Prostaglandin A1 inhibits the phosphorylation of tau via activating protein phosphotase 2A in a michael addition mechanism at the site of cysteine 377

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Research

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Abstract

Background: Prostaglandin (PG) A1 is a metabolic product of cyclooxygenase 2 (COX-2), which potentially involved in regulating the development and progression of Alzheimer's disease (AD). As a cyclopentenone (cy) PG, PGA1 is characterized by the presence of a chemically reactive α, β-unsaturated carbonyl. Although PGA1 is potentially involved in regulating multiple biological processes via michael addition, its specific roles in AD remained unclear.

Methods: The tau^{P301S} transgenic (Tg) mice were employed as *in vivo* AD models and neuroblastoma (N) 2a cells as *in vitro* neuronal models. By intracerebroventricular injected (i.c.v) with PGA1, the binding proteins to PGA1 are analyzed by HPLC-MS-MS. In addition, western blots are used to determine the phosphorylation of tau in PGA1 treated Tg mice in the absence or presence of okadaic acid (OA), an inhibitor of protein phosphotase (PP) 2A. Combining a synthesis of pull down assay, immunoprecipitation, western blots and HPLC-MS-MS, PP2A scaffold subunit A alpha (PPP2R1A) was identified to be activated by directly binding on PGA1 in cysteine 377-dependent manner. Via inhibiting the hyperphosphorylation of tau, morris maze test was employed to determine the inhibitory effects of PGA1 on cognitive decline of tau^{P301S} Tg mice.

Results: By incubation with neuroblastoma (n)2a cells and pull down assay, mass spectra (MS) analysis revealed that PGA1 binds with more than 1000 proteins, among which contains the proteins of AD, especially tau protein. Moreover, short-term administration of PGA1 to tau^{P301S} Tg mice significantly decreased the phosphorylation of tau at the sites of Thr181, Ser202 and Ser404 in a dose-dependent manner. To the reason, it's caused by activating PPP2R1A in tau^{P301S} Tg mice. More importantly, PGA1 has the ability to form michael adduct with PPP2R1A via its cysteine 377 motif, which is critical for the enzymatic activity of PP2A. By activating PP2A, long-term application of PGA1 to tau^{P301S} Tg mice significantly reduced the phosphorylation of tau, which results in improving the cognitive decline of tau^{P301S} Tg mice.

Conclusion: Our data provided the first insights needed to decipher the mechanisms underlying the ameliorating effects of PGA1 on cognitive decline of tau^{P301S} Tg mice via activating PP2A in a PPP2R1A^{C377}-dependent Michael adducting mechanisms.

Introduction

Alzheimer's disease (AD) is an occult and progressive neurodegenerative disease in central nervous system. The main clinical syndrome is the cognitive dysfunction. Although the etiology and pathogenesis of AD have not been thoroughly elucidated, the deposition of β-amyloid protein (Aβ) in β-amyloid plaques (APs) and hyperphosphorylated tau in neurofibribillary tangles (NFTs) are still regarded as the pathological characteristics of AD (1). There are at least 50 million AD patients in the world as a chronic disease and the number of the patients will increase dramatically with the increase of age, which results in heavy burden to the family and society.

Although the pathogenesis of AD is not thoroughly clear, some clinical and experimental studies have shown that the occurrence and development of AD are related to metabolic disorders. For instances, the risk of AD in patients will increase significantly with the metabolic disturbance in glucose and cholesterol (2, 3). Interestingly, metabolic disorders in either glucose or cholesterol will result in the activation of cyclooxygenase-2 (COX-2) (4, 5). As a metabolic enzyme, the activation of COX-2 can significantly accelerate the occurrence and development of AD, including the production and deposition of Aβ and phosphorylated tau proteins, which impaired the learning ability and memory (6). In this process, the inflammatory mechanisms of COX-2 play the key roles in AD (7). The administration of COX-2 specific inhibitor can improve the learning and memory ability of APP/PS1 transgenic (Tg) mice by inhibiting the production and aggregation of Aβ and phosphorylated tau protein, which result in reducing the apoptosis of neurons and promoting the neurogenesis (8). As an important synthases of PGs, the activation of COX-2 will inevitably affect the metabolism of PGs. The over accumulation of PGE_2 in the brain can activate presenilin (PS) 1/2 by upregulating tumor necrosis factor (TNF)-α, leading to promote the production and aggregation of Aβ and ultimately damage the learning and memory ability of APP/PS1 Tg mice (9). PGI_2 promotes the amyloid metabolism of amyloid precursor protein (APP) by activating APH-1α and − 1β and increases the production and deposition of Aβ, which impaired the learning and memory ability of AD mice (10).

Different from PGE_2 and PGI_2, PGD_2 can inhibit the activity of APH-1α/1β and PS1 (11). In this process, DP receptor mediates the action of PGD_2 (11). As the self dehydrating product of PGD_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) also showed inhibition on the activity of APH-1α/1β and PS1 (11). More interestingly, only high concentration of 15d-PGJ_2 can inhibit the activity of γ-secretase, while low concentration of 15d-PGJ_2 activates PS1 and PS2 (9). However, the questions are easily raised why 15d-PGJ_2 played different biological functions from other PGs in AD. Indeed, 15d-PGJ_2 belongs to cyclopentenone (cy) PGs family. A series of reports have proved that 15d-PGJ_2 can bind to transcription factors such as peroxisome proliferators-activated receptor (PPAR) γ or NF-κB (12, 13) and redox-regulated proteins like H-Ras, Keap-1 or GSTP-1 (14–16) to inhibit inflammation. In addition, 15d-PGJ_2 can exert anti-inflammatory functions through noncovalent bonds with PG receptor (such as DP2, etc.) (17).

Apart from 15d-PGJ_2, PGA1 is another important member of cyPGs. Compared to 15d-PGJ_2, the roles of PGA1 in AD are highly overlooked. Since cyPG is different from the classical PGs, PGA1 contains α- and β-unsaturated ketone groups and an electrophilic center, which makes it more likely to have michael addition reaction with other proteins, leading to different physiological functions, including anti-inflammatory, oxidative stress and transcriptional activity (18). As an important member of cyPGs, PGA1 has shown neuroprotective effect (19). PGA1 can also be directly combined with free sulfhydryl group of cysteine residue through michael addition, which results in strong anti-inflammatory effect (20). In SH-SY5Y cells, PGA1 can reduce rotenone-induced cytotoxicity (21). In C6 glioma cells, PGA1 can up regulate the expression of neurotrophic factors (22). These neuroprotective effects of PGA1 are likely to improve the pathological characteristics of AD. Based on this hypothesis, our prior works have found that PGA1 can reduce the production and deposition of Aβ by inhibiting the activity of presenilin enhancer 2 (PEN2)

(23). During this process, PGA1 induces the efflux of cholesterol in ATP-binding cassette transporter A1 (ABCA1)- and PPAR γ-dependent mechanism, which deactivates γ-secretase subunit, PEN2 *in vitro* and *in vivo* (23).

Although PGA1 has shown its effects on inhibiting the production and aggregation of Aβ, there is no evidence to elucidate the mechanisms of PGA1 in the phosphorylation of tau during the course of AD development and progression. As a dephosphorylating enzyame, protein phosphotase (PP) 2A has been reported to be downregulated in AD (24). For a trimeric serine (Ser)/threonine (Thr) protein phosphatase, PP2A is composed of a 65 kDa scaffolding subunit (A), 36 kDa catalytic subunit (C) and more variable regulatory subunit (B), leading to its diverse functions in inflammation and neurodegeneration (25). Moreover, PP2A is the most efficient phosphotase to dephosphorylate tau a the sites of Ser199, Ser202, Thr205, Thr212, Ser214, Ser235, Ser262, Ser396, Ser404 and Ser409 in neuron (26). By inhibiting the activity of PP2A, okadaic acid (OA), I_1^{PP2A} and I_2^{PP2A} treatment induced the phosphorylation of tau, which results in imparing the spatial memory (27–29).

On the basis of these potential clues, we aimed to elucidate the mechanisms of PGA1 in suppressing the phosphorylation of tau via regulating the activity of PP2A in tau^{P301S} Tg mice. Furthermore, the motifs for the activity of PP2A in mediating the effects of PGA1 on inhibiting the phosphorylation of tau were identified by IP and HPLC-MS-MS. Finally, PGA1 shows its effects on suppressing the cognitive decline of tau^{P301S} Tg mice. On the basis of these observations, PGA1 might be a useful chemical candidate for treating AD.

Materials And Methods

Reagents-

PGA1 and biotin-PGA1 (bio-PGA1) were obtained from Cayman Chemical (Ann Arbor, MI, USA). Streptavidin-HRP (str-HRP) and streptavidin-resin (str-resin) were purchased from GenScript Biotech Corp. (Piscataway, NJ, USA). Antibodies against β-actin, p-tau^{Thr181}, p-tau^{Ser202}, p-tau^{Ser404}, tau and HRP-labeled secondary antibodies were obtained from Cell Signalling Technology (Danvers, MA, USA). Antibody specific for PPP2R1A was purchased from Abcam (Shanghai, China). All primers were synthesized by GENEWIZ, Inc. (Suzhou, Jiangsu, China). OA, an inhibitor of PP2A phosphatases, was got from Santa Cruz Biotechnology (Dallas, TX, USA). All reagents used for the quantitative real-time PCR (qRT-PCR) and SDS-PAGE experiments were purchased from Bio-Rad Laboratories (Hercules, CA, USA), and all other reagents were purchased from Thermo Fisher Scientific/Invitrogen (Shenyang, Liaoning, China), unless specified otherwise.

Tg Mice and treatments-

Tau^{P301S} Tg mice (Stock No. 008169) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA), as a model of AD. Genotyping was performed at 1 month after birth. Mice were housed in a standard environment. 3-month-old Tg mice were intranasally instilled with PGA1 (2.5 mg/kg/day) for 6 months. The learning ability of the mice were determined by Morris water maze test or nest construction assay. After behavior assay, the mice were anesthetized, perfused and fixed before collecting the brains for further physiological and biochemical test.

Morris water maze test-

The mice were trained and tested in a Morris water maze after 6-month treatment with PGA1. Briefly, the mice were pretrained in a circular water maze with a visible platform for 2 d. The mice were then allowed to swim freely in a hidden platform pool by adding milk for 6 d. On the last day, the platform was removed and the times that the mice passed through the memorized platform were recorded.

Nest construction assay-

The mice were housed in cages with corncob bedding for 1 w before the nest construction assay. 2 h before the formal experiments, 8 pieces of paper (5×5 cm^2) were put into the cage for nesting. The nest score were determined by the 4-point system: 1, no biting/tearing and random dispersion of the paper; 2, no biting/tearing of the paper, with paper gathered in a corner/side of the cage; 3, moderate biting/tearing of the paper, with gathering in a corner/side of the cage; and 4, extensive biting/tearing of the paper, with gathering in a corner/side of the cage (30).

Intracerebroventricular Injection-

5 μl of vehicle (DMSO), PGA1 (1 μg/μl) and OA (2 ng/μl) was intracerebroventricular injected (i.c.v.) to the mice. Briefly, the mice were fixed on stereotaxic instrument after anesthesia. The coordinate from the bregma was adjusted to mediolateral, − 1.0 mm; anteroposterior, − 0.22 mm; and dorsoventral, − 2.8 mm. 5 μl solution was slowly injected to the ventricles of the mice. After 48 h, the mice were anesthetized, perfused and fixed before collecting the brains for further physiological and biochemical test.

Cell Culture-

Mouse neuroblastoma (n) 2a cells and human embryonic kidney (HEK) 293T cells were grown in 37 °C with 5% CO_2 on 6 cm tissue culture dishes in DMEM and 10% FBS medium. In a separate set of experiments, cells were grown in serum-free medium for an additional 12 h before incubation with 20 nM OA in the absence or presence of 10 μM PGA1.

qRT-PCR-

qRT-PCR assays were performed with the MiniOpticon Real Time PCR detection system (Bio-Rad Laboratories) using total RNA and the Go Taq One-step Real-Time PCR kit with SYBR green (Promega, Madison, WI). Forward and reverse primers for mouse PPP2R1A were 5'-GGACGTTCAGCTTCGTCTCA-3' and 5'-CAGCAGACAGTGCACATACT-3', respectively. The gene expression levels were normalized to GAPDH. The forward and reverse primers were 5'-GCTCATGACCACAGTCCATGCCAT-3' and 5'-TACTTGGCAGGTTTCTCCAGGCGG-3', respectively.

Western Blot Analysis-

Tissues or cells were lysed with RIPA buffer (Thermo Fisher Scientific, Shenyang, Liaoning, China). The protein concentration of the cell lysates was determined with a BCA protein assay kit (Thermo Fisher Scientific, Shenyang, Liaoning, China). The total cell lysates (20 μg) were subjected to SDS-PAGE, transferred to a membrane, and probed with a rabbit polyclonal antibody to PPP2R1A (1:2000,1 μg/μl), rabbit monoclonal antibody to p-tau^{Thr181}, p-tau^{Ser202}, p-tau^{Ser404} or tau (1:2000, 1 μg/μL) as the primary antibodies. Each membrane was probed with only one antibody. β-actin served as a loading control.

Immunofluorescence-

n2a cells were cultivated on cover slips coated with poly-D-lysine (Merck/Sigma-Aldrich, Shanghai, China) in 6-well plates (Corning Incorporated, NY, USA). After treatment with10 μM PGA1or bio-PGA1 for 2 h, cells were washed with PBS (-), fixed with ice-cold methanol, and rehydrated with PBS (-). The cells were then blocked by a blocking buffer containing 2% BSA and 4% goat serum (Merck/Sigma-Aldrich, Shanghai, China). The slides were then stained with 1:200 diluted iFluor™ Streptavidin-555 (Biolegend, San Diego, CA, USA) for 1 h at room temperature. After extensively washing with PBS (-), the cells were counterstained with DAPI (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China). The slides were finally mounted with fluorescent mounting medium (Dako Corporation, Carpinteria, CA, USA) and observed under confocal microscopy (Leica, Beijing, China).

Cloning procedures-

Sequences encoding PPP2R1A, PPP2R1A^{C377A} and PPP2R1A^{C390A} were ligated to the pBobi vector with 3 × flag-tags at amino-terminus using ligation-independent cloning method. Forward and reverse primers for mouse PPP2R1A were 5'-tagagaattcggatccatggcagctgccgacggtgacga-3' and 5'-gcttccatggctcgagggcaagagagagaacagtca-3'; for mouse PPP2R1A^{C377A} were 5'-tggctcagctgaaggatgaggctcctgaagtccgactgaatat-3' and 5'-atattcagtcggacttcaggagcctcatccttcagctgagcca-3'; for mouse PPP2R1A^{C390A} were 5'-atatcatctccaacctggatgctgtgaacgaggtgattggcat-3' and 5'-atgccaatcacctcgttcacagcatccaggttggagatgatat-3'. Sequences encoding PPP2R1A, PPP2R1A^{C377A},

PPP2R1A^{C390A}, PPP2R2A and PPP2CA were ligated to the pET-28a-c (+) vector with 6 × His-tags at carboxy-terminal. These plasmids were used to produce and purify the recombinant proteins by transfecting BL21 E.coli (Agilent Technologies, Beijing, China). Forward and reverse primers for mouse PPP2R1A were 5'-CAGCAAATGGGTCGCGGATCCatggcagctgccgacggtga-3' and 5'-CTCGAGTGCGGCCGCAAGCTTggcaagagagagaacagtca-3'; for mouse PPP2R2A were 5'-CAGCAAATGGGTCGCGGATCCatggcaggagctggaggagg-3' and 5'-CTCGAGTGCGGCCGCAAGCTTattcactttgtcttgaaata-3'; for mouse PPP2CA were 5'-CAGCAAATGGGTCGCGGATCCatggacgagaagttgttcac-3' and 5'-CTCGAGTGCGGCCGCAAGCTTcaggaagtagtctggggtac-3'. His-tagged proteins were induced expression by 1 mM isopropyl thio-β-D-galactoside (IPTG, Merck/Sigma-Aldrich, Shanghai, China) for 12 h at 25 °C. Glutathioneagarose (Merck/Sigma-Aldrich, Shanghai, China) and Ni-NTA agarose (Qiagen, Shanghai, China) were used to purify the recombinant proteins as previously described (31). His-tagged proteins were used for mass spectrum (MS) or *in vitro* PP2A activity assay.

Transfection-

The retroviral vectors encoding the sequences of PPP2R1A, PPP2R1A^{C377A} and PPP2R1A^{C390A} were cotransfected to HEK293T cells with the lentivirus packaging vectors pLP/VSVG, pRSV-Rev and pMDLg/pRRE by a calcium phosphate transfection kit (AMOGENE, Fujian, China). The flag-tagged proteins were used for pull-down (PD) or immunoprecipitation (IP) assay. For knocking down the expression of PPP2R1A, siRNA was transfected to n2a cells by Lipofectamine 2000 (Thermo Fisher Scientific/Invitrogen, Shenyang, Liaoning, China) according to the manufacturer's protocol. The oligonucleotide sequence of siRNA is 5'-GCACUCACCUUCCGAUCUA-3'.

Pull down assay-

The proteins were extracted from cells or brains, which were further incubated with str-resin. Briefly, 100 μg of protein were incubated with str-resin in the buffer containing 1% NP-40 and 0.1% SDS. The unbound proteins were removed by extensive washes with 0.1 mM EDTA, 50 mM Tris-HCl (pH 7.6), 50 mM NaF, 0.1 mM EGTA, 0.1 mM β-mercaptoethanol, 1% NP-40 and 0.1% SDS. The proteins bound on str-resin were collected by centrifugation after boiling for 10 min. The supernatant was used for western blots. The bio-PGA1 conjugated proteins were visualized with a mouse monoclonal antibody against flag-tag (1:2000, 1 μg/μl) or str-HRP (1:5000, 1 μg/μl).

Immunoprecipitation-

PPP2R1A with or without the mutation of Cys377Ala or Cys390Ala was immunoprecipitated with anti-flag antibody using protein A/G agarose (Thermo Fisher Scientific/Invitrogen, Shenyang, Liaoning, China). The proteins bounded on A/G agarose bounded were collected by centrifugation after boiling

10 min. The supernatant was used for western blots. The bio-PGA1 and PPP2R1A were visualized by a str-HRP (1:5000, 1 μg/μl) or mouse monoclonal antibody against flag-tag (1:2000, 1 μg/μl).

Mass spectrum-

1 μg His-tagged PPP2R1A was incubated with 1 μM PGA1 at 37 °C for 1 h. The reacting solution was centrifuged for collecting supernatant, which was then subjected to HPLC-MS-MS analysis. As previously described (32), unassigned ions or those with a charge < 1 + or > 7 + were rejected by MS/MS. Raw data was processed by Thermo Proteome Discoverer (version 2.1) against the SwissProt proteome database using an extracted FASTA file specified for “mouse” taxonomy. The searches were carried out with the maximum 10 ppm and 0.02 Da error tolerances for the survey scan and MS/MS analysis, respectively. The protein identifications were based on at least six amino acids in length and < 1% of the false discovery rate (FDR). On the basis of this analysis, enriched pathways were further identified in the Kyoto Encyclopedia of Genes and Genomes (KEGG) or gene ontology (GO) analysis.

PP2A activity assay-

The cells or tissues were lysed in the lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100. 40 nM PPP2CA was incubated with 50 nM PPP2R1A in TBS with or without mutation of PPP2R1A in the absence or presence of 10 μM PGA1 for 30 min at 37 °C. For determining the activity of PP2A, 60 mM Thr phosphopeptide (KRpTIRR, Merck/Millipore, Shanghai, China) was added to the solution and incubated at 37 °C for 25 min. The free phosphate was measured colorimetrically by malachite green kit (Cayman) according to manufacturer’s instructions.

Animal Committee-

All animals were handled according to the guidelines for the care and use of medical laboratory animals (Ministry of Health, Peoples Republic of China, 1998) and the guide lines of the laboratory animal ethical standards of Northeastern University.

Statistical Analysis-

All data are presented as the means ± S.E. of independent experiments. The statistical significance of the differences between the means was determined using Student’s t test or one-way analysis of variance, where appropriate. If the means were significantly different, multiple pair wise comparisons were performed using Tukey’s post hoc test.

Results

PGA1 suppresses the phosphorylation of tau in tau^{P301S} Tg mice

Prior works have demonstrated that PGA1 is closely associated with AD (23), we thereby continue to elucidate the inherent mechanisms. First of all, n2a cells were incubated with biotin conjugated PGA1 (bio-PGA1) for 2 h. By addition of Streptavidin-555, the distribution of PGA1 was determined under immunofluorescence confocal microscopy. The results demonstrated that PGA1 primarily located in the cytoplasm, but less in the nucleus of neurons (Fig. 1a). As early as the 1970s, PGA1 has been found to be responsible for regulating the physiological and biochemical functions through michael addition (33). After that, a series of proteins including thioredoxin reductase and heat shock protein (Hsp) 90 were fond to form michael adducts with PGA1 (34, 35). However, the binding proteins with PGA1 in AD are quite limited. Therefore, we are promoted to identify the proteins to bind with PGA1 in tau^{P301S} Tg mice. By pull down assay, we found that PGA1 interacted with a variety of proteins, whose molecular weight ranges from less than 26 kDa to more than 180 kDa (Fig. 1b). Moreover, most of proteins concentrated between 43 and 72 kDa in the gel (Fig. 1b). To distinguish these proteins, the lanes of western blots were cut for MS analysis. As a consequence, 1060 proteins were identified to be significantly modificated by PGA1 (10 μM) in tau^{P301S} Tg mice. The proteins were then functionally annotated by Cluster Profiler for analyzing the pathways and biological processes in PGA1-stimulated tau^{P301S} Tg mice. The results of KEGG analysis revealed that the PGA1-modificated proteins are associated with Parkinson, Huntington and Alzheimer's diseases, etc (Fig. 1c). In addition, the results of GO analysis showed that PGA1 has the ability to bind on many proteins, among which tau were identified in PGA1 treated tau^{P301S} Tg mice (Fig. 1d).

To validate these observations, the indicated concentration of 5 μl PGA1 (0, 0.01, 0.1, 1 and 10 μg/μl) was injected to the intracerebroventricles (i.c.v.) of tau^{P301S} Tg mice. After 48 h, the phosphorylation of tau was determined by western blots. The results demonstrated that PGA1 injection (i.c.v.) dose-dependently suppressed the phosphorylation of tau at the sites of Thr181, Ser202 and Ser404 (Figs. 2a, b). By immunohistochemistry (IHC) analysis, we confirmed the fact that 5 μl PGA1 (1 μg/μl) injection (i.c.v.) obviously removed the phosphate groups from the tau protein in Tau^{P301S} Tg mice (Figs. 2c-e).

PP2A mediates the effects of PGA1 on dephosphorylating tau.

As PP2A has been regarded as a canonical enzyme for dephosphorylation (36), we next determine its roles in tau phosphorylation. By treatment of n2a cells with PGA1 (10 μM) in the absence or presence of PP2A inhibitor, OA (20 nM), the activity of PP2A was initially determined by malachite green kit according to the manufacturer's instructions. The results demonstrated that OA treatment thoroughly blocked the effects of PGA1 on stimulating the activity of PP2A (Fig. 3a). In addition, the inhibitory effects of PGA1 on the phosphorylation of tau were reversed by the addition of OA (Fig. 3d). In line with these *in vitro* data,

injection (i.c.v.) of 5 μl OA (2 ng/μl) to the ventricles of tau^{P301S} Tg mice also blocked the suppressive effects of 5 μl PGA1 (1 μg/μl) on the phosphorylation of tau via modulating the activity of PP2A (Figs. 3b, c, e, f). These observations demonstrated that PGA1 suppressed the phosphorylation of tau at the sites of Thr181, Ser202 and Ser404 via activating PP2A.

PPP2R1A is identified as the downstream target of PGA1 in regulating the phosphorylation of tau.

Since PP2A is composed by a series of subunits including A, B and C and the minimal structure of PP2A to dephosphorylate tau is the complex composed of A and C subunits (37), we thereby continue to explore the effects of different subunits of PP2A on the phosphorylation of tau. By MS analysis, PPP2R1A was identified as a potential target of PGA1. In addition, knocking down the expression of PPP2R1A obviously impaired the enzymatic activity of PP2A in PGA1-stimulated n2a cells (Fig. 4a). Once the activity of PP2A was attenuated by knocking down the expression of PPP2R1A, the phosphorylation of tau was restored to the levels of untreated controls (Fig. 4b). Therefore, PPP2R1A is an essential subunit for regulating the activity of PP2A, which mediates the effects of PGA1 on regulating the phosphorylation of tau during the course of AD development and progression. For the reason, we firstly determined if PGA1 has the ability to upregulate the expression of PPP2R1A *in vitro* and *in vivo*. Unfortunately, the mRNA and protein expression of PPP2R1A are not regulated in PGA1-treated n2a cells and tau^{P301S} Tg mice (Figs. 4c, d).

PGA1 usually exerts its biological functions by forming michael adducts with different proteins (38), we therefore speculate if PGA1 can bind with PPP2R1A via michael addition. To this purpose, PPP2R1A with flag-tag was ectopically expressed in HEK293 cells. By pull down assay, we found that PPP2R1A overexpression enhanced the interaction between bio-PGA1 and proteins (Fig. 4e). This result indicated that PGA1 and PPP2R1A might form the michael adducts. To confirm this hypothesis, the PPP2R1A was immunoprecipitated with anti-flag antibody and the combined bio-PGA1 was visualized by western blots. The results demonstrated that PGA1 has the ability to form michael adducts with PPP2R1A (Fig. 4f).

To identify the binding sites of PPP2R1A, HPLC-MS-MS was carried out to analyze the *in vitro* incubated complex of PGA1 and purified PPP2R1A. The results of fragment ions showed that a 336 Da mass shifts were found by analyzing a continuous series of b- and y-type ions, which is corresponding to the adduction of PGA1 on the Cys motif of PPP2R1A (Fig. 5a). By analyzing the protein sequences of PPP2R1A, Cys 377 and Cys 390 are the potential binding sites of PPP2R1A for PGA1. By amino acid mutation, we find that Cys 377, but not Cys390 mutation clearly impaired the binding between PGA1 and PPP2R1A (Figs. 5b, c). Without combination, PGA1 could not affect the enzymatic activity of PP2A anymore (Figs. 5d, e). On the basis of these observations, forming the michael adducts between PGA1 and PPP2R1A is the critical process for affecting the enzymatic activity of PP2A.

Long-term administration of PGA1 improved the cognitive decline of tau ^{P301S} Tg mice via decreasing the phosphorylation of tau in a PGA1-activating mechanism.

Since short-term administration of PGA1 to tau^{P301S} Tg mice has shown its effects on inhibiting the phosphorylation of tau, 3-month-old tau^{P301S} Tg mice were further intranasally instilled with PGA1 (2.5 mg/kg/d) for 6 m. By measuring the enzymatic activity of PP2A and the phosphorylation of tau, we found that PGA1 stimulated the activity of PP2A and inhibited the phosphorylation of tau in either WT or tau^{P301S} Tg mice (Figs. 6a-d). On the basis of these observations, we are promoted to determine if PGA1 has the ability to affect the learning ability of tau^{P301S} Tg mice. By nest construction and morris maze test, we found that PGA1 treatment obviously prevented the cognitive decline of tau^{P301S} Tg mice (Figs. 6e-h). All these observations revealed that long-term administration of PGA1 improved the cognitive decline of tau^{P301S} Tg mice via decreasing the phosphorylation of tau in a PP2A-activating mechanism.

Discussion

AD is associated with the production and deposition of Aβ and phosphorylated tau in APs and NFTs (1). The overloading of aggregated Aβ and phosphorylated tau will induce excessive toxicity to the neurons, which results in impairing the brains of AD patients (39, 40). For neuroprotection, PGA1 has been reported to blunt N-methyl-D-aspartate receptor (NMDAR)-mediated neuronal apoptosis and inhibits enhancement of the intracellular calcium concentration, TXA_2 production, and platelet activation in rodent models of stroke (41). In addition, PGA1 protects striatal neurons against excitotoxic injury in rat striatum (42). In rat models of permanent focal cerebral ischemia, PGA1 also plays neuroprotective effects by inhibiting the activity of NF-κB and upregulating PPARγ (43). In C6 glioma cells, PGA1 was postulated to upregulate the expression of neurotrophic factors, which is beneficial the neurons (44). All these evidences indicated the beneficial effects of PGA1 on AD. By this hypothesis, our previous studies have demonstrated that PGA1 has the ability to inhibit the production and aggregation of Aβ via inducing the efflux of cholesterol from the neurons (23). To extend the prior works, the current investigation studied the mechanisms of PGA1 in regulating the phosphorylation of tau via PP2A-activating mechanisms in tau^{P301S} Tg mice.

As an important member of cyPGs, PGA1 usually modulates biological processes via binding with other functional proteins by michael addition. By MS analysis, PGA1 has been found to form michael adducts with a series of proteins. For example, PGA1 deactivates the activity of the enzyme by binding covalently to Cys299 motif of AKB1B10 (38). Using MALDI-TOF MS, H-Ras^{Cys118} was identified to be the critical site for PGA1 binding by michael addition (45). Although the residues involved in the modification have not been identified, covalent modification of Hsp90 by biotinylated PGA1 (PGA1-B) has been demonstrated *in vitro* (46). Even though the functions of PGA1 adducts have yet to be explored, numerous *in vitro* and *in vivo* studies have revealed the biological effects of cyPG adducts on cellular processes. For instance, the anti-inflammatory roles of cyPG have been revealed by inhibiting the expression of iNOS or COX-2 in

PPARγ- or Nrf2-activating and NF-κB- or AP-1-deactivating mechanisms (47, 48). Apart from its anti-inflammatory effects, cyPGs also have the ability to regulate the cellular redox status (49), proliferation and apoptosis (50).

On the basis of these clues, we extended the prior works to find that bio-PGA1 primarily distributed in the cytosol of n2a cells (Fig. 1a). In line with our observation, PGA1 was also found to be located in the cytosol of NIH3T3 cells (46). These data indicated the potential ability of PGA1 to bind on the cytosolic proteins in neurons. By this hypothesis, the bio-PGA1 conjugated proteins were analyzed by MS. KEGG and GO analysis depicted the PGA1-regulated the physio- or pathological processes, among which includes AD and tau protein (Figs. 1b-d). More importantly, PGA1 was firstly identified to suppress the phosphorylation of tau in tau^{P301S} Tg mice (Fig. 2). Even though there is no direct evidence to support our results, the anti-inflammatory effects of PGA1 might be critical for dephosphorylating tau. For example, IL-1β expression in APP/PS1 Tg mice markedly induced the phosphorylation of tau (51). Blocking the signaling cascade of IL-1 inhibited the tauopathy, which results in improving the learning ability of APP/PS1 Tg mice (52). These *in vivo* observations were further supported by the *in vitro* data showing that IL-1β stimulated tau phosphorylation in cultured microglia, astrocytes, and neurons (53). Apart from IL-1β, IL-18 and IL-6 also has the ability to phosphorylate tau in human SH-SY5Y cells (54) and cultured hippocampal neurons (55). As a potential anti-inflammatory factor (47, 48), PGA1 might inhibit the phosphorylation of tau via suppressing the expression of pro-inflammatory cytokines. As another important member of cyPGs, 15d-PGJ_2 has shown its effects on suppressing the proinflammatory cytokines (56), leading to the phosphorylation of tau *in vitro* and *in vivo* (51–55). Along these lines, these anti-inflammatory pathways might be the mechanisms for mediating the effects of PGA1 on suppressing the phosphorylation of tau.

Even though inflammatory pathways had not been elucidated in our experiments, we found that PGA1 has the ability to activate PP2A (Fig. 3). More importantly, Cys377 is the key motif for PP2A subunit, PPP2R1A to bind with PGA1, which regulates the activity of PP2A (Figs. 5). In agreement with our results, Cys377 on the scaffolding subunit, PPP2R1A was reported to be responsible for the activity of PP2A (57). In addition, PGA1 has the ability to bind on cytosol proteins in NIH3T3 cells (58). Based on the crystal structures of PP2A, Cys377 resides at an interface between three subunits of the core PP2A complex (59), which provides the position advantage of PPP2R1A to regulate the activity of other subunits.

Since PGA1 has the ability to activate PP2A, the question is easily raised if PP2A has the ability to regulate the phosphorylation of tau during the course of AD development and progression. Recently, more and more studies established the relationship between PP2A and AD (60, 61). More accurately, the activity and expression of PP2A including its A, B and C subunits are decreased in the brains of AD patients (61–63). More closely, the expression of PPP2R1A was downregulated in the brains of AD patients compared to that of corresponding normal subjects (63, 64). Specifically in the high aluminum AD model, the activity of PP2A decreased by 59% (65). Further studies showed that the accumulation of zinc ions and the expression of APOE were the pathological factors for decreasing the activity of PP2A (66, 67). Due to the decrease of PP2A, the phosphorylation of tau is increased by activating GSK3β (68).

The use of endogenous PP2A inhibitor obviously induces the phosphorylation of tau (69). Consistently, OA, an inhibitor of PP2A blocked the effects of PGA1 on suppressing the phosphorylation of tau *in vitro* and *in vivo* (Fig. 3). These observations reinforced the critical roles of PP2A in mediating the effects of PGA1 on suppressing the phosphorylation of tau during the course of AD development and progression.

As tau experiences phosphorylation, aggregation and deposition during the course of AD development and progression, it will lose its ability to maintain the stability of microtubes (70). Moreover, the aggregated form of tau will induce neurotoxicity(39), leading to the apoptosis of neurons (71). Long-term potentiation (LTP) was obviously suppressed in tau^{P301S} Tg mice, which potentially affected the memory of the mice (72). Not surprisingly, the results of nest construction and morris maze test demonstrated that the learning ability was obviously impaired in tau^{P301S} Tg mice (Fig. 6). More importantly, PGA1 treatment ameliorated the cognitive decline of tau^{P301S} Tg mice (Fig. 6).

Conclusions

Using the analysis of HPLC-MS-MS, tau protein was firstly identified to bind on PGA1 in the brains of tau^{P301S} Tg mice. By direct interaction, PP2A was activated by PGA1, which results in decreasing the phosphorylation of tau at the sites of Thr181, Ser202 and Ser404 in a dose-dependent manner. Blocking the activity of PP2A by the incubation of PP2A inhibitor, OA restores the phosphorylating levels of tau in tau^{P301S} Tg mice. What's more novel is that cysteine 377 of PPP2R1A is the key motif for the interaction between PGA1 and PP2A, leading to the enzymatic activity of PP2A on depressing the phosphorylation of tau. Beneficially from PP2A activation, PGA1 treatment improved the cognitive decline of tau^{P301S} Tg mice, which might provide a therapeutic strategy to combat AD.

Abbreviations

Prostaglandin A1, PGA1; cyclooxygenase-2, COX-2; Alzheimer's disease, AD; cyclopentenone prostaglandins, cyPGs; okaic acid, OA; protein phosphotase 2A (PP2A); PP2A scaffold subunit A alpha, PPP2R1A; β-amyloid protein (Aβ); β-amyloid plaques, APs; neurofibrillary tangles, NFTs; presenilin, PS; tumor necrosis factor, TNF; amyloid precursor protein, APP; 15-deoxy-$\Delta^{12,14}$-PGJ_2, 15d-PGJ_2; peroxisome proliferators-activated receptor γ, PPAR γ; presenilin enhancer 2, PEN2; ATP-binding cassette transporter 1, ABCA1

Declarations

Conflicts of Interest

The authors declare no conflicts of interests.

Availability of data and materials

All data generated or analyzed in this study are included in the current published article.

Ethics approval and consent to participate

All animals were handled according to the guidelines for the care and use of medical laboratory animals (Ministry of Health, Peoples Republic of China, 1998) and the guide lines of the laboratory animal ethical standards of Northeastern University.

Consent for publication

All the authors have agreed with the publication of the manuscript.

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Author contributions

G.B.X. and P.P.G. conceived and performed all of the experiments, participated in the design of the study and wrote the manuscript. P.W. conceived the experiments, interpreted the data and wrote the manuscript of this study.

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Figures

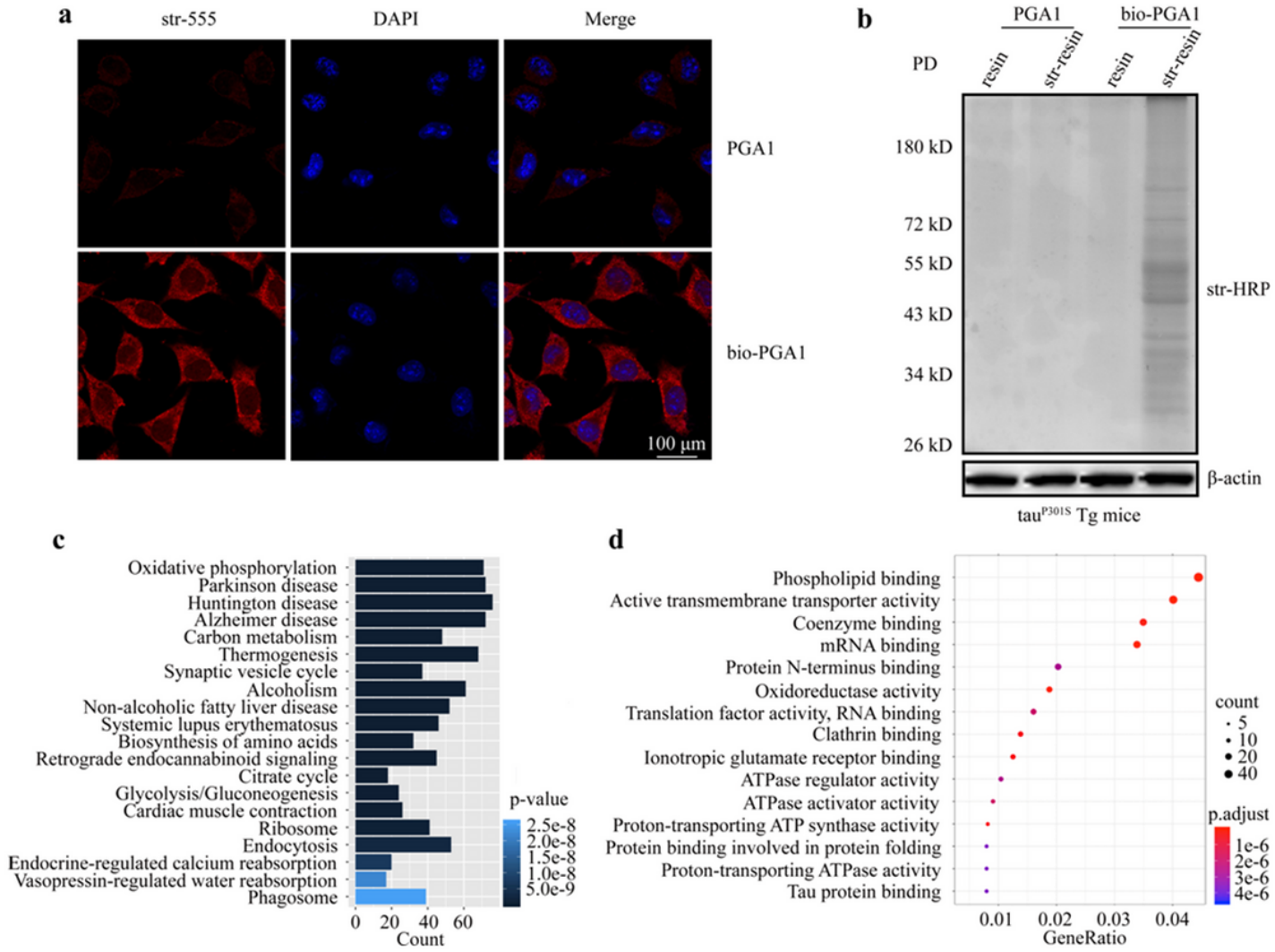


Figure 1

PGA1 regulated multiple physio- and pathological processes. (a) n2a cells were treated with 10 μM PGA1 or bio-PGA1 for 2 h. The cells were then stained with Streptavidin-555 (1:200) before observing under confocal microscopy. (b) 5 μl PGA1 or bio-PGA1 (1 μg/μl) was injected (i.c.v.) to the cerebral ventricles of TauP301S Tg mice for 2 h. Total proteins were extracted for pull down assay with resin or str-resin. The bio-PGA1 conjugated proteins were visualized by str-HRP in western blots. (c, d) The binding proteins on bio-PGA1 were then subjected to MS analysis. (c) The top 20 biological processes were clustered by KEGG pathway analysis. (d) The 56th-70th biological processes were identified by GO pathway analysis.

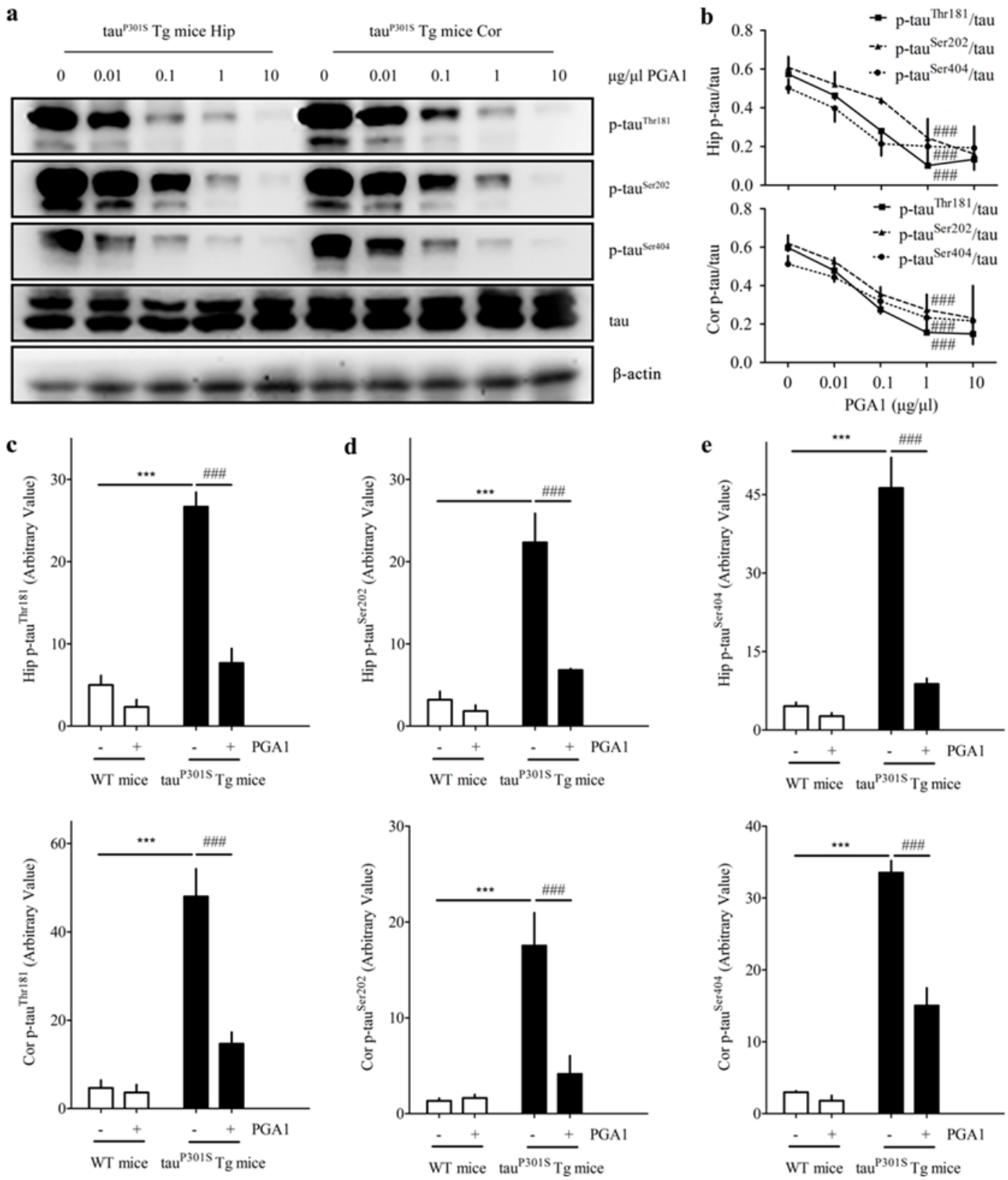


Figure 2

PGA1 decreases the phosphorylation of tau. 9-month-old tauP301S Tg mice was injected (i.c.v.) with 5 μl PGA1 (0, 0.01, 0.1, 1, 10 μg/μl) for 48 h. (a) The levels of phosphorylated tau at the sites of Thr181, Ser202 and Ser404 were determined by western blots. The total tau and β-actin served as internal control. (b) The relative intensity of bands was calculated by the Image J software. (c-e) The immunoreactivities of phosphorylated tau at the sites of Thr181, Ser202 and Ser404 were determined by IHC, which was

further analyzed by the Image J software. The data represent means ± S.E. of independent experiments. ***p < 0.001 compared to vehicle-treated WT mice. ###p < 0.001 compared to vehicle-treated tauP301S Tg mice.

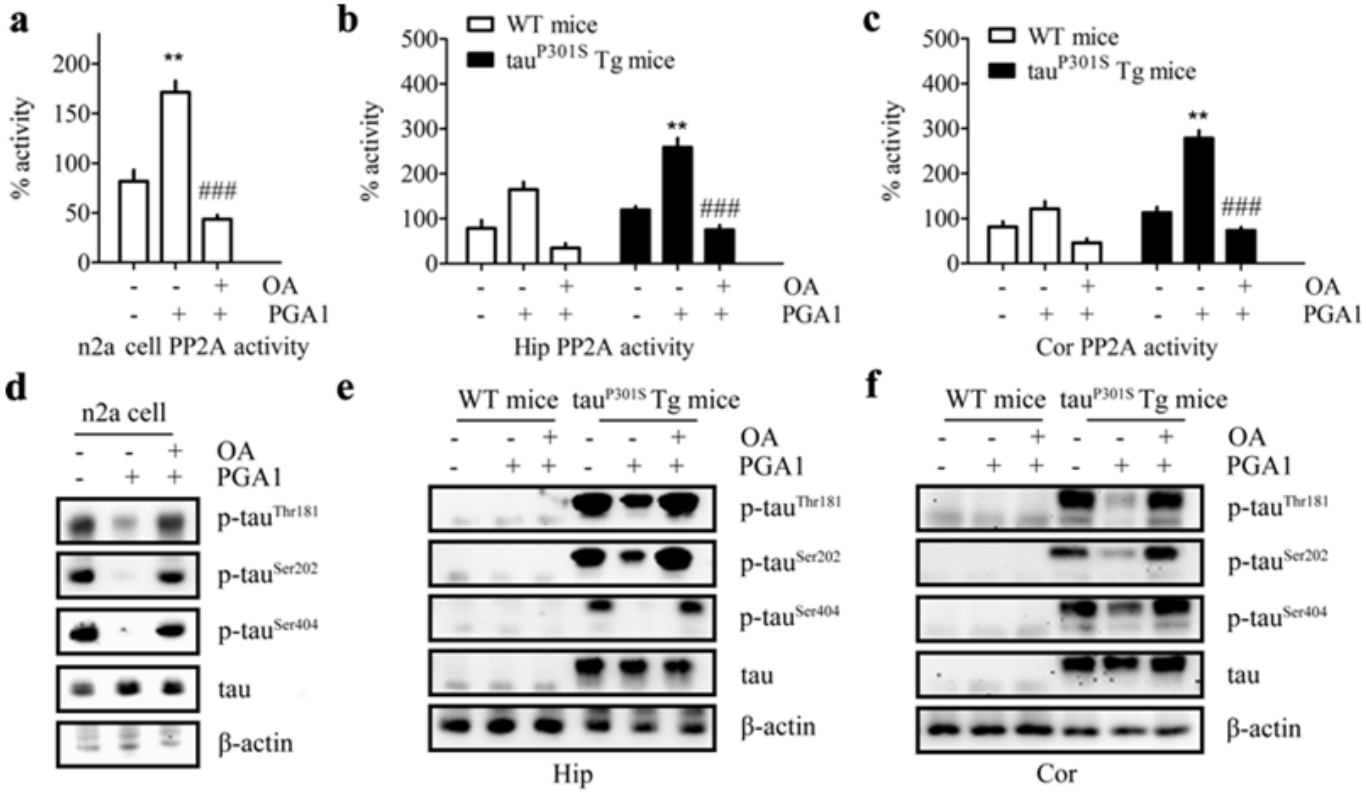


Figure 3

PP2A mediates the effects of PGA1 on suppressing the phosphorylation of tau. (a, d) n2a cells were treated with PGA1 (10 μM) in the absence or presence of OA (20 nM) for 48 h. (b, c, e, f) 9-month-old tauP301S Tg mice was injected (i.c.v.) with 5 μl PGA1 (1 μg/μl) in the absence or presence of OA (2 ng/μl) for 48 h. (a-c) The activity of PP2A was determined by malachite green kit. (d-f) The phosphorylation of tau at the sites of Thr181, Ser202 and Ser404 were determined by western blots. The total tau and β-actin served as internal control. The data represent means ± S.E. of independent experiments. **p < 0.01 compared to vehicle-treated TauP301S Tg mice. ###p < 0.001 compared to PGA1-treated TauP301S Tg mice.

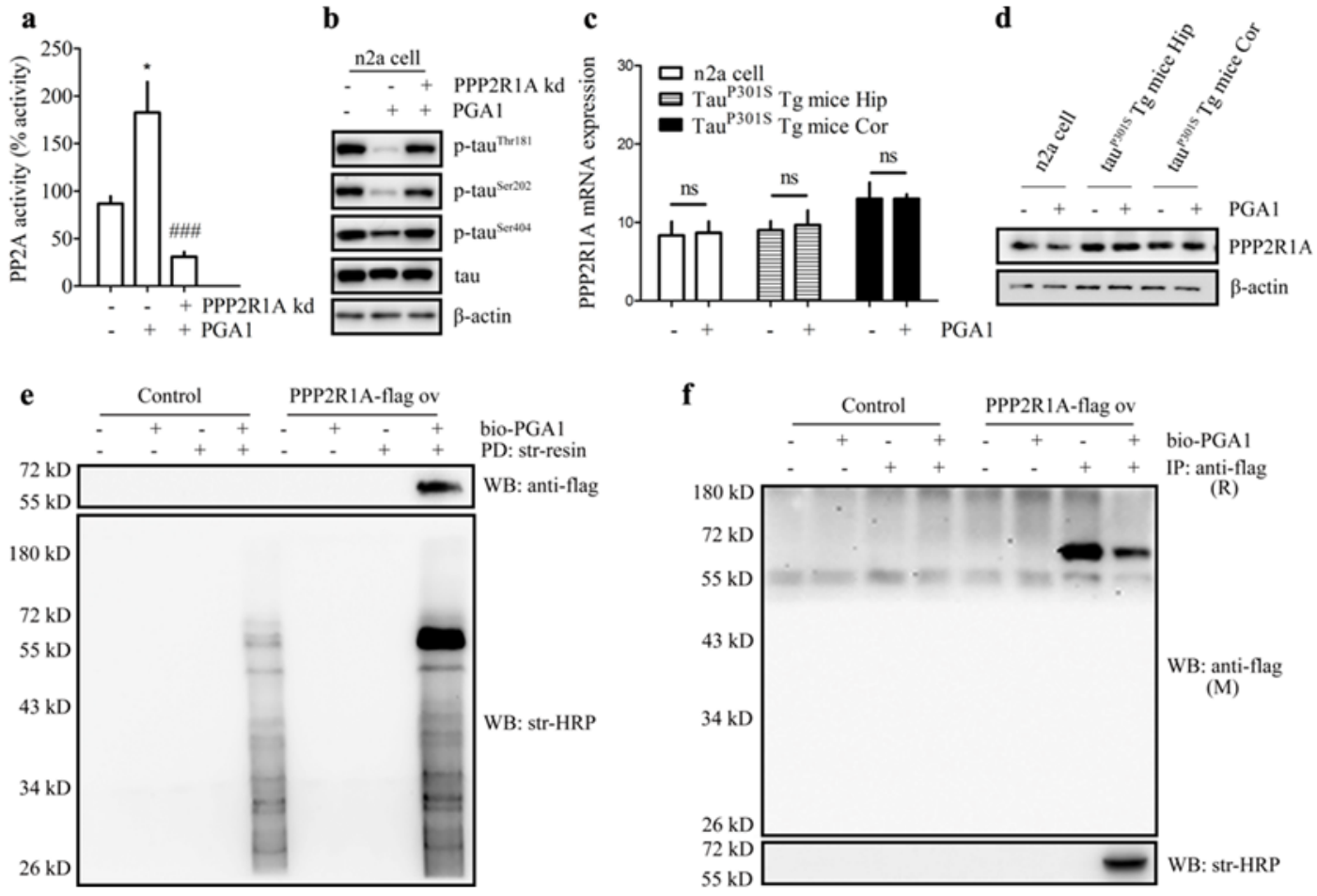
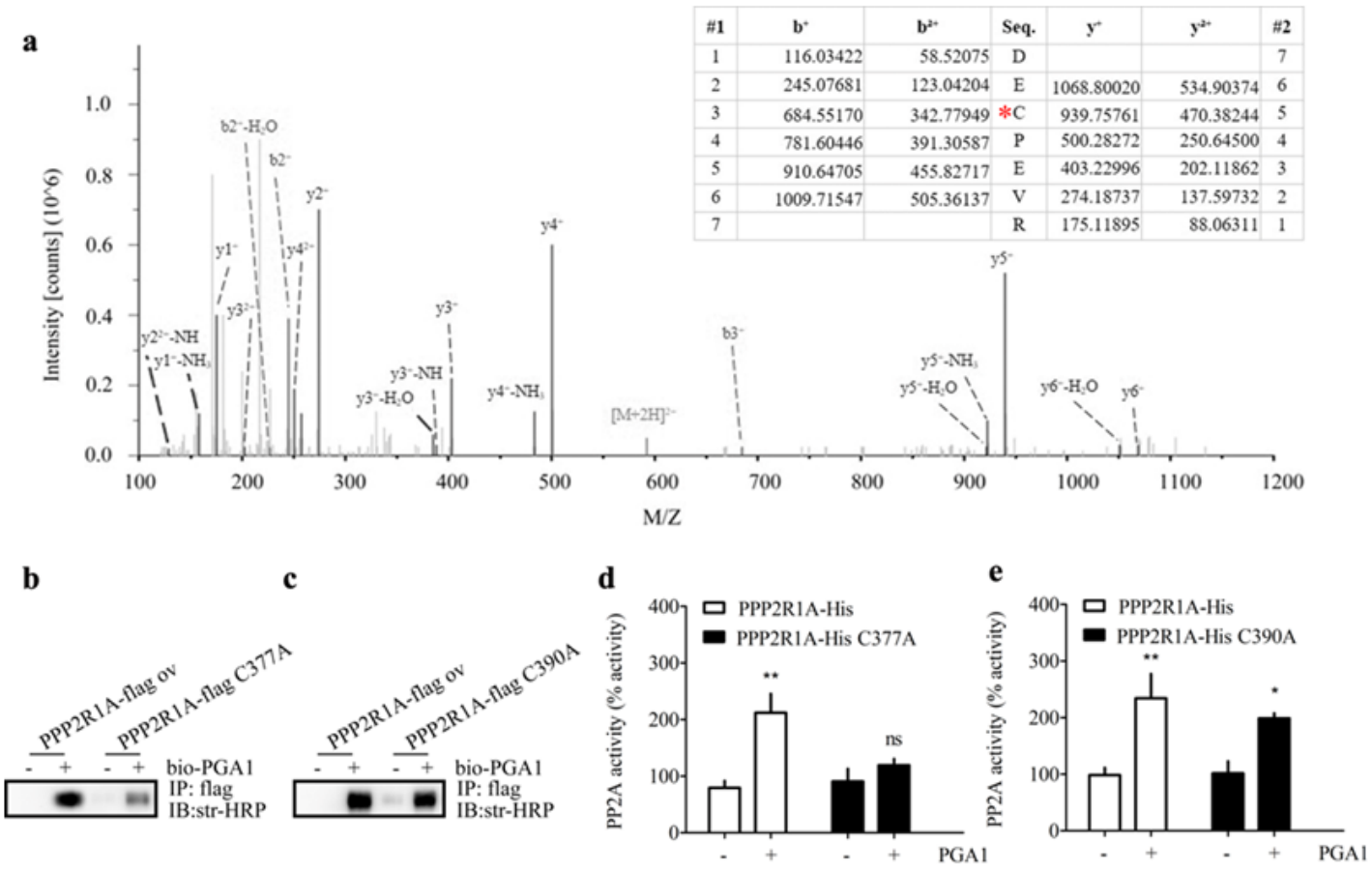


Figure 4

PGA1 activates PP2A via its PPP2R1A subunit. (a) n2a cells were treated with PGA1 (10 μM) in the absence or presence of knocking down the expression of PPP2R1A. The activity of PP2A was determined by malachite green kit. (b) The phosphorylation of tau at the sites of Thr181, Ser202 and Ser404 were determined by western blots. The total tau and β-actin served as internal control. (c, d) n2a cells were treated with PGA1 (10 μM) and 9-month-old tauP301S Tg mice was injected (i.c.v.) with 5 μl PGA1 (1 μg/μl) for 48 h. The mRNA and protein expression of PPP2R1A were determined by qRT-PCR and western blots. GAPDH and β-actin served as internal controls. (e) Protein extracts were subjected to pull down assay with resin or str-resin. The conjugated proteins were then visualized by the specific antibody against flag-tag or str-HRP. (f) Protein extracts were immunoprecipitated with flag-tag antibody. The precipitated proteins were visualized by the specific antibody against flag-tag or str-HRP. The data represent means ± S.E. of independent experiments. *$p < 0.05$ compared to vehicle-treated control; ###$p < 0.001$ compared to PGA1-treated n2a cells.



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5	910.64705	455.82717	E	403.22996	202.11862	3
6	1009.71547	505.36137	V	274.18737	137.59732	2
7			R	175.11895	88.06311	1

Figure 5

PGA1 activate PP2A via Cys377 motif of PPP2R1A. (a) The purified PPP2R1A (1 μg) was incubated with PGA1 (1 μM) for 1 h at 37 °C. The reacting products were subjected to HPLC-MS-MS analysis. (b-e) HEK293T cells overexpressed flag-tagged PPP2R1A with or without mutation of Cys377Ala and Cys390Ala. The cells were then treated with bio-PGA1 in a final concentration of 10 μM for 2 h. (b, c) Protein extracts were immunoprecipitated with flag-tag antibody. The precipitated proteins were visualized by the str-HRP. (d, e) The recombinant PPP2CA (40 nM) was incubated with purified PPP2R2A (50 nM) with or without the motif mutation of Cys377Ala and Cys390Ala in TBS for 30 min at 37 °C. In some cases, the PGA1 at a final concentration of 1 μM was added to the reacting solution. Then, PP2A activity was detected by malachite green kit. The data represent means ± S.E. of independent experiments. *p < 0.05, **p < 0.01 compared to vehicle-treated control.

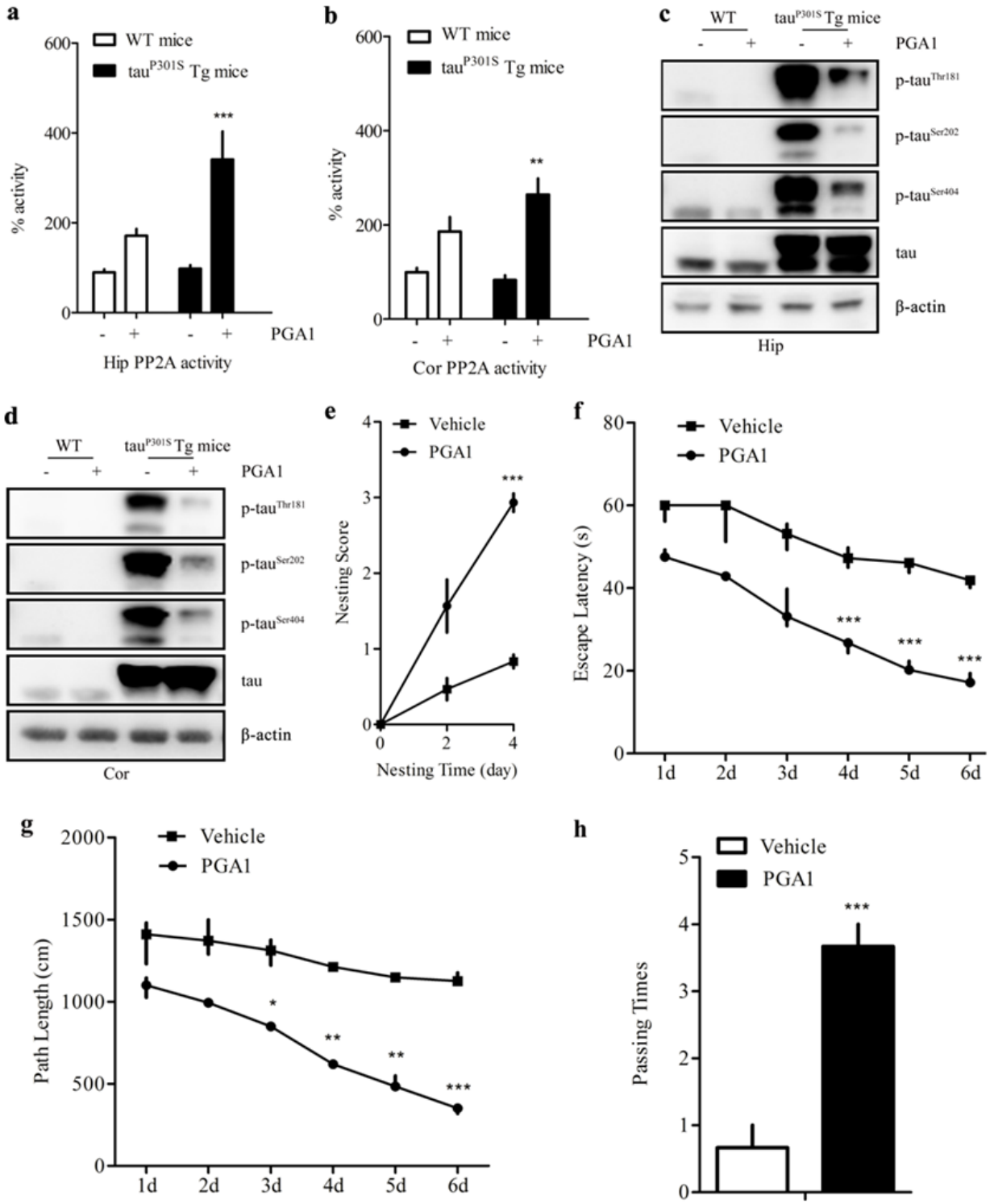

Figure 6

Long-term administration of PGA1 decreased the phosphorylation of tau via activating PP2A, which in turn ameliorates the cognitive decline of tauP301S Tg mice. 3-month-old tauP301S Tg mice were intranasally treated with or without PGA1 (2.5 mg/kg/d) for 6 months. (a, b) The activity of PP2A was determined by malachite green kit. (c, d) The phosphorylation of tau at the sites of Thr181, Ser202 and Ser404 were determined by western blots. The total tau and β-actin served as internal control. (e-h) The

learning ability of tauP301S Tg mice was determined by morris maze test and nest construction assay. The data represent means ± S.E. of independent experiments. **p < 0.01, ***p < 0.001, compared to vehicle-treated control.