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Presenilin 2 deficiency facilitates Aβ-induced neuroinflammation and injury by upregulating P2X7 expression

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Accumulating evidence suggests that β-amyloid (Aβ)-induced neuroinflammation plays a prominent and early role in Alzheimer's disease (AD). In this study, we demonstrated that Presenilin 2 (PS2) deficiency facilitates Aβ-induced neuroinflammation and injury by upregulating P2X7 expression both *in vitro* and *in vivo*. PS2 knockout mice demonstrated increased cognitive impairments and cerebral injury. PS2 deficiency increased the expression of P2X7 both in neurons and microglial cells. Furthermore, extracellular ATP also increased in both Aβ-treated and untreated PS2 knockout microglial cells. Notably, Aβ-induced classical proinflammatory cytokines such as IL-1β, IL-1α and TNF-α were increased in PS2 knockout microglial cells, suggesting a potential role for PS2 in the regulation of neuroinflammation. The expression of P2X7 clearly increased in PS2 knockdown BV2 cells. Consistent with *in vivo* data, Aβ-induced IL-1β production was also clearly enhanced in PS2 knockdown BV2 cells. Additionally, expression of the transcription factor Sp1 was increased in PS2 knockdown cells. When we treated PS2 knockdown cells with the specific Sp1 inhibitor MIT, we observed that enhanced P2X7 expression was significantly rescued. Taken together, these data suggests that PS2 plays a protective role during Aβ-induced neuroinflammation and injury through down-regulation of P2X7 expression.

Presenilin 2, P2X7, Alzheimer's disease, β-amyloid, inflammation

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INTRODUCTION

Alzheimer's disease (AD) is a severe, age-related neurodegenerative disorder and is the most common cause of dementia, affecting more than 45 million people worldwide (Prince, 2015; Shen and Kelleher, 2007). It is clinically characterized by serious behavioral, motor and cognitive impairments, which often lead to the onset of memory loss (Fillenbaum et al., 2008). Although our understanding of AD pathogenesis is far from satisfactory, the presence of intracellular neurofibrillary tangles (NFTs) and extracellular amyloid plaques is commonly recognized as a neuropathological hallmark of AD (Hu et al., 2016; Wes et al., 2016). Extracellular amyloid plaques in the AD brain consist of β-amyloid (Aβ) peptides that are generated via proteolytic processing of the amyloid precursor protein (APP) (Busciglio et al., 1993; Haass et al., 1993). In addition, epidemiologic evidence indicates that nonsteroidal anti-inflammatory drugs (NSAIDs) reduce the risk of Alzheimer's disease, which

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suggests a potential role for neuroinflammation in AD formation (Weggen et al., 2001). Moreover, the deposited Aβ can activate brain-resident astrocytes and microglia to trigger chronic inflammation by increasing levels of inflammatory mediators such as complement factors, reactive oxygen species (ROS), chemokines and cytokines, thus aggravating the progression of AD (Heneka et al., 2015; Rodríguez et al., 2009; Zhang and Jiang, 2015).

Extracellular ATP can act as a type of danger-associated molecular pattern (DAMP) that regulates microglial branch dynamics in the brain, and its release from damaged tissue mediates a rapid microglial immune response to combat injury (Heneka et al., 2015; Davalos et al., 2005). After releasing from damaged cells, ATP specifically activates the P2X receptors, which is a subfamily of ion-gated channel purinergic receptors that comprises seven subtypes (P2X1-7) (Woods et al., 2016). Among them, the P2X7 receptor has received extensive attention due to its wide distribution in the central nervous system (CNS) and its important role in immune regulation during neuroinflammation (Collo et al., 1997; Verderio and Matteoli, 2001). Microglial P2X7 antagonism has been shown to therapeutically inhibit neuroinflammation in the brain in an AD mouse model (Diaz-Hernandez et al., 2012). Moreover, the expression of P2X7 as well as proinflammatory cytokines such as IL-1β was found to be elevated when microglia were exposed to toxic Aβ (McLarnon et al., 2006; Parajuli et al., 2013). Aβ triggers ATP release, IL-1β secretion, and plasma membrane permeabilization in wildtype microglia but not P2X7-deficient microglia, suggesting a key role for P2X7 in the regulation of neuroinflammation in the AD brain (Bhattacharya and Biber, 2016; Lee et al., 2011; Sanz et al., 2009).

As core components of the γ-secretase complex, Presenilins (PS) have been shown to be involved in the cleavage of APP, which leads to the release of Aβ (Selkoe and Wolfe, 2007; Wolfe, 2007). Furthermore, a series of mutations in both PS1 and PS2 have been found in familial AD (FAD) patients (Shen and Kelleher, 2007). Animal models have shown that conditional double knockout mice lacking both PS in the postnatal forebrain exhibit impairments in hippocampal memory and synaptic plasticity, leading to early AD (Saura et al., 2004). Furthermore, subsequent studies examining the brains of PS-conditional knockout mice have shown that PS is associated with neuroinflammation and neurodegeneration (Beglopoulos et al., 2004). Previously, our collaborators' observations also confirmed that conditional PS double knockout mice demonstrated upregulated proinflammatory cytokine levels in the brain in the absence of extra Aβ deposition (Jiang et al., 2009). However, the correlation between PS2 and extracellular nucleotide-regulated neuroinflammation still requires further study to identify new therapeutic targets for Alzheimer's disease. In the present study, we demonstrate that PS2 deficiency facilitates inflammation by upregulating P2X7 expression in Aβ-induced cerebral injury. Insoluble Aβ promotes ATP release, leading to neuroinflammation via activation of the microglial P2X7 receptor. Therefore, our study reveals the potential role of PS2 in the regulation of P2X7 activation, cerebral injury and neuroinflammation.

RESULTS

PS2 deficiency aggravated cerebral injury in an Aβ-induced AD mice model

To understand the correlation between PS2 and impaired memory function in Aβ-injected mice, PS2-deficient and wildtype (WT) mice were analyzed using a Morris water maze. All mice were trained four times per day for five days. As shown in Figure 1A, WT and PS2KO mice were each divided into three groups: a sham operation group, a group injected with artificial cerebrospinal fluid (CSF) and a group injected with Aβ. The mice in the sham group and the CSF group demonstrated shorter escape latency times than the Aβ group after training. Furthermore, compared with the WT Aβ mice, the PS2KO Aβ mice exhibited poor spatial learning. To assess memory maintenance after learning, we performed a probe trial 24 h after the final learning day. Aβ mice spent less time in the target zone than did sham and CSF mice. Moreover, while WT Aβ mice spent 20% of their total time in the target zone, PS2KO Aβ mice spent approximately 10% of their total time in the target zone (Figure 1B). The mice were then sacrificed, and brains were collected for paraffin sectioning. To detect neuronal injury, paraffin-embedded brain sections were stained with Nissl staining solution. As shown in Figure 1C, there were only slightly fewer hippocampal neurons in the Aβ groups compared to the other groups. However, the number of Nissl bodies which reveal the activity of surviving neurons in the CA1, CA2 and CA3 regions in the brains of the PS2KO groups was significantly decreased compared with the WT groups (Figure 1D).

The regulation of purinergic receptors by PS2 in the brain

Previous reports have suggested that purinergic receptors play an important role in neuroinflammation and neurodegeneration (Agrawal et al., 2016), particularly the P2X7 receptor, which induces IL-1β release from microglia (Ferrari et al., 2006; Skaper et al., 2010). In this study, we detected *P2X7* mRNA expression levels in both the frontal cortex and the hippocampus using qRT-PCR. Compared with WT mice, *P2X7* mRNA levels in PS2KO mice were notably increased (Figure 2A and B). To verify that P2X7 expression was upregulated in PS2 knockout mice, the P2X7 protein levels in PS2KO mice were detected by performing immunohistochemical staining (Figure 2C). P2X7 expression levels in the

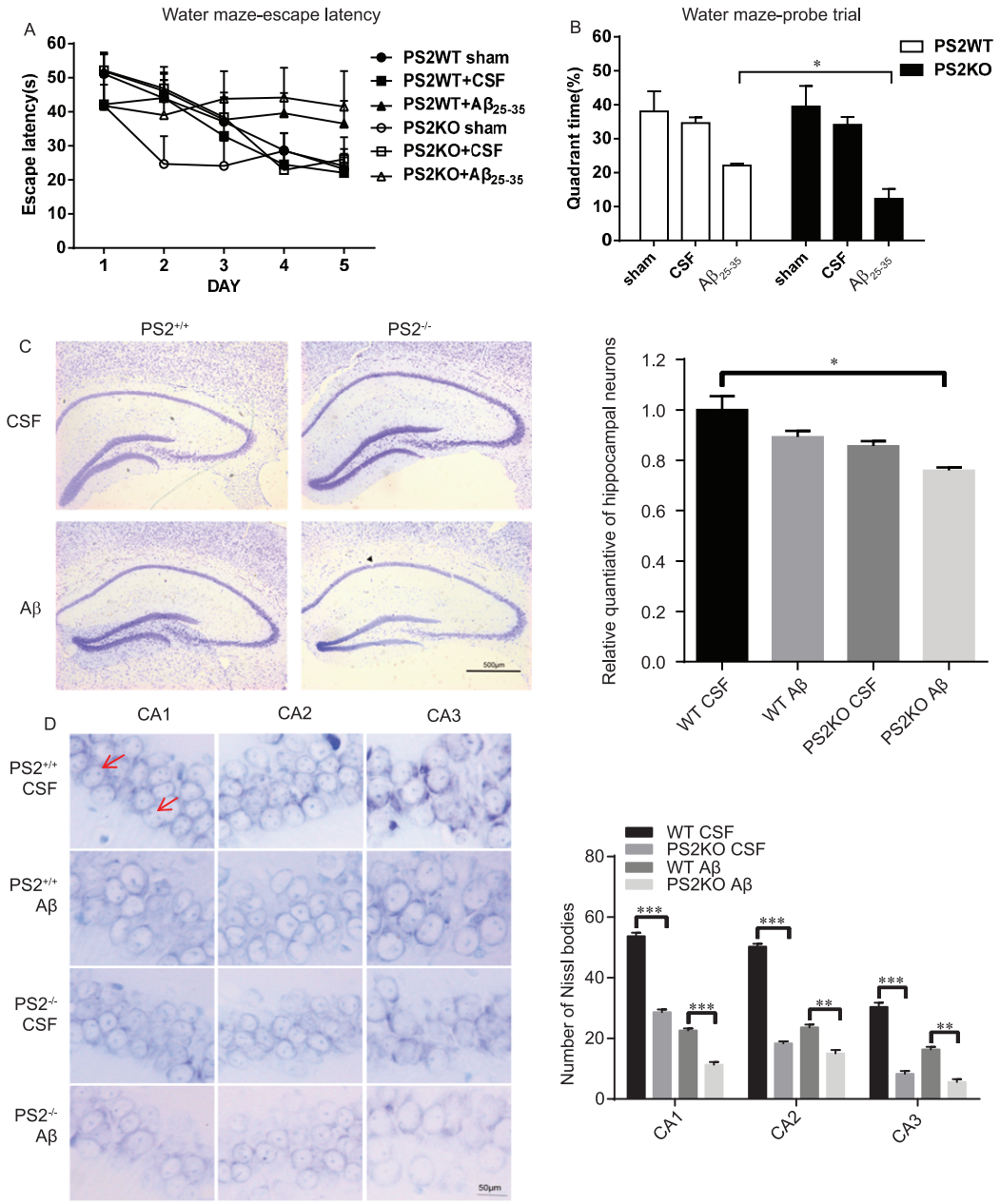

Figure 1 (Color online) Aβ-induced cerebral injury was increased in PS2KO mice. A, The Morris water maze test to examine spatial learning patterns was performed with Aβ-induced wildtype (WT) and PS2$^{-/-}$ Alzheimer's disease mice on days 1–5 following treatment. The escape latency for hind platform searching was recorded. B, Twenty four hours after training, a probe trial was conducted to determine the percentage of time spent in the exact area where the escape platform was located. The results are expressed as the means±SE, and each experiment was independently performed three times with five mice per group. Statistical analysis was performed with Student's *t*-test; *, $P<0.05$. Nissl staining of the hippocampus was performed with 4-μm-thick sections from WT and PS2$^{-/-}$ Aβ-induced AD mice. Representative pictures are presented as a holistic view of the hippocampus (scale bar=500 μm) (C) and details of the CA1, CA2 and CA3 regions (scale bar=50 μm) (D). The number of hippocampal neurons and the number of nissl bodies in CA1, CA2 and CA3 regions were calculated by quantitative analysis. Data are presented as means±SE, statistical analysis was performed with Student's *t*-test; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

cerebral cortex (Figure 2D) and the CA1, CA2, and CA3 regions of the hippocampus were highly elevated (Figure 2E). Similar results were obtained using an immunofluorescent assay (Figure 3A). Furthermore, immunofluorescent co-localization analysis of frozen mouse brain sections was performed to examine P2X7 receptor localization. In PS2 knockout mice, P2X7 expression was increased in neurons (labelled with NeuN) and microglial cells (labelled with Iba1) but not in astrocyte cells (labelled with GFAP) (Figure 3B).

PS2 deficiency promotes ATP release and facilitates inflammation in Aβ-treated primary microglial cells

Microglia are brain-resident macrophages that participate in a variety of neurodegenerative diseases involving inflamma-

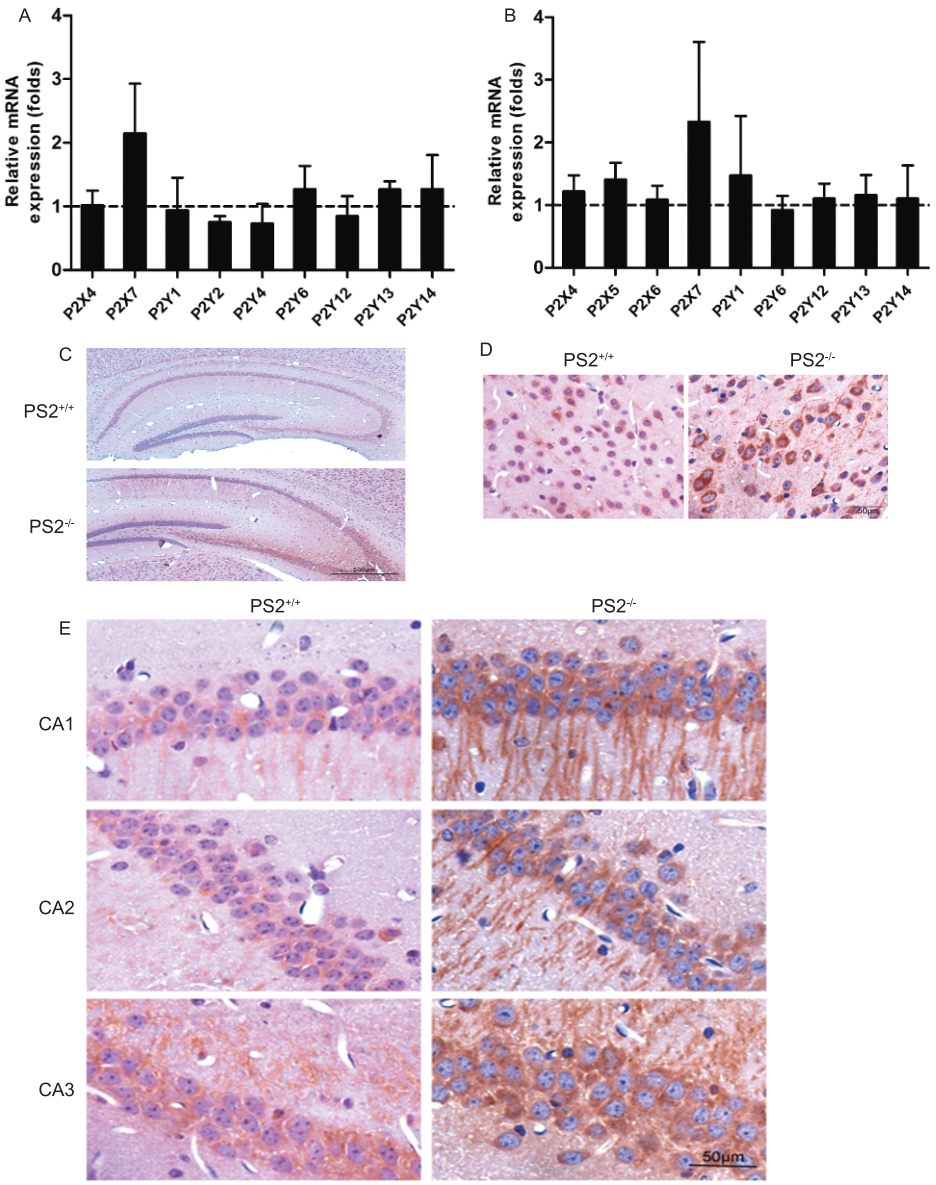

Figure 2 (Color online) The expression of P2X7 in PS2-deficient mice is greatly increased. A quantitative RT-PCR assay was performed to assess P2 family receptor mRNA expression levels in the frontal cortex (A) and hippocampus (B) of PS2-deficient mice, normalized to WT mice. Immunohistochemical staining of the hippocampus (C) and cerebral cortex (D) in WT and $PS2^{-/-}$ mice was performed using an anti-P2X7 antibody. Representative micrographs at magnifications of 5× (left column, scale bar=500 μm) and 20× (right column, scale bar=50 μm) are shown. E, The morphology of P2X7-stained cells in the CA1, CA2 and CA3 regions of the hippocampus for each group of mice.

tion and immune responses (Mandrekar-Colucci and Landreth, 2010). As shown in Figure 4A, many microglial cells were activated adjacent to the hippocampus in mice that were injected with Aβ. Although the number of Iba1 positive cells is little changed between the WT and PS2 KO cerebral cortex, the function of microglia was changed by PS2 deficiency. To confirm the upregulation of P2X7 expression in PS2KO microglia, we detected the release of ATP, which is the ligand that binds the P2X7 receptor. Upon Aβ stimulation, the concentration of ATP released from PS2KO microglia was approximately 200 nmol L^{-1}; however, the ATP concentration released from WT microglia was only approximately 100 nmol L^{-1} (Figure 4B). When P2X7 is activated, inflammation-related factors can be released. Thus, we examined the release of the inflammatory cytokine IL-1, which is associated with the P2X7 receptor. When treated with Aβ, the IL-α and IL-1β levels in PS2KO microglia were higher than those in WT microglia (Figure 4C and D). As demonstrated in a previous report, P2X7 also regulates other inflammatory mediators, including TNF-α (Shieh et al., 2014; Zou et al., 2012); thus, we also measured TNF-α levels in our study (Figure 4E). When cells were treated with OxATP (a specific P2X7 inhibitor), Aβ-triggered inflammation was reduced significantly. These data suggest that PS2 knockout aggravates

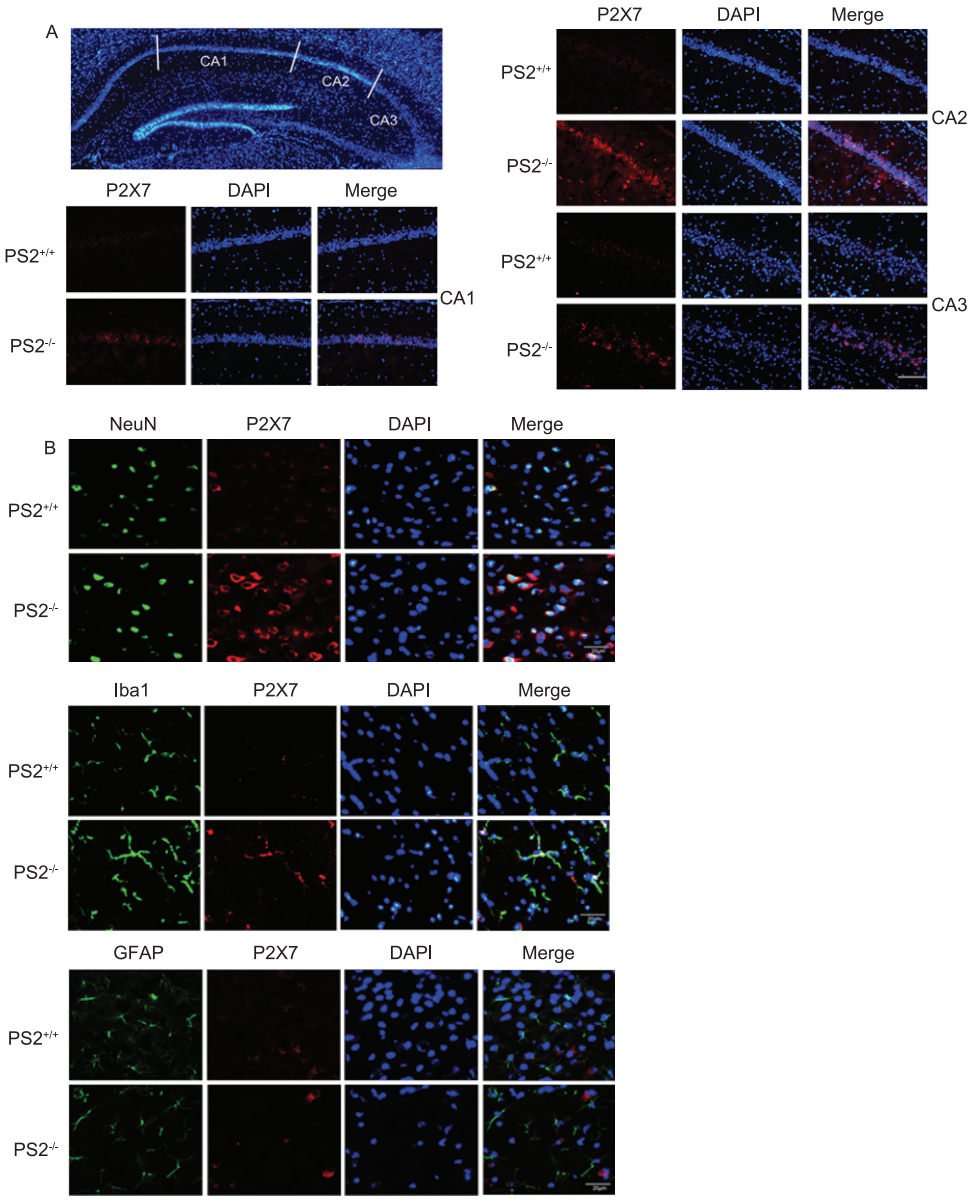

Figure 3 PS2 deficiency increase P2X7 expression in neurons and microglial cells. A, Immunofluorescence staining of (left column) hippocampus specimens from WT and PS2$^{-/-}$ mice with 4′,6-diamidino-2-phenylindole (DAPI) (blue), scale bar=500 μm. Representative photographs of P2X7 (red) immunofluorescence in the (right column) CA1, CA2 and CA3 hippocampal regions. Scale bar=100 μm. B, Double immunofluorescence staining of 10-μm-thick frozen brain sections with antibodies to detect NeuN (green, upper panel), Iba1 (green, intermediate panel), GFAP (green, lower panel) and P2X7 (red) in WT and PS2$^{-/-}$ mice; nuclei are labeled with DAPI (blue). Scale bar=25 μm.

P2X7-mediated inflammation in microglia treated with Aβ.

PS2 knockdown increases inflammation in BV2 cells

To further confirm the function of PS2 in microglial cells, we knocked down *PS2* in the BV2 microglial cell line using shRNA interference. As shown in Figure 5A, PS2 expression levels in PS2 knockdown BV2 cells (BV2KD) were significantly reduced both at the mRNA and protein expression levels. When PS2 was inhibited, the expression of P2X7 improved (Figure 5B) with or without Aβ (Figure 5C). Interestingly, the *IL-1β* levels also increased in Aβ-treated BV2KD cells, and this increase could be reduced by OxATP treatment (Figure 5D). These results were consistent with those observed in primary microglial cells.

PS2 regulates P2X7 expression through Sp1

A previous study reported that Sp1 is involved in the transcriptional regulation of P2X7 receptors in the nervous system (García-Huerta et al., 2012). To clarify whether PS2 regulates the expression of P2X7 through Sp1, we analyzed the Sp1 expression levels. As shown in Figure 6A, Sp1 protein expression was increased in BV2KD cells, which correlated with P2X7 expression. We also detected an additional important transcription factor, CREB (cAMP response element-

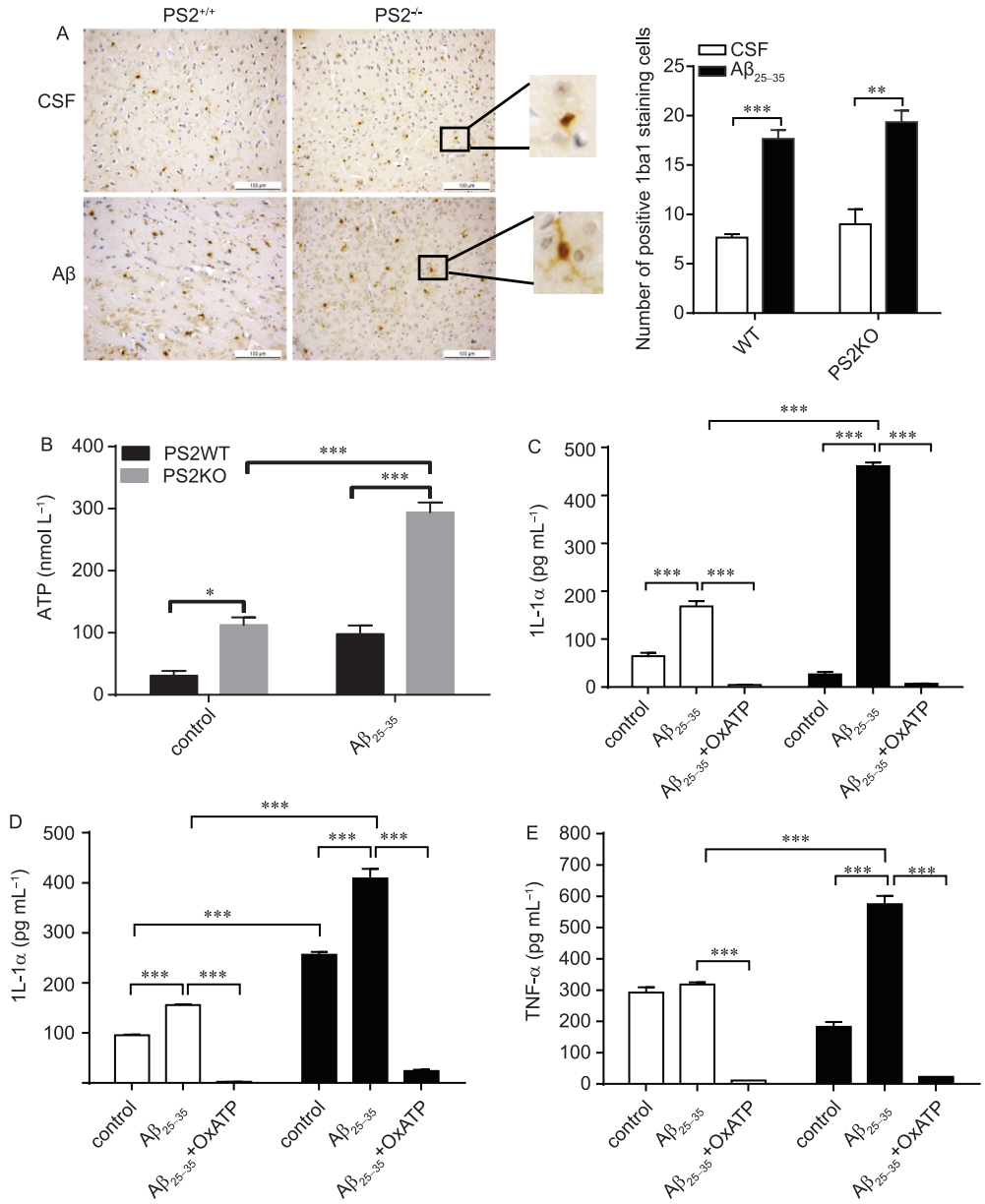

Figure 4 (Color online) ATP/P2X7 facilitates Aβ-induced inflammation in primary microglial cells. A, Representative pictures of immunohistochemical staining for Iba1, a marker of microglia, in cerebral cortex sections from WT and PS2$^{-/-}$ Aβ-induced AD mice. Scale bar=100 μm. The number of Iba1 positive cells was calculated by quantitative analysis. B, Quantification of extracellular ATP is shown for primary microglial cells treated with 60 μmol L^{-1} $A\beta_{25-35}$. Primary microglial cells were incubated with or without a P2X7 selective inhibitor (OxATP, 1 mmol L^{-1}) for 30 min before stimulation with 60 μmol L^{-1} $A\beta_{25-35}$; 12 h later, supernatants were collected to measure IL-1β (C), IL-1α (D) and TNF-α (E) protein levels by enzyme linked immunosorbent assay (ELISA). The results are expressed as the means±SE, and statistical analysis was performed with Student's *t*-test; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

binding protein), which is involved in AD progression; however, the expression levels of phosphorylated CREB did not change (Figure 6B). When BV2KD cells were treated with MIT (mithramycin A, a selective inhibitor of Sp1-mediated transcriptional activation), the PS2 knockdown-mediated upregulation of P2X7 expression levels was down-regulated (Figure 6C). To further investigate whether PS2 knockout affects Sp1 expression, we analyzed the Sp1 levels in mouse brains by employing immunofluorescence. As shown in Figure 6D, compared with WT mice, Sp1 expression levels in PS2KO mice were greatly increased both in neurons and microglial cells, and this result correlated with the expression of P2X7.

DISCUSSION

In this study, we found that PS2 knockout could upregulate expression of the P2X7 receptor by increasing Sp1 to accelerate neuroinflammation in an Aβ-induced AD mouse model. Neuroinflammation plays a central event in the pathological

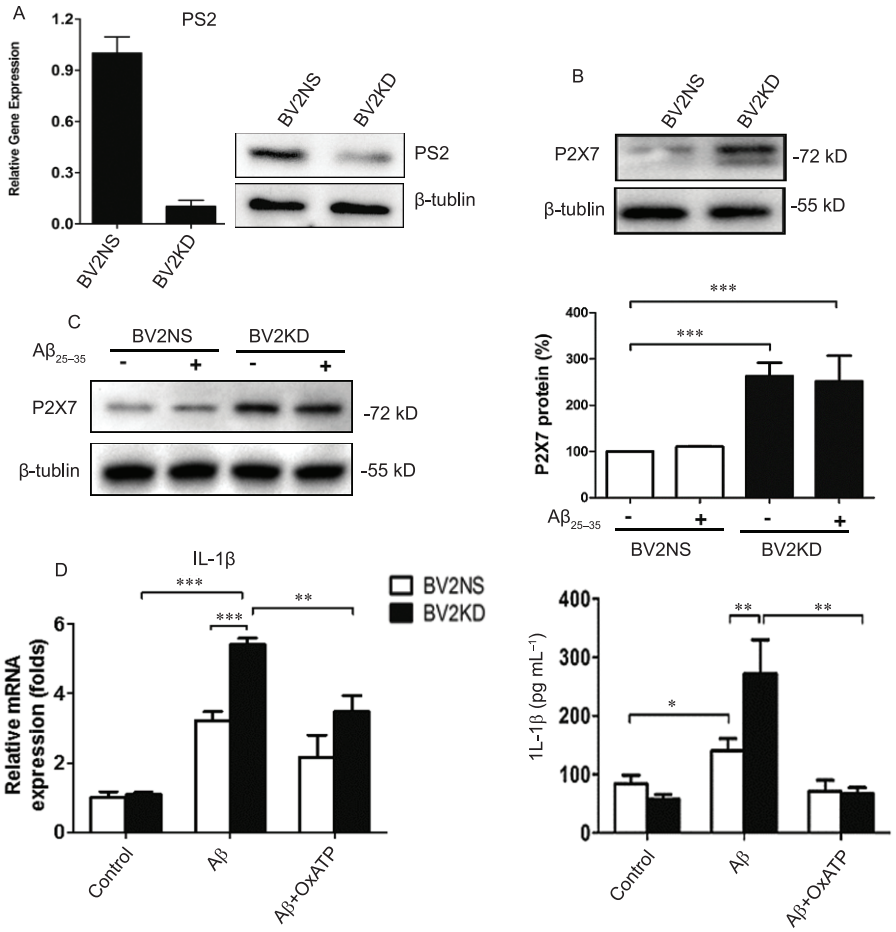

Figure 5 PS2 knockdown enhances P2X7 expression and IL-1β release in BV2 cells. A, Quantitative real-time PCR and an immunoblot assay were performed to assess *PS2* expression in the BV2 mouse microglial cell line after infection with lentivirus-containing medium for PS2 shRNA (KD) and unrelated shRNA (NS). B, The expression of P2X7 in control and PS2 knockdown cells was examined by performing western blots. C, Control and PS2 knockdown BV2 cells were treated without or with 60 μmol L^{-1} $Aβ_{25–35}$ for 30 min and then P2X7 protein levels were assessed by western blotting. Representative figures and semi-quantitative analysis of P2X7 expression are shown. D, Control and PS2 knockdown BV2 cells were incubated with a P2X7 selective inhibitor (OxATP, 1 mmol L^{-1}) for 30 min before stimulation with 60 μmol L^{-1} $Aβ_{25–35}$. *IL-1β* mRNA levels were examined by quantitative PCR, and IL-1β protein levels were examined by ELISA. The data from three independent experiments are presented as the means±SE, and statistical analysis was performed with Student's *t*-test. *, $P<0.05$; **, $P<0.01$, and ***, $P<0.001$ indicate significant differences between groups.

progression of AD (Morales et al., 2014) and leads to serious memory impairment. Some investigators have demonstrated that inflammation mediator levels are increased in the vicinity of Aβ peptide deposits and neurofibrillary tangles (Akiyama et al., 2000), and the inflammation caused by Aβ involves activated glial cells, including microglia and astrocytes (Fernández et al., 2008; Morales et al., 2010). Currently, there are promising targets related to neuroinflammation for the treatment of AD. Interestingly, we also observed increased numbers of activated microglia in brain sections of Aβ-induced mice (Figure 4A).

Microglia are the primary immune cells of CNS and are classified as specialized macrophages (Saijo and Glass, 2011) that participate in the first line of defense against pathogens. After sensing damage or injury signals, pattern-recognition receptors (PRRs), including pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) were activated. Purinergic signaling, particularly the microglial P2X7 receptor signaling pathway, plays a major role in neuroinflammation (Takenouchi et al., 2010). To clarify whether the enhanced inflammation caused by PS2 deficiency occurs via the regulation of purinergic signaling, purinoceptor expression was detected as shown in Figure 2A and B. P2X7 receptor expression was upregulated both in the cortex and hippocampus of PS2KO mice. These observations were confirmed by employing immunohistochemical staining (Figures 2 and 3A). Yang and others have shown that extracellular ATP is released from dying cells to trigger NLRP3 inflammasome activation and IL-1β release through bindinfg P2X7 receptor (Gombault et al., 2013; Yang et al., 2015). In this study, we also found both ATP release and IL-1β production were increased in Aβ-treated PS2KO microglial cells (Figure 4B and C), and this was consistent with the increased expression of P2X7 receptor. The result indicated that in-

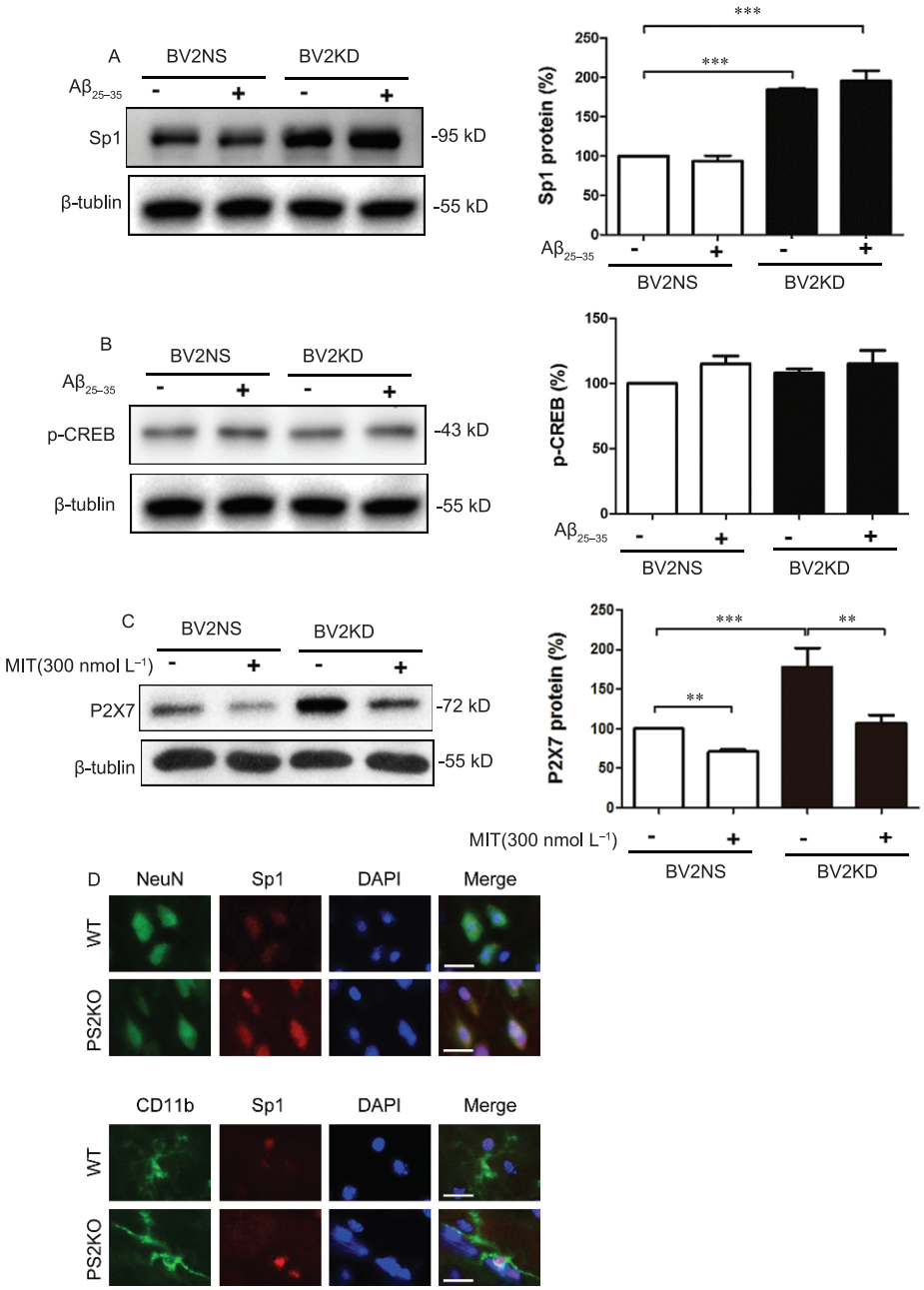

Figure 6 P2X7 expression is regulated by PS2 through Sp1. Control and PS2 knockdown BV2 cells were treated without or with 60 μmol L^{-1} $Aβ_{25–35}$ for 30 min, and then Sp1 (A) and phosphorylated CREB (B) protein levels were assessed by western blotting. C, Cells were pretreated with 300 nmol L^{-1} mithramycin (an Sp1 inhibitor, MIT) for 24 h, then stimulated with $Aβ_{25–35}$ as indicated. P2X7 protein levels were examined by performing western blots using a specific anti-P2X7 Ab. The results are presented as representative figures, and semi-quantitative analysis was performed. Data are presented as the means±SE of three replicates, and statistical analysis was performed with Student's *t*-test; **, $P<0.01$; ***, $P<0.001$. D, Expression in the brain was evaluated by performing immunofluorescence staining with antibodies to detect NeuN (green, upper panel), CD11b (green, lower panel) and Sp1 (red) in brain sections from WT and $PS2^{-/-}$ mice; nuclei are labeled with DAPI (blue). Scale bar=20 μm.

creased inflammation in PS2 knockout mice may be related to inflammasome activation in Aβ-treated microglial cells. Meanwhile, increased inflammation could be reduced via treatment with the P2X7 inhibitor OxATP. These data indicate that PS2 deficiency aggravates inflammation by regulating the expression of P2X7 during Aβ stimulation.

Next, we studied the mechanism underlying the PS2-regulated expression of P2X7 in microglia. The transcription factor Sp1 can activate the P2X7 promoter and mediate transcriptional regulation of P2X7 receptor in the nervous system (García-Huerta et al., 2012). As a proinflammation transcription factor, Sp1 expression is upregulated in the human AD brain and in the cortex and hippocampus of transgenic AD model mice (Citron et al., 2008; Citron et al., 2015; Villa et al., 2013). In this study, Sp1 expression was enhanced when *PS2* was knocked down in the BV2 cell line. However, if we

treated BV2KD cells with the Sp1 inhibitor MIT, P2X7 protein levels decreased (Figure 6C). Furthermore, when compared with WT mice, Sp1 expression in PS2KO neurons and microglia was strengthened. These results suggest that PS2 deficiency increases P2X7 expression by upregulating Sp1 expression levels.

PS2 is a homolog of PS1, and both belong to the PS protein family, whose members are important components of the γ-secretase complex (Smolarkiewicz et al., 2013; Wolfe, 2013). Some studies have reported that *PS1* mutations located on chromosome 14 and *PS2* mutations located on chromosome 1 participate in the pathogenesis of familial Alzheimer's disease (FAD) (Kovacs et al., 1996; Supnet and Bezprozvanny, 2011). To date, many researchers have demonstrated that *PS2* mutations may increase $A\beta_{42}$ production and β-secretase activity through ROS (Canevelli et al., 2014), but the mechanism underlying PS2-regulated inflammation in AD is still not clear.

Here, we found that PS2 is connected to the purinergic signaling, and PS2 deficiency facilitates neuroinflammation by upregulating the P2X7 receptor in an Aβ-induced mice model of AD (Figure 7). Therefore, our study broadens our understanding of the important function of PS2 in neuroinflammation and lays a stable foundation for future studies of PS2 in AD.

MATERIALS AND METHODS

Ethics statement

All animal experiments conformed to the regulations drafted by the Association for the Assessment and Accreditation of Laboratory Animal Care in Shanghai and were performed in direct accordance with the animal care guidelines of the Ministry of Science and Technology of the People's Republic of China. The protocol was approved by the East China Normal University Center for Animal Research (AR2013/08002). All surgeries were performed under anesthesia, and all effort was made to minimize suffering.

Animals

PS2 knockout mice on a C57BL/6 background were generated as previously described (Feng et al., 2004; Herreman et al., 1999; Jiang et al., 2009). Age-matched female C57BL/6 mice were purchased from Shanghai Laboratory Animal Company (Shanghai). All mice used in these experiments were housed under specific pathogen-free (SPF) conditions and were maintained in accordance with institutional guidelines (Laboratory Animal Center, East China Normal University).

Regents and antibodies

Dulbecco's modified Eagle's medium (DMEM), FBS, penicillin-streptomycin and TRIzol reagent were purchased from Invitrogen (Thermo Fisher Scientific, USA). SYBR Premix Ex Taq and PrimeScript RT Master Mix were purchased from TaKaRa (Dalian). The transfection reagent Fugene HD was purchased from Roche (Switzerland). OxATP, LPS and $A\beta_{25-35}$ were obtained from Sigma-Aldrich (USA). Antibodies specific to PS2, CD11b and GFAP were purchased from Abcam (USA). An antibody specific to P2X7 was obtained from Alomone labs (Israel). Antibodies specific to NeuN and Sp1 were purchased from Merk Millipore (USA). An antibody specific to Iba1 was obtained from Wako (Japan). β-Tubulin antibody, HRP-conjugated anti-mouse and anti-rabbit antibodies, and AlexaFluor® s488/555 anti-

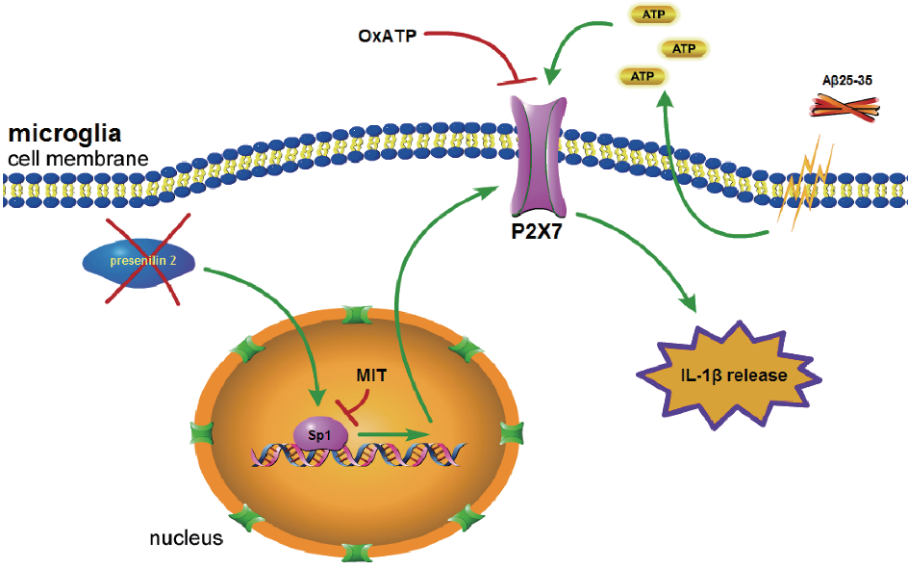

Figure 7 (Color online) Schematic diagram showing the function and mechanism of P2X7 up-regulation due to PS2 deficiency in Aβ-induced Alzheimer's disease mice.

mouse and anti-rabbit secondary antibodies were purchased from Cell Signaling Technology (USA).

Aβ-induced AD mouse model

$A\beta_{25-35}$, which was used to induce AD in mice, was dissolved in artificial cerebrospinal fluid (CSF) at a concentration of 5 mg mL^{-1}, and peptides were aggregated by incubating at 37°C for 7 days. Intracerebroventricular injection was performed as previously described after mice were adapted to their living environment for one week (Yeo et al., 2004). Briefly, mice were intraperitoneally anesthetized with 10% (v/v) choral hydrate (0.1 mL $mouse^{-1}$) and placed in a stereotaxic apparatus (Stoelting, USA). CSF or aggregated $A\beta_{25-35}$ (3 μL $mouse^{-1}$) was injected from the bregma into the lateral ventricle at the following coordinates: 1 mm caudal to the bregma, 1.5 mm lateral to the midline, 3.5 mm from the surface of the cerebral cortex. A microsyringe (Hamilton, USA) was used to control the injection rate at 0.2 μL 15 s^{-1}. The needle was withdrawn 2 min later, and the scalp was sutured. The Aβ-injected animals were housed for one week, and then the mice were subjected to a Morris water maze (MWM) test. For sacrifice after the final MWM test, the mice were anaesthetized and then perfused with 4% paraformaldehyde solution. Then, brains were immediately removed from skulls, and the cortex and hippocampus were dissected on ice. All tissues were stored at −80°C for subsequent analyses.

Morris water maze

The Morris water maze test is a widely accepted method to examine spatial learning in rodents and was employed in the present study as described previously (Vorhees and Williams, 2006). Briefly, a black circular tank (height: 50 cm, diameter: 120 cm) was filled with water (plus titanium pigment dye) and maintained at 21°C. An escape platform (height: 28 cm, diameter: 5 cm) was typically submerged 1−2 cm below the surface of the water. For spatial acquisition, each mouse undertook four trials per day with at least 60 s inter-trial intervals for five consecutive days. Four points equal in length to the hidden platform served as starting positions. The animal was placed in the desired starting position in the maze, facing the tank wall, and was permitted 60 s to locate the submerged platform. If an animal failed to locate the target within 60 s, it was gently guided to the platform and allowed to stand for 30 s before being returned to its home cage. Escape latency was defined as the time required to search for the platform within 60 s. The average speed and the escape latency to reach the goal were recorded for each trial ($n=5$ per group). Twenty four hours after the acquisition phase, a probe trial was performed to assess the spatial reference memory abilities of the mice. The platform was removed, and mice were placed in a novel starting position to swim freely for 60 s. Spatial acuity was expressed as the percentage of time spent in the exact area where the escape platform was located.

Nissl staining

Brain tissues from all mice were collected, fixed in 4% paraformaldehyde, dehydrated in graded ethanol and embedded in paraffin. Four-micrometer sections were obtained using a rotary microtome (Leica Biosystems, Germany) and stained with Nissl Staining Solution (Beyotime Biotechnology, Shanghai), in accordance with the manufacturer's manual. The stained areas were viewed with an optical microscope (Leica Microbiosystems, Germany).

Immunohistochemical staining

The paraffin-embedded brain sections were used to perform immunohistochemical staining. Sections were heated for 2 h before undergoing rehydration through a xylene and graded ethanol series; then, sections were treated with 3% (v/v) hydrogen peroxide and blocked with 10% (v/v) normal goat serum for 30 min at room temperature, followed by incubation with a rabbit anti-P2X7 antibody (1:200, Alomone Labs) overnight at 4°C. After washing in phosphate-buffered saline (PBS), sections were incubated with an HRP-conjugated anti-rabbit antibody (1:1000) for 1 h at room temperature. Sections were stained with diaminobenzidine (DAB) and hematoxylin for 20 min. Finally, slides were mounted and observed under an optical microscope (Leica Microbiosystems).

Immunofluorescence staining

For immunofluorescence staining, brain tissues, which were fixed in 4% paraformaldehyde, were dehydrated in 20% and 30% sucrose solutions and embedded in OCT (Sakura Finetek, USA) at −20°C. The sections were incubated with a rabbit anti-P2X7 antibody (1:200; Alomone Labs), a mouse anti-NeuN antibody (1:200, Merck Millipore, Germany), a mouse anti-Iba1 antibody (1:200, Wako), a mouse anti-Sp1 antibody (1:200, Merck Millipore), a mouse anti-CD11b antibody (1:200, Abcam) or a mouse anti-GFAP antibody (1:200, Abcam) overnight at 4°C. After washing, sections were incubated with AlexaFluor® 488 anti-mouse and AlexaFluor® 555 anti-rabbit secondary antibodies (1:400, Cell Signaling Technology) for 2 h at room temperature. Then, the sections were mounted on coated glass slides. Fluorescence was detected with a fluorescence microscope (Leica Microbiosystems).

Cell culture

The mouse microglial cell line BV2 was obtained from the American Type Culture Collection (ATCC, USA) and cultured in DMEM containing 10% FBS and 100 units mL^{-1} penicillin and 100 mg mL^{-1} streptomycin. The cells were incubated in a 5% CO_2 incubator at 37°C. To obtain primary microglial cells, we performed a procedure as described previously (Saura et al., 2003). The generated primary microglial cells were $CD11b^+$ (purity, 90%).

Western blotting

BV2 cells were seeded in 6-well plates (Corning Costar, USA) and stimulated with $A\beta_{25-35}$ for 1 h. The samples were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). After incubation with P2X7 (1:1000; Alomone Labs) and Sp1 (1:1000, Merck Millipore) antibodies, the PVDF membranes were then incubated with appropriate AlexaFluor® 680 secondary antibodies (Jackson ImmunoResearch, USA). Finally, a Li-COR Odyssey imaging system (Lincoln, USA) was used to detect the proteins. Semi-quantitative analyses of immunoblots were performed using Image J (NIH, USA).

ELISA

To assay cytokine secretion, mouse IL-1α, IL-1β and TNF-α ELISA kits (BioLegend, USA) were used according to the manufacturer's instructions. BV2 cells were grown on 96-well plates (Corning Costar) at 5×10^4 cells well^{-1} and cultured in DMEM overnight. Then, the cells were pretreated with drugs as indicated. After stimulation with $A\beta_{25-35}$ for an additional 6 h using PBS as a negative control, the supernatants and brain homogenates of different groups of mice were obtained to carry out measurements.

PS2 knockdown using a lentiviral system

Lentiviral plasmids containing short hairpin RNA (shRNA) specific for PS2 (pLKO.1-PS2Si) and a non-specific scrambled shRNA (pLKO.1-conSi) were purchased from Sigma-Aldrich. The target sequence of the PS2-specific shRNA is as follows: 5′-CCGGCCTGATATACTCATCTGCCATCTCGAGATGGCAGATGAGTATATCAGGTTTTT-3′. For stable knockdown, the vectors were transfected into HEK293T cells with lentiviral packaging plasmids (psPAX2 and pMD2.G) and Fugene HD transfection reagent (Roche) according to the manufacturer's instructions; then, the lentivirus-containing culture medium was collected and concentrated. The BV2 cells were seeded in 6-well plates (Corning Costar) (1×10^6 cells well^{-1}) 24 h before infection; then, the lentivirus-containing medium for PS2 shRNA (KD) or unrelated shRNA (NS) was individually added into BV2 cells for 24 h. Forty eight hours after infection, cells were selected with puromycin (4 mg mL^{-1}) and pooled for further experiments.

ATP assay

Primary microglial cells (5×10^4 cells well^{-1}) were loaded into 24-well plates (Corning Costar) with DMEM containing 10% FBS for 24 h, then starved in serum-free DMEM for 12 h before the ATP assay was performed. For this assay, primary microglial cells were activated by LPS (100 ng mL^{-1}) for 2 h. Then, 30 min after $A\beta_{25-35}$ treatment, supernatants were harvested to quantify extracellular ATP levels using the ATP Bioluminescent Assay Kit (Sigma-Aldrich) as recommended by the manufacturer. ATP levels were calculated based on an ATP standard curve. All samples were measured with three replicates, and all experiments were repeated at least three times.

RNA isolation and RT-PCR

Total RNA from brains and BV2 cells was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized with 1,000 ng of RNA using a reverse transcription kit (TaKaRa) according to the manufacturer's instructions; then, real-time quantitative PCR analysis employing SYBR Premix Ex Taq (TaKaRa) and the Stratagene Mx3005P Real-Time qPCR System (Agilent Technologies, USA) was carried out. The sequence-specific primers for P2 receptor genes are shown in Table 1. Other primers

Table 1 Sequence-specific primers for P2 receptor genes.

Gene name		Primers (5′–3′)	Size (bp)
P2X4	SENSE:	TACGAGCAGGGTCTTTCCGGA	183
	ANTISENSE:	ACAAGACGTGCTCGGGCAAC	
P2X5	SENSE:	CCACACTATTATTTCAAC	158
	ANTISENSE:	CCATTAACTATCACATCA	
P2X6	SENSE:	CTCAAGCTCTATGGAATC	194
	ANTISENSE:	CTTGCCTCTTCATATTTG	
P2X7	SENSE:	GAACCCAAGCCGACGTTGAAGT	152
	ANTISENSE:	GACAGCCTAGACAGGTCGGAGA	
P2Y1	SENSE:	CGCACACAGGTACAGTGGCGT	150
	ANTISENSE:	TTCCGAGTCCCAGTGCCAGAGT	
P2Y2	SENSE:	GTGCGGGGAACCCGGATCAC	149
	ANTISENSE:	AGCCGCCTGGCCATAAGCAC	
P2Y4	SENSE:	TATTACCTGGCAAGACTG	150
	ANTISENSE:	GAGCTGGTTTCGATACTT	
P2Y6	SENSE:	CCTTCGCTGCTGCCTACAA	149
	ANTISENSE:	TCTCTGCCTCTGCCACTTG	
P2Y12	SENSE:	TCATCTATCTCAAGAACA	150
	ANTISENSE:	CGTACATATTAACATAGAAG	
P2Y13	SENSE:	CTGGAATCACTTGCTAAT	123
	ANTISENSE:	GTCACTAATCAATAGGAACT	
P2Y14	SENSE:	TCTCTTCTATTAACTGGCTTCT	82
	ANTISENSE:	ATACGAGTGTTGCTTGGA	

were: *PS2* sense, 5′-GAGCGAAGAAGACTGTGAAGAG-3′, *PS2* antisense, 5′-GTTAAGCACGGAGTTGAGGAG-3′; *IL-1β* sense, 5′-GCACTACAGGCTCCGAGATGAA-3′, *IL-1β* antisense, 5′-GTCGTTGCTTGGTTCTCCTTGT-3′; *GAPDH* sense, 5′-AGTGGCAAAGTGGAGATT-3′, *GAPDH* antisense, 5′-GTGGAGTCATACTGGAACA-3′.

Statistical analysis

All of the experimental data were imported into GraphPad Prism 6.0 Software and are presented as means±SE. Statistical significance was evaluated with the Student's *t*-test followed by Dunnett's multiple comparison. $P<0.05$, $P<0.01$ and $P<0.001$were considered significant.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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