Neuroscience 354 (2017) 208–220

NEUROPROTECTIVE EFFECTS OF LOW FAT-PROTEIN DIET IN THE P301L MOUSE MODEL OF TAUOPATHY

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Abstract—Tauopathies are a class of neurodegenerative diseases associated with the pathological aggregation of tau protein in the human brain. Although numerous studies in mouse models of Alzheimer disease (AD) have shown a correlation among diet, beta-amyloid and AD onset, little is known about the impact of diet on Tau. We investigated whether a low fat-protein diet (LFPD) may improve lifespan, cognitive and locomotor activity in P301L-tg mouse model of tauopathy. Our data indicate that LFPD has a beneficial effect on these parameters. Tg mice fed with standard diet shown a decrease in body weight, food intake and survival rate if compared to wild type animals. In contrast, LFPD counteracted weight loss, increased mortality and ameliorated cognitive and locomotor performances in tg mice. LFPD also reduced the abnormal accumulation of agglomerates of P-Tau (pathological features of tauopathies) and the expression of apoptotic markers (i.e., TUNEL immunopositive neurons) in the prefrontal cerebral cortex and hippocampus of P301L-tg mice. Interestingly, some of these effects are sex-dependent. For instance, tg females, but not males, fed with LFPD had a significant increase of body weight and a reduction of P-Tau agglomerates compared to tg fed with standard diet. These changes correlated with a more pronounced improvement of cognition and locomotor activity in females than in male tg fed with LFPD. Altogether, these results suggest a sex dependent neuroprotective effect of LFPD in P301L-tg mice, suggesting that lifestyle intervention strategies may be clinically relevant for delaying the onset of cognitive impairment and dementia, especially in females.

Key words: diet, tauopathy, sex difference, hyperphosphorylated Tau, neuronal death, neuroprotection.

INTRODUCTION

Tauopathies are neurodegenerative diseases including several forms of dementia, such as frontotemporal dementia, progressive supranuclear palsy and Alzheimer disease (AD) (Nasreddine et al., 1999; Iqbal et al., 2005). Age, sex and nutrition are the major risk factors for dementia (Launer et al., 1999). In particular, recent epidemiological studies show specific association between nutritional components and the risk for neurodegenerative diseases (Morris and Tangney, 2014; Mosconi et al., 2014; Berti et al., 2015). Moreover, long-term consumption of a high-caloric diet is a risk factor that may significantly contribute to the development of neurological disease (Schroeder and Richardson, 2010).

Indeed, an epidemiological study suggested that individuals following diet with high caloric intake have a 1.5 times greater risk of AD than those with low caloric intake (Luchsinger et al., 2002). Interestingly, a number of studies, investigating the association of n−3 polyunsaturated fats (PUFA) or docosahexaenoic acid (DHA) levels (Kitajka et al., 2002) with AD reported reduction of these molecules in *post-mortem* autopsy brain samples (Soderberg et al., 1991; Guan et al., 1994; Han et al., 2001; Green et al., 2007a,b). Major epidemiological surveys shown that dietary n−3 PUFA and/or DHA significantly reduced the risk of developing AD (Kalmijn et al., 1997; Barberger-Gateau et al., 2002; Morris et al., 2003). Moreover, brain DHA levels were positively associated with cognitive and behavioral performance (McCann and Ames, 2005). Indeed, n−3 PUFA supplementation can improve brain function, especially for complex tasks in normal individuals (Fontani et al., 2005).

Also different macro- and micronutrients as B-vitamins and antioxidants can influence brain structure and function (Bourre et al., 2004; Bourre, 2004, 2006a,b; Weih et al., 2007). For instance, polyphenols, in addition to their antioxidant properties, have been reported to exert neuroprotective effects in tg CRND8 mice (Pantano et al., 2016) by directly modulating cellular pathways related to neuronal processes and synaptic

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Abbreviations: AD, Alzheimer disease; wt, wild type; DHA, docosahexaenoic acid; LFPD, low fat-protein diet; PUFAs, polyunsaturated fatty acids; tg, transgenic.

http://dx.doi.org/10.1016/j.neuroscience.2017.04.027

plasticity (Kawashima et al., 2010). Fatty acids have been implicated in enhancing brain plasticity and cognitive function in healthy adult rodents (Maqsood and Stone, 2016; Wang and Mitchell, 2016; Giles et al., 2016; Weiser et al., 2016) as well as in transgenic mouse models of AD. In fact, enriching the diet of transgenic mice with docosahexaenoic acid (DHA) significantly lowered the synthesis of beta-amyloid peptides and the formation of amyloid plaques, one of the major hallmarks of the disease (Amtul et al., 2011).

The availability of transgenic animal models of AD has permitted investigations of the impact of nutrients and other compounds isolated from foods on AD-related neuropathology, with particular focus on the amyloid-beta peptide production and accumulation in the brain and on behavioral deficits (Kadish et al., 2016; Kothari et al., 2016; Janssen et al., 2016). In a rat AD model caused by injection of amyloid-β ($A\beta_{1-40}$), DHA or eicosapentanoic acid (EPA) were both beneficial agents (Hashimoto et al., 2002, 2006, 2009). Similarly, in various transgenic mouse models supplementation with n−3 PUFA (e.g., DHA) was associated with lower Aβ levels and improved cognition (Green et al., 2007a,b; Fiol-deRoque et al., 2013; Bascoul-Colombo et al., 2016).

The above works indicated that dietary DHA might be beneficial for AD, possibly by alleviating the amyloid pathology.

Different studies have also investigated the role of high-fat diet, high-sugar diet and/or high-cholesterol diet on P-Tau, another pathological hallmark of AD (Refolo et al., 2000; Glöckner et al., 2011; Leboucher et al., 2013). However, the results are conflicting, reporting increased, decreased or unchanged P-tau levels. Therefore, further studies to assess the role of diet on tauopathies are required.

In this work we investigated the impact of a low fat-protein diet on the onset and progress of tauopathy, with a main focus on the phosphorylation of Tau, in the P301L mouse model of tauopathy (Buccarello et al., 2017). In addition, we explored whether a possible sex dimorphic effect may occur. To this purpose, we examined the body weight, food-water intake and survival rate of males and female transgenic P301L-Tau mice fed with standard and low fat-protein diet (LFPD) and wild type mice (wt) fed with standard diet. The 3 months old mice were used for these experiments, and monitored for 12 months. At 15 months of age, mice were tested for behavioral assays to analyze the impact of diet on cognitive/learning and locomotor performance, then sacrificed and processed for immunohistochemical analysis. Together, these results add new perspectives to our understanding on how dietary intake can contribute to AD and Tau-related pathologies, underlying the importance of a correct nutrition in the prevention of neurodegenerative diseases.

MATERIALS AND METHODS

Animals and diets

In this study we used male and female hemizygous P301L-tg mice and age-compatible wild type mice (B6D2F1) of mixed gender as controls. 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 et al., 1996). Mice originated from Taconic Laboratories, USA, were bred at 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%. All mice were provisioned with bedding material (hard wood shavings), ad libitum food and water. Animals were housed (n = 4 per group) in standard mouse cages. Until three months of age all animals were fed with standard rodent chow (**Standard diet**: 18% protein and 5% fat, Envigo Lab. 2018S Tekland global diet, http://www.envigo.com/products-services/teklad/laboratory-animal-diets). Three months old animals were then divided into two experimental groups, balanced for body weight and sex. The first group was fed with a standard rodent chow (**Standard diet**: 18% protein and 5% fat, Envigo Lab. 2018S Tekland global diet, http://www.envigo.com/products-services/teklad/laboratory-animal-diets), while the second group was fed with a low fat protein diet (LFPD) (**Low fat protein diet**: 14% protein and 3.5% fat Envigo Lab. 2014S Tekland global diet, http://www.envigo.com/products-services/teklad/laboratory-animal-diets).

Since the animals were bred in a SPF facility, where all materials introduced are sterilized, we used an autoclavable diet manufactured with high quality ingredients and supplemented with additional vitamins to ensure nutritional adequacy after autoclaving.

The **Standard diet** is a fixed formula, autoclavable diet designed to support gestation, lactation, and growth of rodents. This diet does not contain alfalfa, thus lowering the occurrence of natural phytoestrogens. Typical isoflavone concentrations (daidzein + genistein aglycone equivalents) range from 150 to 250 mg/kg. Exclusion of alfalfa reduces chlorophyll, improving optical imaging clarity. Absence of animal protein and fishmeal minimizes the presence of nitrosamines.

The **Low fat protein diet** (LFPD) is a fixed formula, autoclavable diet designed to promote longevity and normal body weight in rodents. This diet does not contain alfalfa or soybean meal, thus minimizing the occurrence of natural phytoestrogens. Typical isoflavone concentrations (daidzein + genistein aglycone equivalents) range from non-detectable to 20 mg/kg. Exclusion of alfalfa reduces chlorophyll, improving optical imaging clarity. Absence of animal protein and fishmeal minimizes the presence of nitrosamines.

Ethics statement

Procedures involving animals and their care were in accordance 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: 71/2014 B).

Metabolic profile and survival rate

Animals were monitored daily for wellbeing welfare-related disease symptoms. Body weight, food and water consumption were recorded weekly. Based on the daily consumption of food of a mouse (5 g/daily for each mouse), it was decided to administer 200 g of food per cage containing four mice (both standard and LFPD diet), allowing to the animals the food access ad libitum. A decrease in body weight greater than 15% in two consecutive weeks (15% + 15%) was considered as end-point of the study. The data are shown as the averaged values for each experimental group every 4 weeks, except for the metabolic data collected in the first 12 weeks.

Open Field and spontaneous locomotor activity

The Open Field (OF) test 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 (Walsh and Cummins, 1976; Crawley, 2007; Seibenhener and Wooten, 2015). We used a grey 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; Li et al., 2014). The time window between the OF and the NORT was of 24hrs.

Novel-object recognition test

The novel-object recognition test (NORT) is a memory test that relies on spontaneous animal behavior (Ennaceur and Delacour, 1988; Clarke et al., 2010). 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. 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.

Immunohistochemistry

At the end of behavioral tests, animals were euthanized by cervical dislocation (Angus et al., 2008; Carbone et al., 2012); brains were removed and fixed in 10% formalin for 24–48 h with the usual procedure and embedded in paraffin. After deparaffinization brain coronal sections (3 μM thick; three slices per mouse) were stained for immunohistochemistry analysis utilizing specific phospo-Tau primary antibodies AT100 and AT8 (concentration 1:1000, Euroclone). The protocol used for the detection of antibodies was described in Buccarello et al., 2017.

To measure the level of apoptosis in the brain, TUNEL assay were performed using Dead-end™ Colorimetric TUNEL System (Promega, nr G3250) using the protocol described in Buccarello et al., 2017.

Neuronal counts

The accumulation of pathological Tau species (detected with AT100 and AT8 antibodies) and the level of apoptosis (detected with TUNEL) were quantified in the prefrontal cortex (the whole thickness of the prefrontal cortex) and CA1 area of hippocampus (brain coronal sections). Labeled cells were counted by image analysis software (the Olympus DP-software program) 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). Following manual tracing of the prefrontal cortex and hippocampus at the same stereotactic level in all mice, the number of immunopositive staining cells were manually tagged and the software executed

counts. Positive cells' staining was quantified by an operator blind to genotype and treatment.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 6 program. Body weight data, food and water consumption data, NOR data, OF data and neuronal counts data were analyzed using One-way ANOVA, followed by Sidak's post hoc test. Survival ratio was analyzed by Log-rank (Mantel-Cox) test. All data were expressed as mean ± SEM with a statistical significance given at $p < 0.05$.

RESULTS

The low fat protein diet affects body weight, food intake and survival rate of P301L-tg mice

The average body weight of wt and P301L-tg mice fed with the standard diet or the LPFD was monitored from 3 to 15 months of age. Male and female P301L-tg mice fed with standard diet had smaller body weights compared to wt animals (ANOVA, $p < 0.0001$ and $p < 0.001$, respectively; Fig. 1A, B). However, female P301L-tg mice fed with the LFPD had a significant increase in the body weight compared to tg mice fed with the standard diet (ANOVA, $p < 0.001$, Fig. 1A).

Interestingly, the decrease in body weight in tg mice fed with standard diet correlated to a lower food intake (ANOVA main effect of genotype, $p < 0.0001$, Fig. 1C, D). However, both female and male tg mice fed with LFPD exhibited a significantly greater food intake than tg mice fed with standard diet (ANOVA, $p < 0.0001$, Fig. 1C, D). Finally, to investigate the impact of diet on survival of P301L-tg mice, we measured the survival rate. As expected, P301L-tg mice fed with standard diet survived less then wt mice (Chi square = 61.58, $p < 0.0001$, Fig. 1E, Chi square = 44.77 $p < 0.0001$, Fig. 1F). On the contrary, both female and male P301L-tg mice fed with LFPD had a significantly higher percentage of survival compared to tg mice fed with standard diet (Female mice: Chi square = 61.56, $p < 0.0001$, Fig. 1E, Male mice: Chi square = 42.32 $p < 0.0001$, Fig. 1F).

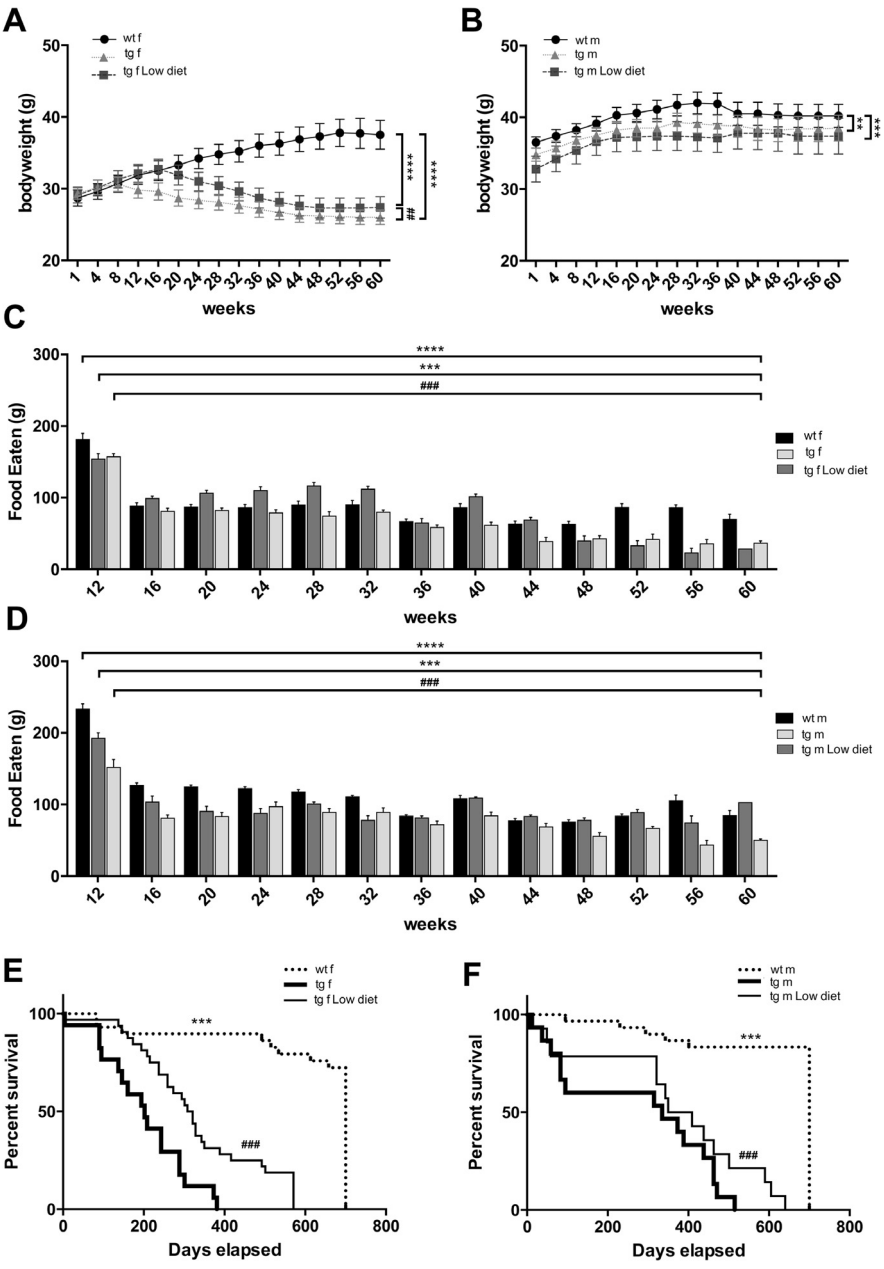

Fig. 1. Effect of low fat-protein diet on body weight, food consumption and survival rate in P301L-tg mice. (A, B) Significant reduction of body weight in female (A) and male (B) tg compared to wt mice fed with standard diet. Tg *vs* wt mice fed with standard diet (both males and females) $^{****}p < 0.001$. Significant increase of body weight in female tg fed with Low Fat protein Diet (LFPD) *vs* tg fed with standard diet. Tg female fed with LFPD *vs* wt mice $^{****}p < 0.001$, tg male fed with LFPD *vs* wt mice $^{****}p < 0.0001$, tg female fed with LFPD *vs* tg mice $^{\#\#}p < 0.01$. (C, D) Significant decrease of food consumption in female (C) and male (D) tg compared to wt mice fed with standard diet. Tg *vs* wt mice fed with standard diet $^{****}p < 0.001$. Significant increase of food consumption in tg fed with LFPD *vs* tg fed with standard diet (both males and females). The tg mice fed with LFPD *vs* ctr mice (both males and female) $^{\#\#\#\#}p < 0.001$. (E, F) Significant reduction of survival rate in female (E) and male (F) tg compared to wt mice fed with standard diet. Significant increase of survival rate in tg fed with LFPD *vs* tg fed with standard diet (both males and females; $n = 30$ for each group). Tg *vs* wt mice $^{***}p > 0.0001$ and tg fed with standard diet *vs* tg fed with low diet $^{\#\#\#}p < 0.0001$ (n = 30 for each group). One-way ANOVA, *Sidak's* post hoc test. Data were shown as mean ± SEM.

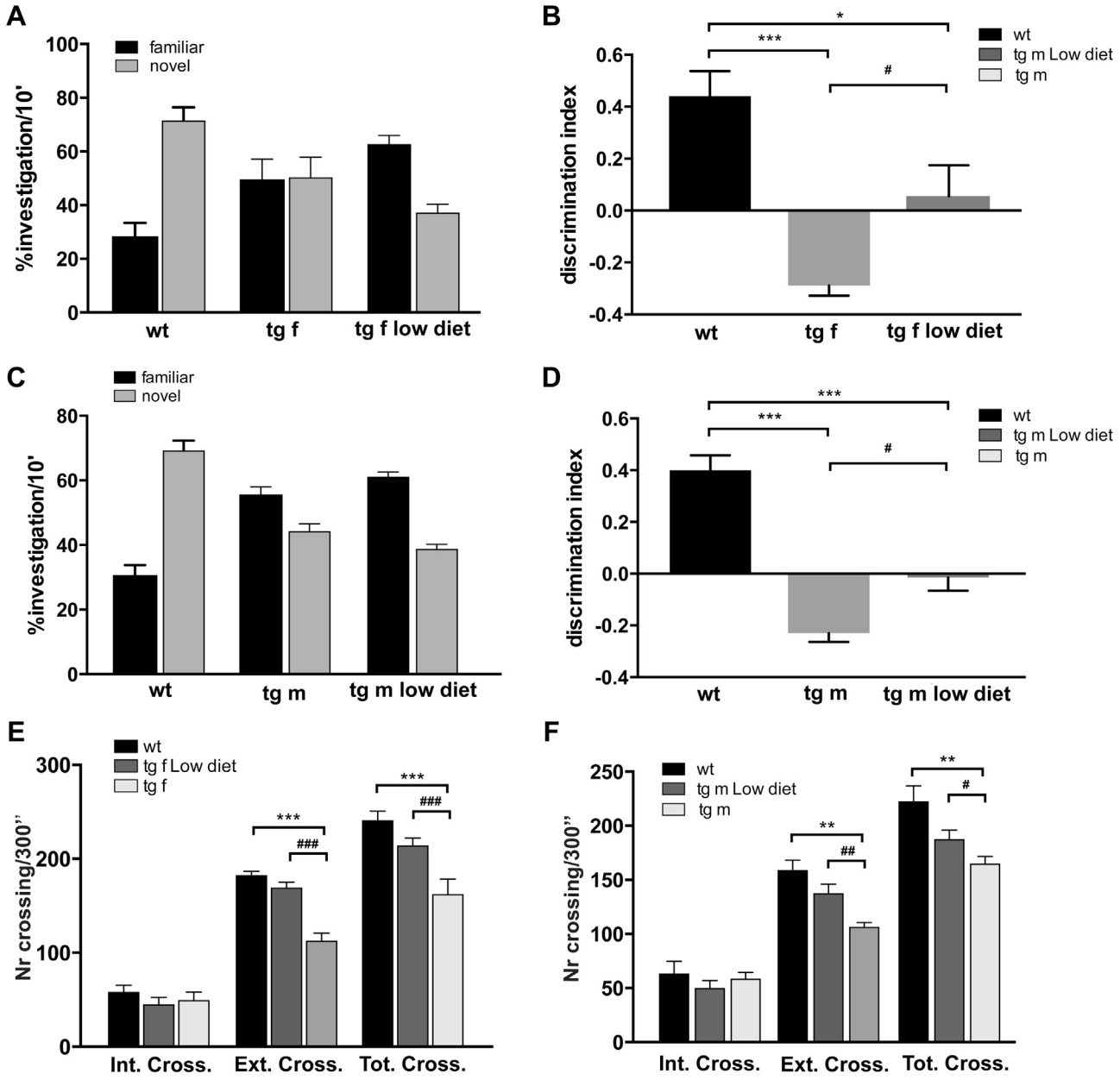

Fig. 2. Effect of low fat-protein diet on cognitive deficits and locomotor performance in P301L-tg mice. (A, D) The Novel Object Recognition Test. Histograms indicate the investigation time (mean ± SEM) spent on the familiar and novel objects in female (A) and male (C) P301L-tg mice fed with LFPD or standard diet *versus* wild type animals fed with standard diet. (B–D) Histograms show mean ± SEM of the DI in female (B) and male (D) P301L-tg and wt mice fed with LFPD or standard diet. There is a significant effect of genotype and diet both in male and female Tg *vs* wt mice fed with standard diet. Both males and females tg *vs* wt $^{****}p < 0.001$, tg female fed with LFPD *vs* wt mice $^{*}p < 0.05$, tg male fed with LFPD *vs* wt mice $^{****}p < 0.001$, tg fed with standard diet *vs* tg fed with LFPD $^{\#}p < 0.05$ by Tukey's post hoc test. (E, F) The Open Field Test. Histograms represent the locomotor activity in female (E) and male (F) P301L-tg mice fed with LFPD or standard diet *versus* wt animals fed with standard diet. External and total crossing: tg female vs wt mice $^{***}p > 0.0001$, tg female fed with standard diet *vs* tg fed with LFPD $^{\#\#\#}p < 0.0001$. External crossing: tg male *vs* wt mice $^{**}p > 0.01$, tg male fed with standard diet *vs* tg fed with LFPD $^{\#\#}p < 0.01$; total crossing: tg male *vs* wt mice $^{***}p > 0.001$, tg male fed with standard diet *vs* tg fed with LFPD $^{\#}p < 0.05$ ($n = 10$ for each group). One-way ANOVA, *Sidak's* post hoc test. Data were shown as mean ± SEM.

The low fat protein diet enhances locomotor and cognitive performances in P301L-tg mice

Two different behavioral tests were performed. The first day mice were tested with the Open field test and the day after with the Novel object recognition test. Data obtained in our previous work (Buccarello et al., 2017) had shown as female P301L mice were more affected than male mice by tauopathy particularly at the final stage of the pathology (with a severe decrease of body weight, food consumption and survival rate). In order to avoid further stress to females, we performed Open field test with a duration of 5 min for each trial, choosing to use this test also as habituation trial for the second test performed the Novel object recognition test.

To investigate the impact of diet on cognitive impairment in P301L-tg mice the locomotor activity was assessed with the Open field test: a genotype effect on locomotor performance was observed (ANOVA, $p < 0.0001$; Fig. 2E, F). In particular, both female and male P301L-tg mice fed with standard diet shown a decrease in the total number of crossing as well as in the number of internal and external crossing if compared to wt mice fed with standard diet (Fig. 2E, F). On the contrary, P301L-tg mice fed with the LFPD shown an improvement of the locomotor performance, more evident in females than male tg mice. Indeed, a significant diet effect was found measuring the number of external crossing (ANOVA female: $p < 0.0001$, Fig. 2E; male: $p < 0.001$, Fig. 2F) and total number of crossing (ANOVA female: $p < 0.0001$, Fig. 2E; male: $p < 0.05$, Fig. 2F).

Concerning the cognitive impairment in P301L-tg mice, cognitive deficits were assessed with the novel object recognition test (NORT). P301L-tg mice fed with standard diet exhibited memory deficits, as they spent less time compare to control (ANOVA $p < 0.0001$, Fig. 2) investigating the novel object compared to the familiar object (Fig. 2A–C), therefore having a lower discrimination index (ANOVA $p < 0.0001$, Fig. 2B, D). Interestingly, the cognitive impairment was more severe in female than male P301L-tg mice fed with standard diet (see Fig. 2B, D). Both female and male P301L-tg mice fed with the LFPD shown a recovery of cognitive deficits (ID) compared to tg mice fed with standard diet (ANOVA $p < 0.05$, Fig. 2B, D). The improvement in cognition due to LFPD was more pronounced in female (87% recovery) than male (50% recovery) (Fig. 2B, D). Analyzing the influence of diet on exploratory activity and anxiety like-behavior of P301L-tg mice, we detected a mainly genotype effect in the parameters analyzed as the number and time spent for rearing (ANOVA, $p < 0.05$; Fig. 3A, B) and the number and time spent for grooming (ANOVA, $p < 0.01$; Fig. 3C, D). In particular, female P301L-tg mice fed with standard diet shown a decrease in the number and time of rearing as well as in the number and time of grooming if compared to wt mice fed with standard diet (Fig. 3A–D).

While we found only in male P301L-tg mice, fed with the LFPD, an improvement in the number and time of grooming (ANOVA: $p < 0.01$, Fig. 3C, D) in comparison with male tg mice fed with standard diet.

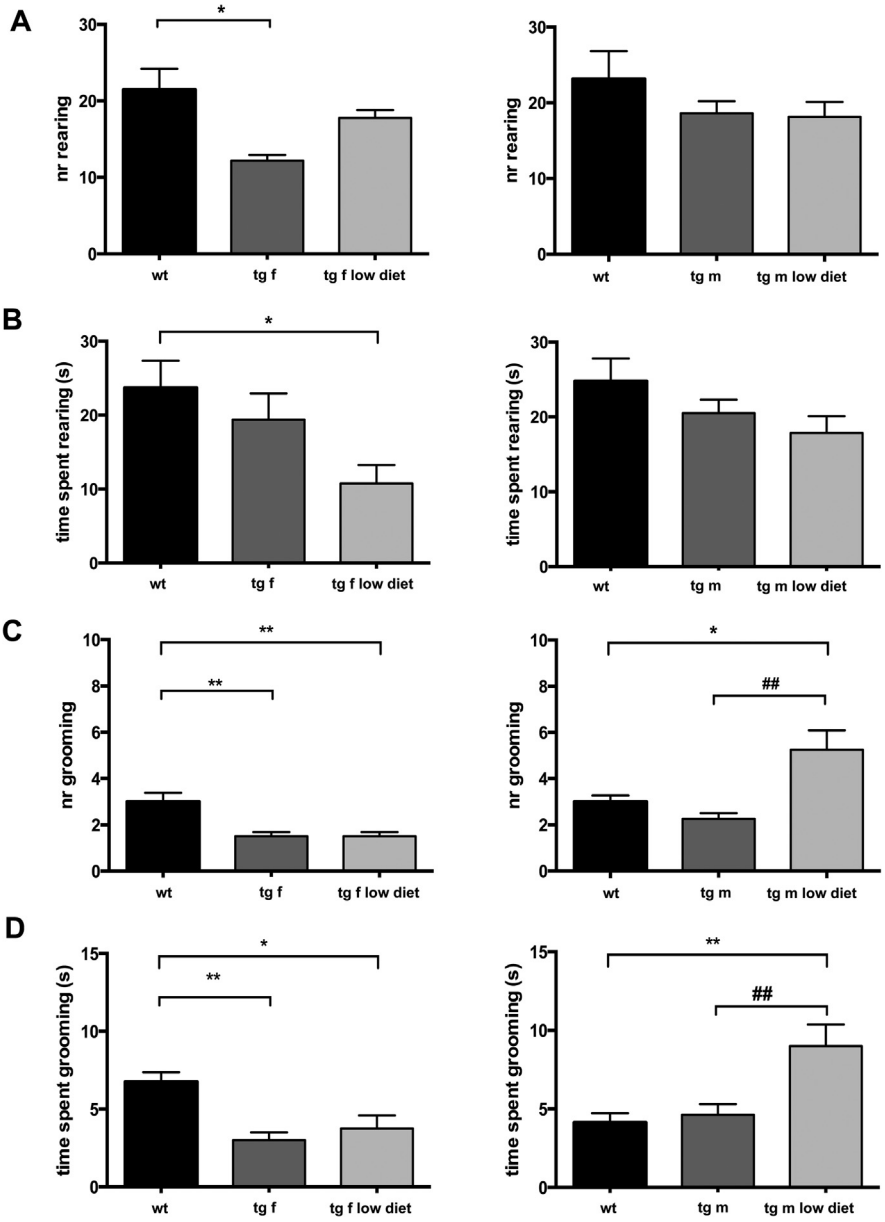

Fig. 3. Effect of low fat-protein diet on anxiety-like behavior in P301L-tg mice. (A-D) The Open Field Test. Histograms represent the anxiety like-behavior in female and male P301L-tg mice fed with LFPD or standard diet *versus* wt animals fed with standard diet. (A) Number of rearings: female wt vs tg mice fed with standard diet $^{*}p > 0.05$. (B) Time spent for rearings: female wt fed with standard diet *vs* tg fed with LFPD $^{*}p > 0.05$. (C) Number of grooming: female wt vs tg mice fed with standard diet $^{**}p > 0.01$; male wt fed with standard diet *vs* tg fed with LFPD $^{*}p > 0.05$; tg male fed with standard diet *vs* tg fed with LFPD $^{\#\#}p < 0.01$. (D) Time spent for grooming: female wt vs tg mice fed with standard diet $^{**}p > 0.01$; female wt vs tg mice fed with standard diet $^{*}p > 0.05$; male wt fed with standard diet *vs* tg fed with LFPD $^{**}p > 0.01$; tg male fed with standard diet *vs* tg fed with LFPD $^{\#\#}p < 0.01$ ($n = 10$ for each group). One-way ANOVA, *Sidak's* post hoc test. Data were shown as mean ± SEM.

The low fat protein diet decreases the agglomerates of P-Tau in cerebral cortex and hippocampus of P301L-tg mice

Sections of both P301L-tg and wt mice brains were screened for P-tau aggregates in the prefrontal cerebral cortex and hippocampal CA1 region (see Figs. 4–5), using the AT100 and AT8 antibodies. The AT100 staining revealed a significant presence of P-Tau agglomerates in both male and female P301L-tg mice fed with standard diet (Fig. 4A, B), revealing a significant genotype effect in either sexes (ANOVA cortex $p < 0.0001$, Fig. 4C; hippocampus: $p < 0.0001$, Fig. 4D). Interestingly, female, but not male, P301L-tg mice fed with the LFPD had a significant decrease of P-Tau agglomerates than P301L-tg mice fed with standard diet (ANOVA cortex $p < 0.0001$, Fig. 4C; hippocampus: $p < 0.0001$, Fig. 4D). The AT8 staining confirmed a significant presence of P-Tau agglomerates in both male and female P301L-tg mice fed with standard diet (Fig. 5A, B), revealing a significant genotype effect in either sexes (ANOVA cortex $p < 0.0001$, Fig. 5C; hippocampus: $p < 0.0001$, Fig. 5D), most marked in females than male tg mice. Interestingly, we observed a significant protective effect of diet in both male and female P301L-tg mice. Tg animals fed with the LFPD had a significant decrease of P-Tau agglomerates than P301L-tg mice fed with standard diet (ANOVA cortex $p < 0.05$, Fig. 5C; hippocampus: $p < 0.05$ and $p < 0.001$, Fig. 5D).

Neuronal death was rescued in P301L-tg mice by the low fat protein diet

The P301L-tg mice exhibited a significant neuronal loss at 10 months of age (Lewis et al., 2001). To investigate whether our dietary manipulation affected neuronal loss in P301L-tg mice, representative sections of both prefrontal cortex and hippocampal CA1 region were stained with the TUNEL. This staining shown the presence of sig-

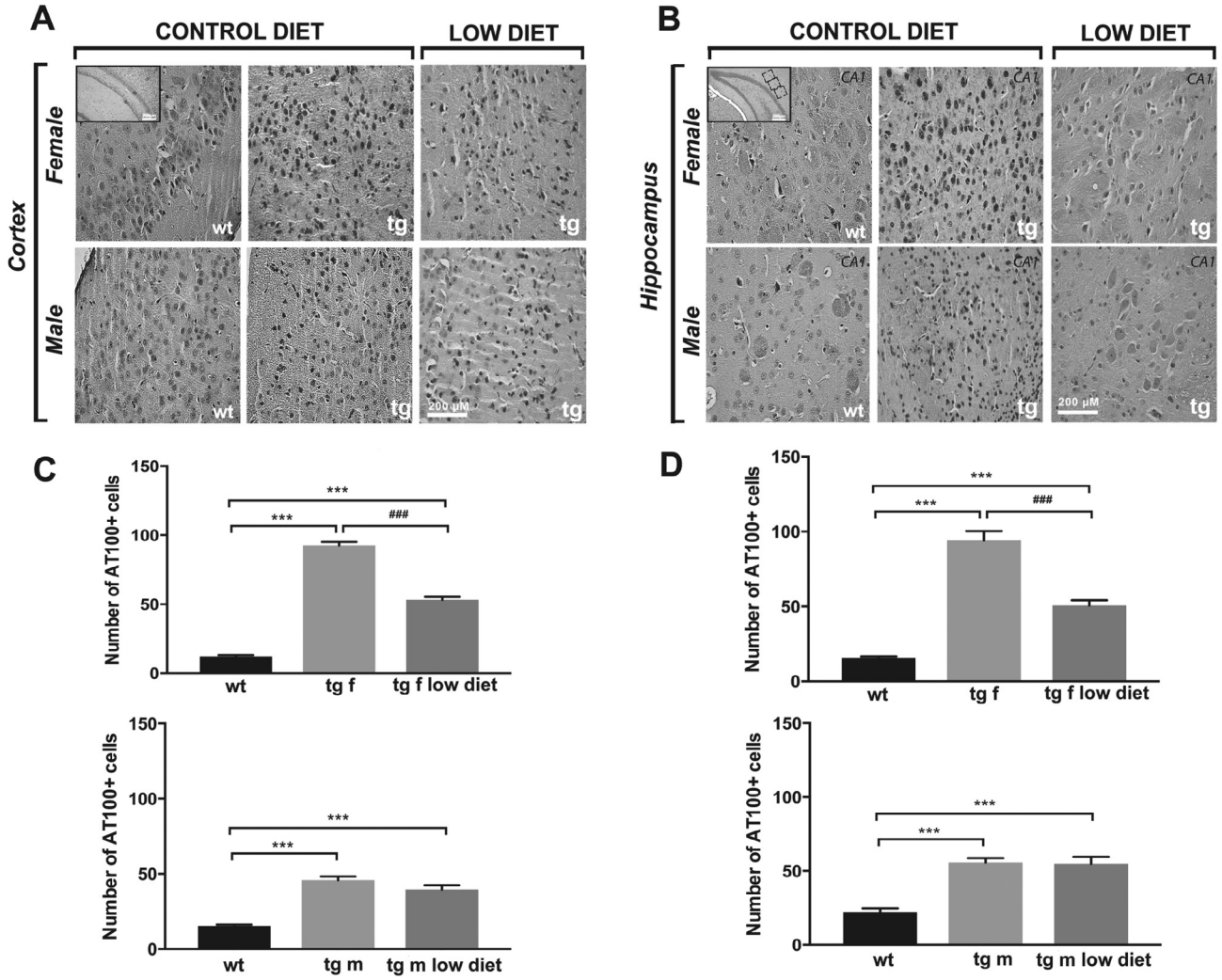

Fig. 4. AT100 immunoreactivity in P301L-tg mice. PThr212/pSer214 (AT100)-stained sections in the cerebral cortex (A) and hippocampus (B) of female and male P301 L-tg mice fed with LFPD or standard diet *versus* wt animals fed with standard diet. Representative sections are shown each group. Scale bar: 200 μm. Quantification of AT100+ neurons in the cerebral cortex (C) and hippocampus (D) of female (up) and male (down) P301L-tg mice fed with LFPD or standard diet *versus* wt animals fed with standard diet. Tg *vs* wt mice fed with standard diet (both males and females) $^{****}p < 0.001$, tg female fed with standard diet *vs* tg fed with LFPDs $^{\#\#\#}p < 0.0001$. One-way ANOVA, *Sidak's* post hoc test. Data were shown as mean ± SEM.

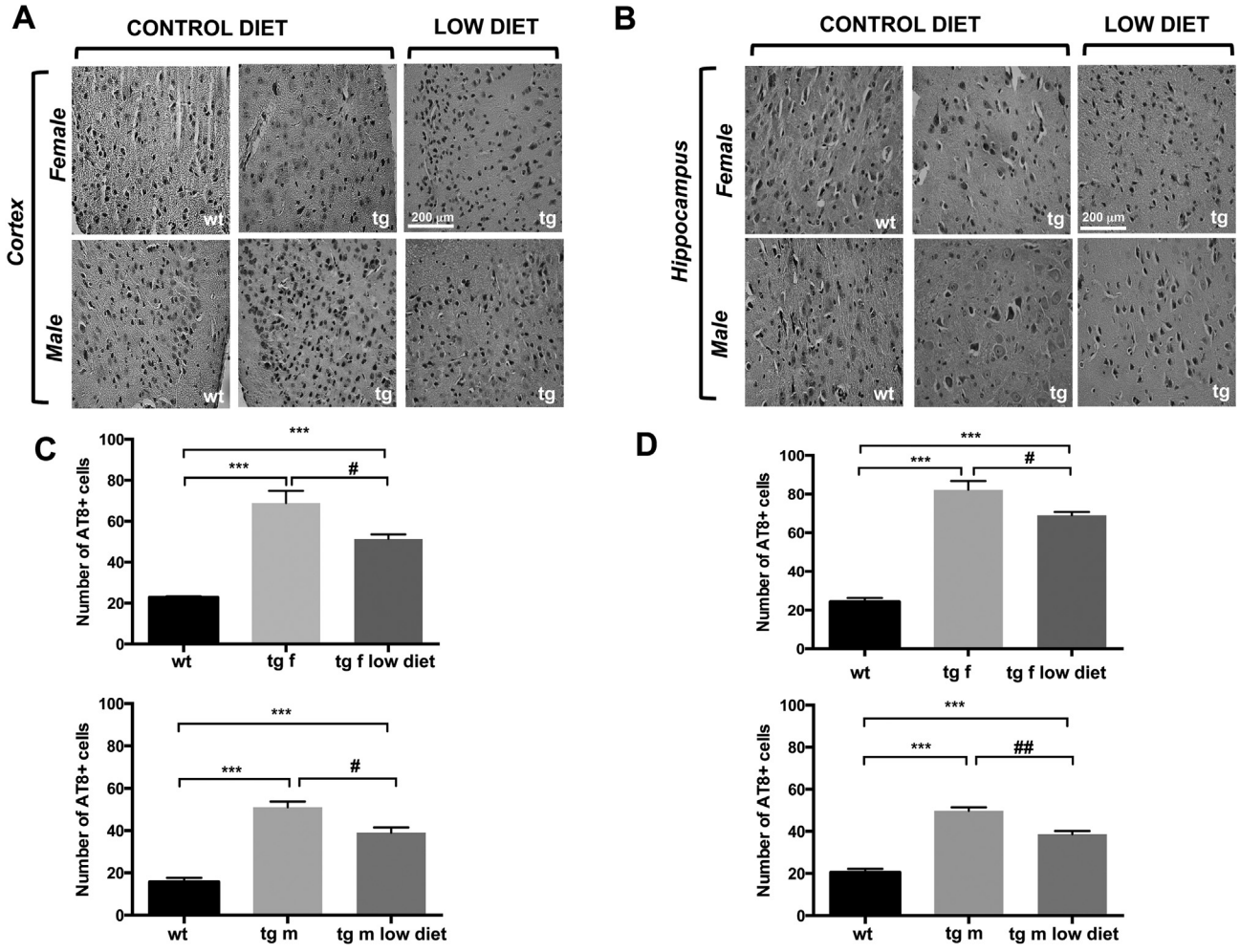

Fig. 5. AT8 immunoreactivity in P301L-tg mice. PSer202/pThr205 (AT8)-stained sections in the prefrontal cerebral cortex (A) and CA1 areas of hippocampus (B) of female and male P301L-tg mice fed with LFPD or standard diet *versus* wt animals fed with standard diet. Representative sections are shown each group. Scale bar: 200 μm. Quantification of AT8+ neurons in the prefrontal cortex (C) and CA1 areas of hippocampus (D) of female (up) and male (down) P301L-tg mice fed with LFPD or standard diet *versus* wt animals fed with standard diet. Tg *vs* wt mice fed with standard diet (both males and females) $^{***}p < 0.001$, tg female fed with standard diet *vs* tg fed with LFPDs $^{\#}p < 0.05$; tg male fed with standard diet *vs* tg fed with LFPDs (cortex) $^{\#}p < 0.05$; (hippocampus) $^{\#\#}p < 0.01$. One-way ANOVA, *Sidak's* post hoc test. Data were shown as mean ± SEM.

nificant neuronal death in the cortex ($p < 0.0001$; Fig. 6C) and hippocampus ($p < 0.0001$; Fig. 6D) of P301L-tg mice fed with the standard diet. As expected, P301L-tg mice fed with the LFPD had a significant decrease in TUNEL labeled neurons if compared to P301L-tg mice fed with the standard diet (ANOVA cortex: female $p < 0.05$, male $p < 0.0001$ Fig. 6C; hippocampus: female $p < 0.05$, male $p < 0.0001$ Fig. 6D).

DISCUSSION

Neurodegenerative diseases are complex diseases often caused by a combination of genetic and environmental risk factors, such as age, sex and nutrition. In particularly, tauopathies cause a devastating and progressive loss of cognitive function and today, no efficient therapies for the treatment of this disease exist.

In the present study, we evaluated whether the LFPD may modify Tau pathology in P301L-tg mice model. As reported by our recent observations, this experimental model, that well replicates the injury found in patients affected by tauopathy, presented agglomerates of hyperphosphorylated-Tau, spine injury, neuronal loss, and synaptopathy that are more severe in females compared to males (Buccarello et al., 2017). Moreover, P301L-tg mice shown cognitive and locomotor defects, tested in the novel object recognition and the open field assay. These deficits are more severe in female P301L-tg mice than males, supporting the hypothesis that synaptic dysfunction (Buccarello et al., 2017) is well correlated with behavioral impairment found in P301L-tg mice. In addition, the metabolic profile of P301L-tg fed with standard diet indicates a significant change in body weight as well as food intake and a lower percentage of survival rate compared to wt animals. These phenotypes correlated with pathological indicators such as P-Tau accumulation and neuronal death as previously described in Buccarello et al. (2017).

In this well characterized mouse model, we assessed the effect of low-fat protein diet (LFPD) on the onset and

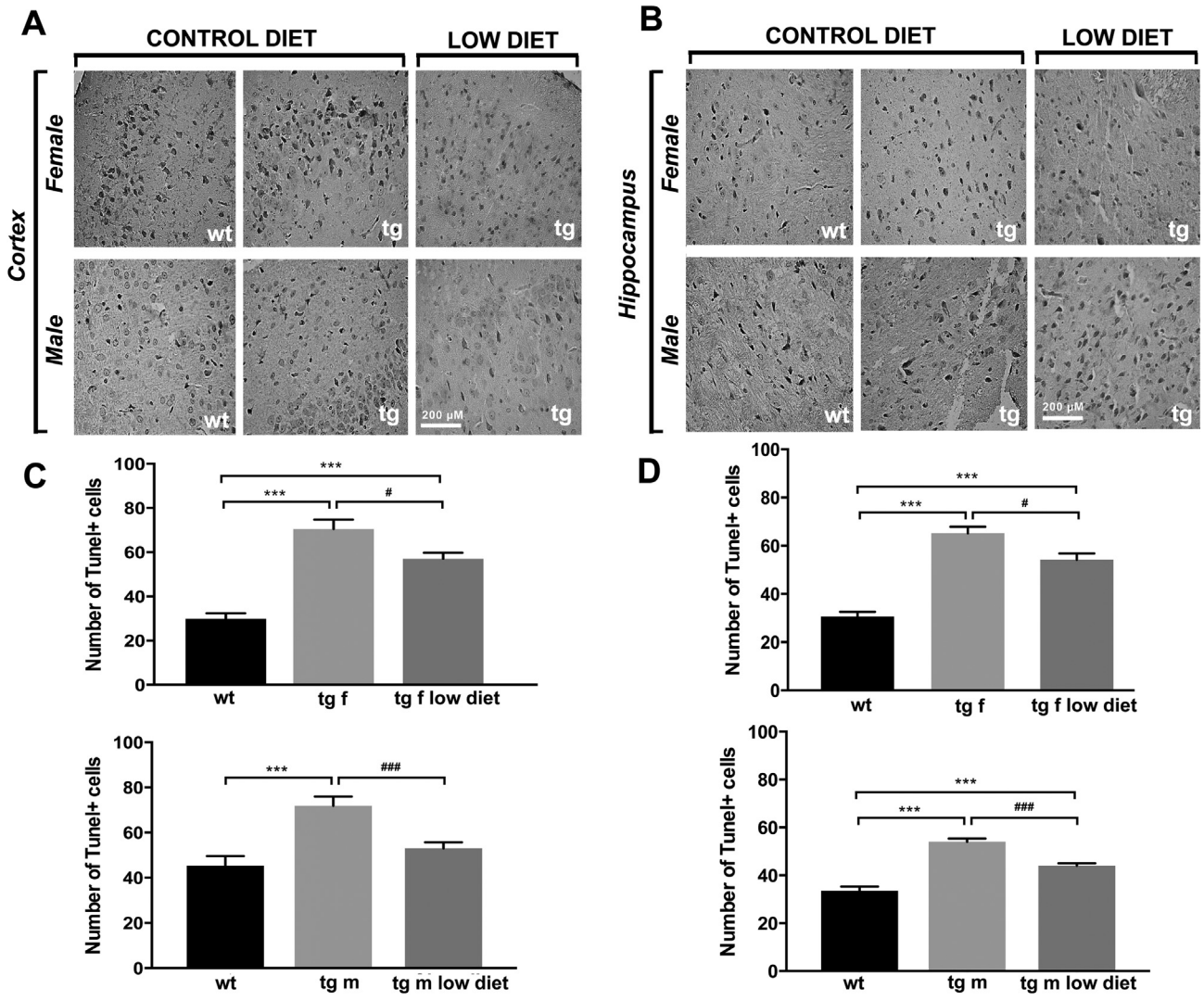

Fig. 6. TUNEL immunoreactivity and apoptotic neuronal counts in P301L-tg mice. TUNEL stained sections stained sections in cerebral cortex (A) and hippocampus (B) of female and male P301L-tg mice fed with LFPD or standard diet *vs* wt animals fed with standard diet. Representative sections are shown for each group. Scale bar: 200 μm. Quantification of TUNEL + neurons in the cerebral cortex (C) and hippocampus (D) of female (up) and male (down) P301L-tg mice fed with LFPD or standard diet *versus* wild type animals fed with standard diet. Tg *vs* wt mice fed with standard diet (both males and females) $^{****}p < 0.001$, tg fed with standard diet *vs* tg fed with LFPDs $^{\#}p < 0.05$, $^{\#\#\#}p < 0.0001$. One-way ANOVA, *Sidak's* post hoc test. Data were shown as mean ± SEM.

development of tauopathy and dementia. LFPD induced an increase in body weight, food intake and lifespan of P301L-tg mice compared to age-matched mice fed with standard diet. A possible hypothesis for body weight and food intake increases in P301L-tg fed with LFPD may be related to the different percentage of macronutrients of the diet.

In agreement with this hypothesis, different observations suggested that dietary constituents and their percentage may be key in modulating neurodegeneration, both in AD and tauopathy (Luchsinger et al., 2002; Calon et al., 2004; Morgan and Gordon, 2008; Takalo et al., 2014; Morris and Tangney, 2014; Berti et al., 2015). For example, Takechi et al., 2013 reported that a diet high in saturated fatty acids was more detrimental than a high cholesterol diet in rodents. In addition, other studies had proved that saturated fatty acids lead to a particularly robust increase in Aβ oligomers (Oksman et al., 2006; Grimm et al., 2012). On the other hand, it is important also to remind that patients with dementia sometimes exhibit significant weight loss, associated with increased intake of calories per kg body weight (Wang et al., 2004). However, in our study we used standard sterilizable diets, without the possibility to change the diet formulation for further study single fat and/or protein effect, this because the animals were housed in a SPF (specific pathogen free) facility and all materials need to be auto-cleavable.

In agreement with observations showing how nutrition profoundly influences lifespan (Solon-Biet et al., 2015), we here report that the main LFPD effect was on the survival rate of P301L-tg mice. Indeed, even if different studies proved that caloric restriction improved age-related health and increased lifespan, more recent data clarify

better that this effect is not due to caloric restriction, but instead to the difference in macronutrient types such as fats and proteins (Lee et al., 2008; Schroeder and Richardson, 2010; Tatar, 2011).

Importantly, the increase in survival rate in P301L tg-mice fed with the LFPD well correlates with improvements in their behavioral performance. In fact, the LFPD induced a significant improvement in cognitive, locomotor and anxiety-like performance compared to P301L-tg fed with standard diet. The P301L-tg fed with the LFPD had a significant higher discrimination index (ID) compared to tg mice, indicating a protective effect of this diet. Interestingly, female impairment is more severe compared to male P301L-tg and the LFPD impacts greater on female cognitive damage.

In parallel the locomotor activity in P301L-tg fed with the LFPD was significantly higher compare to tg mice fed with standard diet (i.e., their total number of crossing was higher) as well as the anxiety-like behavior (i.e., their total number of grooming and time spent for grooming was higher). The locomotor rescue obtained with the diet was again more pronounced in females compare to male tg mice, while for the anxiety state the rescue is related only to male tg mice. The improvement of cognitive and locomotor performances by LFPD here observed is in agreement with what reported by Kadish et al., 2016 showing that a diet with a high fat content exerted a negative effect on cognition in tg animals with AD pathology compared to the standard diet. In addition, another study proved that a LFPD plus high linoleic acid counteracts AD pathology in transgenic mice by reducing Aβ levels (Amtul et al., 2011).

Because diet has an influence on both survival rate and behavioral performance, indicating a protective effect on P301L-tg mice, we then explored whether it also impact the main pathological marker of tauopathy (i.e., P-tau) in the prefrontal cortex and hippocampus (i.e., the most affected brain areas). Female P301L-tg mice fed with the LFPD presented a significant decrease in the level of hyperphosphorylated Tau compared to P301L-tg mice fed with standard diet, while this effect was less powerful in male P301L-tg mice, in fact, just AT8 staining resulted significant for diet effect. Thus, females that presented a more severe tauopathy compare to males tg mice (Buccarello et al., 2017) are also more responsive to dietary effects. In addition, to correlate the powerful LFPD effect on P301L-tg survival rate, we investigated a possible link to the neuronal death in the brain parenchyma by using the TUNEL staining. The LFPD induced a significant reduction of TUNEL immunopositive neurons compared to P301L-tg mice fed with standard diet, both in male and female tg mice, highlighting one more the strong neuroprotective effect of the LFPD. In agreement with the data reported, different studies shown that high fat diet promotes Abeta accumulation, Tau pathologies and impaired cognitive performance in 3xTg-AD (Julien et al., 2010; Knight et al., 2012) and Tg2576 mice (Li et al., 2003; Ho et al., 2004), underling as sex interacts with the metabolic outcomes of high fat diet and, therefore, may alter neuropathological consequences of dietary manipulations inducing a worsening of pathological condition in females, more prone to AD, than males (Wolf et al., 2012; Barron et al., 2013; Moser and Pike, 2016). The sex dimorphic effects reported may be related with the different hormonal environment of peripheral (sex steroid hormones) as well as of neural origin (i.e., neurosteroids). Indeed, it is well demonstrated that these molecules are important sex dimorphic physiological regulators of the nervous functions (Melcangi and Garcia-Segura, 2010). In addition, in several experimental models of neurodegenerative disorders (Melcangi et al., 2016), including AD (Caruso et al., 2013; Overk et al., 2013), the levels of sex steroid hormones and neurosteroids are affected.

An increase of tau pathology has been to be reported in a number of strains of transgenic mice by diet-induced obesity (Julien et al., 2010; Leboucher et al., 2013). Interactions among sex, obesity and AD have been poorly explored. However, a complex interaction among these factors and steroid hormones/neurosteroids in AD development has been recently proposed (Moser and Pike, 2016).

CONCLUSION

In conclusion, the results presented indicate that the LFPD significantly improves lifespan, cognitive and locomotor performance in the P301L mouse model of tauopathy. These effects were correlated to a decrease in the P-Tau agglomerates in the prefrontal cerebral cortex and hippocampus, as well as to a reduction in neuronal death observed with the TUNEL staining. Interestingly, these effects were in some case sex-dependent, with a LFPD improvement of cognitive and locomotor performances most pronounced in female than in male tg mice.

Therefore, these data point towards a great and underestimated effect of nutrition against neurodegenerative diseases and suggest that a correct dietary can be a useful tool for preventing or delaying the onset of tauopathy and related neurodegenerative diseases. In addition, data reported here show that the diet impact on the brain is another important parameter that should be considered for a possible gender medicine to be applied in case of neurodegenerative disorders.

Acknowledgments—This work was supported by ADDF (Alzheimer's Drugs Discovery Foundation) USA grant to TB. We thank D. Corna and I. Bertani for technical help.

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(Received 1 February 2017, Accepted 19 April 2017)
(Available online 27 April 2017)