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Sex Impact on Tau-Aggregation and Postsynaptic Protein Levels in the P301L Mouse Model of Tauopathy

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Abstract. P301L transgenic (tg) mice well mimic features of human tauopathies and provide a good model for investigating the role of tau in neurodegenerative events. We here analyzed the possible interactions among phosphorylation of tau (p-tau), spine injury, neuronal death, and sex in the P301L mouse model of tauopathy. When compared to control mice (ctr), P301L transgenic mice (tg) presented a lower body weight, reduced survival rate, hyperphosphorylated tau, spine injury, and neuronal loss in both cerebral cortex and hippocampus at 15 months of age. Importantly, we found that pathological features were more pronounced in female than male tg mice. Recent reports underline that tau may be localized within both pre- and post-synaptic compartments, suggesting that it may possibly induce or contribute to synaptic dysfunction. Therefore, we focused our attention on tau localization at dendritic spines. We detected high levels of both tau and p-tau in dendritic spine of P301L transgenic mice. In addition, p-tau correlated with a significant reduction of post-synaptic markers, such as GluN2A, GluN2B, GluA1, GluA2, Debrin, and PSD-95, in P301L mice. The p-tau levels are higher in female than in male mice, and the increased p-tau was consistent with a proportional decrease in the post-synaptic marker levels analyzed. The P301L-tg females showed a more severe synaptopathy compared to males. Future investigations on the postsynaptic role of p-tau will be necessary to understand its toxic effects and provide insights into new therapeutic targets for maintaining spine integrity, highlighting the importance of tau toxicity as well as the impact of sex on tau-pathology.

Keywords: Hyperphosphorylated tau, post-synaptic markers, sex, synaptopathy, tauopathy

INTRODUCTION

Tau aggregation is a common feature in Alzheimer's disease (AD), frontotemporal dementia, progressive supranuclear palsy, corticobasal degeneration, familiar frontotemporal dementia (FTDP-17), and other tauopathies [1]. Tauopathies are neurodegenerative disorders caused by abnormal accumulation of tau protein in neurons, leading to cognitive and locomotor dysfunctions. Hyperphosphorylated tau (p-tau) is a principal component of neurofibrillary tangles (NFTs), which are a hallmark of tauopathies.

The identification of tau mutations has helped to establish that tau dysfunction is critical in the

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neurodegenerative processes leading to dementia [2]. The most frequent human tau mutation, *P301L* [3], results in the production of an aggregation-prone hyperphosphorylated form of the protein. This mutation has been used to develop mouse models of tauopathies. In P301L transgenic mouse, the expression of pathogenic P301L-tau is similar to that observed in AD brains. Moreover, it has been reported that P301L-tau promotes assembly and accumulation of abnormal and insoluble tau that triggers neuronal degeneration and loss [4, 5]. However, the role of tau in the neurodegenerative process is not clear.

Tau is a member of the microtubule-associated protein family (MAPs), expressed primarily in axons and playing an important role in microtubule stabilization as well as in neuronal transport [6–11]. However, recent studies have demonstrated that tau is also localized in dendrites and implicated in dendritic 'dying back' [12–13]. Moreover, it has been reported that tau is present in dendritic spines, suggesting that it may have a more direct role in dendritic degeneration as well [14, 15].

Most of available animal models have mutations related to microtubule-associated protein tau (MAPT) gene, responsible of tau synthesis. Among these MAPT mutations, the most common is referred to P301L, a human missense mutation of $Pro^{301} \longrightarrow$Leu [16], which appears to promote the self-assembly of mutant tau protein in the form of filaments and tangles. The P301L-tau mutant mouse mimics well the feature of human tauopathies and provides a good model for investigating the pathogenesis of diseases accompanied by NFTs [17]. These mutant mice exhibit behavioral defects and early NFTs in the brain parenchyma at 6.5 months of age in hemizygous and 4.5 months in homozygotes animals [17]. In a second paper [18], the same authors reported a later onset, around 10 months, with still abundant hyperphosphorylated tau at 10–18 months in the cerebral cortex and hippocampus associated with a maximum symptom manifestation of cognitive impairment. On this basis, we characterized 15-month-old P301L-tg mice by evaluating body weight growth, survival rate, and the localization of p-tau using histological and biochemical fractionation (triton insoluble fraction, TIF) analyses in the cortex and hippocampus. In particular, we explored a possible correlation among p-tau, synaptopathy, and neuronal death in this experimental mouse model of tauopathy. In addition, it is important to remember that in many neurodegenerative disorders, including AD, incidence and neurological outcomes are different in a sex-dimorphic way [19–23]. Indeed sex difference has been reported in AD [24, 25]. For instance, in the rtg4510 mouse model, females showed significantly higher levels of hyperphosphorylated tau than males; this was associated with a more severe impairment in spatial learning and memory as well, underlying a sex-dependent interaction of p-tau [25]. Importantly, in human AD with psychotic disorders, females had significantly higher level of p-tau than males, supporting a sex correlation in AD pathology. In light of these observations, it is important to verify whether pathological changes in tau as well as its sex-interaction may injure dendritic spine and induce consequent neurodegeneration and behavioral alterations typical of tauopathies. We here demonstrated that, in the P301L-tg mouse model, accumulation of p-tau in the post-synaptic compartment correlated with spine injury and ultimately with loss of body weight as well as mouse survival. Moreover, we found that females presented with a more severe synaptopathy compared to male mice, which resulted in a more aggressive disease outcome and a higher mortality rate. These data demonstrate that synaptic dysfunction is a crucial pathogenic event and therefore represents a novel and interesting therapeutic target for the treatment of tauopathies. Moreover, these data underline the importance of targeted sex-therapies against neurodegenerative diseases, due to the elevated vulnerability of females to tau-induced synaptopathy.

MATERIALS AND METHODS

Animals

In this study, we used P301L-tau transgenic mice of both sexes. P301L mice express the mutated form of the human tau protein (P301L), including four-repeats without amino terminal inserts, and driven by the mouse prion promoter 6 (MoPrP) [26]. Age-matched wild-type mice (B6D2F1) of both sexes served as controls. Mice from the Taconic Laboratories, USA, were bred at the IRCCS Mario Negri Institute of Pharmacological Research in a specific pathogen free facility with a regular 12 : 12 h light/dark cycle (lights on 07 : 00 a.m.), at a constant room temperature of $22 \pm 2^{\circ}$C, and relative humidity approximately $55 \pm 10\%$. Animals were housed (4 per group) in standard mouse cages, all with hard wood shavings as bedding material, and *ad libitum* food (Global Diet 2018S, Harlan Italy) and water. No environmental enrichment was used because it tends

to improve the signs of AD pathology in mouse models [27, 28]. Procedures involving animals and their care were in accordance with national and international laws and policies.

The Mario Negri Institute for Pharmacological Research (IRCCS, Milan, Italy) Animal Care and Use Committee (IACUC) approved all protocols, which were conducted according to the institutional guidelines, in compliance with Italian laws. The scientific project was approved by the Italian Ministry of Health (Permit Number 71/2014 B).

Body weight and survival

Animals were monitored daily for wellbeing and welfare-related disease and stress symptoms and their body weights were recorded weekly. Based on the daily consumption of food of a mouse (5 g/day for each mouse), it was decided to administer 200 grams of food per cage containing 4 mice. Body weight loss greater than 15% in two consecutive weeks (> 15% + > 15%) was considered as the humanitarian end-point of the study and the animals were euthanized. During the entire experiment, a large amount of data was recorded, so we decided to group the data showing the average values for each experimental group every four weeks.

Subcellular fractionation (TIF)

Subcellular fractionation was performed as reported in the literature with minor modifications for both cortex and hippocampus from P301L-tg mice [29]. Briefly, tissue was homogenized with a glass-glass potter in 0.32 M ice-cold sucrose buffer containing the following (in mM) concentrations: 1 HEPES, 1 $MgCl_2$, 1 EDTA, 1 $NaHCO_3$, and 0.1 PMSF, at pH 7.4, in the presence of a complete set of protease inhibitors (Complete; Roche Diagnostics, Basel, Switzerland) and phosphatases inhibitors (Sigma, St. Louis, MO). Samples were centrifuged at 1000×g for 10 min. The resulting supernatant (S1) was centrifuged at 3000×g for 15 min to obtain a crude membrane fraction (P2 fraction). The pellet was dissolved in buffer containing 75 mM KCl and 1% Triton X-100 plus protease and phosphatases inhibitors and centrifuged at 100,000×g for 1 h. The supernatant was stored and referred to as TSF (S4). The final pellet (P4) referred to as TIF, was homogenized in a glass potter in 20 mM HEPES in the presence of a complete set of protease and phosphatases inhibitors and stored at –80°C until processing.

Western blot

Protein concentrations were quantified using the Bradford Assay (Bio-Rad Protein Assay 500–0006, Munchen, Germany). 5 μg of TIF extracted proteins were separated by 10% SDS polyacrylamide gel electrophoresis. PVDF membranes were blocked in Tris-buffered saline (5% no fat milk powder, 0.1% Tween20) (1 h, room temperature). Primary antibodies were diluted in the same buffer (incubation overnight, 4°C) using: Phospho-tau (Ser202, Thr205) antibody AT8 (1 : 1000, Euroclone), Phospho-tau (Thr212, Ser214) antibody AT100 (1 : 1000, Euroclone), anti tau-5 (1 : 1000, Millipore Mab 361), anti-NMDA Receptor 2A GluN2A (1 : 2000, Gibco-Invitrogen), anti-NMDA Receptor 2B GluN2B (1 : 2000, Gibco-Invitrogen), anti-Glutamate Receptor 1 (AMPA subtype) GluA1 (1 : 1000, Millipore), anti-Glutamate Receptor 2 (AMPA subtype) GluA2 (1 : 1000, Millipore), anti postsynaptic density protein 95 PSD-95 (1 : 2000, Cayman Chemical Company), anti Debrin (1 : 1000, Millipore), and anti Actin (1 : 5000, Millipore). At least six independent experiments were performed. Blots were developed using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and the ECL chemiluminescence system (Promega). Western blots were quantified by densitometry using Quantity One software (Bio-Rad).

Immunohistochemistry

To perform immunohistochemistry analysis, animals were euthanized by cervical dislocation [30, 31]; brains were removed and fixed in 10% formalin for 24–48 h and embedded in paraffin. After deparaffinization, coronal sections (3 μm thick; three slices per mouse) were stained using different antibodies to detect the typical hallmarks of tauopathy. The sections were incubated for 1 h at room temperature with blocking solutions (AT8 and AT100 : 0.3% Triton X-100 plus 10% NGS) and then overnight at 4°C with the primary antibodies (AT8 and AT100 1 : 1000, Euroclone). After incubation with the biotinylated secondary antibody (1 : 200; 1 h at room temperature; Vector Laboratories, Burlingame, CA, USA), the sections were incubated for 30 min at room temperature with the avidin-biotin-peroxidase complex (Vector Laboratories) and diaminobenzidine (Sigma-Aldrich, Italy). The sections were then lightly counterstained with hematoxylin.

To determine the presence of apoptotic neurons in the brain, a TUNEL assay was done using Dead-end™ Colorimetric TUNEL System (Promega, USA code nr G3250). Briefly, after deparaffinization, sections were immersed at room temperature in a 0.85% NaCl solution for 5 min, washed twice in PBS (5 min), placed in the proteinase-K solution for 10 min at room temperature, and fixed in 10% buffered formalin solution (5 min). After washing in PBS three times (5 min each), sections were immersed in Equilibration buffer (10 min), and incubated with an rTdT reaction mix in a humidified chamber at 37°C for 1 h.

To stop the reaction, sections were immersed in the stop buffer for 15 min at RT and washed twice with PBS (5 min each); to block endogenous peroxidase, the sections were immersed in 0.3% hydrogen peroxide solution for 5 min, washed twice in PBS (5 min each), and covered with Strep-HRP solution for 30 min at RT. After washing twice in PBS (5 min each) to remove the Strep-HRP, sections were immersed in DAB solution for 3 min at room temperature. The sections were then counterstained with hematoxylin for 2 min at room temperature. The specificity of the immunostaining was verified by incubating sections with PBS instead of the specific primary antibodies.

In this tauopathy mouse model, the accumulation of pathological tau species (AT8-, AT100- positive), and the neuronal populations (TUNEL-positive) were quantified in the cortex and hippocampus (coronal sections). Immunoreactive cells were counted by image analysis software in three fields using an Olympus BX51light microscope (Olympus, Italy) equipped with a digital camera (at x400 each field represented a tissue section area of about 0.036 mm^2). After manually tracing the cortex and hippocampus at the same stereotactic level in all mice, the numbers of positive cells were manually tagged and counted. Every section was individually examined for the presence or absence of visible aggregates of hyperphosphorylated tau (AT8- and AT100- immunopositive) and for positive signaling in neuronal populations (TUNEL- immunoreactivities). The observer was blinded to the genotype.

Statistical analysis

Statistical analysis was done using Graph Pad Prism 6 program. Survival rate was analyzed by a Log-rank (Mantel-Cox) test, while other data were analyzed using Two-way ANOVA, followed by Tukey's *post hoc* test. All data were expressed as mean ± SEM with statistical significance given at $p < 0.05$.

RESULTS

Analysis of P301L-tg mice body weight and survival rate

P301L-tg mice showed signs of tauopathy, as previously described in the literature [17, 18]. However, by monitoring the animals' wellbeing and body weight, in addition to what was previously reported by others, we found significant differences not yet reported in the literature.

Ctr mice weighed more than tg mice (see Supplementary Figure 1A), and these changes were more significant in females than males. In fact, the normal age-related body weight curve increase was significantly lower in male and female P301L-tg mice compare to ctr mice during all periods analyzed (ANOVA, $p < 0.0001$, Supplementary Figure 1A). Interestingly, in P301L-tg male mice, the body weight loss was 15%, while in females, it reached 34%. The P301L-tg males weighed more than females (Supplementary Figure 1A). By evaluating food intake in each experimental group, we observed a significant reduction of food consumption in male and female P301L-tg mice compare to ctr mice during all periods analyzed (ANOVA, $p < 0.0001$, Supplementary Figure 1B). In agreement with previous results, we observed that P301L-tg males ate more than females (Supplementary Figure 1B).

We then analyzed the survival rate of ctr and tg mice. In agreement with what previously reported [17, 18], ctr mice had a higher survival rate than tg mice as reported (Chi square 17.30, $p < 0.0001$, Fig. 1A; and Chi square 26.24, $p < 0.0001$, Fig. 1B). However, in analyzing the sex influence, an interesting data was reported. Indeed, when the survival rates of male and female P301L-tg mice were compared, we observed that females had a significantly lower percent survival rate compare to male mice (Chi square 6.150, $p < 0.05$, Fig. 1C). In particular, from 12 to 15 months of age, female mortality increased by 38% and mice did not survive more than 16 months, while males started to die at 15 months (mortality rate of 12%) and easily reached 18 months of age. The increased mortality rate in females is not due to a decrease in food consumption, since female weight never decreased under 15%. In addition, no stress or anxiety-like parameters were observed in females compare to males, nor in ctr mice.

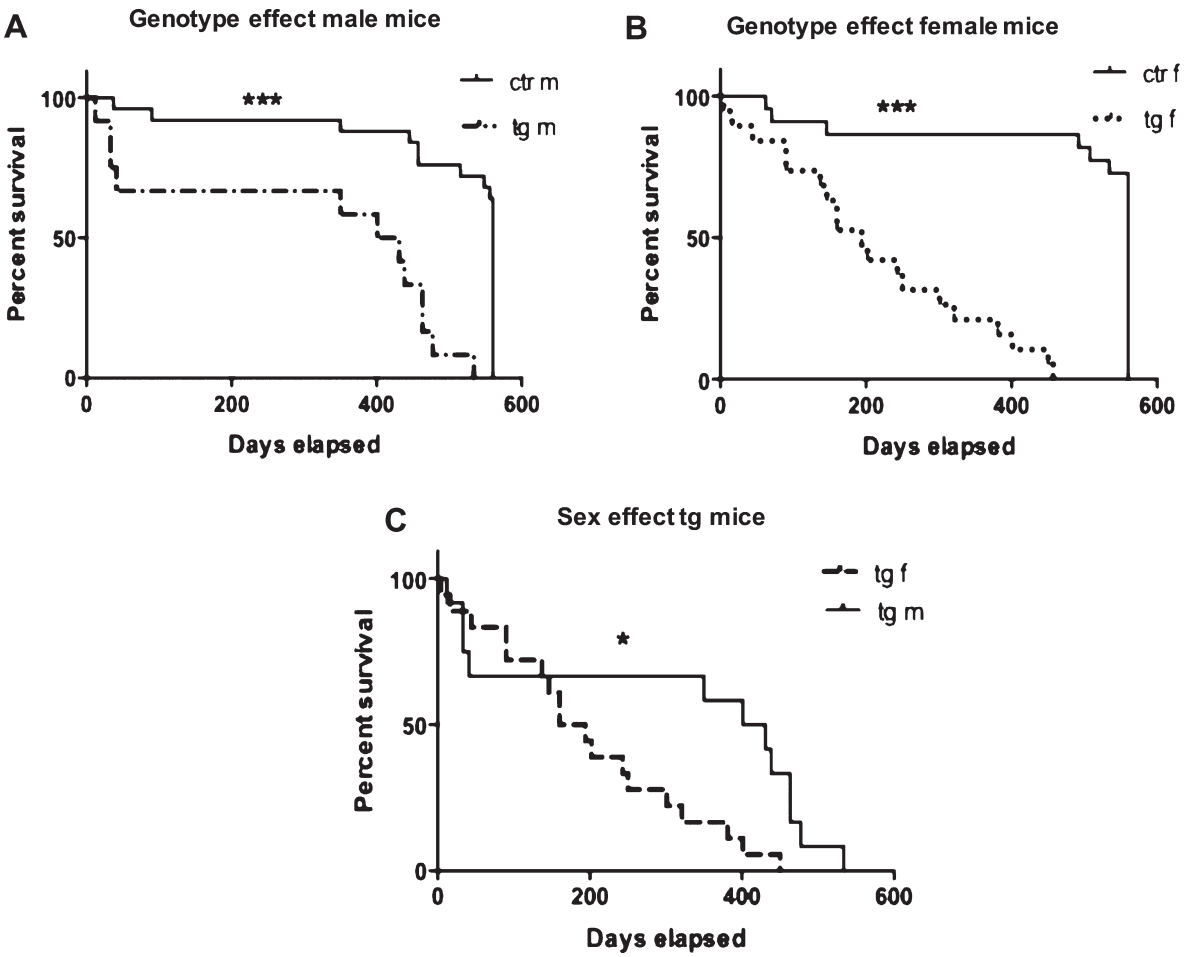

Fig. 1. Survival rate in P301L-tg mice. A-B) Significant decrease in the survival rate in tg males and females compared to ctr in the time windows analyzed ($n = 30$ for each group), male tg versus ctr mice ($^{***}p > 0.0001$) and female tg versus ctr mice ($^{***}p < 0.0001$). C) Significant decrease in the survival rate in female versus male tg mice ($^{*}p < 0.01$). Two-way ANOVA, *Tukey's post-hoc* test. Data were shown as mean ± SEM.

P301L-tg mice reveal sex-specific agglomerates of p-tau in cortex and hippocampus

P301L-tg and ctr mice sections were immunostained for phospho-specific AT8 and AT100 antibodies (Figs. 2 and 3). In both cerebral cortex and hippocampus, neurons positive for AT8 staining were found in P301L-tg mice at 15 months of age but not in ctr mice (Figs. 2B, D, F, H and 3B, D, F, H). By neuronal counts (Fig. 2I, J), a significant increase of tau-positive neurons was found in P301L-tg mice in the cortex ($p < 0.01$; Fig. 2I) and hippocampus ($p < 0.0001$; Fig. 2J). In addition, female P301L-tg showed a greater number of AT8+ neurons compared to males at 15 months of age, indicating a significant sex effect in the cerebral cortex ($p < 0.0001$; Fig. 2I) and in the hippocampus ($p < 0.0001$; Fig. 2J).

In light of the difference found in p-tau levels between female and male tg mice, we checked brain levels of total tau. We performed western blot analysis on brain homogenates from P301L-tg mice and ctr mice, using the total-Tau antibody, which recognizes both phosphorylated and non-phosphorylated epitopes and therefore detects total tau levels. As expected, both males and female P301L-tg mice had higher levels of total tau (Fig. 2K, $p < 0.0001$). Interestingly, we also observed differences in total tau levels induced by sex. In fact, female P301L-tg mice had increased levels of total tau (main effect of sex $p < 0.0001$) compared to males ($p < 0.05$, Fig. 2L).

With the second antibody AT100, the staining and quantification at 15 months of age (Fig. 3A-H and Fig. 3I-J) showed, in agreement with what previously observed with AT8 immuno-staining, a

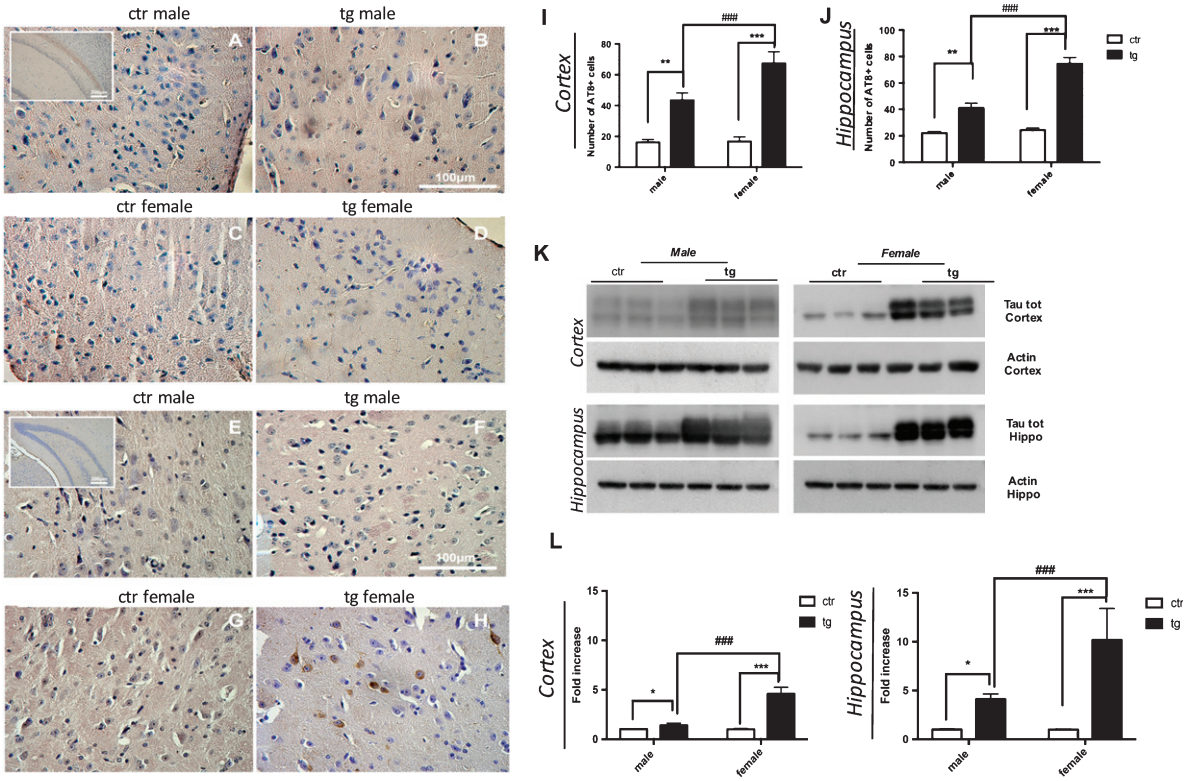

Fig. 2. AT8 immunoreactivity and its immuno-biochemical quantification. PSer202–Thr205 (AT8)-stained sections revealed the accumulation of hyperphosphorylated tau in 15-month-old male and female P301L-tg mice in the cortex (A-D) and hippocampus (E-H). Representative sections are shown. Scale bar: 100 µm. I-J) Quantification of AT8+ neurons in the cortex (I) and hippocampus (J) showed a significant increase in the number of AT8+ neurons in tg compared to ctr mice. The number of AT8+ neurons was also increased in female compared to male tg mice, confirming the presence of a significant gender effect. K-L) Western blot analysis and relative quantification showed accumulation of p-tau in the cortex and hippocampus of tg mice compared to ctr mice, with a greater increase in female versus male tg mice ($n=4$). Significance relative to control $^{*}p<0.05$, $^{**}p<0.001$, $^{***}p<0.0001$, tg male versus tg female $^{\#\#\#}p<0.0001$. Two-way ANOVA, *Tukey's post-hoc* test. Data were shown as mean ± SEM.

significant effect of genotype in the cerebral cortex and hippocampus of P301L-tg mice ($p<0.0001$; Fig. 3A-3J). Moreover, a significant genotype-sex interaction was detected in the cerebral cortex ($p<0.0001$; Fig. 3I) as well as in the hippocampus ($p<0.0001$; Fig. 3J) of female P301L-tg mice. Changes in the levels of p-tau were also detected by western blot using the AT100 antibody (Fig. 3KL). The effect of the genotype was confirmed (tg versus ctr mice: males $p<0.05$, female $p<0.001$, Fig. 3K-L). In addition, female P301L-tg mice had significant higher level of p-tau compared to males (cerebral cortex: $p<0.0001$ and hippocampus: $p<0.05$; Fig. 3L).

These results demonstrate a sex-dependent hyperphosphorylation and accumulation of tau in the cortex and hippocampus of P301L-tg mice.

Neuronal death in P301L-tg mice

To assess neurodegeneration, sections of P301L-tg and ctr mice were stained with TUNEL, for apoptotic neurons. The TUNEL staining revealed more apoptotic neurons in P301L-tg compared to ctr mice, with a greater density of dying neurons in the cerebral cortex than in the hippocampus (Fig. 4A-H). Neuronal death is increased by 55.3% in the cortex, while in the hippocampus, it was around 35% compare to ctr mice. The TUNEL quantification in these two brain areas showed a significant genotype effect (cerebral cortex: $p<0.0001$, Fig. 4I; hippocampus: $p<0.0001$, Fig. 4J). In contrast, no significant genotype-sex interaction was observed in the cerebral cortex ($p=0.1162$, Fig. 4I) or hippocampus ($p=0.6149$, Fig. 4J).

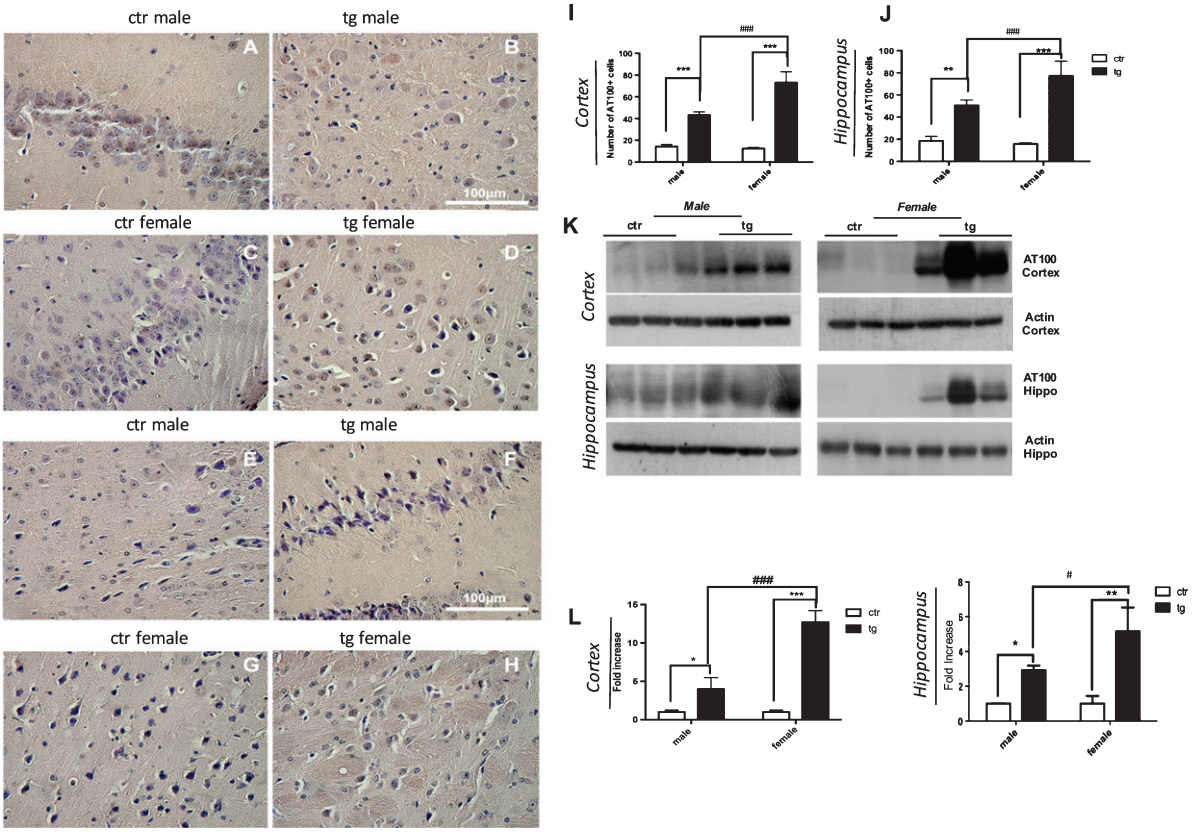

Fig. 3. AT100 immunoreactivity and its immuno-biochemical quantification. Thr212/pSer214 (AT100)-stained sections revealed accumulation of hyperphosphorylated tau in 15-month-old male and female P301L-tg mice in the cortex (A-D) and hippocampus (E-H). Representative sections are shown. Scale bar: 100 µm. I-J) Quantification of AT100+ neurons in the cortex (I) and hippocampus (J) showed an increased in the number of AT100+ neurons in tg compared to ctr mice. The number of AT100+ neurons was also increased in female compared to male tg mice, confirming the presence of a significant gender effect. K-L) Western blot analysis and relative quantification confirmed an increase in p-tau species (AT100) in the cortex and hippocampus of tg compared to ctr mice, with a greater effect in female versus male tg mice ($n=4$). Significance relative to control $^{*}p<0.05$, $^{**}p<0.001$, $^{***}p<0.0001$, tg male versus tg female $^{\#}p<0.05$, $^{\#\#\#}p<0.0001$. Two-way ANOVA, *Tukey's post-hoc* test. Data were shown as mean ± SEM.

Characterization of P301L-tg dendritic spine: Postsynaptic elements

To characterize the synaptic dysfunction in P301L-tg mice, we isolated the postsynaptic elements using a well-established biochemical approach [32]. The triton insoluble fraction (TIF) enriched in postsynaptic proteins was extracted from cerebral cortex and hyppocampus of P301L-tg male and female mice and age-matched ctr mice. To explore if the P301L mutation of tau affects synaptic dysfunction, we measured tau and p-tau levels in the TIF of P301L tg mice and ctr mice. As expected, in the postsynaptic fraction, the level of tau was significantly higher both in the cerebral cortex and hippocampus of tg mice compared to ctr mice (see Figs. 5 and 6). Importantly, total tau levels were greater in females compared to male tg mice ($p<0.0001$, Fig. 5A,B). These results indicate that the P301L mutation induce aggregation of tau in dendritic spines. By analyzing the level of p-tau (with both AT8 and AT100 antibodies), we confirmed both the genotype and sex effect, as previously seen in total brain homogenates (Figs. 5 and 6). With the aim of correlating tau pathological form to synaptic dysfunction, we performed western blot analyses to detect the relative expression of various synaptic proteins in the TIF. In particular, we quantified the levels of NMDA receptor subunits GluN2A and GluN2B, of AMPA receptor subunits GluA1 and GluA2, of Debrin (i.e., a marker of mature spine) and PSD-95 (i.e., a scaffold protein in the post-synaptic density) in the cerebral cortex (see Fig. 5) and hippocampus (see Fig. 6) of P301L-tg and ctr mice.

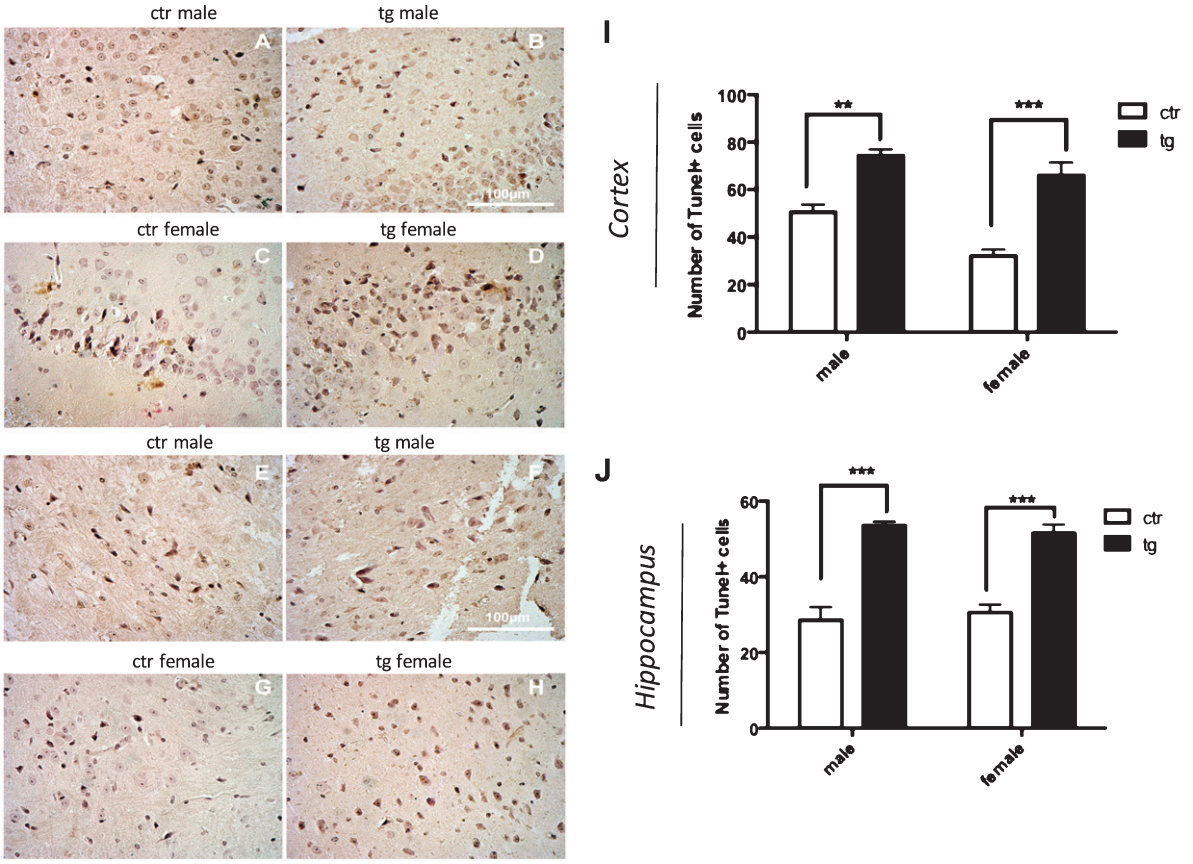

Fig. 4. TUNEL immunoreactivity and apoptotic neuronal counts. TUNEL stained sections of the cortex (A-D) and hippocampus (E-H) of male and female P301L-tg and ctr mice at 15 months of age. Representative sections are shown. Scale bar: 100 μm. I-J) Quantification of TUNEL+ neurons in the cortex (I) and hippocampus (J) showed a significant increase of TUNEL+ neurons in tg compared to ctr mice. Significance relative to control $^{**}p < 0.001$, $^{***}p < 0.0001$. Two-way ANOVA, *Tukey's post-hoc* test. Data were shown as mean ± SEM.

We observed that in the cerebral cortex of P301L-tg male mice, the levels of GluA1 and GluA2 were similar to those of ctr mice, while in females, there was a significant decrease of both GluA1 and GluA2 levels ($p < 0.0001$, Fig. 5C-D) in comparison to ctr mice. The N-methyl-D-aspartate receptor 2A (GluN2A) levels were increased in male P301L-tg versus ctr mice, while in females a decrease of these levels compared to ctr mice was observed. Finally, sex difference was assessed, observing that female P301L-tg mice had an overall lowest level of GluN2A versus male mice. There were no significant differences in the GluN2B in tg versus ctr mice or in female versus male tg mice. In addition, no significant differences in the level of the PSD95 in P301L-tg compared to ctr mice in the cerebral cortex were observed. On the contrary, the levels of Debrin were decreased in P301L-tg, more in females than in males ($p < 0.0001$, Fig. 5D), indicating a low maturity of dendritic spines.

The hippocampus exhibited similar changes in the basal level of synaptic proteins in P301L-tg versus ctr mice as well as in female versus male mice (see Fig. 6). In particular, GluN2A and GluN2B levels were reduced in the hippocampus and in the cerebral cortex, the PSD95 level increased, while those of Debrin decreased in P301L-tg female compared to ctr mice ($p < 0.0001$, Fig. 6D). This may indicate a conversion of mature spines into immature ones. In summary, the P301L-tg mice showed major structural changes in the synaptic proteins and a high levels of p-tau and tau in dendritic spines compared to ctr mice. Interestingly, the deterioration in dendritic spines was more severe in females compared to what observed in male tg mice.

In summary, the tau and p-tau increased levels in the postsynaptic elements are higher in the cerebral

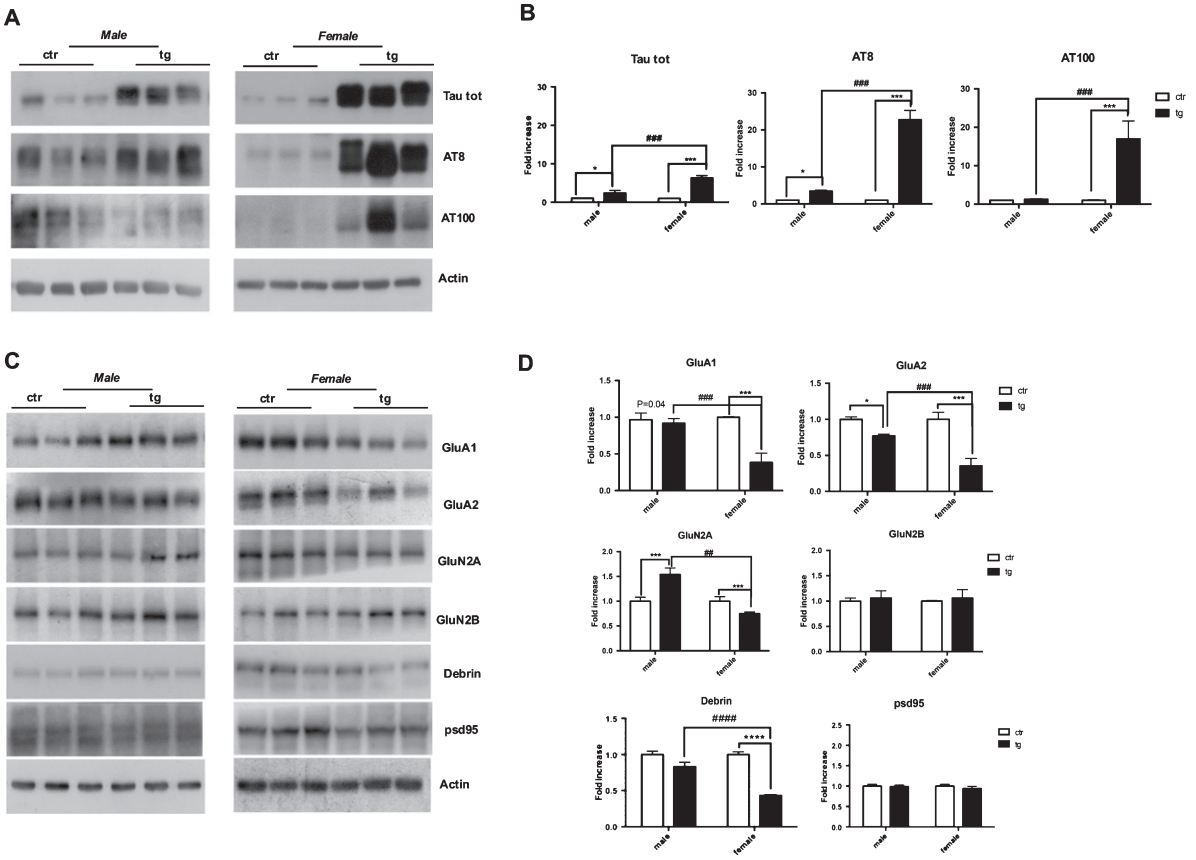

Fig. 5. Synaptopathy occurred in the cerebral cortex of P301L-tg mice, and was more severe in female than male tg mice. A-B) Western blot and relative quantification performed on the TIF fraction of male and female ctr and P301L-tg mice. Tg mice showed a significant increase of p-tau species (AT8, AT100 and total tau) compared to ctr mice, with a greater effect in female versus male tg mice ($n = 4$). C-G) Western blot and relative quantification performed on the TIF fraction of male and female ctr and P301L-tg mice. Tg mice showed a significant reduction in the PSD levels of GluR1 and of GluR2 (D) subunits of AMPAr, as well as reduction of GluN2A (E) subunit of NMDAr, and debrin (F) if compared with age-matched ctr mice. The level of these post-synaptic receptors was significantly lower in female versus male tg mice. No statistically difference was found for the levels of GluN2B (E) and PSD-95 (G). Significance relative to control $^{*}p < 0.05$, $^{***}p < 0.0001$, tg male versus tg female $^{\#\#}p < 0.001$, $^{\#\#\#}p < 0.0001$ ($n = 6$). Two-way ANOVA, *Tukey's post-hoc* test. Data were shown as mean ± SEM.

cortex compared to the hippocampus and these correlated well with the more severe spine dysfunction found (drop levels in biochemical markers in the PSD region).

DISCUSSION

Expression of human tau containing FTDP-17 mutation (P301L) in mice mimics features of human tauopathies and provides a model for investigating the pathogenesis of the disease [17, 18]. Tau aggregation coincides with clinical symptoms and is thought to mediate neurodegeneration [33]. Thus, our findings corroborate the concept that pathological tau aggregation (p-tau) plays a critical role in the neurodegenerative process. Our data underline that a genotype-dependent as well as a sex-dependent increase in the levels of both tau and p-tau occur in P301L-tg mice. In particular, female P301L-tg mice have a significant body weight decrease and a lower percentage of survival rate when compared to male mice and this powerfully correlates with an accumulation of both tau and p-tau in the brain areas most affected by tauopathy. Since it has been already reported that female P301L-tg mice showed enhanced neurofibrillary pathology as compared to male mice, and impairment in females was more severe than in males [17, 34], we here investigated tau and p-tau expression both female and male P301L-tg mice.

These analyses confirmed a sex-dependent accumulation of both tau and p-tau in both cerebral cortex

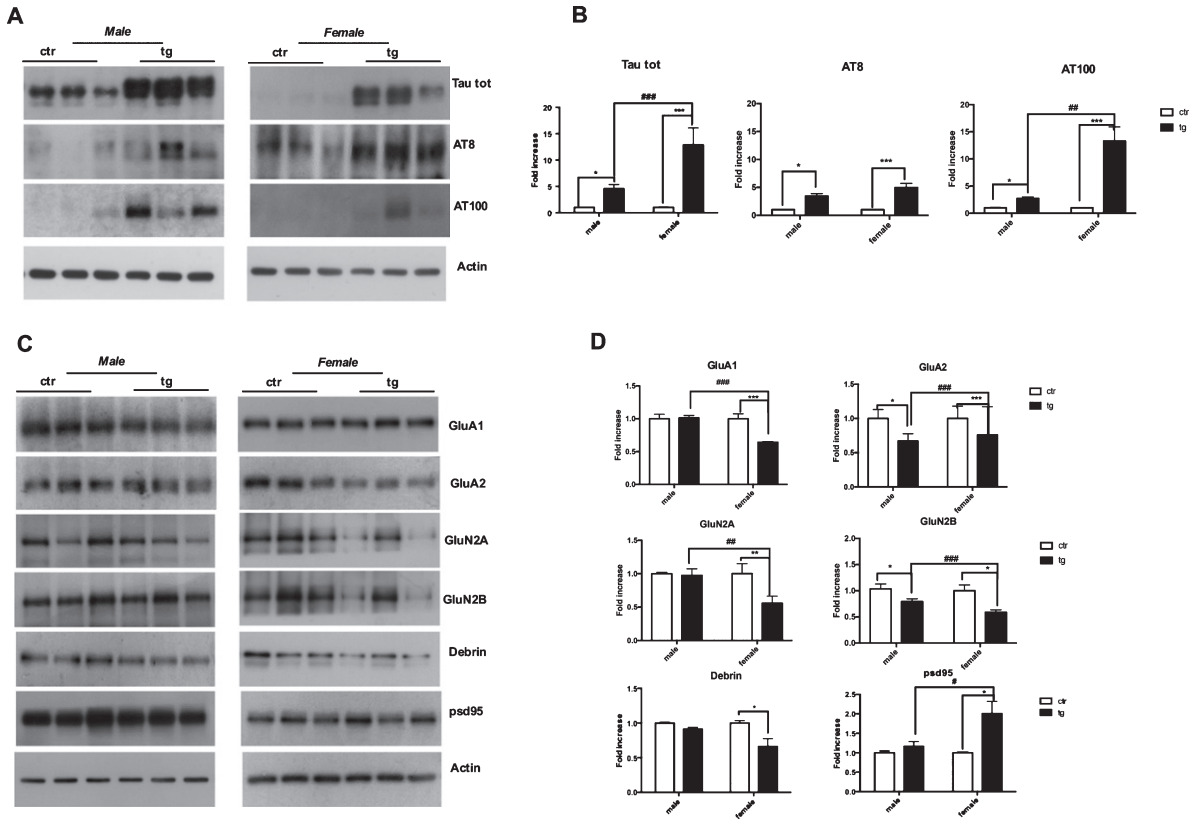

Fig. 6. Synaptopathy occurred in the hippocampus of P301L-tg mice, and was more severe in female than male tg mice. A-B) Western blot and relative quantification performed on the TIF fraction of male and female ctr and P301L-tg mice. Tg mice showed a significant increase in p-tau species (AT8, AT100, and total tau) compared to ctr mice, with a greater effect in female versus male Tg mice ($n=4$). C-G) Western blot and relative quantification performed on the TIF fraction of male and female ctr and P301L TG mice. Tg mice showed a significant reduction in the PSD levels of GluR1 and of GluR2 (D) subunits of AMPAr, as well as reduction of GluN2A and GluN2B (E) subunits of NMDAr, a reduction of debrin (F), and an increase in the PSD-95 level (G), when compared to age-matched ctr mice. There was also a significant further reduction of the level of these post-synaptic receptors in female versus male tg mice. Significance relative to control $^{*}p<0.05$, $^{**}p<0.001$, $^{***}p<0.0001$, tg male versus tg female $^{\#}p<0.05$, $^{\#\#}p<0.001$, $^{\#\#\#}p<0.0001$ ($n=6$). Two-way ANOVA, *Tukey's post-hoc* test. Data were shown as mean ± SEM.

and hippocampus; however, the p-tau stained was not directly correlated to neuronal death. In fact, neuronal death was similar in female and male mice.

To deeper investigate this discrepancy, we studied the effect of tau accumulation on synaptopathy. In fact, synaptic decline is one of the first pathogenic events characterizing a variety of neurodegenerative disease, including tauopathies. Synapse loss occurs prior to neuronal death and better correlates with cognitive dysfunction [35, 36]. Since proper synaptic function is essential in a healthy brain for memory formation or learning [37], dementia such as AD, FTPD, and many neurological diseases may be attributed to progressive synaptic dysfunction [38].

Despite many observations linking amyloid-β (Aβ) oligomers to synapse loss in AD, only recently it has been proposed that tau toxicity might be a key player in synaptic dysfunction [39]. Our hypothesis is that mislocalization of tau and p-tau into dendritic spines leads to synaptic dysfunction in early stages of the disease, in a similar way as Aβ oligomers contribute to synaptopathy in AD. In support of this hypothesis, it has been reported in the literature that tau impacts synaptic activity in several ways. Indeed, tau can localize in dendrites, where it directly interacts with post-synaptic signaling complexes, regulating the content of glutamatergic receptors in dendritic spines [40], and influencing targeting and function of synaptic mitochondria [40]. Moreover, accumulation of both tau and p-tau in dendritic spines (TIF) was reported to correlate with cognitive deficits in P301L mice [40, 41]. However, none of the previous studies

investigated the possible relationship between p-tau mislocalization and biochemical changes in the levels of post-synaptic markers in P301L-tg mice. Therefore, we analyzed a possible relationship between increased tau and p-tau levels in dendritic spines and synaptic dysfunction in P301L-tg mice.

We here report that tau and p-tau accumulated in dendritic spines of P301L-tg mice. Moreover, tau and p-tau mislocalization in dendrites significantly reduces the level of NMDAR subunits (GluN2A and GluN2B) and AMPAR subunits (GluA1 and GluA2), as well as other post-synaptic proteins (Debrin and PSD-95) in the post-synaptic compartment of P301L mice. Increased levels of p-tau in dendritic spines were also found in other mouse models of degenerative disorders, such as AD. Thus, tau pathology seems to have a broader impact on spine injury in neurodegenerative diseases.

Our results prove that the P301L mutation in human tau leads to accumulation of tau in dendrites and induces anomalies of the postsynaptic compartment, that might correlate to memory impairment in P301L-tg mice [17, 18]. These data suggest that memory deficits imply dendritic spine defects/injury. However, further experiments are needed to better study alterations in spine morphology in this model and their correlation to behavioral alterations. Moreover, the molecular mechanisms underlying spine disease and p-tau mislocalization are still unclear and will need further investigations.

Importantly, we here proved that the dendritic spine alterations in tau pathology are a sex-specific feature. Indeed, females showed significantly higher accumulation of p-tau and tau in dendritic spines than males, as well as major structural changes in the post-synaptic biomarkers. These molecular changes correlate to a more severe phenotype in female P301L-tg mice, which showed higher mortality rate and increased body weight loss if compared to males. These data suggest an important impact of sex on tauopathy. Recently, another study [42] reported a sex-genotype interaction in a mouse model of tauopathy, describing how environmental stress triggered memory impairments in females, but not in male P301L-tg mice. These observations linked memory deficits with hyperphosphorylation and accumulation of tau. Also, in a triple transgenic AD mouse model, in which animals show progressive cognitive decline [43], female animals performed worse than males at 6 months of age [44]. Noteworthy, also in the rtg4510 mouse model of tauopathy, the onset of abnormal tau biochemistry is sex-dependent, with females being affected earlier and more aggressively than males [25]. In addition, psychotic AD is associated with accumulation of p-tau in a sex-specific manner [24]. This feature may represent a background for the emerging idea that females are inherently more vulnerable to the first degenerative event in neurodegenerative disorders (i.e., synaptopathy).

Neuroactive steroids (i.e., steroid hormones synthesized in peripheral glands and neurosteroids synthesized in the nervous system) are important sex dimorphic regulators of nervous function as well as protective agents in several experimental models of neurodegenerative disorders, including AD [45–53]. Therefore, a possible hypothesis for the sex-dimorphism ascertained in the experimental model of tauopathy described here may be related to the neuroactive steroid environment. Indeed, in an animal model of AD, such as the 3xTgAD mice [54, 55] as well as in postmortem brain tissue of AD patients, altered levels of neuroactive steroids and steroidogenic enzymes have been reported [56–58]. In agreement, as demonstrated in cell cultures as well as in animal models (e.g., 3xTgAD mice), neuroactive steroids, such as 17β-estradiol, progesterone, and testosterone are able to rescue aberrant tau, by acting on enzymes involved in tau phosphorylation, such as protein kinase A (PKA) or glycogen synthase kinase 3β (GSK3 β) [58, 59]. In addition, it is also important to highlight that epidemiologic studies as well as animal models have shown strong association between obesity, metabolic dysfunction, and neurodegeneration, including AD, suggesting a key role not only for neuroactive steroids as mentioned above but also for hormones such as leptin, ghrelin, insulin, and IGF-1 [60]. For instance, leptin deficient mice (ob/ob) and leptin receptor deficient mice (db/db) show cognitive impairments and increased tau phosphorylation [61–64]. In agreement, leptin has been shown to reduce tau phosphorylation in neuronal cells by a mechanism dependent on the activation of AMP-activated protein kinase [65]. Leptin and insulin have been also demonstrated to activate PI3K/AKT/mTOR signaling pathway that has been reported to be involved in hyperphosphorylation and aggregation of tau protein [66, 67].

In addition, tau pathology is increased by diet-induced obesity in a number of strains of transgenic mice [68–70] and treatment with streptozotocin (i.e., an animal model for type I diabetes) increases tau phosphorylation in the brain [71–73]. Indeed, on the basis of all these findings, a complex interaction among apolipoprotein E, inflammation, obesity, sex,

and neuroactive steroids in AD development has been recently proposed [74, 75].

In conclusion, the data reported here may have important clinical implications and suggests the importance of targeted sex-therapy against neurodegenerative diseases. In particular, in light of current AD clinical trials aiming to block tau aggregation, the P301L-tau transgenic model offers a good model of AD-like disease and highlights the important interplay between sex-genotype interaction in tauopathy.

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SUPPLEMENTARY MATERIAL

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