Polyphyllin I attenuates cognitive impairments and reduces AD-like pathology through CIP2A-PP2A signaling pathway in 3XTg-AD mice

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Abstract
Polyphyllin I (PPI) is a natural phytochemical drug isolated from plants which can inhibit the proliferation of cancer cells. One of the PPI tumor-inhibitory effects is through downregulating the expression of Cancerous Inhibitor of PP2A (CIP2A), the latter, is found upregulated in Alzheimer's disease (AD) brains and participates in the development of AD. In this study, we explored the application of PPI in experimental AD treatment in CIP2A-overexpressed cells and 3XTg-AD mice. In CIP2A-overexpressed HEK293 cells or primary neurons, PPI effectively reduced CIP2A level, activated PP2A, and decreased the phosphorylation of tau/APP and the level of Aβ. Furthermore, synaptic protein levels were restored by PPI in primary neurons overexpressing CIP2A. Animal experiments in 3XTg-AD mice revealed that PPI treatment resulted in decreased CIP2A expression and PP2A re-activation. With the modification of CIP2A-PP2A signaling, the hyperphosphorylation of tau/APP and Aβ overproduction were prevented, and the cognitive impairments of 3XTg-AD mice were rescued. In summary, PPI ameliorated AD-like pathology and cognitive impairment through modulating CIP2A-PP2A signaling pathway. It may be a potential drug candidate for the treatment of AD.

Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; Aβ, amyloid-β; BSA, bovine serum albumin; CCK8, cell counting kit 8; CL, cell lysate; CIP2A, cancerous inhibitor of PP2A; CS, conditioned stimulus; DMEM, Dulbecco's modified eagle medium; DMSO, dimethyl sulfoxide; ELISA, Enzyme-Linked Immunosorbent Assay; F12, Ham's F 12 nutrient medium; FA, formic acid; FBS, fetal bovine serum; FCM, fear condition memory; FCT, fear conditioning test; ITI, intertrial interval; MWM, morris water maze; NFT, neurofibrillary tangles; NOR, novel object recognition; OLM, object location memory; ORM, object recognition memory; PPI, polyphyllin I; PP2A, protein phosphatase 2A; PVDF, polyvinylidene fluoride; PSD95, postsynaptic density 95; RIPA, radio immunoprecipitation assay; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; Syn, synaptophysin; Syn I, Synapsin I; Tg, transgenic; WT, wild type.

Ying Zhou, Dichen Yang and Hao Chen contributed equally to this study
1 | INTRODUCTION

Alzheimer’s disease (AD) is a typical cause of dementia which is characterized by the development of progressive cognitive dysfunction. The main pathologic features in AD brains include amyloid plaques formed by excessive accumulation of amyloid-β (Aβ) peptides, and intraneuronal fibrillar aggregates consisting of abnormally hyperphosphorylated tau. There is an increasing body of evidences showing that both Aβ and hyperphosphorylated tau accumulate and spread even years before clinical symptoms appear.

The Amyloid Precursor Protein (APP) is a type I transmembrane protein with extracellular and intracellular domains. Aβ is produced by the continuous cleavage of APP by BACE-1 and γ-secretase complex. One characteristic of AD is the overproduction of Aβ40 and Aβ42 peptide caused by abnormal APP processing and cleavage. Excessive Aβ accumulation and deposition in the brain may lead to impaired neuronal function and cognitive decline, and reducing Aβ production is a focus in AD drug development for a long period.

An important function of tau is to promote the assembly and stability of microtubules. Hyperphosphorylation of tau results in loss of tau normal function and gain of toxicity of tau aggregates in different configurations. Hyperphosphorylation of tau starts from the inner olfactory cortex and hippocampus and gradually spreads to the entire cortex. Accumulation of hyperphosphorylated tau in the brain can lead to neuronal death and loss of synapses. Studies indicate that inhibiting tau hyperphosphorylation may be an effective therapeutic strategy for AD treatment.

In AD brains, Protein Phosphatase 2A (PP2A) inactivation is closely linked to tau hyperphosphorylation and Aβ accumulation. It has been reported dephosphorylation activity of most hyperphosphorylated tau in neurons is related to the subunit PPP2R2A in PP2A holoenzyme. PP2A dephosphorylates hyperphosphorylated tau in patients’ brains while restoring tau’s ability to promote microtubule assembly. Inhibition of PP2A in the brain leads to hyperphosphorylation of tau at lots of sites related to AD. At the same time, PP2A inactivation also contributes to the hyperphosphorylation of APP at T668 and the following overproduction of Aβ.

Cancerous Inhibitor of PP2A (CIP2A) is an endogenous PP2A inhibitor with relatively high expression in the human brains. In our previous study, we reported an increased CIP2A inhibitor in AD brains, and identified the role of CIP2A as a key molecular linking both tau pathology and Aβ overproduction through regulating the PP2A activity. CIP2A enhances APP phosphorylation at T668 by inhibiting PP2A, and thus promotes the β-secretase processing of APP and Aβ production. It also induces tau hyperphosphorylation by inhibiting PP2A. Many studies indicated that CIP2A regulates PP2A in a substrate-dependent manner, which suggests that...
targeting CIP2A may be an effective and useful strategy for the treatment of AD.

Polyphillin I (PPI), which is mainly extracted from the Rhizoma of Paris polyphylla, is a bioactive phytochemical with molecular weight of 855.02 Da, and its chemical formula is C_{44}H_{70}O_{16}.^{57} The rhizome of Paris polyphylla is known as Chong-lou in Chinese and is one of the important components in various medicine, such as “Yunnan Baiyao.” Paris polyphylla has been found to have antibacterial, anti-cancer, and anti-inflammatory effects.^{28} In addition, studies showed that PPI has preclinical anticancer effect in various types of cancer,^{29-32} by reducing cancer-related symptoms as well as adverse drug reactions, and inhibiting tumor growth.^{33,34} Recently several labs have shown that PPI can inhibit tumor progression by inhibiting the expression of CIP2A.^{35,36} Whether PPI can modulate the CIP2A expression thus ameliorating AD-related pathologic changes in AD is worthy exploration and identification.

In the present study, we have demonstrated both in vitro and in vivo that PPI is effective in reducing the CIP2A expression which rescues the CIP2A-mediated PP2A inhibition, therefore, the hyperphosphorylation of tau/APP and Aβ production are decreased, synaptic impairment is ameliorated, and the cognitive functions of 3XTg-AD mice are improved. Our findings suggest the potential of PPI as a new drug candidate for AD treatment.

2 | MATERIALS AND METHODS

2.1 | Materials

All primary antibodies used in this study were as follow: CIP2A (Cell Signaling Technology, Cat#14805), pT668(Cell Signaling Technology, Cat#6986), APP (Cell Signaling Technology, Cat#2452), GluA1 (Cell Signaling Technology, Cat#13185), pS396 (Signalway Antibody, Cat#11102), pS404 (Signalway Antibody, Cat#11112), Tau-5 (Abcam, Cat ab80579), Synaptophysin (Abcam, Cat ab32127), β-actin (Abcam, Cat ab8226), PP2Ac (Millipore, Cat#05-777), Synapsin 1 (Millipore, Cat S193), GAPDH (Proteintech, Cat 60004-1-lg), ERK (Cell Signaling Technology, Cat #4695), p-EKR (Cell Signaling Technology, Cat #4370), p38 (Multi Sciences, Cat 100-401-G26), and p-p38 (Multi Sciences, Cat 600-401-C90).

CIP2A plasmid and tau plasmid were generous gifts from Prof. Rong Liu (Department of Pathophysiology, Key Laboratory of Ministry of Education for Neurological Disorders, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China). Wild-type APP770 plasmid was generous gift from Prof. Angela Ho (Boston University, Boston, MA, USA). pAAV-SYN-CIP2A-EGFP-3FLAG and control vector were purchased from Obio Technology (Shanghai, China).

3XTg AD male mice (10 months old, 30 ± 2 g) were provided by Jackson Laboratory. All the mice can take food and water freely in an air-conditioned room (22 ± 2°C, 12 hours light/dark cycle). Mice were treated with intraperitoneal administration of 1 mg/kg of PPIs twice a day when saline served as control for 1 month. The behavior tests were performed on their active hours. PPI (CAS No.: 50773-41-6, purity: 98%) was purchased from MedChemExpress Co., Ltd. (New Jersey, USA) and dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), stored at −20°C. Neofect DNA transfection reagent was purchased from Neofect Biological Technology Co., Ltd (Beijing, China). DMEM-high and protein marker were purchased from Invitrogen (Grand Island, NY, USA). Serine/Threonine Phosphatase Assay kit was purchased from Promega Company (Madison, Wisconsin, USA). DMEM/F12 and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, New York, USA). Human or rat or mouse Aβ40/Aβ42 ELISA kits were purchased from Elabscience Biotechnology (Wuhan, China). Cell Counting Kit (CCK8) was purchased from Yeason Biotechnology Co., Ltd. (Shanghai, China).

2.2 | Methods

2.2.1 | Cell culture and transfection

For HEK293-T cell culture, the cells were cultured in DMEM-high medium supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37°C in the presence of 5% CO2. The cells were cultured to 60%-70% confluence in 6-well plates and changed to fresh medium, then transfected with relevant plasmids by Neofect DNA transfection reagent (Neofect Biological Technology, Beijing, China). After 24 or 48 hours, cells and culture media were collected for further detections. For primary neuron culture, cortical neurons were isolated from embryonic day (E) 18 Sprague Dawley rats and cultured as previously described.^{37} At the end of treatments, cells were collected and lysed in radio immunoprecipitation assay (RIPA) buffer for further biological detections.

2.2.2 | Aβ40/42 assay by ELISA

Cells were lysed in RIPA buffer and centrifuged for 5 minutes at 3000 g at 4°C, the supernatants containing soluble Aβ40 and Aβ42 were collected. The pellets were further dissolved in formic acid (FA) for detection of Aβ. The levels of Aβ40 and Aβ42 in RIPA-soluble and FA-soluble fractions were detected.
by ELISA following the construction offered by the assay kit manufacturer (Elabscience Biotechnology, Wuhan, China).

### 2.2.3 PP2A activity and CCK-8 assay

PP2A activity was measured according to the protocol provided by the manufacturer (V2460 kit; Promega). The CCK-8 assay was performed using a cell counting kit (Yeasen) according to the manufacturer’s instructions.

### 2.2.4 RNA isolation and reverse transcription PCR

Total RNA was extracted using a RNeasy mini kit (Qiagen) according to the manufacturer’s protocol. Reverse transcription reactions were performed using murine leukemia virus reverse transcriptase and oligo-dT primers (Fermentas). Conventional PCR was performed using LC Taq (Fermentas). The sequences of CIP2A are as follows: F: TGCGGCACTTGGAGGTAATTTC and R: AGCTCTACAAGGCAACTCAAGC.

### 2.2.5 Coimmunoprecipitation

For immunoprecipitation assays, cells were lysed using Pierce IP lysis buffer (Thermo Fisher Scientific) supplemented with protease inhibitor cocktail (Roche). The cell lysates were centrifuged, and then, immunoprecipitated overnight at 4°C using the indicated primary antibodies followed by incubation with Dynabeads Protein G (Life Technologies) for 1 hour. The immunocomplexes were washed twice with IP lysis buffer before being resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with indicated antibodies.

### 2.2.6 Western blotting

For Western blotting, cells were collected and lysed in 1 × RIPA buffer. After centrifugation at 15,000 g for 5 minutes at 4°C, we collected the supernatant as the protein lysate. Protein samples were separated by SDS-PAGE, transferred to a 0.45-μm pore-sized polyvinylidene fluoride (PVDF) membrane (Millipore). The membranes were blocked with 5% BSA and then, incubated with certain primary and secondary antibodies.

### 2.2.7 Golgi staining

The mice were anesthetized by isoflurane and perfused intracardially with 400 mL of normal saline containing 0.5% sodium nitrite, followed with 400 mL 4% formaldehyde and the Golgi dye solution containing 5% chloral hydrate, 4% formaldehyde, and 5% potassium dichromate. After being perfused, the brains were dissected into 5 mm three 5 mm sections and transferred to a vial containing Golgi dye solution for 3 days in the dark, then immersed in solution containing 1% silver nitrate for another 3 days. The brains were serially sectioned into 100-mm-thick slices using a vibrating microtome (Leica, VT1000S, Germany). Images were observed under the microscope (Nikon, Tokyo, Japan).

### 2.2.8 Nissl staining

Thirty micrometers of coronal sections were mounted on gelatin-coated slides. Then, the sections were incubated in Cresyl violet for 3 minutes at room temperature, following with dehydration through 50%, 75%, 95%, and 100% alcohol, cleaning in xylene, and cover slipped with neutral balsam. Images were observed using light microscope.

### 2.2.9 Behavior tests

**Novel objective recognition test**

Novel object recognition (NOR) tests were performed in a 50 cm × 50 cm × 50 cm white plastic box referring to Shentu's study (Shentu et al., 2018). The mice were habituated to the arenas (50 cm *50 cm*50 cm plastic container) for 5 minutes without objects 24 hours prior to the test. Arenas were cleaned with 70% ethanol between each habituation period. The day after the mice reentered the arenas from the same starting point, they were granted 5 minutes to familiarize themselves with A object and B object. After each familiarization period the arena and objects were cleaned with 70% ethanol. Exactly 1 hour after the familiarization period, B object was replaced with C object, and the mice were granted 5 minutes to explore both objects. After 24 hours, C object was replaced with D object, and the mice were granted 5 minutes to explore both objects. The behavior was recorded by a video camera positioned above the arena. The recognition index was calculated by TA/(TA + TB), TB/(TA + TB), TC/(TA + TC), and TD/(TA + TD). The discrimination index was calculated by (TC − TA)/(TA + TC), (TD − TA)/(TA + TD). TA, TB, TC, and TD were, respectively, the time mice explored the objects A, B, C, and D.

**Object location memory**

After a rest for 5 days, the same animals underwent an Object Location Memory (OLM) task referring to Li’s study.38 To this end, the animals were habituated to an empty white box (30 cm × 26 cm × 30 cm) for 30 minutes per day for three consecutive days and 10 minutes at the 4th day. During the training session at the 5th day, the mice freely explored the floor of the box that contained two different objects. To get
a measure of the OLM, one object was moved 24 cm to a
new position and the total time of exploration of the familiar
and novel object localization was measured 24 hours after the
training. The objects and boxes were cleaned with ethanol
after every training or test. An outline of the procedures are
depicted in Figure 4A,B.

Fear conditioning test
The fear conditioning test paradigm was performed follow-
ing the methods previously described. In brief, the test was
conducted in a conditioning chamber (33 cm × 33 cm × 33 cm)
equipped with white board walls, a transparent front door, a
speaker, and a grid floor. On day 1, mice were placed into the
conditioning chamber and allowed free exploration for 2 min-
utes before the delivery of the conditioned stimulus (CS) tone
(20 seconds, 80 dB, 2000 Hz) paired with a foot shock uncon-
ditioned stimulus (US; 2 seconds, 0.95 mA) through a grid
floor at the end of the tone. A total of five CS-US pairs with a
60-s intertrial interval (ITI) were presented to each animal in
the training stage. The mouse was removed from the chamber
1 minute after the last foot shock and placed back in its home
cage. The contextual fear conditioning stage started 48 hours
after the training phase, when the animal was put back inside
the conditioning chamber for 5 minutes. The animal's freezing
responses to the environmental context were recorded. The
animal was placed back into the same chamber with different
contextual cues, including green wall, smooth plastic floor,
and vinegar drops condition for 5 minutes, and the animal's
freezing responses to the altered context were recorded. The
tone fear conditioning stage started 2 hours after the differ-
ent contextual stage. After 2 minutes of free exploration, the
mouse was exposed to the exact same 3-CS stones with 20-s ITI
in the training stage without the foot shock, and its freezing
responses to the tones were recorded.

Morris water maze test
Spatial reference memory was detected by the Morris water
maze (MWM) test. Morris Water Maze (MWM) was used to
detect spatial reference memory.40 A circular arena
(120 cm × 50 cm) filled with water (23 ± 2°C) was used for
MWM. Then, an escape platform (10 × 10 × 15 cm) was
placed on the tank, 1.5 cm below the water surface. A white
titanium dioxide was added to the water. The walls of the test
room were pasted with some pictures for permanent extra-
maze cues. The trajectory of the mice was monitored by a
video-tracking camera (placed 200 cm above the center of the
pool surface). Latency time (s) to find the hidden platform
were recorded during each trial. If the mice found the platform
within 60 seconds, it was left on the platform for 20 seconds.
If the mice was not able to find the platform within the time
limit, it was gently placed on the platform 20 seconds. Probe
tests were performed 1 or 48 hours after acquisition. Learning
consisted of six consecutive daily acquisition sessions, each
of them consisting of four trials. Probe tests were carried out
1 or 48 hours after the last acquisition session.

2.2.10  |  Statistical analysis

Data are expressed as mean ± SEM and analyzed using
Graphpad Prism 8 statistical software (GraphPad Software).
The one-way ANOVA was used to determine the differences
among groups. For the comparison between two groups, the
Student's t test was used. The significance was assessed at
P < .05. All results shown correspond to individual represen-
tative experiments.

3  |  RESULTS

3.1  |  PPI Reduces the phosphorylation levels
of tau through inhibiting CIP2A expression
and promoting PP2A Activity in CIP2A and
tau co-expressed HEK293-T cells

CIP2A is a specific inhibitor of PP2A which is the most im-
portant tau phosphatase, and PPI can inhibit the expression
of CIP2A protein in tumor.35,36 To investigate whether PPI
dephosphorylates tau by inhibiting CIP2A expression, the
CIP2A and tau co-transfected HEK293-T cells were incub-
ated with or without PPI (0.125, 0.25, 0.5, 1.0, or 2.0 μM)
incubation for 48 hours and with or without PPI (0.5 μM)
incubation for 6, 12, 24, 48, or 72 hours, and CIP2A expression,
PP2A activity, and tau phosphorylation were detected. The
PPI chemical structure was showed (Figure S1A). During the
period of our experiment, CIP2A overexpression inhibited
PP2A activity, and incubation of PPI (0.125 and 0.25 μM)
for 48 hours did not inhibit the CIP2A overexpression, as a
result, they did not reverse the PP2A activity. However, in-
cubation of PPI (0.5, 1.0, or 2.0 μM) for 48 hours inhibited
the CIP2A overexpression and restored the PP2A activity
(Figure S1B,D,E). Incubation of 0.5 μM PPI for 6, 12, and
24 hours did not inhibit the CIP2A expression and restore
the PP2A activity (Figure S1F,H,I). PPI at all concentrations
and time points had no cytotoxicity to cells (Figure S1C,G).
In the following study, PPI treatments (0.5 or 1.0 μM) for
24 or 48 hours were used. The data showed CIP2A overex-
pression inhibited the PP2A activity, and incubation of PPI (0.5 or 1.0 μM) for 24 hours did not reverse the PP2A activ-
ity, however, incubation of PPI (0.5 or 1.0 μM) for 48 hours
restored the PP2A activity (Figure 1A). PPI with our con-
centrations and two time points had no cytotoxicity to cells
(Figure 1B). We further found that PPI (0.5 or 1.0 μM) for
24 hours could not inhibit CIP2A mRNA expression, but PPI
(0.5 or 1.0 μM) for 48 hours could rescue the CIP2A mRNA
expression (Figure 1C). Besides, we revealed the interaction
FIGURE 1  PPI Reduces the Phosphorylation Levels of Tau through Inhibiting CIP2A Expression and Promoting PP2A Activity in CIP2A and tau co-expressed HEK293-T cells. HEK293-T cells were co-transfected with pFUW vector and tau plasmids, or pFUW-CIP2A and tau plasmids with or without PPI (0.5 or 1.0 μM) incubation for 24 or 48 h. A, Cell lysates of HEK293-T cells were subjected to PP2A activity assay. (Con + Tau: 100.00 ± 0.71; CIP2A + Tau: 45.35 ± 1.28; CIP2A + Tau + 0.5 μM PPI-24 h: 49.04 ± 1.61; CIP2A + Tau + 1.0 μM PPI-24 h: 50.98 ± 2.03; CIP2A + Tau + 0.5 μM PPI-48 h: 80.85 ± 1.51; CIP2A + Tau + 1.0 μM PPI-48 h: 89.56 ± 0.40; Con + Tau vs CIP2A + Tau: P < .001; CIP2A + Tau vs CIP2A + Tau + 0.5 μM PPI-24 h: P = .6250; CIP2A + Tau vs CIP2A + Tau + 1.0 μM PPI-24 h: P = .0805; CIP2A + Tau vs CIP2A + Tau + 0.5 μM PPI-48 h: P < .001; CIP2A + Tau vs CIP2A + Tau + 1.0 μM PPI-48 h: P < .001; *P < .05, **P < .01, ***P < .001. Data are mean ± SEM, n = 9 wells per group). B, The cell viability was detected by CCK8 assay kit. (Con + Tau: 100.00 ± 1.20; CIP2A + Tau: 107.90 ± 5.67; CIP2A + Tau + 0.5 μM PPI-24 h: 109.90 ± 3.31; CIP2A + Tau + 1.0 μM PPI-24 h: 102.50 ± 2.58; CIP2A + Tau + 0.5 μM PPI-48 h: 96.07 ± 4.38; CIP2A + Tau + 1.0 μM PPI-48 h: 114.90 ± 4.12; Con + Tau vs CIP2A + Tau: P = .9381; CIP2A + Tau vs CIP2A + Tau + 0.5 μM PPI-24 h: P > .9999; CIP2A + Tau vs CIP2A + Tau + 1.0 μM PPI-24 h: P = .9980; CIP2A + Tau vs CIP2A + Tau + 0.5 μM PPI-48 h: P = .5289; CIP2A + Tau vs CIP2A + Tau + 1.0 μM PPI-48 h: P = .9740; Data are mean ± SEM, n = 3 wells per group). C, Quantitative analysis of mRNA levels of CIP2A was detected by RT-PCR. (Con + Tau: 100.00 ± 0.00; CIP2A + Tau: 949.40 ± 67.68; CIP2A + Tau + 0.5 μM PPI-24 h: 753.10 ± 88.50; CIP2A + Tau + 1.0 μM PPI-24 h: 678.90 ± 77.43; Con + Tau vs CIP2A + Tau: P < .0001; CIP2A + Tau vs CIP2A + Tau + 1.0 μM PPI-48 h: P < .0001; *P < .05, **P < .01, ***P < .001. Data are mean ± SEM. n = 3 wells per group). D, The interaction of Tau, CIP2A, and PP2Ac was examined by co-immunoprecipitation (CO-IP) assay. E, HEK293-T cells were co-transfected with pFUW vector and tau plasmids, or pFUW-CIP2A and tau plasmids, with or without PPI (0.5 or 1.0 μM) incubation for 48 h. Cells were collected for Western blot. Representative blots of CIP2A, PP2Ac, S396-Tau, S404-Tau, and Tau-5. β-actin was used as a loading control. F, Quantitative analysis of the normalized CIP2A, PP2Ac, S396-Tau, S404-Tau, and Tau-5 intensities in (E). (CIP2A: Con + Tau: 100.00 ± 30.59; CIP2A + Tau: 728.90 ± 145.10; CIP2A + Tau + 0.5 μM PPI: 268.40 ± 52.22; CIP2A + Tau + 1.0 μM PPI: 165.00 ± 47.75; Con + Tau vs CIP2A + Tau: P = .0038; CIP2A + Tau vs CIP2A + Tau + 0.5 μM PPI: P = .0247; CIP2A + Tau vs CIP2A + Tau + 1.0 μM PPI: P = .0076; PP2A: Con + Tau: 100.00 ± 14.53; CIP2A + Tau: 125.20 ± 8.53; CIP2A + Tau + 0.5 μM PPI: 103.20 ± 6.25; CIP2A + Tau + 1.0 μM PPI: 130.80 ± 2.13; Con + Tau vs CIP2A + Tau: P = .8236; CIP2A + Tau vs CIP2A + Tau + 0.5 μM PPI: P = .8879; CIP2A + Tau vs CIP2A + Tau + 1.0 μM PPI: P > .9999; 396: Con + Tau: 100.00 ± 31.14; CIP2A + Tau: 238.60 ± 12.62; CIP2A + Tau + 0.5 μM PPI: 103.30 ± 34.71; CIP2A + Tau + 1.0 μM PPI: 238.60 ± 12.62; CIP2A + Tau + 0.5 μM PPI: 103.30 ± 34.71; CIP2A + Tau + 1.0 μM PPI: 62.26 ± 8.21; Con + Tau vs CIP2A + Tau: P = .02635; CIP2A + Tau vs CIP2A + Tau + 0.5 μM PPI: P = .0267; CIP2A + Tau vs CIP2A + Tau + 1.0 μM PPI: P = .0056; 404: Con + Tau: 100.00 ± 27.06; CIP2A + Tau: 388.50 ± 50.09; CIP2A + Tau + 0.5 μM PPI: 209.70 ± 13.012; CIP2A + Tau + 1.0 μM PPI: 76.62 ± 3.81; Con + Tau vs CIP2A + Tau: P = .0007; CIP2A + Tau vs CIP2A + Tau + 0.5 μM PPI: P = .0152; CIP2A + Tau vs CIP2A + Tau + 1.0 μM PPI: P = .0004; Tau-5: Con + Tau: 100.00 ± 11.87; CIP2A + Tau: 123.60 ± 6.23; CIP2A + Tau + 0.5 μM PPI: 99.70 ± 4.49; CIP2A + Tau + 1.0 μM PPI: 129.00 ± 23.77; Con + Tau vs CIP2A + Tau: P = .8394; CIP2A + Tau vs CIP2A + Tau + 0.5 μM PPI: P = .8320; CIP2A + Tau vs CIP2A + Tau + 1.0 μM PPI: P > .9999; *P < .05, **P < .01, ***P < .001. Data are mean ± SEM, n = 3 wells per group)
of CIP2A, PP2A, and tau5 through coimmunoprecipitation (Co-IP) (Figure 1D). PPI (0.5 or 1.0 μM) for 48 hours was valid in inhibiting CIP2A expression, therefore, PPI (0.5 or 1.0 μM) for 48 hours was used in the next experiments. Furthermore, PPI decreased the phosphorylation of tau at S396 and S404 (Figure 1E,F). These results indicate that PPI reduces tau hyperphosphorylation by inhibiting the expression of CIP2A mRNA and protein, then activating PP2A in CIP2A and tau co-expressed HEK293-T cells.

3.2 PPI reduces the phosphorylation and β-secretion of APP through inhibiting CIP2A expression and promoting PP2A activity in CIP2A and APP co-expressed HEK293-T cells

To examine whether PPI participates in CIP2A inhibition in CIP2A and APP co-expressed HEK293-T cells, cells were incubated without or with PPI (0.5 or 1.0 μM) for 24 or 48 hours, then CIP2A expression, PP2A activity, APP phosphorylation, and β-secretion were detected. CIP2A overexpression inhibited the PP2A activity, and incubation of PPI (0.5 or 1.0 μM) for 24 hours did not reverse the PP2A activity, however, incubation of PPI (0.5 or 1.0 μM) for 48 hours restored the PP2A activity (Figure 2A). PPI with our concentrations and two time points had no cytotoxicity to cells (Figure 2B). Meanwhile, incubation of PPI (0.5 or 1.0 μM) for 24 hours did not decrease the CIP2A mRNA expression, but incubation of PPI (0.5 or 1.0 μM) for 48 hours decreased the CIP2A mRNA expression (Figure 2C). We further detected the interaction of CIP2A, PP2A, and APP by CO-IP (Figure 2D). Furthermore, CIP2A increased the phosphorylation of APP at T668, and PPI (0.5 or 1.0 μM) for 48 hours rescued the hyperphosphorylation (Figure 2E,F). Actually, CIP2A raised the β-secretion of APP (Aβ40 and Aβ42), and PPI also declined Aβ40 and Aβ42 both in cell supernatant (CS) or cell lysate (CL) (Figure 2G,H). These findings indicate PPI promoted APP dephosphorylation and Aβ clearance.
by inhibiting CIP2A and promoting PP2A activity in CIP2A and APP770 co-expressed HEK293-T cells.

3.3 | PPI reduces tau and APP phosphorylation, Aβ production and rescues loss of synaptic proteins in CIP2A-overexpressed primary neurons

As we know, AD is a neurodegenerative disease, in addition to tau hyperphosphorylation, APP hyperphosphorylation, and Aβ aggregation, loss of some synaptic proteins results in loss of neuronal function. To investigate whether PPI rescue loss of some synaptic proteins in CIP2A-overexpressed primary neurons, neurons were infected with CIP2A lentivirus for 6 days, incubated with 0, 0.5, or 1.0 (μM) PPI for 24 or 48 hours, then CIP2A expression, PP2A activity, tau and APP phosphorylation, β-secretion were detected. We found that the overexpression of CIP2A inhibited the PP2A activity, PPI (0.5 or 1.0 μM) for 24 hours did not reverse the PP2A activity, however, incubation of PPI (0.5 or 1.0 μM) for 48 hours restored the PP2A activity (Figure 3A) in primary hippocampal neurons. Cytotoxicity results showed that the use of PPI did not affect the growing metabolism of neurons (Figure 3B). Similarly, incubation of PPI (0.5 or 1.0 μM) for 24 hours did not decrease the CIP2A mRNA expression, but incubation...
of PPI (0.5 or 1.0 μM) for 48 hours decreased the CIP2A mRNA expression (Figure 3C) in CIP2A-overexpressed primary neurons. Then, PPI (0.5 μM) for 48 hours was adopted in the further experiments. We found the overexpression of CIP2A-induced tau and APP hyperphosphorylation, while PPI rescued tau and APP hyperphosphorylation (Figure 3D,E). In the same time, the overexpression of CIP2A decreased the synapse-related proteins including Syn, Syn I, PSD95, and
FIGURE 3 PPI Reduces Tau and APP phosphorylation, Aβ production, and rescues loss of synaptic proteins in CIP2A-overexpressed Primary Neurons. Primary hippocampal neurons were infected with CIP2A lentiviruses for 6 days, then incubated with 0, 0.5, or 1.0 (μM) PPI for 24 h or 48 h. A. Cell lysates of primary neurons were subjected to PP2A activity assay. n = 9 wells per group. (Con:100.00 ± 4.70; CIP2A: 35.18 ± 1.39; CIP2A + 0.5 μM PPI: 23.50 ± 2.55; CIP2A + 1.0 μM PPI: 20.76 ± 1.14; CIP2A + 0.5 μM PPI-48 h: 79.71 ± 30; Con vs CIP2A: P = .0001; CIP2A vs CIP2A + 0.5 μM PPI-24 h: P > .9999; CIP2A vs CIP2A + 1.0 μM PPI-24 h: P = .9995; CIP2A vs CIP2A + 0.5 μM PPI-48 h: P = .0001; CIP2A vs CIP2A + 1.0 μM PPI-48 h: P = .0001. Data are mean ± SEM, n = 9 wells per group). B. The cell viability of primary neurons was detected by CCK8 assay kit. n = 9 wells per group. (Con: 100.00 ± 0.82; CIP2A: 97.17 ± 2.52; CIP2A + 0.5 μM PPI-24 h: 98.48 ± 0.25; CIP2A + 1.0 μM PPI-48 h: 103.60 ± 5.75; Con vs CIP2A: P > .9999; CIP2A vs CIP2A + 0.5 μM PPI-24 h: P = .9999; CIP2A vs CIP2A + 1.0 μM PPI-48 h: P = .9990; CIP2A vs CIP2A + 0.5 μM PPI-48 h: P = .9866; Data are mean ± SEM, n = 9 wells per group). C. Quantitative analysis mRNA levels of CIP2A was detected by RT-PCR, n = 3 per group. (Con: 100.00 ± 0.00; CIP2A: 826.50 ± 153.60; CIP2A + 0.5 μM PPI-24 h: 520.70 ± 64.30; CIP2A + 1.0 μM PPI-24 h: 478.8 ± 9.043; CIP2A + 0.5 μM PPI-48 h: 226.5 ± 38.62; CIP2A + 1.0 μM PPI-48 h: 160.1 ± 35.59; Con vs CIP2A: P = .0002; CIP2A vs CIP2A + 0.5 μM PPI-24 h: P = .1462; CIP2A vs CIP2A + 1.0 μM PPI-24 h: P = .0703; CIP2A vs CIP2A + 0.5 μM PPI-48 h: P = .0010; CIP2A vs CIP2A + 1.0 μM PPI-48 h: P = .0004; Data are mean ± SEM, n = 3 wells per group). D, Cells were collected for Western blot. Representative blots of CIP2A, PP2Ac, S396-tau, S404-tau, Tau-tau, Tau-5, T668-APP, and APP. β-actin was used as a loading control. E. Quantitative analysis of the proteins level in (D). (CIP2A: Con: 100.00 ± 33.25; CIP2A: 856.00 ± 184.84; CIP2A + 0.5 μM PPI: 232.33 ± 76.39; Con vs CIP2A: P = .0114; CIP2A vs CIP2A + 0.5 μM PPI: P = .0277; PPI: 169.40 ± 36.96; Con vs CIP2A: P = .014; CIP2A vs CIP2A + 0.5 μM PPI: P = .9999; 396: Con: 100.00 ± 0.00; CIP2A: 973.40 ± 76.39; CIP2A + 0.5 μM PPI: 358.50 ± 46.24; Con vs CIP2A: P = .0001; CIP2A vs CIP2A + 0.5 μM PPI: P = .0251; APP: Con: 100.00 ± 22.48; CIP2A: 174.43 ± 33.72; CIP2A + 0.5 μM PPI: 83.88 ± 11.40; Con vs CIP2A: P = .2049; CIP2A vs CIP2A + 0.5 μM PPI: P = .1121; *P < .05, ***P < .001. Data are mean ± SEM, n = 3 wells per group). F. Representative blots of Syn, Syn I, PSD95, and GluA1. β-actin was used as a loading control. G. Quantitative analysis of the proteins level in (F) (Syn: Con: 100.00 ± 8.90; CIP2A: 21.85 ± 3.36; CIP2A + 0.5 μM PPI: 32.43 ± 2.48; Con vs CIP2A: P = .0002; CIP2A vs CIP2A + 0.5 μM PPI: P = .5531; Syn I: Con: 100.00 ± 6.97; CIP2A: 35.18 ± 1.39; CIP2A + 0.5 μM PPI: P = .9995; Con vs CIP2A: P = .0001; CIP2A vs CIP2A + 0.5 μM PPI: P = .0407; PSD95: Con: 100.00 ± 0.91; CIP2A: 10.80 ± 3.13; CIP2A + 0.5 μM PPI: 25.01 ± 1.80; Con vs CIP2A: P = .0001; CIP2A vs CIP2A + 0.5 μM PPI: P = .0102; GluA1: Con: 100.00 ± 3.96; CIP2A: 12.37 ± 1.88; CIP2A vs CIP2A + 0.5 μM PPI: 29.23 ± 2.06; Con vs CIP2A: P = .0001; CIP2A vs CIP2A + 0.5 μM PPI: P = .0158; *P < .05, ***P < .001. Data are mean ± SEM, n = 3 wells per group). H and I, The relative content of Aβ40 and Aβ42 in cell supernatant (CS) or cell lysate (CL) were detected by ELISA. (Aβ40 in CS: Con: 100.00 ± 16.66; CIP2A: 486.00 ± 23.76; CIP2A + 0.5 μM PPI: 358.50 ± 46.24; Con vs CIP2A: P < .0001; CIP2A + 0.5 μM PPI: P = .0245; Aβ42 in CS: Con: 100.00 ± 4.70; CIP2A: 255.80 ± 14.98; CIP2A + 0.5 μM PPI: 143.20 ± 10.22; CIP2A + 0.5 μM PPI-48 h: 140.30 ± 6.37; CIP2A + 0.5 μM PPI-48 h: 114.30 ± 4.81; Con vs CIP2A: P = .0031; CIP2A vs CIP2A + 0.5 μM PPI: P = .0251; Aβ42 in CL: Con: 100.00 ± 8.63; CIP2A: 179.40 ± 9.12; CIP2A + 0.5 μM PPI: 118.80 ± 6.95; Con vs CIP2A: P < .0001; CIP2A vs CIP2A + 0.5 μM PPI: P < .0001; *P < .05, ***P < .01, ***P < .001. Data are mean ± SEM, n = 9 wells per group).

3.4 PPI makes an improvement in performance of AD transgenic mice (3XTg) both in object recognition memory (ORM) and object location memory (OLM) tests

To further explore the effect of PPI on cognitive function in vivo, we adopted 3XTg mice. We found that CIP2A was increased in 3XTg mice (Figure S2A,B). Then, PPI (1 mg/kg) was injected into 3XTg mice (10 months old) by intraperitoneal injection once a day for 1 month. The behavior tests and postmortem experiments were performed. We did ORM and OLM tests first (Figure S2C). In ORM, we designed 4-day experiment, the animals were allowed to explore a round black barrel (diameter: 26 cm and height 38 cm) for 30 minutes on the 1st day for habituation. On the 2nd day, the animals were trained to memorize two objects (A and B) with 3 minutes. After 24 and 48 hours retention interval, a novel object with a different shape and color (C or D) was taken to replace the object B for test (Figure 4A). The recognition index was no significant difference in the three groups including Wild-type mice(WT), APP/PS1/Tau triple transgene mice (3XTg), and PPI-treated triple transgene mice (3XTg + PPI) in the acquisition trial (Figure 4C). In the test, mice in WT and 3XTg + PPI groups showed increased interest to the new object with comparable recognition index, however, recognition index to the
new object was significantly decreased in 3XTg group mice, discrimination index was significant difference (Figure 4D,F), indicating ORM is impaired in 3XTg mice and PPI rescue ORM of 3XTg mice. Subsequently, the same animals underwent an OLM task. To this end, the animals were habituated to an empty white box (30 cm × 26 cm × 30 cm) for 30 minutes on the 5th day. The same objects (E1 and E2) were placed in the corners of the white box and the animals were trained for 3 minutes on the 6th day. The location of E2 was changed day by day after habituation and training (Figure 4B). The recognition index was no significant difference in the three groups (WT, 3XTg, and 3XTg + PPI) during the acquisition trial (Figure 4G). In two tests, both WT and 3XTg + PPI mice were more interest in E1 at the same place and E2 at new place (Figure 4H,I). Moreover, the discrimination index was declined in 3XTg mice, but the discrimination index was raised in 3XTg + PPI mice in OLM testing on Day 7 and Day 8 (Figure 4J). These results indeed confirmed the memory impairment were rescued by PPI.

3.5 | PPI improves spatial learning and memory deficits in AD transgenic mice (3XTg)

To examine whether PPI improves spatial learning and memory deficits in 3XTg mice, we performed the MWM test on three groups of mice (WT, 3XTg, and 3XTg + PPI) (Figure 5A). In the last 2 days of the acquisition phase, 3XTg mice had significantly longer escape latency than the control and escape latency was substantially shortened in 3XTg + PPI mice (Figure 5B,C). In the probe test after 24 hours, compared with the 3XTg mice, the 3XTg + PPI mice showed increased Crossing Times and Time in target quadrant (Figure 5D,F,G) with close swimming speed (Figure 5E). These results suggest that PPI is beneficial to spatial learning and memory decline.

3.6 | PPI rescues fear condition memory (FCM) impairment in AD transgenic mice (3XTg)

Proper function of the hippocampus formation is the basis of the FCM. Therefore, we performed fear conditioning test (FCT) by measuring the freezing time for three groups of mice (WT, 3XTg, and 3XTg + PPI) after they were previously given mild electrical foot shocks and tone during training phase (Figure 6A). In FCM, 3XTg mice showed a significant reduction both in freezing time and freezing times, however, PPI-treated 3XTg mice heightened the freezing time and freezing times in both the context test, and tone test (Figure 6B,D). Whatsoever, there were no obvious difference in the altered context test (Figure 6C) in the three groups. Together, these results indicate that 3XTg mice have conditional fear memory deficits and PPI strongly improves conditional fear memory.
FIGURE 4 PPI rescues memory impairments of AD transgenic mice (3XTg) both in Object Recognition Memory (ORM) and Object Location Memory (OLM) tests. A and B, The schematic diagram indicates the sequence of the object recognition and object location tasks. C, Recognition index for object A and B in the acquisition trial on Day 2. (Recognition index for object A of WT mice: 0.50 ± 0.009; Recognition index for object B of WT mouse: 0.49 ± 0.009; Recognition index for object A of 3XTg mice: 0.495 ± 0.03; Recognition index for object B of 3XTg mice: 0.51 ± 0.027; Recognition index for object A of 3XTg + PPI mice: 0.48 ± 0.014; Recognition index for object B of 3XTg + PPI mice: 0.52 ± 0.014; WT: A vs B: P = .5546; 3XTg: A vs B: P = .7966; 3XTg + PPI: A vs B: P = .0626; n = 6 mice per group). D, Object B was replaced by a new object C, recognition index for object A and C was detected on Day 3. (Recognition index for object A of WT mice: 0.22 ± 0.019; Recognition index for object B of WT mice: 0.78 ± 0.02; Recognition index for object A of 3XTg mice: 0.50 ± 0.015; Recognition index for object B of 3XTg mice: 0.498 ± 0.015; Recognition index for object A of 3XTg + PPI mice: 0.25 ± 0.031; Recognition index for object B of 3XTg + PPI mice: 0.75 ± 0.03; WT: A vs C: P < .0001; 3XTg: A vs C: P = .8477; 3XTg + PPI: A vs C: P < .0001; ***P < .001. Data are mean ± SEM, n = 6 mice per group). E, Object C was replaced by a new object D, and recognition index for object A and D was detected on Day 4. (Recognition index for object A of WT mice: 0.24 ± 0.02; Recognition index for object B of WT mice: 0.76 ± 0.02; Recognition index for object A of 3XTg mice: 0.49 ± 0.01; Recognition index for object B of 3XTg mice: 0.51 ± 0.01; Recognition index for object A of 3XTg + PPI mice: 0.28 ± 0.03; Recognition index for object B of 3XTg + PPI mice: 0.72 ± 0.03; WT: A vs D: P < .0001; 3XTg: A vs D: P = .3043; 3XTg + PPI: A vs D: P < .0001; *P < .05, **P < .01. Data are mean ± SEM, n = 6 mice per group). F, Discrimination index in Object Recognition Memory testing on Day 3 and Day 4. (Discrimination index on Day 3: WT: 0.57 ± 0.04; 3XTg: 0.02 ± 0.01; 3XTg + PPI: 0.50 ± 0.062; WT vs 3XTg: P < .0001; 3XTg vs 3XTg + PPI: P < .0001; Discrimination index on Day 4: WT: 0.68 ± 0.025; 3XTg: 0.033 ± 0.054; 3XTg + PPI: 0.585 ± 0.074; WT vs 3XTg vs 3XTg + PPI: P < .0001; *P < .05, **P < .001. Data are mean ± SEM, n = 6 mice per group). G, Recognition index for the objects in place E1 and E2 in the acquisition trial (Day 6). (Recognition index for object E1 of WT mice: 0.49 ± 0.011; Recognition index for object E2 of WT mice: 0.51 ± 0.011; Recognition index for object E1 of 3XTg mice: 0.505 ± 0.017; Recognition index for object E2 of 3XTg mice: 0.495 ± 0.017; Recognition index for object E1 of 3XTg + PPI mice: 0.490 ± 0.023; Recognition index for object E2 of 3XTg + PPI mice: 0.510 ± 0.023; WT: E1 vs E2: P = .3329; 3XTg: E1 vs E2: P = .6747; 3XTg + PPI: E1 vs E2: P = .5634; Data are mean ± SEM, n = 6 mice per group). H, The position E2 were changed, and recognition index for the objects at place E1 and E2 were detected on Day 7. (Recognition index for object E1 of WT mice: 0.220 ± 0.017; Recognition index for object E2 of WT mice: 0.780 ± 0.017; Recognition index for object E1 of 3XTg mice: 0.498 ± 0.015; Recognition index for object E2 of 3XTg mice: 0.502 ± 0.015; Recognition index for object E1 of 3XTg + PPI mice: 0.282 ± 0.027; Recognition index for object E2 of 3XTg + PPI mice: 0.718 ± 0.027; WT: E1 vs E2: P < .0001; 3XTg: E1 vs E2: P = .8394; 3XTg + PPI: E1 vs E2: P < .0001; ***P < .001. Data are mean ± SEM, n = 6 mice per group). I, The position of E2 were changed again, and recognition index for the objects at place E1 and E2 were detected on Day 8. (Recognition index for object E1 of WT mice: 0.236 ± 0.013; Recognition index for object E2 of WT mice: 0.764 ± 0.013; Recognition index for object E1 of 3XTg mice: 0.497 ± 0.015; Recognition index for object E2 of 3XTg mice: 0.503 ± 0.015; Recognition index for object E1 of 3XTg + PPI mice: 0.300 ± 0.037; Recognition index for object E2 of 3XTg + PPI mice: 0.700 ± 0.037; WT: E1 vs E2: P < .0001; 3XTg: E1 vs E2: P = .7538; 3XTg + PPI: E1 vs E2: P < .0001; ***P < .001. Data are mean ± SEM, n = 6 mice per group). J, Discrimination index in Object Location Memory testing on Day 7 and Day 8. (Discrimination index on Day 7: WT: 0.561 ± 0.035; 3XTg: 0.004 ± 0.029; 3XTg + PPI: 0.436 ± 0.053; WT vs 3XTg: P < .0001; 3XTg vs 3XTg + PPI: P < .0001; Discrimination index on Day 8: WT: 0.528 ± 0.025; 3XTg: 0.007 ± 0.031; 3XTg + PPI: 0.399 ± 0.074; WT vs 3XTg: P < .0001; 3XTg vs 3XTg + PPI: P < .0001; ***P < .001. Data are mean ± SEM, n = 6 mice per group).

3.7 | PPI increases neuron and spine numbers, restores PP2A activity, and reduces Aβ and Tau(APP) phosphorylation levels in the hippocampus of AD Transgenic mice (3XTg)

To study whether PPI affects neuron loss in vivo, we did Nissl staining and Golgi staining in hippocampus. Compared with WT mice, the number of Nissl body was decreased in 3XTg mice, but PPI increased the number of Nissl body (Figure 7A,C). At the same time, Golgi staining results show that dendritic spines of hippocampal neurons have also been decreased in 3XTg mice, PPI increased the number of dendritic spines in 3XTg + PPI mice (Figure 7B,D). Furthermore, to investigate how PPI rescues the loss of hippocampal neurons in 3XTg mice, we found CIP2A, the phosphorylation of tau (S396 and S404) and APP (T668) increased in 3XTg mice, and they decreased in 3XTg + PPI mice (Figure 7E,F). We also noticed that the quantity of synapse-related proteins including Syn, Syn I, PSD95, and GluA1 in 3XTg + PPI mice hippocampus were much more than those in 3XTg mice hippocampus (Figure 7G,H). The PP2A activity was declined in 3XTg mice hippocampus and was increased in 3XTg + PPI mice hippocampus (Figure 7I). Besides, Aβ40 and Aβ42 levels in 3XTg + PPI mice hippocampus were lower than those in 3XTg mice hippocampus (Figure 7J,K). These results indicate that PPI can increase the Nissl body and dendrites of neurons, rescue the PP2A activity, promote the dephosphorylation of tau and APP, and reduce the accumulation of Aβ in 3XTg mice hippocampus.

4 | DISCUSSION

AD is a progressive neurological degenerative disease characterized by the deposition of amyloid plaques, neurofibrillary
It is estimated that the global number of AD cases will reach more than 13.8 million by 2050. Annual health-care costs for Alzheimer’s and other dementia patients are expected to increase from US$277 billion in 2018 to US$1.1 trillion in 2050. As the number of Alzheimer’s patients increases, it becomes more urgent to find effective treatment to the disease. The accumulation of hyperphosphorylated tau-containing neurofibrillary tangles (NFTs) is most closely related to cognitive decline. Tau hyperphosphorylation induces its dissociation from microtubules, which may cause synaptic dysfunction. Therefore, inhibiting tau hyperphosphorylation is considered an promising way to treat AD.

PP2A is a key protein phosphatase in dephosphorylating tau, and its activity is decreased in the brain of AD patients. PP2A inactivation will lead to abnormal tau hyperphosphorylation. Furthermore, APP dephosphorylation at T668 is also regulated by PP2A. PP2A inhibition resulted in increased APP-T668 phosphorylation and enhanced β-secretase processing of APP, which promoting Aβ production.

Thus, strategy aiming at reactivating PP2A in AD is catching more attention.

Since PP2A is widely expressed in different cells and tissues, and it participates in lots of signaling pathways through dephosphorylating numerous substrates, a relatively specific intervention of PP2A activity toward tau/APP is a precondition for possible drug development in AD. CIP2A has been proven as an endogenous PP2A inhibitor, and it inhibits PP2A in a substrate-dependent manner. We have identified that CIP2A is overexpressed in AD patients’ brain, and CIP2A-mediated PP2A inhibition drives tau hyperphosphorylation and Aβ overproduction, suggesting that CIP2A is a potential target for modulating PP2A in AD treatment.

In many tumors, CIP2A is also upregulated and promotes cancer development through inhibiting PP2A. In a scanning of antitumor drugs which may act on CIP2A expression, we noticed that PPI may be a potential candidate drug for AD. PPI is a compound extracted from the rhizomes of Paris polyphylla and is widely used to treat inflammation, cancer,
and other diseases. Previous studies have shown that PPI can inhibit the expression of CIP2A and accelerate its decomposition, which suggests that PPI is an effective inhibitor of CIP2A. CIP2A is an inhibitor of PP2A, and PPI can re-activate PP2A by inhibiting CIP2A. Thus, we explored the effects of PPI both in cells and AD animal models.

By applying PPI to CIP2A and tau or CIP2A and APP co-expressed HEK293-T cells, we found that PPI can significantly reduce the expression of CIP2A and increase the relative activity of PP2A. We also found that PPI can dephosphorylate tau at AD-related S396 and S404 sites, dephosphorylate APP at T668, and reduce Aβ levels. Same results
were observed in primary neurons overexpressed CIP2A. More importantly, PPI effectively prevented the loss of synaptic proteins, indicating the efficacy of PPI in neuronal protection. This effect was further identified in 3XTg-AD mice. First, PPI improved the learning and memory ability of the mice in several behavior tests such as ORM, OLM, spatial learning, and memory and fear conditioning memory test. In biochemical analysis of brain tissues, the same
FIGURE 7  PPI increases neuron and spine numbers, reduces Aβ and Tau/APP phosphorylation levels in the hippocampus of AD Transgenic mice (3XTg). The hippocampus of the mice were sliced for Nissl staining and Golgi staining, or homogenized for ELISA and immunoblotting. A. Representative Nissl staining images of the brain slices. B. Representative images of dendritic spines in hippocampal neurons. C. Quantitative analysis of the Nissl body numbers in CA3 region of (A). (WT: 100.00 ± 5.270; 3XTg: 65.65 ± 3.72; 3XTg + PPI: 85.33 ± 0.420; WT vs 3XTg: P = .0026; 3XTg vs 3XTg + PPI: P = .0381; *P < .05, **P < .01, Data are mean ± SEM, n = 3 hemi-brains per group). D. Quantitative analysis of (B). (WT: 17.67 ± 0.88; 3XTg: 10.00 ± 1.16; 3XTg + PPI: 14.67 ± 0.88; WT vs 3XTg: P = .0044; 3XTg vs 3XTg + PPI: P = .0448; *P < .05, **P < .01, Data are mean ± SEM, n = 3 hemi-brains per group). E. Representative blots of CIP2A, PP2Ac, S396-tau, S404-tau, Tau-5, T668-APP, and APP. β-actin was used as a loading control. F. Quantitative analysis of the proteins level in (E) (CIP2A: WT: 100.00 ± 11.65; 3XTg: 1250.46 ± 420.47; 3XTg + PPI: 384.256 ± 35.21; WT vs 3XTg: P = .0008; 3XTg vs 3XTg + PPI: P = .0069; PP2A: WT: 100.00 ± 6.00; 3XTg: 127.85 ± 28.05; 3XTg + PPI: 91.63 ± 8.89; WT vs 3XTg: P = .9992; 3XTg vs 3XTg + PPI: P = .7919; 396: WT: 100.00 ± 9.14; 3XTg: 370.621 ± 111.80; 3XTg + PPI: 253.13 ± 107.24; WT vs 3XTg: P = .026; 3XTg vs 3XTg + PPI: P = .3432; 404: WT: 100.00 ± 23.23; 3XTg: 697.64 ± 104.81; 3XTg + PPI: 298.98 ± 29.97; WT vs 3XTg: P = .0018; 3XTg vs 3XTg + PPI: P = .0139; Tau-5: WT: 100.00 ± 8.83; 3XTg: 89.92 ± 6.12; 3XTg + PPI: 61.42 ± 3.36; WT vs 3XTg: P = .1944; 3XTg vs 3XTg + PPI: P = .599; 668: WT: 100.00 ± 8.65; 3XTg: 187.56 ± 8.73; WT vs 3XTg: P < .0001; 3XTg vs 3XTg + PPI: P < .0001; APP-WT: 100.00 ± 5.77; 3XTg: 158.09 ± 27.80; 3XTg + PPI: 94.256 ± 2.937; WT vs 3XTg: P = .1345; 3XTg vs 3XTg + PPI: P = .198; *P < .05, **P < .01, Data are mean ± SEM, n = 3 hemi-brains per group). G. Representative blots of Syn, Syn I, PSD95, and GluA1 in the hippocampus. β-actin was used as a loading control. H. Quantitative analysis of the proteins level in (G). (Syn: WT: 100.00 ± 17.9420; 3XTg: 32.14 ± 12.33; 3XTg + PPI: 86.03 ± 8.29; WT vs 3XTg: P < .0002; 3XTg vs 3XTg + PPI: P = .0020; Syn I:WT: 100.00 ± 5.26; 3XTg: 17.15 ± 1.47; 3XTg + PPI: 55.99 ± 10.62; WT vs 3XTg: P < .0001; 3XTg vs 3XTg + PPI: P = .0266; PSD95: WT: 100.00 ± 12.39; 3XTg: 19.50 ± 8.29; 3XTg + PPI: 84.12 ± 14.80; WT vs 3XTg: P < .0001; 3XTg vs 3XTg + PPI: P < .0331; GluA1: WT: 100.00 ± 12.93; 3XTg: 19.50 ± 8.29; 3XTg + PPI: 74.12 ± 14.80; WT vs 3XTg: P < .0001; 3XTg vs 3XTg + PPI: P = .0018; *P < .05, **P < .001, Data are mean ± SEM, n = 3 hemi-brains per group). I. Hippocampus lysates of mice were subjected to PP2A activity assay. (WT: 100.00 ± 4.30; 3XTg: 53.33 ± 5.23; 3XTg + PPI: 75.32 ± 2.48; WT vs 3XTg: P = .0006; 3XTg vs 3XTg + PPI: P = .0288; *P < .05, **P < .001, Data are mean ± SEM, n = 3 hemi-brains per group). J and K. The relative content of Aβ40 and Aβ42 in hippocampus detected by ELISA. (Aβ40: WT: 100.00 ± 9.49; 3XTg: 207.60 ± 19.86; 3XTg + PPI: 154.30 ± 9.44; WT vs 3XTg: P < .0001; 3XTg vs 3XTg + PPI: P = .0346; Aβ42: WT: 100.00 ± 5.47; 3XTg: 325.40 ± 20.15; 3XTg + PPI: 187.90 ± 20.13; WT vs 3XTg: P < .0001; 3XTg vs 3XTg + PPI: P = .0006; *P < .05, **P < .001, Data are mean ± SEM, n = 3 hemi-brains per group).

ACKNOWLEDGMENTS

The authors thank the members of the Shentu and Liu labs for helpful comments on the manuscript. This work was supported by the National Natural Science Foundation of China (grants 31900685, 31771189), Natural Science Foundation of Zhejiang Province of China (LQ19H050002), and the Wenzhou Municipal Science and Technology Bureau of China (Y20201001, ZS2017008 and Y20180159).

CONFLICT OF INTEREST

The authors declare no competing financial interest.

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AUTHOR CONTRIBUTIONS

YPST and RL had the initial concept and managed the study. YPST and YZ were involved in analysis data and drafting. DCY and YPST wrote the initial draft of the manuscript. Wang All authors read and approved the final manuscript. YZ, YPST, DCY, HC, HJ, XYL, SMY, CF, SSS, NJ, ZYZ, and SQM performed all experiments.
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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Zhou Y, Yang D, Chen H, et al. Polyphyllin I attenuates cognitive impairments and reduces AD-like pathology through CIP2A-PP2A signaling pathway in 3XTg-AD mice. *The FASEB Journal*. 2020;34:16414–16431. [https://doi.org/10.1096/fj.202001499R](https://doi.org/10.1096/fj.202001499R)