **B** and **C:** Details of combined amyloid and tau pathology in neocortex revealed by IHC with different combinations of antibodies: mAbs 3D6 and MC1 (**B**) and pAb Pan-A β and mAb AT100 (**C**). Note tauopathy (blue) as highly fibrillar tangles in pyramidal neurons and diffuse in dystrophic neurites around amyloid plaques (brown). Pictures are from different APP-V717I $\times$ Tau-P301L bigenic mice aged 14 to 17 months. **D:** Tauopathy revealed by IHC with mAb AT100 in layer V of cerebral cortex at high magnification to illustrate neurons containing highly fibrillar tangles (arrowheads) and dystrophic neurites around amyloid plaques (thin arrows). **E:** Protein tau phosphorylated at tyrosine 18³⁵ in neurons of old APP-V717I $\times$ Tau-P301L bigenic mouse (14 months), absent in Tau-P301L mice (see also Figure 4D).³⁸ **F:** Tauopathy revealed by IHC with mAb AT100 on sagittal brain sections of old APP-V717I $\times$ Tau-P301L bigenic mouse (17 months) in different planes relative to bregma to illustrate the extensive tauopathy in different brain regions, particularly hippocampus, entorhinal, and piriform cortex. **G:** Histological staining with compound X34³⁹ of brain section of old APP-V717I $\times$ Tau-P301L bigenic mouse (17 months) showing tangled neurons (arrowheads), amyloid plaques (thin arrows), and vascular amyloid (asterisk). Scale bars: 1 mm (**Aa**, **F**); 50 μ m (**Ab–Ac**, **B–D**); 100 μ m (**E**, **G**).

denly of unknown causes, most of them at age 9 to 11 months and without exception before 12 months.³⁸ The Tau-P301L mice develop a severe neuronal tauopathy with intraneuronal tangles in all major brain regions, beginning in midbrain, brainstem, and spinal cord. With progressing age, the tauopathy spreads to forebrain, but it remains there always less conspicuous than in hindbrain.³⁸ This morbid and moribund phenotype compares to similar mouse models that express frontotemporal dementia (FTD)-mutant tau.^{33–38,44}

APP-V717I $\times$ Tau-P301L Bigenic Mice with Combined AD-Like Pathology

The APP-V717I mice develop the full complement of defects, typical for amyloid pathology, and moreover early defects in cognition and LTP.²⁹ Like all amyloid models they

do not develop tauopathy, even at very old age. Therefore, we generated APP-V717I $\times$ Tau-P301L bigenic mice (biAT) that develop combined amyloid and tau pathology with abundant amyloid plaques and neurofibrillary tangles in hippocampus and cortex (Figure 1; Supplementary Figures S1 and S2, see <http://ajp.amjpathol.org>). The combined amyloid and tau-pathology in aging biAT mice recapitulates the postmortem pathology of AD in relevant brain regions, ie, hippocampus, neocortex, entorhinal cortex, piriform cortex, and amygdala (Figure 1A). Amyloid accumulation begins intracellularly at young age (Supplementary Figure S1, see <http://ajp.amjpathol.org>), which decreases with aging and is progressively replaced by extracellular amyloid depositions, first as diffuse plaques (10 to 12 months) followed by senile plaques (12 to 15 months) and vascular amyloid (15 to 18 months) (Figure 1; Supplementary Figure S2, see <http://ajp.amjpathol.org>).

Neuronal tangles and neuropil treads in different brain regions of biAT mice were visualized histologically (thioflavinS, X34, silver impregnation) and immunohistochemically (IHC) with antibodies specific for all known pathological tau epitopes, including the conformational epitope MC1 (Figure 1, B–E; Supplementary Figures S1 and S2, see <http://ajp.amjpathol.org>). Significantly, in old biAT mice (13 to 18 months) many neurons in hippocampus area CA1 contained neurofibrillary tangles, which were rare or absent in CA1 of the parental Tau-P301L mice (Figure 3; Supplementary Figure S2, see <http://ajp.amjpathol.org>).³⁸

Forebrain and particularly hippocampus tauopathy became very abundant later (13 to 18 months) and notably much more intense than in the parental Tau-P301L mice, even in their hindbrain.³⁸ The obvious caveat here is, however, that Tau-P301L mice never reached the old age of the biAT mice,³⁸ although, to our surprise, the bigenic mice with the combined pathology survived longer (see below). Significantly, the time course and relative intensity of the amyloid pathology in the biAT mice closely compared to that in the parental APP-V717I mice^{29,30,32} and appeared not to be affected by the concurrently developing tau pathology. The amyloid pathology sets in at 10 to 12 months, ie, before tauopathy in forebrain of biAT mice because tangled neurons became notable at approximately age 13 months. We interpret these findings to mean that even in models that express two human AD-related mutant proteins, aging remains the most decisive factor in the development of combined AD-like pathology. We conclude that the biAT mice are an experimental model that recapitulates, with aging, the combined amyloid and tau pathology observed in the brain of AD patients, including diffuse and senile plaques, vascular amyloid, and neurofibrillary tangles (Figure 1G).

GSK-3 β $\times$ Tau-P301L Bigenic Mice with Dramatic Tauopathy

To define the eventual contribution of GSK-3 β to the tauopathy, we generated and characterized in parallel to the biAT mice, also GSK-3 β $\times$ Tau-P301L bigenic mice (biGT) by crossing our previously characterized GSK-3 β transgenic mice¹⁵ with the same Tau-P301L strain³⁸ as in the biAT mice. Thereby both bigenic strains have identical Tau-P301L components and can directly be compared to the parental Tau-P301L mice. Dramatic tauopathy developed in the brain of aging biGT mice (14 to 18 months). Their forebrain became literally inundated with neurons that contained highly fibrillar tau inclusions, particularly in the hippocampus and specified cortical regions (Figure 2; Supplementary Figures S2 and S3, see <http://ajp.amjpathol.org>). The neurofibrillary tangles were authenticated histologically with ThioflavinS (Figure 2A, e–g) and compound X34 (Figure 2Ah; Supplementary Figure S3, see <http://ajp.amjpathol.org>). Immunohistologically they stained for all pathological phospho-epitopes, known from AD and primary tauopathies (Figure 2C, a–e; Supplementary Figures S2 and S3, see <http://ajp.amjpathol.org>). Besides all typical pathological epitopes (AT100, PHF1, PG5, AD2, among others) the conformational epitope

MC1 was evident in tangled, but also in pretangled neurons (Figure 2Aa; Supplementary Figure S2, see <http://ajp.amjpathol.org>).

As noted for the biAT mice, the tauopathy was much more severe in the forebrain of aging biGT mice than we ever observed in the parental Tau-P301L mice,³⁸ which never reached the old age of the biGT mice, however. Again to our surprise, the bigenic mice, despite their very severe forebrain tau pathology, survived longer than the parental Tau-P301L mice (see below).³⁸ The biGT mice are proposed to represent an experimental model for forebrain tauopathy, demonstrating besides aging, the major contribution of GSK-3 β to the development of tau pathology *in vivo*.

Defects in Behavior and Cognition and Clinical Features

Groups of biAT and biGT mice, 4 to 6 months of age, were analyzed in four different experimental settings to test for different behavioral and cognitive characteristics. Not surprisingly, considerable cognitive deficits were observed in biAT and biGT mice, even in the young adults tested, ie, before onset of combined AD pathology or tau pathology, respectively (Figure 3). Although the cognitive analysis will be extended to other paradigms, the available data demonstrate that biAT and biGT mice are severely impaired, even in less demanding tests of novel object recognition and passive inhibitory avoidance (Figure 3). Lesser defects were observed in the conditional taste aversion test, but still reaching significance for the biGT mice (Figure 3). Conversely, the open field test did not reveal inhibition of exploration or anxiety, whereas the biGT mice tended to be the more active or exploring, or less inhibited or anxious (Figure 3).

Clinically, aging bigenic mice of both types became progressively less mobile, but were not markedly motor impaired as judged by normal walking and absence of limb-clasping (data not shown), two typical symptoms in Tau-P301L mice in later stages.^{38,41} Nonetheless, aging progressively impacted negatively on the general appearance of the bigenic mice, particularly the biAT mice, with progressive reduction in body weight, less activity in the home cage, and less grooming resulting in poor fur condition (results not shown). Remarkably, despite their much more dramatic tau pathology, all biGT mice remained in relatively better condition than age-matched biAT mice. When the condition of aging mice deteriorated, they had to be euthanized for ethical reasons, particularly the biAT mice (13 to 18 months) most likely attributable to their combined AD pathology. The ethical considerations, imposing euthanasia, precluded the collection of authentic mortality data for Kaplan-Meier survival curves.

We conclude that early forms of amyloid and tau pathology that precede the actual physical deposition of amyloid peptides or tau proteins in brain, cause synaptic defects that underlie the cognitive and behavioral problems in young biAT and biGT mice. At old age the amyloid pathology appears to constitute an additional pathological burden over the tauopathy, imposing extra clinical

Figure 2. Dramatic tauopathy in forebrain of old biGT mice. **A:** Extensive neuronal tauopathy in old GSK-3 β $\times$ Tau-P301L bigenic mice revealed by IHC with mAb MC1 in piriform cortex (**a**) and with mAb AT100 (**b**) in amygdala (**bottom left**) and subiculum (**top right**). Details at higher magnification from same regions to illustrate the highly fibrillar nature of the tau aggregates in the somata (**c**) and neuropil treads (**d**). Histological staining with ThioflavinS of hippocampal subregion CA1 (**e**) and piriform cortex (**f**) with higher magnification (**g**, of boxed area in **e**). Histological staining with compound X34 of hippocampus CA1 at higher magnification (**h**). **B:** Tau pathology revealed by IHC with mAb AT100 in forebrain of old GSK-3 β $\times$ Tau-P301L bigenic mouse (17 months) in hippocampus (**a**), piriform cortex (**b**), and lateral nucleus of olfactory tract (**c**). **C:** Tauopathy in hippocampus of old GSK-3 β $\times$ Tau-P301L bigenic mice (14 to 18 months) revealed by IHC with antibodies specific for different pathological phospho-epitopes of human protein tau, ie, mAb AT8 (**a**), pAb AP422 (**b**), mAb PHF1 (**c**), mAb PG5 (**d**), and pAb PY18 (**e**). Sections in **b–e**, but not in **a**, are counterstained with hematoxylin to reveal cell nuclei. Scale bars: 100 μ m (**Aa**, **Ab**, **Ae–Af**, **B**, **C**); 50 μ m (**Ac**, **Ad**, **Ah**).

Figure 3. Cognitive impairment of young biAT and biGT mice in different behavioral tasks. Young biAT and biGT mice 4 to 6 months of age were compared in different behavioral and cognitive tasks to age- and sex-matched nontransgenic mice (WT), in groups of six to eight mice depending on the task, with equal numbers of male and female mice in each group. Data shown are from 1) open field test (OFT) with fractional residence time in center zone, whereby transgenic mice do not significantly differ from nontransgenic mice proving equal exploratory drive and no increased anxiety in the transgenic mice; 2) novel object recognition task (ORT) measured at 3.5 hours after familiarization with old object; 3) passive inhibitory avoidance test (PIA); 4) conditioned taste aversion test (CTA). The biGT mice are significantly impaired in these three tasks that test for different cognitive capacities, whereas the biAT mice are not impaired in the conditioned aversion test, although they show a trend in the same direction as the biGT mice. **Asterisks** denote statistically significant differences: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

problems that necessitated earlier euthanasia of biAT mice, thereby exposing the contribution of amyloid deposition in parenchyma and vasculature, which is most severe at that age.

Increased Survival Is Related to Alleviated Brainstem Tauopathy

As stated already, the most surprising observation of biAT and biGT mice was that they both maintained a relative good health condition, at the age when Tau-P301L mice became moribund (10 to 12 months). The biAT mice survived up to 18 months, whereas biGT mice surpassed 20 months and longer, ie, the oldest biGT mouse analyzed with postmortem confirmed extensive tauopathy was 26 months old. Because this is far beyond the age at which the parental Tau-P301L mice became terminal (8 to 12 months),³⁸ we authenticated the genetic status of all bigenic mice reported here by extra genotyping. Moreover, postmortem biochemical analysis certified the expression in their brain of the human transgenic proteins concerned, at the expected levels relative to the parental single transgenic mice (results not shown).

The limited lifespan of Tau-P301L mice, and of similar models reported in the literature,^{33–35} was ascribed to the severe hindbrain tauopathy, particularly in brainstem areas that control autonomous vital systems, such as breathing, swallowing, or blood pressure (results not shown).³⁸ We compared therefore the tauopathy in different brain regions and in brainstem of both bigenic models relative to the parental Tau-P301L mice (Figure 4). Strikingly, tauopathy was significantly less in brainstem of biGT and biAT mice (Figure 4C), in sharp contrast to the dramatic tauopathy in their forebrain (Figure 4, A, B, and D). Brainstem tauopathy was thereby demonstrated to be inversely related to the relative lifespan, in line with the hypothesis that premature death of Tau-P301L mice was caused by disturbed vital autonomic functions controlled by various brainstem nuclei and centers.^{38,44} This issue needs to be defined experimentally.

Biochemical Analysis of Tau and GSK-3 Isozymes

The combined observations in both bigenic strains (Figures 1 to 3; Supplementary Figures S1 and S2, see <http://ajp.amjpathol.org>) points to similar underlying mechanisms and firmly supports the hypothesis that amyloid activated the GSK-3 β kinase. This activation leads eventually to tauopathy by increased phosphorylation of tau by GSK-3 β , in concert with other kinases, eg, stress-activated kinase/cJun N-terminal kinase (SAPK/JNK-P) (Supplementary Figure S2, see <http://ajp.amjpathol.org>) or undefined tyrosine kinases (Figures 1E and 2Ce). Interestingly, phosphorylation of protein tau on tyrosine18 was evident in both bigenic mouse strains (Figures 1E and 2Ce) a molecular trait notoriously absent in the parental Tau-P301L mice (Figure 4D, left).³⁸ The findings

demonstrated that amyloid and GSK-3 β caused very similar phosphorylation of protein tau, directly by GSK-3 β and indirectly by activating other kinases or inhibiting phosphatases. Strikingly, the quantitative aspects of the tauopathy, ie, more intense and widespread in the biGT than in the biAT mice, is in line with the anticipated lesser activation of GSK-3 β by amyloid than by transgenic GSK-3 β expression.

Because amyloid is expected to activate GSK-3 β not only in forebrain but in neurons in all brain regions, the models imply that the mechanisms that regulate tau phosphorylation by GSK-3 β in response to activation by amyloid are actually different in different brain regions. Whereas region-specific responses are a normal physiological fact and typical for brain, in the case of activation of GSK-3 β by amyloid it is not only physiologically significant, but will have important therapeutic implications when GSK-3 β is targeted pharmacologically in AD.

We addressed the brain-regional issue experimentally in the parental APP-V717I mice, by comparative biochemical analysis of hippocampus and brainstem. We determined the phosphorylation status of endogenous protein tau at its preferred GSK-3 β target site, ie, S396/S404. In the same mice, we also analyzed the levels of GSK-3 β and its phosphorylation at Ser9 and at Tyr216, residues that become phosphorylated and responsible, respectively, for inactivation and activation of the kinase.

Protein extracts from hippocampus and brainstem of adult (6 to 8 months) and old (13 to 14 months) APP-V717I transgenic mice, ie, before and after amyloid deposition^{29,30,32} were prepared with great care to preserve the *in vivo* phosphorylation patterns.^{15,38} Western blotting for total tau levels with mAb Tau-5 revealed more protein tau was present with the typical slower electrophoretic mobility in protein extracts of hippocampus and brainstem of APP-V717I mice than of wild-type mice (Figure 5). This electrophoretic feature of protein tau is diagnostic for the increased phosphorylation of tau, whereby the slower migrating species (Figure 5; denoted with arrowheads on Western blots and as high MW in histograms) represents highly phosphorylated murine protein Tau-4R as we demonstrated previously.^{15,38,41,43} The concentration of these phosphorylated tau isoforms was significantly elevated in the hippocampus of old APP-V717I transgenic mice, but also in the younger APP-V717I mice relative to age-matched nontransgenic mice (WT) (Figure 5, top right). These biochemical observations corroborate the presence of endogenous mouse tau phospho-epitopes in the brain of APP-V717I mice, observed previously by IHC^{29,30} although authentic tauopathy does not develop, even at old age in the APP-V717 amyloid mice.

Amyloid Activates Both GSK-3 Isozymes by Tyrosine Phosphorylation

Total protein levels of GSK-3 β (Figure 5) and levels of the isoform phosphorylated at S9 (results not shown) were not different in hippocampus or brainstem of APP-V717I mice compared to nontransgenic mice (WT) in both age

Figure 4. Comparison of tauopathy in biAT and biGT mice relative to parental Tau-P301L mice. Each horizontal panel (A–D) compares by representative pictures, different aspects of the tauopathy in different brain regions of APP-V717I × Tau-P301L mice (**middle**) and GSK-3β × Tau-P301L mice (**right**) relative to the parental Tau-P301L mice (**left**). IHC with mAb AT100 shown for hippocampus CA1 (A), cortex (B), and brainstem (C) and with pAb PY18 in cortex (D). Note: mAb AT100 always yields some nuclear staining because of weak cross-reaction with a nuclear phospho-protein present in all brain cells, not related to protein tau. **Insets in B** show higher magnifications of single neurons to illustrate the highly neurofibrillary aspect of tangles in both bigenic mouse strains at old age (14 to 18 months) as opposed to the more diffuse aspect in the parental Tau-P301L mice, which are evidently younger (and see text).²⁹ The fibrillar morphology of the tangles is demonstrated by ultrastructural analysis of tau filaments in GSK-3β × Tau-P301L bigenic mice (E) and after immunogold labeling with mAb AT8 (F). Scale bars: 100 μm (A–D); 5 nm (E); 200 nm (F).

Figure 5. Biochemical analysis of tau and GSK-3 in young and old APP-V717I transgenic mice. **Left:** Western blots for (from **top** to **bottom**) total protein tau (Tau-5); tau phosphorylated at S396/S404 (AD2 epitope); GSK-3 α and GSK-3 β isozymes phosphorylated at tyrosine Y279 and Y216, respectively; total GSK-3 β . Extracts from hippocampus (**top**) and brainstem (**bottom**) from nontransgenic (WT) and APP-V717I transgenic mice at ages 6 to 8 and 13 to 14 months, as indicated. Representative Western blots are shown. All blots were quantified by densitometric scanning and image analysis (**right**, mean $\pm$ SEM, $n = 4$). Significant statistical differences ($P < 0.01$) denoted with **asterisk**.

groups. In contrast, phosphorylation of the essential tyrosine residue Y216 in GSK-3 β proved to be significantly increased in the hippocampus of old and young APP-V717I mice (Figure 5, top) consistent with increased GSK-3 β activity. In parallel, we observed increased phosphorylation of tau at residues S396/S404 (Figure 5, top) that constitute the AD2 epitope as the most typical GSK-3 β target in protein tau. This is a physiologically important modification that regulates tau-microtubule interactions as demonstrated *in vivo* and *in vitro*.^{15,45}

Similar to human AD brain, no direct spatial relation between amyloid plaques and tangled neurons was evident in the biAT mice (Supplementary Figure S4, see <http://ajp.amjpathol.org>). On the other hand, tyrosine phosphorylated GSK-3 isozymes co-localized with tauopathy in neurons in both bigenic mouse models (Supplementary Figure S5, see <http://ajp.amjpathol.org>), as observed in AD and FTD brain.¹³ Unexpectedly, the equivalent tyrosine residue Y279 in the GSK-3 α isozyme was also more phosphorylated in brain of young and old APP-V717I mice than in age-matched nontransgenic mice (Figure 5). Thereby, both GSK-3 isozymes are dem-

onstrated to be activated in the amyloid mouse model, even at young age before any amyloid deposition is discernable. Interestingly, both GSK-3 isozymes were more phosphorylated at their critical tyrosine residues in the hippocampus of old APP-V717I mice, but also in old nontransgenic mice relative to young nontransgenic mice (Figure 5, top; Western blots). Thereby, not only amyloid pathology but also aging is accompanied by increased activation of the GSK-3 isozymes, which explains why both pathologies still take a long time in these bigenic models to become established.

Phosphorylation of the same specified tyrosine residues in the GSK-3 isozymes was increased in brainstem of APP-V717I mice (Figure 5, bottom) although less marked than in the hippocampus of the same mice (Figure 5, top). The biochemical analysis revealed, however, neither a marked increase in total phosphorylation of protein tau, nor in AD2 or other phospho-epitopes of tau in brainstem of APP-V717I mice relative to nontransgenic mice (Figure 5, bottom; results not shown).

The combined data thereby corroborate the primary hypothesis that GSK-3 β is activated in brain of APP-V717I

mice, and particularly in forebrain, and increasingly phosphorylates protein tau as the necessary first step into its aggregation. Interestingly, activation of GSK-3 isozymes by amyloid appears not to be sufficient to induce tauopathy in the APP-V717I mice, implying other factors that remain to be identified. At this moment, FTD-associated tau mutants remain the most effective, if not the only factor capable to generate tauopathy in combination with amyloid in transgenic mouse brain (this study)^{35,37} for reasons that still elude a molecular or structural explanation at the level of the mutant tau.

Discussion

The comparative characterization of two novel transgenic mouse models for combined amyloid and tau pathology in AD, and for tau pathology in primary tauopathies, revealed a most extensive parallel in a large number of clinical and phenotypic traits. In contrast to the evident increase in tauopathy imposed by amyloid, was the dramatic increase in tauopathy by transgenic GSK-3 β less expected. The overall parallel in both models adds considerable weight to the major conclusion of our study that amyloid triggers the tauopathy by increasing GSK-3 activity. This conclusion was substantiated biochemically by the observed tyrosine phosphorylation of GSK-3, which is diagnostic for its activation in the parental APP-V717I mice, even at young age when they are known to be defective in cognition and LTP.^{29,40}

Although the conclusion is in line with the amyloid cascade hypothesis, the explicit implication of GSK-3 β is far-reaching in terms of physiological mechanisms and pathological and therapeutic implications. Moreover, the biochemical data for the primarily neglected GSK-3 α isozyme, raise or revive some important and unsettled questions about the GSK-3 isozymes, ie, their specific or overlapping functions in normal adult and aging brain, as well as their precise contribution to AD.¹¹

The observation that despite the vastly increased forebrain pathology in both bigenic models, both survived longer than the parental Tau-P301L mice was unexpected. The correlation to decreased hindbrain tau pathology is striking and the exact physiological systems affected remain to be defined to understand the early mortality that is typical for mutant FTD-tau models.^{38,44} The documented vast decrease in tauopathy in brainstem in both bigenic models, paralleling prolonged survival, is completely in line with the hypothesis that the brainstem tauopathy was responsible for early death (<12 months) of the parental Tau-P301L mice.³⁸ Pains-taking pathological analysis and electrophysiological measurements in the complex architecture of the brainstem of the parental Tau-P301L mice is ongoing, and preliminary data support the hypothesis (results not shown). Nevertheless, the data are as surprising as they are informative, because they underline and confirm the extra close parallel in the actions of amyloid and GSK-3 β in the two independent models.

These differential effects of amyloid and GSK-3 β on tauopathy in hindbrain and forebrain underline the notion

that mechanisms controlling the phosphorylation of protein tau differ in different brain regions, which is not unexpected. Alternatively or in addition, the consequences of increased phosphorylation of tau are anticipated to depend on different factors that are brain-region specific, entailing distinct clinical and brain-regional aspects in pathology in the many human tauopathies.

Whereas FTD is a primary tauopathy with mutant tau as the driving force, AD is a secondary tauopathy caused or triggered by amyloid. Nevertheless, it is unclear why overexpression of GSK-3 β itself did not cause tau pathology in mouse brain, despite increased phosphorylation of mouse tau.^{14,15} Only very high expression of GSK-3 β results in hyperphosphorylated tau and neurodegeneration, albeit without neurofibrillary tangles.^{11,16,17} It is equally unclear why all amyloid-only mouse models, even those co-expressing wild-type human protein tau fail to develop significant tauopathy, which is effectively obtained by inclusion of mutant human tau (this study).^{36,37}

Because the biAT mice recapitulate the combined pathology that is diagnostic for AD, we can extrapolate the role for GSK-3 β to the aging, degenerating human brain. The question is then raised what form of amyloid is activating GSK-3 β and by what mechanism? As briefly stated in the introduction, amyloid remains the prime candidate as pathological agent in AD, but their earliest mode of action and the exact physical form and cellular sites remain all but resolved.¹⁻³ The defective cognition in young adult (4 to 6 months) bigenic mice, excludes amyloid plaques and neurofibrillary tangles as direct causes, consistent with observations in other models. In the parental APP-V717I mice we were first to report defects in cognition and in LTP at young age, ie, before deposition of amyloid,²⁹ ascribed recently to synaptic or intracellular amyloid.⁴² Consequently, the combined data make soluble amyloid peptides the prime cause of early defects in cognition and LTP, and then also for the tauopathy by activating GSK-3. The reason why it takes a relatively long time before depositions of amyloid and tau in aging mouse brain remains unknown, and understanding the molecular nature of the increased GSK-3 β activity in old nontransgenic mice reported here, is anticipated to become informative in this respect. In the absence of conclusive experimental data, we cannot but speculate about the nature of the activation of GSK-3, which can be mediated by amyloid peptides in any physical form, acting directly or indirectly via other kinases or phosphatases.

The tau-P301L mice appear not to fit the overall picture, because they perform better in cognitive tasks and have improved LTP at young age.⁴¹ Nevertheless, biGT mice have impaired cognition and because their brain does not contain appreciable amounts of amyloid in noxious forms, the conclusion must be that also phosphorylated mutant tau affects cognition negatively. We were unable to detect specific multimeric forms of tau in brain of the biGT mice (results not shown), leaving the hypothesis that the peculiar phosphorylation status of tau is the determining factor. The finding of increased phosphorylation at tyrosine18 in protein tau in both bigenic models, and the activation of Jnk kinase exposes not only extra parallels between the two models, but provides interesting leads to other protein kinases involved.^{46,47} More-

over, given the intimate link between phosphorylation and conformation, the conformationally changed tau defined by antibody MC1 is another candidate, which moreover could link observations in mouse models (this study)³⁸ to observations in the yeast model lacking the GSK-3 β orthologue.⁴⁵

The combined data tighten the link between increased neuronal GSK-3 β activity and its role in defects in cognition and hippocampal LTP in AD models.^{23,25–28} The deepening pathological implication of GSK-3 in neurodegeneration and in AD in particular is, however, not yet paralleled by our understanding of its normal physiological role in adult neurons, ie, in synaptic functions underlying cognition and LTP.^{48,49}

We conclude by pointing out some important consequences and therapeutic implications of the apparent contradictions raised by the bigenic mice, particularly by the novel GSK-3 β $\times$ Tau-P301L mice. Their dramatic forebrain tauopathy caused far less neurodegeneration than anticipated, if any, because neuronal cell death was conspicuously absent by a number of histological and immunohistological methods and criteria (data not shown). Particularly in the biGT mice the massive number of neurons loaded with tangles did not induce obvious indications of neurodegeneration. Although occasional ghost tangles were evident in brain of both models, most tangled neurons retained their nucleus and their normal appearance. Moreover, despite the severe tauopathy in hippocampus and cortex, we were unable to define clear signs of excessive neurodegeneration in the biAT model relative to the parental APP-V717I amyloid model. This implies that a factor is still lacking in the models, eg, the inflammatory component of the human brain, or that the time span in mice is simply too short. Arguments for both theses can be construed.

The brain-regional differences in response to amyloid between forebrain and brainstem, uncovers a potential major hurdle for the therapeutic use of inhibitors of GSK-3. These might be beneficial by decreasing tauopathy in forebrain, but conversely, are predicted to promote tauopathy in hindbrain, with potential autonomic and motoric repercussions. In addition, in contrast to the alleviation of axonopathy and motoric defects by activating GSK-3 β ,¹⁵ inhibiting its activity could promote axonopathy as in Tau-4R mice treated with lithium ions (results not shown).

The action of GSK-3 β on tauopathy is evident in both directions, ie, increased GSK-3 β activity aggravates tauopathy (current study) whereas inhibition of GSK-3 β improves tauopathy (results not shown).¹⁷ The combined data maintain GSK-3 as valid therapeutic targets in primary tauopathies, and in the secondary tauopathy of AD, despite the problems. In addition, the GSK-3 isozymes also maintain their reputation of interesting, enigmatic kinases⁵⁰ probably explaining the slower than expected progress in pharmacological applications.

Finally, mouse models with higher expression levels of APP and tau than the current models³⁶ or containing a third mutant human protein (presenilin-1) show earlier onset of combined amyloid and tau pathology³⁷ than the late-onset bigenic mice presented here. The current

models do offer, however, an interesting wider prepathology window for analysis that is being explored to define molecular signals that act upstream and downstream of, or in parallel with the GSK-3 isozymes.

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