Estrogens Inhibit Amyloid--Mediated Paired Helical Filament-Like Conformation of Tau Through Antioxidant Activity and miRNA 218 Regulation in hTau Mice

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Estrogens Inhibit Amyloid-β-Mediated Paired Helical Filament-Like Conformation of Tau Through Antioxidant Activity and miRNA 218 Regulation in hTau Mice

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Abstract.

Background: The risk of developing Alzheimer's disease as well as its progression and severity are known to be different in men and women, and cognitive decline is greater in women than in men at the same stage of disease and could be correlated at least in part on estradiol levels.

Objective: In our work we found that biological sex influences the effect of amyloid-β_{42} ($A\beta_{42}$) monomers on pathological tau conformational change.

Methods: In this study we used transgenic mice expressing the wild-type human tau (hTau) which were subjected to intraventricular (ICV) injections of Aβ peptides in nanomolar concentration.

Results: We found that $A\beta_{42}$ produces pathological conformational changes and hyperphosphorylation of tau protein in male or ovariectomized female mice but not in control females. The treatment of ovariectomized females with estradiol replacement protects against the pathological conformation of tau and seems to be mediated by antioxidant activity as well as the ability to modulate the expression of miRNA 218 linked to tau phosphorylation.

Conclusion: Our study indicates that factors as age, reproductive stage, hormone levels, and the interplay with other risk factors should be considered in women, in order to identify the best appropriate therapeutic approach in prevention of cognitive impairment.

Keywords: Alzheimer's disease, antioxidants, estradiol, miRNA, tau protein

INTRODUCTION

Hallmarks of Alzheimer's disease (AD) are the accumulation of amyloid-β (Aβ) peptides in amyloid plaques, and the aggregation of tau protein to form neurofibrillary tangles.

Aβ derives from the amyloid-β protein precursor (AβPP) through β site APP cleaving enzyme (BACE) 1 and γ-secretase processing that generates multiple C-termini, most ending at residue 40 and 42. $A\beta_{42}$ aggregates more quickly and stably than $A\beta_{40}$ through sequential phases: first Aβ

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monomers aggregate into soluble oligomers that then form insoluble oligomers, generating protofibrils and fibrils [1]. Recent studies indicate that plaques are not toxic but rather a reservoir of Aβ molecules: small soluble oligomers are the key to Aβ toxicity and in contrast, monomers have been suggested to be involved in physiological processes [2, 3].

Several data support the amyloid hypothesis: accumulation of Aβ peptides is the primary and early event that induces neuronal degeneration, characterized by conformational altered aggregated tau. We have developed a powerful system based on male mice expressing the wild-type human tau (hTau) which were subjected to intraventricular (ICV) injections of Aβ peptides in nanomolar concentration. We discovered that $A\beta_{42}$ monomers, but not oligomers: 1) produce paired helical filament-like conformation of tau protein, and 2) induce two phosphorylated epitopes which are not present in normal tau (Ser396 and Ser422) through the activation of GSK3β, JNK, and ERK 1/2 kinases [4].

Recent epidemiological studies showed that two-thirds of AD patients are women [5], and this fact cannot be attributed only to their higher life expectancy. In this connection, the loss of estradiol might be one of the factors leading to declining cognitive function in women [6].

Of note, we found that oxidative stress, together with important oxidative stress-related risk factors related to AD, such as hypoxia, hyperglycemia, and hypercholesterolemia, are potential causes of the increased BACE1 activity [7]. In AD, estrogen neuroprotective activity is exerted at multiple levels. Preclinical data showed that, in addition to their action against neuroinflammation and oxidative stress, estrogens are able to influence both the main players of neurodegeneration, Aβ, and tau [8].

In this paper, we pursue the hypothesis that biological sex influences the effect of $A\beta_{42}$ monomers on pathological tau conformational change. Our data revealed that $A\beta_{42}$ monomers produce the pathological conformational changes and hyperphosphorylation of tau protein in male or ovariectomized female mice but not in control female. The treatment of ovariectomized females with estradiol replacement protects against the pathological conformation of tau. The hypothesized protective mechanism is mediated both by their antioxidant activity and by their ability to modulate the expression of miRNA 218 linked to tau phosphorylation.

MATERIALS AND METHODS

Mice and ICV

hTau mice ($Mapt^{tm1(EGFP)Klt}$Tg(MAPT) 8cPdav/J; #004808, Jackson Laboratory) were crossed with tau knock-out (KO) mice ($Mapt^{tm1(EGFP)Klt}$/J; #004779, Jackson Laboratory), to obtain pregnant females carrying hTau features as described by Andorfer and colleagues, 2003 [9]. Mice were genotyped by PCR assay using the following primers: human tau transgene (forward 5'-ACTTTGAACCAGGATGGCTGAGCCC-3', reverse 5'-CTGTGCATGGCTGTCCCTACCTT-3'), mouse tau gene (forward 5'-CTCAGCAT CCCACCTGTAAC-3', reverse 5'-CCAGTTGT GTATGTCCACCC-3'), and disrupted tau gene (forward 5'-CAGGCTTTGAACCAGTATGG-3', reverse 5'-TGAACTTGTGGC CGTTTACG-3'). Mice were maintained on a Swiss Webster/129/SvJae/C57BL/6 background [9]. Animals were kept on a 12 h light/dark cycle with food and water available *ad libitum*. All experimental procedures on live animals were performed under the supervision of a licensed veterinarian, according to: 1) European Communities Council Directive (November 24, 1986; 86/609/EEC), 2) Italian Ministry of Health and University of Torino's institutional guidelines on animal welfare (DL 116/92 on Care and Protection of living animals undergoing experimental or other scientific procedures; authorization No. 17/2010-B, June 30, 2010), and 3) *ad hoc* Ethical Committee of the University of Turin (http://www.unito.it/unitoWAR/page/istituzionale/ricerca1/Ricerca_comitato1).

Two groups of 2-month-old male and/or female mice were treated for 3 h ($n = 60$). Under isoflurane O_2/N_2O anesthesia, hTau mice ($n = 80$) were ICV injected with Aβ peptides or saline. Coordinates used for injection were: anteroposterior, –0.5 mm; lateral, 1.2 mm relative to Bregma and dorsoventral, 1.7 mm from the dural surface. The method was validated by injecting one mouse with Trypan blue (1 μl).

Ovariectomy

Two groups of 2-month-old female mice underwent bilateral ovariectomy (OVX) except for the sham-operated groups. The bilateral ovaries of anaesthetized female mice were exposed through a midline incision in the dorsal skin and muscle layer. After

ligating the uterine horn, the bilateral ovaries were removed. In the sham group, mice underwent the procedures of anesthesia, incisions, bilateral ovaries exposure and incision closure without bilateral ovaries removing. After the surgery procedure, OVX female mice were fed with phytoestrogen-free feed.

Treatments

Mice were injected with 0.2 μM $A\beta_{42}$ peptides (#20276, Anaspec). The lyophilized synthetic peptides were dissolved in 1% of NH_4OH to get a clear solution and stored at –20°C in aliquots. Monomeric preparations were brought to 0.2 μM (final concentration) with sterile double distilled water, centrifuged at 10000 g for 10 min to remove possibly aggregates and then intraventricularly injected. The quality of Aβ preparations was controlled using atomic force microscopy (AFM). AFM was carried out on a Multimode AFM with a Nanoscope V system operating in Tapping Mode using standard antimony(n)-doped Si probes (T: 3.5–4.5 mm, L: 115–135 mm, W: 30–40 mm, f0:313–370 kHz, k: 20–80 N/m) (Bruker). The scan rate was tuned proportionally to the area scanned and was kept in the 0.5–1.2 Hz range. The sample was then diluted to 5 μM with PBS, and 50 μl of solution was spotted onto a freshly cleaved muscovite mica disk and incubated for 5 min. The disk was then washed with ddH_2O and dried under a gentle nitrogen stream. Samples were analyzed with the Scanning Probe Image Processor (SPIP Version 5.1.6 released April 13, 2011) data analysis package (Nanoscience Instruments, Phoenix, AZ, USA). SPIP software was used to analyze the distribution of the molecular assemblies of the different populations in terms of height and diameter, as previously described [10]. Our controls were hTau mice ICV injected with saline. The experiments were done four weeks after ovariectomy. After two weeks from ovariectomy surgery, one group of female mice was subjected to a daily subcutaneous injection of 17β-estradiol (E_2) for three weeks (1 μg/Kg) [11]. Animals were allowed to recover for at least three weeks before experiments were performed and subsequently were subjected to intracerebroventricular injection of $A\beta_{42}$ monomers (200 nM) or saline and sacrificed after 3 h.

Antibodies and immunoblot analysis

Immunoblot analysis was performed using the following antibodies: MC1 (kind gift from Dr. P Davies, Albert Einstein College of Medicine, New York, 1:500); Tau5 (Millipore, #577801, 1:500); AT8 (Innogenetics, #90206, 1:500); Tau 46 (Abcam, #22261, 1:1000), TaupS396 (Invitrogen, #44752G, 1:1000); TaupS244 (Invitrogen, #44764G, 1:1000); GSK3α/β tot (1:1000, Invitrogen, #44610, 1:1000); GSK3βpS9 (Novex, #710100, 1:1000); pJNK1/2 (Cell Signaling Technology, #9251, 1:500); JNK1/2 (Cell Signaling Technology, #9252, 1:500); pERK1/2 (Cell Signaling Technology, #43765, 1:1000); ERK1/2 (Santa Cruz Biotechnology, Sc-93, 1:1000; β-actin (Sigma Aldrich).

Fresh frozen brains were mechanically homogenized in ice-cold buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM PMSF, phosphatase and protease inhibitors) and then centrifuged at 10,000 g for 15 min at 4°C to isolate soluble proteins. Supernatants (2 mg/ml solution) were collected and incubated with detergent sarkosyl (5% final concentration) overnight at 4°C. The sarkosyl mixtures were then centrifuged in Beckman SW 55 Ti rotor at 35,000 rpm for 1 h at 4°C. Pellets were resuspended in 100 μl sample buffer to obtain sarkosyl-insoluble proteins. Lysates (20 μg) were run on 3–8% Tris-HCl gradient PAGE gel (Invitrogen) and then transferred to nitrocellulose membrane. Blots were blocked (5% no fat milk) and incubated overnight at 4°C with primary antibodies. Peroxidase-conjugated secondary antibodies were incubated 1 h at room temperature (RT) and developed with Luminata Forte Western substrate (WBLUF0100, Millipore). Densitometric values were normalized to β-actin.

Total antioxidant capacity

To evaluate the antioxidant capacity of mice brain tissues, we performed the total antioxidant capacity (TAC) dosage kit (ab65329, Abcam). The analysis was performed on total extracts according to the manufacturing protocol.

Quantitative determination of 17β-estradiol

To quantify E2 levels, blood mice was collected to obtain plasma. To perform the measuring, we used a commercially available ELISA kit from ENZO (Catalog # ADI-901-174) according to the manufacturing protocol.

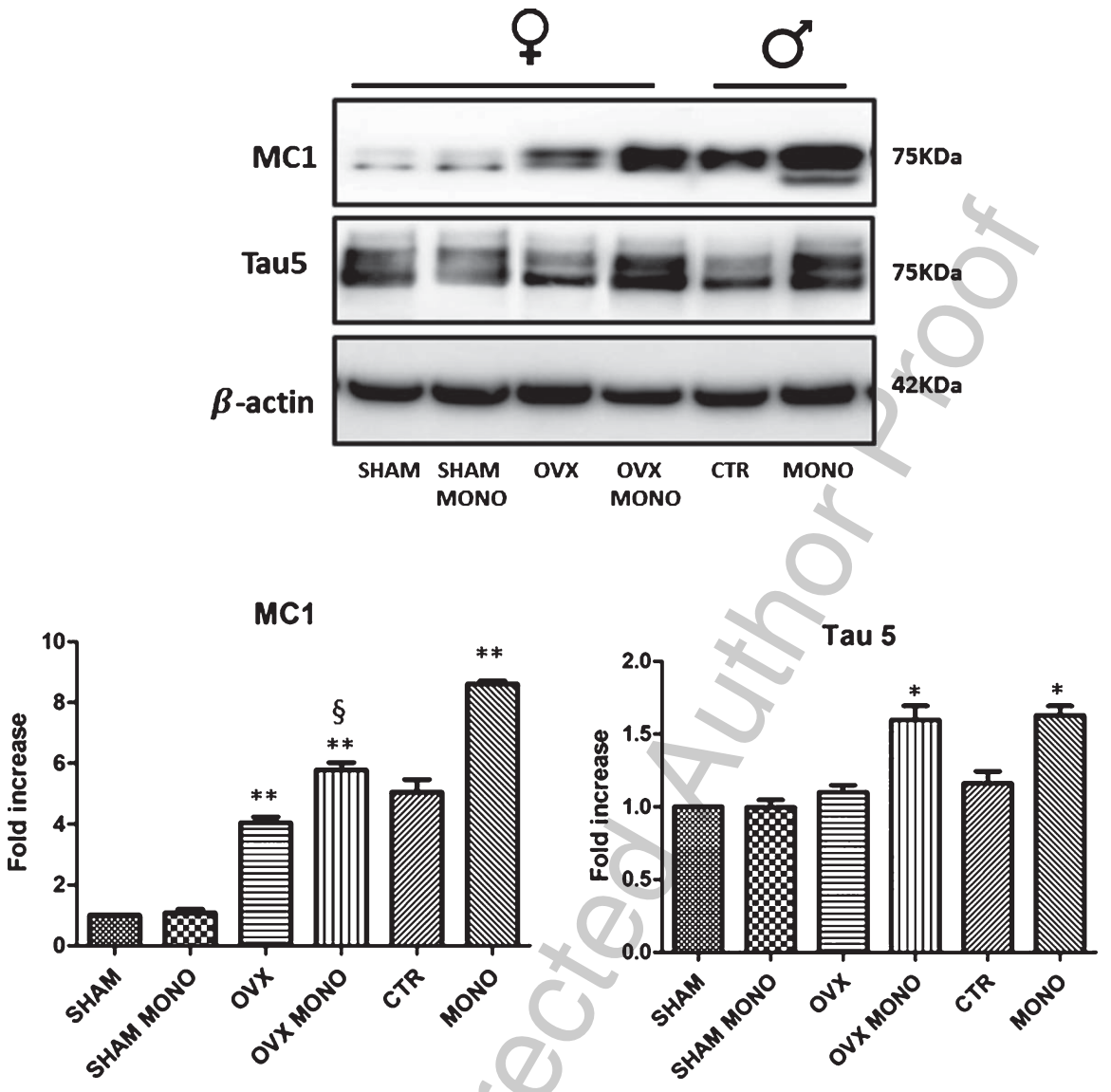

Fig. 1. Intracerebroventricular treatment with $A\beta_{42}$ causes a change in tau conformation only in male or ovariectomized female mice. Representative western blot of brain samples from control (saline) and treated $A\beta_{42}$ peptides for ICV male and female hTau mice using a conformational tau antibody (MC1) and a total tau antibody (Tau 5) for detection. Some female mice were subjected to ovariectomy. β-actin served as loading control. Densitometric quantification shows an increase of the total protein level of both MC1 and Tau 5 in male and ovariectomized female mice injected or not with $A\beta_{42}$. The data are mean ± standard error of the mean (SEM); $^{*}p < 0.05$; $^{**}p < 0.01$ versus control; $^{\S}p < 0.05$ versus OVX by one-way ANOVA followed by Bonferroni post test $n = 5$.

MicroRNA isolation and quantitative real time PCR

MicroRNA was isolated from brains of female mice using the MagMAXmirVana kit and according to manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Subsequently, cDNA synthesis was performed using the TaqMan Mi-croRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and a RT-primer pool containing microRNA-specific stem-loop primers for miR218 (Mature miRNA Sequence: UUGUGCUUGAUCUAACCAUGU) and for snRNA U6 (Control Sequence: GTGCTCGCTTCGGCAGCACATATACTAAAATTGGAACGATACAGAGAAGATTAGCATGGCCCCTGCGCAAGGATGACACGCAAATTCGTGAAGCGTTCCATATTTT).

Each qPCR contained 1.3 μL transcribed cDNA, 1 μL 20X TaqMan MicroRNA Assay and 10 μL 2X TaqMan Universal PCR MasterMix (Applied

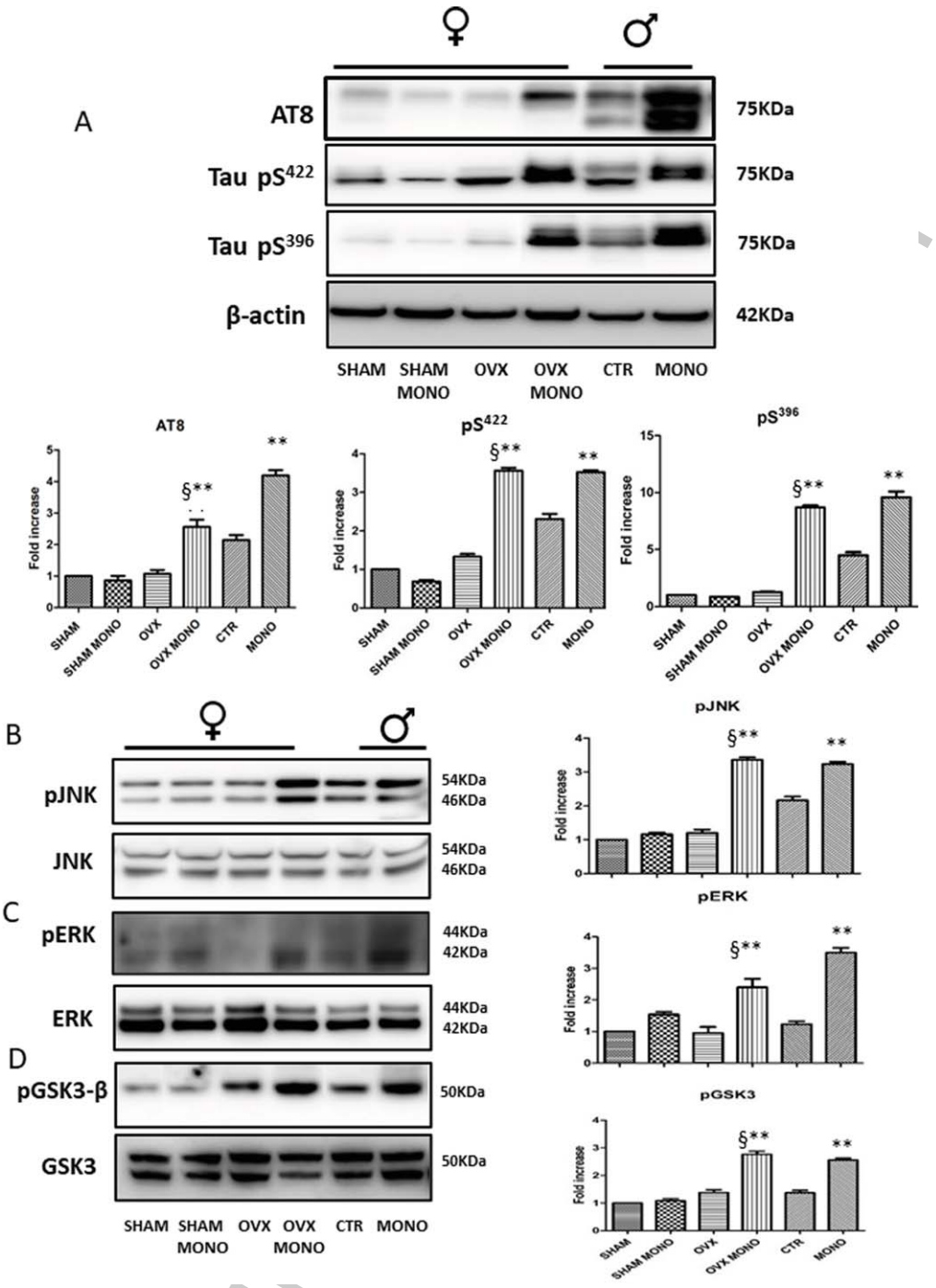

Fig. 2. The conformational change mediated by $A\beta_{42}$ is induced by protein hyperphosphorylation. A) Representative western blot of brain extracts from control (saline) and treated $A\beta_{42}$ peptides for ICV male and female hTau mice using antibodies specific for the detection of pathological tau phosphorylation sites such as AT8, pS422, and pS396. Some female mice were subjected to ovariectomy. βactin served as loading control. Densitometric analysis shows a significant increase of total protein levels of AT8, pS422, and pS396 only in male mice and ovariectomized female mice. B–D) Representative western blot of brain extracts from control (saline) and treated $A\beta_{42}$ peptides for ICV male and female hTau mice using p JNK (B), pERK1/2 (C), and pGSK3β (D). Some female mice were subjected to ovariectomy. β-actin served as loading control. Densitometric analysis shows a significant increase of total protein levels of all the three kinases only in male mice and ovariectomized female mice. The data are mean ± standard error of the mean (SEM); $^{*}p<0.05$; $^{**}p<0.01$ versus control; $^{\S}p<0.05$ versus OVX by one-way ANOVA followed by Bonferroni post test $n=5$.

Biosystems) in a total volume of 20.3 μL. Each sample was processed in doublets for 2 min at 50°C, 10 min at 96% and then for 40 cycles of 95°C for 15 s and 60°C for 60 s using the StepOnePlus Real Time PCR (Applied Biosystems Foster City, CA, USA). The mean Ct-values were technically normalized using the snRNA-U6, and the expression level calculated as $2^{-\Delta\Delta Ct}$ (ΔCt= Ct miR218− Ct snRNA-U6and$\Delta\Delta Ct$= ΔCt miR218−ΔCt calibrator).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.0 (GraphPad software, San Diego). All values were presented as mean ± standard error of the mean (SEM). Means were compared by one or two-way analysis of variance (ANOVA) with Bonferroni as a *post-hoc* test. Values of $*p < 0.05$ were considered significant, $**p < 0.01$ very significant, and $***p < 0.001$ extremely significant.

RESULTS

Intracerebroventricular treatment with $A\beta_{42}$ causes a change in tau conformation only in male or ovariectomized female mice

Figure 1 shows that, as previously demonstrated, the intracerebroventricular injection of 200 nM $A\beta_{42}$ in male hTau mice is able to determine a pathological conformational change of the tau protein. The same treatment is able to determine this effect only in female mice after ovariectomy. The same result was obtained by evaluating the total tau protein levels. As can be seen, treatment with $A\beta_{42}$ significantly increases total tau levels in male (1.5-fold increase) and ovariectomized female mice (1.5-fold increase) with respect to control female mice injected or not with $A\beta 1_{42}$.

The conformational change mediated by $A\beta_{42}$ is induced by protein hyperphosphorylation

To understand if the conformational change of tau was due to hyperphosphorylation, we measured the phosphorylation of some specific sites related to the pathology by western blotting. Phosphorylation levels were studied through the use of the AT8 antibody (which recognizes the Ser 202/Thr 205 epitopes), the antibody S396 and S422. These are all phosphorylation sites closely associated with disease progression.

In Fig. 2A, we show that the treatment with $A\beta_{42}$ induces phosphorylation of the sites taken into consideration in male and ovariectomized female mice, while in control females, phosphorylation of the sites is not observed. The increase in phosphorylation is extremely significant for all the phosphorylation sites studied: (+4–5-fold increase AT8; 3–4-fold increase S422; and +4–5-fold increase S396). We previously showed that these sites are phosphorylated by GSK3β, ERK, and JNK kinases [4]. We therefore studied the kinase levels in nuclear lysates. Figure 2B shows that $A\beta_{42}$ treatment significantly increases pJNK levels in nuclear extracts of male mice (+3-fold increase) or ovariectomized females (+3-fold increase). A similar result was obtained by measuring the nuclear levels of pERK and pGSK3β which increase in the same groups by about three times compared to the controls (Fig. 2C, D).

Estradiol hormone therapy protects against $A\beta_{42}$-mediated tau conformational change

To confirm that the presence of estrogens is involved in the different effect exerted by the treatment with $A\beta_{42}$ on the pathological conformational change of tau, groups of female mice, ovariectomized or not were subcutaneously treated with estradiol (E_2) (1 μg/kg) and fed with a soy-free diet for three weeks. We first tested the validity of the treatment by measuring the levels of estradiol in the experimental groups. As can be seen, oophorectomy significantly decreases circulating estradiol levels (–60%), while E2 treatment determines an increase in levels that becomes significantly higher with respect to control females (+100%) (Fig. 3). Figure 4A shows that the treatment with estradiol completely protects both the pathological conformational change and the increase of total tau mediated by $A\beta_{42}$ in ovariectomized females. Then, to further confirm the protective role of estradiol, we studied the insoluble fraction by sarkosyl detergent technique, and the results showed a tau band at approximately 75 kDa molecular weight revealed with Tau 46 antibody after injection of $A\beta_{42}$ in ovariectomized female, whereas the therapy with estradiol blocks the aggregation of tau protein (Fig. 4B).

Estradiol therapy protects female hTau mice against $A\beta_{42}$-mediated tau hyperphosphorylation

Figure 5 shows that enrichment with estradiol is also followed by complete protection of $A\beta_{42}$-

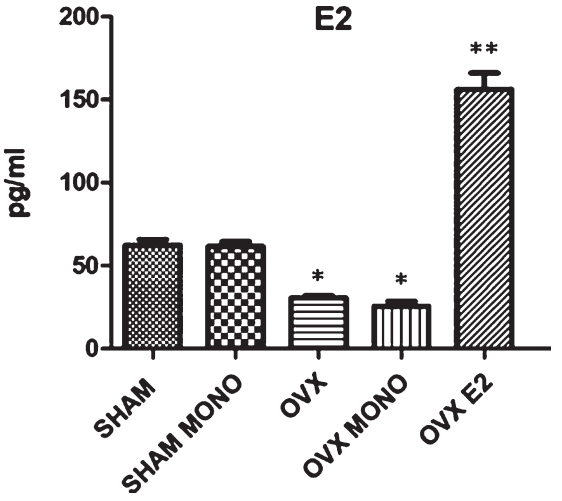

Fig. 3. Estradiol hormone therapy significantly increases E_2 levels. Groups of female mice, ovariectomized or not, were subcutaneously treated with E_2 (1 μg/kg) and fed with a soy-free diet for three weeks. To test the validity of the treatment, we measured E2 levels in serum of our experimental groups. We observed that the ovariectomy induces a significant decrease in hormone levels, whereas the treatment protects the decrease of E2 levels, that significantly increased with respect to controls. The data are mean ± standard error of the mean (SEM); $*p < 0.05$; $**p < 0.01$ versus control by one-way ANOVA followed by Bonferroni post test $n = 6$.

mediated phosphorylation, after oophorectomy, of pathology-related sites (Fig. 5A). As expected, the kinases involved in the phosphorylation of the above sites do not appear induced; thus, as observed in Fig. 5B, the levels of nuclear pJNK, pERK, and pGSK3β are absolutely comparable to the levels of the control females.

Estrogen hormone therapy protects male hTau mice against $A\beta_{42}$-mediated tau conformational change and hyperphosphorylation

To further confirm the protective role of estradiol on the pathological conformational change of tau and its hyperphosphorylation, we also treated hTau male mice with estradiol, following the same protocol as the females. As can be seen in Fig. 6, the treatment with estradiol is able to completely protect both the conformational change of tau as revealed by the significant increase of the band revealed with MC1 antibody as well as its hyperphosphorylation revealed by using AT8 antibody that recognizes Ser202/Thr205 phospho-epitopes (Fig. 6).

Estradiol hormone therapy protects against oxidative stress and downregulate miRNA 218

It is well known that E2 treatment, at least during the early stage of AD pathology, significantly promotes the recovery of cognitive function and upregulated neurogenesis-related mediators in $A\beta_{42}$ mice and that these effects may have been due, at least in part, to decreased levels of oxidative stress via reductions in the production of nitric oxide and reactive oxygen species [12]. Thus, we tested the total antioxidant capacity in our experimental groups. As shown in Fig. 6A, ovariectomy is capable of causing a significant decrease in antioxidant capacity (–50%) and the simultaneous intracerebroventricular injection of $A\beta_{42}$ induces a further deterioration of the parameter (–70%). Treatment with estradiol protects the drop-in antioxidant capacity by bringing it back to control values (Fig. 6A).

Finally, we measured levels of miR218, since recent discoveries demonstrate that estrogen receptors are able to modulate the expression of microRNA involved in tau phosphorylation [13]. In particular it has been found that increase of miRNA 218 reduces level of target protein tyrosine phosphatase α with consequent enhancement of tau phosphorylation.

We observed that levels of miR218 are significantly higher in ovariectomized female mice, injected or not with $A\beta_{42}$, whereas the E2 treatment is followed by a total protection of the miRNA increase (Fig. 6B). It is interesting to note that the results obtained on male hTau mice treated with Aβ are similar to those obtained in females after oophorectomy (Fig. 6C, D).

DISCUSSION

In our work we pursued the hypothesis that gender influences the effect of $A\beta_{42}$ monomers on pathological tau conformational change. Our data revealed that $A\beta_{42}$ produced pathological conformational changes and hyperphosphorylation of tau protein in male or ovariectomized female mice but not in control female. The risk of developing AD as well as its progression and severity are known to be extremely different in men and women [14]. A link between the drop-in estrogen and the pathology is confirmed by data indicating that early menopause increases the risk of developing dementia [5]. Furthermore, cognitive decline is greater in women than in men at the same stage of disease and this is evidently correlated with estrogen levels [15]. The debate on the therapeutic potential of estrogen has been very

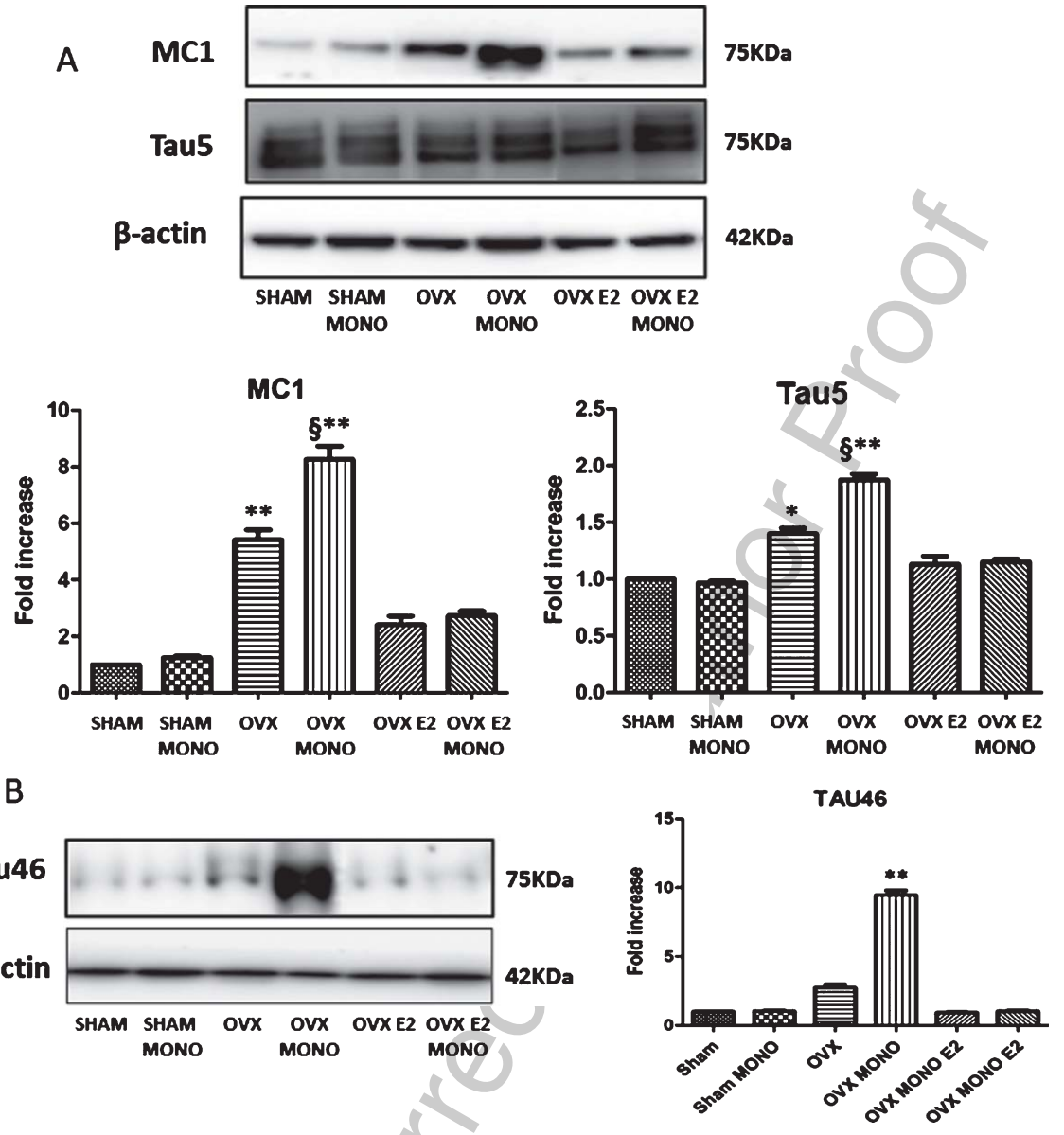

Fig. 4. Estradiol hormone therapy protects against $A\beta_{42}$-mediated tau conformational change. A) Representative western blot of brain samples from control (saline) and treated $A\beta_{42}$ peptides for ICV female hTau mice using a conformational tau antibody (MC1) and a total tau antibody (Tau 5) for detection. Some female mice were subjected to ovariectomy and or to E_2 (1 μg/kg) and soy-free diet for three weeks. β-actin served as loading control. Densitometric quantification shows an increase of the total protein level of both MC1 and Tau 5 in ovariectomized female mice injected or not with $A\beta_{42}$; the treatment with estradiol completely protects the conformational change and the increase of tau protein. B) Representative western blot of insoluble tau fraction by sarkosyl detergent technique extracts from control (saline) and treated $A\beta_{42}$ peptides for ICV female hTau using Tau 46 antibody for detection. β-actin served as loading control. After injection of $A\beta_{42}$ in ovariectomized females, we showed a band at approximately 75 kDa molecular weight revealed with Tau 46 antibody, whereas estradiol blocks the aggregation of tau protein. The data are mean ± standard error of the mean (SEM); $^{*}p < 0.05$; $^{**}p < 0.01$ versus control; $^{\S}p < 0.05$ versus OVX by one-way ANOVA followed by Bonferroni post test $n = 5$.

active in the last years and recently it has taken new life, thus finding new therapeutic approaches for AD is one of the most important challenges of modern medicine. Numerous experimental evidences have shown that estrogens have protective effects on the induction of neuroinflammation and neurodegeneration [16–18]. The encouraging results obtained *in vitro* clashed with clinical trials outcomes. In 2003,

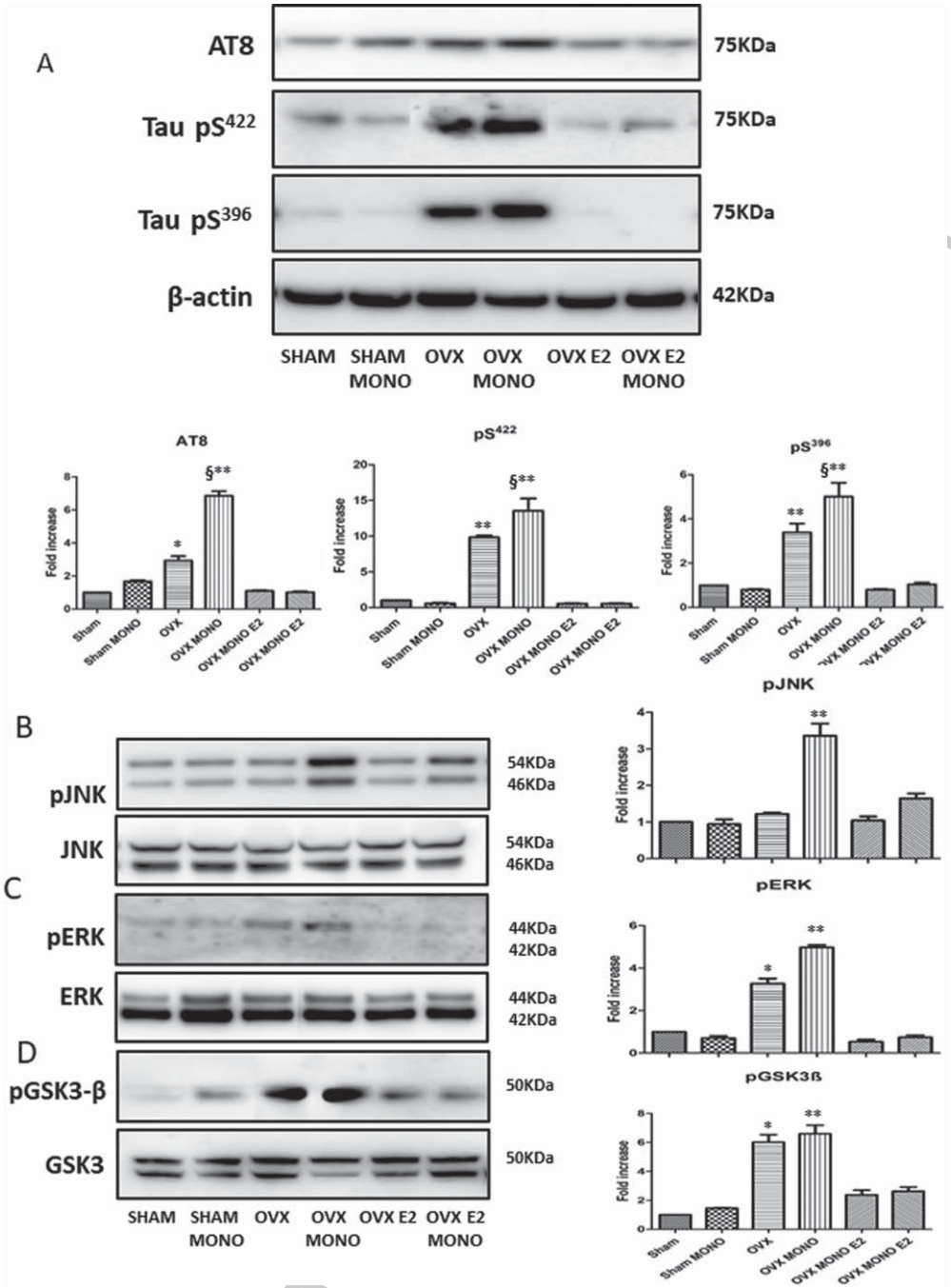

Fig. 5. Estradiol hormone therapy protects against $A\beta_{42}$-mediated tau hyperphosphorylation. A) Representative western blot of brain extracts from control (saline) and treated $A\beta_{42}$ peptides for ICV female hTau mice using antibodies specific for the detection of pathological tau phosphorylation sites such as AT8, pS422, and pS396. Some female mice were subjected to ovariectomy and or to E_2 (1 μg/kg) and soy-free diet for three weeks. β-actin served as loading control. Densitometric analysis shows an increase of total protein levels of AT8, pS422, and pS396 in ovariectomized female mice injected or not with $A\beta_{42}$; the treatment with estradiol completely protects the hyperphosphorylation of tau protein. B–D) Representative western blot of brain extracts from control (saline) and treated $A\beta_{42}$ peptides for ICV female hTau mice using p JNK (B), pERK1/2 (C), and pGSK3β (D). Some female mice were subjected to ovariectomy and or to E_2 (1 μg/kg) and soy-free diet for three weeks. β-actin served as loading control. Densitometric analysis shows an increase of total protein levels of all the three kinases in ovariectomized female mice injected or not with $A\beta_{42}$; the treatment with estradiol completely protects the activation of the kinases. The data are mean ± standard error of the mean (SEM); $^{*}p < 0.05$; $^{**}p < 0.01$ versus control; $^{\S}p < 0.05$ versus OVX by one-way ANOVA followed by Bonferroni post test $n = 5$.

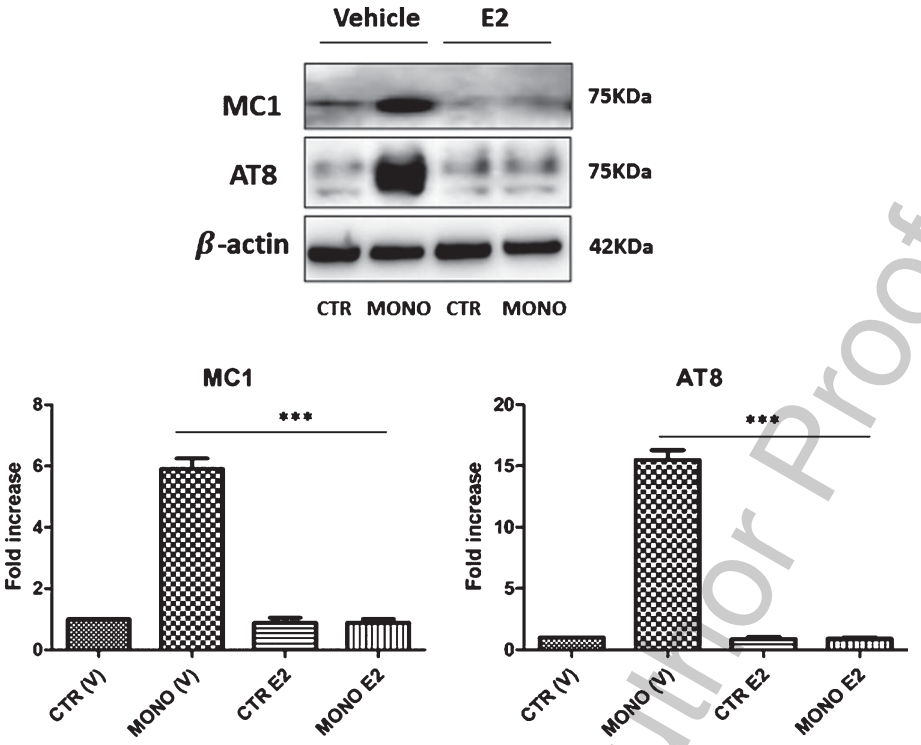

Fig. 6. Estradiol hormone therapy protects male mice against $A\beta_{42}$-mediated tau conformational change as well as hyperphosphorylation. Representative western blot of brain samples from control (saline) and treated $A\beta_{42}$ peptides for ICV male hTau mice using a conformational tau (MC1) and a specific antibody for tau pathological phosphorylation (AT8) for detection. Some male mice were subjected to E_2 (1 μg/kg) and soy-free diet for three weeks. β-actin served as loading control. Densitometric quantification shows an increase of the total protein level of both MC1 and AT8 in mal mice injected or not with $A\beta_{42}$; the treatment with estradiol completely protects the conformational change and the increase of tau phosphorylation. The data are mean ± standard error of the mean (SEM); $^{**}p < 0.01$ versus control by one-way ANOVA followed by Bonferroni post test $n = 5$.

clinical trials had shown that the use of replacement therapy significantly increased the risk of dementia and cognitive decline [19, 20]. These studies suggested that patients aged 65 and over, already in menopause for a long time, did not represent an adequate experimental group, because replacement therapy is indicated for women who have just gone through menopause, and suggested the presence of a therapeutic window useful for this type of therapeutic approach [21]. More recently, emerging evidence suggests that protein tau could be a potential target for estrogens. It has been demonstrated that 17β estradiol promotes tau dephosphorylation *in vitro* in rat cortical neurons and SH-SY5Y neuronal cells [22]. Other authors confirmed these results showing that E2 prevent the phosphorylation of tau in an estrogen receptor-mediated and dose-dependent manner [23]. *In vivo* studies have shown that estrogenic treatment increases GSK3β phosphorylation in Ser 9/21, a site that inactivates the kinase activity, protecting the phosphorylation of pathological sites related to disease progression [24]. Moreover, literature data suggest that estrogens exert their protective action through their alpha-type receptor, which interacts with insulin-like growth factor 1 receptor (IGF-R1) by incorporating itself into a macromolecular complex that includes phosphoinositol 3 kinase (PI3K) and protein kinase A (AKT). The activation of these signal pathways leads to an inhibition of GSK3β and therefore to a reduction in tau phosphorylation [25]. Our results confirm the protective role of estrogens on the pathological conformation and hyperphosphorylation of tau mediated by intracerebroventricular injection with $A\beta_{42}$ in hTau female mice. From our results, it cannot be determined if estradiol acts as an antioxidant compound and, therefore, in a mode independent of its receptor or modulates at the receptor level protective signal pathways. We showed that it inhibits the kinases that, under Aβ treatment, hyperphosphorylate tau; these results suggest a cellular signaling effect of estradiol.

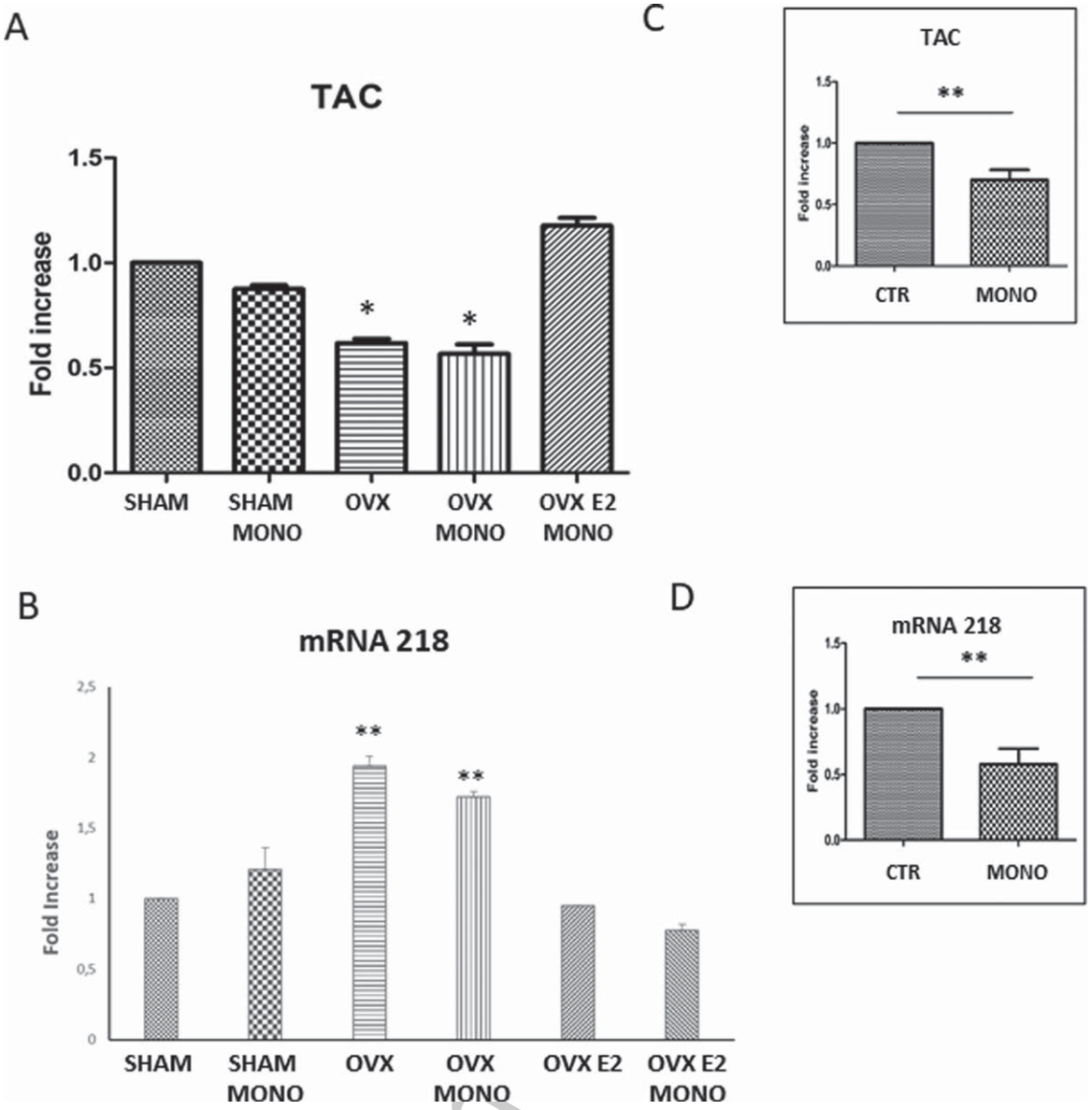

Fig. 7. Estradiol hormone therapy protects against oxidative stress and downregulate miRNA 218. A) Evaluation of the total antioxidant capacity in female mice subjected to ovariectomy and or to E_2 (1 μg/kg) and soy-free diet for three weeks. We show that ovariectomy significantly decreases the antioxidant capacity respect to control females, whereas the treatment with estradiol protects the drop-in antioxidant capacity. B) miRNA 21 levels capacity in female mice subjected to ovariectomy and or to E_2 (1 μg/kg) and soy-free diet for three weeks. We show that ovariectomy significantly increases the levels of miRNA 218 with respect to controls female, the treatment with estradiol completely protects the increase. C) Evaluation of the total antioxidant capacity in male mice treated or not with $A\beta_{42}$. We show that $A\beta_{42}$ decreases the antioxidant capacity respect to control males. D) miRNA 21 levels capacity in male mice treated or not with $A\beta_{42}$. We show that $A\beta_{42}$ significantly decreases miRNA level respect to control males. $^*p < 0.05$; $^{**}p < 0.01$ versus control; $^{\S}p < 0.05$ versus OVX by one-way ANOVA followed by Bonferroni post test $n = 5$.

A lot of protective mechanisms relating to estrogens have been described in the literature [26]. Most recent studies have revealed that estrogens exert an antioxidant action not only by direct chemical neutralization of reactants, but also by modulating the expression of antioxidant enzymes that control levels of biological reducing agents [27]. In our experimental models, we found that the decrease of estrogenic levels was followed by a decrease in the total antioxidant activity and that this event is blocked by the

estrogenic therapy in ovariectomized female hTau mice. Although it is not clear how oxidative stress alters tau phosphorylation and aggregation, it is well known that tau phosphorylation is related to the balance between kinases and phosphatases activities. Thus, oxidative stress may alter this balance in favor of the activation of kinases that induces the hyperphosphorylation of tau. This statement is in agreement with the evidence that the estrogenic drop is followed by the activation of stress related kinases, as well as JNK and ERK 1/2, while the replacement therapy reports the phosphorylated nuclear levels to those of control.

Of note, we found that oxidative stress, together with important oxidative stress-related risk factors related to AD such as hypoxia, hyperglycemia, and hypercholesterolemia, are potential causes of the increased BACE1, the crucial enzyme for Aβ production, activity [7].

Recently emerging evidence suggests that estrogens are involved in regulation of microRNAs in many pathological conditions [28]. Rao et al. showed that estradiol regulates particular target miRNAs in a specific tissue and age manner in ovariectomized rats [29]. Furthermore, the deprivation of estrogens caused the progressive loss of regulation of the miRNA, leading to a lack of regulation even after the reintroduction of the estrogens [30]. In our work we focused our attention on the miRNA 218, because it is implicated in the phosphorylation of tau upon estrogen receptor (ER) α and β activation. There are two known ERs, usually referred to as ERα and ERβ, and both are widely distributed in the brain [31]. In the brain of patients with AD, both ERα and ERβ are defective. Mitochondrial ERβ is reduced in the frontal cortex of female patients with AD [32], and the alternative splicing of ERα mRNA is decreased in the AD brain especially in female patients [33]. Moreover, in the hippocampus of AD patients the ERα-expressing neurons are reduced [34], whereas ERβ immunoreactivity is increased [35]. These findings indicate a potential role of these two receptors in the pathogenesis of AD. Then it has been reported that the neuroprotection against Aβ toxicity by estrogens requires the expression of both receptors and the activation of mitogen-activated protein kinase pathway [36]. Specifically, Xiong et al. demonstrated opposite effects of these two receptors on tau phosphorylation. ERα overexpression increased miRNA 218 expression and the hyperphosphorylation of tau, whereas ERβ decreased miRNA 218 expression and tau phosphorylation [13]. Interestingly, a number of miRNA 218 targets multiple components of receptor tyrosine signaling pathways [37]. The increase in miRNA 218 downregulates protein tyrosine phosphatase α that promotes tau phosphorylation [13]. This finding is in agreement with our results that demonstrate higher levels of miRNA 218 in ovariectomized female mice in comparison to control females.

Our study indicates that factors as age, reproductive stage, hormone levels, and the interplay with other risk factors should be considered in women, in order to identify the best appropriate treatment with estrogens in prevention of cognitive impairment.

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REFERENCES

[1] Hubin E, van Nuland NA, Broersen K, Pauwels K (2014) Transient dynamics of Aβ contribute to toxicity in Alzheimer's disease. *Cell Mol Life Sci* **71**, 3507-3521.

[2] Nimmrich V, Ebert U (2009) Is Alzheimer's disease a result of presynaptic failure? Synaptic dysfunctions induced by oligomeric beta-amyloid. *Rev Neurosci* **20**, 1-12.

[3] Puzzo D, Privitera L, Fa' M, Staniszewski A, Hashimoto G, Aziz F, Sakurai M, Ribe EM, CM, Mercken M, S Jung SS, Palmeri A, Arancio O (2011) Endogenous amyloid-β is necessary for hippocampal synaptic plasticity and memory. *Ann Neurol* **69**, 819-830.

[4] Manassero G, Guglielmotto M, Zamfir R, Borghi R, Colombo L, Salmona M, Perry G, Odetti P, Arancio O, Tamagno E, Tabaton M (2016) Beta-amyloid 1-42 monomers, butnotoligomers, produce PHF-like conformation of Tau protein. *Aging Cell* **15**, 914-923.

[5] Beam CR, Kaneshiro C, Jang JY, Reynolds CA, Pedersen NL, Gatz M (2018) Differences between women and men in incidence rates of dementia and Alzheimer's disease. *J Alzheimers Dis* **64**, 1077-1083.

[6] Laws KR, Irvine K, Gale TM (2016) Sex differences in cognitive impairment in Alzheimer's disease. *World J Psychiatry* **6**, 54-65.

[7] Tamagno E, Guglielmotto M, Monteleone D, Tabaton M (2012) Amyloid-β production: Major link between oxidative stress and BACE1. *Neurotox Res* **22**, 208-219.

[8] Merlo S, Spampinato SF, Sortino MA (2017) Estrogen and Alzheimer's disease: Still an attractive topic despite disappointment from early clinical results. *Eur J Pharmacol* **817**, 51-58.

[9] Andorfer C, Kress Y, Espinoza M, de Silva R, Tucker KL, Barde YA, Duff K, Davies P (2003) Hyperphosphorylation and aggregation of tau in mice expressing normal human tau isoforms. *J Neurochem* **86**, 582-590.

[10] Messa M, Colombo L, del Favero E, Cantù L, Stoilova T, Cagnotto A, Rossi A, Morbin M, Di Fede G, Tagliavini F, Salmona M (2014) The peculiar role of the A2V mutation

in amyloid-β (Aβ) 1-42 molecular assembly. *J Biol Chem* **289**, 24143-24152.

[11] López-Grueso R, Gambini J, Abdelaziz KM, Monleón D, Díaz A, El Alami M (2014) Early, but not late onset estrogen replacement therapy prevents oxidative stress and metabolic alterations caused by ovariectomy. *Antioxid Redox Signal* **20**, 236-246.

[12] Nilsen J (2008) Estradiol and neurodegenerative oxidative stress. *Front Neuroendocrinol* **29**, 463-475.

[13] Xiong YS, Liu FF, Liu D, Huang HZ, Wei N, Tan L, Chen JG, Man HY, Gong CX, Lu Y, Wang JZ, Zhu LQ (2015) Opposite effects of two estrogen receptors on tau phosphorylation through disparate effects on the miR-218/PTPA pathway. *Aging Cell* **14**, 867-877.

[14] Pike CJ (2017) Sex and the development of Alzheimer's disease. *J Neurosci Res* **95**, 671-680.

[15] Ruitenberg A, Ott A, van Swieten JC, Hofman A, Breteler MM (2001) Incidence of dementia: Does gender make a difference? *Neurobiol Aging* **22**, 575-580.

[16] Villa A, Vegeto E, Poletti A, Maggi A (2016) Estrogens, neuroinflammation, and neurodegeneration. *Endocrinol Rev* **37**, 372-402.

[17] Khan M, Ullah R, Rehman SU, Shah SA, Saeed K, Muhammad T, Park HY, Jo MH, Choe K, Rutten BPF, Kim MO (2019) 17β-estradiol modulates SIRT1 and halts oxidative stress-mediated cognitive impairment in a male aging mouse model. *Cells* **8**, 928.

[18] Pandey R, Shukla P, Anjum B, Gupta HP, Pal S, Arjaria N, Gupta K, Chattopadhyay N, Sinha RA, Bandyopadhyay S (2020) Estrogen deficiency induces memory loss via altered hippocampal HB-EGF and autophagy. *J Endocrinol* **244**, 53-70.

[19] Rapp SR, Espeland MA, Shumaker SA, Henderson VW, Brunner RL, Manson JE, Gass MLS, Stefanick ML, Lane DS, Hays J, Johnson KC, Coker LH, Dailey M, Bowen D, WHIMS Investigators (2003) Effect of estrogen plus progestin on global cognitive function in postmenopausal women: The Women's Health Initiative Memory Study: A randomized controlled trial. *JAMA* **289**, 2663-2672.

[20] Shumaker SA, Legault C, Rapp SR, Thal L, Wallace RB, OckeneJK,Hendrix SL, Jones BN 3rd, Assaf AR, Jackson RD, Kotchen JM, Wassertheil-Smoller S, Wactawski-Wende J; WHIMS Investigators (2003) Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: The Women's Health Initiative Memory Study: A randomized controlled trial. *JAMA* **289**, 2651-2662.

[21] Henderson VW (2014) Alzheimer's disease: Review of hormone therapy trials and implications for treatment and prevention after menopause. *J Steroid Biochem Mol Biol* **142**, 99-106.

[22] Alvarez-de-la-Rosa M, Silva I, Nilsen J, Pérez MM, García-Segura LM, Avila J, Naftolin F (2010) Estradiol prevents neural tau hyperphosphorylation characteristic of Alzheimer's disease. *Ann N Y Acad Sci* **1052**, 210-224.

[23] Zhang Z, Simpkins JW (2010) Okadaic acid induces tau phosphorylation in SH-SY5Y cells in an estrogen-preventable manner. *Brain Res* **1345**, 176-181.

[24] Goodenough S, Schleusner D, Pietrzik C, Skutella T, Behl C (2005) Glycogen synthase kinase 3beta links neuroprotection by 17beta-estradiol to key Alzheimer processes. *Neuroscience* **132**, 581-589.

[25] Hansberg-Pastor V, González-Arenas A, Piña-Medina AG, Camacho-Arroyo I (2015) Sex hormones regulate cytoskeletal proteins involved in brain plasticity. *Front Psychiatry* **6**, 165.

[26] Vegeto E, Villa A, Della Torre S, Crippa V, Rusmini P, Cristofani R, Galbiati M, Maggi A, Poletti A (2019) The role of sex and sex hormones in neurodegenerative disease. *Endocr Rev* **41**, 273-319.

[27] Cervellati C, Bergamini CM (2016) Oxidative damage and the pathogenesis of menopause related disturbances and diseases. *Clin Chem Lab Med* **54**, 739-753.

[28] Reddy PH, Tonk S, Kumar S, Vijayan M, Kandimalla R, KuruvaCS, Reddy AP (2017) A critical evaluation of neuroprotective and neurodegenerative MicroRNAs in Alzheimer's disease. *Biochem Biophys Res Commun* **483**, 1156-1165.

[29] Rao YS, Mott NN, Wang Y, Chung WC, Pak TR (2013) MicroRNAs in the aging female brain: A putative mechanism for age-specific estrogen effects. *Endocrinology* **154**, 2795-2806.

[30] Rao YS, Shults CL, Pinceti E, Pak TR (2015) Prolonged ovarian hormone deprivation alters the effects of 17β-estradiol on microRNA expression in the aged female rat hypothalamus. *Oncotarget* **6**, 36965-36983.

[31] Perez SE, Chen EY, Mufson EJ (2003) Distribution of estrogen receptor alpha and beta immunoreactive profiles in the postnatal rat brain. *Brain Res Dev Brain Res* **145**, 117-139.

[32] Long J, He P, Shen Y, Li R (2012) New evidence of mitochondria dysfunction in the female Alzheimer's disease brain: Deficiency of estrogen receptor-β. *J Alzheimers Dis* **30**, 545-558.

[33] Ishunina TA, Swaab DF (2012) Decreased alternative splicing of estrogen receptor-α mRNA in the Alzheimer's disease brain. *Neurobiol Aging* **33**, 286-296.e283.

[34] Hu XY, Qin S, Lu YP, Ravid R, Swaab DF, Zhou JN (2012) Decreased estrogen receptor-alpha expression in hippocampal neurons in relation to hyperphosphorylated tau in Alzheimer patients. *Acta Neuropathol* **106**, 213-220.

[35] Savaskan E, Olivieri G, Meier F, Ravid R, Muller Spahn F (2001) Hippocampal estrogen beta-receptor immunoreactivity is increased in Alzheimer's disease. *Aging Cell* **908**, 113-119.

[36] Fitzpatrick JL, Mize CB, Wade CB, Harris JA, Shapiro RA, Dorsa DM (2002) Estrogen-mediated neuroprotection against beta-amyloid toxicity requires expression of estrogen receptor alpha or beta and activation of the MAPK pathway. *J Neurochem* **82**, 674-682.

[37] Mathew LK, Skuli N, Mucaj V, Lee SS, Zinn PO, Sathyan P, Imtiyaz HZ, Zhang Z, Davuluri RV, Rao S, Venneti S, Lal P, Lathia JD, Rich JN, Keith B, Minn AJ, Simon MC (2014) miR-218 opposes a critical RTK-HIF pathway in mesenchymal glioblastoma. *Proc Natl Acad Sci U S A* **111**, 291-296.