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ORIGINAL PAPER

MicroRNA-26b/PTEN Signaling Pathway Mediates Glycine-Induced Neuroprotection in SAH Injury

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Abstract

Subarachnoid hemorrhage (SAH) is a form of stroke associated with high mortality and morbidity. Despite advances in treatment for SAH, the prognosis remains poor. We have previously demonstrated that glycine, a non-essential amino acid is involved in neuroprotection following intracerebral hemorrhage via the Phosphatase and tensin homolog (PTEN)/protein kinase B (AKT) signaling pathway. However, whether it has a role in inducing neuroprotection in SAH is not known. The present study was designed to investigate the role of glycine in SAH. In this study, we show that glycine can reduce brain edema and protect neurons in SAH via a novel pathway. Following a hemorrhagic episode, there is evidence of downregulation of S473 phosphorylation of AKT (p-AKT), and this can be reversed with glycine treatment. We also found that administration of glycine can reduce neuronal cell death in SAH by activating the AKT pathway. Glycine was shown to upregulate miRNA-26b, which led to PTEN downregulation followed by AKT activation, resulting in inhibition of neuronal death. Inhibition of miRNA-26b, PTEN or AKT activation suppressed the neuroprotective effects of glycine. Glycine treatment also suppressed SAH-induced M1 microglial polarization and thereby inflammation. Taken together, we conclude that glycine has neuroprotective effects in SAH and is mediated by the miRNA-26b/PTEN/AKT signaling pathway, which may be a therapeutic target for treatment of SAH injury.

Keywords Subarachnoid hemorrhage · Glycine · miRNA-26b · AKT · PTEN · Microglia

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Introduction

Subarachnoid hemorrhage (SAH) is a form of stroke in which blood flows into the subarachnoid space between the arachnoid membrane and pia mater [1, 2]. SAH frequently occurs due to a ruptured aneurysm. It is associated with a high mortality rate and in cases of primary rupture, 30% of patients die prior to admission. This rate increases to 50% following a secondary rupture [3]. To improve prognosis for patients, there has been considerable interest in reducing early brain damage associated with blood–brain barrier (BBB) disruption and cerebral edema [4, 5]. Despite numerous advances, SAH continues to be associated with high morbidity and mortality rates. It is thus imperative to develop new therapies to alleviate early brain damage in these patients.

Glycine is a non-essential amino acid that acts as a neurotransmitter in the central nervous system (CNS) [6]. It is a bioactive molecule with opposing effects [7]. On one hand, glycine is an important inhibitory neurotransmitter in the mammalian nervous system [8]. However, it is also an agonist of the *N*-methyl-D-aspartate (NMDA) receptor and is essential for its' activation [9]. Glycine is also essential for the synthesis of many biomolecules such as creatine, porphyrins and purine nucleotides [10]. As a therapeutic agent, it has been proven to be useful in patients with ischemic stroke and can act as a neuroprotective agent in many CNS injuries [11]. We have previously demonstrated that glycine protects in neurons in intracerebral hemorrhage via the phosphatase and tensin homolog deleted on chromosome 10 (PTEN)/protein kinase B (AKT) signaling pathway, however the function of glycine in SAH remains unclear.

PTEN is a known tumor suppressor gene, which acts by negative regulation of the AKT (also known as protein kinase B) signaling pathway [12]. Inhibition of PTEN promotes cell proliferation and cell survival [13]. PTEN is a dual-specificity phosphatase, and acts as both a lipid phosphatase and protein phosphatase [14]. PTEN relies on its lipid phosphatase activity to inhibit the phosphatidylinositol-3-kinase (PI3K)/AKT pathway, which plays a key role in promoting cell survival and growth [15]. Meanwhile, inhibition of PTEN protein phosphatase activity, leads to inhibition of the extra-synaptic NMDA receptor subunit GluN2B, a known mediator of neuroprotection [16]. Inhibition of PTEN also preserves Gamma-Aminobutyric Acid (GABA) alpha receptor expression and function to prevent ischemia–reperfusion-induced neuronal death [17].

In this study, we investigated the neuroprotective effects of glycine in SAH injury. Our results show that glycine can induce neuroprotection by activating the AKT pathway. Glycine-regulated AKT activation appears to be mediated by the microRNA-26b/PTEN signaling pathway.

Methods

The experimental design of this study is depicted in Fig. 1a, b.

Verification In Vivo

Animal

Adult male Sprague–Dawley rats, weighing between 260 and 280 g, were randomly divided into the following groups: Sham, SAH + Vehicle, and SAH + Glycine. Five rats were housed per cage on a 12 h light/dark cycle in a

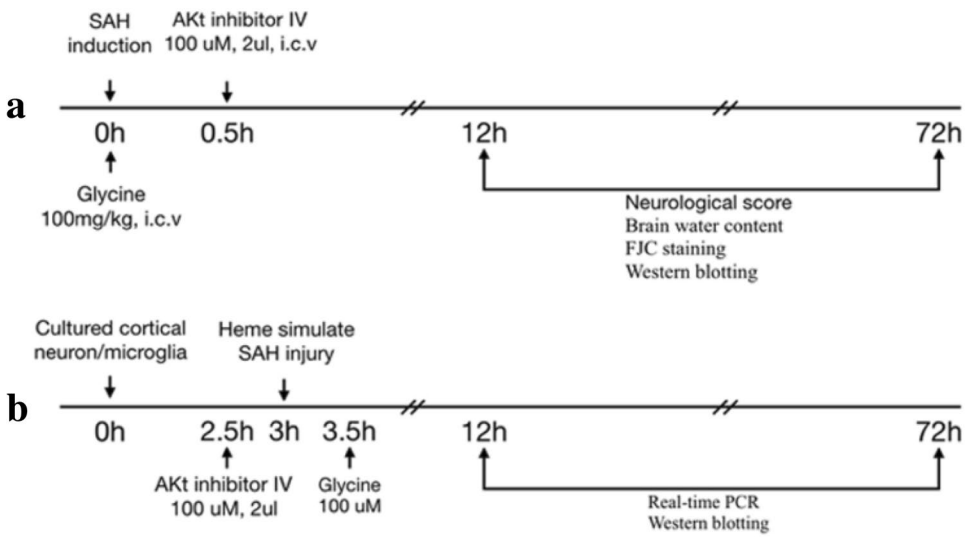

Fig. 1 Experimental design **a** In vivo. **b** In vitro

temperature-controlled room (23–25 °C) with free access to water and food. Animals were given 7 days to acclimatize prior to any procedures taking place. All experimental protocols were approved and carried out in compliance with the IACUC guidelines and the Animal Care and Ethics Committee of Renmin Hospital of Wuhan University (approval number: 2018 K-C017). Experimental groups were subject to randomization and investigators were blinded to the groups for which each animal was assigned.

SAH Model

SAH was induced by intravascular perforation using previously described techniques [18]. Briefly, animals were anesthetized with intraperitoneal injection of chloral hydrate (350 mg/kg). Intraoperative temperature was maintained at 37 °C with a heating blanket. A midline skin incision was performed to expose the left common carotid artery, external carotid artery and the internal carotid artery. The left external carotid artery was ligated and incised, leaving a 3 mm stump. A 3-0 monofilament nylon suture was then inserted through the external carotid artery stump into the left internal carotid artery. Intravascular perforation was performed at the anterior cerebral artery and middle cerebral artery bifurcation.

Intracerebroventricular Injection (i.c.v) Administration

Rats were anaesthetized with a mixture of 4% isoflurane in 30% oxygen (O_2) and 70% Nitrous oxide (N_2O) in a sealed perspective box. The rat head was firmly held using the stereotaxic frame. A midline sagittal incision was made to expose the bregma. A 23-guage needle attached via polyethylene tubing to a Hamilton microsyringe was used to inject the cerebral ventricle, 1.5 mm lateral to the bregma at a depth of 3.5 mm. To ensure that the needle position was correct, few microliters of clear cerebrospinal fluid was withdrawn into the Hamilton microsyringe before commencing drug infusion at a rate of 1.0 µL/min.

SAH Grade

Blinded measurements of SAH severity were performed using the grading scale described by Sugawara et al. [19].

Neurological Scores

The modified neurological severity score was performed on rats as previously reported [20]. These are a series of tests to measure sensation, motor function and coordination. Neurological function scores ranged from 0 to 18, with 0 indicating normal and 18 indicating maximum neurological dysfunction [20].

Brain Water Content

The brain water content was measured 24 h after surgery by calculating the percentage of moisture content (wet weight − dry weight)/wet weight × 100%).

Fluoro Jade–C (FJC) Staining

Rats were euthanized using isoflurane overdose followed by intracardial perfusion with 0.9% saline. Following harvest, the brains were placed in 4% paraformaldehyde (PFA) at 4 °C for 24 h, and then transferred into 30% sucrose solution in 100 mol/mL phosphate buffer at 4 °C for 72 h. The brain tissue was dissected into 15 µm sundial sections by the Leica VT1000S vibratome (Leica Microsystems AG, Walnut Alley, Germany). The brain slices were immersed in 1% sodium hydroxide and soaked in 80% ethanol for 5 min. They were then rinsed with 70% ethanol for 2 min, distilled water for 2 min and finally soaked in 0.06% high potassium potassium citrate solution for 10 min. Brain tissue sections were transferred to 0.0001% FJC solution in 0.1% acetic acid for 10 min. The brain tissue was rinsed with water for 1 min three times, air-dried at 50° C for at least 5 min, and finally immersed in xylene for at least 1 min. Brain sections were mounted using DPX media (Sigma Aldrich, USA). Brain slices were imaged under an Olympus fluorescence microscope (IX51, Olympus, Japan). A series of micrographs of ×20 target and FJC-positive cells in three regions of the ipsilateral side cerebral cortex were taken using Image J software (Image-pro Plus 6.0, USA). The data is expressed as unit/mm^2.

Verification In Vitro

Cell Viability and LDH Release

Assessment of cell viability in primary neuronal cultures was assessed using the thiazolyl blue tetrazolium bromide (MTT assay) (PowerWave X, Bio-Tek, Winooski, State, USA). The LDH assay Colorimetric Toxin 96 Cytotoxicity Kit (Promega, USA) was used to measure the presence of cell damage.

Cortical Neuron Culture

Cortical neuron cultures were prepared from female SD rats at 17 days of gestation [21]. Pregnant rats were euthanized with cervical dislocation. Embryos were obtained and the brain tissue was completely harvested. Cerebral cortical tissue was isolated under a microscope and placed in fresh frozen media with supplements (Neurobasal medium, 0.5% FBS, 2% B-27 supplement, 25 mM glutamate and 0.5 mM L-glutamine Amide). Cortical neurons were seeded

on Petri dishes coated with poly-D-lysine (PDL) and suspended in media. The media was changed every 3 days using Neurobasal medium, 0.5 mM L-glutamine and 2% B-27 supplements. Cortical neurons were cultured for approximately 12 days.

Cortical Microglia Culture

Cortical microglia cultures were prepared from female SD rats at 17 days of gestation. Pregnant rats were euthanized with cervical dislocation. Embryos were obtained and the brain tissue was completely harvested. Pelleted cells were re-suspended in warmed DMEM culture medium completed with 10% heat inactivated fetal bovine serum (FBS), 1% antibiotic–antimycotic, and 5 ng/mL carrier-free recombinant mouse Granulocyte/macrophage-colony stimulating factor (GM-CSF). We seeded approximately 1.3×10^6 cells into tissue culture grade poly L-lysine coated T75 cell culture treated flasks and placed in a 37 °C incubator with relative humidity. We replaced the supernatant from culture twice weekly with 10 mL fresh completed medium until confluency of cells was observed at approximately 3 weeks.

Western Blotting Analysis

Western blotting was performed as previously described [22]. Briefly, the polyvinylidene difluoride (PVDF) membrane by Millipore (USA) was incubated with a primary antibody against AKT (Mouse, 1:1000), phospho-AKT (Ser^{473}) (Rabbit, 1:2000), PTEN (Rabbit, 1:1000), Actin (Rabbit, 1:2000) from Cell Signaling Technology (MA, USA). Primary antibodies were labelled with secondary antibody, protein bands were imaged using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA). The EC3 Imaging System (UVP, LLC, Uplant, USA) was used to obtained blot images directly from the PVDF membrane. The data of western blot was quantified using Image-Pro Plus Version 6.0, USA.

Cell Culture

SHSY5Y cells and U251 cells were purchased from the Chinese Academy of Sciences Cell Bank. U251 cells were seeded in a 6—well plate (8 × 105 cells per well) in DMEM supplemented with 10% heat-inactivated FBS, penicillin G (100 U/mL), streptomycin (100 mg/mL) and L-glutamine (2.0 mM) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air.

Treatment

Cultured cortical neurons and microglia were treated with standard estrous cow serum (ECS) for 60 min and followed by glycine (100 μM) for 60 min. Cell replacement medium was used for subsequent experiments.

Once the confluence of SH-SY5Y cells reached 60–70%, cells were transfected with human PTEN siRNA (siRNApten) and non-targeting control siRNA (NsiRNA) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 8 h. The sequence of human PTEN siRNA (siRNApten) was 5′-CTGCTAGCCTCTGGATTTGA-3′ and non-targeting control siRNA (NsiRNA) was 5′-CTTCTGGCATCCGGTTTAGA-3′, as previously described [23]. The medium was then replaced with normal growth medium for 24 h. On the following day, the cells were treated with standard ECS for 60 min and then treated with glycine for 60 min. The cells were then collected for western blot analysis.

We established the PTEN wild type (WT) and phosphatase domain mutant: PTEN G129E (lacks lipid phosphatase activity while retaining protein phosphatase activity) (TaiHe Biotechnology Co, LTD) [24]. Once the confluence of U251 cells reached 60–70% on the treatment day, cells were transfected with human PTEN plasmid pCDNA3.1(+)-PTEN-WT (WT PTEN), pCDNA3.1(+)-PTEN-G129E (PTEN G129E) and pCDNA3.1(+) [Empty vector (EV)] for 8 h. The medium was then replaced with normal growth medium for 24 h. On the following day, the cells were treated with standard ECS for 60 min and then treated with glycine (100 μM) for 60 min. The cells were then collected for western blot analysis.

Cultures were washed with ECS for 10 min and then treated with serum-free medium supplemented with 1.0 μM of the miRNA-26b agomir, antagomir and respective controls (RiboBio) at 5% CO_2, 95% humidity and 37 °C for 2 h. After incubation, the culture was further cultured in maintenance medium for 24 h.

Real-Time PCR

Total RNA was isolated from pseudo-brain and ischemic brains using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions; the first strand of cDNA was synthesized using 5 μg of Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). PCR was performed on the Opticon 2 real-time PCR detection system (Bio-Rad) using the corresponding primers (Table 1) and SYBR gene PCR master mix (Invitrogen). The cycle time value was normalized to GAPDH of the same sample. The mRNA expression level was then reported as fold change compared to the control group.

Statistics

Student's independent *t* test or analysis of variance (ANOVA) was used where appropriate to examine the statistical significance of the differences between groups of

Table 1 Primers for RT-PCR

Gene	Primer
M1	
IL-1β	SENS: GAGGACATGAGCACCTTCTTT REVS: GCCTGTAGTGCAGTTGTCTAA
TNF-α	SENS: ACCACGCTCTTCTGTCTACT REVS: GTTTGTGAGTGTGAGGGTCTG
CD32	SENS: AATCCTGCCGTTCCTACTGATC REVS: GTGTCACCGTGTCTTCCTTGAG
M2	
Arg-1	SENS: TCACCTGAGCTTTGATGTCG REVS: CTGAAAGGAGCCCTGTCTTG
CD206	SENS:CAAGGAAGGTTGGCATTTGT REVS: CCTTTCAGTCCTTTGCAAGC
YM-1	SENS:CAGGGTAATGAGTGGGTTGG REVS: CACGGCACCTCCTAAATTGT

data. All results are presented as mean ± SE. Significance was placed at $P < 0.05$.

Results

Glycine Relieves Brain Damage and Neuronal Death After SAH Injury

We administered glycine (100 mg/kg) via intracerebroventricular (i.c.v) injection in rats immediately after SAH. The SAH grade scores were not significantly different in the glycine treated group versus control 24 h after SAH (Fig. 2a). However, the neurological scores were significantly improved with glycine treatment 24 h after SAH injury (Fig. 2b). Subarachnoid blood clots were predominantly observed around the circle of Willis and ventral brainstem (Fig. 2c) with evidence of surrounding brain edema, which was ameliorated by glycine treatment (Fig. 2d). Together, these results demonstrate that glycine exhibits neuroprotective effects in SAH. Fluoro-Jade C (FJC) staining was performed in rat brain slices to determine the effect of glycine. The number of FJC-positive degenerating neurons in the cortical regions were increased after SAH injury.(Figure 2e). We treated cultured neurons with heme to simulate SAH injury. We observed that glycine treatment led to reduced heme-induced neuronal death (Fig. 2f, g).

Glycine Induces Neuroprotection in SAH by Activating AKT

AKT transmits cellular signals regulating cell survival and proliferation [25]. Hyperactivation of AKT promotes cell survival and prevention of apoptosis. Following induced SAH, there appears to be reduced levels of p-AKT in vivo (Fig. 3a). Glycine treatment increases the level of p-AKT expression following SAH injury (Fig. 3b). These results suggest that AKT can be activated by glycine following SAH injury. We observed a similar expression pattern in cultured cortical neurons in vitro (Fig. 3c). To determine whether glycine-induced neuroprotection in SAH injury is mediated by AKT activation, we used AKT inhibitor IV (1 μM) to treat cultured cortical neurons 30 min before heme stimulation in vitro (Fig. 3d). Glycine induced neuroprotection is reversed by AKT inhibition suggesting that Glycine mediates its protective effects via AKT activation (Fig. 3e, f).

Glycine-Regulated AKT Activation is Mediated by PTEN Inhibition

PTEN is a tumor suppressor [12], which negatively regulates AKT activation [17]. Western blotting analysis did not reveal significant differences in PTEN expression following SAH injury (Fig. 4a). However, this was reduced with glycine treatment (Fig. 4b). Similar results were observed on cultured cortical neurons (Fig. 4c). To determine whether glycine-mediated AKT activation is dependent on PTEN, we down-regulated PTEN by siRNApten in SHSY5Y cells. Downregulation of PTEN was associated with AKT activation (Fig. 4d, e). In addition using U251 cells (Fig. 4f), a PTEN deficient cell line, we show there is no expression of p-AKT in control and after glycine treatment (Fig. 4g). Following induced expression of PTEN in U251 cells, we treated the cells with glycine to reduce PTEN levels, this led to increased levels of p-AKT (Fig. 4h, i). Together, these results indicate that the glycine-mediated AKT activation in SAH is dependent on PTEN inhibition.

Glycine Reduces Neuronal Death in SAH by Modulating Expression of miRNA-26b

Previous studies have demonstrated that miRNA-26b can directly bind the predicted 3′UTR target sites of PTEN and reduce PTEN expression in tumors [26, 27]. Using RT-PCR of miRNA-26b, we observed reduced levels of miRNA-26b after SAH injury in vivo and in vitro (Fig. 5a, b), and this was increased after treatment of glycine (Fig. 5c). Following miRNA-26b inhibition, there was increased expression of PTEN and reduced expression of p-AKT in cultured cortical neurons. Activation of miRNA-26b led to reduced PTEN expression and increased p-AKT expression (Fig. 5d, e). However, no effect on p-AKT expression was observed in U251 cells following miRNA-26b modulation (Fig. 5f). Moreover, miRNA-26b inhibition followed by glycine treatment in cultured neurons, did not significantly affect the expression of PTEN or p-AKT (Fig. 5g). Inhibition of miRNA-26b was associated with increased heme-induced neuronal death, which was not reversed following glycine

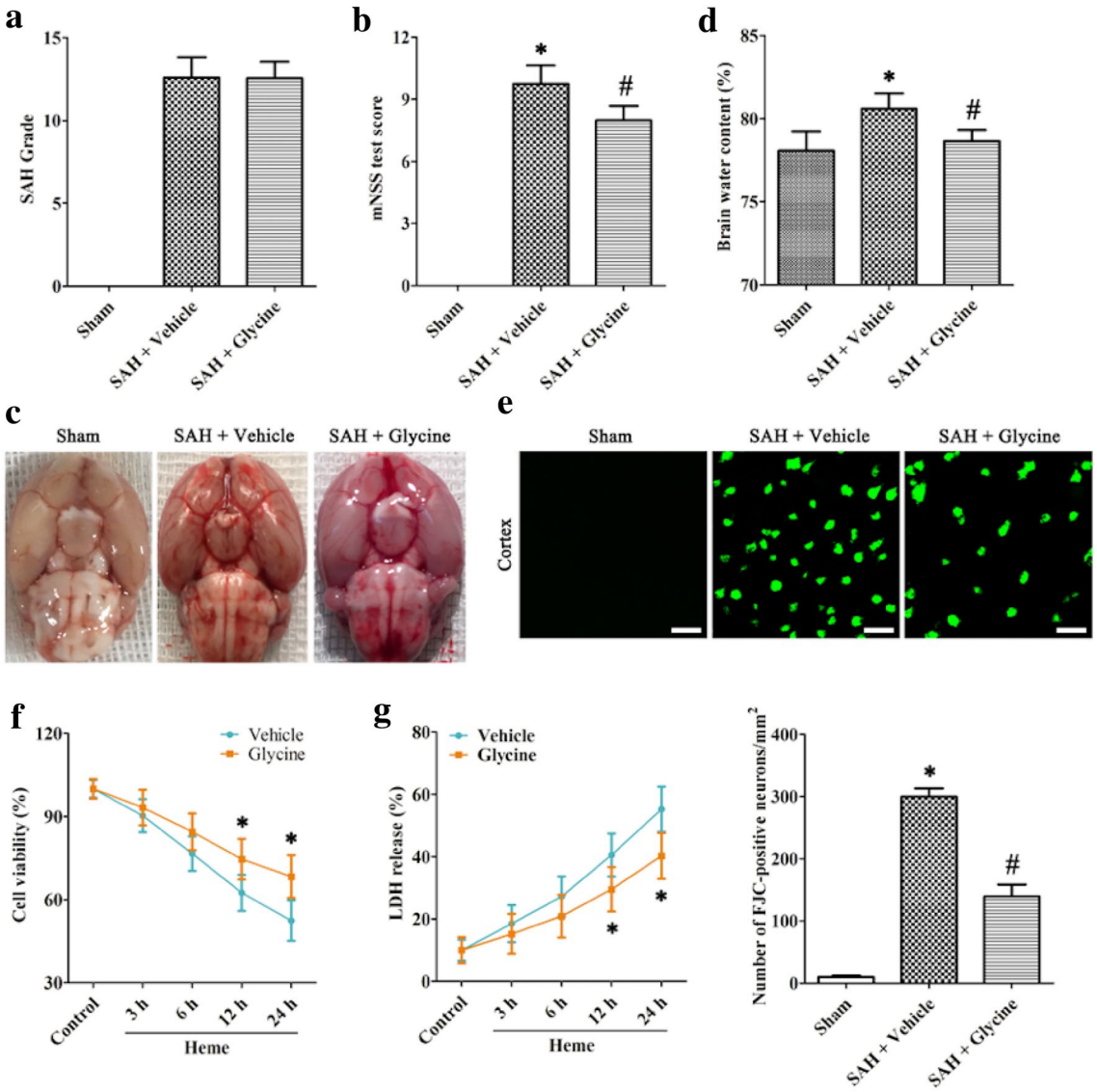

Fig. 2 Glycine attenuates neuronal death and edema after SAH injury. **a**The SAH grade was not significantly different among the groups (n = 10). **b** Glycine treatment reduces SAH-induced neurological dysfunction (n = 10, $^{*}P < 0.05$ vs. the sham, $^{\#}P < 0.05$ vs. the SAH + Vehicle). **c** The severity of SAH was similar in all groups 24 h after SAH injury (n = 10). **d** Glycine alleviated brain edema caused by SAH injury (n = 10, $^{*}P < 0.05$ vs. the sham, $^{\#}P < 0.05$ vs. the SAH + Vehicle). **e** FJC staining and quantification analysis show that glycine decreased the number of FJC-positive neurons 24 h after h (n = 6 at each group, $^{*}P < 0.05$ vs. the sham, $^{\#}P < 0.05$ vs. the SAH + Vehicle). Scale bar, 100 μm. **f**, **g** Glycine reduces heme-induced neuronal death (n = 6 at each group, $^{*}P < 0.05$ vs. the Vehicle)

treatment (Fig. 5h, i). We therefore demonstrate that glycine—induced neuroprotection is mediated via the effects of miRNA-26b on the PTEN/AKT pathway.

Glycine Suppresses M1 Microglial Polarization and Indirectly Promotes Neuronal Survival After SAH Injury

Neuroinflammation plays an important role in SAH injury [28]. Microglia is an innate immune cell of the CNS [29]. Activated microglia have two phenotypes: classically activated (M1 microglia) and alternatively activated (M2 microglia) as described. RT-PCR analysis of M1 and M2 microglial markers showed that SAH is associated with increased inflammation, which could be reduced with glycine treatment (Fig. 6a, b). Western blotting analysis of p-AKT in cultured cortical microglia showed that heme treatment reduced p-AKT expression, which was revered by glycine treatment (Fig. 6c). RT-PCR analysis of M1 and M2 microglial markers in cultured cortical microglia

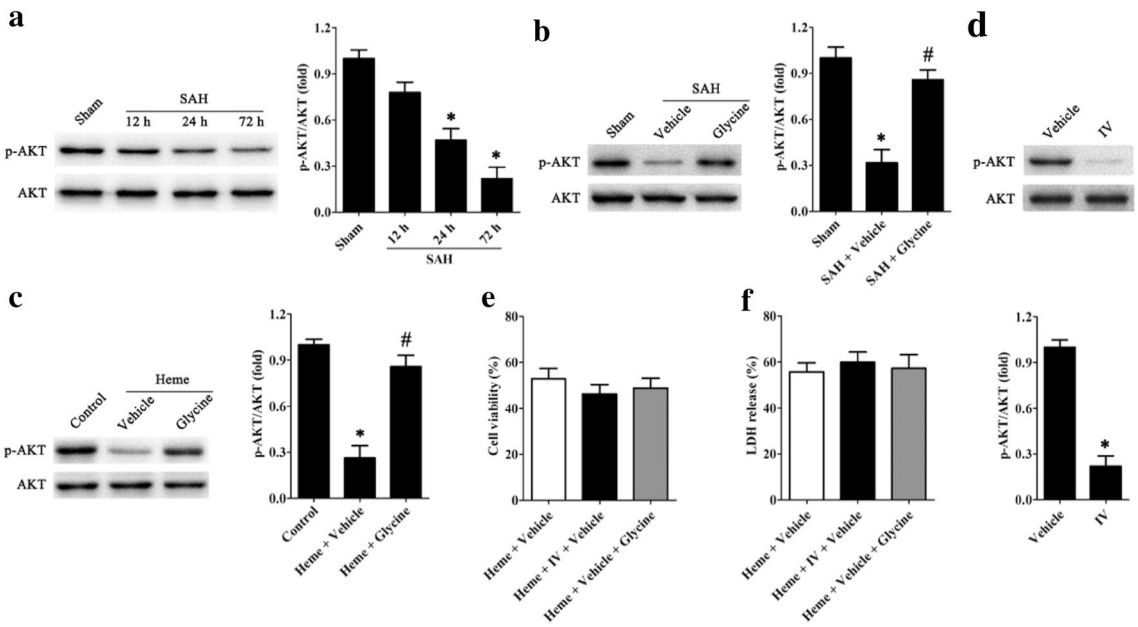

Fig. 3 Glycine reduces neuronal death after SAH by activating AKT. **a** Western blotting analysis reveals p-AKT down-regulation in SAH (n = 6 at each group, $^{*}P < 0.05$ vs. the Sham). **b** Glycine treatment increases p-AKT expression 24 h after SAH injury (n = 6 at each group, $^{*}P < 0.05$ vs. the SAH + Vehicle). **c** Heme-induced p-AKT down-regulation was reversed with glycine treatment in cultured cortical neurons (n = 6 at each group, $^{*}P < 0.05$ vs. the Control, $^{\#}P < 0.05$ vs. the Heme + Vehicle). **d** Treatment with IV reduces the level of p-AKT (n = 6 at each group, $^{*}P < 0.05$ vs. the Vehicle). **e**, **f** Glycine induced neuroprotection is reversed by AKT inhibition (n = 6 at each group)

in vitro showed that glycine inhibited heme-induced M1 microglial polarization, and this was revered with AKT inhibition (Fig. 6d, e). These results suggest that glycine reduces SAH-induced inflammation by regulating AKT activation. In addition, we recovered the microglia cultured medium (MCM) 24 h after heme treatment and mixed it with DMEM in a 1:1 ratio to culture neurons. Treatment with MCM led to greater neuronal death in cells compared to cells treated with standard treatment. This effect was reversed with the use of glycine (Fig. 6f, g).

Neuroprotection of Glycine in SAH is Mediated by miRNA-26b/PTEN/AKT Signal Pathway

We confirmed glycine-induced neuroprotection is mediated by miRNA-26b/PTEN/AKT signal pathway in SAH injury in vitro. In our in vivo experiments, we used IV, an AKT inhibitor (100 μM, 2 μL) in vivo 1 h before SAH injury (Fig. 7a), however this did not significantly affect the SAH grade scores and subarachnoid blood clots (Fig. 7b, d). However, AKT inhibition reduced the observed benefits of glycine in reducing brain edema and neurological scores (Fig. 7c, e).

Discussion

Aneurysmal SAH accounts for 2–9% of all strokes [2] and is associated with high mortality and disability. Glycine is a non-essential amino acid and a precursor of proteins. In the CNS, glycine is an inhibitory neurotransmitter that binds to glycine receptors, leading to inhibition of postsynaptic neurons. It is also a co-agonist of the excitatory NMDA receptor. It has been shown to have neuroprotective effects in ischemic stroke injury [22, 30].

In a recent study, we have demonstrated the beneficial effects of glycine in a stroke model where the administration of glycine was associated with reduction in brain edema and improved neurological outcome following induced hemorrhage [31]. However, the role of glycine in SAH is unclear. In this study we sought to understand whether glycine could have similar effects in SAH and we identified a novel pathway involved in the pathophysiology of SAH. We demonstrate that glycine protects against SAH-induced neuronal damage. Glycine inhibits PTEN and activates AKT by activating miRNA-26b, which reduces SAH-induced neuronal death. In addition, glycine treatment suppresses M1 microglial polarization through activation of AKT following SAH injury.

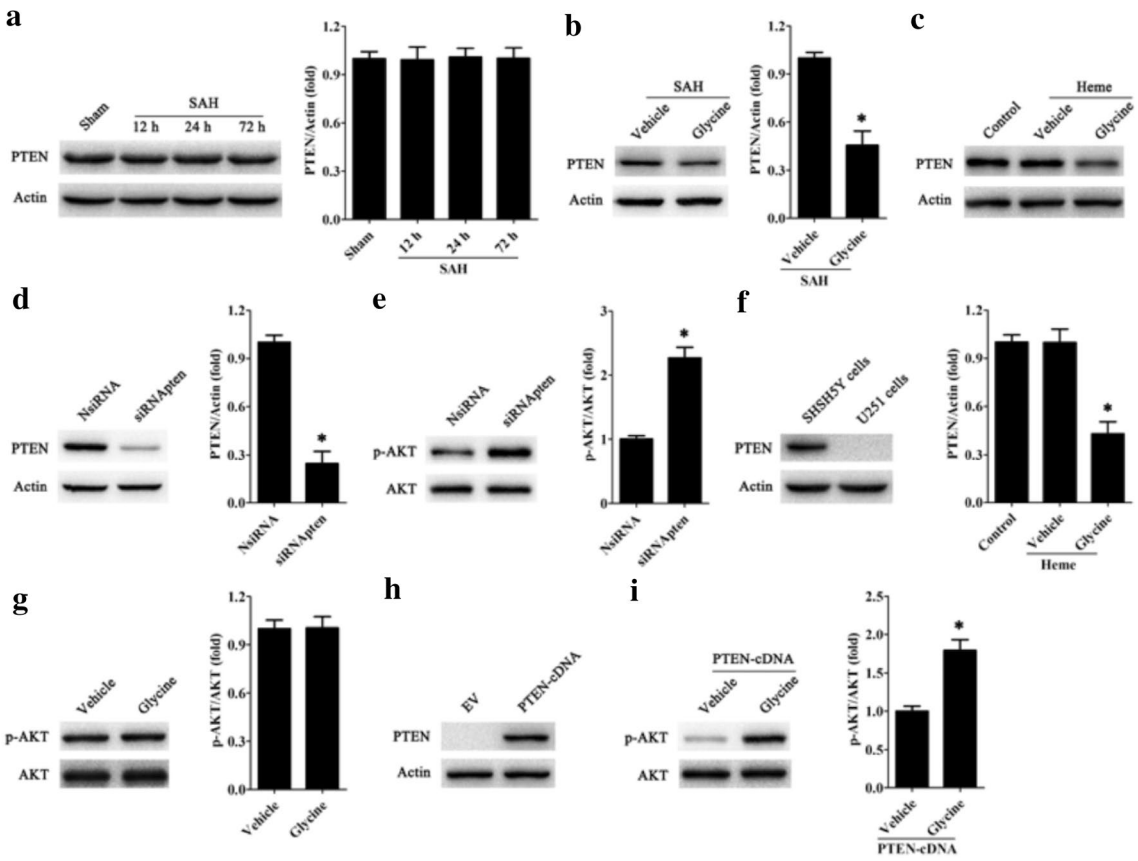

Fig. 4 Glycine-regulated AKT activation is mediated by inhibition of PTEN. **a** Western blotting analysis demonstrates that PTEN expression at different time points following SAH is not significantly different (n = 6,t each group). **b** Glycine treatment reduces the level of PTEN expression (n = 6 at each group, $^*P < 0.05$ vs. the SAH + Vehicle). **c** Glycine reduces PTEN expression after heme treatment in cultured cortical neurons (n = 6 at each group, $^*P < 0.05$ vs. the Heme + Vehicle). **d** PTEN expression is reduced in SHSY5Y cells using siRNApten (n = 6 at each group, $^*P < 0.05$ vs. the NsiRNA). **e** p-AKT expression was increased in SHSY5Y cells following PTEN inhibition (n = 6 at each group, $^*P < 0.05$ vs. the NsiRNA). **f** U251 cells do not express PTEN(n = 6 at each group). **g** Expression of p-AKT in U251 cells was not significantly different after glycine treatment (n = 6 at each group). **h** PTEN expression was induced in U251 cells (n = 6 at each group). **i** Glycine treatment increases expression of p-AKT in U251 cells following forced PTEN expression (n = 6 at each group, $^*P < 0.05$ vs. the Vehicle)

AKT is a member of the AGC kinase family, which is a key intracellular mediator of many biological processes and cellular functions, including proliferation, migration, cell growth and metabolism [32]. Activation of PI3K by hormones and growth factors stimulates the enzymatic activity of AKT protein kinase [28]. AKT activation can directly phosphorylate many substrates in several subcellular compartments, and they can further influence long-term effects on gene expression, cell viability, division or differentiation [33]. Phosphorylation levels of AKT (p-AKT) has been shown to decrease after SAH injury. However, in this study we showed that AKT phosphorylation increases after glycine treatment. PTEN plays a major role in tumor suppression [23], interestingly loss of PTEN can contribute to neuronal protection [34]. PTEN is a phosphatase with both lipid and protein activity [14]. The lipid phosphatase function of PTEN is inversely related to AKT activation by inhibiting the PI3K/AKT signaling pathway [35]. On the other hand, downregulation of PTEN protein phosphatase inhibits the extra-synaptic GluN2B subunit of NMDA receptors suggesting that PTEN plays an important role in neuronal function. Following SAH injury, the expression levels of PTEN do not significantly decrease, however, we show that administration of glycine can downregulate PTEN expression levels. We therefore confirm that the neuroprotective effect of glycine in SAH injury is mediated by the PTEN/AKT signaling pathway.

MicroRNAs are non-coding small RNAs (21–23 nucleotides) that regulate many biological processes, mainly by regulating gene expression at the post-transcriptional level [36]. Previous studies have demonstrated that the predicted 3′UTR target sites of PTEN can be directly bound by miRNA-26b [27, 37], which reduces the expression of PTEN. We found that the level of miRNA-26b is

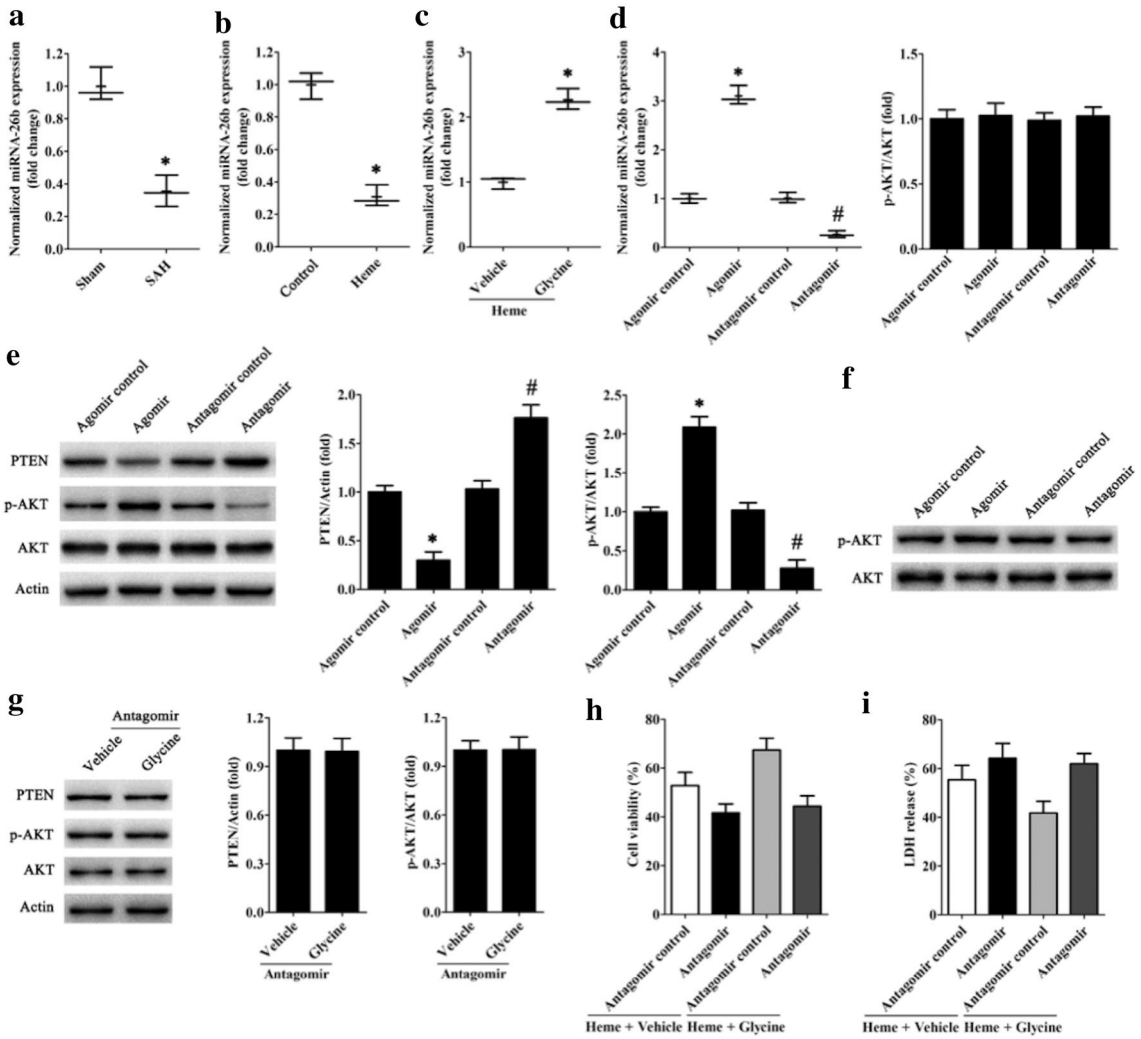

Fig. 5 Glycine reduces neuronal death in SAH by modulating the PTEN/AKT/miRNA-26b pathway. **a** RT-PCR analysis reveals reduced levels of miRNA-26b in SAH (n = 6 at each group, $^{*}P < 0.05$ vs. the Sham). **b** Heme treatment reduces the level of miRNA-26b (n = 6 at each group, $^{*}P < 0.05$ vs. the Control). **c** Glycine treatment increases the level of miRNA-26b (n = 6 at each group, $^{*}P < 0.05$ vs. the Heme + Vehicle). **d** miRNA-26b and p-AKT expression following treatment of cultured cortical neurons with miRNA-26b agomir, antagomir and respective controls (n = 6 at each group, $^{*}P < 0.05$ vs. agomir control, $^{\#}P < 0.05$ vs. antagomir control). **e** Western blotting analysis of PTEN and p-AKT in cultured cortical neurons after treatment with miRNA-26b agomir, antagomir and respective controls (n = 6 at each group, $^{*}P < 0.05$ vs. agomir control, $^{\#}P < 0.05$ vs. antagomir control). **f** Expression of p-AKT in U251 cells after treatment with miRNA-26b agomir, antagomir and respective controls t (n = 6 at each group). **g** Expression of PTEN and p-AKT in cultured cortical neurons after treatment with miRNA-26b antagomir were not significantly different after glycine treatment (n = 6 at each group). **h**, **i** miRNA-26b antagomir reverses glycine induced neuroprotection in cultured cortical neurons (n = 6 at each group)

down-regulated after SAH injury and up-regulated by glycine treatment. Activation of miRNA-26b leads to reduced PTEN expression and increased p-AKT expression, and thereby reduces neuronal damage in SAH.

Microglia is an innate immune cell of CNS, and it is characterized by resident macrophages in the brain [38]. Microglia are the main mediators of neuroinflammation [39]. Neuroinflammation plays an important role in SAH-induced brain damage. We show that the M1 phenotype microglia are activated following SAH injury. Glycine treatment suppresses the SAH-induced M1 microglial polarization and reduces inflammation. We confirm that glycine-mediated microglial polarization is mediated by AKT activation.

In conclusion, the results of our present study demonstrate that glycine exhibits neuroprotective effects in SAH injury. We show that glycine-induced neuroprotection is mediated by AKT activation. Glycine upregulates miRNA-26b, leading to PTEN downregulation and AKT activation. In addition, glycine also indirectly protects neurons in SAH by reducing inflammation through activation of AKT. Taken together, these findings show that glycine may act as a potential therapeutic agent in SAH injury. It is a widely available,

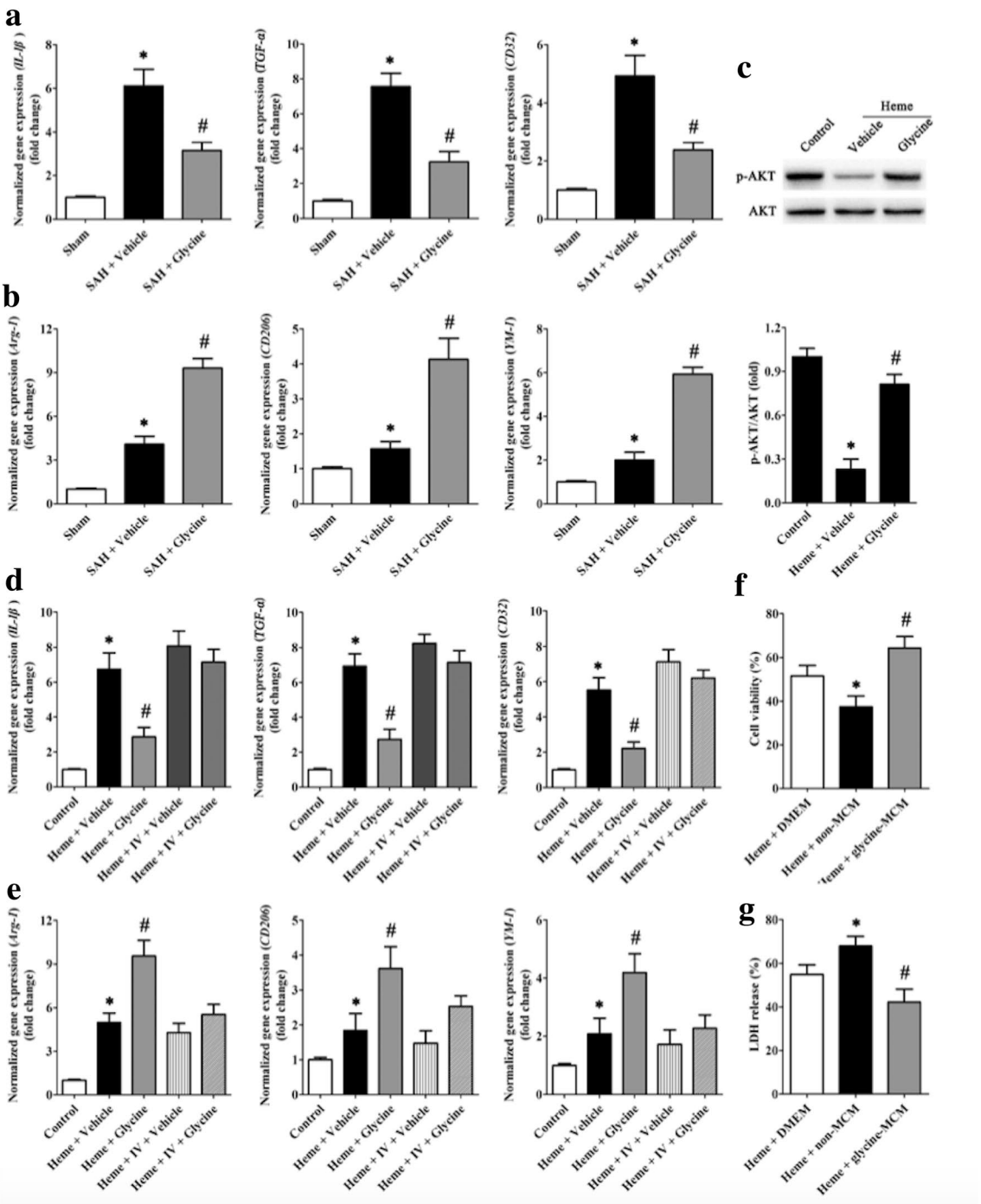

Fig. 6 Glycine suppresses M1 microglial polarization and indirectly promotes neuronal survival after SAH injury. **a**, **b** RT-PCR analysis of *IL-1β*, *TGF-α*, *CD32*, *Arg-1*, *CD206* and *YM-1* in rats 24 h after SAH injury (n = 6 at each group, $^{*}P < 0.05$ vs. Sham, $^{\#}P < 0.05$ vs. SAH + Vehicle). **c** Western blotting analysis of p-AKT in cultured cortical microglia showed that glycine reverses heme-induced downregulation of p-AKT (n = 6 at each group, $^{*}P < 0.05$ vs. Control, $^{\#}P < 0.05$ vs. Heme + Vehicle). **d**, **e** RT-PCR analysis of *IL-1β*, *TGF-α*, *CD32*, *Arg-1*, *CD206* and *YM-1* in cultured cortical neurons 24 h after Heme treatment (n = 6 at each group, $^{*}P < 0.05$ vs. Control, $^{\#}P < 0.05$ vs. Heme + Vehicle). **f**, **g** Cell viability and LDH release in cultured cortical neurons after treatment of MCM (n = 6 at each group, $^{*}P < 0.05$ vs. Heme + DMEM, $^{\#}P < 0.05$ vs. Heme + non-MCM)

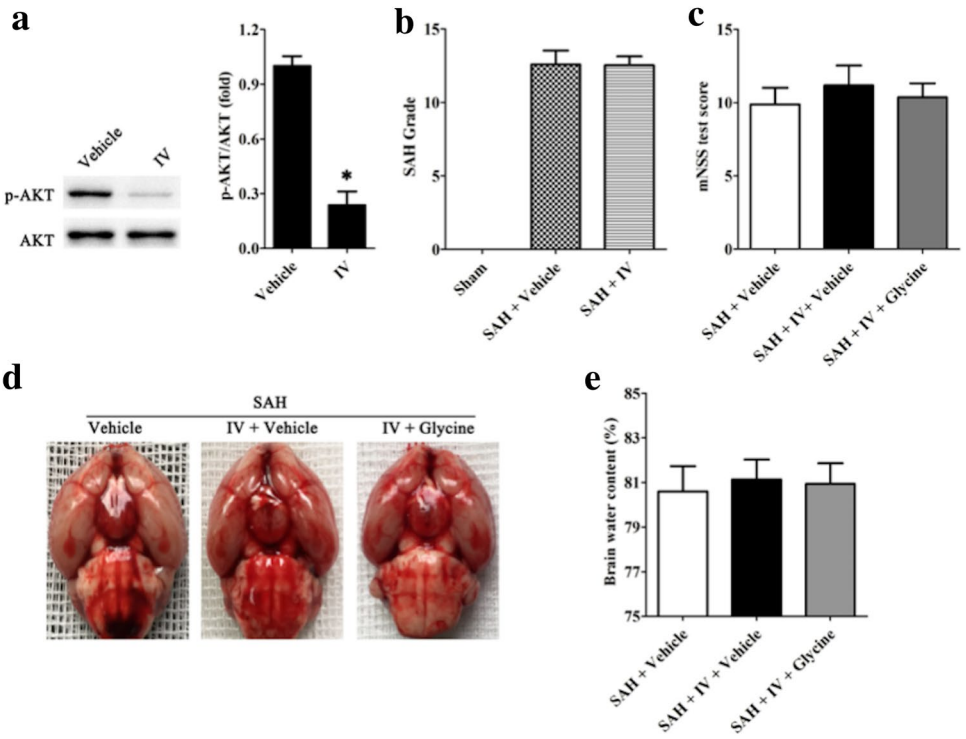

Fig. 7 Neuroprotection of glycine in SAH is mediated by miRNA-26b/PTEN/AKT signal pathway. **a** Western blotting analysis confirms downregulation of p-AKT following treatment with an AKT inhibitor IV (n = 6 at each group, $*P < 0.05$ vs. Vehicle). **b** The SAH grade was not significantly different after IV treatment (n = 6 at each group). **c** Glycine-induced neuroprotection was reduced with IV treatment (n = 6 at each group). **d** The severity of SAH bleeding was similar in all groups 24 h after SAH injury (n = 6 at each group). **e** SAH-induced brain edema was not reduced by glycine in the presence of an AKT inhibitor (n = 6 at each group)

safe and cheap nonessential amino acid and therefore has potential to be used in the clinical setting.

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Compliance with ethical standards

Conflict of interest No conflict of interest exists in the submission of this manuscript. I would like to declare on behalf of my co-authors that the work described is original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.

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