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Title: Role of PrP^C expression in tau protein levels and phosphorylation in Alzheimer's disease evolution.

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ABSTRACT

Alzheimer's disease (AD) is characterized by the presence of amyloid plaques mainly consisting of hydrophobic β-amyloid peptide (Aβ) aggregates and neurofibrillary tangles (NFTs) composed principally of hyperphosphorylated tau. Aβ oligomers have been described as the earliest effectors to negatively affect synaptic structure and plasticity in the affected brains, and cellular prion protein (PrP^C) has been proposed as receptor for these oligomers.

The most widely accepted theory holds that the toxic effects of Aβ are upstream of change in tau, a neuronal microtubule-associated protein that promotes the polymerization and stabilization of microtubules. However, tau is considered decisive for the progression of neurodegeneration, and indeed tau pathology correlates well with clinical symptoms such as dementia. Different pathways can lead to abnormal phosphorylation, and, as a consequence, tau aggregates into Paired Helical Filaments (PHF) and later on into NFTs.

Reported data suggest a regulatory tendency of PrP^C expression in the development of AD, and a putative relationship between PrP^C and tau processing is emerging. However the role of tau/PrP^C interaction in AD is poorly understood.

In this study we show increased susceptibility to Aβ derived diffusible ligands (ADDLs) in neuronal primary cultures from PrP^C knock-out mice, compared to wild-type, which correlates with increased tau expression. Moreover, we found increased PrP^C expression that paralleled with tau at early ages in an AD murine model, and in early Braak stages of AD in affected individuals. Taken together, these results suggest a protective role for PrP^C in AD by down-regulating tau expression, and they point to this protein as being crucial in the molecular events that lead to neurodegeneration in AD.

Keywords: Alzheimer's disease, microtubule-associated protein tau, cellular prion protein, Aβ-oligomers.

INTRODUCTION

The cellular prion protein (PrP^{C}) is a glycosylphosphatidylinositol (GPI)-anchored cell surface protein highly expressed by neurons and glial cells in the adult central nervous system (CNS) [1-3]. Its physiological functions are poorly understood but a relation between the protein and neuroprotection has been reported [4]. The abnormal processing of PrP^{C} gives rise to a proteinase-k-resistant misfolded isoform termed PrP^{SC}, which is the etiologic agent of transmissible spongiform encephalopathies (TSEs) [5]. These encephalopathies are characterized by profound histological changes, including extensive neuronal death, reactive gliosis, and neuroinflammation, together with the extracellular accumulation of aggregated PrP^{SC} in affected brains [6,7].

As with prionopathies, the most prevalent neurodegenerative dementia, Alzheimer's disease (AD), presents extracellular deposition of an abnormally processed protein, the amyloid precursor protein (APP). Individuals suffering AD are characterized by a progressive cognitive decline and behavioral changes, and the main neuropathological hallmarks include the presence of senile plaques, enriched in β-amyloid (Aβ) peptide, and intracellular hyperphosphorylated tau protein which aggregates in neurofibrillary tangles (NFTs) (e.g., [8,9]). Tau is a neuronal microtubule-associated protein that promotes the polymerization and stabilization of microtubules [10] under the regulatory control of several kinases and phosphatases. In this sense, glycogen synthase kinase 3 (GSK3β) and the cyclin-dependent kinase 5 (Cdk5) are the main kinases implicated in the phosphorylation of some tau epitopes observed in AD [11-14]. When hyperphosphorylated, tau detaches from microtubules, leading to increased instability, impaired axonal transport, and profound deficits in synaptic function. In fact, today it is recognized that some of the kinases misregulated in AD (e.g., GSK3β) affect both Aβ production [15] and tau hyperphosphorylation [16].

Several TSEs show an abnormal processing of tau protein, in parallel to the presence of PrP^{SC}. In fact, increased levels of tau protein have been detected in the cerebrospinal fluid (CSF) and serum of Creutzfeldt-Jakob's disease (CJD) patients [17,18]. In parallel, Wang and coworkers have described several changes in expression and phosphorylation levels of tau in infected hamsters. This occurs in parallel with the alteration of Cdk5 and GSK3 transcription levels [19]. In addition, other studies have

associated GSK3β and Cdk5 activity with neurotoxic processes induced by the $PrP_{(106-126)}$ peptide, a synthetic peptide widely used as a model of neurotoxicity in prion research (e.g., [20,21]). In parallel, PrP^C may bind with several intracellular proteins including tubulin [22,23]. And more relevantly, Wang *et al.* described physical interaction between tau and PrP^C hypothesizing that the presence of phosphorylated tau in some TSEs is the result of a differential affinity between tau and the N-terminal octarepeat (OR) domain of PrP^C [24]. Moreover, the PrP^C mutation associated with Gerstmann-Sträussler-Scheinker syndrome (GSS), *P102L*, resulting in an increased number of ORs, shows greater tau interaction [25]. Taken together, these observations suggest a role for PrP^C in tau changes related to TSEs.

The widely accepted amyloid cascade hypothesis posits that the deposition of Aβ in the brain parenchyma triggers a sequence of events leading to tau dysfunction [26,27]. In this sense, Lambert and coworkers attributed higher toxic potential to Aβ-derived diffusible ligands (ADDLs) than to insoluble forms of Aβ [28]. ADDL treatment is proapoptotic, inducing synaptic alterations and interfering with long-term potentiation [29,30]. Lauren and coworkers point to PrP^C as being responsible for this phenomenon [31]. In this sense, transgenic AD mouse models require endogenous PrP^C to induce several deficits in learning and memory [32]. The effect of deleting PrP^C on mouse transgene phenotypes is similar to that of deleting tau expression, with a lessening of Aβ neuronal dysfunction in both cases [33]. At nanomolar concentrations, neurotoxicity of ADDLs depends on membrane receptors and in turn on the kinase Fyn, which induces the recruitment of oligomers in 'lipid rafts' of plasma membrane [34]. Both PrP^C and tau are localized in these membrane microdomains [35]. In parallel, antibody-mediated aggregation of PrP^C on the cellular surface activates Fyn and other kinases *in vitro* initiating a signaling transduction cascade that induces tau phosphorylation in tyr^{18}, an epitope implicated in AD [36-38]. In this scenario, several studies have suggested that oligomeric Aβ cytotoxicity in AD depends on the level of tau [39] and is mediated by PrP^C, which in turn promotes the activation of Fyn and the consequent hyperphosphorylated tau [40-42].

Additionally, several studies have reported changes in PrP^C expression in aging and in the development of AD. Higher PrP^C levels have been described in old healthy mice compared with younger animals [43]. Moreover, decreased expression of PrP^C in AD cases [43] with increased levels at the start of the disease and in other neurodegenerative disorders [44,45] has been also reported. A recent study has

shown regulation of Aβ production by the prion protein [46]. Taken together, these observations suggest a protective role for PrP^C in AD reinforcing the hypothesis regarding a neuroprotective function of the protein [4]. Taking into account the physical interaction between PrP^C and both ADDL and tau, we investigated the role of the tau/PrP^C relationship in the phatogenic cascade of AD, and our results indicate that PrP^C plays an important role in the molecular events that lead to neurodegeneration in AD, by regulating total tau levels.

MATERIAL AND METHODS

Mouse strains and genotyping

We used four transgenic mouse lines at different ages. In addition, embryos from pregnant females of the different genotypes were also used. Mating day was considered to be embryonic day 0 (E0) and the offspring was genotyped according to the genotype of the crossed progenitor.

Prnp$^{0/0}$ mice bred from a C57BL6J/129, Ola/Sv background [47] were purchased from EMMA (Monterotondo, Italy). We backcrossed to C57BL/6J for at least 15 generations to obtain *Prnp*$^{0/0}$ and control littermates *Prnp*$^{+/+}$. 9 adult mice and 17 pregnant females were used in this study. Specific primers to *Prnp* genotyping were designed in our laboratory based on the original P3 and P10 primers as described [47]: neo: 5'-gccttctatcgccttcttgac-3'; 3'NCnew: 5'-gctacaggtggataacccctc-3' and P10new: 5'-cataatcagtggaacaagccc-3'. The 40 cycling conditions were 45" 95º C, 45" 62º C, and 1' 72º C, followed by a final extension at 72º C for 5 minutes.

Prnp overexpressing mice (Tg20) were purchased from EMMA. They were generated from *Prnp*$^{0/0}$ mice as described by Marek *et al.* [48]. 11 pregnant females were used in this study. For Tg20 mice, the transgene was detected by using primers specific to the Tg20 alleles 5'-caaccgacgtgaagcattctgccta-3' and 5'-cctgggactccttctggtaccgggtgacgc-3' as indicated [49].

APP/PS1 transgenic mouse line expresses human APP Swedish, and presenilin 1 deleted in exon 9 mutations, and is named B6.Cg-Tg (APPSwe, PSEN1dE9). These mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). We used 13 transgenic mice and non-transgenic littermates as

controls for each age. Also, 14 pregnant females were used in this study. Mice were genotyped as by Jankowsky [50] using three primers; one antisense primer matching sequence within PrP^C (5'-gtggataccccctcccccagcctagacc-3'), one sense primer specific to the transgene (PS1: 5'-caggtggtggagcaagatg-3' or APP: 5'-ccgagatctctgaagtgaagatggatg-3'), and a third sense primer specific to the genomic PrP^C (5'-cctctttgtgactatgtggactgatgtcgg-3').

TgTP6.3 mouse line is a transgenic mouse carrying a tau-tagged green fluorescence protein (GFP) transgene generated by [51]. Mice carrying the tau-GFP transgene were identified by fluorescence microscopy of tail tissue. 3 adult mice were used in this study.

All experiments were performed under the guidelines and protocols of the Ethical Committee for Animal Experimentation (CEEA) at the University of Barcelona, and the protocol for the use of animals in this study was reviewed and approved by the CEEA at the University of Barcelona (CEEA approval# 115/11).

Human cases

Human cases comprise 6 non-AD and 31 AD postmortem brains from the Institute of Neuropathology and University of Barcelona/Hospital Clinic Brain Banks. All brains were obtained following the Code of Ethics of the World Medical Association and the protocols of the local Ethical Committee. Basic patient data are shown in Table 1. Cases with and without clinical neurological disease were processed in the same way following the same sampling and staining protocols. At autopsy, half of each brain was fixed in 10% buffered formalin, while the other half was cut in coronal sections 1 cm thick, frozen on dry ice, and stored at -80°C until use. In addition, 2 mm thick samples of the cerebral isocortex, cingulum, hippocampus and entorhinal cortex, and brainstem were fixed with phosphate buffered 4% paraformaldehyde for 24 hours, cryoprotected with 30% sucrose, frozen on dry ice, and stored at -80°C until use.

Following neuropathological examination, AD cases were categorized as stages I to VI of Braak and Braak [52,8]. Healthy cases (non-AD) did not show neurological or metabolic disease, and the neuropathological examination, carried out in similar regions and with the same methods as in AD cases,

did not show lesions. In particular, no amyloid or tau deposits were seen in the regions examined. The group is represented by middle-aged adults because most individuals over 65 years old might have preclinical signs of AD [53].

Primary embryonic neuronal cultures

E15-16 mouse embryo brains were dissected and washed in ice-cold 0.1 M phosphate-buffered saline (PBS) containing 6.5 mg/ml glucose. The meninges were removed and the cortical lobes isolated. Tissue pieces were trypsinized for 15 minutes at 37ºC. After addition of horse serum and then centrifugation, cells were dissociated by trituration in 0.1 M PBS containing 0.025% DNase with a polished pipette (all from Sigma, MO, USA). Dissociated cells were plated at ~3,000 cells/mm^2 on plates (Nunc, Denmark) coated with poly-D-lysine (Sigma). The culture medium was Neurobasal supplemented with 2 mM glutamine, 6.5 mg/ml glucose, antibiotics (Pen./Strept.), 5% horse serum, and B27 (Invitrogen-Life Technologies, Belgium). After 72 hours, AraC 5 μM (Cytosine β-D-arabinofuranosidehydrochloride (Sigma) was added for 48 hours to inhibit the growth of dividing non-neuronal cells. Cultures were used after 7 days *in vitro*.

Treatment of cortical cultures with Aβ peptides

Aβ1–42 peptide was purchased from Bachem (Bubendorf, Switzerland). Different aliquots of Aβ1–42 were prepared as described previously [54,55] and stored desiccated at -80ºC. To obtain soluble oligomers (ADDLs), a working solution of 100 μM was prepared in culture medium and incubated at 4ºC for 24 hours to treat the cultures with a final concentration of 10 μM. For the fibril-forming conditions we chose the acidic pH method using 10 mM HCl with 24 hours incubation at 37ºC, and cultures were treated with a final concentration of 10 μM.

Peptide-treated cells were scraped in homogenization buffer containing 50 mM Hepes, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, and 1x protease inhibitor cocktail (Roche Diagnostic, Switzerland) and 1 μM okadaic acid (Merck Millipore, MA, USA), 0.1 M sodium fluoride, 10 mM sodium pyrophosphate and 1 mM sodium orthovanadate (Sigma) as phosphatase inhibitors.

Transmission electron microscopy (TEM) procedures

For TEM, peptide solutions incubated for 24 hours for each condition, both in culture medium at 4ºC and in 10 mM HCl at 37ºC, were fixed to carbon-forward-coated copper supports. Negative staining was performed using a 2% uranyl acetate stain (pH 7.4) (Sigma), after which samples were placed in silica-based desiccant for a minimum of 2 hours. Finally, we proceeded to TEM observation using a Leica microscope (Wetzlar, Germany) at the Electron Microscopy Service, University of Barcelona, Barcelona, Spain.

Lentiviral vector production

Lentiviral particles were produced by transient transfection of 293FT cells with Lipofectamine 2000 (Invitrogen-Life Technologies) using Mission shRNA prion protein NM_011170 (Sigma), the second-generation packaging construct psPAX2 (Tronolab, Switzerland), and the envelope plasmid pMD2G (Tronolab). 293FT cells were cultured in Dulbecco-modified Eagle medium (DMEM) (Invitrogen-Life Technologies) supplemented with 10% fetal calf serum (FCS) (Invitrogen-Life Technologies) and without antibiotics before transfection. Medium was changed and supplemented with antibiotics after 6 hours. Vector supernatants containing viral particles were harvested approximately 48 and 72 hours later and concentrated by ultracentrifugation (2 hours at 26,000*rpm* at 4°C, in an Optima LK98, SW-28 rotor).

Western blotting techniques.

Soluble extract from human hippocampal samples, mouse cortex brains, and cultured cells were processed for western blot. The collected samples were homogenized in lysis buffer: 50 mM Hepes, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100 or 100 mM Tris pH 7.0, 100 mM NaCl, 10 mM EDTA, 0.5% NP-40, and 0.5% sodium deoxycolate, and in both cases with supplemental 1x protease inhibitor cocktail and phosphatase inhibitors as indicated above. After this, samples were centrifuged at 13,000 x *g* for 20 minutes at 4ºC. The resulting supernatant was normalized for protein content using BCA kit (Pierce Biotechnology, USA). Cell extracts were boiled in Laemmli sample buffer [56] at 96ºC for 5 minutes, followed by 10% SDS-PAGE electrophoresis, and they were then electrotransferred to nitrocellulose membranes for 1 hour at 4ºC. Membranes were blocked with 5%

non-fat milk in 0.1 M Tris-buffered saline (pH 7.4) for 2 hours and incubated overnight in 0.5% blocking solution containing primary antibodies. After incubation with peroxidase-tagged secondary antibodies (1:2000 diluted), membranes were revealed with the ECL-plus chemiluminescence western blot kit (Amersham-GE Healthcare, UK).

In our experiments, each nitrocellulose membrane was used to detect consecutive antigens. To perform this sequential incubation, membranes were incubated in 25 ml of stripping solution (2% SDS, 62.5 mM Tris pH 6.8 and 100 mM 2-mercaptoethanol) for 30 minutes at 65ºC and then extensively washed before reincubation with blocking buffer and antibodies for re-blotting. Some samples were dephosphorylated using γ-phosphatase (New England Biolabs, MA, USA) as described [57].

Primary antibodies

Several antibodies were used for immunohistochemistry and western blot. To detect amyloid species we used 6E10 (Covance, NJ, USA), which specifically recognizes the first 16 amino acids of the Aβ peptide. Monoclonal anti-PrP 6H4 (Prionics, Zurich, Switzerland) was used to determine PrP^{C} levels. Different phosphorylation sites of tau were checked with anti-ptau thr^{181} (Signalway Antibody, MA, USA), anti-ptau $ser^{199/202}$ (pSpS) (Invitrogen-Life Technologies), anti-ptau ser^{202} (AT8) (Thermo Scientific, MA, USA) and anti-ptau $ser^{396/404}$ (PHF-1), a gift from Peter Davies (Albert Einstein College of Medicine, Bronx, New York, USA). Total levels of the tau protein were checked with monoclonal tau 5 antibody (Invitrogen-Life Technologies). The monoclonal anti-β-actin antibody (Merck Millipore) was used as internal control in western blots.

Aβ(1-42) quantification by ELISA

The brain homogenates obtained as described above were diluted 1:50 in albumin-PBS-Tween buffer (5% Bovine Serum Albumin, 0.03% Tween-20 in PBS) before centrifugation (16,000 x *g* for 20 minutes 4ºC), and the supernatant was used shortly after preparation. Brain amyloid burden was measured using the Aβ42 Human ELISA kit (Invitrogen-Life Technologies) following the manufacturer's protocol. Microwell plate absorbance at 450 nm was read with Opsys MR microplate reader (Dynex Technologies, VA, USA).

RT-qPCR

Quantitative real time PCR was performed on total RNA extracted with mirVana's isolation kit (Ambion, TX, USA) from the cortex of analyzed mice or from cultured cells. Purified RNAs were used to generate the corresponding cDNAs, which served as PCR templates for mRNA quantification. The primers used in this study for RT-qPCR validation were: (5'-ccccctaagtcaccatcagctagt-3') and (5'-cactttgctcaggtccaccggc-3) for mouse tau [58], (5'-agtcgttgccaaaatggatca-3') and (5'-aaaaaccaacctcaagcatgtgg-3') for mouse PrP^C [59].

PCR amplification and detection were performed with the ROCHE LightCycler 480 detector, using 2x SYBR GREEN Master Mix (Roche) as reagent, following the manufacturer's instructions. The reaction profile was denaturation-activation cycle (95ºC for 10 minutes) followed by 40 cycles of denaturation-annealing-extension (95ºC for 10 seconds, 55ºC for 15 seconds and 72ºC for 20 seconds). mRNA levels were calculated using the LightCycler 480 software. Data were analyzed with SDS 1.9.1 Software (Applied Biosystems, USA) following the $2^{-\Delta\Delta CT}$ method of Applied Biosystems [60]. The results were normalized by the expression levels of the housekeeping gene, *gapdh* (5'-aggtcggtgtgaacggatttg-3') and (5'-tgtagaccatgtagttgaggtca-3'), which were quantified simultaneously with the target gene [61].

Immunohistochemical procedures

For immunohistochemistry, postnatal mice were anesthetized and perfused with 4% paraformaldehyde (PFA) in PBS pH 7.4. After perfusion, brains were removed and post-fixed in the same solution for 12 hours, cryoprotected in 30% sucrose in PBS, and sectioned (30 μm) on a freezing microtome (Leica). They were then processed for the immunohistochemical detection of Aβ, tau, and PrP^C, respectively, following an immunoperoxidase protocol. Briefly, free-floating sections from different developmental stages were processed in parallel. Sections were rinsed in 0.1 M PBS, and endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide (H_2O_2) and 10% methanol dissolved in 0.1 M PBS. For amyloid plaque detection, tissue was incubated in 70% formic acid for 30 minutes. After extensive rinsing, sections were incubated in 0.1 M PBS containing 0.2% gelatin, 10% normal goat serum, 0.2% glycine, and 0.2% Triton X-100, for 1 hour at room temperature. Afterwards, sections were incubated for 36 hours at 4°C with primary antibodies. After that, sections were incubated

with the appropriate secondary biotinylated antibodies (2 hours, 1:200 diluted) and Streptavidin-Horseradish peroxidase complex (2 hours, 1:400 diluted). Peroxidase activity was revealed with 0.025% diaminobenzidine (DAB) and 0.003% H_2O_2. After rinsing, sections were mounted onto slides and dehydrated, and coverslipped with Eukitt™ (Merck). Immunohistochemical controls, including omission of the primary antibody or its replacement by normal serum, were devoid of staining. Images were obtained using an Olympus BX61 microscope equipped with cooled DP72L digital camera.

Densitometry and statistical processing

For the quantification of western blots, developed films were scanned at 2,400 x 2,400 dpi (i800 MICROTEK high quality film scanner, Hsinchu, Taiwan) and the densitometric analysis was performed using Quantity One Image Software Analysis (Biorad, Barcelona, Spain). Statistical analysis of the resulting data was performed using STATGRAPHICS plus 5.1 and Origin 8™ programs using ANOVA test. Asterisks in the histograms indicate the following *p* values of significance: (*) $p<0.05$; (**) $p<0.01$.

RESULTS

Increased susceptibility of tau phosphorylation to ADDLs in cortical cultures lacking PrP^C

In vitro ADDL treatment led to hyperphosphorylated tau in several residues as also occurs in the brain parenchyma of AD patients [11,62]. Indeed, although there were some contradictory results (i.e., ser^{404} residue), these tau phospho-epitopes have been largely reported [63,64].

Initially we aimed to determine whether acute changes of *Prnp* expression might affect cellular tau levels before analyzing levels of phosphorylated tau (ptau). Thus, primary cultures from $Prnp^{+/+}$, $Prnp^{0/0}$, and Tg20 embryos were prepared. In parallel, primary cultures from $Prnp^{+/+}$ embryos were treated with siRNA-PrP^C. Western blot analysis indicated that tau levels remained constant in all three genotypes and under silencing PrP^C expression (data not shown). In addition, further *MAPT* analysis of previously obtained microarray results using neuroblastoma N2a cells [65] reinforced these results showing constant tau levels in this cell line independently of PrP^C expression [65].

Next, to investigate whether PrP^C expression modulates the phosphorylation of tau induced by ADDL treatment, primary cortical neurons from *Prnp*$^{0/0}$, *Prnp*$^{+/+}$, and Tg20 embryos were incubated with 10 μM ADDLs or vehicle alone for 3, 6, and 24 hours after 7 DIV (Fig. 1 and Suppl. Fig 1d). In all experiments, peptide aggregation prior to treatment was corroborated both by western blot (Suppl. Fig. 1a) and by Transmission Electron Microscopy (TEM) observation (Suppl. Fig. 1b) (see also Material and methods for details). Densitometric analysis of revealed films demonstrated that *Prnp*$^{0/0}$ cortical neurons incubated with ADDLs showed an increase in tau phosphorylation (phosho-tau/total tau ratio) in all residues studied (ser$^{396/404}$, ser^{202}/thr^{205}, and thr^{181}) (Fig. 1a-c) at 3 and especially at 6 hours. In contrast, no significant changes in tau phosphorylation were found in cultured *Prnp*$^{+/+}$ cells treated with ADDLs for 3 and 6 hours (Fig. 1a-c). In addition, in cultured neurons from Tg20 mice incubated with ADDLs, there was no significant increase in phosphorylation of the tau epitopes (ser$^{396/404}$, ser^{202}/thr^{205}, and thr^{181}) when compared with *Prnp*$^{+/+}$ cultures (Fig. 1b,c).

Synergistic effect of absence of PrP^C and ADDL treatment in tau protein levels in cultured neurons

Next we aimed to determine whether there is an increase in tau protein levels after ADDL treatment and its putative relationship with the presence or absence of PrP^C. Results showed that after incubation of ADDLs, levels of tau (total tau/actin ratio) increased by ~12% at 3 hours and ~23% at 6 hours from treatment in *Prnp*$^{0/0}$ cortical cultures (Fig. 1a,d). Moreover, an increase, although smaller, was observed in Tg20-derived cortical cultures after ADDL treatment (~8% at 3 hours and ~11% at 6 hours) (Fig. 1b,d). In parallel, PrP^C levels showed an increase at 3 and 6 hours after ADDL treatment only when a total tau increase was detected in Tg20 cultures (~15% increase at 3 hours and ~24% increase at 6 hours, respectively) (Fig. 1b). More relevantly, both ptau and total tau levels were unchanged after 24 hours of ADDL treatment in these phenotypes (Suppl. Fig. 1d). These data correlate with the observations that Aβ aggregated for 24 hours was unable to induce changes in tau levels (Suppl. Fig. 2) and that after 24 hours in culture conditions (37ºC) ADDLs can form fibrils (Suppl. Fig. 1c). Taking this into account, further experiments were performed at 6 hours from ADDL treatment.

Given that the tau 5 antibody recognizes both native and phosphorylated forms of tau, some samples were dephosphorylated after 6 hours following ADDL treatment using γ-phosphatase (see

Material and methods for details) and then analyzed with western blotting. Our results showed an increase in total tau level independently of phosphorylation, suggesting that ADDLs cause an increase in tau expression (Fig. 1e), probably prior to hyperphosphorylation. Moreover, increased levels of tau in *Prnp*$^{0/0}$ cultures treated with ADDLs were also corroborated with RT-qPCR at 6 hours (Fig. 1f).

To discriminate between putative differences in genetic background of *Prnp*$^{0/0}$ *versus* Tg20 [66] or differences due to intrinsic levels of PrP^{C}, we performed similar experiments in *Prnp*$^{+/+}$ treated with lentiviral vector neurons carrying a small interfering RNA (siRNA) for *Prnp* (see Material and methods for details). Cells infected with these lentivirus showed a 15-30% decrease in PrP^{C} expression according to different experiments and compared with control cultures infected with enhanced green fluorescent protein (GFP)-encoding lentiviral particles. After exposure to ADDLs for 6 hours, treated cultures showed a statistically significant ~14% increase in tau expression only when PrP^{C} expression was decreased (Fig. 1g). This finding suggests that a small decreased expression of PrP^{C} is enough to compromise the increased tau expression in the presence of ADDLs. Lastly, we also stimulated cultured *Prnp*$^{0/0}$ cells with both ADDLs and/or Aβ fibers for 6 hours to determine whether the increased tau was associated exclusively with ADDLs. Indeed, our results showed a significant increase in total tau levels with western blot only in cortical *Prnp*$^{0/0}$ cells treated with ADDLs for 6 hours (Suppl. Fig. 2). In conclusion, PrP^{C} seems to be a relevant factor in controlling tau levels after ADDL treatment.

Parallel progression of PrP^{C} and tau expression in APP/PS1 mice

Several studies have reported progressive development of AD-like pathology in an APP/PS1 mouse model including both Aβ deposition at 4 months and the presence of hyperphosphorylated tau in affected neurons at 6 months of age (e.g., [67,68]). First, we analyzed the presence of amyloid plaques in the APP/PS1 model and amyloid burden in our mouse colony (Suppl. Fig. 3). Increased levels of Aβ could be detected in our APP/PS1 Tg mice at 3 months of age and increasing numbers of plaques were observed in the cerebral cortex from 4 to 9 months (Suppl. Fig. 3). Next, we aimed to analyze the correlation among tau phosphorylation, total tau levels, and PrP^{C} expression in the cerebral cortex of APP/PS1 Tg mice at different postnatal ages (3, 6, and 9 months) using immunohistochemistry and western blot (Fig. 2). The immunohistochemical results showed an increase in total tau immunoreactivity

(Fig. 2b) in correlation with higher PrP^C expression (Fig. 5d) in 3-month-old transgenic mice when compared with wild-type (Fig. 5a and 5c respectively). This correlates with western blot results (Fig. 2e). Densitometric analysis of developed films showed a ~13% increase in total tau levels of 3 month-old Tg APP/PS1 with respect to non-transgenic mice (Fig. 2e,f) and a correlated ~16% increase in PrP^C levels in the same animals (Fig. 2e,g). This increase of tau and PrP^C was not observed at 6 and 9 months of age. Then analysis of phosphorylation for several tau epitopes ($ser^{396/404}$, ser^{202}/thr^{205}, and thr^{181}) was performed in the same animals by western blot. Our results showed non-significant increase in phospo-tau/total tau ratio for any residue studied (Fig. 2e). Data from protein levels were corroborated by RT-qPCR demonstrating the peak of both mRNAs at 3 months and their decline in the cerebral cortex of APP/PS1 mice at 6 and 9 months (Fig. 2h,i).

Next we aimed to determine whether the absence of *Prnp* modified tau expression in APP/PS1 mice. Triple APP/PS1/$Prnp^{0/0}$ Tg mice [69] showed a ~22% increased expression of tau at 9 months of age compared with non-Tg APP/PS1/$Prnp^{0/0}$ mice (Fig. 3a,b), in contrast to APP/PS1 expressing normal levels of PrP^C with the same tau levels (data not shown). Lastly, we performed a reduction of *Prnp* by lentiviral vectors in APP/PS1-derived cultures (Fig. 3c). Data revealed that decreased PrP^C increased levels of endogenous tau, similar to what happens with ADDL treatment of $Prnp^{0/0}$ neurons (see Fig. 3 c,d). This reinforces the notion that PrP^C participates in the control of tau expression in AD.

Next we aimed to determine whether the induced tau synthesis was regulated through the tau promoter. To this end, we generate a triple transgenic resulting from crossing TgTP6.3 mouse, which overexpress tau-GFP under the β-actin promoter, with APP/PS1. Primary neuronal cultures from embryos of these mice were treated with siRNA-PrP^C. In these cultured cells analyzed with western blot, a band corresponding to endogenous tau and the fusion protein tau-GFP could be detected at 55 and 95 kDa, respectively (Fig. 4). Revealed blots demonstrated an increase of both proteins (tau and tau-GFP) after ADDL treatment, indicating that PrP^C modulation of tau expression in ADDL-treated neurons is not specific to the tau promoter.

Correlative progression of PrP^C and tau expression in human AD samples

Several studies have shown a decrease in PrP^C levels in different areas of AD-affected brains when compared with controls [70,71]. However these studies were focused on advanced AD stages (from III to VI). In contrast, McNeill and coworkers demonstrated increased levels of the PrP^C in a short duration case of AD [44]. Taking into account the relation between levels of PrP^C and tau protein reported in APP/PS1 mice and *in vitro* models, we analyzed levels of both proteins at different stages. Developed films showed a mean ~46% increase in total tau levels in AD (stage I) cases with respect to healthy middle-aged individuals (Fig. 5a) and a progressive decline in protein levels in accordance with the increased PrP^C expression (from 12% to 27% between AD stages I and III) (Fig. 5b,c).

DISCUSSION

In this study, our experiments using $Prnp^{0/0}$, Tg20, and siRNA-lentivirus treatment support the notion that *Prnp* dosage does not influence total tau protein levels in cultured cortical neurons. This reinforces previously reported microarray data in N2a cells [65] and PrP^C mutant mice at younger stages compared with adults (2-3 months) [72-74]. However, these observations are partially in contrast with some recently published results, using human neuroblastoma [75] and HEK293 cells [74]. In these studies, forced PrP^C expression in transfected cells with low expression levels of endogenous PrP^C decreased total tau protein levels [75].

It is relevant to note that increased expression of PrP^C increased its association with lipid rafts which may in turn trigger increased intracellular oxidative stress [76,22], thereby affecting tau expression and phosphorylation [74,77]. In contrast to the expected result, decreased PrP^C expression in our experiments correlated with increased tau phosphorylation (ptau/total tau as well as ptau/actin ratios) after *in vitro* treatment with ADDLs. Thus levels of tau, determined by tau 5 antibody, with or without γ-phosphatase treatment (to avoid simultaneous detection of some ptau epitopes with tau 5 antibody), demonstrated that ADDL treatment is able to induce a relevant increase in tau in the absence of PrP^C as well as in overexpressing cultures. In this sense, it is important to note that both a lack of PrP^C expression and overexpression of the protein may impair PrP^C function [73].

In addition, this is exclusively associated with ADDL treatment since aggregated Aβ is unable to induce these effects in treated neurons. In fact, aggregated peptides in fibrillar forms failed to activate tau phosphorylation (e.g., tyr^{18} residue) in contrast to the antibody-mediated PrP^C aggregation in the membrane [37]. Thus PrP^C reduces the effects of ADDLs in tau level modulation as well as their phosphorylation. Schmitz *et al.* showed that increased PrP^C expression in transfected cells decreased ptau levels (determined using AT8 antibody) [74]. This was also observed in $Prnp^{0/0}$ mice (FVB background) with increased tau phosphorylation at earlier stages [72]. Similar data have described tyr^{18} phosphorylation of tau after ADDL treatment [41]. Our data confirm but also expand these observations to all the phospho-epitopes studied. Concerning different phospho-epitopes, it has been shown that, after Aβ oligomer treatment, wild-type primary neurons increased specifically AT8-associated phospho-epitopes as well as those recognized with 12E8 antibody ($ser^{262/356}$) and 9G3 antibody (tyr^{18}). In contrast, $ser^{396/404}$ (PHF1 antibody) remained unaltered (i.e, [78]). In our experiments, the absence of *Prnp* increased the phosphorylation of all ptau residues analyzed: (PHF1 antibody) $ser^{396/404}$, (AT8 antibody) ser^{202}/thr^{205}, and (phospho-thr181 antibody) thr^{181}, in contrast to $Prnp^{+/+}$ and Tg20 primary cultures. In this respect, it has recently been shown that thr^{181} and ser^{396} residues act as putative PrP^C interacting domains of tau [74]. Thus it is reasonable to consider that these residues were not phosphorylated by intracellular kinases in wild-type and Tg20 cultures after ADDL treatment.

Several studies have analyzed the putative role of PrP^C expression in the survival of various models of neurodegenerative diseases (e.g., [79,69]. In fact, PrP^C seems not to play a crucial role in survival in Parkinson, taupathies, or APP/PS1 mouse models [79,69]. Although tau phosphorylation was not analyzed, no differences were observed in the tangle levels in the brain parenchyma of P301L mice with different *Prnp* dosages [79]. Only one study describes in detail propagation of tau pathology in the same mouse model [80]. In contrast, the participation of PrP^C in the appearance and evolution of the hallmarks of AD (i.e, Aβ deposits) has been reported by several laboratories [81,82]. In fact, PrP^C was detected with immunohistochemistry close to Aβ deposits [83] and accumulated in dystrophic neurites in AD postmortem brain sections [84].

With respect to PrP^C levels, most of these studies have been focused on the changes of PrP^C protein levels in advanced stages of the disease (mainly stage III until VI) (i.e., [70]) probably due to

neuronal loss [44]. In our study we demonstrated that in APP/PS1 mouse models as well as in postmortem AD brains (ranking from stage I to stage VI) there is an increase of PrP^C protein in the brain at early stages of the disease that peaks around stage III in AD brains and at ~3 months old in APP/PS1 mice. Thereafter PrP^C levels decreased inversely until the evolution of the clinical symptoms in both cases. Considering that low levels of PrP^C may increase tau expression, which may contribute to the AD pathology, and that PrP^C may act as an anti-oxidant agent as has been widely demonstrated [85,74], it is reasonable to consider that overexpression of PrP^C in AD might be part of a protective response in the evolution of the disease.

PrP^C expression, ADDLs, tau phosphorylation and neuroprotection

Phosphorylated form of tau protein is the main constituent of NTFs in AD and its appearance correlates with neurodegeneration in the disease (see [86] revised in [87]). It has been reported that the single overexpression of human tau in a mouse model is robust enough to induce tau phosphorylation [88] and neuronal aggregation [89]. In fact, the expression of a truncated form of tau reduces the excitotoxicity mediated by Aβ [90]. In our study, the absence of PrP^C in an APP/PS1 mouse and in cultured APP/PS1-derived neurons led to increased tau levels. It has been described how PrP^C-Fyn complex is responsible for pathological changes induced by ADDLs in tau phosphorylation [41]. In this context, Fyn co-localizes with ser/thr phosphorylated forms of tau in AD brains [91], and a direct interaction between PrP^C and Fyn has been described [92,41]. Furthermore, Fyn is up-regulated in AD brains and it interacts with tau, phosphorylating it at tyr^{18} epitope [38], implicated in the formation of tau aggregates and NFTs [93]. Along these lines, we recently reported increased phosphorylation levels of tyr^{18} in older (~ 9 months) but not younger (3-6 months) APP/PS1 mice overexpressing PrP^C [69]. The data presented more recently by Larson *et al.*, indicating a dependence of tyr^{18} phosphorylation on PrP^C, reinforce our previously reported data [41]. In addition, we also observed increased PrP^C in Tg20-derived cultures after ADDL treatment which may also contribute to the increased levels of ptau (tyr^{18}) observed in our previous study [69] and the study by Larson *et al*. in 14-month-old mice [41]. However, we were unable to determine differences in ptau epitopes (other than tyr^{18}) in old APP/PS1 mice with different PrP^C dosages, although we did demonstrate an increase in tau levels in APP/PS1 mice lacking PrP^C with respect to APP/PS1-$Prnp^{+/+}$ as also reported by Larson *et al.* [41]. In fact, our present results indicate that levels of PrP^C are largely

reduced in APP/PS1 mice of 9 months of age by 28% (Fig. 2). This correlates with western blotting data in human studies (see above). Taken together, these results suggest that the control of tau expression by PrP^C under the effects of ADDLs declines along the development of the disease or with age in the mouse model. This decline correlates with increased appearance of NFTs enriched in ptau in AD brains.

Are the effects of PrP^C mediating tau levels in ADDL-treated neurons specific?

Our data indicate that tau levels are increased in the absence of PrP^C in the presence of ADDLs. In fact, it has been proposed that PrP^C suppresses tau transcription, probably though modulation of the Fyn pathway [75]. However when we crossed APP/PS1 mice with a mouse model of tau overexpression under a β-actin promoter, reduction of PrP^C levels by siRNA induced increased presence of both endogenous (regulated by tau promoter) and exogenous tau-GFP (regulated by β-actin promoter) (Fig. 4). This might indicate that the absence of PrP^C also regulates the activity of different promoters, or, more likely, that PrP^C may intervene in the aggregative state of the protein or its turnover. In this sense phosphorylation of tau results in decreased tau degradation by the proteasomes that appear to play a major role in the turnover of the protein [94]. Also, cortical neurons overexpressing tau alter intracellular distribution of PrP^C and promote accumulation in the cell body, forming insoluble aggregates that in turn might damage the proteasome [95]. Taken together, these data suggest decreased degradation of tau in some pathological cases and probably modified synthesis/degradation rates. Further study is warranted to examine these possibilities and to determine biochemically whether PrP^C modulates tau levels in both healthy and AD-affected neurons.

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FIGURE LEGENDS

Fig. 1

Analysis of effect of ADDLs on tau. **a, b** Cortical cultures from *Prnp*$^{0/0}$ or *PrnP*$^{+/+}$ mice (a) and Tg20 mice (b) were treated with either ADDL (10 μM) (n = 6 *Prnp*$^{0/0}$, n = 6 *PrnP*$^{+/+}$, and n= 6 Tg20) or vehicle alone for 3 and 6 hours (n = 6 *Prnp*$^{0/0}$, n = 6 *PrnP*$^{+/+}$, and n= 6 Tg20; each case). Samples were analyzed with western blot using different anti-phospho tau or total tau antibodies in parallel with anti-PrP^{C} antibody to reveal PrP^{C} expression in each sample. Membranes were retested with an antibody against actin for normalization. **c** Densitometric analysis showing the increase in phosphorylated tau levels (epitopes ser$^{396/404}$, ser^{202}/thr^{205}, and thr^{181}, respectively) only in *Prnp*$^{0/0}$ mouse samples after 3 or 6 hours of ADDL treatment when compared to the vehicle control. **d** Densitometric analysis showing the increase in total tau levels only in *Prnp*$^{0/0}$ mouse samples at 3 and 6 hours post-treatment and the slight increase in total tau levels in Tg20 mice at the same time of stimuli. Data on tau-phosphorylated and total tau protein levels in control samples (vehicle alone) were considered as the unit at each experimental time and the rest of the data were corrected as an increase of this initial value. **e, f** Analysis of tau expression in protein extracts of cortical *Prnp*$^{0/0}$ cultures treated with ADDLs for 6 hours after treatment with λ-phosphatase with western blot (e) (n = 4) or analyzed by RT-qPCR (n = 3) (f). Densitometric values in (e) are standardized with vehicle treatment, and quantification was represented as fold increase ± SEM. **g** Neuronal primary cultures from wild-type mice were also stimulated in the same conditions after silencing PrP^{C} expression with a lentivirus. The western blot analysis showed an increase in tau expression only in the silenced ones compared with the wild-type ones. Densitometric values in c, d and f are standardized with vehicle treatment, and quantification was represented as fold change ± SEM. Bars represent the mean of three independent experiments. * $p< 0.05$, ** $p< 0.01$; (ANOVA test).

Fig. 2

a-d Immunohistochemical characterization of mouse brain sections. Anti-total tau antibody (monoclonal Tau 5) (a, b) and anti-PrP^{C} antibody (monoclonal 6H4) (c, d) were used to detect an increased

immunoreaction in APP/PS1 mice (n=4) (b, d) in contrast to wild-type animals (n=4) (a, c). Abbreviations including CA1 and CA2, cornus ammonis I and II; DG dentate gyrus; GCL, granular cell layer; H, hilus; ML, molecular layer; SLM, stratum lacunosum-moleculare; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum. Scale bar = 200 μm. **e** Western blot analysis of tau and PrP^C in brains from wild-type (WT) and APP/PS1 mice (Tg) at different postnatal ages (3, 6, and 9 months respectively). Anti-actin monoclonal antibody was used for standardization. **f, g** Histograms showing the densitometry study of each protein, tau (f) and PrP^C (g), respectively. **h, i** Histograms showing the RT-qPCR analysis of expression of each protein, tau (h) and PrP^C (i), respectively. 9 animals were examined in each case and bars represent the mean of each group of transgenic animals normalized with control group at the same age. * $p< 0.05$, ** $p< 0.01$; (ANOVA test).

Fig. 3

a, b Tau expression in brains from triple transgenic mice APP/PS1/$Prnp^{0/0}$ (n = 9) compared to $Prnp^{0/0}$ (n = 9) at 9 months old. An example of western blot analysis of two animals of each genotype (a) and the respective histogram quantification of tau expression (b). Anti-actin monoclonal antibody was used for standardization. * $p< 0.05$ (ANOVA test). **c, d** Cortical cultures from APP/PS1 mice after silencing PrP^C expression with a lentivirus (n = 4). The western blot analysis showed increased levels of endogenous tau when PrP^C expression was decreased (c). Densitometric analysis of developed films (d). * $p< 0.05$ (ANOVA test).

Fig. 4

Analysis of tau expression in cortical primary cultures from a triple transgenic APP/PS1/TgTP6.3 mouse. Western blot analysis shows an increase in tau expression when levels of PrP^C are reduced with lentiviral system. Both tau-GFP, which migrated to 95 KDa, and endogenous tau, around 55 KDa, are overexpressed. Anti-actin monoclonal antibody was used for standardization. Densitometric values are standardized with vehicle treatment, and quantification was represented as fold increase ± SEM of three independent experiments (n = 10 cases).

Fig. 5

Immunoblot analysis of hippocampal necropsies from AD patients compared to healthy (middle-aged adults) cases. **a** Example of western blot determination of total tau levels and PrP^{C} expression. Braak and Braak stage progression is indicated (I-VI). Membranes were retested with an antibody against actin for standardization. **b** Histograms showing the densitometric study for all cases showed in Table 1. Asterisks indicate significant differences between AD groups and healthy cases, whose average represents the unit. * $p< 0.05$, ** $p< 0.01$; (ANOVA test). Increased levels of tau can be seen in patients from ADI to ADIII group (a, b) and a correlative increment in PrP^{C} expression is also shown (a, c).

Supplementary Fig. 1

a Electrophoretic characterization of different Aβ1-42 amyloid species. Peptide was matured under protocols for ADDLs or fibril formation prior to peptide culture treatments. Immunoblot was done with 6E10 monoclonal antibody. **b, c** TEM analysis of Aβ1-42 24 hours post-dissolution under oligomer- (b) or fibril-forming conditions (c). Scale bar: 200 nm. **d** Western blot analysis of total tau level or phosphorylated tau level (epitopes $ser^{396/404}$, ser^{202}/thr^{205}, and thr^{181}, respectively) in cortical cultures from *Prnp*$^{0/0}$, *PrnP*$^{+/+}$, and Tg20 mouse samples (n = 6, *Prnp*$^{0/0}$; n = 6 *PrnP*$^{+/+}$ and n = 6 Tg20) after 24 hours of ADDL treatment.

Supplementary Fig. 2

Comparative analysis of cortical primary culture from *Prnp*$^{0/0}$ mice treated with ADDLs or fibrillar Aβ1-42, respectively, for 6 hours (n = 3 ADDLs and n = 4 Aβ1-42). Note that tau expression increases only when cells are treated with oligomers.

Supplementary Fig. 3

Characterization of the APP/PS1 mouse model used in this study. **a-d** Immunohistochemical analysis of 9-month-old animals with 4G8 monoclonal antibody for detection of amyloid plaques. Scale bars a, b, c = 200 μm Scale bar d = 40 μm. Abbreviations including H, hilus; GCL, granular cell layer; ML, molecular layer; SLM, stratum lacunosum-moleculare; SR, stratum radiatum; SP, stratum pyramidale; SO, stratum oriens; WM, white matter and I–VIb, neocortical layers. **a** Absence of amyloid plaques in brain sections of wild-type mice in contrast to Aβ deposits found in transgenic mice (b, c). **d** Higher magnification of

the immunolabeled plaque framed in c. **e** Increasing levels of soluble Aβ1-42 amyloid with age quantified by ELISA. 9 animals were analyzed for each age.

Table
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Table 1. Control and AD patients, categorized as stages I to VI of Braak and Braak, used in this study.

Case number	Braak and Braak stage	Gender	Age	Age (mean ± s.d.m)	Post-mortem delay
C1	-	M	39	46.5±5.3	9h 15min
C2	-	F	46		14h 15min
C3	-	M	53		7h 25min
C4	-	M	46		15h
C5	-	M	43		4h 35min
C6	-	M	52		3h
AD1	I	M	61	67.3±7.7	3h 40min
AD2	I	M	53		6h 15 min
AD3	I	M	74		4h
AD4	I	M	71		11h 30min
AD5	I	M	64		2h 15min
AD6	I	F	79		3h 35min
AD7	I	M	65		5h 15min
AD8	I	F	75		4h 55min
AD9	I	M	63		6h
AD10	I	M	68		10h 55min
AD11	II	F	77	69.6±6.7	11h
AD12	II	M	65		5h
AD13	II	M	66		4h 55min
AD14	II	M	72		8h 45 min
AD15	II	M	71		5h 15min
AD16	II	M	66		5h
AD17	II	F	60		9h 40 min
AD18	II	F	80		3h 30min
AD19	III	F	81	76.3±5.0	1h 30min
AD20	III	M	71		7h 15min
AD21	III	F	77		11h 30min
AD22	IV	F	80	81±2.1	2h 45min
AD23	IV	F	81		12h
AD24	IV	M	84		12h 45min
AD25	IV	M	79		50min
AD26	V	M	87	80.3±5.4	7h 5min
AD27	V	M	75		11h 30min
AD28	V	M	82		3h 45min
AD29	V	M	77		16h
AD30	VI	M	86	76.5±13.4	20h 35min
AD31	VI	M	67		8h

F: female; *M*: male;

Figure 1

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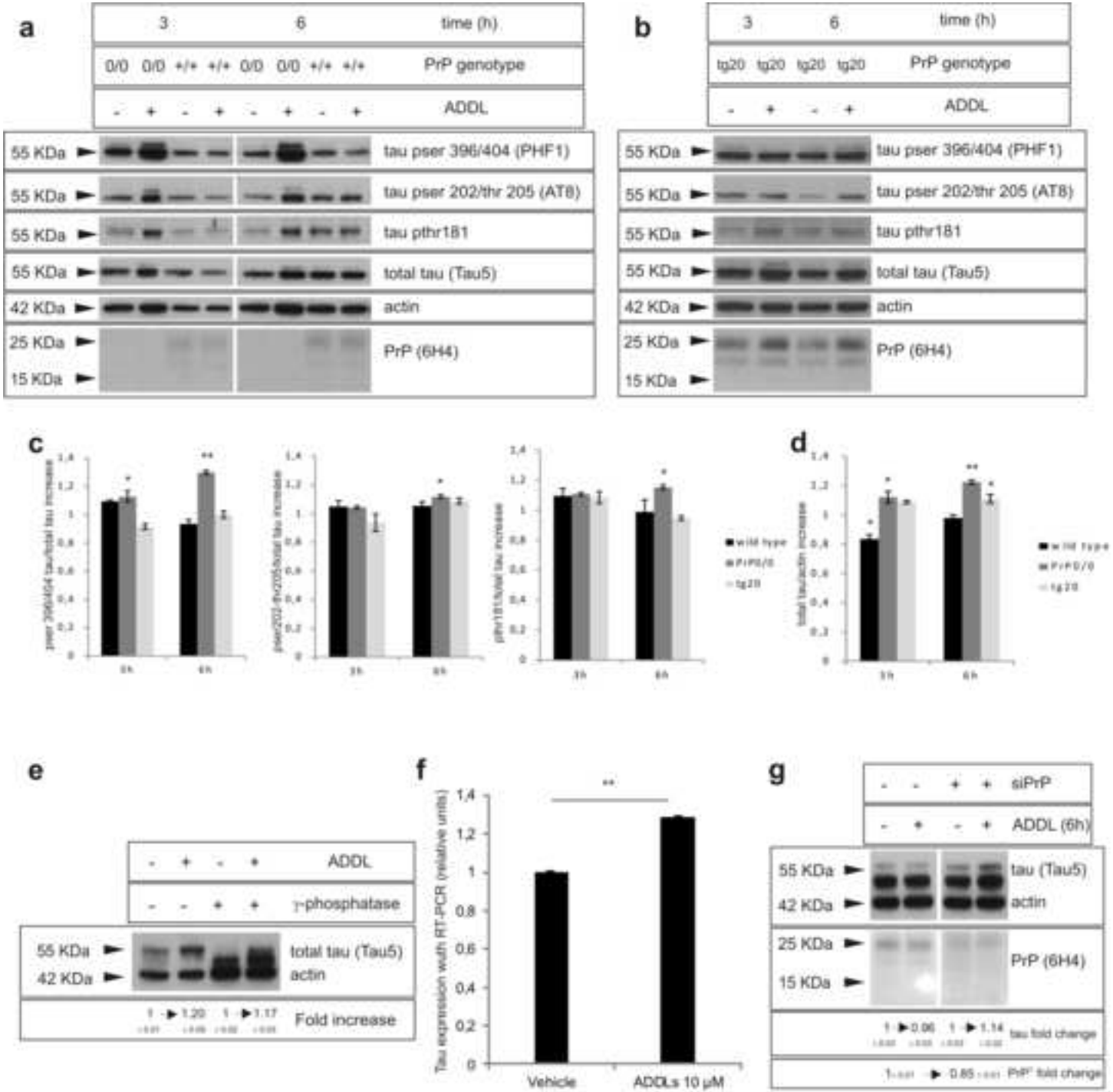

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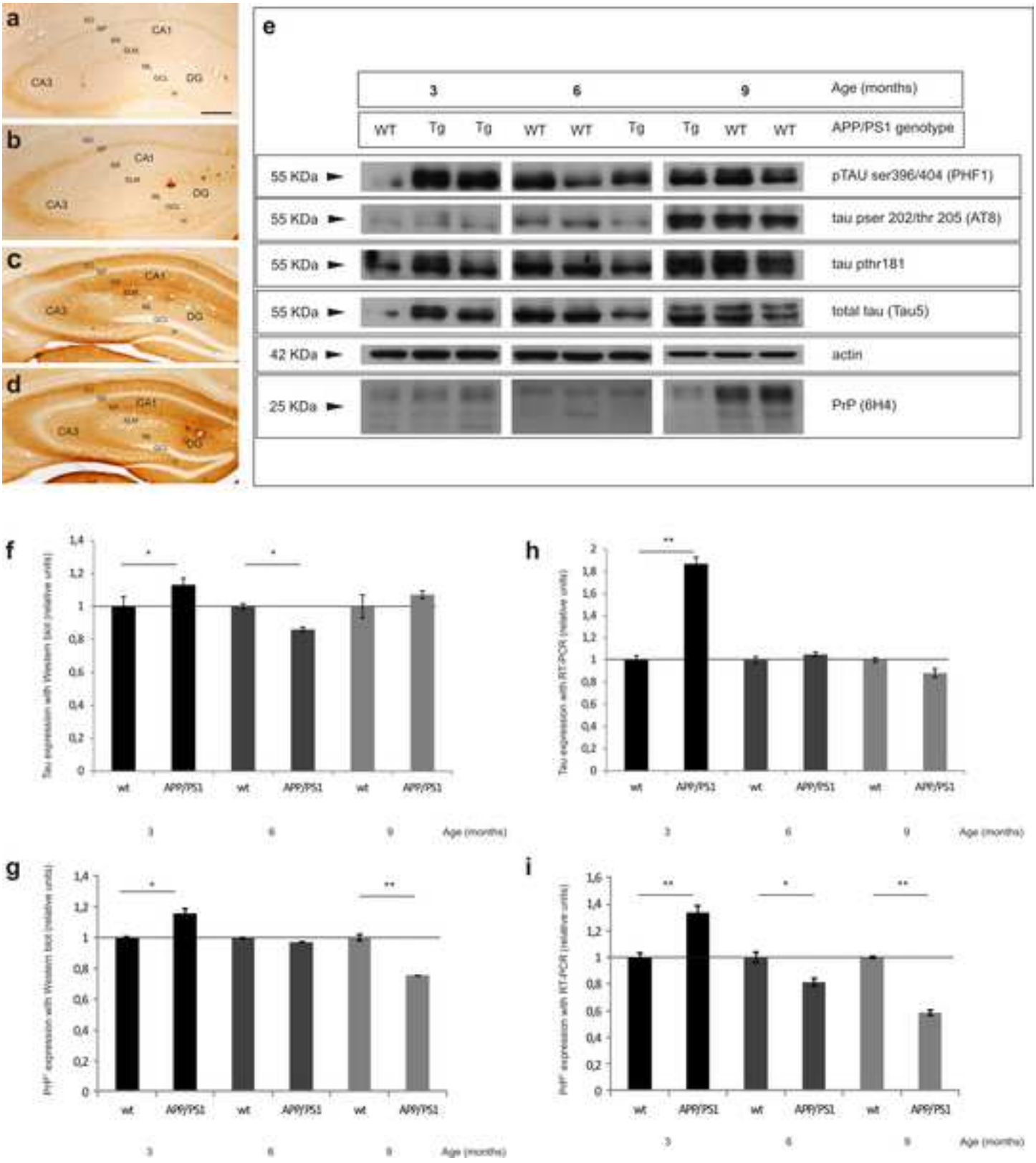

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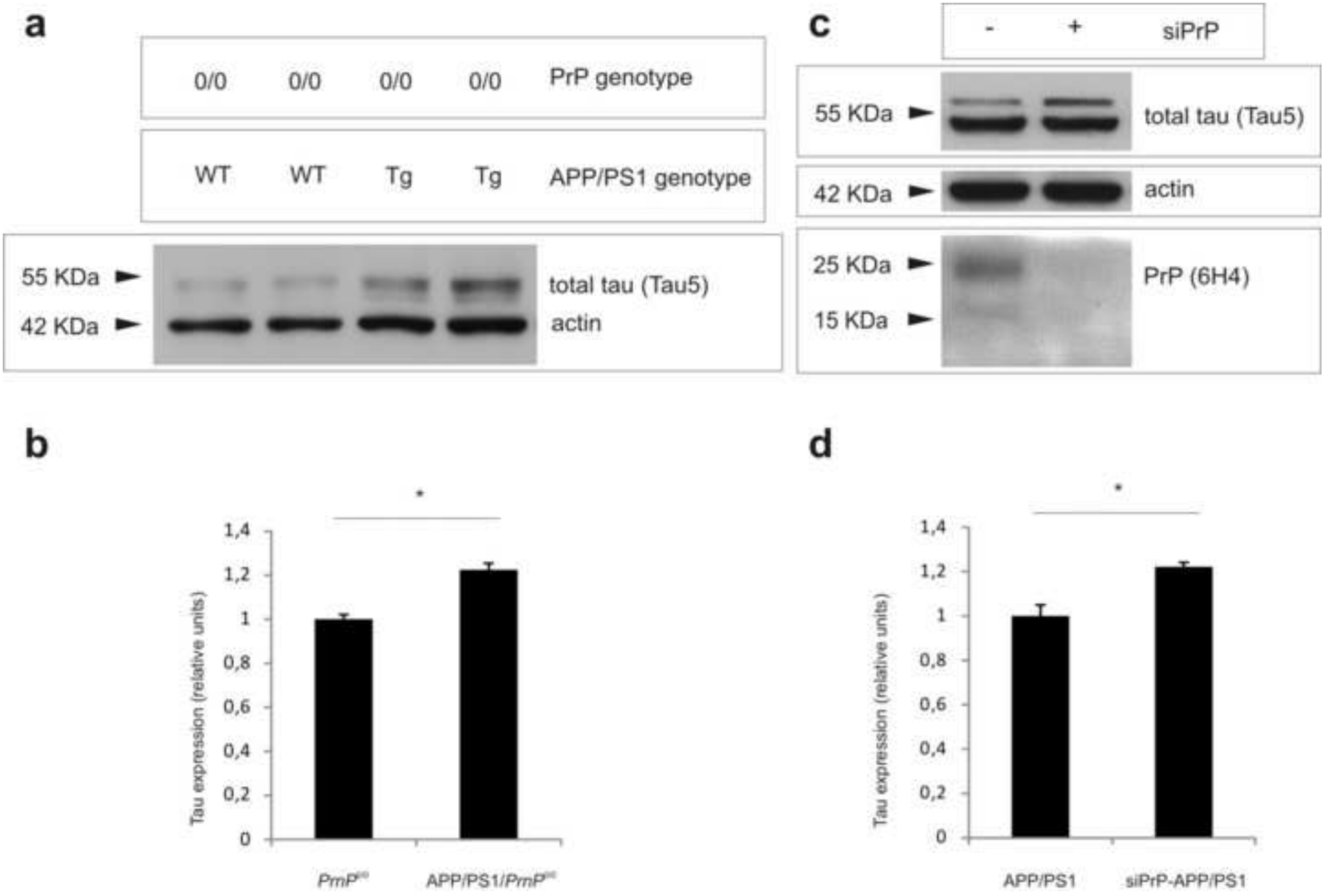

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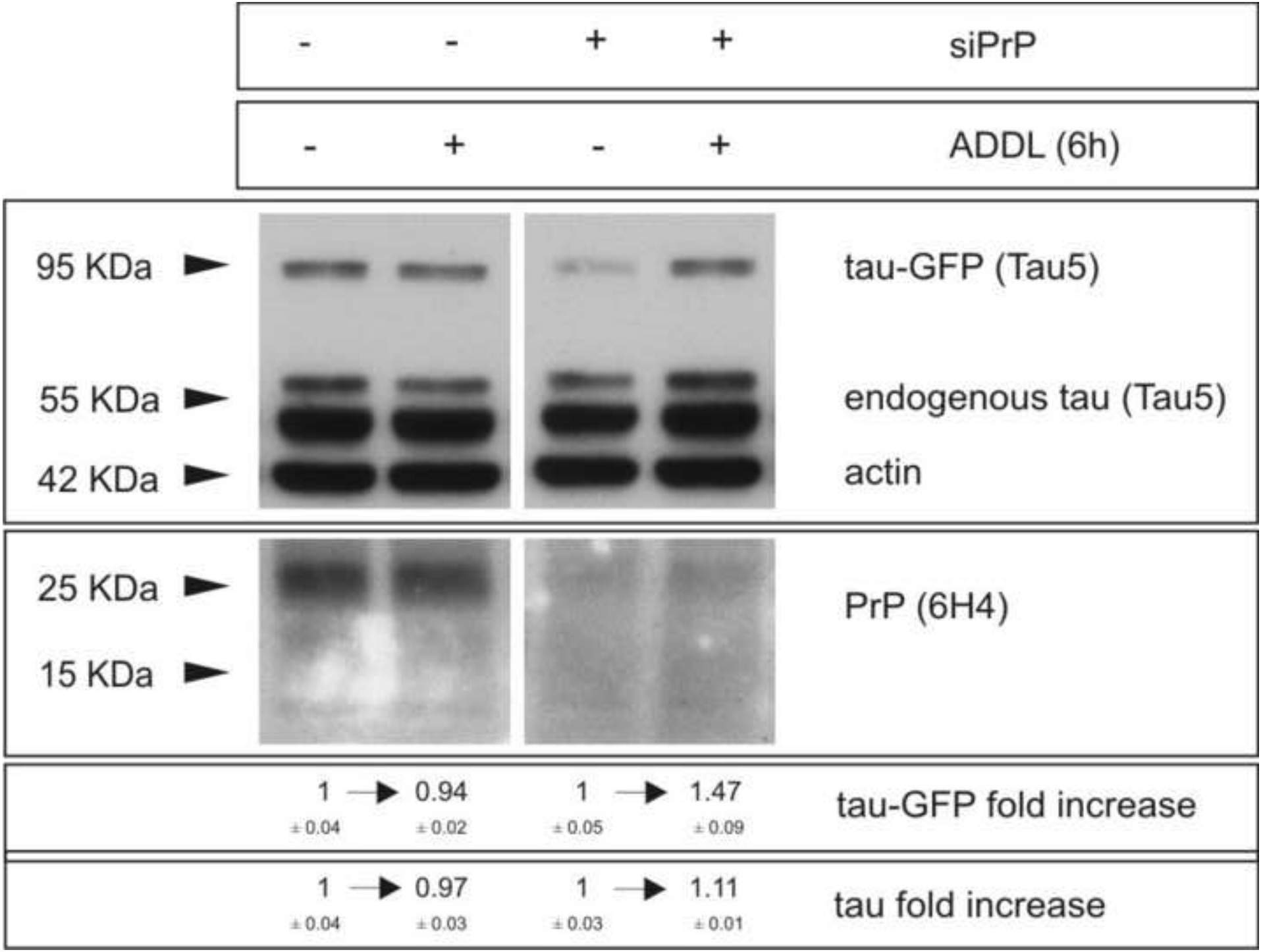

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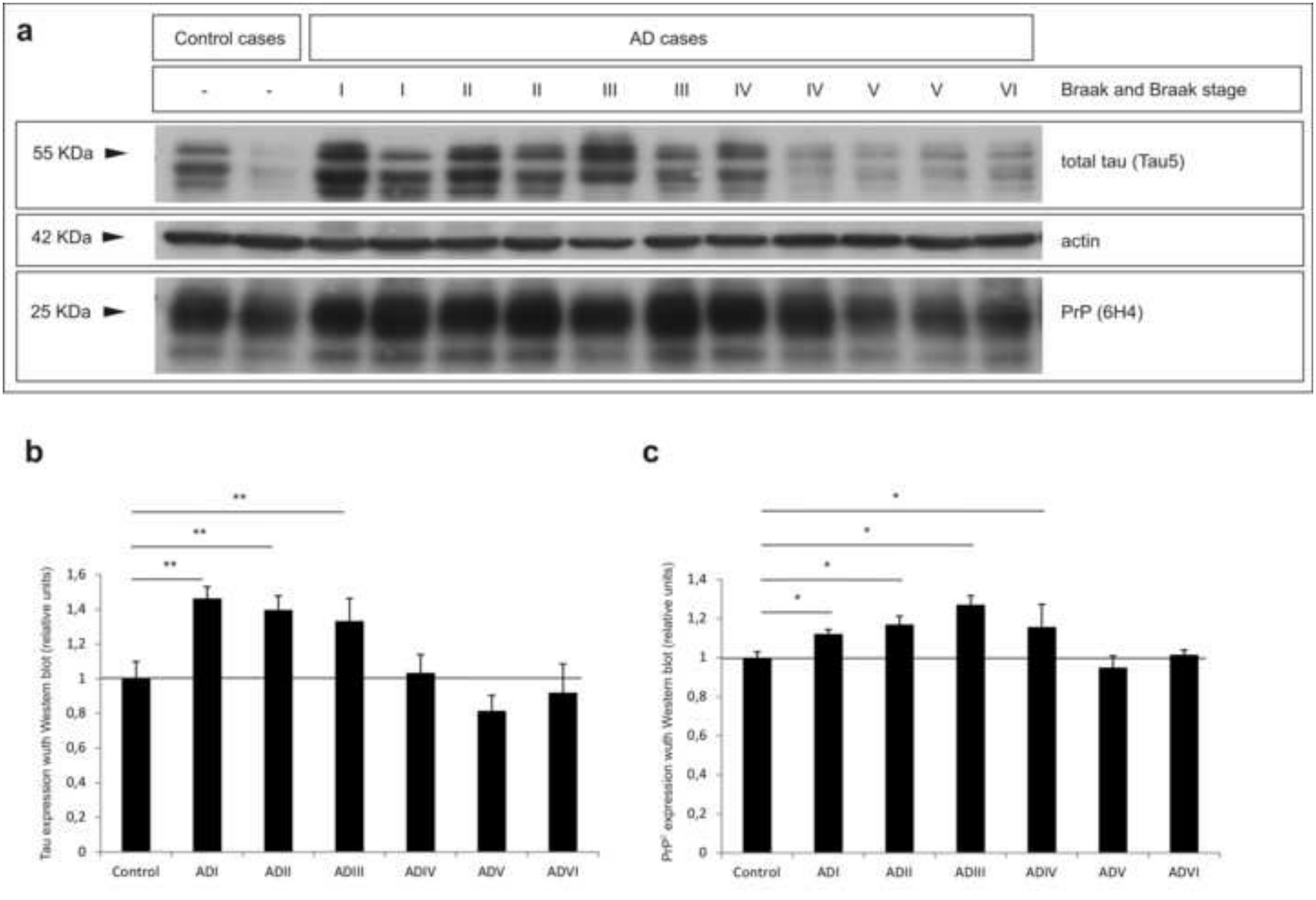

Supplementary Figure 1

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