Intraneuronal accumulation of C99 contributes to synaptic alterations, apathy-like behavior, and spatial learning deficits in 3×TgAD and 2×TgAD mice

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ABSTRACT

The triple transgenic mouse model (3×TgAD: APP^{swe}, Tau^{p301L}, PS1^{M146V}) recapitulates both amyloid β (Aβ)- and tau-related lesions as well as synaptic and memory deficits. In these mice, we reported an early apathy-like behavior and alterations in synaptic plasticity appearing concomitantly with intraneuronal accumulation of C99 in the subiculum. To delineate the genuine contribution of C99 on the above phenotypes, we generated double transgenic mice (2×TgAD: APP^{swe}, Tau^{p301L}) that accumulate C99 without Aβ deposition or hyperphosphorylation of tau and compared them to 3×TgAD mice. Here, we show that both TgAD mice display similar decreases in long-term potentiation and in spontaneous locomotor activity measured by actimetry suggesting that the synaptic alterations and the apathy-like behavior were likely linked to C99 rather than Aβ. However, spatial learning alterations, assessed by the Morris water maze task, are more pronounced in 3×TgAD than in 2×TgAD, suggesting that both Aβ and C99 contribute to defects in the acquisition of spatial information. Finally, even if similar results are observed in males, cognitive and non-cognitive deficits are more severe in females.

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1. Introduction

Alzheimer's disease (AD) is an age-related disorder responsible for the most common form of dementia in the world. Histologically, it is characterized by two abnormal protein deposits: extracellular senile plaques mainly composed of hydrophobic amyloid β (Aβ) peptides and neurofibrillary tangles made of intracellular filamentous aggregates of the hyperphosphorylated tau protein. One of the etiological hypotheses concerns the amyloid cascade that proposes a key role of Aβ in AD setup and progression (Hardy and Higgins, 1992; Selkoe and Hardy, 2016). Aβ peptides are produced from their precursor, the β-amyloid precursor protein (βAPP) in the amyloidogenic pathway. βAPP is first cleaved by the β-secretase to release a C-terminal fragment, C99, which then undergoes subsequent hydrolysis by the γ-secretase to yield not only Aβ but also the cytosolic APP Intracellular Domain (AICD) that controls the

transcription of several genes related to AD (Pardossi-Piquard and Checler, 2012). An alternative non-amyloidogenic pathway by α-secretase generates another C-terminal fragment, C83. This cleavage precludes Aβ production but does not impair γ-secretase-mediated production of AICD.

Because senile plaques are poorly correlated with the severity of the disease, it has been suggested that the early occurrence of intracellular Aβ and soluble Aβ oligomers rather than senile plaques could be responsible for the first pathological events (LaFerla et al., 2007; Larson and Lesne, 2012; Wirths et al., 2004). However, several lines of evidence suggest that some Aβ-independent mechanisms could also contribute to AD-related anatomical and functional alterations (Pimplikar et al., 2010).

We have recently reported that the intraneuronal aggregation of C99 induces an Aβ-independent lysosomal-autophagic dysfunction, in vivo, in triple transgenic mice (3×TgAD). Interestingly, this phenotype can be mimicked by the viral expression of C99 in wild-type mice (AAV-C99 mice), and it is exacerbated by the pharmacological blockade of γ-secretase (Lauritzen et al., 2016). These findings agreed with previous studies showing that C99 accumulation induces axonal transport impairment and morphological and

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functional abnormalities of early endosomes (Jiang et al., 2010; Kim et al., 2016). Although these studies support the idea that C99, independently to A β , could contribute to molecular and cellular dysfunctions taking place in AD pathology, little is known concerning the contribution of C99 on cognitive and behavioral deficits observed in AD mouse models.

The 3 $\times$ TgAD mouse model first generated and described by LaFerla is broadly used in AD field. These mice expressing mutated amyloid precursor protein and tau proteins (APP_{swe} and Tau_{P301L}), and physiological levels of mutated PS1 protein (PS1_{M146V}), develop both amyloid plaques and neurofibrillary tangles (Oddo et al., 2003b). Synaptic dysfunction and cognitive deficits occur before plaque or tangle formation and could be temporally correlated with intraneuronal A β -related immunoreactivity rather than with extracellular A β deposits (Billings et al., 2005). However, by both genetic and pharmacological approaches, we have demonstrated that this intracellular staining did not correspond to A β itself but rather to its direct precursor C99 (Lauritzen et al., 2012). Moreover, we found that this early C99 accumulation was both spatially and temporally associated with the first synaptic alterations impacting long-term potentiation (LTP) (Pardossi-Piquard et al., 2016). Interestingly, 3 $\times$ TgAD mice displayed an early and drastic decrease of spontaneous activity measured by actimetry that could be assimilated to an apathy-like phenotype (Pardossi-Piquard et al., 2016). This neuropsychiatric symptom is the most frequent and earliest non-cognitive behavior observed in AD patients (Zhao et al., 2016) and in clinical trials, apathy is associated with a decreased activity that can be monitored by actigraphy (David et al., 2012; Konig et al., 2014).

By crossing 3 $\times$ TgAD and non transgenic wild-type (WT) mice, we developed another AD mice model, the double transgenic mice (2 $\times$ TgAD) harboring both APP_{swe} and Tau_{P301L} mutant proteins but expressing endogenous wild-type PS1. Similarly to 3 $\times$ TgAD mice, 2 $\times$ TgAD mice exhibit early accumulation of C99 in the subiculum. However, in contrast to 3 $\times$ TgAD mice, 2 $\times$ TgAD mice display undetectable A β and fail to produce extracellular plaques, even at very late ages of the pathology (Lauritzen et al., 2012). Moreover, hyperphosphorylation of tau is not observed in 2 $\times$ TgAD mice even at 20 months of age. Thus, the comparison between 3 $\times$ TgAD and 2 $\times$ TgAD mice seems particularly adequate to investigate the contribution of C99 to synaptic and behavioral alterations independently of A β and tau pathology. Here, we report that 3 $\times$ TgAD and 2 $\times$ TgAD mice have similar decreases in spontaneous activity and LTP suggesting that apathy-like behavior and synaptic alterations are not associated with A β but more likely with C99. In contrast, learning impairment assessed by the Morris water maze (MWM) task is stronger in 3 $\times$ TgAD compared to 2 $\times$ TgAD mice indicating that besides C99, A β could also contribute to deficient acquisition of spatial information. Finally, our study did not unravel major sex-related differences, even if we observed more pronounced phenotypes in females.

2. Materials and methods

2.1. Animals

3 $\times$ TgAD (APP_{swe}, Tau_{P301L}, and PS1_{M146V}) and nontransgenic (WT) mice colonies were maintained from breeding pairs generously provided by Dr LaFerla (Oddo et al., 2003b). As described previously, 2 $\times$ TgAD expressing β APP_{swe}, Tau_{P301L}, and wild-type PS1 were obtained in our laboratory by crossing the 3 $\times$ TgAD with these WT mice (Lauritzen et al., 2012). Therefore, the same WT mice with the original hybrid background (129-C57BL/6) were used as control for both 2 $\times$ TgAD and 3 $\times$ TgAD mice. Each strain line is maintained by intercross breeding and backcrossed with the original strain every 10 generations. Males and females were housed with a 12:12 hour light/

dark cycle and were given free access to food and water. All experimental procedures were in accordance with the European Communities Council Directive of 22 September 2010 (2010/63/EU) and approved by the French Ministry of Higher Education and Research (project number #00253.02).

2.2. Actimetry

Spontaneous locomotor activity was measured using an actimeter apparatus (Actimeter system, Imetronic, France), as previously described (Pardossi-Piquard et al., 2016). Briefly, the cages were illuminated 12 hours per day starting from 8 AM. Each cage was equipped with infrared captors at each side of the box and connected to an electronic interface that allowed the automatic measurement of spontaneous locomotor activity (rearing, front activity, back activity, and locomotion). Phenotypes obtained in 12-month-old WT (28 females and 16 males), 2 $\times$ TgAD (2AD, 24 females and 16 males), and 3 $\times$ TgAD (3AD, 22 females and 16 males) mice were statistically analyzed by 2-way analysis of variance (ANOVA) (genotype $\times$ sex) applied with Benjamini-Hochberg correction using InVivoStat statistical software.

2.3. Morris water maze

At least 2 weeks after actimetry analysis, a group of 12-month-old WT (16 females and 16 males), 2 $\times$ TgAD (2AD, 13 females and 15 males), and 3 $\times$ TgAD (3AD, 16 females and 16 males) mice was assessed in the MWM task conducted in a circular pool 90 cm in diameter filled with water made opaque with nontoxic white dye opacifier (Viewpoint). Pool temperature was maintained at 24 °C. The maze was located in a room containing several distal extramaze posters helping mice to orientate. The circular escape platform, 10 cm in diameter, was submerged in the center of 1 quadrant of the pool and remained in the same position throughout the learning trials. During 5 days, each mouse performed 4 trials per day. For each trial, the mouse was placed into the pool at one of 3 different designed start positions located in all quadrants not housing the platform. Mice were allowed to find the platform during 60 seconds. If the mouse found the platform before 60 seconds, it was left 20 seconds on the platform then removed from the pool. If the platform was not found after 60 seconds of swimming, the mouse was manually guided to the platform and allowed to reorient to the distal visual cues for an additional 30 seconds before being removed from the pool. For each day, the latency to find the platform was averaged and statistically analyzed by mixed model ANOVA (genotype $\times$ sex $\times$ training day) using InVivoStat statistical software. The means of mice swim speed were calculated for each group during the last trial and analyzed by 1-way ANOVA using InVivoStat statistical software. The visual cued test was performed in females after the hidden platform training. During the visual cued test, each mouse performed 3 consecutive trials in the pool filled with opaque water. The mouse was given 60 seconds to find the platform placed above the water level and made visible using a flag mounted above the platform. For each trial, the mouse was placed at the same start position, and the visible platform was moved to different locations between each trial. Latency to find the visible platform was averaged for 12-month-old WT (16 females), 2 $\times$ TgAD (2AD, 13 females), and 3 $\times$ TgAD (3AD, 16 females) mice and analyzed by 1-way ANOVA using InVivoStat statistical software.

2.4. Rotarod test

Before the MWM task, motor performance was measured using a Rotarod apparatus (Bioseb, model LE8200). Mouse was placed on the rotating rod, and the latency to fall was measured while the

speed was accelerating from 4 to 40 rpm. The mean of the latency to fall obtained during 3 independent trials for 12-month-old WT (16 females and 16 males), 2×TgAD (2AD, 13 females and 15 males), and 3×TgAD (3AD, 16 females and 16 males) was analyzed by 2-way ANOVA (genotype × sex) using InVivoStat statistical software.

2.5. Electrophysiological analysis

LTP measurements were performed by E-PHY-SCIENCE, Sophia Antipolis, France. Briefly, 8-month-old females (6 WT, 6 2×TgAD, 6 3×TgAD) were anesthetized with isoflurane, and then brain slices (400 µm thick) were prepared for LTP measurement as previously described (Pardossi-Piquard et al., 2016). Field excitatory post-synaptic potential (f-EPSP) was recorded from the subicular pyramidal neurons after stimulation in CA1. For the test pulses, the intensity of the stimulation corresponded to one-third of the maximal response. The LTP-induction protocol consisted of 4 trains of 100 stimuli at 100 Hz repeated every 20 seconds as previously described (Attar et al., 2012). The signal was amplified with an Axopatch 200B amplifier (Molecular Devices, Union City, CA), digitized by a Digidata 1200 interface (Molecular Devices) and sampled at 10 kHz with Clampex 10 (Molecular Devices). For each animal, f-EPSPs obtained from several slices presenting a stabilized baseline were averaged and followed during 60 minutes after tetanus. The mean values obtained for each mouse during the last 40 minutes were analyzed by 1-way ANOVA using InVivoStat statistical software.

2.6. Immunohistochemistry

Animals were deeply anesthetized with pentobarbital (Dolethal, 145 mg/kg) and perfused transcardially with cold phosphate-buffered saline (PBS) followed by 4% paraformaldehyde/PBS for fixation. Brains were then embedded in paraffin, and coronal sections (8 µm) were cut on a microtome. After deparaffinization, sections were treated with formic acid 2 minutes at room temperature for APP-ct and AT8 immunostaining and with formic acid 2 minutes followed by 10 µg/mL Proteinase K in Tris 50 mM ethylenediaminetetraacetic acid 5 mM 30 minutes at 37 °C for 82E1/glia fibrillary acidic protein (GFAP) coimmunostaining. Sections were then blocked with 5% bovine serum albumin/0.05% Tween-20 during 1 hour and then incubated at 4 °C overnight with the primary antibodies: rabbit polyclonal anti-APPct (1/1000, kind gift from Paul Fraser, Toronto, Canada), rabbit monoclonal anti-GFAP (1/1000, Abcam, ab33922), and mouse monoclonal AT8 raised against tau phosphorylated at serine 202 and threonine 205 (1/1000, ThermoFisher Scientific) or 82E1 targeting the human Aβ residues 1–16 ([Horikoshi et al., 2004], 1/100, IBL). After washes, sections were incubated with secondary antibodies: anti-mouse horseradish peroxidase antibody (for 3,3'-Diaminobenzidine, DAB staining) or fluorescent Alexa Fluor antibodies (Alexa 488 and Alexa 594 conjugated, Molecular Probes, 1:1000) at room temperature during 1 hour. Immunofluorescence was visualized using a confocal microscope (Fluoview 10, Olympus). For DAB development, slides were incubated with DAB-impact (Vector), rinsed with PBS, counterstained 3 minutes with cresyl violet and observed using an optical light microscope (DMD108, Leica). For Aβ detection, 12-month-old WT, 2×TgAD, and 3×TgAD mice (2 females and 1 male for each group) were analyzed, whereas additional 20-month-old females (1 female for each group) were analyzed for phosphorylated-tau immunostaining.

2.7. Preparation of soluble and insoluble brain fractions

Dissected hippocampi from 12-month-old WT, 2×TgAD and 3×TgAD mice (5 females and 5 males for each group) were homogenized in radioimmunoprecipitation assay buffer (Tris 50 mM;

pH 7.4 containing NaCl [150 mM], ethylenediaminetetraacetic acid [1 mM], Triton X100 [1%], deoxycholate [0.5%], sodium dodecyl sulfate (SDS) [0.1%] and protease inhibitor cocktail as previously described (Pardossi-Piquard et al., 2016). After sonication, proteins were centrifuged (100,000 × g, 1 hour, 4 °C), and supernatants were considered as soluble fractions. Pellets containing insoluble material were dissociated in formic acid (70%), centrifuged (100,000 × g, 1 hour, 4 °C), and then supernatants were neutralized to pH 7.5 with Tris-HCl (1M, pH 10.8) containing betaine (25 mM) (25 times dilution) and kept as the insoluble fraction.

2.8. SDS/PAGE and Western blot analyses

Soluble fractions (50 µg of total protein) were dissolved in SDS sample buffer, separated on Tris-Tricine gels 16% (for C83 and C99 detection) or Tris-Glycine gels 10% (for βAPP full-length protein [APP-FL] and actin detection), and transferred to nitrocellulose membranes. Proteins were then detected by enhanced chemiluminescence (WesternBright Quantum or Sirius HRP substrate from Advanta) with a mouse monoclonal anti-actin (Sigma) or a rabbit polyclonal anti-APPct antibody (kind gift from Paul Fraser, Toronto, Canada). The original full scans of western-blots are provided in Supplementary data.

2.9. Sandwich ELISA analysis

Human Aβ40 and Aβ42 levels were measured in the aforementioned soluble and insoluble fractions using sandwich enzyme-linked immunosorbent assay kits (BioSource [Invitrogen], France). Detection limits were <10 pg/mL for Aβ42 and <6 pg/mL for human Aβ40.

2.10. Experimental design and statistical analysis

Experiments were designed to compare the behavior of WT, 2×TgAD, and 3×TgAD mice. All statistical analyses were conducted using InVivoStat software (Bate and Clark, 2014). For each statistical analysis, normal distribution of residuals and homogeneity of variance were tested by checking normal probability plot and residual versus predicted plot. For Western blot analysis, each statistical replicate represents the level of detected proteins extracted from 1 mouse, and data were analyzed independently in males and females using the non-parametric Kruskal–Wallis test, appropriated for a low number of samples, followed by Mann–Whitney tests adjusted by Benjamini–Hochberg's method. For LTP assessment, each statistical replicate represents the mean of f-EPSPs obtained from several hippocampal slices of 1 mouse, and data were analyzed in females using a 1-way ANOVA and multiple comparisons adjusted by Benjamini–Hochberg's method. For MWM task, escape latency was analyzed by mixed model ANOVA with repeated measure (genotype × sex × training day) and followed by Benjamini–Hochberg's correction. Concerning statistics of actimetry and Rotarod, the influences of both sex and genotype were considered by 2-way ANOVAs (genotype × sex) with multiple comparisons adjusted by Benjamini–Hochberg's method. For visible cue test, we used a 1-way ANOVA to compare only the genotype factor. For actimetry statistical analyses, data were log-10 transformed to respect homoscedasticity assumptions.

3. Results

3.1. 2×TgAD and 3×TgAD mice accumulate similar levels of intraneuronal C99 within the subiculum in both males and females

We have previously shown that C99 levels increased similarly and progressively with age in both 2×TgAD and 3×TgAD females

(Lauritzen et al., 2012, 2016) and confirmed in the present study, C99 accumulation in both males and females. As expected, due to transgene expression, level of APP-FL was higher in both 2×TgAD and 3×TgAD than in WT mice but remain similar in the two transgenic mouse models (Fig. 1A–B). Moreover, the levels of C99 and to a lesser extent C83 were also increased in both TgAD mice, whatever the sex examined. Compared to WT mice, the levels of C99 are increased in both 2×TgAD and 3×TgAD females and males (Fig. 1A–B; +786% between WT and 2AD females, +747% between WT and 3AD females, $p = 0.0441$ for both, +533% between WT and 2AD males, +483% between WT and 3AD males, $p = 0.0292$ for both, Mann–Whitney). We confirmed these findings by immunohistochemical analysis using the same antibody directed toward the C-terminal region of β APP (APPct antibody). In both male and female 2×TgAD and 3×TgAD mice, we observed an increased APP-like immunoreactivity corresponding to APP-FL and APP carboxy-terminal fragments, C99 and C83 (Fig. 2A and B). In both 2×TgAD males and females, the APPct-related staining was strictly intracellular and membrane associated. In 3×TgAD mice, an additional immunoreactivity appears more extracellular and granular when localized closely to senile plaques. This extracellular staining is more pronounced in 3×TgAD females because they display a stronger amyloid plaque burden than males at this stage of the pathology as described below.

3.2. 3×TgAD but not 2×TgAD mice produce A β peptides and display A β plaques and hyperphosphorylated tau with a more severe phenotype in 3×TgAD females

We showed that A β plaques deposits and hyperphosphorylation of tau are virtually undetectable in 12-month-old females and males 2×TgAD. Thus, unlike is the case in 3×TgAD mice (see Fig. 3A and B, black bars), measurements of A β 40 and A β 42 levels using highly

sensitive and specific enzyme-linked immunosorbent assay revealed barely detectable amounts of A β in both soluble and insoluble fractions from male and female 2×TgAD mouse hippocampi (Fig. 3A and B). The absence of A β was corroborated by histological analysis showing that, unlike 3×TgAD, 2×TgAD mice totally lacked amyloid plaques (Fig. 4A and B). In male and female 2×TgAD mice, the staining obtained with 82E1 antibody (epitope 1–16 of A β) was exclusively intracellular. Although this antibody targeting the N-terminal part of A β can recognize both A β and C99 (but does not stain uncleaved APP-FL), we previously demonstrated that this intracellular staining corresponded to aggregated forms of C99 (Lauritzen et al., 2016). In contrast, in the subiculum of 3×TgAD mice, the 82E1 antibody recognized additional extracellular deposits and amyloid plaques surrounded by GFAP-positive astrocytes (Fig. 4A and B). A β -related pathology appeared more aggressive in 3×TgAD females than in males, in accordance with previous studies (Carroll et al., 2010; Hirata-Fukae et al., 2008). The extensive charge of amyloid plaques in 3×TgAD females was observed in the same region of the subiculum where the extracellular APP-ct-specific staining was detected (Fig. 2A). This confirmed the presence of hippocampal neuroinflammation and histological lesions in 3×TgAD female brains that are less expanded in age-matched 3×TgAD male brains (Fig. 4A and B). Interestingly, tau pathology is observed only in 3×TgAD old mice. AT8 phospho-tau antibody that specifically targets phosphorylated serine 202 and threonine 205 was used to evaluate phosphorylation of tau in 2×TgAD and 3×TgAD females. As reported previously, despite expression of mutated tau protein (TauP301L), hyperphosphorylation of tau in 3×TgAD mice is observed only lately and after A β deposits (Oddo et al., 2003a,b). AT8-positive neurons were barely detectable in 12-month-old 3×TgAD mice while AT8 immunostaining was readily apparent in 20-month-old 3×TgAD females. In contrast, even at 20 months of age, 2×TgAD females did not display evident AT8-positive staining (Fig. 4C).

Fig. 1. Female and male 2×TgAD and 3×TgAD mice both display C99 and C83 accumulations. APP-FL, C99, C83, and actin levels from 12-month-old WT (females $n = 4$, males $n = 5$), 2×TgAD (2AD, females $n = 4$, males $n = 5$), and 3×TgAD (3AD, females $n = 4$, males $n = 5$) mice hippocampi are measured by Western blot using anti-APPct and anti-actin antibodies. Actin is used as loading control (A). Data in (B) are expressed as percentage of control (WT) and represent means $\pm$ SEM. * $p < 0.05$; according to Kruskal–Wallis test (for C99 immunoreactivity: statistic 7.41, $p = 0.0246$ for females, statistic 8.63, $p = 0.0134$ for males) followed by Mann–Whitney comparison test applied with Benjamini–Hochberg correction. Abbreviations: 3×TgAD, triple transgenic mice; AD, Alzheimer's disease; WT, wild type; 2×TgAD, double transgenic mice.

Fig. 2. C99 accumulates in neurons of the subiculum of 2×TgAD and 3×TgAD mice. Low (upper panel, scale bar = 300 μm) and high (lower panel, scale bar = 50 μm) magnification of immunohistochemical staining obtained using anti-APPct antibody on coronal brain slices from 12-month-old WT, 2×TgAD (2AD), and 3×TgAD (3AD) mice at the level of the subiculum in females (A) and males (B). Abbreviations: 3×TgAD, triple transgenic mice; AD, Alzheimer's disease; WT, wild type; 2×TgAD, double transgenic mice.

3.3. Both 2×TgAD and 3×TgAD mice display decreased spontaneous activity, a phenotype more severely affected in females than in males

We have recently described an apathy-like behavior in 3×TgAD mice starting at 4 months of age and persisting in older mice. This phenotype, which was characterized by an early decrease in spontaneous locomotor activity assessed by actimetry, was found to appear concomitantly to intraneuronal C99 accumulation (Pardossi-Piquard et al., 2016). Because 2×TgAD and 3×TgAD mice accumulate similar levels of intracellular C99, we compared the spontaneous activity of 12-month-old male and female 2×TgAD and 3×TgAD mice. The actimeter system measures the total and continuous activity of the mice during 3 days including frontal activity, back activity, rearing, and locomotion and allows us to visualize the circadian rhythm with higher activity during the night

(8 PM–8 AM, Fig. 5A–D). In females, total activity of both 2×TgAD and 3×TgAD mice were significantly decreased compared to age-matched WT mice (Fig. 5E, –29% between WT and 2AD, $p < 0.001$; –32% between WT and 3AD, $p < 0.001$, 2-way ANOVA [genotype × sex]). This decrease was even more pronounced when we examined rearing activity (Fig. 5F, –59% between WT and 2AD, $p < 0.001$; –69% between WT and 3AD, $p < 0.001$, 2-way ANOVA [genotype × sex]). In males, although the reduction of total activity in both TgAD mice failed to reach statistical significance (Fig. 5G, –17% between WT and 2AD, $p = 0.0887$ and –16% between WT and 3AD, $p = 0.0887$, 2-way ANOVA [genotype × sex]), the decline of the rearing activity was significant in both 2×TgAD and 3×TgAD males (Fig. 5H, –39% between WT and 2AD, $p = 0.0362$; –44% between WT and 3AD, $p = 0.0048$, 2-way ANOVA [genotype × sex]). It should be noted that sex interferes with the

Fig. 3. Aβ levels are detected in 3×TgAD but not 2×TgAD hippocampi. Aβ40 and Aβ42 levels are measured by ELISA in soluble (sol) and insoluble (insol) fractions prepared from 12-month-old WT, 2×TgAD (2AD), and 3×TgAD (3AD) hippocampi of both females (A) and males (B). Data are expressed in pg (Aβ)/mg of wet tissue (means ± SEM, $n = 5$). Abbreviations: 3×TgAD, triple transgenic mice; AD, Alzheimer's disease; Aβ, amyloid-β peptide; WT, wild type; 2×TgAD, double transgenic mice.

genotype effect for rearing activity [genotype × sex interaction: $F(2,116) = 3.18$; $p = 0.0453$] but not for total activity [genotype × sex interaction: $F(2,116) = 1.13$; $p = 0.3264$]. Thus, the apathy-like behavior observed in both 2×TgAD and 3×TgAD mice is likely linked to C99 accumulation (present in both 2×TgAD and 3×TgAD mice) rather than to Aβ or abnormal phosphorylation of tau (present only in 3×TgAD mice).

3.4. Both 2×TgAD and 3×TgAD mice display altered LTP

It is well established that 3×TgAD mice display early and progressive synaptic dysfunction occurring long time before plaque and tangle formations. A recent article from LaFerla's group described a significant reduction in dendritic spines and an early impaired AMPA signaling in the hippocampus of 3×TgAD mice (Baglietto-Vargas et al., 2018). This decline illustrated by an alteration of LTP was first correlated with the accumulation of intraneuronal Aβ (Oddo et al., 2003b). However, as mentioned previously, we have demonstrated that the first intracellular Aβ-like immunoreactivity observed in the subiculum corresponds to C99

rather than Aβ (Lauritzen et al., 2012). More recently, we found that this early C99 accumulation was spatially and temporally associated with the first synaptic LTP alterations measured in the subiculum (Pardossi-Piquard et al., 2016). Here, the LTP was measured in the subiculum of both 2×TgAD and 3×TgAD females at 8 months of age, when brain slices remain technically usable for electrophysiological measurements and Aβ levels clearly detectable in 3×TgAD mice (Lauritzen et al., 2012). We obtained identical decreases in LTP in both 2×TgAD and 3×TgAD mice (Fig. 6A and B –19% between WT and 2AD, $p = 0.0184$; –16% between WT and 3AD, $p = 0.0340$, 1-way ANOVA), indicating that LTP alterations could not be accounted for Aβ, but should rather be linked to intracellular C99 accumulation. Furthermore, because hyperphosphorylated tau started to be slightly detectable only after 12 months, it appears unlikely that LTP alterations observed in younger 3×TgAD and 2×TgAD mice could be accounted for or linked to tau abnormal phosphorylation.

3.5. 3×TgAD mice and to a lesser extent 2×TgAD mice present learning deficits in the MWM task

To determine if the LTP impairments observed in both 2×TgAD and 3×TgAD females could be correlated to spatial learning deficits, we used the MWM task to compare performances of 12-month-old WT, 2×TgAD, and 3×TgAD males and females. A 3-way repeated measures mixed model ANOVA analysis revealed strong interaction of mice's genotype in results depending on the day of training [genotype × training day interaction: $F(8,344) = 2.19$, $p = 0.0037$]. As expected, we first observed a strong impairment in the acquisition of spatial information in 3×TgAD females. In WT mice, the escape latency was significantly improved during training, whereas this was not the case for 3×TgAD females (Fig. 6C, –79% latency to find the hidden platform between J1 and J5 for WT, –32% between J1 and J5 for 3AD; $p = 0.3593$ comparing WT and 3AD at J1 and $p < 0.001$ comparing WT and 3AD at J5, mixed model ANOVA). In contrast, trained 2×TgAD females improved their ability to find the escape platform even if their performance remained lower than those observed for WT mice, particularly after 3 and 4 days of training (Fig. 6C, latency to find the hidden platform on fourth day training for WT = 9.6 ± 2.0 seconds, 2AD = 27.1 ± 3.8 seconds, and 3AD = 40.2 ± 3.8 seconds. Comparison between WT and 2AD, $p < 0.001$; between WT and 3AD, $p < 0.001$; and between 2AD and 3AD $p = 0.0131$, mixed model ANOVA). In 3×TgAD males, the spatial learning impairment was found to be much less pronounced than in 3×TgAD females [Fig. 6C and D, genotype × sex × training day interaction: $F(8,344) = 2.78$, $p = 0.058$; mixed model ANOVA). These mice improved their spatial capacity by training, but the delay to find the escape platform at day 3 and 4 of training remained higher than for WT mice (Fig. 6D, latency to find the hidden platform on fourth day of training for WT = 12.2 ± 1.7 seconds and 3AD = 25.3 ± 4.0 seconds, $p = 0.0086$; mixed model ANOVA). The 2×TgAD males displayed the same learning curve than WT mice during the first 3 days but presented a significant delay in the escape latency at 4 days of training (Fig. 6D latency to find the hidden platform on fourth day of training for WT = 12.2 ± 1.7 seconds, 2AD = 22.6 ± 3.6 seconds, $p = 0.0485$; mixed model ANOVA). Therefore, spatial learning acquisition seems less altered in 2×TgAD mice than in 3×TgAD mice suggesting a contribution of Aβ besides C99.

To examine any lack of escape motivation or influence of any visual and/or motor alterations that might be present particularly in 12-month-old mice, we performed a visual cue test in females that present a much more pronounced phenotype than males. All mice find the platform with the same latency [Fig. 6E, genotype effect: $F(2,42) = 1.50$, $p = 0.2339$; 1-way ANOVA]. In agreement with the

Fig. 4. A β plaques, microglia activation, and tau hyperphosphorylation are observed in 3 $\times$ TgAD but not in 2 $\times$ TgAD brain. (A–B) Coimmunofluorescence obtained with the 82E1 antibody targeting both C99 and A β (red) and anti-GFAP for activated astrocytes (green) in 12-month-old WT, 2 $\times$ TgAD (2AD), and 3 $\times$ TgAD (3AD) coronal brain slices from females (A) and males (B). Low-magnification images of 82E1 immunostaining (top panels, scale bar = 300 μ m), high magnification of subiculum with 82E1, GFAP immunostaining (middle panels, scale bar = 50 μ m), and merged images (bottom panels, scale bar = 50 μ m). (C) DAB staining obtained with anti-AT8 antibody targeting phosphorylated tau protein in 12-month-old (upper panels) and 20-month-old (lower panels) female WT, 2 $\times$ TgAD (2AD), and 3 $\times$ TgAD (3AD) coronal brain slice (scale bar = 300 μ m). Abbreviations: 3 $\times$ TgAD, triple transgenic mice; AD, Alzheimer's disease; A β , amyloid- β peptide; GFAP, glial fibrillary acidic protein; WT, wild type; 2 $\times$ TgAD, double transgenic mice. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

absence of motor deficit, no difference in swimming speed between all groups during the last trial of training in MWM was detected [Fig. 6F, genotype $\times$ sex interaction: $F(2,86) = 2.12$, $p = 0.1260$; genotype effect $F(2,86) = 0.14$, $p = 0.8703$; 2-way ANOVA). In addition, we performed the Rotarod performance test in females and males to evaluate locomotor abilities. In males, we observed no difference in the Rotarod retention time between all groups (Fig. 6G, $p = 0.5093$ between WT and 2AD, $p = 0.4644$ between WT

and 3AD; 2-way ANOVA) while in females, the latency to fall off the Rotarod is even longer in both 2 $\times$ TgAD and 3 $\times$ TgAD mice compared to WT (Fig. 6G, latency to fall +91% between WT and 2AD, $p < 0.001$; +43% between WT and 3AD, $p = 0.0493$; 2-way ANOVA). Although the increased Rotarod retention time observed in both 2 $\times$ TgAD and 3 $\times$ TgAD females is unexpected, it is already reported in 3 $\times$ TgAD mice (Filali et al., 2012; Stover et al., 2015) and more importantly it confirms the lack of motor impairment in these

Fig. 5. Spontaneous locomotor activity is decreased in both 3×TgAD and 2×TgAD mice. Mean of total activity (A–B) or rearing (C–D) waveform recorded each 20 minutes for 12-month-old WT (n = 28), 3×TgAD (3AD, A, C, n = 22), or 2×TgAD (2AD, B, D, n = 24) females. Bars represents the mean ± SEM of total activity (E, G) or rearing (F, H) recorded during 70 hours in females (WT n = 28, 3AD n = 22, 2AD n = 24) and males (n = 16 for each group), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ according to a 2-way ANOVA (genotype × sex) applied with Benjamini-Hochberg correction. Abbreviations: ANOVA, analysis of variance; 3×TgAD, triple transgenic mice; AD, Alzheimer's disease; WT, wild type; 2×TgAD, double transgenic mice.

mice. One explanation could come from the high rearing activity recorded in WT mice that could induce more unbalance movements compared to 2×TgAD and 3×TgAD mice.

4. Discussion

While most of the publications in the field have focused on A β , we are interested in the pathological contribution of its direct precursor, the C99 fragment. Although several studies have provided direct or indirect evidences for C99-mediated toxicity, few have aimed to discriminate between A β and C99 itself. We have recently shown that C99 accumulation triggers A β -independent lysosomal-autophagic dysfunction (Lauritzen et al., 2016). In

addition, we have demonstrated a spatial and temporal correlation between the early intraneuronal C99 accumulation and a defective LTP measured specifically in the subiculum of 3×TgAD mice (Pardossi-Piquard et al., 2016). In the present work, we provide new evidence for the contributions of C99 on synaptic plasticity, spatial learning, and non-cognitive deficits such as apathy-like behavior. We compared 2 mouse lines, 3×TgAD and 2×TgAD mice, maintained on the same original 129-C57BL/6 hybrid background but presenting different biochemical features. 2×TgAD mice (APP^{swe}, TauP301L) bearing wild-type instead of mutated presenilin accumulate similar levels of C99 than 3×TgAD mice but without any A β buildup or abnormal phosphorylation of tau. Thus, our findings showing the same decrease in total spontaneous locomotor activity,

Fig. 6. LTP and spatial learning capacity are impaired in 3xTgAD and 2xTgAD mice. (A–B) LTP measurements. f-EPSP slopes recorded in subiculum of 6 females per group: WT, 2xTgAD (2AD), and 3xTgAD mice (3AD). Graph in (B) represents LTP magnitudes calculated 20–60 minutes after high-frequency stimulation from graphs in (A) with statistical analysis (* $p < 0.05$; 1-way ANOVA with Benjamini-Hochberg correction). Error bars represent SEM. (C–D) MWM test. Learning curve obtained in 12-month-old female (C) and male (D) WT ($n = 16$ males and females), 2xTgAD (2AD, $n = 13$ females, $n = 15$ males), and 3xTgAD (3AD, $n = 16$ males and females) mice for 5 days. Data are expressed as means of 4 trials per day $\pm$ SEM. ** $p < 0.01$ and *** $p < 0.001$ comparing WT and 3AD groups; \$ $p < 0.05$ and \$\$\$ $p < 0.001$ comparing 2AD and 3AD groups; # $p < 0.05$ and ### $p < 0.001$ comparing WT and 2AD groups, according to a mixed model ANOVA (genotype $\times$ sex $\times$ day training), Benjamini-Hochberg correction. (E) The visible cue test was performed after the hidden platform training. Latency to reach the visible platform was average for females WT ($n = 16$), 2xTgAD (2AD, $n = 13$), and 3xTgAD (3AD, $n = 16$) mice. Data are expressed as means of 3 trials $\pm$ SEM. Genotype effect: $F(2,42) = 1.50$, $p = 0.2339$ using 1-way ANOVA, Benjamini-Hochberg correction. (F) Mean speed (m/s) of mice on the fifth day of MWM (average of 4 trials/mice $\pm$ SEM). Genotype effect: $F(2,86) = 0.14$, $p = 0.8703$, 2-way ANOVA (genotype $\times$ sex), Benjamini-Hochberg correction. (G) The Rotarod performance test was performed before the MWM. Latency to fall off the Rotarod apparatus was expressed as means of 3 trials/mouse $\pm$ SEM for WT ($n = 16$ females, $n = 16$ males), 2xTgAD (2AD, $n = 13$ females, $n = 15$ males), and 3xTgAD (3AD, $n = 16$ females, $n = 16$ males) mice. Genotype $\times$ sex interaction: $F(2,86) = 3.8$, $p = 0.0262$, 2-way ANOVA Benjamini-Hochberg correction. * $p < 0.05$ comparing WT and 3AD females; \$ $p < 0.05$ comparing 2AD and 3AD females; ### $p < 0.001$, comparing WT and 2AD females. Abbreviations: ANOVA, analysis of variance; 3xTgAD, triple transgenic mice; AD, Alzheimer's disease; f-EPSP, field excitatory postsynaptic potential; LTP, long-term potentiation; MWM, Morris water maze; WT, wild type; 2xTgAD, double transgenic mice; ns, not statistically significant.

the same decline of rearing motion, and the same LTP impairments in both 3×TgAD and 2×TgAD mice suggest a functional link between these AD-related dysfunctions and the intraneuronal accumulation of C99. However, A β also contributes to the acquisition of spatial information. Indeed, our result showing a stronger phenotype in the 3×TgAD than in 2×TgAD mice in MWM suggests that A β -related pathology exacerbates the cognitive deficits initially due to C99 accumulation. Even if we cannot exclude the possibility that alterations observed in both 3×TgAD and 2×TgAD mice might also be linked to mutated tau dysfunction, our conclusion agrees with the results from LaFerla's group showing an absence of LTP impairment in mice expressing PS1^{M146V} and Tau^{P301L} but lacking APP^{Swe} mutation, suggesting that altered LTP observed in 3×TgAD and 2×TgAD mice is associated to APP transgene (Oddo et al., 2003b).

The main risk factor for AD is age, but it is also sex related. Several clinical and epidemiological studies have demonstrated a higher prevalence and incidence of AD in women than in men (Pike, 2017; Podcasy and Epperson, 2016). This sex difference is likely linked to the drastic reduction of estrogens in women at menopausal stage (Gandy and Duff, 2000; Vina and Lloret, 2010). As previously reported by other groups (Carroll et al., 2010; Hirata-Fukae et al., 2008), the 3×TgAD mouse model mimics this sex difference because 3×TgAD females exhibit a stronger A β deposition and more pronounced alteration of AD-related behavior than age-matched males. We did not examine hormone levels or estrous cycle in 2×TgAD and 3×TgAD mice and cannot exclude the fact that these parameters might influence the AD-related phenotypes observed. However, we observed a more severe phenotype in 2×TgAD and 3×TgAD females compared to males in both actimetry and MWM task. In addition, we reported that sex influences and statistically worsens the genotype effect on cognitive and non-cognitive impairments.

One of the earliest and most frequent AD-associated neuropsychiatric syndromes is a lack of emotion, interest and motivation designated as an apathetic behavior. In clinical trials, it is well known that apathy is associated with a decline in motor activity that can be assessed by ambulatory actigraphy. The wrist actigraphy is indeed used to evaluate apathy in several neurological disorders, not only in AD but also in schizophrenia, stroke, and so on (David et al., 2012; Goldfine et al., 2016; Kluge et al., 2018; Kuhlmei et al., 2013). By analogy, we used actimetry to evaluate the mouse spontaneous locomotor activity during several days. Our previous studies have reported an early apathy-like phenotype in 4- and 6-month-old 3×TgAD mice as revealed by a drastic reduction in spontaneous mouse locomotor activity appearing concomitantly with hippocampal C99 accumulation. Here, we confirmed this phenotype in older 3×TgAD mice. Despite a consistent A β pathology in the 3×TgAD mice at this age (12 months of age), we demonstrated that the apathy-like behavior remains independent to A β deposition because 12-month-old 2×TgAD mice lacking A β display the same decrease in total activity. Importantly, the locomotor performances assessed in the Rotarod test were not altered in 2×TgAD and 3×TgAD mice. Therefore, the loss of activity measured by actimetry in these mice could not be explained by impairment in locomotor ability but more likely by a decrease in voluntary activity. In addition, we observed a drastic alteration in rearing behavior in both 2×TgAD and 3×TgAD mice. Many mammals, including rodents, rear on their hind legs in response to novelty, and this is considered as a good indicator for mice curiosity and environmental exploration. Thus, the drastic decrease of rearing activity observed in both 2×TgAD and 3×TgAD males and females is consistent with the apathy-like behavior described in 3×TgAD mice (Pardossi-Piquard et al., 2016). It should be emphasized that this phenotype is unrelated to A β or tau pathology

because biochemical (Fig. 3) and anatomical (Fig. 4) evaluation of 2×TgAD mice brain failed to uncover any A β - or tau-related stigmata. This fully agreed with our observation that 3×TgAD mice presented the same apathy-like phenotype at 4 months of age, that is, much before any detection of A β peptides, phosphorylated tau, or related lesions (Lauritzen et al., 2012; Pardossi-Piquard et al., 2016). Spontaneous rearing in mice is generally associated to exploration, curiosity of environmental novelty, and/or escape motivation, increasing the knowledge of their surroundings. Several lines of evidence suggest that the hippocampal system governs rearing motion in mammals (Lever et al., 2006). For instance, strain rats with an increased number of open-field rearings also presented a higher electroencephalogram hippocampal power (van Lier et al., 2003) and a higher activity of Na⁺/K⁺-ATPase specifically into the hippocampus (Alves et al., 2005). Similarly, mice genetically selected for high rearings showed morphologically larger intrapyramidal and infrapyramidal mossy fiber projections (Crusio et al., 1989). Thus, the drastic decrease of rearing and impairment of hippocampal functions observed in our TgAD mouse models could be linked to the hippocampal accumulation of C99. This is also in good agreement with other studies showing that γ -secretase inhibitors (Bittner et al., 2009; Mitani et al., 2012) or presenilins inactivation (Saura et al., 2004; Zhang et al., 2010) promote C99 accumulation and trigger undesirable effects on synapses (Bittner et al., 2009; Zhang et al., 2010), as well as negative effects on cognitive function in mice (Mitani et al., 2012; Saura et al., 2004).

Unexpectedly, in the present study, we could not strictly correlate LTP alterations with MWM deficits. Thus, while the 2×TgAD and 3×TgAD mice displayed same LTP impairment, the former TgAD mice better performed in the MWM. Spatial memory assessed by the MWM performances is usually well correlated with LTP and N-methyl-D-aspartate receptor function. This technique is therefore considered as a good behavioral readout to investigate hippocampal circuitry (Vorhees and Williams, 2014). Most studies report a positive correlation between LTP and spatial memory. However, dissociation between LTP and spatial memory has also already been observed (Gerlai, 2002; Zamanillo et al., 1999), and several reports have shown increased LTP in mutant mice associated with impaired learning or memory (Gerlai et al., 1998; Kaksonen et al., 2002; Pineda et al., 2004). This may illustrate the complexity of learning paradigm that involves not only brain multifunctional neurobiological systems (Hunsaker and Kesner, 2017) but also many compensatory mechanisms or external factors such as motivation or stress perception that could easily interfere with mice behavior.

Overall, our study indicates that C99 per se, in addition to A β peptides, contributes to several AD-related phenotypes such as apathy-like phenotype, synaptic dysfunction, and learning deficits observed in both 3×TgAD and 2×TgAD mice and reinforce the idea that the 2×TgAD mice are an excellent model to assess the respective contribution of C99 and A β peptides.

Disclosure statement

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.neurobiolaging.2018.06.038>.

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