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The Stress c-Jun N-terminal Kinase Signaling Pathway Activation Correlates with Synaptic Pathology and Presents A Sex Bias in P301L Mouse Model of Tauopathy

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Abstract—Pathological Tau (P-Tau) leads to dementia and neurodegeneration in tauopathies, including Alzheimer's disease. The P301L transgenic mice well mimic human tauopathy features; P-Tau localizes also at the dendritic spine level and this correlates with synaptic markers down-regulation. Importantly, tg female present a more severe pathology compared to male mice. We describe JNK activation in P301L-tg mice, characterizing by P-JNK and P-c-Jun, cleaved-Caspase-3, P-PSD95 and P-Tau (direct JNK-targets) increased levels in tg vs control mice. These data indicate that JNK stress pathway is involved in neuronal degenerative mechanisms of this mouse model. In addition, P-JNK level is higher in female compared to male tg mice, underlying a sexual dimorphism in the JNK pathway activation. The behavioral studies highlight that tg female present major cognitive and locomotor defects, strongly correlated with a more severe synaptic injury, in comparison to tg male. Notably, at the dendritic spine level, JNK is powerfully activated and its level reveals a sexual dimorphism that is coherent with behavioral defects and spine pathology. The P301L's synaptic pathology is characterized by a strong increase of P-PSD95/PSD95 and P-JNK/JNK ratios and by an augmented level of cleaved-Caspase-3 and a decrease of Drebrin level in the post-synaptic elements. These results suggest that JNK plays a key role in synaptopathy of P301L mice. Importantly, until now, there are any efficient treatments against synaptic pathology and JNK could represent an interesting target to tackle P-Tau-induced synaptic pathology. It will be important to test specific JNK inhibitors to verify their potential neuroprotective effect. © 2018 IBRO. Published by Elsevier Ltd. All rights reserved.

Keywords: synaptopathy, behavioral defects, synaptic dysfunction, Drebrin, cleaved-caspase-3, P-Tau protein.

INTRODUCTION

Tauopathies are a class of neurodegenerative diseases associated with the pathological aggregation of Tau protein in the human brain (Delacourte and Buée, 2000; Ingram and Spillantini, 2002; Yancopoulou and Spillantini, 2003; Yoshiyama et al., 2007). Tauopathies are characterized by abnormal accumulation of Tau protein in neurons, leading to cognitive and locomotor dysfunctions. Hyper-phosphorylated Tau (P-Tau) is a principal component of neurofibrillary tangles (NFTs), which represent a typical hallmark of tauopathies. Major diseases with Tau pathology are: Alzheimer disease (AD), Pick disease (PD), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP) (Yoshiyama et al., 2007), frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) (Yancopoulou and Spillantini, 2003). Recent studies underline the Tau role in neurodegeneration, such as in Alzheimer's disease (Hoover et al., 2010), is underestimated. In the tauopathies, Tau is hyper-phosphorylated and the pathological Tau (P-Tau) aggregates into neurofibrillary tangles at the somatic level in an age-dependent manner (Avila et al., 2004; Gendron and Petrucelli, 2009). In addition, P-Tau causes key morphological changes in neurons, such significant alterations in dendritic length and their complexities (Crimins et al., 2011, 2012; Rocher et al., 2010) as well as the reduction in dendritic spine number (Kopeikina et al., 2013; Thies and Mandelkow, 2007), impairs cellular trafficking (Roy et al., 2005; Shemesh et al., 2008; Thies and Mandelkow, 2007) and synaptic activity alteration (Hoover et al., 2010; Yoshiyama et al., 2007).

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Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; NFTs, neurofibrillary tangles; JNK, c-Jun N-terminal kinase; NORT, novel-object recognition test; OFT, open-field test; P-Tau, phospho-Tau.

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In this contest, we examined P301L-tg's synaptopathy and its related intracellular pathways, focusing on the stress c-Jun N-terminal Kinase (JNK) that directly phosphorylated Tau (Ploia et al., 2011) and has a pivotal role in AD's synaptic injury (Sclip et al., 2013, 2014). The synaptic dysfunction is the first neurodegenerative event and it is a core feature of many different brain disorders, underlying a common and initially reversible mechanism in these pathologies. Until now, no efficient treatment exists against synaptic pathology. Investigate the molecular mechanism of this crucial degenerative event is important to develop novel therapeutical strategies against many different neurological disorders. The P301L transgenic mouse model (JNPL3 mice) well mimic the feature of human tauopathies (Lewis et al., 2000, 2001) and provide a good tool for investigating not only the pathogenesis of this disease, but also the intracellular mechanisms underline P-Tau-synaptopathy (Buccarello et al., 2017a).

The P301L's tauopathy showed a clear sex difference: females were more severely affected then male mice, resulting in a more severe tau-synaptopathy and a higher mortality rate in female vs male tg mice (Buccarello et al., 2017a). This is particularly interesting because, in human, the dementia incidence is 16% higher in females then males and, in addition, females with dementia or with psychotic disorders had significantly higher level of phosphorylated Tau compared to males (Koppel et al., 2014; Murray et al., 2014), supporting the theory of sexual dimorphism in this brain pathology. Here we correlate the P301L's synaptopathy to: 1 – the behavioral impairments, the cognitive and locomotor defects, and 2 – the activation of stress JNK signaling pathway and the P-Tau accumulation at the dendritic spine level. We find a genotypic effect on behavioral tests and, importantly, females presented greater cognitive and locomotor impairments then male tg mice, confirming the strong synaptic damage previously detected in P301L females mice (Buccarello et al., 2017a). These data indicate, for the first time, that JNK is involved in P301L synaptopathy and the synaptic dysfunction is stronger in female then male tg mice. These results suggest that JNK is playing a key role in Tau-induced synaptic injury and underline once more the importance of sex/gender therapies against neurodegenerative diseases.

EXPERIMENTAL PROCEDURES

Mice

Male and female P301L-tg mice were purchased from Taconic Laboratories, USA, and bred on a B6D2F1 background in the IRCCS Mario Negri Institute of Pharmacological Research in a Specific Pathogen free (SPF) facility with a regular 12:12-h light/dark cycle (lights on 07:00 a.m.), at a constant room temperature of 22 ± 2 °C, and relative humidity approximately 55 ± 10%. As a classic tauopathy model, hemizygous P301L-tg mice carry the mutant form of human tau protein (P301L), which includes four-repeats without amino terminal inserts, and driven by the mouse prion promoter 6 (MoPrP) (Borchelt and Sisodia, 1996; Borchelt et al., 1996).

P301L-tg and control mice were provisioned with bedding material (hard wood shavings), ad libitum food (Envigo Lab. 2018S Tekland global diet) and water.

All animal experiments were performed according to the national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1 Dec.12, 1987; NIH Guide for the Care and use of Laboratory Animals, U.S. National Research Council, 2011). The Mario Negri Institute for Pharmacological Research (IRCCS, Milan, Italy) Animal Care and Use Committee (IACUC) approved the experiments, which were conducted according to the institutional guidelines, which are in compliance with Italian laws (D.L. no. 116, G.U. suppl. 40, Feb. 18, 1992, Circular No. 8, G.U., July 14, 1994). The scientific project was approved by Italian Ministry of Health (Permit Number: 71/2014B). At the age of 15 months, all animals performed behavioral tests and, 24 hours after the tests, mice were sacrificed for biochemical analysis.

Behavioral tests: open-field test and spontaneous locomotor activity

The Open-Field test (OFT) is used to examine the general locomotion, as well as exploration activities, and consequent level of anxiety by exposing mice to a novel and open space (Crawley, 1999; Seibenhener and Wooten, 2015; Walsh and Cummins, 1976). We used a gray Perspex OF box (40 × 40 × 40 cm) with the floor divided into 25 (8 × 8 cm) squares. After allowing the animals to acclimatize to the testing room for 30 min, the mice were placed into the behavioral room in order to decrease their reactions to a novel environment. Mice were placed into the center of the floor defined as a 'starting point' and their behavior video-recorded for 5 min. This short time period was chosen to avoid further stress to tg mice. The parameters analyzed as measure of spontaneous locomotor activity, exploratory activity and state of anxiety were: the duration of locomotion divided into the number of internal (the nine central squares) and external (the sixteen peripheral squares) square crossed, the time spent in the central and external area of the open field, the number and duration of rearing (standing on the hind paws with the front limbs either against the wall or freely in the air (Streng, 1974); the number and duration of self-grooming (rubbing the body with paws or mouth and rubbing the head with paws). The animals in immobile state longer than 4 minutes were excluded from statistical analysis. The time window between the OF and the NORT was of 24hrs (Buccarello et al., 2017a) and we used $n = 10$ animals for each experimental group.

Behavioral tests: novel-object recognition test

The novel-object recognition test (NORT) is a memory test that relies on spontaneous animal behavior (Clarke et al., 2010; Ennaceur and Delacour, 1988) without the need for stressful elements such as food or water deprivation or foot-shock (Antunes and Biala, 2012). The NORT

was conducted in an open-field arena (40 × 40 × 40 cm) with floor divided into 25 squares by black lines; three stimulus objects of similar size were used: a black plastic cylinder (4 × 5 cm), a glass vial with a white cup (3 × 6 cm), and a metal cube (3 × 5 cm). The first phase of the NORT is the *habituation trial* during which the animals were placed in the empty arena for 5 min, and their movements were recorded as the number of line crossings, which provided an indication of locomotion motor activities. In the next day, mice were re-placed in the same arena containing two identical objects (familiarization phase/ second phase). The objects were randomly selected to avoid bias among animals and between groups. Objects and positions were counterbalanced across experiments and behavioral trials. Exploration was recorded in a 10-min trial by an investigator blinded to the genotype and treatment. Sniffing, touching, and stretching the head toward the object at a distance of no more than 2 cm were scored as object investigation. In the Novel object phase, twenty-four hours later (3 phase), mice were placed again in the arena containing two objects: one already presented during the familiarization phase (familiar object) and a new different one (novel object). The time spent exploring the two objects was recorded for 10 min; animals with a time of investigation equal to 0 or minor to 2 s were excluded from the statistical analysis. Results were expressed as percentage time of investigation on objects per 10 min or as discrimination index (DI), i.e., (seconds spent on novel – seconds spent on familiar)/(total time spent on objects). Animals with no memory impairment spent a longer time investigating the novel object, giving a higher DI. In order to avoid further stress to tg mice, we decided to use the OF test as habituation trial for the Novel object recognition test and we used $n = 10$ animals for each experimental group.

Subcellular fractionation (TIF)

After the behavioral test, animals were sacrificed and brains were removed for biochemical analysis. Subcellular fractionation was performed as reported in the literature with minor modifications for both cortex and hippocampus from P301L-tg mice (Buccarello et al., 2017a). Briefly, cortex and hippocampi were dissected and homogenized in 0.32 M ice-cold sucrose buffer containing the following (in mM): 1 HEPES, 1 MgCl2, 1 EDTA, 1 NaHCO3 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, USA). Samples were centrifuged at 1000×*g* for 10 min. The resulting supernatant (S1) was centrifuged at 13000×*g* for 15 min to obtain a crude membrane fraction (P2 fraction). The pellet was resuspended in 1 mM HEPES plus protease and phosphatase inhibitor in a glass potter and centrifuged at 100 000×*g* for 1 h. The pellet (P3) was resuspended in buffer containing 75 mM KCl and 1% Triton X-100 and centrifuged at 100 000×*g* for 1 h. The final pellet (P4) referred to as TIF was homogenized in a glass potter in 20 mM HEPES 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% non-fat milk powder, 0.1% Tween20, 1 h, room temperature). Primary antibodies were diluted in the same buffer (incubation overnight, 4 °C) using: c-Jun (cat. #9165, 1:1000, Cell Signaling Technology, Danvers, MA, USA), P-c-Jun [Ser63] (cat. #9164, 1:1000, Cell Signaling Technology, Danvers, MA, USA), P-JNK (cat. #9251, 1: 1000, Cell Signaling Technology, Danvers, MA, USA), JNK (cat. #9252, 1: 1000, Cell Signaling Technology), anti-postsynaptic density protein 95 PSD95 (cat. #10009506, 1:2000, Cayman Chemical Company), anti phospho-PSD95 (cat. #10011435, 1:2000, Cayman Chemical Company), Caspase 3 (Anti-Caspase-3 cat. #AB13847, 1:500, AbCAM), anti-Drebrin (cat. # AB10140, 1:1000, Millipore), anti P-Tau (cat. #MABN388, 1:1000, Millipore, Billerica, MA, USA), anti-Actin (cat. #MAB1501, 1:5000, Millipore, Billerica, MA, USA) and at least six independent experiments were performed. Blots were developed using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and the Clarity Western ECL Blotting Substrates (Bio-Rad). Western blots were quantified by densitometry using ImageLab 6.0 software (software associated to ChemiDoc MP images, Bio-Rad) and was based on at least three independent experiments, using $n = 10$ animals for each experimental group.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 6 program. All data were analyzed using Two-way ANOVA, followed by Tukey's *post hoc* test, and were expressed as mean ± SEM with a statistical significance given at $p < 0.05$. For the calculation of the "Sample size" estimated with the following formula: $n = 2\sigma 2f\ (a,\ \beta)/\Delta 2$, for the behavioral analysis the number calculated by experimental group is $n = 10$ and for biochemical analysis $n = 6$.

RESULTS

The cognitive deficits and locomotor impairments in P301L-tg mice

To correlate P301L-tg's synaptopathy, previously described (Buccarello et al., 2017a), with functional defects, we examined cognitive and locomotor impairments in this mouse model, analysing separately females and male mice. The P301L-tg's females and male mice were assessed with two different tests to reveal their impairments compared to control mice. In the Novel Object Recognition test (NORT), P301L-tg showed strong cognitive deficits compared to ctr mice (evaluating discrimination index: DI). In fact, P301L-tg exhibited severe memory deficits, as they spent less time, compared to control mice, investigating the novel object compared to

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the familiar object (Two-way ANOVA, $p < 0.05$, $p < 0.001$; Fig. 1A). Therefore, females were more severely affected then males, having a lower discrimination index compared to male mice (Two-way ANOVA, $p < 0.05$, Fig. 1A), while, there was no statistically significant difference between male and female in ctr mice (see Fig. 1A).

Concerning the valuation of locomotor activity, a significant genotype effect on these performances was observed (Two-way ANOVA, $p < 0.01$, $p < 0.001$; Fig. 1B-D), as expected. In particular, female P301L-tg showed a greater decrease in the total number of crossing ($p < 0.001$; Fig. 1B) as well as in the number of internal and external crossing (Two-way ANOVA, $p < 0.01$, $p < 0.001$; Fig. 1C-D) compared to P301L-tg male. There was no statistically significant difference between male and female ctr mice (see Fig. 1B-D).

The cognitive and locomotor impairments presented a clear sex bias, this well correlated with PSD biochemical markers deregulation previously detected (Buccarello et al., 2017a).

The activation of JNK signaling pathway in P301L-tg mice

We here analyzed the JNK involvement in P301L-tg pathology in total homogenates. To quantify JNK activation, we measured both the action of JNK on its elective target c-Jun (P-c-Jun/c-Jun ratio) and the phosphorylation state/activation of the kinase itself (P-JNK/JNK ratio). In the cortex and hippocampus, P-c-Jun/c-Jun and P-JNK/JNK ratios were significant higher in P301L-tg compared to ctr mice (see Fig. 2A-D), indicating the activation of the stress-JNK signaling in tg mice. In particular, in the cortex we observed an increased level of P-c-Jun/c-Jun ratio in males (Two-way ANOVA, $p < 0.05$, Fig. 2A-B) and females P301L-tg (Two-way ANOVA, $p < 0.001$, Fig. 2A-B) vs ctr mice and a strong increase of P-JNK/JNK ratio (Two-way ANOVA, $p < 0.001$, Fig. 2A-B) in both sexes compared to ctr mice. In the hippocampus, P-c-Jun/c-Jun and P-JNK/JNK ratios were higher in both sexes of P301L-tg in comparison with ctr mice (Two-way ANOVA, $p < 0.01$, $p < 0.001$; Fig. 2C-D). As for the behavioral

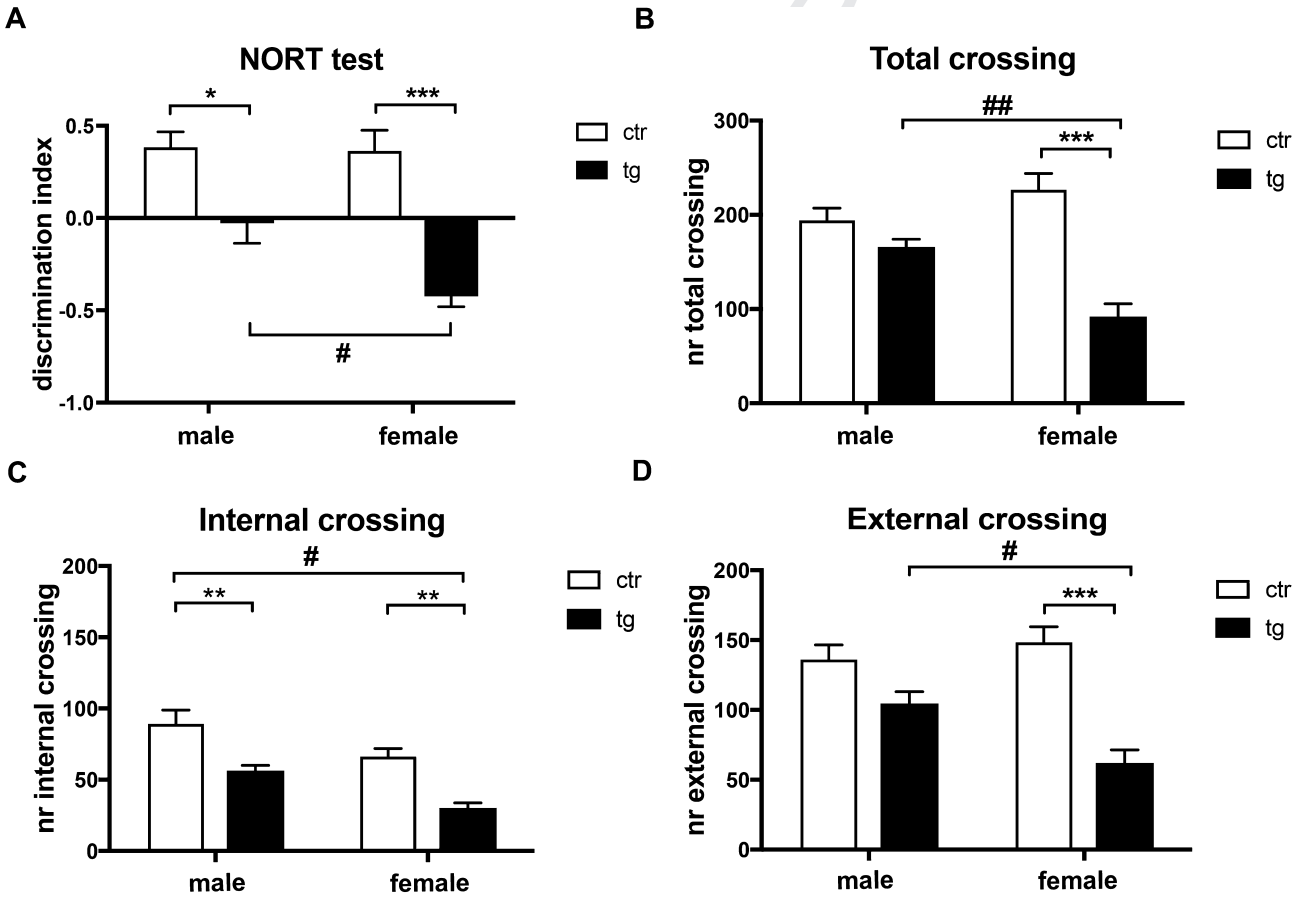

Fig. 1. Cognitive and locomotor deficits in P301L-tg mice. A) The Novel Object Recognition Test. Histograms indicate the time percentage (mean ± SEM) of investigation on the familiar and novel objects performed by female and male ctr and P301L-tg mice. There is a significant reduction of discrimination index in both male and female tg mice compared with the age-matched ctr mice, with a greater DI decrease in female vs. male tg mice. Significant relative to control $^{*}p < 0.05$, $^{***}p < 0.001$; tg female vs. male $^{\#}p < 0.05$. B-D) The Open Field Test. Histograms represent the locomotor activity in 15-months-old female and male ctr and P301L-tg mice. Both male and female tg mice had a significant reduction of locomotor performance if compared with ctr mice, with a greater decrease in female vs male tg mice. Total crossing: significant relative to control $^{***}p < 0.001$; tg female vs male $^{\#\#}p < 0.01$. Internal crossing: significant relative to control $^{**}p < 0.01$; tg female vs male $^{\#}p < 0.05$. External crossing: significant relative to control $^{***}p < 0.001$; tg female vs male $^{\#}p < 0.05$ [$n = 10$]. Two-way ANOVA, *Tukey's* post-hoc test. Data were shown as mean ± SEM.

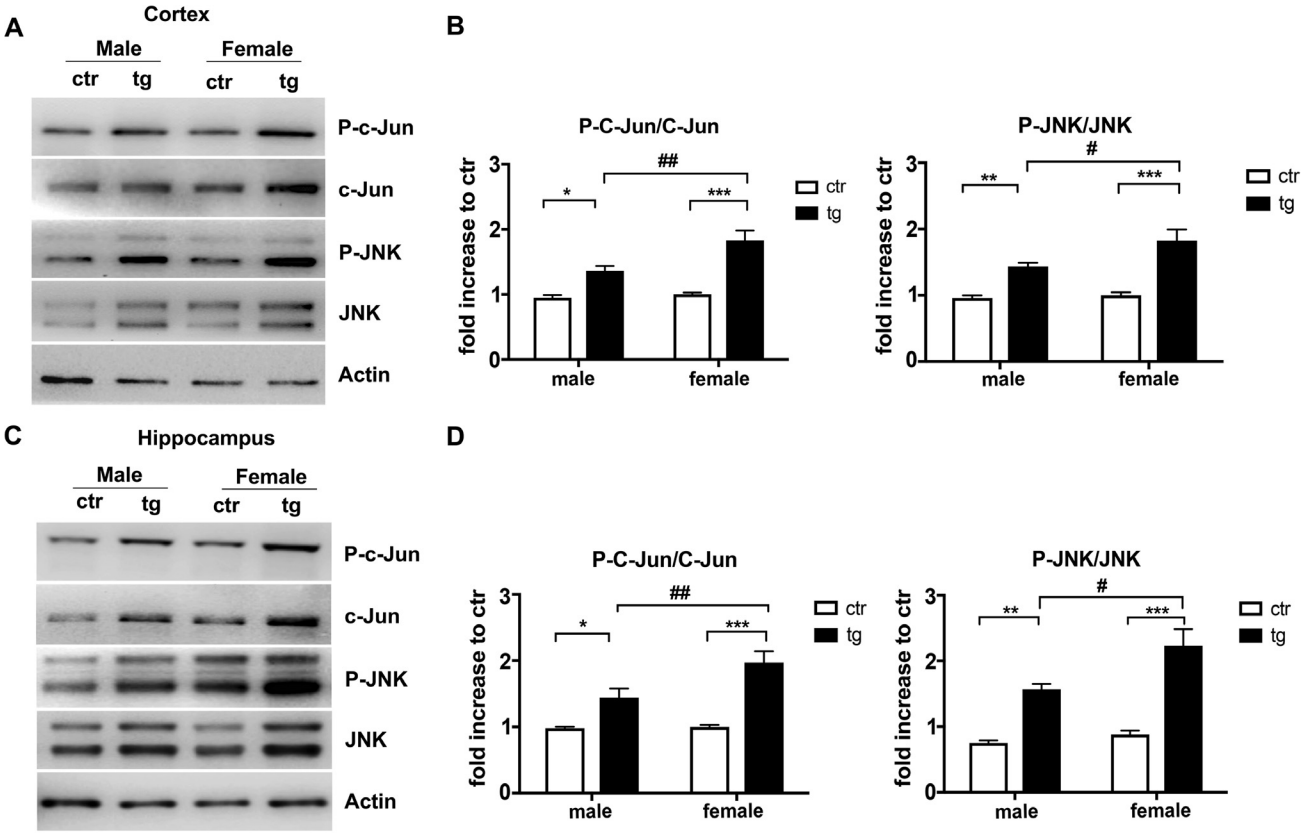

Fig. 2. Characterization of JNK pathway in the cerebral cortex and hippocampus of P301L-tg mice. A-C) Western blots and (B-D) relative quantifications showing P-JNK, JNK, P-c-Jun and c-Jun in the cerebral cortex and hippocampus from 15-month-old male and female ctr and P301L-tg mice. P-JNK/JNK and P-c-Jun/c-Jun ratios were increased in both male and female tg mice compared to the age-matched ctr mice in both brain areas analyzed. Considering the sex bias in both brain areas, P-c-Jun/c-Jun and P-JNK/JNK ratios were significant increase in female vs male tg mice. Cortex: significant relative to control $^{*}p < 0.05$, $^{***}p < 0.001$, tg female vs. male $^{\#}p < 0.05$, $^{\#\#}p < 0.01$. Hippocampus: significant relative to control $^{**}p < 0.01$, $^{***}p < 0.001$, tg female vs. male $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ [$n = 10$]. Two-way ANOVA, *Tukey's* post-hoc test. Data are shown as mean ± SEM.

tests, we separated females and male mice to study the sex difference in JNK pathway activation. In the cortex, the P-c-Jun/c-Jun and the P-JNK/JNK ratios were significant increase in females (Two-way ANOVA, P-c-Jun/c-Jun $p < 0.01$, P-JNK/JNK $p < 0.05$, Fig. 2A-B) compared to male tg mice. In the hippocampus, the P-c-Jun/c-Jun (Two-way ANOVA, $p < 0.01$, Fig. 2C-D) and P-JNK/JNK (Two-way ANOVA, $p < 0.05$, Fig. 2C-D) ratios were higher in female vs male tg mice as well. Thus, in summary the stress signaling JNK pathway is more activated in females P301L-tg mice in total homogenates.

P-Tau- and Caspase-3 activation in P301L-tg mice

Tau is a direct JNK's target (Ploia et al., 2011) and the hyper-phosphorylation of Tau is the main pathological feature in tauopathy. The total P-Tau (P-Tau_{tot}) level was measured in order to correlate this parameter to JNK pathway activation in this model. The P301L-tg mice presented a significant increased level of P-Tau_{tot} compared to ctr mice in both cortex and hippocampus (Two-way ANOVA, $p < 0.05$ and $p < 0.001$, see Fig. 3A-D). In addition, P-Tau_{tot} level was higher in female vs male tg mice in both brain areas analyzed (Two-way ANOVA, $p < 0.05$, Fig. 3A-D). Thus, females showed higher P-Tau_{tot} level compared to male P301L-tg mice, as previously reported by using different anti-bodies against specific sites of Tau phosphorylation (Buccarello et al., 2017a). Moreover, also caspase-3 is a direct JNK's target and it is implicated in neurodegenerative processes as well (D'Amelio et al., 2011; Sclip et al., 2014). Thus, we analyzed the activation of caspase-3 (cleaved-caspase-3) in P301L-tg total homogenates: cleaved-caspase-3 level was significantly higher in P301L-tg compared to ctr mice in both cortex and hippocampus (Two-way ANOVA, $p < 0.001$, Fig. 3A-D). Concerning the sexual dimorphism, the cleaved-caspase-3 level was higher in female vs male tg mice in both brain areas (Two-way ANOVA, $p < 0.01$ and $p < 0.05$, Fig. 3B-D). As a result, there is a sex-genotype effect on total P-Tau and cleaved caspase-3 levels in P301L-tg mice.

The dendritic spine pathology: JNK activation

We analyzed at the dendritic spine level the JNK activation to characterize the synaptic dysfunctionality/spine pathology of P301L-tg mice, never studied yet in this mouse model. The P-JNK/JNK ratio was significantly increased in P301L-tg compared to ctr mice

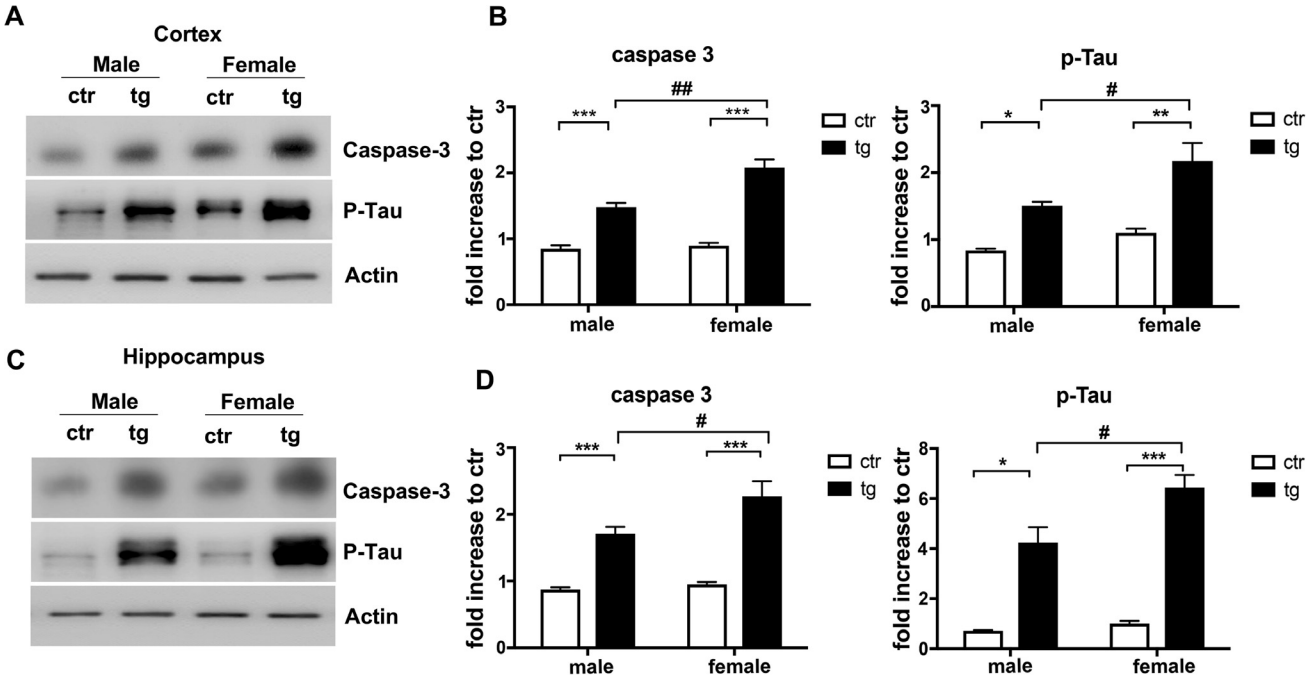

Fig. 3. Caspase-3 activation and P-Tau deposition in P301L-tg mice. (A-C) Western blots and (B-D) relative quantifications showing cleaved-caspase-3 and P-Tau$_{tot}$ in the cerebral cortex and hippocampus from 15-month-old male and female ctr and P301L-tg mice. The cleaved-caspase-3 and P-Tau$_{tot}$ levels were increased in both males and female tg mice compared to ctr mice in both the cortex and hippocampus, with a greater increase in females vs male tg mice in both brain areas analyzed. For cortex: significant relative to control $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; tg female vs male $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$. For Hippocampus: significant relative to control $^{*}p < 0.05$, $^{***}p < 0.001$, tg female vs male $^{\#}p < 0.05$ [$n = 10$]. Two-way ANOVA, *Tukey's* post-hoc test. Data are shown as mean ± SEM.

in the post-synaptic elements (see Fig. 4A-D), indicating that also at this level, the JNK signaling is powerfully activated. In particular, in the cortex, we observed a strong increase of P-JNK/JNK ratio in both sexes (Two-way ANOVA, $p < 0.001$, Fig. 4A-B) compared to ctr mice and, in the hippocampus, the P-JNK/JNK ratio was higher in males (Two-way ANOVA, $p < 0.01$; Fig. 4C-D) and females (Two-way ANOVA, $p < 0.001$; Fig. 4C-D) compared to ctr mice. We confirmed the sexual dimorphism previously observed, finding a stronger JNK activation in female vs male tg mice (Two-way ANOVA, $p < 0.001$, Fig. 4A-D). To link the spine dysfunctionality to the pathological Tau species, we studied P-Tau$_{tot}$ missorting at the dendritic spine level. The P-Tau$_{tot}$ level was significantly higher in tg vs control mice in both brain areas analyzed (Two-way ANOVA, cortex: $p < 0.05$ and $p < 0.001$, hippocampus: $p < 0.001$, Fig. 4A-D), and females showed higher P-Tau$_{tot}$ level vs male tg (Two-way ANOVA, cortex: $p < 0.05$ and hippocampus: $p < 0.001$, Fig. 4A-D), indicating a sex bias. In addition, we measured the phosphorylation of JNK on PSD95, a direct JNK's target and the most abundant scaffold protein of the PSD region. We already demonstrated PSD95 altered level in this model (Buccarello et al., 2017a); however, here we measured the P-PSD95/PSD95 ratio, studying the phosphorylation of PSD95 specifically mediated by JNK. The P-PSD95/PSD95 ratio was higher in P301L-tg compared to ctr mice, indicating a significant genotypic difference in the cortex as well as in the hippocampus (Two-way ANOVA, cortex: $p < 0.01$, $p < 0.001$ and hippocampus: $p < 0.001$; Fig. 4A-D). Importantly, dendritic spines presented a higher increase of P-PSD95/PSD95 ratio in female vs male tg mice in both brain areas (Two-way ANOVA, cortex: $p < 0.05$ and hippocampus: $p < 0.001$, Fig. 4A-D), denoting that there is a significant sex difference. To further characterize the spine pathology and its correlation with the JNK stress pathway, we analyzed the also the other JNK target, measuring the cleaved-caspase-3 level. Such as in the whole homogenate, we found a significant genotypic difference of its level: in fact, cleaved-caspase-3 level is higher, in both males and females P301L-tg mice compared to ctr mice in both brain areas analyzed (Two-way ANOVA, cortex and hippocampus: $p < 0.05$, $p < 0.001$, Fig. 4A-D), proving a genotypic effect. Concerning the sexual dimorphism, we observed an increased cleaved-caspase-3 level in females compared to male P301L-tg mice (Two-way ANOVA, $p < 0.05$, $p < 0.001$, Fig. 4A-D) in both brain areas examined. At the dendritic spine level, there was a sex effect on the caspase-3 activation in P301L-tg mice. We then examined Drebrin, an actin-binding protein highly concentrated within dendritic spines (Koganezawa et al., 2017), implicated in spine morphological changes. The Drebrin level was lower in tg compared to ctr mice in both brain areas analyzed (Two-way ANOVA, cortex: $p < 0.001$; hippocampus: $p < 0.01$ and $p < 0.001$, Fig. 4A-D), indicating smaller or less-plastic spines. Intriguingly, females showed lower Drebrin level vs male tg mice (Two-way ANOVA, cortex and hippocampus: $p < 0.001$, Fig. 4A-D), confirming the sexual dimorphism in P301L mouse model.

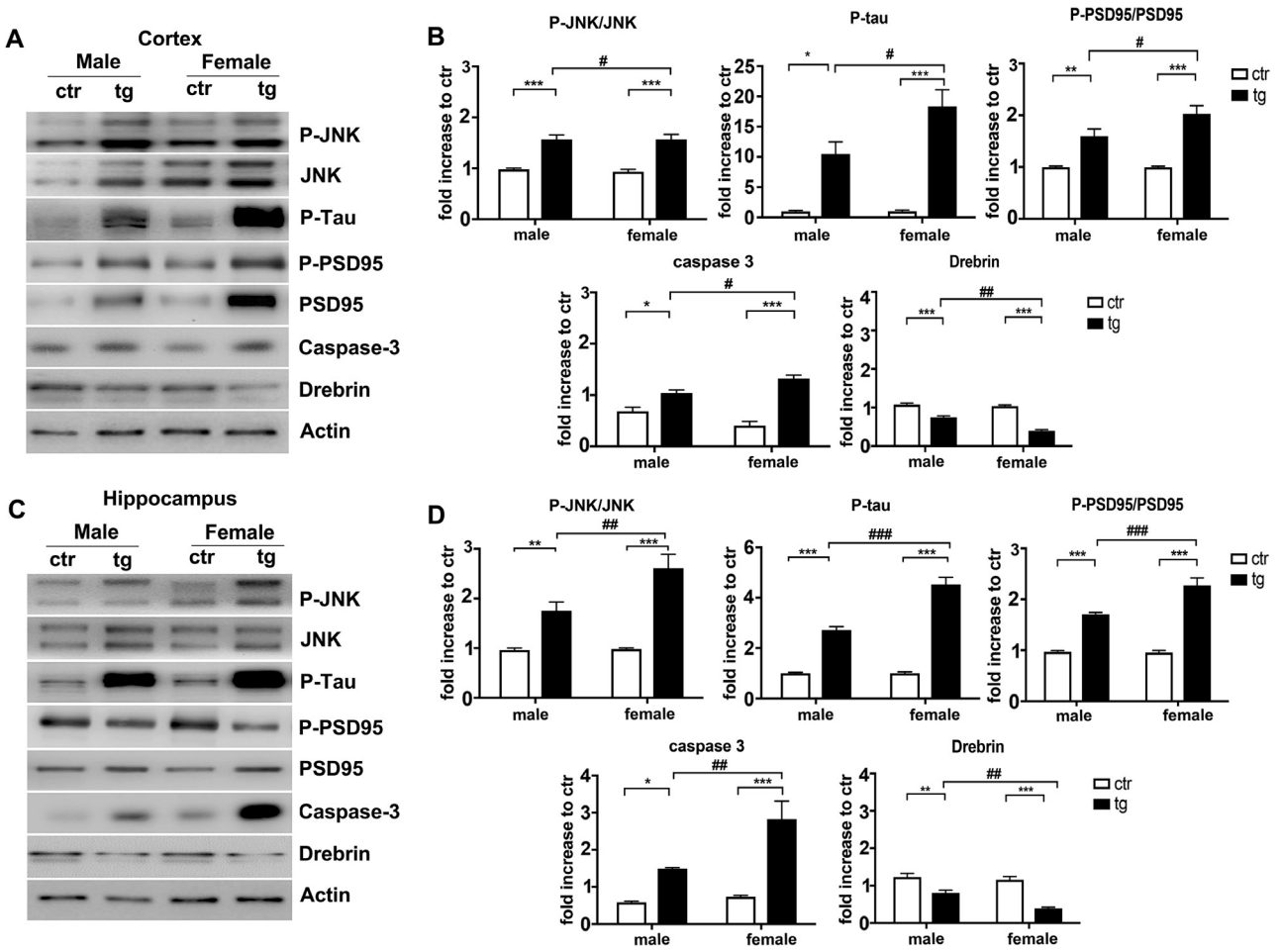

Fig. 4. The P301L-tg synaptopathy. A-C) Western blots and (B-D) relative quantifications performed on the TIF fraction showed in cortex and in hippocampus a significant increase of P-JNK/JNK and P-PSD95/PSD95 ratios in both females and male tg compared to ctr mice. It was found also in both brain areas a significant increase of P-Tau$_{tot}$ and cleaved-Caspase-3 level in both male and female tg mice compared to ctr mice and a strongly increase in female vs male tg mice. Drebrin level was decreased in male and female tg vs ctr mice in both the cortex and hippocampus, with a powerful reduction in females vs male tg mice in both brain areas analyzed. For P-JNK/JNK and P-PSD95/PSD95 ratios, Cortex: significant relative to control $^{**}p < 0.01$, $^{***}p < 0.001$, tg female vs male $^{\#}p < 0.05$; Hippocampus: significant relative to control $^{**}p < 0.01$, $^{***}p < 0.001$; tg female vs. male $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$. For Caspase 3, P-Tau$_{tot}$ and Drebrin, cortex: significant relative to control $^{*}p < 0.05$, $^{***}p < 0.001$; tg female vs male $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; Hippocampus: significant relative to control $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, tg female vs. male $^{\#\#}p < 0.01$ [$n = 10$]. Two-way ANOVA, *Tukey's* post-hoc test. Data are shown as mean ± SEM.

DISCUSSION

The main pathological hallmark of tauopathies is hyperphosphorylated Tau aggregates (NFTs) in the somato-dendritic compartment (Avila et al., 2004; Gendron and Petrucelli, 2009); more recently, P-Tau species has been identified also at the dendritic spine level (Haass and Mandelkow, 2010; Hoover et al., 2010; Menkes-Caspi et al., 2015). In particular, P301L-tg mice presented P-Tau and Tau missorted at the PSD region and this has been previously associated with PSD marker reduction levels (Buccarello et al., 2017a; Hoover et al., 2010), suggesting a role of P-Tau in spine pathology. Likewise, in primary neurons derived from P301L-tg mice, the mutant Tau (P-Tau) located to dendritic spines more than in control mice (Hoover et al., 2010). Thus, P-Tau accumulated into dendritic spines, but its pathological role, at this level, is not clear yet.

We find a correlation between behavioral impairments (cognitive and locomotor tests NORT and OF) and synaptic pathology (biochemical marker changes and the accumulation of toxic P-Tau) in P301L-tg mice. The behavioral tests demonstrated cognitive and locomotor impairments and, importantly, in both tasks the pathology was more severe in females compared to male tg mice. The functional analysis corroborates the previous metabolic results, confirming the most severe pathological phenotype in tg female associated to a significant body weight decrease and a lower percentage of survival rate compared to male mice (Buccarello et al., 2017a). In agreement with our data, Kandimalla and co-workers (Kandimalla et al., 2018) found that hippocampal learning/memory, motor learning and coordination were impaired in P301L mice, highlighting as hippocampal accumulation of P-Tau is responsible for abnormal mitochondrial dynamics, dysmorphogenesis

of dendritic spines and behavioral defects in this tauopathy mouse model.

The functional data on behavioral impairments completed a coherent picture, in which, females are more severe affected than male mice. Concerning the pathological mechanism that underling hyper-phosphorylated-Tau and its aggregates species in P301L, we investigated the possible involvement of the stress JNK signaling pathway. Notably, JNK not only hyper-phosphorylates Tau (Buccarello et al., 2017b; Orejana et al., 2013; Ploia et al., 2011), but it is also a key player in the AD's synaptic pathology, indicating a possible role of this stress pathway in this tauopathy model too. Here, analysing the JNK activation using a double approach for its quantification (evaluating the phosphorylation of JNK kinase itself and of its main target c-Jun), we proved a powerful JNK activation in P301L-tg mice.

Importantly, phospho-JNK was associated to hyperphosphorylated Tau in human samples (Atzori et al., 2001), this give a translational value to JNK role in Human. Moreover, neuropathological and biochemical findings reported in patients affected by frontotemporal dementia associated with a P310L, Tau accumulation in neurons associated with increased expression of different kinases, linked with tau phosphorylation such as p38, cdkl5 and GSK-3 beta (Ferrer et al., 2003).

P-Tau$_{mut}$ and P-JNK increased levels may be due to a wide range of stress pathway activations, among these ER and mitochondria play important roles in this cascade. How P-Tau$_{mut}$ triggers downstream JNK activation is not clear jet. We speculate that this may be due to microtubule disaggregation induced by the hyperphosphorylation of Tau, that leads to c-Jun N-terminal kinase-interacting protein 1 (JIP1) detachment from microtubules. JIP1 is a scaffold protein involved in focusing and accelerating JNK activity; if JIP1 is free from the microtubules, can link JNK and accelerate and focus its phosphorylation (inducing and increasing P-JNK level). Further experiments are needed to investigate this issue.

In more details, in P301L mice P-JNK is higher in hippocampus (hippocampus 28% more than cortex) compared to the cortex, suggesting that these two brain areas are differentially affected by the Tau-pathology. This difference has already been previously reported in this transgenic mouse model (Buccarello et al., 2017b). Interestingly, comparing the JNK activation between sexes in whole homogenate, in both brain areas, we found a sex bias underling a stronger activation of JNK in female vs male P301L-tg mice. To better clarify the JNK role in tauopathy, we analyzed different JNK targets implicated in neurodegenerative processes (Antoniou et al., 2011; Borsello et al., 2003; Repici and Borsello, 2006). In whole homogenate, we studied the elective JNK target c-Jun and cleaved-caspase-3, a direct JNK's target and an important executor in the apoptotic degenerative pathway (D'Amelio et al., 2011; Sclip et al., 2013) implicated in neuronal death, these are significantly higher in tg compared to ctr mice. Notably, the c-Jun and cleaved-caspase-3 levels are higher in female vs male P301L-tg mice, indicating that the death pathway activation is prominent in female vs male tg mice, as expected. Therefore, females are more severely affected by tauopathy then male mice in P301L mouse model (Buccarello et al., 2017a; Hunsberger et al., 2014; Katsuse et al., 2006) as well as in human patients (Narasimhan et al., 2017).

Being synaptic pathology a key feature in many brain diseases, we focus on studying the mechanism of spine degeneration. We examined JNK activation at the dendritic spine level, since in polarized cell, like neurons, the localization/cellular compartments of the kinase activity are even more important.

In addition, we focused on studying the mechanism of spine pathology. JNK is strongly activated at the spine level as well. This according to our knowledge is the first data indicating JNK involvement in Tau-induced spine injury.

In this contest, P-JNK/JNK ratio was increased in P301L-tg compared to ctr mice, and its level was significantly higher in females vs male P301L-tg, indicating an important sexual dimorphism. Notably, we investigated also the P-Tau$_{tot}$ at the dendritic spine level to correlate toxic Tau species with JNK activation at the dendritic spine. The P-Tau$_{tot}$ level presented a sex-genotype effect, in line with JNK activation.

Concerning the JNK targets at this level, PSD95 and cleaved-capsase-3 showed an increased in P301L-tg compared to ctr mice. Importantly, females exhibited higher levels in comparison with male P301L-tg, indicating an important sexual dimorphism. Finally, the Drebrin marker is in line with P-Tau-induced spine pathology: in fact, P301L-tg mice showed lower Drebrin level compared to ctr mice and females showed a further reduction then male tg mice. The decreased Drebrin level indicates smaller or less-plastic dendritic spines and this was more pronounced in females, indicating a stronger impairment of their synapses. All together these data suggest that dendritic spine pathology presents an important sex dimorphism: females showed a higher activation of JNK and its targets with a lower Drebrin level revealing a more severe dendritic spine dysfunction/loss in female vs male P301L-tg mice. Notably, the stronger synaptopathy in female vs male tg mice is well correlate with the severe cognitive/locomotor impairments observed in females.

Summarizing, these results suggest that the synaptic dysfunction and memory impairments are caused by abnormal accumulation of P-Tau that this is associated to JNK signaling pathway activation at the spine level, which could be targeted by inhibitory drugs to tackle spine pathology in many different brain diseases.

However how Tau mutation in P301L correlated with the stress JNK pathway activation is not clear jet, since JNK is a kinase activated by many different stressors.

Importantly, the sex bias described for JNK signaling is a very interestingly aspect, going in the direction of the gender differences found in many brain diseases. It will be fundamental now to verify the translational value of these results studying patient's tissues, since JNK

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could be a new target and also an important predictive biomarker of synaptopathy. In fact, recently encouraging evidences had showed that JNK3 is detected in MCI and AD liquors as a new indicator of dementia (Gourmaud et al., 2015).

CONCLUSION

JNK sexual dimorphic activation can have important implications for future target therapeutic directions and underlines the need of personalized medicine, an important aspect of which is the sex and gender differences, which are still underestimate.

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CONFLICTS OF INTEREST

The authors declare no actual or potential conflicts of interest.

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