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Neuroprotective effect of AGGF1 against isoflurane-induced cognitive dysfunction in aged rats through activating the PI3K/AKT signaling pathways

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ABSTRACT

Purpose: This study aimed to evaluate and identify the value and explore the mechanisms of Angiogenic Factor with G-patch and FHA domains 1 (AGGF1) in postoperative cognitive dysfunction (POCD). *Methods:* Rats were separated into four different groups, namely sham, isoflurane, isoflurane + recombinant human Aggf1 (rh-Aggf1) (5 μg kg^{-1}), and isoflurane + rh-Aggf1 (10 μg kg^{-1}). qPCR and western blot assays were applied to detect the correlation between the expression of AGGF1 and isoflurane administration. Then, the Morris water maze (MWM) test was applied to evaluate the effect of AGGF1 on improving the POCD rats. Subsequently, TUNEL assay was applied and the cell apoptosis-related proteins were tested to reveal the anti-apoptotic effect of AGGF1 in POCD rats. Furthermore, the mRNA and protein levels of TNF-α, IL-6, and IL-1β were also detected by qPCR and ELISA to verify the anti-inflammatory effects of AGGF1 on POCD rats. Besides, the protein expression levels of PI3K, Akt, and NF-κB in each group were examined by western blot. *Results:* In this study, the results revealed that isoflurane induced a decrease in AGGF1 expression in the hippocampus of aged rats. In addition, exogenous AGGF1 attenuated POCD in aged rats. Meanwhile, exogenous AGGF1 had anti-apoptotic and anti-inflammatory effects in POCD rats. Further research indicated that AGGF1 activated the PI3K/Akt pathway. *Conclusion:* AGGF1 has neuroprotective effect against isoflurane-induced cognitive dysfunction in aged rats via activating the PI3K/AKT signaling pathways.

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KEYWORDS

AGGF1, POCD, PI3K, AKT, isoflurane, aged rats

INTRODUCTION

Postoperative cognitive dysfunction (POCD) is a serious neurological complication characterized by a slight decrease in memory, attention, and information processing speed after patients suffering from anesthesia and surgery [1–3]. It is more common in older people [4]. However, little is known about the etiology of POCD.

Neuroinflammation has been reported to contribute to the progress of POCD [4, 5]. Clinical studies and experimental evidence have confirmed that anti-inflammatory therapy can reduce POCD [6–8]. In the aged brain, microglia may become overactive in an inflammatory state, which impairs cognitive function [6, 7]. Volatile anesthetics such as isoflurane have been reported to induce increased inflammatory responses in the brain of aged rats and cognitive impairment in elderly mice [8]. In addition, anesthesia-induced apoptosis of neurons is also one of the causes of POCD [9, 10]. Numerous studies have revealed that anesthesia can induce apoptosis in the rat hippocampus [11]. Therefore, relieving neuroinflammation and neuronal apoptosis induced by anesthetics is a potential strategy for treating POCD.

As a vascular endothelial-derived protein, angiogenic factor with G-patch and FHA domains 1 (AGGF1) has the function of promoting angiogenesis. A growing number of evidence suggests that AGGF1 plays a number of roles in various pathological processes including inflammation, autophagy, apoptosis, and tumorigenesis [12]. For example, overexpression of AGGF1 can reduce cell apoptosis, alleviate inflammatory response and increase angiogenesis in the myocardial ischemia model [13]. AGGF1 inhibits the production of inflammatory cytokine in dental pulp cells and promotes angiogenesis [14]. AGGF1 treatment can effectively block the formation of new intima after vascular injury [15]. In addition, increased AGGF1 expression has a neuroprotective role through decreasing the neuroinflammation and blood-brain barrier destruction induced by subarachnoid hemorrhage and improves the neurological function recovery by activating the PI3K/Akt pathway [16]. Numerous studies have revealed that activation of the PI3K/Akt pathway can improve cognitive dysfunction. For example, overexpressed C1q/TNF-Related Protein 3 (CTRP3) can activate the PI3K/Akt pathway to play a neuroprotective role against cognitive dysfunction induced by sevoflurane anesthetic in elderly rats [17]. Disintegrin and metallopeptidase domain 2 (ADAM2) knockout also activated the P13K/Akt pathway in immature rats, thus alleviating POCD [18]. Therefore, we speculated that AGGF1 may play a neuroprotective role in mitigating cognitive dysfunction through regulating the P13K/Akt pathway.

SUBJECTS AND METHODS

Animals

20-month-old male Sprague-Dawley rats were provided by BIORAY LABORATORIES Inc. (Shanghai, China). All the protocols used in this study were approved by the Animal Ethics

Committee of the Chengdu First People's Hospital in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines [19].

Establishment of POCD model

A total of 108 Sprague-Dawley rats weighing 400–450 g were used in animal experiments, of which 12 Sprague-Dawley rats were randomly divided into two groups ($n = 6$), namely Group (1) sham, Group (2) isoflurane, to detect mRNA and protein expression of AGGF1 in the hippocampus of rats. The remaining 96 Sprague-Dawley rats were used in four separate experiments, with 24 Sprague-Dawley rats in each experiment. Twenty-four Sprague–Dawley rats were divided into four groups ($n = 6$) at random, designated Group (1) sham, Group (2) isoflurane, Group (3) isoflurane + recombinant human Aggf1 (rh-Aggf1) (5 μg kg^{-1}), and Group (4) isoflurane + rh-Aggf1 (10 μg kg^{-1}). Rats in the isoflurane-treated groups underwent isoflurane anesthesia by 2.5% isoflurane in 100% O_2 of 1.6 L min^{-1} for 2 h according to the protocol [20]. No animals died accidentally during the experiment.

Drug administration

First, isoflurane was administered to rats in different groups. Then, the rats in the isoflurane + rh-Aggf1 (5 μg kg^{-1}) and isoflurane + rh-Aggf1 (10 μg kg^{-1}) groups received rh-Aggf1 via tail intravenous injection on the next day, and 0.9% saline was administered to rats that served as controls in the sham and isoflurane groups.

Morris water maze (MWM) test

One day after isoflurane administration, the MWM test was performed [21]. Briefly, each rat was kept staying in the water facing the maze wall in one of the three quadrants without a platform. The time for rats to search and mount the platform and their swim speed were detected and recorded for five days. On the sixth day, rats were subjected to MWM test without the platform. The data were collected and processed by the Morris software. After the MWM test, and the rats in each group were sacrificed and the skulls were opened to take out the hippocampus. Collected tissues were snap frozen and stored in a −80 °C refrigerator or liquid nitrogen.

qPCR detection

Trizol reagent (Invitrogen, Grand Island, NY, USA) was used to extract the total RNA from hippocampus tissue. RNA integrity and quantity were detected using a Nano Drop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.). The mRNA expression of AGGF1, TNF-α, IL-6, and IL-1β was detected by qPCR by using SYBR Premix EX Taq (Takara, Japan). The relative expressions of AGGF1, TNF-α, IL-6, and IL-1β were analyzed by the $2^{-\triangle\triangle Ct}$ method. Primer sequences used for quantification of cytokines mentioned above are shown in Table 1.

Western blot

Briefly, RIPA lysis buffer was used to extract the total protein from hippocampus tissues (Beyotime, Shanghai, China). The BCA kit (CoWin Biotechnology) was used to determine the concentration of protein, which was then electrophoresed by SDS-PAGE. Proteins were next

Table 1. Primers for AGGF1, TNF-α, IL-6, IL-1β, and reference genes

Gene	Primer	Sequence ($5'\rightarrow3'$)
AGGF1	Forward	GGACTAGTTTGCGAA AACATGGGTAGTG
	Reverse	CCCAAGCTTTGCCTGCAATGGTCTTTATC
IL-6	Forward	CACATGTTCTCTGGGAAATCG
	Reverse	TTGTATCTCTGGAAGTTTCAGATTGTT
IL-1β	Forward	ACCTTCCAGGATGAGGACATGA
	Reverse	CTAATGGGAACGTCACACACCA
TNF-α	Forward	GCCACCACGCTCTTCTGTCTAC
	Reverse	GGGTCTGGGCCATAGAACTGAT
β-actin	Forward	GTGACGTTGACATCCGTAAAGA
	Reverse	GCCGGACTCATCGTACTCC

transferred to PVDF membranes (Millipore, Boston, MA, USA) followed by immersion in 5% non-fat milk for 1 h. The membranes were then incubated with different specific primary antibodies at 4 °C for 12 h. The antibodies were against AGGF1 (ab203680, 1:1200; Abcam), Cleaved Caspase-3 (ab214430, 1:5000; Abcam), Bax (ab53154, 1:3000; Abcam), Bcl-2 (ab182858, 1:1200; Abcam), p-PI3K (ab182651, 1:3000; Abcam), PI3K (ab ab154598, 1:3000; Abcam), p-AKT (ab38449, 1:3000; Abcam), AKT (ab8805, 1:2200; Abcam), p-NF-κB p65 (ab86299, 1:2200; Abcam), NF-κB p65 (ab16502, 1:2200; Abcam), and β-actin (ab8227, 1:1,200; Abcam). The membranes were next incubated with HRP-conjugated secondary antibody (goat anti-rabbit IgG, ab205718, 1:1800; Abcam) and the blots were visualized using ECL chemiluminescence reagent (Beyotime). β-actin was used as a normalized standard. ImageJ software (NIH, version 1.8.0) was employed for the semiquantitative analysis of protein expression.

Immunofluorescent staining

Briefly, hippocampus tissues were treated with 4% PFA and 0.15% Triton X-100 for 25 min. Cells were next treated with 5% BSA for 1.5 h and incubated with primary antibody against AGGF1 (11889-1-AP, 1:2200; Proteintech, Rosemont, IL, USA). Finally, cells were incubated with florescent-dye conjugated secondary antibody (Beyotime) for 2 h followed by staining with DAPI (Sigma-Aldrich, blue).

TUNEL staining

Briefly, hippocampus tissues were treated with 4% PFA and 0.15% Triton X-100 for 25 min. Cells were then treated with Biotin-dUTP and Streptavidin-HRP for 60 min. Finally, cells were incubated with 0.5 ml DAB for 30 min (Abcam, red).

ELISA

For detecting the TNF-α, IL-6, IL-1β protein levels, ELISA kits (TNFα kit, ab100785; Abcam; IL-6 kit, ab100772; Abcam; IL-1β kit, ab100768; Abcam) were used. The production of TNF-α, IL-6, and IL-1β in the **hippocampus tissue** of rats was detected following the manufacturer's instructions.

Statistical analysis

All data are expressed as mean ± standard error with 6 independent repeats. Six Sprague-Dawley rats were included in each group of each experiment. Comparisons among different groups with one independent variable were performed using one-way ANOVA and comparisons among different groups with two independent variables were performed using two-way ANOVA. *P* values of <0.05 (two-tailed) were considered as a statistically significant difference. GraphPad Prism 5 (GraphPad Software, Inc.,) was used for analysis.

RESULTS

Isoflurane induced the decrease of AGGF1 expression in aged rat hippocampus

In this research, the animal postoperative cognitive dysfunction (POCD) model was first established by administration of isoflurane according to the protocol. In order to detect the relationship between AGGF1 expression and isoflurane administration, the expression of AGGF1 mRNA and protein in hippocampus of rats was checked by qPCR, western blot, and immunofluorescent staining, respectively. The result revealed that the expression of AGGF1 mRNA and protein was significantly decreased in the isoflurane-treated group compared to the sham group (Fig. 1A, B, and C). These results verified that isoflurane induced a decrease in AGGF1 expression in aged rat hippocampus.

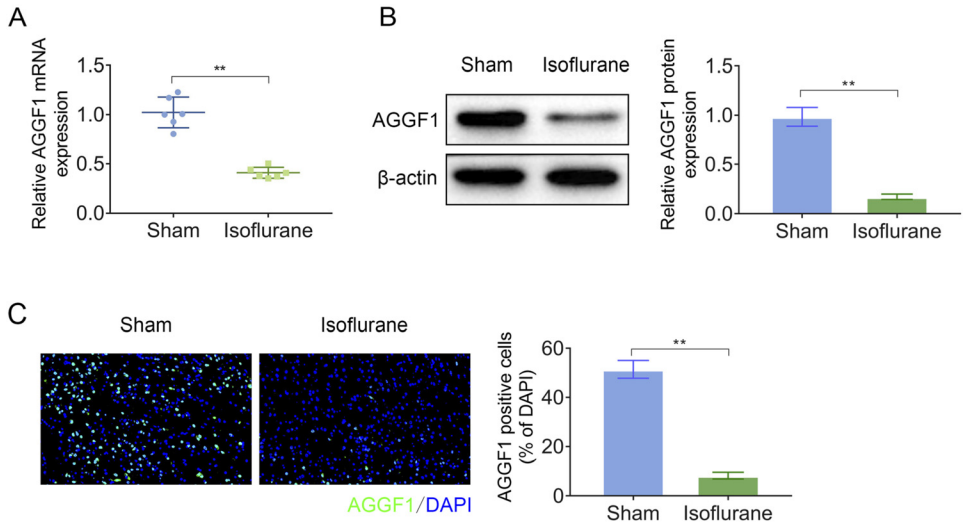

Fig. 1. Isoflurane induced a decrease in AGGF1 expression in the hippocampus of aging rats (A) qPCR was applied to detect the mRNA expression levels of AGGF1 in the sham group and the isoflurane-treated group. (B and C) Western blot and immunofluorescent staining were performed to detect the protein expression of AGGF1 in the sham group and the isoflurane-treated group. Data are presented as the mean ± Standard Error with six independent experiments. $^{**}p < 0.01$ versus sham group.

Exogenous AGGF1 attenuated POCD in aged rats

To explore the role of exogenous AGGF1 in attenuating POCD in rats, animal behavioral tests were used to observe the behavioral performance of rats receiving isoflurane. The Morris water maze (MWM) test showed that the rats in the isoflurane-treated group spent more time on locating the hidden platform than those in the sham group on the sixth day, suggesting that anesthesia led to spatial learning disabilities in the aged rats. However, these conditions were rescued after the rat received increasing doses of exogenous AGGF1 recombinant protein (Fig. 2A). Besides, in the probe trial, rats treated with isoflurane exhibited a decrease in the percentage of time spent in the target quadrant and a reduction in the number of platform crossings, indicating that isoflurane also impaired spatial memory in the aged rats. However, exogenous AGGF1 recombinant protein treatment improved isoflurane-induced short residence time in the target quadrant and the decreased number of platform crossings, with significant therapeutic effects at high doses, suggesting that exogenous AGGF1 recombinant protein can rescue the isoflurane-induced declines in spatial learning and memory in the aged rats (Fig. 2B, C, and D). These results showed that exogenous AGGF1 attenuated POCD in aged rats.

Exogenous AGGF1 inhibited isoflurane-induced apoptosis in aged rat hippocampus

To explore the effect of exogenous AGGF1 on inhibiting apoptosis in POCD rats, TUNEL assay was performed, and the results revealed that isoflurane administration dramatically increased the number

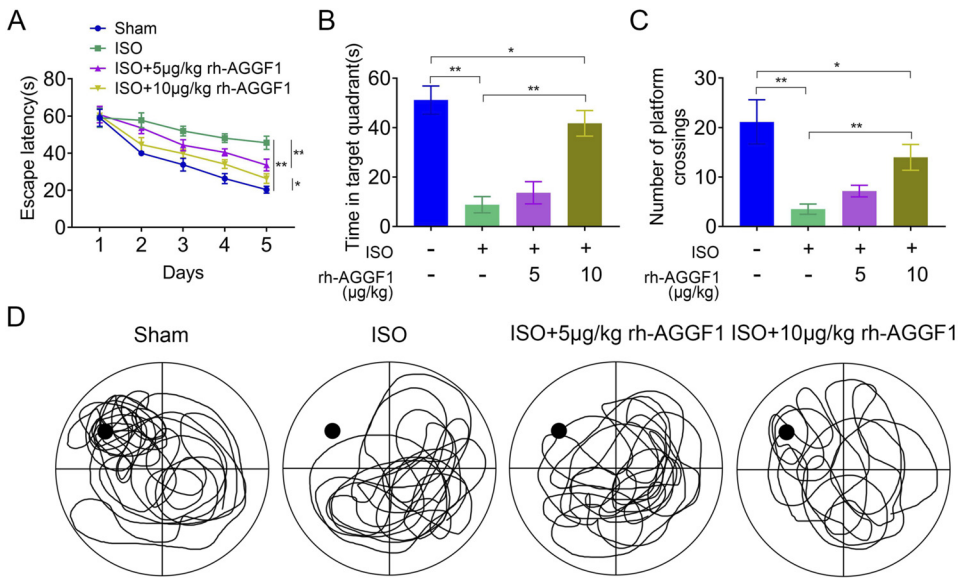

Fig. 2. Exogenous AGGF1 attenuated isoflurane-induced cognitive dysfunction in aging rats
(A) Escape latency in the MWM. (B) The time spent in the target quadrant in the probe trial of the MWM. (C) The number of platform crossings in the probe trial of the MWM. (D) Representative swim paths in the MWM. Data are presented as the mean ± Standard Error with six independent experiments. $^{**}p < 0.01$ versus sham group or isoflurane group.

of TUNEL-positive neurons, which was decreased by exogenous AGGF1 recombinant protein treatment, especially at high doses (Fig. 3A). Then, cell apoptosis-related proteins were analyzed and the results indicated that isoflurane administration markedly increased the expression of Bax and cleaved caspase 3, but decreased the expression of pro-caspase 3 and Bcl-2. However, the expression of Bax and cleaved caspase 3 were both observably downregulated in the isoflurane-treated group supplied with exogenous AGGF1 recombinant protein in a dose-dependent manner, whereas the expression of pro-caspase 3 and Bcl-2 showed an opposite trend (Fig. 3B). These results revealed that exogenous AGGF1 inhibited isoflurane-induced apoptosis in the aged rat hippocampus.

Exogenous AGGF1 alleviated isoflurane-induced inflammatory response in aged rats

Next, the production and mRNA levels of inflammatory cytokines in hippocampus tissue of rats, such as TNF-α, IL-6, and IL-1β, were detected by qPCR and ELISA to confirm the anti-

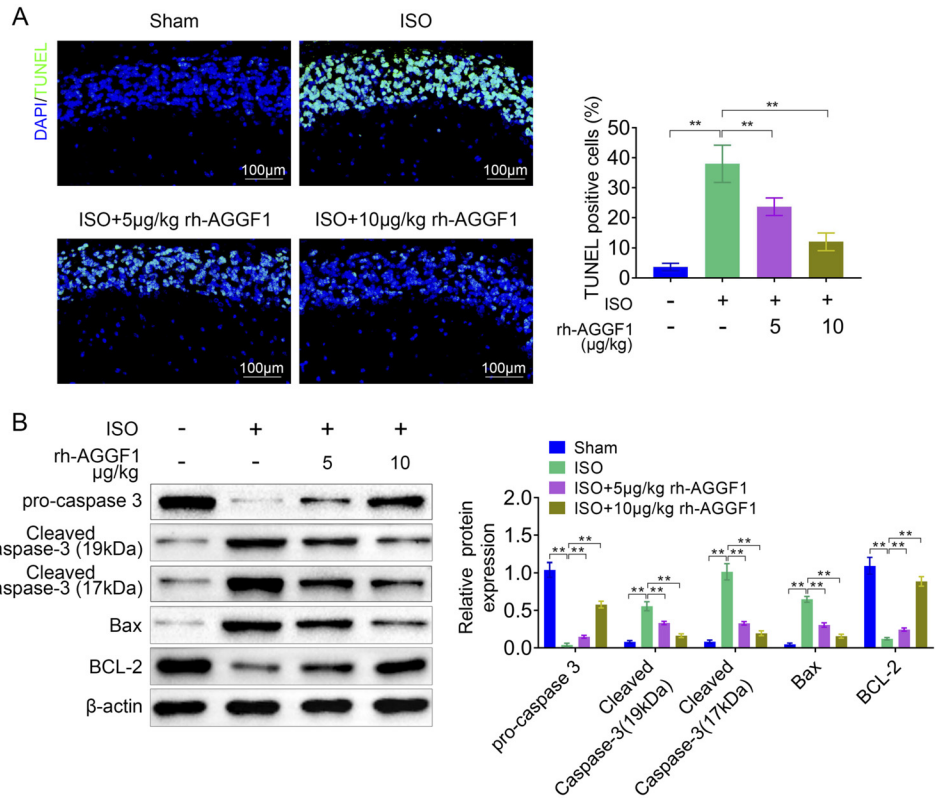

Fig. 3. Exogenous AGGF1 inhibited isoflurane-induced apoptosis in aging rat hippocampus (A) TUNEL assay was applied to determine cell apoptosis in each group. (B) Western blot was applied to detect the expression of cell apoptosis-related proteins in each group. Semi-quantitative results were determined by Image J as shown in histogram. Data are presented as the mean ± Standard Error with six independent experiments. $^{**}p < 0.01$ versus sham group or isoflurane group.

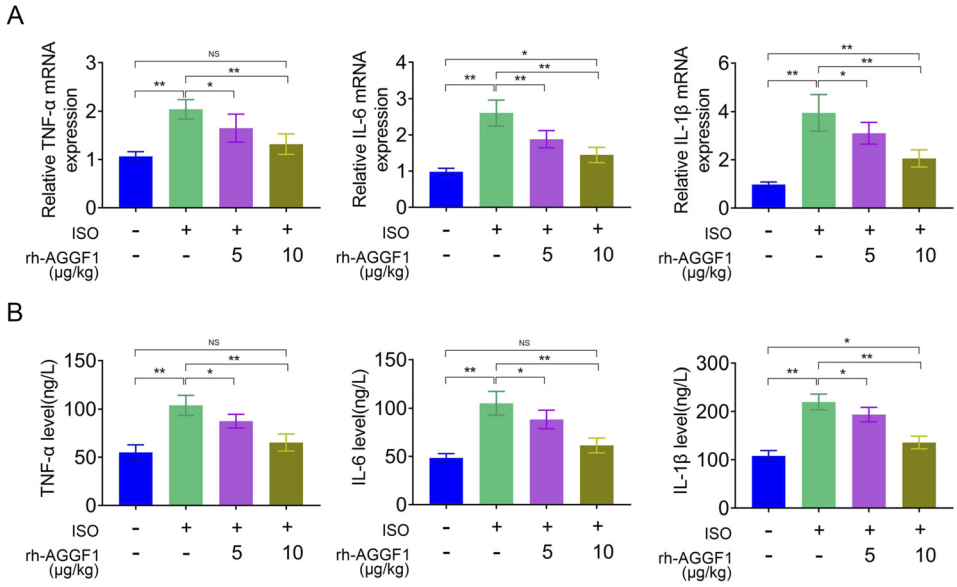

Fig. 4. Exogenous AGGF1 reduced isoflurane-induced inflammatory response in aged rats
(A) The expression of TNF-α, IL-6, and IL-1βmRNA in rats of each group. (B) The expression of TNF-α, IL-6, and IL-1β protein in rats of each group. Data are presented as the mean ± Standard Error with six independent experiments. $^{**}p < 0.01$ versus sham group or isoflurane group, and $^{*}p < 0.05$ versus isoflurane group.

inflammatory role of exogenous AGGF1 in POCD rats. Both qPCR and ELISA results suggested that the mRNA expression and protein level of TNF-α, IL-6, and IL-1β were strikingly increased in the isoflurane-treated group compared to the sham group, indicating that isoflurane administration induced an inflammatory response. However, exogenous AGGF1 recombinant protein markedly reduced the expression of TNF-α, IL-6, and IL-1β both on the mRNA and protein level (Fig. 4A and B). These results revealed that exogenous AGGF1 recombinant protein exhibited a significant anti-inflammatory effect on isoflurane-treated aged rats.

AGGF1 activated the PI3K/Akt pathway

To evaluate the mechanism of AGGF1 in attenuating POCD in aged rats by ameliorating neurotoxicity through regulating the PI3K/AKT pathway, western blot was used to detect the expression of Akt, PI3K, and NF-κB in POCD rats. The results hinted that phosphorylated PI3K and Akt were decreased, whereas the phosphorylation of NF-κB gave opposite results in isoflurane-treated hippocampus. However, the expression of phosphorylated PI3K, Akt, and NF-κB was reversed by exogenous AGGF1 recombinant protein treatment in a dose-dependent manner in the isoflurane-treated group, indicating that AGGF1 activated PI3K and Akt signaling but inhibited the NF-κB pathway (Fig. 5).

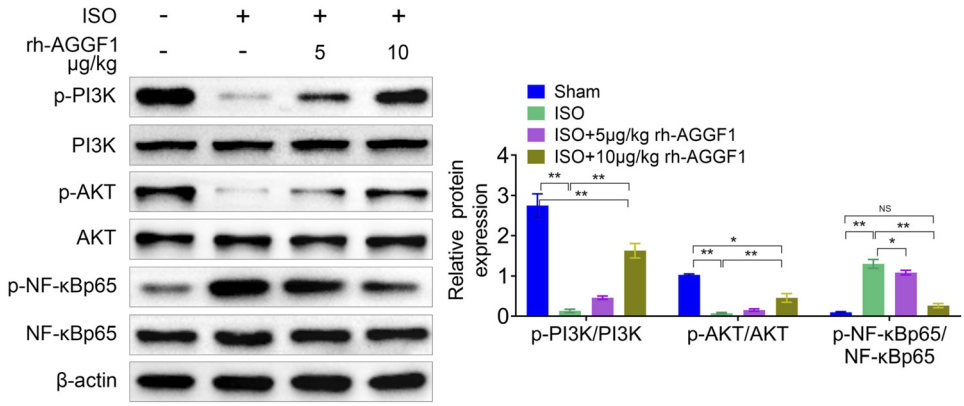

Fig. 5. AGGF1 activated the PI3K/Akt pathway
The protein levels of PI3K, Akt, and NF-κB in each group. Semi-quantitative results were determined by Image J as shown in histogram. Data are presented as the mean ± Standard Error with six independent experiments. $^{**}p < 0.01$ versus sham group, $^{\#}p < 0.05$, and $^{\#\#}p < 0.01$ versus isoflurane group. $^{**}p < 0.01$ versus sham group or isoflurane group, and $^{*}p < 0.05$ versus isoflurane group.

DISCUSSION

Despite great progress made in medical sciences, the molecular mechanism of postoperative cognitive dysfunction (POCD) is not well understood. Recently, several studies have suggested that AGGF1 plays a crucial role in anti-inflammation and anti-apoptosis [14, 16, 22–24]. However, the molecular regulatory mechanism of AGGF1 in the determination of POCD fate remains unclear. Therefore, it is necessary to uncover the mechanism of AGGF1 in regulating POCD, which may provide a novel therapeutic target for treating the disease.

AGGF1 was originally identified as a vascular endothelial-derived protein that promotes angiogenesis. It has been reported that AGGF1 exhibited anti-inflammatory and anti-apoptotic properties [22]. In addition, AGGF1 has been proved to be able to markedly improve symptoms of hepatic diseases, nerve injury, or other diseases [25, 26]. Specifically, AGGF1 can induce endothelial progenitor cell repair after vascular defect [27]. Recently, it was confirmed that AGGF1 may induce poor prognosis and metastasis of colorectal cancer [28]. Moreover, AGGF1 has been found to promote angiogenesis in mouse limb ischemia [29]. AGGF1 may have a promising prospect in the development of new and safe drugs due to its multi-targeted actions. However, few studies focus on investigating the role of AGGF1 in POCD and the potential mechanism remains elusive. This study disclosed that exogenous AGGF1 attenuated POCD in aged rats through its anti-apoptotic and anti-inflammatory effects, which suggested that AGGF1 may contribute to improving the symptoms of POCD.

A growing number of studies have shown that AGGF1 exerts its functions by regulating the expression of target mRNAs. As revealed in a previous study, AGGF1 promotes the invasion and migration of gastric cancer via activating the Wnt/β-catenin pathway [30]. Moreover, AGGF1 was reported to attenuate the activation of hepatic stellate cells and hepatic inflammation through repressing Ccl2 transcription [24]. In addition, AGGF1 was

reported to protect glioblastoma angiogenesis via the MCM3AP-AS1/miR-211/KLF5 axis [31]. The novelty of this study was that PI3K and AKT proteins were first identified as the targets of AGGF1. In our present study it was demonstrated that AGGF1 can target PI3K and AKT as shown by western blot results. The decrease in phosphorylated PI3K and AKT protein induced by isoflurane administration was gradually reversed with increasing AGGF1 dose, indicating that AGGF1 alleviated symptoms of POCD by activating the PI3K/Akt pathway.

This study was the first to explore the neuroprotective effects of AGGF1 and its potential rescue mechanism in POCD rats. Our new findings are: (1) isoflurane could decrease AGGF1 expression in aged rat hippocampus, (2) exogenous AGGF1 could improve isoflurane-induced cognitive dysfunction in rats, (3) exogenous AGGF1 could also inhibit isoflurane-induced apoptosis, (4) exogenous AGGF1 could reduce isoflurane-induced inflammation in mice, (5) AGGF1 could activate the PI3K/AKT pathway.

So far, it has not been determined whether there were any specific intermediate molecules between AGGF1 and the PI3K/AKT signaling pathway. However, a previous study reported that AGGF1 could bind to the membrane of epithelial cells and further regulate downstream signaling pathways [32]. Thus, there may be an AGGF1 receptor on the cell membrane to regulate downstream signaling pathways. Future studies may be required to further clarify the presence of intermediate molecules between AGGF1 and the PI3K/AKT signaling pathway.

CONCLUSION

Our study still has some limitations. In this study, rh-AGGF1 was only injected by tail vein administration route, using a single administration time (one day after ISO treatment), and the best administration route and the potential treatment window for POCD were not evaluated. Many studies have shown that AGGF1 exerts a variety of protective effects on various pathological processes, including anti-inflammatory and anti-apoptotic activities. In this study, we only studied the neuroprotective effect of AGGF1 after ISO treatment in rats. Therefore, the possibility that other AGGF1-mediated effects may play an indirect neuroprotective role in POCD rats cannot be ruled out. Further studies are needed to explore its other functions and possible underlying mechanisms in the future.

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Competing interests: The authors state that there are no conflicts of interest to disclose.

Ethics approval: Ethical approval was obtained from the Ethics Committee of Chengdu First People's Hospital.

Contribution of authors: Xiaoping Wu and Xuan Zhang designed the study, supervised the data collection, Lei Zhao analyzed the data, interpreted the data, Shan Jiang prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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Not applicable.

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