ChK1 activation induces reactive astrogliosis through CIP2A/PP2A/STAT3 pathway in Alzheimer's disease

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Abstract
Cancerous Inhibitor of PP2A (CIP2A), an endogenous PP2A inhibitor, is upregulated and causes reactive astrogliosis, synaptic degeneration, and cognitive deficits in Alzheimer's disease (AD). However, the mechanism underlying the increased CIP2A expression in AD brains remains unclear. We here demonstrated that the DNA damage-related Checkpoint kinase 1 (ChK1) is activated in AD human brains and 3xTg-AD mice. ChK1-mediated CIP2A overexpression drives inhibition of PP2A and activates STAT3, then leads to reactive astrogliosis and neurodegeneration in vitro. Infection of mouse brain with GFAP-ChK1-AAV induced AD-like cognitive deficits and exacerbated AD pathologies in vivo. In conclusion, we showed that ChK1 activation induces reactive astrogliosis, degeneration of...
1 | INTRODUCTION

Alzheimer’s disease (AD) is a common neurodegenerative disease with complex unclear underlying mechanisms. The pathological characteristics of AD are Aβ deposition, tau hyperphosphorylation, neurodegeneration, as well as reactive gliosis.\(^1\,^2\) Glia, in cells number, are 5 to 10 times of neurons, and an increasing body of studies have shown that abnormal activation of glial cells participated in the development of neurodegenerative diseases through neuroinflammation and disturbed glia-neuron interaction.\(^3\,^4\) Astrocytes as the major type of glial cells also play a crucial role in neurodegenerative diseases. Studies have reported that in the early stage of AD, activation of astrocytes would lead to loss of neurons and synapses, and accumulation of pathogenic proteins.\(^5\,^6\) However, the mechanisms underlying the activation of astrocytes in AD have not been completely understood.

Cancerous inhibitor of PP2A (CIP2A) is an endogenous inhibitor of protein phosphatase 2A (PP2A),\(^7\,^8\,^9\) the most important tau phosphatase. In AD brains, PP2A activity has been reported to be decreased.\(^10\,^11\) In our previous study, we found that CIP2A is upregulated in the brains of AD. Increased CIP2A levels correlated with PP2A inhibition, and CIP2A overexpression in neurons induces hyperphosphorylation of PP2A substrates tau and APP, neurodegeneration, and cognitive deficits in mice.\(^12\) Furthermore, CIP2A upregulation specifically in astrocytes can induce reactive astrogliosis which promotes synaptic impairments both in cells and mouse brains.\(^13\) As an oncprotein, the expression of CIP2A is upregulated by activation of checkpoint kinase 1 (Chk1) upon DNA damage in tumor cells.\(^14\) However, whether Chk1 activation also participates in CIP2A upregulation and astrocytes activation in AD remains unanswered.

STAT3 (Signal transducer and activator of transcription 3) as a signal transducer is associated with many biological processes including cell survival, differentiation, growth, and immune response.\(^15\) PP2A inhibition results in STAT3 phosphorylation and activation, leading to the proliferation of tumor cells.\(^16\,^18\) These findings indicate that STAT3 is negatively regulated by PP2A. In AD patients and transgenic AD mouse brains, the activity of STAT3 is upregulated. Recently, Reichenbach et al, showed that in STAT3 knock-out 3XTg-AD mice, astrocytes had better ability to internalize Aβ and produced less inflammatory cytokines,\(^19\) indicating a role of STAT3 in astrogliosis in AD.

Taking all these events together, we speculate that Chk1-CIP2A-PP2A-STAT3 signaling is activated in astrocytes in AD brains and might participate in AD-like astrogliosis. In the present study, we aimed to investigate the change in the Chk1-CIP2A-PP2A-STAT3 signaling pathway in 3XTg-AD mice and to further confirm the hypothesis in both cell and animal models.

2 | MATERIALS AND METHODS

2.1 | Materials

The primary antibodies used in this study were as follow: yH2A.X (Proteintech, Cat#10856-1-ap), S345 (Gene Tex, Cat#39233), Chk1 (Bios, Cat ac01184523; Abclonal, Cat#a7653), S727-STAT3 (Affinity, Cat#af3294), STAT3 (Zenbio, Cat#385805), CIP2A (Cell Signaling Technology, Cat#14805), GluA1 (Cell Signaling Technology, Cat#13185), Synaptophysin (Abcam, Cat#ab32127), β-actin (Abcam, Cat#ab8226), Synapsin 1 (Millipore, Cat#S193), GAPDH (Proteintech, Cat#60004-1-Ig).

Chk1 plasmid and GFAP-Chk1-AAV were from Shanghai Genechem Co. Ltd. (Shanghai, China). Neofect DNA transfection reagent was purchased from Neofect Biological Technology Co. Ltd (Beijing, China). Neurobasal,
B27, DMEM-high, and protein marker were from Invitrogen (Grand Island, NY, USA). DMEM/F12 and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, New York, USA). Serine/Threonine Phosphatase Assay kit was purchased from Promega Company (Madison, Wisconsin, USA). Human or rat or mouse Aβ40/Aβ42 ELISA kits were purchased from Elabscience Biotechnology (Wuhan, China). Nissl staining solution was from Beyotime Biotechnology (Shanghai, China). Golgi staining solution was from GENMED SCIENTIFICS INC. USA.

Human brain samples were from China Brain Bank (Zhejiang University School of Medicine). Details of human brain samples were listed as follows.

| Sample | Age | sex | Braak stage | Brain region |
|--------|-----|-----|-------------|--------------|
| Con-1  | 72  | male | 6           | hippocampus  |
| Con-2  | 83  | male | 4           | hippocampus  |
| Con-3  | 86  | male | 4           | hippocampus  |
| Con-4  | 98  | female | 4         | hippocampus  |
| Con-5  | 83  | female | 5         | hippocampus  |
| AD-1   | 78  | male | 4           | hippocampus  |
| AD-2   | 71  | female | 4         | hippocampus  |
| AD-3   | 73  | male | 6           | hippocampus  |
| AD-4   | 79  | male | 5           | hippocampus  |
| AD-5   | 99  | female | 5         | hippocampus  |

### 2.2 Methods

#### 2.2.1 Animals and AAV delivering

For animal experiments, Sprague-Dawley rats and C57BL/6J mice were purchased from Zhejiang Vital River Experimental Animal Technology Co. LTD Tongxiang branch. 3xTg-AD mice carrying human mutated APP, PS1, and tau genes were from Jackson Lab. All animals have free access to food and water in an air-conditioned room (22 ± 2°C, 12 h light/dark cycle). The behavior tests were performed during their active hours.

For AAV injection, C57 mice were deeply anesthetized with isoflurane. AAV virus particles (2.0 μl at 0.4 μl/min) were injected into the lateral ventricle (interaural 3.58 mm, bregma 0.22 mm, depth 0.30 mm). After the injection, the mice were kept under standard laboratory conditions.

#### 2.2.2 HEK 293-T cell culture and transfection

For HEK293-T cell culture, the cells were cultured in DMEM-high glucose 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 50%-60% confluency in 6-well plates and changed to fresh medium, then transfected with relevant plasmids using Neofect DNA transfection reagent (Neofect Biological Technology, Beijing, China). After 48 h, cells and culture media were collected for further analysis.

#### 2.2.3 Primary astrocyte and neuron culture

Primary astrocyte culture and neuron culture were performed following a previously described method. At the end of treatments, cells were collected and lysed in RIPA buffer for further biological detections or fixed with 4% paraformaldehyde for immunofluorescence imaging.

#### 2.2.4 Western blotting and Co-IP

For Western blotting, brain tissue homogenates or cell lysates were boiled at 100°C for 5 min in the loading buffer (50 mM Tris-HCl, pH 7.6, 2% SDS, 10% glycerol, 10 mM DTT, and 0.2% bromphenol blue). The proteins were electrophoresed in 10% SDS-polyacrylamide gel and the separated proteins were transferred to nitrocellulose membranes (Amersham Biosciences). The membranes were then blocked with 5% nonfat milk dissolved in TBS-Tween-20 (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.2% Tween-20) for 1 h and probed with primary antibody at 4°C overnight. Then, the blots were detected using secondary antibodies at room temperature for 1 h and visualized using the Odyssey Infrared Imaging System (LicorBiosciences, Lincoln, NE, USA). The protein bands were quantitatively analyzed by Image J software (Rawak Software, Inc. Germany).

To analyze protein-protein interactions, Co-IP experiments were performed. 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 h. The immunocomplexes were washed twice with IP lysis buffer before being resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with indicated antibodies.

#### 2.2.5 Fluorescence imaging and confocal microscopy

For cultured neurons, cells were fixed with 4% paraformaldehyde for 15 min, permeabilized in 0.5% Triton
X-100 for 10 min, followed by incubation with 3% bovine serum albumin (BSA) to block nonspecific sites. For mouse brain slices, sectioned slices were incubated with 3% bovine serum albumin (BSA) to block nonspecific sites. After blocking, primary antibody incubation was performed overnight at 4°C. Alexa 488-conjugated secondary antibody (1:200, A-21206, Invitrogen) was used for fluorescence labeling. All the images were observed under the LSM710 confocal microscope (Zeiss, Germany).

For cultured astrocytes, the previous steps were as above. After blocking, primary antibodies incubation was performed overnight at 4°C. Alexa 488 and 543-conjugated secondary antibodies (1:200, A-21206, A-10036, Invitrogen) were used for double fluorescence labeling. All the images were observed under the LSM710 confocal microscope (Zeiss, Germany).

2.2.6 | ChK1 and PP2A activity and ELISA assay

ChK1 and PP2A activity was measured according to the protocols provided by the manufacturers (ChK1, GMSS0155.2 GENMEDSCIENTIFICS INC. USA; PP2A, V2460 kit; Promega). The culture medium or brain tissue homogenates that were lysed in RIPA buffer were collected. The levels of IL-6, TNF-α, Aβ40, and Aβ42 were detected by ELISA following the instructions offered by the assay kit manufacturer (Elabscience Biotechnology, Wuhan, China).

2.2.7 | Behavior tests

**Novel objective recognition test (NORT)**
The mice were habituated to the arenas (50 cm x 50 cm x 50 cm wooden container which was from Techman Software Co., Ltd., Chengdu, China) for 5 min without objects 24 h prior to the test. Arenas were cleaned with 70% ethanol between each habituation period. The day after, the mice re-entered the arenas from the same starting point and were granted 5 min to familiarize themselves with the A object and B object. One hour after the familiarization period, object B was replaced with a C object, and the mice were granted 5 min to explore both objects. After 24 h, the C object was replaced with a D object, and the mice were again granted 5 min to explore both objects. All the behaviors were recorded by a camera 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 of the mice exploring the object A, B, C, and D.

**Object location test (OLT)**
The animals were habituated to a container that is the same as in the novel objective recognition test. During the training session on the 6th day, the mice freely explored the floor of the box that contained two different objects. To get a measure of the OLT, one object was moved 45 cm to a new position, the total time of exploration of the familiar and novel object localization was measured 24 and 48 h after the training. The objects and boxes were cleaned with ethanol after every training or test.

**Morris water maze test**
Spatial learning and memory were detected by Morris Water Maze (Techman Software Co., Ltd., Chengdu, China). Briefly, a circular arena (120 cm x 50 cm) was filled with water (23 ± 2°C). An escape platform (10 x 10 x 15 cm) was placed into the pool, 1.5 cm below the water surface. The water was made opaque by the addition of a white material (titanium dioxide). The test room contained several permanent extra-maze cues such as posters, a flag, or other objects on the walls. A video-tracking camera above the center of the pool surface monitored the trajectory of the mice. The learning period consisted of six consecutive daily acquisition sessions, each of them consisting of four trials, with a maximum trial duration of 60 s. Latency time (s) to find the hidden platform was recorded during each trial of each learning session. If the mice found the platform within the maximum trial time allowed, it was left on the platform for 20 s. If the mice did not find the platform within the time limit, it was gently guided and placed on the platform for a 20 s. Probe tests were carried out 1 or 2 days after acquisition.

**Fear conditioning test**
The fear conditioning test paradigm was performed following the methods previously described. In brief, the test was conducted in a conditioning chamber (33 cm x 33 cm x 33 cm) equipped with whiteboard walls, a transparent front door, a speaker, and a grid floor (Techman Software Co., Ltd., Chengdu, China). On day 1, mice were placed into the conditioning chamber and allowed free exploration for 2 min before the delivery of the conditioned stimulus (CS) tone (20 s, 80 dB, 2000 Hz) paired with a foot shock unconditioned stimulus (US; 2 s, 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 min
after the last foot shock and placed back in its home cage. The contextual fear conditioning stage started 24 h after the training phase when the animal was put back inside the conditioning chamber for 5 min. 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 a blue wall, smooth plastic floor, and alcohol drops condition for 5 min, and the animal’s freezing responses to the altered context were recorded. The tone fear conditioning was evaluated 24 h after the different contextual evaluations. After 2 min of free exploration, the mouse was exposed to the exact same 3-CS tones with 20-s ITI as in the training stage without the foot shock, and its freezing responses to the tones were recorded.

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 min at room temperature, followed by dehydration through 50%, 75%, 95%, and 100% alcohol, clearing in xylene, and coverslipped with neutral balsam. Images were observed using a light microscope.

2.2.9 | Golgi staining

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

2.2.10 | Statistical analysis

Data are expressed as mean ± SEM and analyzed using GraphPad Prism 8 statistical software (USA, GraphPad Software). The one-way analysis of variance (ANOVA) procedure followed by the Tukey test was used to determine the differences among groups. For the comparison between two groups, the Student’s t-test was used. The significance was set at p < .05. All results shown correspond to individual representative experiments.

3 | RESULTS

3.1 | Increased DNA damage, ChK1 activation, and CIP2A upregulation in AD human and 3xTg-AD mice brains

To explore the role of ChK1 in astrogliosis in AD, we first examined whether ChK1 is activated or not in the brains of 3xTg-AD mice and AD patients’ brains. Compared to the wild type (WT), DNA damage marker γH2A.X, the active form of ChK1 (ChK1 phosphorylated at S345), and CIP2A levels were all increased in 3xTg-AD mouse brains (Figure 1A,B). Of note, after linear fitting, we found that γH2A.X and ChK1- S345 were highly correlated (Figure 1C). ChK1- S345 and CIP2A correlation exhibited similar results (Figure 1D). In AD human brains, compared to the control, the protein level of γH2A.X was increased slightly, however, the active form of ChK1 (ChK1 phosphorylated at S345), and CIP2A levels were all increased significantly (Figure 1E,F). Taking together, our data show that in AD brains, DNA damage, ChK1 activation, and CIP2A upregulation occur simultaneously, indicating a potential relationship of these events in AD development.

3.2 | Aβ induces DNA damage, ChK1-CIP2A-PP2A-STAT3 signaling axis activation, and astrogliosis in primary astrocytes

To further confirm the signal transduction pathway from ChK1 to STAT3 in astrocytes and astrogliosis in AD, we detected the protein molecules in the signaling axis in primary astrocytes treated with Aβ, with or without pre-incubation of ChK1 inhibitor SB. Compared to the non-treated or Aβ42-1-treated negative control, Aβ incubation increased γH2A.X, ChK1-S345, CIP2A, the active form of STAT3 (S727 phosphorylated STAT3) and GFAP levels, and SB treatment reversed γH2A,X elevation, ChK1 activation, CIP2A overexpression, STAT3 activation as well as astrogliosis induced by Aβ (Figure 2A,B). We also found that the expression level of GFAP highly correlated with ChK1-S345, indicating that ChK1 activation might promote astrogliosis (Figure 2C). ChK1 activation and PP2A inhibition by Aβ was confirmed by ChK1/PP2A activity assays results. Again, SB treatment partially reversed these changes (Figure 2D,E). Reports from previous studies showed that activated astrocytes can release
toxic factors to induce neuronal damage and death.\textsuperscript{21} To evaluate whether those neurotoxic factors that are released from Aβ-activated astrocytes also promote neurodegeneration, we incubated rat primary neurons (DIV 8) with conditioned medium from Aβ- treated astrocytes (Aβ- ACM) or Aβ+SB- treated astrocytes (Aβ+SB- ACM) for 4 days. Synaptic proteins including Syn, Syn I, PSD95, and GluA1 were all decreased upon Aβ- ACM incubation, while ACM from SB+Aβ- treated astrocytes showed less toxicity to neurons (Figure S1A,B). Therefore, the above results suggest that activated ChK1 which is induced by Aβ probably promotes the proliferation and activation of astrocytes through CIP2A- PP2A- STAT3 signaling pathway, leading to neuronal degeneration.

3.3 Overexpression of ChK1 induces CIP2A-PP2A-STAT3 signaling axis activation and reactive astrogliosis in primary astrocytes

To further confirm the hypothesis that ChK1 promotes astrogliosis through activating STAT3, we overexpressed ChK1 through Lenti-virus infection with GFAP promoter in primary astrocytes. Immunofluorescence imaging of astrocytes with anti-GFAP illustrated ChK1-induced reactive astrogliosis characterized by hypertrophic cell bodies and more processes in ChK1-overexpressed astrocytes. Interestingly, SB prevented the reactive astrogliosis induced by ChK1 overexpression (Figure 3A). Moreover, the interaction of CIP2A, STAT3, and ChK1 was demonstrated through coimmunoprecipitation (Figure 3B). Western blotting showed that overexpression of ChK1 increased ChK- S345, CIP2A, STAT3- S727, and GFAP protein levels, which indicate the activation of astrocytes. But in the SB treatment group, CIP2A- STAT3 signaling activation and reactive astrogliosis were prevented or at least attenuated (Figure 3C,D). We also found that the expression level of CIP2A protein rose along with the ChK1 levels (Figure 3E). It has been shown that reactive astrocytes may acquire toxicity by releasing pro-inflammatory cytokines.\textsuperscript{22} We thus measured the release of cytokines from ChK1-overexpressed astrocytes. The results showed that cytokines like IL-6 and TNF-α in the culture media were significantly increased in the ChK1 group compared to the control, and SB effectively reduced the levels of released cytokine (Figure 3F). Reactive astrocytes can also produce and release considerable levels of Aβ.\textsuperscript{23,24} Thus,
we measured the Aβ levels in the culture medium. Both Aβ40 and Aβ42 levels were dramatically increased in the ChK1 group, an effect that was reversed by ChK1 inhibitor SB (Figure 3G). In summary, these data indicate that overexpression of ChK1 causes reactive astrogliosis, accompanied by secretion of inflammatory cytokines and Aβ through altering the ChK1-CIP2A-STAT3 signaling pathway.

3.4 | ChK1-ACM induces neurodegeneration

To figure out whether neurotoxic factors released from ChK1-activated astrocytes also promote neurodegeneration, we incubated rat primary neurons (DIV 8) with conditioned medium from ChK1-overexpressed astrocytes (ChK1-ACM) or ChK1+SB-treated astrocytes (ChK1+SB-ACM) for 4 days. At the end of incubation, after labeling neurons with MAP-2 antibody, we observed a decrease in neurites that occurred in the ChK1 group which was reversed by SB (Figure 4A). Western blotting analysis revealed a significant reduction in synaptic proteins including Syn, Syn I, PSD95, and GluA1 in the ChK1 group, but not in the SB+ChK1 group (Figure 4B,C). In conclusion, these results indicate that ChK1-ACM can promote synaptic degeneration.

3.5 | Overexpression of ChK1 in astrocytes induces cognitive deficits in mice

To further determine the role of astrocytic ChK1 activation in AD, we explored the effect of specific ChK1 activation in astrocytes on cognition in animal models. GFAP-ChK1-AAV was injected into the lateral ventricle of C57/BL6 mice aged 8–10 weeks before behavioral
FIGURE 3  Overexpression of Chk1 induces CIP2A-PP2A-STAT3 pathway activation and reactive astrogliosis in primary astrocytes, which can be reversed by the Chk1 inhibitor. (A) The primary astrocytes (DIV10) were infected with Lenti-virus with GFAP promoter, with or without SB treatment. EGFP (green), immunofluorescence staining of GFAP (red), and Hoechst fluorescence (blue) in Vector, Chk1, Chk1+SB groups. Scale bar: 20 μm. (B) The interaction of CIP2A, STAT3, and Chk1 was examined by Co-IP assay. (C) Representative immunoblots of total Chk1, Chk1-S345, CIP2A, active STAT3-S727, total STAT3, GFAP, and β-actin in the Vector, Chk1, and Chk1+SB groups. (D) Quantitative analysis of the proteins levels in (C). (E) Linear correlation between CIP2A and Chk1. (F and G) Quantitative analysis of IL-6, TNF-α, Aβ40 and Aβ42 in the Vector, Chk1, Chk1+SB groups. n = 3 per group, *p < .05, **p < .01, ***p < .001, Data are mean ± SEM
In order to induce specific expression of target protein in astrocytes, we constructed an AAV vector expressing ChK1 under the direct control of a human GFAP promoter (Figure 5C). Four weeks after the virus infection, ChK1 overexpression in the hippocampus was confirmed by immunofluorescence staining of brain slices (Figure 5D). Among a battery of behavioral tests, ORM and OLM tests were firstly performed (Figure 5E,F). A 4 days experiment was tailored for the mice in ORM, including one day for habituation, one day for the training with objects A and B, and two days for the test. After 24 h retention interval, a novel object with a different shape and color (C) was used to replace object B, then after 48 h, object D was used to replace C for testing memory retention (Figure 5E). There was no difference between the two groups in recognition index in the acquisition trial (Figure 5G). As for the two tests, the control group showed exploratory preference toward the new objects (C and then D) with comparable recognition and discrimination index, an effect that was absent in the ChK1 mice group (Figure 5H–J), indicating ORM is impaired in ChK1 mice. Subsequently, the same mice underwent an OLM task. To this end, the animals were habituated to an empty white box on the 5th day. Two objects (E and F) were placed in two different corners of the white box and the mice were trained on the 6th day. The location of F was changed once a day after habituation and training (Figure 5F). The recognition index showed no significant difference in the habituation task (Figure 5K). The analysis of the discrimination index in the following two test trials showed that ChK1 overexpression also impaired the discrimination of different locations (Figure 5L–N). These results confirmed that ChK1 overexpression in astrocytes induced visual episodic memory impairment.
FIGURE 5  Overexpression of ChK1 in astrocytes induces deficits in novel object recognition test (NORT) and object location test (OLT) in C57BL/6J mice. (A) Timeline of the experiment. (B) GFAP-ChK1-AAV was injected into the lateral ventricle. (C) The structure diagram of GFAP-ChK1-AAV. (D) Immunofluorescence staining of brain slices four weeks after virus infection. (E and F) The schematic diagram of the novel object recognition test (NORT) and object location test (OLT) tests. (G) Recognition index for objects A and B in the acquisition trial on Day 2. (H) Object B was replaced by a new object C, and the recognition index for objects A and C was calculated on Day 3. (I) Object C was replaced by a new object D, and the recognition index for objects A and D was detected on Day 4. (J) Discrimination index in Object Recognition Memory test on Day 3 and Day 4. (K) Recognition index for the objects in place E and F in the acquisition trial on Day 6. (L) The position F was changed, and the recognition index for the objects at place E and F were detected on Day 7. (M) The position of F was changed again, and the recognition index for the objects at place E and F were detected on Day 8. (N) Discrimination index in Object Location Memory test on Day 7 and Day 8. \( n = 12 \) mice/group, ***\( p < .001 \). Data are mean ± SEM.
We have shown that ChK1 overexpression induces episodic visual memory impairments. To investigate whether astrocytic ChK1 activation also impairs spatial memory in mice, we used the Morris Water Maze (MWZ) to evaluate the reference spatial learning and memory (Figure 6A). In the acquisition phase, the escape latency showed no significant difference between Con and ChK1 mice (Figure 6B). However, in the probe test after 24 and 48 h, compared with the control group, the ChK1 mice showed decreased crossing times and time in the target quadrant (Figure 6C,D). These results suggest that ChK1 causes disruption in spatial learning and memory.

Proper function of the hippocampus formation poses the physiological basis of the fear condition memory (FCM). Next, we performed the fear conditioning test (FCT) by measuring the freezing time and freezing number in the training phase when mice were given mild electrical foot shocks following a conditioned stimulus (tone) (Figure 6H). In the fear conditioning test, mice in the ChK1 group exhibited a marked reduction in freezing time and number both in the contextual and the tone conditioning paradigms (Figure 6I–K). Whatsoever, there was no obvious difference in the altered context test (Figure 6L) between the two groups. Together, these results suggest that ChK1 causes disruption in spatial learning and memory.

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3.6 Overexpression of ChK1 in astrocytes causes PP2A inhibition, increased inflammatory cytokines and Aβ production, and neuronal and synaptic impairments

To prove that the molecular signaling pathway of ChK1-CIP2A-PP2A-STAT3 is indeed activated in ChK1 overexpression mice, we evaluated the ChK1 and PP2A activity, and the protein levels of CIP2A and active STAT3 in mouse brain homogenates. ChK1 overexpression led to increased ChK1 activity and decreased PP2A activity (Figure 7A,B). At the same time, CIP2A and S727-phosphorylated STAT3 levels were upregulated (Figure 7C,D), indicating the activation of the ChK1-CIP2A-PP2A-STAT3 signaling axis in mouse brains. Coinciding with the ChK1-CIP2A-PP2A-STAT3 signaling pathway activation, the astrocytes were activated as evidenced by the increased GFAP level (Figure 7C,D), accompanied by increased cytokine and Aβ levels (Figure 7E,F) in ChK1 overexpression mice.

To evaluate whether ChK1-activated astrocytes promote neurodegeneration in vivo, we used Golgi staining and Nissl staining to show neuron integrity. Golgi staining showed that the dendritic spine number in the ChK1 group was substantially decreased compared with the control group (Figure 7L). Moreover, Nissl staining displayed that the number of Nissl bodies was decreased in the hippocampal CA1 region in the ChK1 group (Figure 7K,L). Consistent with these findings, synaptic proteins including Syn, Syn I, PSD95, and GluA1 in the hippocampus of ChK1 mice were found to be much lower than those in the hippocampus of control mice (Figure 7M,N). Together, ChK1 overexpression in astrocytes triggers the release of pro-inflammatory cytokines and Aβ production and results in synapses and neurons impairments in vivo.

4 | DISCUSSION

The pathological characteristics of Alzheimer’s disease (AD) include the deposition of senile plaques (SPs), formation of neurofibrillary tangles (NFTs), loss of spines and neurons, as well as abnormal activation of glial cells. Astrocytes are the major glial cells and play important roles in regulating various physiological neuronal functions, including neuronal migration, synaptogenesis, and neuroplasticity. Astrocytes are also deeply involved in regulating neuronal functions under pathological conditions, including neuroinflammation and oxidative stress. Reactive astrogliosis, characterized by increased proliferation and activation of astrocytes, is an early event in the AD brains. PET using 11C-Deuterium-L-Deprenyl (11C-DED) showing the astrocytosis revealed increased 11C-DED binding throughout the brain of the 11C-PIB+MCI patients whose cognition remain normal, suggesting that astrogliosis is an early phenomenon in AD development. The activation and proliferation of astrocytes lead to increased Aβ internalization, inflammatory cytokines production, and cognitive dysfunction. Reducing astrocyte activation in an AD mouse model ameliorated AD-like pathology. Thus, astrogliosis is an important part of AD pathogenesis. However, the mechanisms underlying early astrogliosis in AD remain largely unclarified.

We have previously reported that the upregulated PP2A endogenous inhibitor, CIP2A, in astrocytes in AD mice brain resulted in astrogliosis, AD-like neurodegeneration, and cognitive dysfunction, while in cancer cells, DNA damage response kinase, ChK1, upregulates CIP2A level and inhibits PP2A activation. In the present study, we filled in the gap between these two events by revealing that ChK1 activation upregulates CIP2A and induces astrogliosis. The latter promotes neuronal and synaptic degeneration and induces cognitive impairment.

STAT3 is an important transcription factor. Activation of STAT3 regulates reactive astrogliosis, induces GFAP expression, and production of pro-inflammatory factors such as TNFα and IL-6. However, the molecular
Overexpression of ChK1 in astrocytes induces deficits in the reference spatial memory and fear memory in C57BL/6J mice. (A) Experimental design of Morris Water Maze (MWM). (B) The escape latency of mice during the 5-day training. (C) The representative traces during the 24 h and 48 h probe test in which the platform was removed. (D) Times of crossing the position of the platform after removing the platform during the 24-h probe trial. (E) Time of swimming in target quadrant after removing the platform during the 24-h probe trial. (F) Time of swimming in target quadrant after removing the platform during the 48-h probe trial. (G) Time of swimming in target quadrant after removing the platform during the 48-h probe trial. (H) The schematic diagram of fear conditioning test (FCT). (I) The freezing time and freezing times of the mice in the Context test (Day 2). (J) The freezing time and freezing times of the mice in the Altered Context test (Day 3). (K) The freezing time and freezing times of the mice in the Altered Context test and tone test (Day 4). 

n = 9 mice/group, *p < .05, **p < .01, ***p < .001, Data are mean ± SEM
FIGURE 7  Overexpression of ChK1 in astrocytes causes ChK1-CIP2A-PP2A-STAT3 signaling axis activation, astrogliosis, AD-like Aβ overproduction, inflammatory factors release, and neurodegeneration in C57BL/6J mice. (A and B) The brain hippocampal tissues of the mice were collected and homogenized for ChK1 and PP2A activity assay. (C) Representative immunoblots of Chk1, CIP2A, S727-STAT3, total STAT3, GFAP, and GAPDH in the hippocampus of the Con and Chk1 groups, GAPDH was used as a loading control. (D) Quantitative analysis of the proteins level in (C). (E and F) The brain homogenates of the hippocampus were used for ELISA assay of Aβ40 and Aβ42. (G and H) The brain homogenates of the hippocampus were used for ELISA assay of IL-6 and TNF-α. (I) Typical images of neuronal spines in hippocampal neurons. (J) Quantitative analysis of neuronal spines in CA1 region of (I). (K) Representative Nissl staining images of brain slices. (L) Quantitative analysis of Nissl bodies in CA1 region of (K). (M) Representative immunoblots of Syn, Syn I, PSD95, GluA1, and GAPDH in the hippocampus of the Con and Chk1 groups, GAPDH was used as a loading control. (N) Quantitative analysis of the proteins level in (M). n = 3 per group, *p < .05, **p < .01, ***p < .001, Data are mean ± SEM
mechanism of STAT3 activation in astrocytes is not clear. In tumor cells, PP2A inactivation increases phosphorylation levels of STAT3-S727, activates STAT3, and leads to the proliferation of tumor cells.\(^\text{16,18}\) Again, we linked these two events (PP2A inhibition and STAT3 activation) together and disclosed a new mechanism underlying astrogliosis in AD.

The results from this study showed that, in the brains of 3XTg-AD mice and AD patients, γH2A.X, ChK1-S345 (the active form of ChK1), and CIP2A expression levels are much higher than those in the controls. Similarly, in Aβ-treated primary astrocytes, we observed DNA damage, ChK1 activation, CIP2A upregulation, as well as PP2A inhibition. Together with these changes, the activity of astrocytes was found to be upregulated. Further evaluations indicated that STAT3 phosphorylation at S727 and activation was involved in this process. After applying ChK1 inhibitor SB, the alterations in CIP2A, PP2A, S727 phosphorylated STAT3, and GFAP were all completely or partially reversed, suggesting that the cascade signaling pathway involving ChK1-CIP2A-PP2A-STAT3 participates in astrogliosis in the AD brain. Notably, ChK1 inhibition by SB also resulted in alleviated DNA damage, which was manifested by decreased level of γH2A.X. DNA damage is upstream of ChK1 activation and can be triggered by Aβ. While in astrocytes, just as we showed, ChK1 overexpression induced over-production of Aβ, which may form a vicious cycle. Thus, we suspect that inhibition of ChK1 breaks this cycle and reduces DNA damage.

To further confirm the role of ChK1-CIP2A-PP2A-STAT3 signaling in astrogliosis, we overexpressed ChK1 to directly activate this kinase in primary astrocytes. Consistent with the previous findings, ChK1 activation through its overexpression resulted in CIP2A upregulation, STAT3 activation, and astrogliosis, which could be reversed by the ChK1 inhibitor. Accompanied with astrocytes activation, cytokine and Aβ levels in the media were increased, and neurons treated with the astrocyte-conditioned media showed degeneration characterized by decreased synaptic protein levels and impaired neurite morphology. Taken together, these findings imply that ChK1 activation in astrocytes can induce astrogliosis through the ChK1-CIP2A-PP2A-STAT3 signaling pathway and thereby promote neurodegeneration.

We have shown that specific overexpression of CIP2A in astrocytes in mouse brains induces neurodegeneration and cognitive impairments.\(^\text{13}\) ChK1 activation promotes CIP2A overexpression and cell proliferation in cancer cells.\(^\text{33}\) Based on the finding that ChK1 is also upstream of CIP2A in astrocyte activation, we specifically overexpressed ChK1 in astrocytes in the mouse brain to investigate whether ChK1 activation might promote astrocyte activation and neurodegeneration in vivo. Interestingly, the results were consistent with the data obtained in cell experiments. ChK1 overexpression resulted in CIP2A upregulation, PP2A inhibition, and STAT3 activation. These coincide with increased astrocytic cytokine and Aβ production, as well as synaptic and neuronal degeneration. Moreover, animals overexpressing ChK1 showed learning and memory deficits in several behavior tests.

These data conclusively identified the role of ChK1 activation in promoting astrogliosis in AD. In a parallel study, we also found that activation of ChK1 in neurons leads to AD-like tau hyperphosphorylation, Aβ overproduction, neurodegeneration, and cognitive impairment (unpublished).

In a summary, we demonstrated, for the first time, that ChK1 activation as a result of DNA damage, or ChK1 overexpression, in astrocytes leads to CIP2A upregulation, PP2A inactivation, and STAT3 activation, induces reactive astrogliosis, thus resulting in neurodegeneration, synaptic and cognitive impairments in AD. These provide an insight into new mechanisms in AD pathogenesis and highlights ChK1 as a promising drug target in AD therapy.

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DISCLOSURES
The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS
Ying Zhou, Xiaoyuan Liu and Shuqing Ma, Nan Zhang performed most of the experiments, including PP2A activity assay, ChK1 activity assay, ELISA, Golgi staining, Nissl staining, cell culture, and cell experiments. They also wrote the manuscript. Dichen Yang, Simin Ye, Shiyi Wang, and Nan Jiang performed behavioral tests (OPF, NOR, FCT, and MWM). Yangping Shentu, Ying Zhou, and Ling Wang analyzed the data. Ying Zhou and Xiaoyuan Liu injected AAV into the lateral ventricles of mice. Zongyuan Zhao, Jing Ruan, Jun Ma, and Shujue Zhao collected and sectioned mice brain samples. Shuqing Ma and Zongyuan Zhao assisted the primary neuron culture and Immunofluorescence. Chenfei Zheng, Xiaofang Fan, Yongsheng Gong, Junming Fan, Jingye Pan, and Xiaochuan Wang provided oversight.
and technical support. Wenting Hu performed the Western blotting of AD patients’ brain samples. Mahaman Yacoubou Abdoul Razak extensively revised the grammar and spelling of the revision. Jianmin Li, Rong Liu, and Yangping Shentu designed experiments, secured funding, directed the project, reviewed experimental results, and edited the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All experimental procedures were approved by the Ethics Committee for Experimental Animal Use and Care of Wenzhou Medical University.

DATA AVAILABILITY STATEMENT

All data associated with this study are present in the paper and AAV was constructed by Shanghai Genechem Co. (Shanghai, China).

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

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