Tau Protein as a New Regulator of Cellular Prion Protein Transcription

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Abstract

Cellular prion protein (PrP^C) is largely responsible for transmissible spongiform encephalopathies (TSEs) when it becomes the abnormally processed and protease resistant form PrP^{Sc}. Physiological functions of PrP^C include protective roles against oxidative stress and excitotoxicity. Relevantly, PrP^C downregulates tau levels, whose accumulation and modification are a hallmark in the advance of Alzheimer's disease (AD). In addition to the accumulation of misfolded proteins, in the initial stages of AD-affected brains display both increased reactive oxygen species (ROS) markers and levels of PrP^C. However, the factors responsible for the upregulation of PrP^C are unknown. Thus, the aim of this study was to uncover the different molecular actors promoting PrP^C overexpression. In order to mimic early stages of AD, we used β -amyloid-derived diffusible ligands (ADDLs) and tau cellular treatments, as well as ROS generation, to elucidate their particular roles in human *PRNP* promoter activity. In addition, we used specific chemical inhibitors and site-specific mutations of the *PRNP* promoter sequence to analyze the contribution of the main transcription factors involved in *PRNP* transcription under the analyzed conditions. Our results revealed that tau is a new modulator of PrP^C expression independently of ADDL treatment and ROS levels. Lastly, we discovered that the JNK/c-jun-AP-1 pathway is involved in increased *PRNP* transcription activity by tau but not in the promoter response to ROS.

Keywords Cellular prion protein · Tau · Promoter · Alzheimer's disease · Tauopathies

Introduction

During the progression of prionopathies, the abnormal processing of cellular prion protein (PrP^C) gives rise to a protease-resistant form of the protein, PrP^{Sc} [1, 2], responsible for the transmissible spongiform encephalopathies (TSEs) that

include Creutzfeldt-Jacob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI) in humans. Thus, TSE clinical phenotypes should be considered as additional effects of the loss of function of natural PrP^C together with the neurotoxic effects of pathogenic prions [3].

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Furthermore, physiological functions of PrP^C, which remain under study [4], include several roles in neuroprotection (e.g., [5]). In this sense, it has been shown that PrP^C protects against oxidative stress through copper interaction [6] or modulation of endogenous superoxide dismutase (SOD) [7] and glutathione reductase (GR) activities [8], as well as against excitotoxicity through modulation of glutamate receptors [9, 10]. In experimental models of Alzheimer's disease (AD), PrP^C plays roles at two levels: (1) interacting with β -amyloid-derived diffusible ligands (ADDLs), the most toxic species of A β 1–42 [11, 12]; and (2) downregulating tau levels [13, 14]. In this respect, tau modification and accumulation are observed in AD and also in other tauopathies including progressive supranuclear palsy (PSP) and frontotemporal lobar degeneration-tau (FTDP-tau) [15–17].

At the genomic level, PrP^C is encoded by a single-copy gene (*PRNP*) located on chromosome 20 in humans [18], comprised of two exons. The first, untranslated of 134 nucleotides, contains a single major transcriptional start site in its 5'-flanking region, while the second, separated by an intron of 12,696 bases, encodes the entire 759 bp ORF in 2355 nucleotides [18, 19]. The *PRNP* gene is homologous in numerous species with a high level of conservation in mammals [20]. Its transcription is constitutive in a wide range of tissues with greater expression in CNS and lymphoid tissues [21], and it is decreased during normal aging [22].

Because the level of PrP^C expression is known to influence the initiation and progression of prion diseases [23], and expression of physiological levels may be involved in neuroprotection, relevant efforts have been made to improve understanding of the transcriptional regulation of the *PRNP* gene. In this sense, a 1-kb region around *PRNP* exon 1 forms a "CpG island" containing several putative transcription factor-binding sites including transcriptional activators Sp1, AP-2, and HSE. Therefore, the most active region of the human *PRNP* promoter in neurons has been determined to be between –148 and +125 bp, with an AP-1 transcription factor-binding site at –120 [24]. In addition, other transcription factors involved in upregulation of PrP^C have been defined in response to several exogenous factors. One of the most studied physiological conditions triggering upregulation of PrP^C is the presence of a high level of oxidative stress. In this regard, copper is the main actor in triggering increased reactive oxygen species (ROS) [25, 26], pointing to Sp1 and MTF-1 transcription factors mediating the increased *PRNP* activity [27]. In addition, the ataxia-telangiectasia mutated (ATM) pathway is also a target of unbalanced copper and promotes binding of Sp1 and p53 to a consensus sequence in *PRNP* promoter [26]. Therefore, Sp1 can act as a single upregulator of *PRNP* transcriptional levels under hypoxia or copper overload (see, e.g., [28]).

ROS generation plays a role during AD progression [29]. In addition, AD brains presented A β deposition that includes

ADDLs and higher quantities of normal and abnormally phosphorylated tau than unaffected controls [30, 31]. Likewise, AD-affected brains also present, although this is subject to debate [32], increased levels of PrP^C at initial stages of the disease (Braak I-II) in contrast to a decrease in protein level at advanced disease stages [13, 33, 34]. However, a detailed description of the factors able to upregulate PrP^C at initial stages of AD has yet to be settled upon.

Therefore, in this study, we aimed to uncover the specific roles of β -amyloid (A β) species and tau vs ROS generation in the transcriptional control of *PRNP* promoter. We discovered that tau is a novel modulator of PrP^C expression independently of ROS generation, in both the overexpression and the uptake of non-fibrillary variants of tau-enhanced *PRNP* activity. Lastly, we determined AP-1 to be the main transcription actor mediating tau effects in parallel to non-specific factors (Sp1).

Materials and Methods

Mouse Strains and Genotyping

Three transgenic mouse lines and wild type (WT) C57BL/6J were used in this study. For primary cultures, we worked with embryos from pregnant females. Mating day was considered to be embryonic day 0 (E0), and the offspring was genotyped according to the genotype of the crossed progenitor.

Mapt^{−/−} mice were generated as previously described [35]. Heterozygous (*Mapt*^{+/-}) mice were crossed in order to obtain homozygous tau knockout mice (*Mapt*^{-/-}) and WT control littermates.

TgTP6.3 mouse line is a transgenic mouse carrying a tau-tagged green fluorescence protein (GFP) transgene generated by [36]. Mice carrying the tau-GFP transgene were identified by fluorescence microscopy of tail tissue.

P301S transgenic mice harbor the T34 isoform of tau with one N-terminal insert and four microtubule binding repeats (1N4R) encoding the human P301S on a B6C3H/F1 genetic background [37]. P301S or non-transgenic littermates (WT control) were used to obtain brain extracts as well as purified tau fibers from sarkosyl-insoluble fraction.

Cell Culture and Transfection

HEK293 human embryonic kidney cells (CRL-1573, American Type Culture Collection (ATCC)) were maintained in Advanced Dulbecco's Modified Eagle's Medium (AddMEM) supplemented with 10% fetal bovine serum (FBS), 0.5% glutamine, and 1% penicillin/streptomycin (Invitrogen-Thermo Fisher Scientific, MA, USA) in 75-cm² culture bottles (Nunc, Denmark) in a 5% CO₂ atmosphere at 37 °C. One day before transfection, cells were cultured in

AdDMEM supplemented with 10% FBS and without antibiotics, on poly-D-lysine (Sigma-Aldrich, Darmstadt, Germany)-coated plates (Nunc).

C17.2 mouse multipotent neural progenitor cells (ECACC 07062902, Sigma-Aldrich) were cultured as described previously [38]. One day before transfection, cells were cultured in DMEM supplemented with 10% FBS, 5% HS, and 1% glutamine, and without antibiotics, on poly-D-lysine (Invitrogen-Thermo Fisher Scientific)-coated plates (Nunc).

Transfection was performed using Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific) according to the manufacturer's instructions.

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 min at 37 °C. After addition of horse serum followed by centrifugation, cells were dissociated by trituration in 0.1-M PBS containing 0.025% DNase with a pipette (all from Sigma-Aldrich). Dissociated cells were plated at ~3000 cells/mm² on plates (Nunc) coated with poly-D-lysine (Sigma-Aldrich). The culture medium was Neurobasal supplemented with 2 mM glutamine, 6.5 mg/ml glucose, antibiotics (Pen./Strept.), 5% horse serum, and B27 (Invitrogen-Thermo Fisher Scientific). After 72 h, 5 μ M cytosine β -D-arabino-furanoside hydrochloride (AraC) (Sigma-Aldrich) was added for 48 h to inhibit the growth of dividing non-neuronal cells. Cultures were used after 7 days in vitro.

Stereotaxic Surgery

Three-month-old mice anesthetized with 50 mg/kg pentobarbital sodium were unilaterally injected (at a rate 0.5 ml/min) with 3 μ l of ADDLs (10 mM) into the dorsal hippocampus (coordinates with respect to bregma in mm (AP-2.15, ML 2.0, DV 2.0)), according to the stereotaxic atlas of mouse brain [39]. Seven days after injection, bilateral hippocampal sections of brain were collected (10% w/v) in ice-cold saline buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, pH 7.5) containing 1% (w/v) Triton X-100, 10% (w/v) glycerol, and 1 $\times$ cocktail of protease and phosphatase inhibitors, using a homogenizer in ice.

Plasmids and Construction of PRNP Promoter-Deleted Forms

A 1.05-kb *SacI* fragment in the immediately upstream promoter region of Exon I of *PRNP* (GenBank Ac. Number AJ289875) was excised from the pPrPS2.7 plasmid, a gift from Prof. John Collinge [24], and was cloned in the correct

orientation into the promoter-less luciferase vector pGL2-Basic (Promega, Madison, WI, USA) to generate construct pPrP-pGL2.

To generate deletion constructs Δ AP-1, Δ Sp1, and Δ AP-1/Sp1, the pPrP-pGL2 encoding plasmid was used as a template for inverse PCRs, and the inserts obtained were fused. Briefly, a phosphorylated primer set was designed for each construct in order to amplify the region of *PRNP* promoter for deletion. Primers (Sigma-Aldrich) are detailed as follows: p Δ Ap1 (F: 5'-Phospho-caccctttaactaaacctcg-3, R: 5'-Phospho-gagtgagattcgctgaac-3') to Δ AP-1 mutant; p Δ Sp1 (F: 5'-Phospho-tccgagccagtcgctgac-3, R: 5'-Phospho-cgaggtttaagttaaagggtg-3) to Δ Sp1 mutant. Primers p Δ Ap1R and p Δ Sp1F were used to generate the double-deleted mutant. After amplification with AccuPrime™ Taq DNA Polymerase high fidelity (Invitrogen-Thermo Fisher Scientific), the blunt ends of the amplimers were religated (T4 DNA Ligase, New England Biolabs, MA, USA). An aliquot of each ligation reaction was electroporated into *Escherichia coli* DH5 α , and transformants were selected for ampicillin resistance. Twenty-five candidates were selected and screened by sequence analysis (Terminator Big Dye™ v3.1, Applied Biosystems, Thermo Fisher Scientific).

Both tau cDNA constructs were cloned into the eukaryotic expression vector pSG5 (Stratagene, Agilent, CA, USA) under the control of SV40 early promoter. Human tau expression plasmids pSGT30 and pSGT42 have been previously described [40]. Tau isoform nomenclature defines the length of the microtubule binding repeat (3R or 4R) and the presence or absence of exons 2 and 3 at the N-terminus (0 or 2).

In addition, Bcl-2-pSG5-encoding plasmid was constructed using standard subcloning procedures.

All plasmid DNA was purified with QIAprep Miniprep Kit (Qiagen, Hilden, Germany). DNA concentrations were determined with a UV spectrophotometer.

Tau Variant Purification

The recombinant tau protein isoform (2N4R tau) containing 2N-terminal inserts was isolated as described previously [41]. Phosphorylated isoform of 2N4R tau was obtained from an insect cell culture (Sf9) infected with baculovirus containing the longest tau isoform present in the central nervous system. Baculoviral construction and Sf9 cell culture infection have been described previously [42]. Briefly, Sf9 cells were grown at 27 °C in a spinner culture bottle in Grace's Insect Medium (Invitrogen-Thermo Fisher Scientific), supplemented with 10% FBS, 100 μ g/ml penicillin/streptomycin, and 0.05% (v/v) Pluronic F68 (Invitrogen-Thermo Fisher Scientific). Monolayer cultures of Sf9 cells were infected with recombinant baculoviruses at a multiplicity of infection of 1–5. The samples were taken at 72 h post-infection and tau protein was purified; the levels of tau phosphorylation were measured with

antibody AT8 (Invitrogen-Thermo Fisher Scientific) as described before [43].

Soluble oligomeric tau and ptau (O-tau/O-ptau) were prepared by incubating tau/ptau solution (1 $\mu\text{g}/\mu\text{l}$) at room temperature for 2 days. For insoluble fibril assembly, tau/ptau solution (1 $\mu\text{g}/\mu\text{l}$) was incubated with 1 $\mu\text{g}/\mu\text{l}$ heparin (Invitrogen-Thermo Fisher Scientific) for 2 days at room temperature, to generate fibrillary forms of tau (F-tau and F-ptau, respectively).

Brain extracts from 2, 3, and 9-month-old P301S mice were obtained (10% w/v) in ice-cold saline buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl_2 , 1 mM EGTA, pH 7.5) containing 1% (w/v) Triton X-100, 10% (w/v) glycerol, and 1 $\times$ cocktail of protease and phosphatase inhibitors, using a homogenizer in ice. In addition, sarkosyl-insoluble tau from 9-month-old P301S animal brain homogenates was isolated as described in [44].

Pharmacological and A β Peptide Treatments

MEK1/2 inhibitor U0126 was obtained from Promega and JNK inhibitor SP600125 was purchased from Sigma-Aldrich.

A β 1–42 peptide was purchased from Bachem (Bubendorf, Switzerland). Different aliquots of A β 1–42 were prepared as described previously [12, 45] and stored desiccated at -80°C . To obtain the toxic globular oligomers of A β 1–42, ADDLs, a working solution of 100 μM was prepared in culture medium and incubated at 4°C for 24 h. Cultures were treated with a final concentration of 10 μM for 6 h.

Western Blotting Techniques

Soluble extracts from 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, 0.5% sodium deoxycolate, and 1 μM okadaic acid (Merck Millipore, MA, USA), 0.1 M sodium fluoride, 10 mM sodium pyrophosphate, and 1 mM sodium orthovanadate (Sigma-Aldrich) as phosphatase inhibitor. After this, samples were centrifuged at 13,000g for 20 min at 4°C . The resulting supernatant was normalized for protein content using BCA kit (Pierce Biotechnology, Waltham, MA, USA). Cell extracts were boiled in Laemmli sample buffer [46] at 100°C for 10 min, followed by 10% SDS-PAGE electrophoresis, and they were then electrotransferred to nitrocellulose membranes for 1 h at 4°C . Membranes were blocked with 5% non-fat milk in 0.1 M Tris-buffered saline (pH 7.4) for 1 h 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).

For western blot quantification, developed films were scanned at 2400×2400 dpi (i800 MICROTEK high-quality film scanner), and the densitometric analysis was performed using Fiji™ software (National Institutes of Health, USA).

Primary Antibodies

Several antibodies were used for western blot. To detect A β species, we used 6E10 (Covance, NJ, USA), which specifically recognizes the first 16 amino acids of the A β 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 thr¹⁸¹ (Signalway Antibody, MA, USA), anti-ptau ser^{199/202} (pSpS) (Invitrogen-Thermo Fisher Scientific), anti-ptau ser²⁰²/thr²⁰⁵ (AT8), and anti-ptau ser^{396/404} (PHF1), a gift from Peter Davies (Albert Einstein College of Medicine, Bronx, NY, USA). Total levels of the tau protein were checked with monoclonal Tau5 antibody (Invitrogen-Thermo Fisher Scientific). Different tau isoforms 0N3R and 2N4R were checked with monoclonal RD3 and RD4 antibodies, respectively (Merck Millipore). Anti-pERK1/2 ser²⁰²/tyr²⁰⁴, anti-pJNK thr¹⁸³/tyr¹⁸⁵, and anti-JNK were from Cell Signaling Technology (Beverly, MA, USA). panERK was from Transduction Laboratories (Lexington, KY, USA). Anti-p-c-jun ser73 and anti-c-jun were from Abcam (Cambridge, MA, USA). Anti-Sp1 and the monoclonal anti- α -actin antibody (used as internal control in western blots) were obtained from Merck Millipore. Anti-Bcl-2 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Immunocytochemical Procedures

For immunocytochemistry, fixation of cultures was performed with 4% paraformaldehyde (Sigma-Aldrich) for 10 min at 4°C . Between each step described below, permeabilization and washing of coverslips were carried out three times in phosphate buffered saline (PBS)-Triton™ X-100 0.1% (Sigma-Aldrich). Non-specific sites were blocked by treating cells with a blocking solution with 10% FBS in 0.1-M PBS for 1 h. Then, primary antibodies were diluted in the blocking solution using a dilution of 1:500 monoclonal anti-PrP 6H4. Coverslips were incubated with primary antibodies for 16 h at 4°C . After incubation with primary antibodies, cells were incubated with the pertinent Alexa Fluor-tagged secondary antibodies (Alexa-488 goat anti-mouse 1:500, Invitrogen-Thermo Fisher Scientific). Finally, cells were stained with 0.1 μM DAPI (Sigma-Aldrich), diluted in 0.1 M PBS, mounted on Mowiol™ (Calbiochem, San Diego, CA, USA), and viewed using an Olympus BX61 epifluorescence microscope.

To determinate internalization of tau protein, HEK293 cells were trypsinized with trypsin-EDTA 0.25% (Invitrogen-Thermo Fisher Scientific) for 3–5 min to remove surface-bound tau aggregates. Cells were centrifuged at 1100g for 5 min, re-plated in media, and allowed to recover for 6 h at 37 °C before fixation for immunocytochemistry. Monoclonal anti-tau antibody (Tau5) (Invitrogen-Thermo Fisher Scientific) was used to detect total tau protein, and cells were viewed using a Zeiss confocal microscope with Spectral Detection LSM 800. Confocal stacks of images were obtained (z -axis interval, 2 μ m), and Z-projections were analyzed using the ZEN Blue Edition software (Zeiss). Corrected total cell fluorescence (CTCF) measurement was performed using Fiji™ software following published instructions <http://sciencetechblog.com/2011/05/24/measuring-cellfluorescence->. See also [47] for details. CTCF values were determined using the following formula. CTCF = Integrated Density – (Area of selected cell $\times$ Mean fluorescence of background readings).

Determination of PRNP Promoter Activity

To determine *PRNP* promoter activity, 4.5×10^4 HEK293 or C17.2 cells were seeded 24 h before transfection into 24-well plates. Cells were co-transfected with Renilla luciferase reference control plasmid pRL-SV40 (Promega) or tau overexpression constructs in some cases. Cell lysates were prepared in passive lysis buffer following the manufacturer's instructions, and luciferase reported activity was measured with an Infinite M200 Pro Microplate Reader luminometer using the dual-luciferase assay system (Promega). Background luminosity was subtracted from untransfected cells and luciferase readings normalized by dividing the reported gene firefly luciferase by the reference Renilla luciferase activity. Each construct was transfected in triplicate on three different days.

Determination of ROS Production

ROS generation was analyzed with two methods. For the first, cells were incubated with fluorescence probe Dihydroethidium (DHE, Sigma-Aldrich) at 1 μ g/ml, and 610 nm emission was monitored with an Infinite M200 Pro Microplate Reader. For the second, Cys-oxidation biotin switch analysis was performed firstly with the alkylation of reduced cysteines, followed by reducing and biotin labeling of reversibly oxidized cysteines. Cell lysates were blocked in 200 mM Tris (pH 8.5), 5 mM EDTA, 6 M Urea (Sigma-Aldrich), 0.05% SDS, and 20 mM S-Methyl methanethiosulfonate (MMTS, Sigma-Aldrich) for 20 min at 50 °C. Then, they were incubated with 20% TCA (Sigma-Aldrich) for 30 min at –20 °C in order to precipitate proteins. After centrifugation for 10 min at 20,000g, the pellets were washed twice with acetone and

centrifuged for 5 min at 20,000g, at 4 °C. Oxidized cysteines were reduced by resuspending pellets in 200 mM Tris (pH 8.5), 5 mM EDTA, 6 M Urea (Sigma-Aldrich), 0.05% SDS, and 10 mM DL-Dithiothreitol (DTT, Sigma-Aldrich), and then incubating them for 30 min at 30 °C. Following incubation with 20% TCA (Sigma-Aldrich) for 30 min at –20 °C and centrifugation for 10 min at 20,000g, the pellets were washed twice with acetone and centrifuged for 5 min at 20,000g, at 4 °C. Biotin labeling was performed by resuspending pellets in 200 mM Tris (pH 8.5), 5 mM EDTA, 6 M Urea (Sigma-Aldrich), and 0.05% SDS, followed by incubation with 4 mM EZ-Link™ HPDP-Biotin (Invitrogen-Thermo Fisher Scientific) in N-Dimethylformamide (DMF, Sigma-Aldrich) for 2 h at room temperature. Proteins were precipitated by incubation with 20% TCA (Sigma-Aldrich) for 30 min at –20 °C and centrifugation for 10 min at 20,000g. Finally, pellets were washed twice with acetone, centrifuged for 5 min at 20,000g, at 4 °C, and resuspended in 200 mM Tris (pH 8.5), 5 mM EDTA, 6 M Urea (Sigma-Aldrich), and 0.05% SDS.

HPDP-Biotin labeled samples were transferred to nitrocellulose membranes and blocked with 5% non-fat milk in 0.1-M Tris-buffered saline (pH 7.4) for 1 h. After incubation with streptavidin-biotinylated peroxidase complex (1:5000 diluted) (Amersham-GE Healthcare), membranes were revealed with ECL-plus chemiluminescence (Amersham-GE Healthcare). For quantification, developed films were scanned at 2400 $\times$ 2400 dpi (i800 MICROTEK high-quality film scanner, Hsinchu, Taiwan), and the densitometric analysis was then made using Fiji software.

Transmission Electron Microscopy Procedures

For transmission electron microscopy (TEM), tau solutions were fixed to carbon-forward-coated copper supports. Negative staining was performed using a 2% uranyl acetate stain (pH 7.4) (Sigma-Aldrich), after which samples were placed in silica-based desiccant for a minimum of 2 h. Finally, we proceeded to TEM observation using a Jeol JEM-1010 microscope at the Electron Microscopy Service, Centros Científicos y Tecnológicos de la Universidad de Barcelona (CCITUB), Barcelona, Spain.

Statistical Processing

Data analysis was performed using the Prism 8.0 (GraphPad Software, CA, USA). Data were obtained using Student *t* test. Differences with $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$ were considered significant. All data are expressed as mean $\pm$ standard deviation of the mean (SD).

Results

Parallel Increase of Tau and PrP^C After ADDL Treatment In Vitro and In Vivo

First, we aimed to determine the role of ADDLs on PrP^C levels in vitro and in vivo in mouse models. Thus, primary cultures from WT embryos were treated with 10 μ M ADDLs or vehicle for 6 h as reported [13], and collected samples were analyzed with western blot. Results revealed that PrP^C protein levels increased $\sim$ 17.97% after ADDL treatment (Fig. 1a, Supplementary Fig. 1a). This

was also in correlation with a significant $\sim$ 12.02% increase in total tau (Fig. 1a), although p-ser^{396/404}, p-ser^{202/thr²⁰⁵}, and p-thr¹⁸¹ epitopes were unchanged in these conditions (data not shown). Next, five young-adult *Prnp*^{+/+} mice were unilaterally injected with 13.5 μ g of ADDLs into the dorsal hippocampus (see the “Materials and Methods” section). Western blot analysis demonstrated a $\sim$ 54.10% increase in PrP^C levels in ipsilaterally injected hemisphere and a parallel tau level increase (around $\sim$ 17.32%) compared with contralateral hippocampus (Fig. 1b, Supplementary Fig. 1b). Changes in tau phosphorylation were not observed.

Fig. 1 Changes in PrP^C levels by ADDLs or tau. **a–d** Western blot analysis using total anti-tau antibody (monoclonal Tau5) in parallel with anti-PrP^C antibody (monoclonal 6H4) in each case. Actin detection was used as control loading protein. **a** Representative primary cortical cultures from WT mice at 7 DIV treated with either ADDL or vehicle alone ($n = 4$ each case). **b** Representative hippocampal extracts from two ADDL-injected mice ($n = 5$). Contralateral hemisphere was used as control. **c** Representative brain extracts from WT (*Mapt*^{+/+}) and *Mapt*^{-/-} mice ($n = 3$ in each case). **d** C17.2 culture transfected with mock or human 0N3R tau or 2N4R tau expression plasmids (pSG5, pSGT30, or pSGT42, respectively) ($n = 3$ in each case). **e–h** Immunocytochemical detection of PrP^C expression from primary cortical cultures of WT (**e–f**) or tau-tagged GFP mice (TgTP6.3) (**g–h**). Arrows in **g** and **h** point to some cells expressing high levels of PrP^C (Alexa-488 emission) and low GFP emission, thus ruling out spectral overlapping in the two images. Scale bar = 100 μ m in **h**

PrP^C Levels Are Mediated by Tau Expression in the Absence of ADDL Treatment

As the increase in tau and PrP^C levels correlated in the two models, we analyzed PrP^C expression in *Mapt*^{-/-} mouse brain samples with western blot in loss-of-function experiments (Fig. 1c). Results showed a significant ~29.92% decrease in PrP^C expression in *Mapt* knockout mice. Interestingly, PrP^C levels remained constant in primary cortical cultures from *Mapt*^{-/-} mice after ADDL treatment for 6 h in contrast to primary cultures from *Mapt*^{+/+} littermates (Supplementary Fig. 2). Conversely, we developed gain-on-function experiments by overexpressing tau in cellular models and analyzing PrP^C levels in the absence of ADDL treatment. To do this, we developed two strategies: first, levels of PrP^C were analyzed in transiently transfected C17.2 cells with human 0N3R tau and 2N4R tau expression vectors with western blot (Fig. 1d), and second, we measured PrP^C levels in TgTP6.3 primary cultured neurons that overexpress tau-GFP transgene with immunocytochemistry (Fig. 1e–h). Densitometric analysis of developed films of tau-transfected C17.2 cells showed an increase in PrP^C levels of ~47.87% (for tau3R) and ~50.15% (for tau4R) when compared with non-transfected cells (Fig. 1d, Supplementary Fig. 1c). In addition, CTCF was measured in PrP^C immunocytochemistry images using Fiji™ (see the “Materials and Methods” section for details). In this sense, levels of PrP^C (red channel) were measured in 20 cells from three independent images belonging to different cultures, and results showed a significant ~80.29% increase in TgTP6.3 primary cultures when compared with WT (Fig. 1f, h and Supplementary Fig. 1d).

Human PRNP Promoter Activation by Tau, Independently of ROS Levels

To further explore the role of tau in regulating human *PRNP* gene expression, we used HEK293 cells as an additional non-neuronal cell line to avoid potential noise due to the endogenous expression of tau in C17.2 cells. First, we analyzed the endogenous PrP^C levels in this model to ensure that it possessed the necessary transcriptional machinery. Developed films showed detectable PrP^C expression in HEK293 cells at 2 days in vitro (DIV) (Supplementary Fig. 3). So, to explore the human *PRNP* promoter response to tau, HEK293 cells were transiently transfected with -901 to +144 5' flanking sequence of the *PRNP* gene cloned into the luciferase reporter vector pGL2 Basic. In addition, cells were transfected with pRL-SV40 vector for Renilla luciferase expression. Then, overexpression of tau was induced by co-transfection with 2N4R tau or 0N3R tau expression vectors, respectively, and compared with ADDL-treated cells. In parallel, promoter activation by ROS was induced as a positive control by treating cells with 1 mM H₂O₂ for 30'. Thirty hours after transfection,

cell lysates were collected in passive lysis buffer, and dual-luciferase reporter assay was performed by sequentially measuring the Firefly and Renilla luciferase activity in each sample. Normalized quantification of results showed that *PRNP* promoter activity did not increase after ADDL treatment when compared with vehicle treatment, whereas tau transfection (both 0N3R tau and 2N4R tau) increased luciferase activity by 2.06 ± 0.14 and 2.11 ± 0.37 times when compared with empty vector pSG5 (mock transfection) (Fig. 2a). This increase is also similar to the oxidative stress induced by H₂O₂ treatment in our model (2.10 ± 0.19 times when compared with control) (Fig. 2a). Levels of tau expression after transfection were confirmed by western blot in cell lysates (Fig. 2b).

Subsequently, we demonstrated the specificity of tau in modulating *PRNP* promoter activity through transfection of Bcl-2 expression vector in the same system. Overexpression of unrelated protein Bcl-2 showed no significant change in luciferase activity (Supplementary Fig. 4).

In a parallel set of experiments, C17.2 cell line was used to confirm *PRNP* promoter response to tau overexpression in a neuronal cell line. Luciferase activity showed an increase with both tau transfection vectors (1.87 ± 0.02 for 0N3R tau and 1.68 ± 0.08 for 2N4R tau) and 1.82 ± 0.20 for H₂O₂ treatment when compared with the empty vector or non-treated cells, respectively, as observed in HEK293 cells (Supplementary Fig. 5). So, subsequent experiments were performed in a HEK293 cells.

Finally, we checked whether either 0N3R tau or 2N4R tau was able to induce per se ROS generation. Thus, we incubated tau-transfected HEK293 cells with the fluorescence probe DHE to monitor ROS generation in our treatment. In addition, the imbalance in the levels of ROS results in reversible cysteine oxidation (Cys_{OX}) [48]. On account of this, identification of total reversible Cys_{OX} was analyzed in our samples using a biotin switch assay. Analysis of three independent experiments showed ROS generation only in cells treated with H₂O₂ (Fig. 2c, e) and a correlative higher content of Cys_{OX} (Fig. 2d), indicating that activation of *PRNP* promoter by tau is not a consequence of oxidative stress but rather works in an independent way.

The Amount of Intracellular Tau Correlates with PRNP Promoter Activity After Exposure to Extracellular Tau

As tau expression enhances *PRNP* promoter activity, we aimed to explore the participation of extracellular tau species in *PRNP* transcription. Both ADDLs and extracellular tau species play a crucial role during AD evolution. Thus, in a first set of experiments, we used the homogenate brain extract from 9-month-old transgenic P301S mice [49]. P301S brain extract (7 µl, 8 µg/µl) as well as sarkosyl-insoluble fractions (7 µl) (Fig. 3a) was added to HEK293 cells for 24 h to analyze *PRNP* promoter activity. WT mouse brain extract and the

Fig. 2 Functional analysis of the *PRNP* promoter in HEK293 cells after H_2O_2 or ADDL treatment and tau transfection. **a** Bars represent the mean $\pm$ SD of relative light units (RLU) standardized to untreated cells (control) obtained from $n = 6$ experiments performed in triplicate. **b** Western blot analysis showing the two tau isoforms (tau3R and tau 4R) in cell extracts after 0N3R tau and 2N4R tau transfection in HEK293 cells. Actin detection was used as control loading protein. **c, d** Histograms illustrating quantitative ROS generation by the quantification of fluorescence nuclei after dihydroethidium (DHE) incubation (**c**) or cysteine oxidation (Cys_{ox}) (**d**) in samples analyzed in **a**. Bars represent the mean $\pm$ SD of three independent experiments. **e** Phase contrast and parallel fluorescence of cells incubated with DHE (quantitative results showed in **c**). Scale bar = 100 μm . Differences between ADDLs vs vehicle were not statistically significant. However, the differences between groups (H_2O_2 vs control or tau vs mock transfection) were considered statistically significant at **** $p < 0.0001$, *** $p < 0.001$, and * $p < 0.05$ (t test)

vehicle of the sarkosyl extraction were used as controls in each case. Surprisingly, only brain extracts from P301S but not from sarkosyl-insoluble fractions showed a 1.65 ± 0.04 increase in *PRNP* promoter activity (Fig. 3b). In addition, cells incubated with the fluorescence probe DHE after P301S brain extract treatment showed no fluorescence increase (Fig. 3c). This ruled out the generation of ROS as being responsible for the observed changes in *PRNP* promoter activity. In addition, increased *PRNP* promoter activity correlated with an increased intracellular presence of tau only after P301S brain homogenate treatment but not in sarkosyl-insoluble fractions

or WT treatments measured with western blot (Fig. 3d), nor with CTCF microscopic analysis (Fig. 3e, f), suggesting that only internalized tau was able to increase *PRNP* transcription. In this sense, CTCF measurement of tau levels (green channel) was obtained in all cells from three random images and from three independent experiments (Fig. 3f). Lastly, to correlate tau levels and their capability of internalization with subsequent *PRNP* promoter activation, we incubated HEK293 cells with brain extracts from younger P301S mice (2–3 months old), since their expression of the mutated form of tau is lower compared with older mice (> 9 months old)

Fig. 3 Soluble fractions from P301S mouse brain promotes *PRNP* promoter activation by internalization. **a** Western blot analysis of mouse brain fractions used in functional analysis of *PRNP* promoter: soluble brain extracts from WT mice (1), soluble brain extracts from P301S mice (2), and sarkosyl-insoluble fractions from P301S mice (3). **b** Histograms illustrating the *PRNP* promoter activity of HEK293 cells treated with P301S brain extract or P301S sarkosyl-insoluble fraction and compared with untreated cells (control), control brain extract (WT), and vehicle (VH), respectively. Bars represent the mean $\pm$ SD of relative light units (RLU) standardized to untreated cells (control) obtained from $n=2$ experiments performed in triplicate. **c** Phase contrast and parallel fluorescence of HEK293 cells incubated with DHE after soluble brain extract treatments (WT and P301S, respectively). H_2O_2 treatment was taken as positive control of ROS generation. Scale bar = 100 μ m. **d–f** Correlation between tau internalization and increased *PRNP* promoter

activity was made with western blot (**d**) and confocal microscopy (**e**, **f**), respectively. Samples analyzed in **d** correspond to HEK293 lysates after WT (1) and P301S (2) brain extract or sarkosyl-insoluble fraction (3) treatments, and immunoblotted with total anti-tau antibody (Tau5) and anti-actin as control loading protein. **e** Z-stacks projection confocal microscopy images showing internalized tau (Tau 5 antibody) in HEK293 cells. Note the presence of tau protein (green) only after P301S brain extract treatment but not after P301S sarkosyl-insoluble fraction treatment. WT and VH represent control treatments in each case. Scale bar = 50 μ m. **f** CTCF values derived from the analysis of the tau-derived immunofluorescence in the cytoplasm of HEK293 cells after P301S brain extract and sarkosyl-insoluble treatments, respectively, relative to their control in each case. Differences between groups were considered statistically significant at **** $p < 0.0001$, *** $p < 0.001$, and ** $p < 0.01$ (t test)

[50]. As shown in Supplementary Fig. 6, extracts from 2 to 3 month-old mice were unable to increase *PRNP* promoter activity despite presenting the mutated protein (Supplementary Fig. 6a). This suggests that only if there are high enough levels of protein, independently of the mutation, can it internalize and activate the *PRNP* promoter.

Additionally, it is well known that tau internalization largely depends on its quaternary structure and aggregative stage [51–53]. Indeed, tau internalization is enhanced by its phosphorylation [54]. Thus, in the following experiments, we used oligomeric (O-tau/O-ptau) or fibrillary (F-tau/F-ptau) forms from 2N4R tau and phospho-2N4R recombinant tau (Fig. 4a) (see the “Materials and Methods” section) to elucidate their effects on *PRNP* promoter activity. The different aggregative state was analyzed with TEM (Fig. 4b) and western blot (Supplementary Fig. 7), respectively. To perform the experiment, 65 nM of each tau variant was added to HEK293 cells for 24 h before promoter activity was measured, as above. Results indicated that only O-ptau was able to increase *PRNP* promoter activity by 1.38 ± 0.05 times when compared

with its vehicle (Fig. 4c). Western blot analysis confirmed that only these oligomeric phospho-tau variants were capable of being internalized by the plasma membrane in our conditions (Fig. 4d). This was also corroborated by CTCF analysis from confocal microscopy images, following the procedure described for Fig. 3e, f (Fig. 4e, f).

The Promoter Regulatory Elements in Tau Triggered *PRNP* Transcription

The most active consensus DNA-binding sites in *PRNP* promoter region include TGACTCA for transcription factor complex AP-1 (mapped in position – 120) and a GC box element for Sp1-binding (mapped in – 62, + 6, and + 35) (Fig. 5a). In fact, AP-1 binding may be involved in higher levels of *PRNP* expression in neurons (see above) where neuronal protein tau promotes assembly and stabilization of microtubules. Likewise, Sp1 is regulated by oxidative stress, and an abnormal expression of this transcription factor has been reported in human tauopathies, including AD and PSP [55]. Therefore,

Fig. 4 Internalized non-fibrillary species of recombinant tau promotes *PRNP* promoter activation. **a**, **b** Recombinant tau and ptau used to perform functional analysis of *PRNP* promoter. **a** Western blot analysis showing phosphorylated and non-phosphorylated tau species before fibrillation protocol. Total tau antibody (Tau5) and anti-ptau ser^{396/404} (PHF1) were used in both cases. **b** Transmission electron microscopy images of negatively stained tau protein after oligomeric tau (O-tau/O-ptau) and fibrillary tau (F-tau/F-ptau) formation with species shown in **a** (phosphorylated and non-phosphorylated, respectively). Scale bar = 200 nm. **c** Histograms illustrating the *PRNP* promoter activity of each analyzed tau variant and their corresponding vehicles when compared with control. Bars represent the mean $\pm$ SD of relative light units (RLU) standardized to untreated cells (control) obtained from $n = 3$ experiments

performed in triplicate. **d–f** Correlation between tau internalization and increased *PRNP* was made with western blot (**d**) and confocal microscopy (**e**, **f**), respectively. Samples analyzed in **d** correspond to HEK293 lysates after ptau treatments, both O-ptau and F-ptau, and immunoblotted with Tau5 antibody and actin as control loading protein. **e** Z-stack confocal microscopy images showing internalized tau (Tau 5 antibody) in HEK293 cells after O-ptau treatment in contrast to other tau variants (O-tau, F-tau, and F-ptau) or vehicle treatments. Scale bar = 50 μ m. **f** CTCF values derived from the analysis of the tau-derived immunofluorescence in the cytoplasm of HEK293 cells relative to their vehicle control in each case. Differences between groups were considered statistically significant at $**p < 0.01$ (*t* test)

we first analyzed Sp1 levels and one of the components of the AP-1 complex, c-jun, in HEK293 cells transfected with 0N3R tau and 2N4R tau or treated with 1 mM H₂O₂. Because c-jun is activated through double phosphorylation of serines 63 and

73 by JNK [56], and *PRNP* promoter activation in a Sp1-dependent manner involves ERK1/2 phosphorylation [57], we decided also to analyze activation of JNK and ERK1/2 with western blot. Results showed a slight increase in Sp1

Fig. 5 Determination of the more relevant binding sites in *PRNP* promoter after tau transfection. **a** Scheme of the 5' flanking region of *PRNP* (−901 to +144), excised from pPrPS2.7 and cloned in the luciferase, reported vector pGL2basic. *PRNP* promoter sequence contents AP-1 and Sp1 binding sites. **b, c** Western blot analysis showing Sp1 upregulation (**b**) in parallel to ERK1/2 activation (**c**) after H₂O₂

treatment and c-jun phosphorylation (**b**) in parallel to JNK activation (**c**) after tau transfection. **d, e** Densitometric analysis of data obtained from $n = 3$ experiments and shown in **b**. Differences between groups (H₂O₂ vs control and tau vs mock transfection) were considered statistically significant at ** $p < 0.01$ and * $p < 0.05$ (t test)

protein levels in tau-overexpressing cells (1.38 ± 0.12 for 0N3R tau and 1.24 ± 0.07 for 2N4R tau) in contrast to the almost threefold increase observed after H_2O_2 treatment (2.67 ± 0.28) (Fig. 5b, d). In addition, a correlated ERK1/2 activation of 8.71 ± 1.50 times was only observed after H_2O_2 treatment (Fig. 5c). However, for c-jun, there was a global decrease of total protein but a slight relative increase of its phosphorylated form only in cells overexpressing tau (1.27 ± 0.07 for 0N3R tau and 1.18 ± 0.04 for 2N4R tau) (Fig. 5b, e), in correlation with an increase in phosphorylated levels of JNK (1.13 ± 0.005 for 0N3R tau and 1.16 ± 0.03 for 2N4R tau) (Fig. 5c). We next aimed to determine the participation of ERK1/2 and JNK pathways in transactivation of *PRNP* promoter by using specific kinase inhibitors. Thus, U0126 (50 μ M) and SP600125 (5 μ M) were used to block ERK1/2 and JNK pathways, respectively, in tau-transfected cells and in H_2O_2 -treated cells. Results showed that U0126 but not SP600125 treatment was able to block the increase in *PRNP* transcription triggered by H_2O_2 (Fig. 6a). In contrast, although U0126 reduced *PRNP* promoter activity after tau transfection, only SP600125 promoted a significant decrease in tau-transfected HEK293 cells (Fig. 6b, c).

Lastly, we developed functional analysis of *PRNP* promoter activity using site-directed mutagenesis of relevant AP-1 and Sp1-binding sites. In this sense, Δ AP-1/*PRNP*-pGL2basic and Δ Sp1/*PRNP*-pGL2basic constructs were generated by deletion of 43 residues (mapped between -131 and -88 in *PRNP* promoter sequence) and 139 residues (mapped between -67 and +72) in *PRNP*-pGL2basic vector (Fig. 7a). In addition, the double mutant Δ AP-1/Sp1/*PRNP*-pGL2basic construct was generated by deletion of 203 residues (mapped between -131 and +72 in *PRNP* promoter sequence). Then, each of the three constructs was co-transfected with 2N4R tau and 0N3R tau expression vectors, respectively, or, alternatively, treated with 1 mM

H_2O_2 for 30' to analyze *PRNP* promoter activity. Results from single mutants demonstrated that *PRNP* activity after H_2O_2 treatment was unaffected by the absence of AP-1 (Fig. 7b) but increased when Sp1-binding sites were lacking (Fig. 7c). In contrast, the absence of Sp1, but more relevantly AP-1-binding sites, decreased the *PRNP* promoter activity induced by tau expression (both 0N3R tau and 2N4R tau), by $\sim 48.01\%$ and $\sim 55.53\%$ in each case when compared with full-length *PRNP*-pGL2basic (Fig. 7b, c). In conclusion, tau did not induce significant promoter activity in the absence of AP-1-binding sites while it showed significant promoter activity in the absence of Sp1-binding sites for 2N4R tau and a tendency for 0N3R. After analyzing the promoter activity of the double mutant Δ AP-1/Sp1, we observed a loss of activity of $\sim 35.10\%$ after H_2O_2 although it still showed the ability to increase promoter activity significantly. For tau transfection, neither 0N3R tau nor 2N4R tau was able to activate the double-deleted promoter, showing a decrease in activity similar to single Δ AP-1 mutant.

We next aimed to determine the participation of AP-1 and Sp1-binding sites in *PRNP* promoter activation by internalized tau. HEK293 cells were transfected with single deleted constructs and treated with P301S or WT brain extracts, as described before. Results showed a significant luciferase signal decrease of $\sim 28.92\%$ and $\sim 25.10\%$ in each case when compared with full-length *PRNP*-pGL2basic (Supplementary Fig. 8). This suggests that transfected tau and internalized tau might act in the same pathway, at least partially.

These observations lend support to the idea that the transcription factor AP-1 downstream JNK pathway may mediate tau regulation of the human *PRNP* gene as an independent response of the promoter to oxidative stress. Also, Sp1 may participate in this.

Fig. 6 Effect of kinase inhibitors on *PRNP* promoter activity after H_2O_2 treatment and tau transfection (**a–c**) U0126 and SP600125 were used to block ERK1/2 and JNK pathways, respectively. Bars represent the fold increase in *PRNP* promoter activity after H_2O_2 treatment (**a**), 0N3R tau (**b**), and 2N4R tau transfection (**c**) in the presence or absence of kinase

inhibitors and related to control cells. Data were obtained from $n = 3$ experiments performed in triplicate and are represented by mean $\pm$ SD. Differences between groups were considered statistically significant at $*p < 0.05$ (t test)

Fig. 7 Functional analysis of *PRNP* activity using site-directed mutagenesis of AP-1 and Sp1 binding sites after tau transfection. **a** Scheme of the deletion mutant constructs of *PRNP*-pGL2basic. **b**, **c** Quantitative RLU standardized to untreated cells (control) obtained for each experimental group in full-length *PRNP* promoter construct (*PRNP*-pGL2basic) vs AP-

1 (**b**), Sp1 (**c**), and AP-1/Sp1 (**d**) deleted mutants. Plotted data represent results from $n = 3$ experiments performed in triplicate (mean $\pm$ SD). Differences between full-length *PRNP* promoter and deleted forms were considered statistically significant at *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ (t test)

Discussion

In the present study, we investigated the factors that regulate overexpression of PrP^C in the presence of the two hallmark proteins of AD, ADDLs (resulting from sequential β and γ -secretase activity), and tau [58], as well as the ROS commonly observed in affected brains [59]. To date, both oxidative stress and γ -secretase have been reported as activating factors of the *PRNP* promoter [60, 61]. Our experiments using ADDL treatments, driving overexpression of tau [13] and the present results, revealed intracellular tau levels to be a novel molecular factor involved in *PRNP* transcription. In fact, tau has recently been reported to be responsible for modifying the transcriptome in AD brains [62], so it is possible that this acts on the *PRNP* promoter.

This idea was corroborated by analyzing *PRNP* promoter activity in HEK293 and C17.2 cells through a dual-luciferase reporter assay system, which also confirmed that ROS can activate *PRNP* promoter independently of tau. Then, both transfection with full-length tau (0N3R or 2N4R) and extracellular tau treatments were the two systems we used to induce PrP^C overexpression in cultured cells. Also, we analyzed JNK and ERK1/2 phosphorylation or downstream changes in transcriptional factors c-jun and Sp1 after inducing *PRNP* promoter activation. While the results showed only slight correlations between c-jun and JNK for tau transfection, and between

ERK1/2 and Sp1 in H₂O₂ treatment, subsequent experiments supported our conclusion. So, pharmacological inhibition of ERK1/2 and JNK pathways, as well as promoter disruption of AP-1 and Sp1 binding sites in *PRNP* promoter, demonstrated that after tau overexpression, AP-1 is the most relevant downstream effector modulating *PRNP* promoter through activation of JNK-c-jun signaling. However, we cannot completely rule out a role for ERK1/2-Sp1 signaling in the *PRNP* promoter activity triggered by tau since, although we did not observe significant activation of the ERK1/2 kinases after tau overexpression, this was likely because the activation peak would have decreased by the time of sample collection; U1026 treatments and experiments using the *PRNP* promoter lacking the SP1-binding site showed that promoter activation by tau was reduced. This is also corroborated by results from deleted mutants treated with WT or P301S brain extracts where promoter activity is affected by deletion of the two binding sites, AP-1 and Sp1, respectively, although in no case was there a total loss of the promoter's function. Interestingly, AP-1 deletion promotes a baseline decrease in *PRNP* transcription, pointing up this element as one of the more relevant transcriptional factors in maintaining endogenous PrP^C levels.

Furthermore, neither inhibition of JNK nor deletion of AP-1-binding sites affects *PRNP* activity under ROS. Surprisingly, although inhibition of ERK1/2 signaling reduces *PRNP* activation through ROS, ablation of Sp1 binding sites

enhances *PRNP* transcription triggered by H_2O_2 . So, there may exist parallel transcriptional factors activated under oxidative stress conditions. In this sense, *PRNP* promoter regulation by Sp1 has been widely reported [26, 27, 63], and Sp1 is a target of ERK1/2 in increasing *PRNP* promoter activation [26, 57]. However, additional transcriptional factors involved in *PRNP* transcription have been reported (e.g., HSE, MTF-1) [26, 27, 64]. In addition, Sp1 plays a negative regulatory role in MTF element [65] which responds to a variety of stress signals including H_2O_2 [66] and presents one putative binding site at -90, near a deleted region in Sp1 mutant [27]. In this sense, Sp1-deleted mutant may increase MTF response to ROS above the levels found in the full *PRNP* promoter. However, single deletion of AP-1 or double mutant AP-1/Sp1 drag may be a collateral effect of the loss of this MTF-MRE-binding site. As *PRNP* promoter activity induced by H_2O_2 was not affected by AP-1 deletion but was strongly reduced in the absence of the two binding sites, results confirm the independence of AP-1 from ROS *PRNP* promoter response. Even so, the absence of Sp1 is not enough to abolish the activity of the H_2O_2 -induced *PRNP* promoter, so H_2O_2 may need additional transcription factors to Sp1 in addition to MTF.

In AD-affected brains, Sp1 is overexpressed, and it has been pointed up as a putative target in the disease [67]. However, Santpere et al. suggested a sequestration of Sp1 by tau aggregates in AD and other tauopathies [55]. Because PrP^C presents antioxidant activity [7, 8, 68, 69] and has a role in the regulation of copper homeostasis [6, 70], commonly altered in AD [71], it is encouraging to know that other molecular factors can enhance *PRNP* transcription in this scenario.

An increase in the amount of intracellular tau, either by overexpression or by internalization, may be associated with post-translational modifications of the protein such as hyperphosphorylation and subsequent aggregation in NFTs, a common factor in all tauopathies associated with toxic effects (e.g., [72, 73]). In AD, tau overproduction mediated by ADDLs might induce tau release to extracellular space [43]. In addition, although not all tauopathies have been linked to mutations in *MAPT* gene, these diseases usually present alterations in the ratio of 3R and 4R isoforms resulting from the alternative splicing [74]. Thus, we tried to determine under what conditions tau protein has the ability to activate the promoter. Our results showed firstly that the phosphorylation level of tau, as well as aggregation state, plays at least an indirect role in *PRNP* activation, as only O-ptau and P301S brain extracts from 9-month-old mice, which present high levels of ptau protein, lead to *PRNP* promoter activation. In both cases, we observed a correlation between promoter activation and intracellular levels of tau by western blot or confocal microscopy. And neither monomeric nor fibrillar forms promoted the transcription despite being phosphorylated.

Although we have not done specific experiments to block the entry of tau into cells, relevance of the degree of phosphorylation in internalization has previously been demonstrated [51–54, 75]. In this sense, extracellular variants of tau may promote overexpression of PrP^C only when internalized into cells, and this depends not only on phosphorylation but also on aggregative state, with the O-ptau having greater capability. In fact, the role of tau aggregation intermediates in neurodegeneration has been reported [76] as occurring with oligomeric species of other self-aggregative polypeptides such as A β [77] and several prion peptides [78]. In this sense, endocytic capability of O-ptau may be advantageous at initial stages of AD by promoting overexpression of PrP^C. Later, in the progression of AD, tau fibers' pull may increase and with this the inability to be internalized and to promote *PRNP* promoter activation. Additionally, injection of ADDLs in mouse brains as well as WT cortical cultures treated with ADDLs suggests that non-phosphorylated variants of tau may be able to increase the expression of PrP^C, as there is no detectable tau phosphorylation upon PrP^C overexpression. However, we only analyzed three epitopes in these samples, p-ser^{396/404} (which represents an early event in AD [79]), p-ser²⁰²/thr²⁰⁵, and p-thr¹⁸¹. So, additional experiments are needed to rule out a direct role of phosphorylation, as well as aggregation state, in *PRNP* transcription. Secondly, our results also demonstrate that overexpression of both isoforms, 3R and 4R, can promote the activation of the *PRNP* promoter, which would indicate that in any of the known tauopathies, in which there is an imbalance between the different isoforms, it is possible that there is an overexpression of PrP^C at the beginning of the disease. However, this observation needs to be further analyzed, since to date, studies of PrP^C levels have been performed only in patients at advanced disease states [17]. And thirdly, intracellular increase of the WT protein, after transfection with 2N4R tau or treatment with recombinant protein, has the same activating capacity of the *PRNP* promoter as the P301S tau gene mutation which causes rapidly lethal and early-onset FTDP-17 [80]. In this sense, results of promoter activity induced by brain extracts from young P301S mice have demonstrated that *PRNP* promoter activation does not depend on the mutation of the protein but rather on the internalization capability of tau, which in turn depends on protein levels in addition to the state of phosphorylation and aggregation. This suggests that the role of PrP^C in tau may be a physiological mechanism to control tau level and is not related exclusively to tauopathies.

Taken together, our results indicate that the cellular elevation of tau levels (by overexpression or internalization) promotes *PRNP* promoter activation independently of ROS generation. Also, AP-1 transcription factor is involved only in *PRNP* promoter response induced by tau but not by oxidative stress. In conclusion, our results describe a new regulator of *PRNP* transcription with a significant role in tauopathies such

as AD. However, additional studies are required to examine tau function in *PRNP* promoter in healthy brains.

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Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

Ethics Approval 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# 276/16).

Consent to Participate Not applicable.

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