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Inhibition of PMCA activity by tau as a function of aging and Alzheimer's neuropathology

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ABSTRACT

Ca^{2+}-ATPases are plasma membrane and intracellular membrane transporters that use the energy of ATP hydrolysis to pump cytosolic Ca^{2+} out of the cell (PMCA) or into internal stores. These pumps are the main high-affinity Ca^{2+} systems involved in the maintenance of intracellular free Ca^{2+} at the properly low level in eukaryotic cells. The failure of neurons to keep optimal intracellular Ca^{2+} concentrations is a common feature of neurodegeneration by aging and aging-linked neuropathologies, such as Alzheimer's disease (AD). This disease is characterized by the accumulation of β-amyloid senile plaques and neurofibrillary tangles of tau, a protein that plays a key role in axonal transport. Here we show a novel inhibition of PMCA activity by tau which is concentration-dependent. The extent of inhibition significantly decreases with aging in mice and control human brain membranes, but inhibition profiles were similar in AD-affected brain membrane preparations, independently of age. No significant changes in PMCA expression and localization with aging or neuropathology were found. These results point out a link between Ca^{2+}-transporters, aging and neurodegeneration mediated by tau protein.

1. Introduction

Aging involves a physiological neurodegenerative process which includes functional and biochemical changes before a significant cell death occurs [1]. However, these changes are frequently observed in neuropathologies where aging is also a risk factor.

Neurodegeneration is primarily related with a lack of function in cells where functional polarity and cytoskeleton organization is crucial. Several aging-associated pathologies are related with cytoskeleton-associated proteins such as tauopathies, a group of disorders where the function of tau protein is affected. Tau is a microtubule associated protein involved in the assembly and stability of microtubules in neurons [2]. Its function is dependent on its phosphorylation state [3], e.g. when tau is dephosphorylated, microtubules can elongate promoting vesicular transport, but when tau is phosphorylated, microtubules are more stable and elongation is reduced. In pathological conditions, tau protein may result hiperphosphorylated generating aberrant aggregates that are toxic to neurons. Thus, the presence of these tau aggregates, known as neurofibrillary tangles (NFTs), is a hallmark of different tauopathies [4].

Neural aging is also well associated to Ca^{2+} dyshomeostasis [5–9]. That would involve alteration of Ca^{2+}-mediated signaling pathways that may affect critical activities as membrane excitability, synaptic plasticity or cytoskeleton reorganization [10]. In fact, tau phosphorylation can be Ca^{2+}-dependent since kinases such as glycogen synthase kinase 3β (GSK3β) increase their activity when cytosolic Ca^{2+} levels are high [11], contributing to the disorder.

Among aging-related neuropathologies, Alzheimer´s disease (AD) is one where both, the presence of NFTs of tau [12,13] and Ca^{2+} dysregulation [5,14–16] have been associated to the neurodegenerative process. AD is characterized by a progressive loss of cognitive abilities that implies an important social impact. In addition, AD presents another histological hallmark such as a large number of senile plaques of the neurotoxic amyloid β-peptide (Aβ). The relationship between Aβ and Ca^{2+} dyshomeostasis has been well documented [17–20] including alteration of Ca^{2+}-regulatory proteins. Thus, it has been reported an inhibitory effect of Aβ in the activity of plasma membrane Ca^{2+}-ATPase (PMCA) [21,22] and an alteration of amyloid β production by SERCA in CHO cells [23]. Ca^{2+}-ATPases are high affinity active transporters that pump Ca^{2+} ions through the plasma membrane (PMCA) or organelle membranes of sarco(endo)plasmic reticulum (SERCA) and secretory pathway (SPCA) (reviewed in [24,25]). Their activities in neurons have been well documented in many important processes depending

Abbreviations: AD, Alzheimer´s disease; Aβ, amyloid-β peptide; MV, membrane vesicles; NFTs, neurofibrillary tangles; PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; SPCA, secretory pathway Ca^{2+}-ATPase

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of Ca^{2+}, in physiological [26–28] and pathological conditions where optimal intracellular Ca^{2+} levels are affected [29–31], including the Aβ-Ca^{2+}-ATPases interactions mentioned above. However, Ca^{2+} regulation trough a functional connection between tau and Ca^{2+}-transporters has not yet been shown. In this work we have used kinetic and immunological assays to look for a functional relationship between tau and Ca^{2+}-ATPases in adult and aged mouse brain, and also in AD-affected human brain, in order to link Ca^2 dyshomeostasis and tau protein in aging and AD.

2. Material and methods

2.1. Materials

Biotinylated-calmodulin was from Calbiochem, Sulfo-NHS biotin from ProteoChem and GenJuice transfection reagent from Novagen. Tau protein used in this work was a recombinant tau protein expressed in *E. coli* containing 2 N-terminal inserts and 4 microtubule binding repeats, that is usually named as tau 42 according to [32]. The anti-SERCA2b and anti-SPCA1 polyclonal antibodies, and cDNA of SERCA2b cloned in the pSV45 and SPCA1a cloned in the pMT2 plasmids were kindly provided by Dr. F. Wuytack, (Katholieke Universiteit Leuven, Belgium). The cDNA of PMCA4b cloned in pMT2 was kindly supplied by Dr. E.E. Strehler (Mayo Clinic, USA). Monoclonal antibodies 5F10 (which recognizes all PMCA isoforms) was from Affinity Bioreagents, anti-β-tubulin from Sigma, AT8 (anti-phospho-tau) from Innogenetics, Tau1 (anti-non-phospho tau) from Chemicon, Tau5 (anti-total tau) from Calbiochem and anti-GAPDH from Santa Cruz Biotechnology. Nine adult Swiss mice (3-month-old) and 9 aged mice (15-month-old) were obtained from the Animal Facility at Universidad de Extremadura (Badajoz, Spain). Adult and aged mice were anaesthetized and decapitated in accordance with the policies on the use of animals in research. Each mouse's brain was sagitally divided into two halves; one half was used to prepare membrane vesicles for biochemical studies and blots and the other half was immediately fixed for histochemical analysis. Twelve samples of human tissues from medial frontal gyrus of brains affected by AD (60-91 years old, Braak stages 5 and 6) and eleven tissue samples from nondemented age-matched controls (60-91 years old) were obtained from the Netherlands Brain Bank (Amsterdam, The Netherlands).

2.2. Preparation of membranes

Membrane vesicles (MV) were prepared from adult or aged mice brains, from postmortem human brain of normal and Alzheimer' disease affected donors or from overexpressing-COS cells, following the protocol described by [33]. Briefly, tissues were homogenized in 10 mM HEPES/KOH pH 7.4, 0.32 M sucrose, 0.5 mM $MgSO_4$, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM 2-mercaptoethanol, and protease inhibitor cocktail solution (Roche). The homogenate was centrifuged for 10 min at 1500×*g*, the resulting supernatant was centrifuged for 45 min at 100 000×*g* and the final pellet was resuspended in 10 mM HEPES/KOH pH 7.4, 0.32 M sucrose and stored at −80 °C until use. The resulting fraction (MV) contained a mixture of plasma and organellar membranes. The protein content was evaluated by the Bradford method [34] using bovine serum albumin as a protein standard.

2.3. Over-expression of PMCA4b, SERCA2b y SPCA1a

COS cells were seeded in 100-mm culture plates at a density of 2.5×10^6 cells/plate and transfected using GenJuice transfection reagent with respective plasmids (see Materials). After incubation for 60 h at 37 °C in the presence of 5% CO_2, cells were harvested from plates for membrane vesicle preparation. High transfection efficiency and high levels of protein overexpression were previously confirmed respect to transfection with empty-plasmid by immunocytochemistry and Western blot (not shown).

2.4. Ca^{2+}-ATPase activity

Total Ca^{2+}-ATPase activities or specific PMCA, SERCA and SPCA activities were determined in MV by using a coupled enzymatic assay at 37 °C as described in [35]. Briefly, the reaction was started with 1 mM ATP, followed by addition of tau when required. Subsequent activity measurements were done after independent additions of 100 nM thapsigargin (to inhibit SERCA activity), 2 μM vanadate (to selectively inhibit PMCA activity) and 3 mM EGTA (to measure Mg^{2+}-ATPase activity). The total Ca^{2+}-ATPase activity was calculated after subtraction of the Mg^{2+}-ATPase activity to the activity obtained in the presence of ATP. The SERCA activity was calculated by subtracting the activity measured in the presence of thapsigargin, which includes PMCA, SPCA, and Mg^{2+}-ATPase activities, from the total (Ca^{2+}-Mg^{2+})-ATPase activity. The SPCA activity was calculated by subtracting the Mg^{2+}-ATPase activity from the activity in the presence of thapsigargin and vanadate; and the PMCA activity was calculated by subtracting the Mg^{2+}-ATPase and SPCA activities from the ATPase activity in the presence of thapsigargin.

2.5. Tau and PMCA biotinylation

Tau biotinylation was performed using a 15:1 molar ratio of biotin to tau (equivalent to 0.33 mol of biotin per lysine). Briefly, a 0.01 mg/ml stock solution of Sulfo-NHS biotin (ProteoChem) was freshly prepared in 50 mM sodium bicarbonate buffer (pH 8.0). Then 0.22 μg of biotin was mixed with 1.8 μg of tau and incubated in 200 μl final volume for 2 h on ice. Free biotin was removed through a 30 kDa cut-off microcone centrifuge filter (Millipore). PMCA biotinylation was carried by mixing 2.7 μg of freshly made biotin (0.1 mg/ml stock solution) with 22.5 μg of purified pig brain PMCA [36] (38:1 molar ratio of biotin to PMCA, equivalent to 0.33 mol of biotin per lysine) in 200 μl final volume. After 2 h incubation on ice free biotin was removed through a 50 kDa cut-off microcone centrifuge filter.

2.6. Overlay binding assay

Purified PMCA and recombinant tau were subjected to a Dot blot or to a 10% SDS-PAGE gel and transferred to nitrocellulose membranes. After blocking with phosphate-buffered saline-1% Tween 20 (PBS-T), containing 2% (w/v) low-fat milk for 1 h at RT and following several washes with PBS-T, membranes were incubated for 2 h at 37 °C with 0.5 μg/μl biotinylated-tau (to detect PMCA) or with 0.3 μg/μl biotinylated-PMCA in PBS-T in presence of 0.5 mM $CaCl_2$ (to detect tau). Following extensive washing steps with PBS-T to remove unbound biotinylated proteins, the membranes were incubated with ExtrAvidin-Peroxidase (1:2000, Sigma) in PBS-T for 1 h at RT. After washing, membranes were developed with ECL substrate and signal was visualized with a Chemidoc™ XRS + Imaging System (BioRad) and quantified with Image Lab™ software 3.0 (BioRad).

2.7. Western blotting

Electrophoresis was performed in 10% (w/v) SDS-polyacrylamide gels according to the method of [37]. Proteins (20 μg) were electrotransferred to nitrocelullose membranes using a Trans-Blot SD semidry system (Bio-Rad). After blocking in Tris-buffered saline (TBS) containing 2% (w/v) of low-fat milk for 1 h, immunostaining reactions were performed by incubating the membranes for 3 h at room temperature or overnight at 4 °C with the following primary antibodies diluted in TBS-0.05% (v/v) Tween 20: AT8 (1:500 dilution), Tau1 (1:1000), 5F10 (1:3000), anti-SERCA2b (1:1000), and anti-SPCA1 (1:1000). Afterwards, membranes were incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies (1:3000, Bio-Rad)

and developed with ECL substrate. The monoclonal anti-GAPDH antibody (1:1000) was used as control of protein loading. Protein bands were visualized as mentioned above.

2.8. Histochemistry

Half of each mouse brain samples were fixed by immersion in 4% (w/v) paraformaldehyde in PBS for 24 h at 4 °C. After rinsing with PBS, tissue was cryo-protected in 10% (w/v) sucrose in PBS for 2 days, and then embedded in 10% (w/v) gelatine, 10% (w/v) sucrose in PBS. The blocks were frozen for 2 min in isopentane, cooled at −70 °C by dry ice, and stored at −80 °C. Serial para-sagittal sections of 20 μm were collected on Super-Frost Plus slides using a cryostat Leica CM1900. Paraffin-embedded human brain tissue sections were cut from blocks at 8 μm thickness in a microtome Microcut H and dried at 37 °C until use.

Cresyl violet staining was carried out with a solution containing 0.01% cresyl violet, 0.01% thionin, 0.6% acetic acid, and 1.25% sodium acetate during 5 min at 37 °C and differentiated with 80% ethanol and 2 drops of acetic acid. Sections were mounted with Eukitt. Alternatively, several sections without staining were covered in FluorSave mounting medium for observation of lipofuscin autofluorescence in a Nikon E600 microscope.

2.9. Immunohistochemistry

Cryostat or deparaffinized tissue sections were permeabilized by immersion in PBS-0.05% (v/v) Triton X-100 (PBS-Tx) for 15 min. Endogenous peroxidase activity was quenched with PBS-0.5% (v/v) H_2O_2 for 45 min. Sections were blocked in a solution containing 0.2% (w/v) gelatin, 0.25% (v/v) Triton X-100 in PBS (PBS-G-Tx) and 0.1 M lysine for 1 h. Afterwards, sections were incubated overnight at room temperature in a humidified chamber with 5F10 (at 1:500 dilution), anti-SERCA2b (1:500), anti-SPCA1 (1:500) or tau5 (1:500) primary antibodies diluted in PBS-G-Tx. Sections were subsequently washed in PBS-Tx and incubated with biotinylated goat anti-mouse or anti-rabbit antibodies (1:200 dilution, Sigma) and then with ExtrAvidin-peroxidase (1:200, Sigma). The immunodetection of peroxidase activity was carried out using 0.03% (w/v) 3,3-diaminobenzidine tetrahydrochloride. Sections were dehydrated and mounted with Eukitt for their observation under the microscope.

2.10. Behavioural tests

Two tests were performed: in the Morris Water Maze [38], the mouse was placed into a small pool (100 cm in diameter, 20 cm high) half-filled with water to 10 cm in depth, which contained an escape platform hidden a few millimetres below the water surface. Colour symbols as visual cues were placed around the pool in plain sight of the animal. After starting the task, mouse swimed around the pool in search of an exit and the time taken to reach the platform was registered. The experiment was performed in 2 steps; in the learning stage, after subsequent trials (1 per day for 6 days), mouse were able to locate the platform increasingly rapidly. In the evaluation stage (day 7), platform was hidden using coloured water, and time required to find the platform, as result of learning and memory relative to visual cues, was recorded. An Objects Recognition Test was also carried out according to [39], in two steps: in the learning stage, the mouse was allowed to explore a box (55×35×20 cm) for 5 min, twice per day for 2 days. After that, 2 equal objects (A1 y A2) were introduced in the box, and the mouse was allowed to explore them for 5 min. In the testing phase, one of the objects was changed to a new object different in shape and colour (B). The mouse was introduced in the box and the time required for recognizing objects (nose close to the object) was registered in order to calculate a Memory Index (MI). The MI was defined as the time required to recognize the new object B related to the time to recognize A and B, in percentage (MI = (tB/tA + tB)×100). Both tests were used for evaluation of spatial learning and memory associated to hippocampal lesions.

2.11. Data processing and statistical analysis

Data are represented as mean ± SE and significant differences determined by an unpaired Student t-test using the SigmaPlot v10 software (SPSS Inc., Chicago, IL). A value of $p \leq 0.01$ was considered statistically significant.

3. Results

3.1. Aging and neurodegeneration-dependent impairment of plasma membrane Ca^{2+}- ATPase activity in the presence of tau protein

In order to determine if tau affects the functional properties of Ca^{2+} pumps and if this modulation is aging and/or neurodegeneration dependent, Ca^{2+}-ATPase activity assays were carried out in the presence of tau on brain membrane vesicles from young adult (3-months-old) and aged (15-months-old) mice (Fig. 1). As shown in Fig. 1A, tau inhibited total Ca^{2+}-ATPase activity in a concentration-dependent manner in both, adult and aged-mice membranes. Solid lines represent a single 3-parameter exponential decay equation fit to the ATPase inhibition data. As can be seen, inhibition of ATPase activity by tau was aging-dependent, reaching maximum inhibition levels of 53.2 ± 3 % and 34 ± 3% at 5 nM tau. The IC_{50} values (concentration producing half-maximum inhibition) were 1.54 nM and 2.62 nM in adult and aged mice membranes, respectively. A more selective assay (described in the Methods section) was performed to measure the sensitivity of each type of Ca^{2+} pump (PMCA, SERCA and SPCA) to tau (Fig. 1B). As shown, only PMCA activity was significantly inhibited by tau, being this inhibition higher in adult than in aged-mice membranes. Experiments were carried out with non-phosphorylated tau (non-P-tau), although similar results were obtained with phosphorylated tau (P-tau) (not shown).

As described elsewhere, tau is a biological marker of AD, a neurodegenerative disorder closely associated to aging. Therefore, functional effects of tau on Ca^{2+}-transporters were also assayed in membrane vesicles from human brains diagnosized with AD and age- (around 76-82 years old) and sex-matched controls. As shown (Fig. 2A), tau also inhibited the Ca^{2+}-ATPase activity in these membranes, in a concentration-dependent manner, reaching a maximum 62.5% inhibition in both, control and AD membranes. Interestingly, the inhibition profile and pattern were similar in both membrane preparations. Specific kinetic assays (Fig. 2B) showed that plasma membrane Ca^{2+}-ATPase activity was exclusively inhibited by tau. Although non P-Tau was used in these experiments, the PMCA activity was equally reduced in the presence of P-tau (results not shown). The effect of tau was also analyzed in membranes from control and AD human brains samples of different age groups (60 and 91 years). As shown (Fig. 3A) in control membranes the maximal inhibitory effect of tau on Ca^{2+}-ATPase activity was lower in the oldest group, as found in adult and aged mice membranes, whereas AD samples exhibited an age-independent inhibition of ATPase activity by tau (Fig. 3B). Similar age-related changes in the regulation of PMCA activity by tau were also found in other species, e.g. chicken at hatching (P0) respect to 7 months-old (Fig. S1).

The specific inhibition of the plasma membrane Ca^{2+}-ATPase activity by tau was confirmed by performing similar kinetic assays with membrane vesicles from COS cells overexpressing PMCA, SERCA or SPCA (Fig. 4A). Only PMCA activity was inhibited by tau, as obtained in mice membranes, being this inhibition dependent on tau concentration (results not shown). Besides, the presence of 7.5 nM tau on PMCA activity measured as a function of Ca^{2+} concentrations did not show any significant effect on the Ca^{2+}-dependence of ATPase activity (Fig. 4B). The Kact values estimated in the presence and absence of tau were about 0.19 μM, albeit the Vmax value was around 41% lower

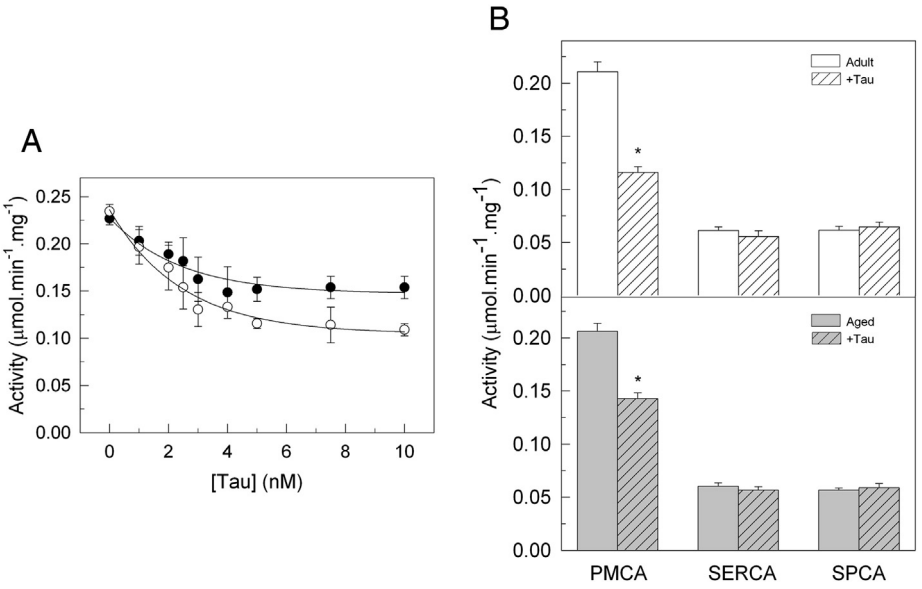

Fig. 1. Effect of tau on Ca^{2+}-ATPase activity in membrane vesicles of adult and aged mice brain. A) The total Ca^{2+}-ATPase activity was measured in 10 μg of MV from adult (○) and aged (●) mice brain as described in Methods, in the presence of the indicated concentrations of tau. B) The total Ca^{2+}-ATPase activity and specific PMCA, SERCA and SPCA activities were assayed in 10 μg of MV from adult (white bars) and aged (grey bars) mice brain in the absence (empty bars) and presence (dashed bars) of 7.5 nM tau. Data are mean ± SE values obtained from 6 experiments performed in 3 adult and 3 aged mice preparations.

in the presence (0.42 ± 0.03 μmol.min^{-1}.mg^{-1}) than in the absence (0.71 ± 0.03 μmol.min^{-1}.mg^{-1}) of tau.

To test for a possible interaction between tau and PMCA we performed binding studies by Dot-blot overlay immunoassays. Thus, purified PMCA, recombinant tau and BSA were spotted onto nitrocellulose membrane and overlaid with tau (Fig. 5A). Later incubation with an anti-tau antibody revealed a positive tau-PMCA binding. Tau and BSA were also spotted as positive and negative controls, respectively. Alternative overlay assays were also done after SDS-PAGE separation of proteins and transfer to nitrocellulose membrane. PMCA and tau were overlaid with biotinylated-PMCA or biotinylated-tau, respectively and interactions were visualized with ExtrAvidin-peroxidase. Fig. 5B clearly shows binding of biotinylated-tau to ~130 kDa PMCA and biotinylated-PMCA to ~60 kDa tau.

3.2. Age-related morphological changes in mice hippocampal regions

In order to analyze and compare brains of adult- and aged-mice used in this study, histological assays were performed. Age-related changes were observed mainly in the hippocampus (Fig. 6), while no significant changes were observed in other areas such as cerebral cortex or cerebellum (results not shown). Cresyl violet staining in hippocampus (Fig. 6A) showed a narrowing of the pyramidal cell layer of the CA1 region of the Ammon´s horn in aged mice compared to the same region in adult mice.

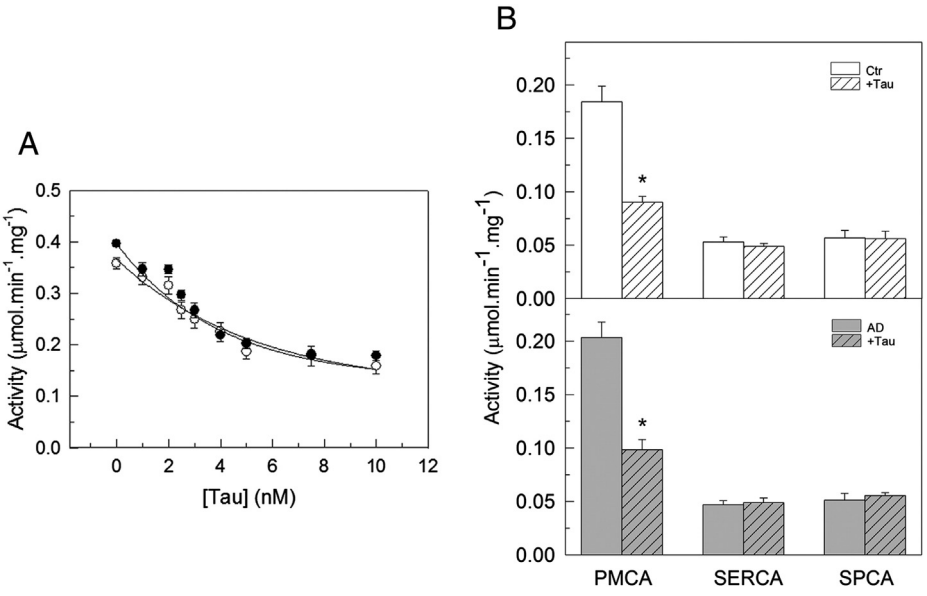

Fig. 2. Effect of tau on Ca^{2+}-ATPase activity in membrane vesicles from Alzheimer's disease (AD) and age-matched control (Ctr) human brain tissues. A) The total Ca^{2+}-ATPase activity was measured in 20 μg of MV from Ctr (○) and AD (●) brain, as described in Methods, in the presence of the indicated concentrations of tau. B) Specific PMCA, SERCA and SPCA activities were assayed in 20 μg of MV from Ctr (white bars) and AD (grey bars) human brain in the absence (empty bars) and presence (dashed bars) of 7.5 nM tau. Data are mean ± SE values obtained from 6 experiments performed in membrane fractions obtained from AD cases ($n = 7$) and age-matched controls ($n = 6$).

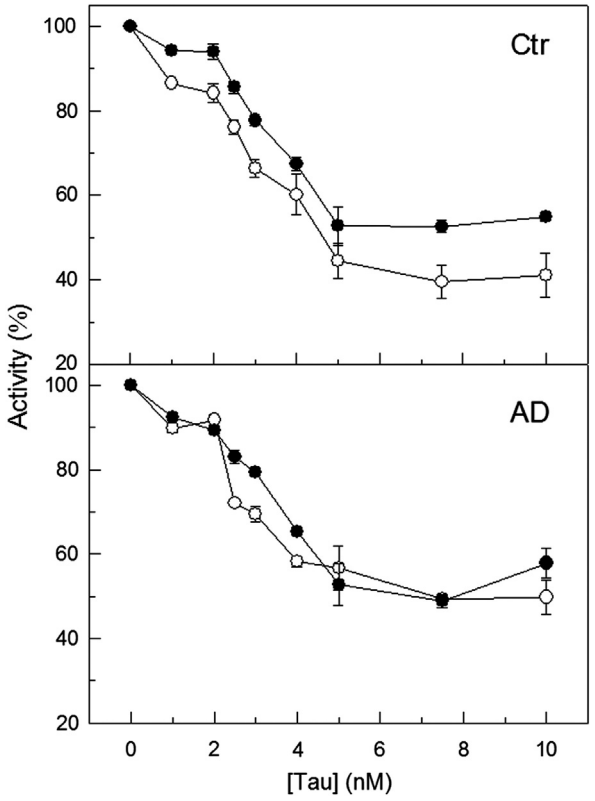

Fig. 3. Effect of tau on Ca^{2+}-ATPase activity in membranes from control (Ctr) and AD affected human brain samples at different ages. The total Ca^{2+}-ATPase activity was measured in 20 μg of MV from Ctr and AD human brain tissue from individuals aged 60 (○) and 91 (●), as described in Methods, in the presence of the indicated concentrations of tau. The 100% activity values correspond to 0.302 ± 0.010 and 0.363 ± 0.010 μmol.min^{-1}.mg^{-1}, for 60 and 91 years old control membranes respectively, and 0.409 ± 0.010 and 0.408 ± 0.010 μmol.min^{-1}.mg^{-1}, for 60 and 91 years old AD membranes respectively. Data represent means ± SE values of 6 experiments performed with different membrane preparations obtained from AD cases (n = 4) and age-matched controls (n = 4).

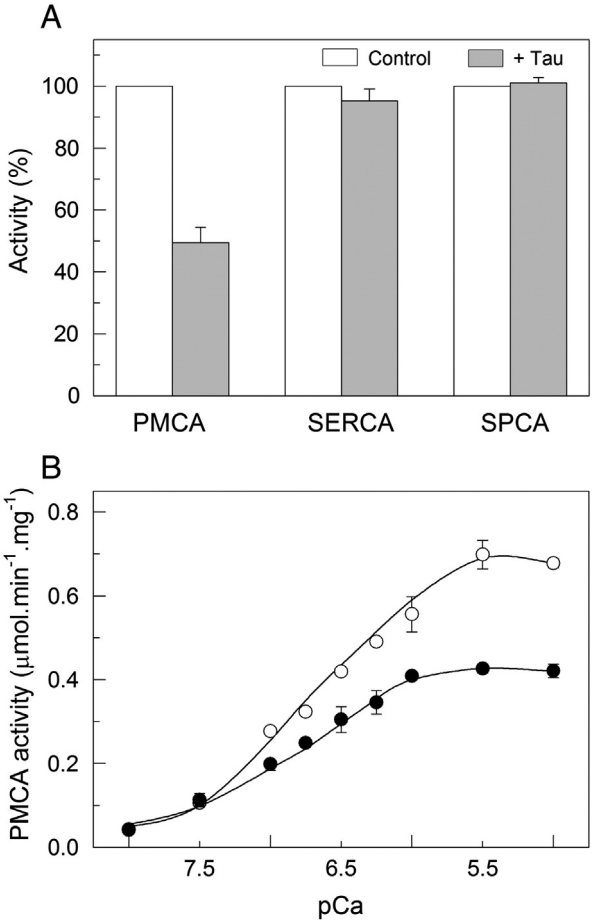

Fig. 4. (A) Effect of tau on Ca^{2+}-ATPase activities of overexpressed hPMCA4b, SERCA2b and SPCA1 in COS cells. The Ca^{2+}-ATPase activity was measured in 10 μg of MV from COS cells overexpressing hPMCA4b, SERCA2b and SPCA1 as described in Methods, in the absence (white bars) and the presence (grey bars) of 7.5 nM tau. Data are mean ± SE values obtained from 5 experiments performed in 4 preparations. The 100% activity values correspond to 0.32 ± 0.015, 0.088 ± 0.010 and 0.33 ± 0.040 μmol.min^{-1}.mg^{-1}, respectively, in the absence of tau. (B) Effect of tau on the Ca^{2+} dependence of ATPase activity of overexpressed hPMCA4b. The activity was measured in 10 μg of membranes, in the absence (○) or presence (●) of 7.5 nM tau, as described in the Methods. Free Ca^{2+} concentration, expressed as pCa, was adjusted by the appropriated addition of BAPTA. Data represent mean ± SE values from four experiments and with two different preparations.

Besides, these cells in CA1 showed a more intensely stained cytoplasm, probably as a result of its acidification during degeneration. Detection of autofluorescence by the presence of lipofuscin pigments, also known as age-pigments, showed a remarkable increase also in the CA1 region of aged mice compared to no signal in adult mice (Fig. 6B). To correlate these histological changes in hippocampus with functional defects, memory tests were performed. Fig. 6C shows results obtained with the Morris Water Maze task, widely used to measure hippocampal-dependent learning and memory. After 6 days of learning stage, the mice behaviour was evaluated on day 7. As shown, aged-mice required more time to reach the platform (Fig. 6C). Additionally, an object recognition test revealed that adult mice spent significantly more time recognizing the novel object, whereas aged mice failed to distinguish between old and new objects, as can be seen by a significant reduction in the memory index (Fig. 6D), confirming hippocampal alterations.

Considering that tau aggregation and Ca^{2+} dyshomeostasis are hallmarks of aging and aging-related neurodegerative disorders, we analyzed in adult and aged mice the expression of tau and the three families of Ca^{2+} pumps, highly involved in the maintenance of optimal cytosolic Ca^{2+} concentration. Immunohistochemical studies were performed in the CA1 region of the hippocampus, the most affected area by aging and AD (Fig. 6E). In adult mice, Tau 5 antibody (that recognizes total tau) showed tau expression in the neuropil of CA1, while in aged mice it was highly concentrated in the soma of pyramidal cells of CA1. Neither histological changes nor differences in tau expression were significantly detected in the CA2 region by aging (results not shown). The localization and expression of the three families of Ca^{2+}-ATPases PMCA, SERCA and SPCA were not altered by aging.

Differences with respect to histology, lipofuscin content and expression of tau and Ca^{2+}-ATPases were also analyzed in control and AD-affected human brains used in this study (Fig. 7). While no significant differences were observed after haematoxylin staining (Fig. 7A), visualization of lipofuscin-associated autofluorescence showed to some extent an increase in AD brains with respect to control tissue (Fig. 7B). Immunohistochemistry assays with Tau5 revealed localization of tau in the cytoplasm of pyramidal cells soma in control groups and in neurofibrillary tangles in AD tissues (see magnifications of tau staining). Concerning to ATPases distribution (Fig. 7C), PMCA protein showed its typical localization in the plasma membrane in pyramidal cells and in the neuropil whereas SERCA2b and SPCA1 proteins were found in the somatic cytoplasm of these cells, in accordance to intracellular ATPases distribution in endoplasmic reticulum and Golgi complexes, respectively (as it has been reported in other species). No significant changes in the expression of these pumps were found in AD-affected tissue.

3.3. Tau and Ca^{2+}-ATPases content in membrane vesicles of adult and aged mice brains and in AD and control human brains

Western blots were used to determine expression levels of non-P-tau and P-tau (Fig. 8A), and Ca^{2+}-ATPases (Fig. 8B) in adult and aged

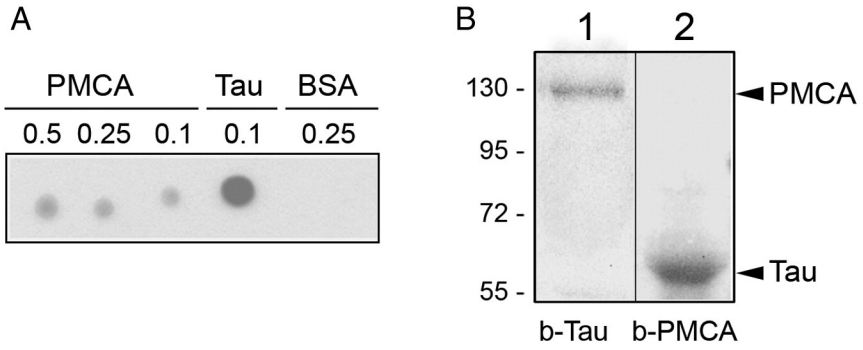

Fig. 5. Tau and PMCA interaction. (A) Dot blot assay: pig brain purified PMCA, recombinant tau and BSA were applied onto a nitrocellulose membrane at the indicated amounts (μg). After blocking and washing, the membrane was incubated for 2 h at 37 °C with 0.18 μg of tau, followed by several washes to remove unbound tau and overnight incubation at 4 °C with Tau5 antibody (1:1000), which binds to total tau. The presence of bound tau was detected by chemiluminiscence. (B) Five μg of pig brain PMCA (lane 1) or 2.5 μg of recombinant tau (lane 2) were subjected to 10% SDS-PAGE and subsequently transferred onto nitrocellulose membranes and incubated with biotinylated-tau (b-Tau) or biotinylated-PMCA (b-PMCA) as indicated in Methods (lanes 1 and 2, respectively). After washing away unbound protein, membranes were incubated with ExtrAvidin-Peroxidase and binding was detected by chemiluminescence.

mice membrane vesicles. These blots showed that expression levels of non P-tau (probed with Tau1 antibody) were almost the same in both adult and aged groups, while aged mice showed a significant increase in P-tau levels (81 ± 6%, probed with AT8 antibody) compared to adult mice. Conversely, the estimation of Ca^{2+}-ATPases content using antibodies against PMCA, SERCA and SPCA (Fig. 8B) showed an age-independent expression of the three families of Ca^{2+} pumps. Expression levels of tau and ATPases were also evaluated by Western blots in membrane vesicles from human brains affected by AD and aged-matched controls (Fig. 9). Levels of non P-tau (Fig. 9A) were about 65.2 ± 10% higher in control than in AD-affected brains, while P-tau content was barely detectable in control samples and highly expressed in AD membranes (85 ± 15% increase in AD with respect to control brain samples). On the other hand, Western blots performed with ATPases specific antibodies (Fig. 9B) showed that PMCA was less abundant in membrane vesicles from AD-affected brain than in matched controls, while expression levels of intracellular ATPases did not change with neurodegeneration. In addition to this, we have seen that there are differences among human control samples regarding the aging status, showing that non P-tau content was independent of age while P-tau levels appear to be increased in the oldest group (Fig. S2). This is in agreement with our previously mentioned results in mice.

4. Discussion

This study describes a novel inhibition of PMCA pump by tau, a protein that in its aberrant state is actively involved in neurodegenerative processes and aging. Besides, the percentage of inhibition seems to be aging- but not neurodegeneration-dependent.

We did not find significant age or neurodegeneration-dependent changes in PMCA activity, neither in SERCA nor in SPCA, but we observed a specific decline of PMCA activity by both, P-tau and non P-tau. This functional effect, which is probably due to intermolecular interactions, as shown by overlay assays, could reflect a protein-protein interaction under physiological but also under pathological conditions. The presence of tau had no significant effect on the Ca^{2+} dependence of PMCA activity, suggesting that tau did not affect the affinity of PMCA for Ca^{2+}. Similarly, other PMCA inhibitors, such as spermine [40] and thioridazine [41] had no effect on the affinity of the ATPase for Ca^{2+}.

Although tau is a cytosolic protein, several studies have reported that it may interact with the plasma membrane [42–45]. We have also seen tau localization close to the plasma membrane in mice and human tissues used in the present work (result not shown). Besides, it has been shown that tau is also present in lipid rafts [46], cell surface microdomains which also associate to PMCA [47–49]. All the aforementioned results support a tau-PMCA interaction. As previously reported PMCA activity is inhibited by Aβ, a peptide that clumps to form the senile plaques, while intracellular Ca^{2+} pumps are not affected [21,22]. Therefore, the plasma membrane Ca^{2+}-ATPase can be functionally modulated by molecular components of the two pathological hallmarks of AD associated with memory impairment [50]. Further studies are being undertaken to go in depth into Aβ, tau and PMCA functional relationship.

It is worth to note that tau showed more affinity for PMCA in adult than in aged mice, as in youngest human membranes when compared to the oldest samples not affected by AD. Thus, a 30-year age difference was a wide enough age gap to find a different inhibitory effect of tau on PMCA in control human brain membranes. However, the PMCA inhibition pattern by tau was similar in the AD group independently of the age. Considering all this together we can conclude that the inhibitory effect of tau on PMCA increases with cognitive decline associated to normal aging, but it seems to be independent of cognitive decline and other neurodegenerative processes linked to AD pathology.

The differential effect of tau with aging could be explained by the fact that either, PMCA or its membrane environment, may present age-related structural changes that make PMCA less sensitive to its inhibition by tau. Several reports have shown that PMCA and its endogenous activator calmodulin are very susceptible to oxidative stress with aging [51,52]. Also, calmodulin content was significantly reduced in AD affected brains [53]. On the other hand, increase of hyperphosphorylated tau protein is linked to the formation of NFTs, which could affect functional PMCA-tau interaction. Besides, membranes from aged mice or from the oldest healthy human samples were less sensitive to tau inhibition. This could be due to the higher P-tau content already present in these tissues, which could be responsible for partial PMCA activity inhibition before exogenous tau was added to the assay.

Intriguingly tau inhibition profile found in AD membranes was similar to that found in control oldest human membranes. This suggests that somehow the molecular mechanisms involved in AD lead to structural and/or conformational changes in the enzyme to a state with similar affinity for tau, independently of age.

Although further experiments are required in order to elucidate the PMCA inhibition mechanisms mediated by tau, we could speculate that PMCA-tau binding involve electrostatic interactions. Indeed, tau is negatively charged in both, N- and C-terminus and positively charged in the microtubule binding domain. It has been reported that tau interacts with the neural plasma membrane through its amino-terminal projection domain, being a mediator of microtubule-plasma membrane interactions [42,43]. Likewise, tau may use the N-terminus to interact trough the plasma membrane with PMCA. On the other hand, given the ionic nature of the transport ligands Ca^{2+} and H^{+}, the substrates Mg^{2+} and

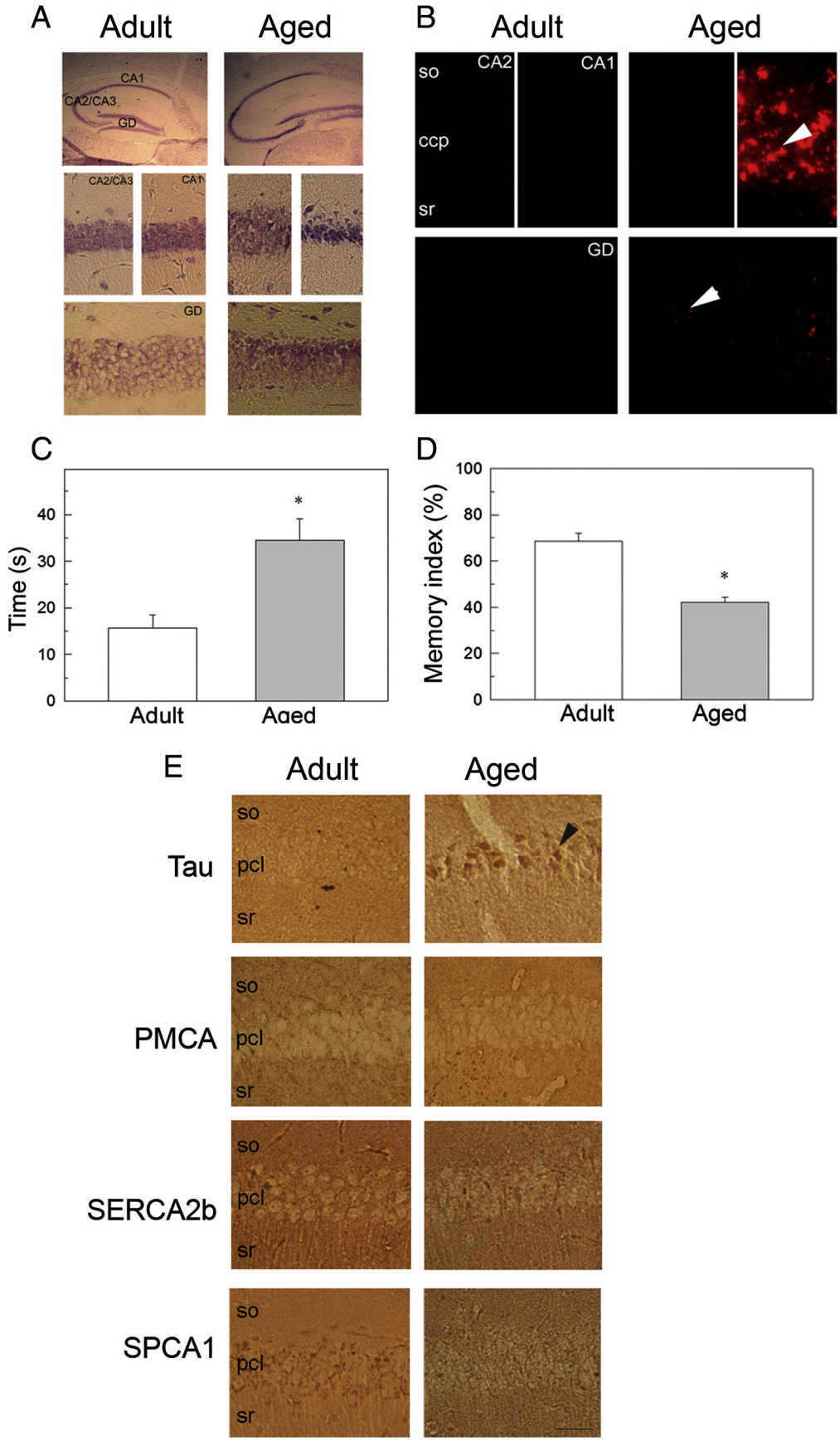

Fig. 6. Effect of aging on the morphology and lipofuscin fluorescence of mice hippocampus and memory tests. Immunolocalization of tau and Ca^{2+}-ATPases. Criostat sections of hipoccampus were analyzed by cresyl violet staining (A) and lipofuscin-associated autofluorescence (B). Morris Water Maze (C) and Objects Recognition (D) tests were performed as indicated in Methods. (E) Hippocampal CA1 sections of adult and aged-mice were stained with Tau5 (that recognizes phosphorylated and non-phosphorylated Tau), 5F10 (that recognizes all PMCA isoforms), a-SERCA2b and a-SPCA1 antibodies. pcl: pyramidal cell layer; so: stratum oriens; sr: stratum radiatus. Scale bar: 15 μm.

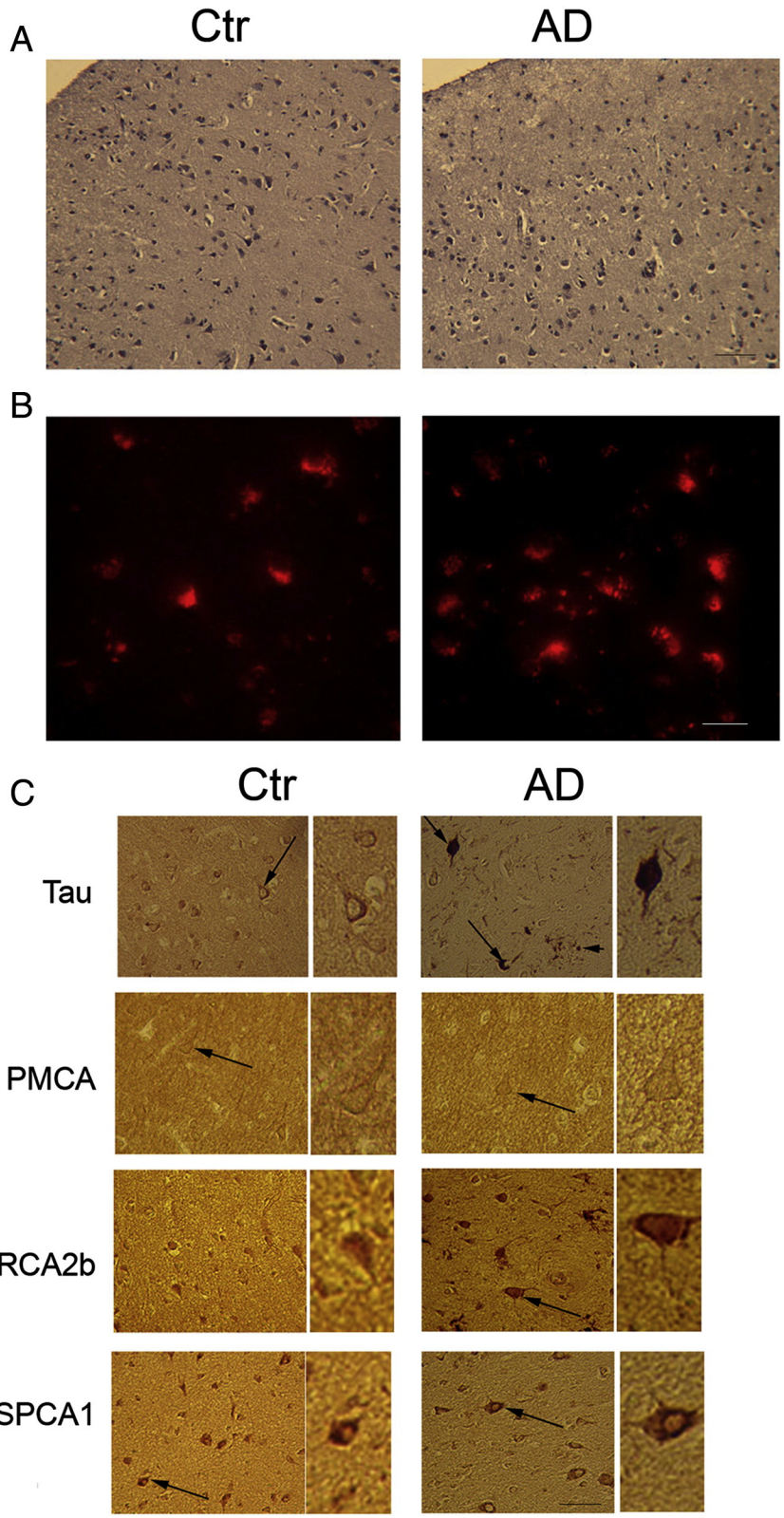

Fig. 7. Effect of neurodegeneration on the morphology and lipofuscin fluorescence and immunolocalization of tau and Ca^{2+}-ATPases in control and Alzheimer´s disease affected human brain. Paraffin sections of medial frontal gyrus of control (Ctr) and AD-affected human brain tissues were analyzed by haematoxylin staining (A) and lipofuscin-associated autofluorescence (B). No significant differences in histology were observed between Ctr and AD brain tissues, while lipofuscin autofluorescence levels were slightly higher in AD. Immunohistochemistries (C) were performed in similar sections with Tau5 (that recognizes total tau), 5F10 (that recognizes all PMCA isoforms), a-SERCA2b and a-SPCA1 antibodies. Scale bar: 15 μm.

ATP, it is highly likely that electrostatic interactions may play a significant role in PMCA reaction. An important manifestation of electrostatic interactions is the ionic strength dependence. Therefore this can be exploited to shed light into the nature of PMCA-tau interaction. If we assume that PMCA-tau binding is trough ionic interactions, the inhibitory effect of tau on PMCA activity would be expected to decrease by

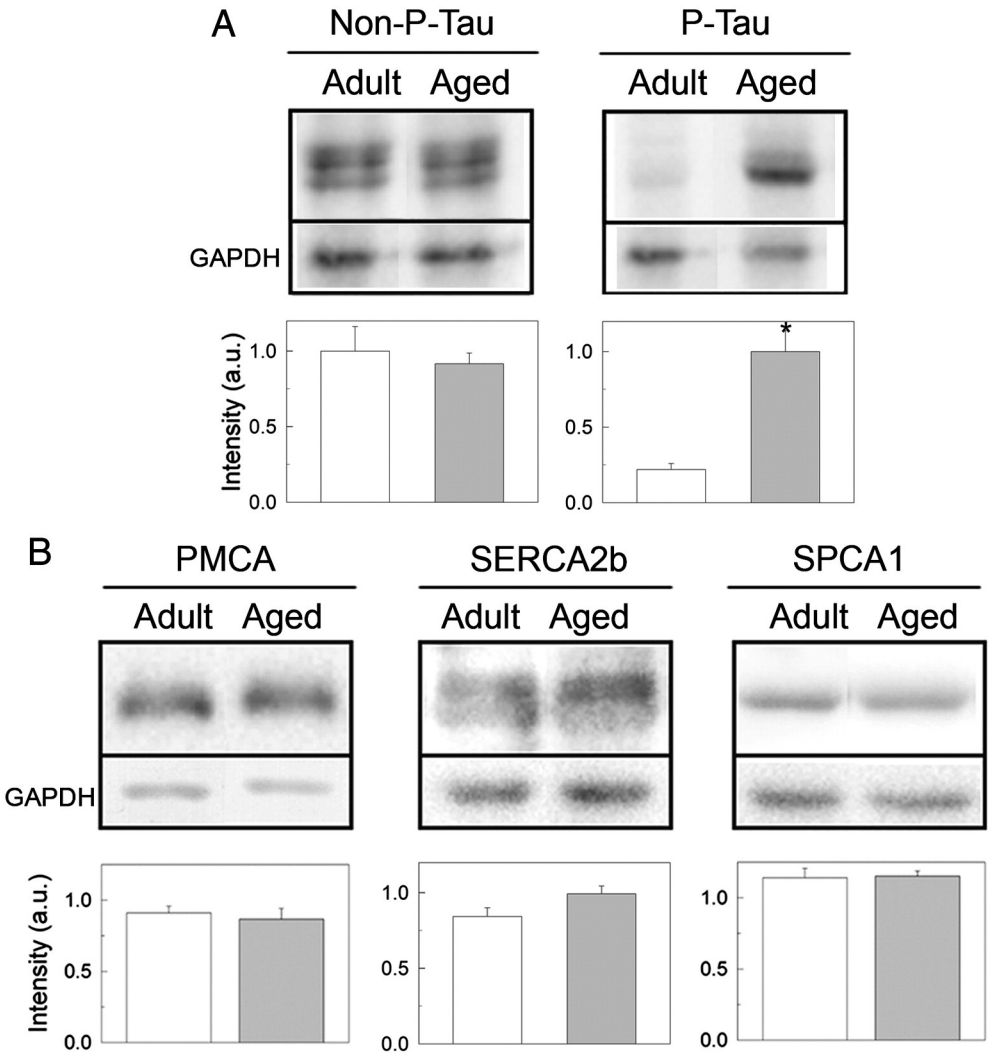

Fig. 8. Levels of non-phosphorylated tau (non-P-Tau), phosphorylated tau (P-Tau), and Ca^{2+}-ATPases (PMCA, SERCA2b and SPCA1) in membrane vesicles of adult and aged mouse brain. MV (20 μg) from adult and aged mice were subjected to Western blot as described in Methods. The blots were probed with Tau-1 and AT8 antibodies to identify the presence of non-P-Tau and P-Tau, respectively (A) and with 5F10, a-SERCA2b, and a-SPCA1 antibodies to detect levels of the 3 families of ATPases (B). Representative Western blots are shown. Protein levels were quantified relative to GAPDH expression to correct for loading differences (data are shown as mean ± SE relative values (arbitrary units) obtained from 2-4 blots performed in 9 different preparations of each group).

increasing the ionic strength of the assay medium and then to be reversible. This is what we observed when activity assays were done at increasing salt concentrations (see Fig. S3). Consequently we may suggest an ionic nature for PMCA-tau interaction. This binding seems to be reversible, since salt can affect the interaction between water and protein side chains or backbone by masking charged residues which are important for PMCA-tau binding. Besides, we suggest that tau binding site on PMCA may be located somewhere at its C-terminal cytosolic domain, and that it may involve the calmodulin binding domain or another domain close to it. Thus, overlay assays with biotynilated-calmodulin on PMCA previously incubated with increasing concentration of tau shows (Fig. S4) that calmodulin binding to PMCA becomes weaker when the pump is previously preincubated with increasing concentrations of tau. Current functional studies are being undertaken in order to elucidate tau inhibitory mechanism.

Neural aging is a physiological condition in life characterized by different processes, including memory and orientation problems. We have confirmed by behavioral tests that aged mice used in this study showed cognitive impairments and histological changes associated with aging, mainly in hippocampus. These results are in agreement with other studies which have reported a fine correlation between memory defects and hippocampus alteration [54,55]. In fact, rats with damage in fimbria or dentate gyrus did not show good results in the Morris test [38,56]. Similar correlation was found using tests in human patients [57]. We used cresyl violet staining as a routine technique to analyze neural tissue [58,59], especially in neurodegenerative processes which involve an acidification of cytoplasm as a consequence of altered cellular ion homeostasis which implies different dye affinity. Results showed hippocampal degeneration of these aged-mice mainly in the pyramidal cell layer of CA1 and dentate gyrus. It has been reported that CA1 cells are especially sensitive to neurotoxic agents decreasing sensitivity in CA2 and CA3 [60–62], which could explain the reduced effect observed in these areas with aging. Dentate gyrus as well showed changes with aging, being an area that is also affected in age-related diseases such as tauopaties [63]. Both regions showed an increase in lipofuscin fluorescence, a hallmark of normal senescence [64,65] and symptomatic of numerous age-related diseases, including AD [64,66]. Changes in tau localization were observed in both mice and human samples, in accordance to the involvement of this protein in aging and AD, albeit we did not observe any evident histological changes or lipofuscin increase in AD samples with respect to age-matched controls. Indeed, tau was localized in the soma of pyramidal cells in aged mice, and in neurofibrillary tangles in AD-affected human samples. A differential distribution of tau in neurons has been described depending on its phosphorylation

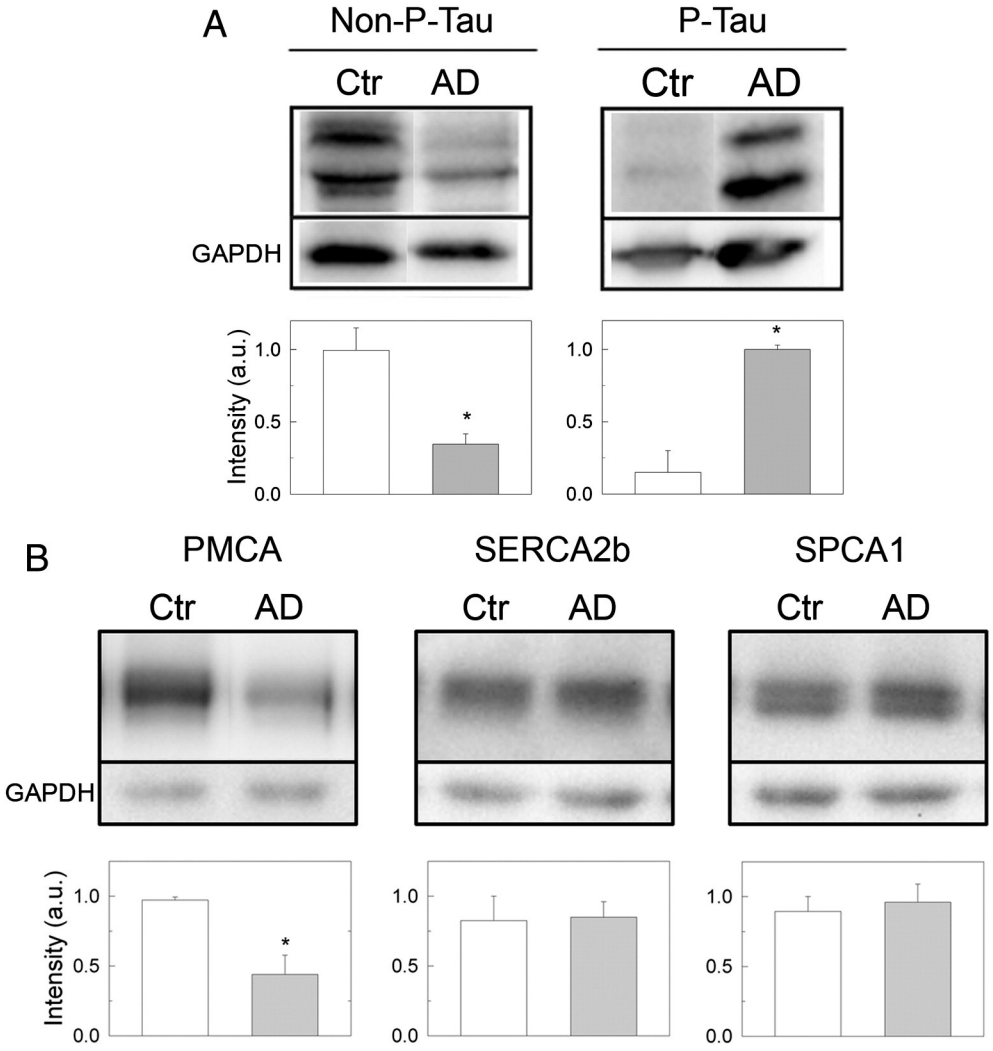

Fig. 9. Levels of non-phosphorylated tau (non-P-Tau), phosphorylated tau (P-Tau), and Ca^{2+}-ATPases (PMCA, SERCA2b and SPCA1) in membrane vesicles of control (Ctr) and Alzheimer´s disease-affected human brain (AD). MV (20 μg) were subjected to Western blot as described in Methods. The membranes were probed with Tau-1 and AT8 antibodies to identify the presence of non-P-Tau and P-Tau, respectively (A) and with 5F10, a-SERCA2b, and a-SPCA1 antibodies to detect levels of the 3 families of ATPases (B). Representative Western blots are shown. Quantification of proteins relative to GAPDH levels are shown as mean ± SE relative values (arbitrary units) obtained from 2-4 experiments performed in 9 different preparations of each group.

state, being dephosphorylated tau located primarily in distal regions of the axon, and phosphorylated tau being present in the somatodendritic compartment [67], which is in accordance with our results.

As we have shown, expression levels of non P-tau seem to be age-independent, both in mice and human control samples. However, P-tau levels increased with aging and neurodegeneration. Similar results have been reported by [68] in aged mice and could be related to the reported accumulation of abnormally hyperphosphorylated tau in AD human brain [69,70] that could be either associated to age-related decrease in levels and activity of protein phosphatase 2a [71–73] and see reviews [74,75] or to an increase in GSK3β activity [76–78]. In fact, experiments with mice overexpressing GSK-3β have shown an increase of phosphorylated tau especially in hippocampus [79], the region where we observed a more concentrated localization of tau linked to age-related degeneration in mice. Western blots revealed that AD samples contained significant levels of P-tau whereas only traces of P-tau were detected in membranes from control cases, as previously found in [80]. Similar profiles were also observed by [81] and [82].

Emerging studies have associated aging related disorders with increased resting levels of intracellular Ca^{2+} that lead to neuronal loss [83,84]; see review [85]. However, evidences are mainly focused in Ca^{2+} influx trough the plasma membrane (from NMDAR, L-VGCC or SOC channels [86]) or the endoplasmic reticulum (from InsP3R and RyanR (reviewed in [87,88])), but do not pay particular attention to Ca^{2+} extrusion mechanisms. Considering the key role that Ca^{2+} pumps play in Ca^{2+} extrusion mechanisms due to their high Ca^{2+} affinity, we analyzed Ca^{2+}-pumps with respect to neurodegeneration in aging mice or to neuropathology in AD human tissue. However our data did not show differences in terms of distribution although a specific reduction in PMCA protein levels associated to neurodegeneration was observed in AD-affected membranes, and interestingly, only the Ca^{2+}-ATPase activity coupled to PMCA could be inhibited by tau in a concentration-dependent manner.

5. Conclusions

In summary, our data provide the first evidence that tau protein acts as a PMCA inhibitor by directly interacting with this Ca^{2+}-transporter. Besides, the inhibitory effect of tau on PMCA activity is aging dependent in animal species and control human membranes but not in AD-affected human membranes. Overall, these results lead to consider PMCA-tau an interaction that deregulates intracellular Ca^{2+} levels. Further work needs to be done in order to elucidate these differences, since looking for molecular sites of interactions could make PMCA a target for

development of therapeutic approaches to AD and other tau-related pathologies.

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Transparency Document

The Transparency Document associated with this article can be found, in the online version.

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