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Rh-relaxin-2 attenuates oxidative stress and neuronal apoptosis via ERK-nNOS-NO pathway after germinal matrix hemorrhage in rats

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Abstract

Oxidative stress and neuronal apoptosis could be an important factor leading to post-hemorrhagic consequences after germinal matrix hemorrhage (GMH). Previously study have indicated that relaxin 2 receptor activation initiates anti-oxidative stress and anti-apoptosis in ischemia-reperfusion injury. However, whether relaxin 2 activation can attenuate oxidative stress and neuronal apoptosis after GMH remains unknown. To investigate the beneficial effect of relaxin 2 on oxidative stress injury and neuronal apoptosis by GMH, a total of 150 rat pups were subjected to GMH by an intraparenchymal injection of bacterial collagenase. Recombinant human relaxin-2 (rh-relaxin-2) was administered intraperitoneally injections at 1 h and 13 h after GMH. Lenti-virus with sgRXFP1 and sgCtrl was administered intracerebroventricular (i.c.v.) on the left side of the brain to inhibit the RXFP1 at 2d prior to GMH induction, and LY321499, ERK inhibitor, was administered by i.c.v. injection at 1 h on the left side of the brain prior to GMH induction, respectively. Co-immunoprecipitation, immunofluorescence, TUNEL, Fluoro-Jade C, DHE staining, western blot, Nitrix Oxide (NO) quantification and side effect experiments were performed to evaluate post-GMH. We found endogenous relaxin-2 interacts with RXFP1 and both protein colocalized in neurons on the first day after GMH. Additionally, RXFP1 activation with rh-relaxin-2 significantly inhibited oxidative stress and neuronal apoptosis in GMH + rh-relaxin-2 group compared with GMH + vehicle group. Moreover, rh-relaxin-2 treatment significantly inhibited the phosphorylation of ERK and nNOS, as well as upregulated expression of Bcl2 and NO and downregulated expression of Bax and Romo 1. The beneficial effects of rh-relaxin-2 were reversed by i.c.v. injection of lenti-virus with sgRXFP1 and LY321499, respectively. Furthermore, the side effect experiment showed rh-relaxin-2

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did not affect neurological behavior and the function of liver and kidney. In conclusion, our finding showed that rh-relaxin-2 attenuated oxidative stress and neuronal apoptosis after GMH through RXFP1-ERK-nNOS-NO signaling pathway.

Keywords Relaxin-2, Germinal matrix hemorrhage, Oxidative stress, Neuronal apoptosis

Introduction

Germinal matrix hemorrhage (GMH), termed as periventricular / intraventricular hemorrhage, is the most common neurological disorder of newborns and is a major cause of brain injury in preterm infants [1–3]. Germinal matrix is a highly vascularized zone beneath ependymal or periventricular region that constitutes a capillary bed with particular structure fragility. Anatomical and physiological characteristics of periventricular vascular structures is tend to cause primary and secondary injury, activate microglia, generate oxidative stress (mediated by free oxygen and nitrogen radicals) and apoptosis of neurons, glutamate toxicity, energetic failure and so on [4]. Among all of those, oxidative stress and neuronal apoptosis could be an important factor leading to post-hemorrhagic consequences [5]. Therefore, inhibition of the oxidative stress and neuronal apoptosis of neurons is critical importance at the early stage after GMH.

GMH leads to acute induction of inflammation cytokines, followed by oxidative stress at 24 h and sustained 7 days after GMH induction [3]. Oxidative stress was concentrated in white matter, adjacent to the lateral ventricular, where the apoptotic pathways was activated and nitric oxide synthase (NOS) was induced to increase cerebral blood flow via NO-mediated vasodilation [6, 7]. Therefore, developing antioxidant and anti-apoptosis strategies to effectively combat brain injury in GMH appears to be necessary.

Relaxin 2, a 6-KD member of insulin-like peptide family, binds to its receptor Relaxin Family Peptide Receptor 1(RXFP1) with high affinity. Previous studies have found that RXFP1 was expressed on neurons, astrocytes and macrophages/microglia, and was increased post-stroke, but was expressed to a lower extent in astrocytes [8, 9]. Growing evidence reported that relaxin 2 can be involved in the pathophysiology of arterial hypertension and heart failure, in the molecular pathways of fibrosis and cancer, and in angiogenesis and remodeling [10–12]. Currently, it was confirmed that therapeutic action of relaxin 2 is dependent on NOS by reducing oxidative stress during hypertension [13]. It is also reported that relaxin 2 significantly decreased ischemia-reperfusion injury (IRI) via both antioxidant and anti-apoptotic mechanisms [14]. In addition, relaxin 2 exerts a protective effect in lung ischemia/reperfusion, the detailed analysis of NO gene expression and activity revealed that the relaxin 2-induced early and moderate NOS stimulation is the extracellular signalpregulated kinase-1/2 (ERK1/2) [15]. Our previous results demonstrated that rh-relaxin-2 treatment with GMH pups suppressed mast cell activation, consequently reduced the secretion of proinflammatory cytokines, improved neurological function in the short and long term and ameliorated post-hemorrhagic hydrocephalus (PHH). All these beneficial effects was mediated by PI3K-AKT/TNFAIP3/NF-κB signaling pathway [16]. However, little is known on rh-relaxin-2's antioxidant and anti-apoptotic effects after GMH.

Based on the above mentioned evidence, in the current study, we assess the hypothesis that rh-relaxin-2 treatment attenuates oxidative stress and neuronal apoptosis through ERK-nNOS-NO pathway after GMH in rats.

Materials and methods

Animals

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Southern Medical University and were strictly complied with the national guidelines for the use of animal in neuroscience research. One hundred Fifty P7 Sprague-Dawley neonatal pups (weight = 12–14 g, Harlan, Livermore, CA) were randomly divided into Sham ($n = 24$) and GMH ($n = 126$) groups. All pups were housed under standard conditions with controlled temperature and 12-h-light/dark cycle and access to food and water ad libitum. All rats (up to 21 days old) were returned to their home cages with mothers after surgery, drug administration, and behavior tests.

GMH model and experimental protocol

The procedure for the GMH model using collagenase infusion was performed as our previously described [16]. In detail, pups were anesthetized with isoflurane (3.0% induction, 1-1.5% maintenance) and placed on a stereotaxic frame. Using aseptic technique, a 1.5 mm longitudinal incision was made on the skin overlying the skull and the bregma was exposed. Then, a 27-gauge needle with 0.3 U clostridial collagenase (0.3 unites of clostridial collagenase VII-S, Sigma-Aldrich, MO) was injected at stereotaxic coordinates (1.6 mmL, 1.5 mmA, and 2.7mmV), and infused (1 μl/min) using a 10-μl Hamilton syringe (Hamilton Co, Reno, NV, USA) guided by a microinfusion pump (Havard Apparatus, Holliston, MA). The needle was remained in position for a further 10 min to avoid leakage before being withdrawn slowly at a speed of 0.5 mm/min. The pups were allowed to recover on a heated pad for several minutes after infusion and then

returned to their mothers, and euthanized at different time points according to the experimental design.

We have previously described the drug administration to the brains by Intracerebroventricular injection and the coordinates were at 1mmA, 1mmL, and 1.7mmV.

Recombinant human relaxin-2 (rh-relaxin-2, Sigma-Adrich) was dissolved in sterile phosphate-buffered saline (PBS). Pups were administered at 60 μg/kg via intraperitoneal eall. The Sham group was treated with the same volume of solvent (PBS, 2 μl) of clostridial collagenase by stereotaxic injections as control.

ERK inhibitor LY3214996 (Selleckchem, TX) was infused by i.c.v. injection at 1 h prior to GMH induction.

Study design

Seven separated experiments were conducted as follows (Fig. 1s).

Experiment 1

To study the time course of endogenous relaxin-2 and its receptor RXFP1 in the whole brain, thirty-six rats whole brains were collected after perfusion with cold PBS at 0 (naive), 0.5, 1, 3, 5, and 7 days after GMH($n = 6$/time point) for western blot. An additional 3 rats at 1 day after GMH was used to detect cellular localization of relaxin-2/RXFP1 and NeuN by double immunofluorescence staining on neurons. Another 3 rats at 1 day after GMH was performed by Co-Immunoprecipitation (co-IP) to explore the interaction between relaxin-2 and RXFP1.

Experiment 2

Considering the drug safety profile and according to our previously conclusion, we chose the medium dose of rh-relaxin-2 (60 μg/kg) for the following studies. To evaluate the effect of rh-relaxin-2 on the oxidative stress and neuronal apoptosis after GMH, rats were randomly divided and assigned to three groups: Sham ($n = 6$), GMH + vehicle ($n = 6$), and GMH + rh-relaxin-2 ($n = 6$). Eighteen rat brains were conducted western blot analysis on the first day post-GMH and after being perfusion with cold PBS.

Experiment 3

Dihydroethidium (DHE) staining, and Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL), were performed to evaluate oxidative stress and neuronal apoptosis in the brain cortex at 1 day after GMH. Fluoro-Jade C staining was conducted at 28 d after GMH. Thirty-six rats were randomly divided into Sham, GMH + vehicle, and GMH + rh-relaxin-2 ($n = 6$ rats/group).

Experiment 4

To explore the effects of RXFP1 in vivo on neurons after administration of rh-relaxin-2 post GMH, lenti-virus with sgRXFP1 and sgCtrl was administered i.c.v. on the left side of the brain to inhibit the RXFP1 at 2 d prior to GMH induction. The whole brain samples were collected to perform western blot analysis on the first day post-GMH and after being perfused with cold PBS. The pups were randomly divided into five groups: Sham, GMH + vehicle, GMH + vehicle + rh-relaxin-2 (i.p. 60 μg/kg), GMH + sgCtrl + rh-relaxin-2 (i.p. 60 μg/kg), and GMH + sgRXFP1 + rh-relaxin-2 (i.p. 60 μg/kg) ($n = 6$ rats/group).

Experiment 5

To assess the role of ERK pathway in vivo on neurons after administration of rh-relaxin-2 post-GMH, LY321499 was administered by i.c.v. injection at 1 h on the left side of the brain prior to GMH induction. The whole brains were collected for western blot on the first day post-GMH after being perfused with cold PBS. The pups were divided randomly into five groups as following: Sham, GMH + vehicle, GMH + vehicle + rh-relaxin-2 (i.p. 60 μg/kg), GMH + corn oil + rh-relaxin-2 (i.p. 60 μg/kg), and GMH + LY321499 + rh-relaxin-2 (i.p. 60 μg/kg) ($n = 6$ rats/group).

Experiment 6

To explore the side effect of rh-relaxin-2 (i.p. 60 μg/kg) administered, the rats were divided randomly in two groups: naïve and rh-relaxin-2 treatment. Open field test and T maze were performed on the 28th day after treatment rh-relaxin-2 [2]. The body weight of rats between two groups were weighed before GMH induction and at the 28th day after treatment. The rat blood samples were collected to quantify the level of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatinine after behavior test.

Experiment 7

The level of NO were determined in the brain using the nitrite assay kit (Griess Reagent, sigma) as the manufacturer's instruction [17]. The brain tissue lysate was from experiment 2, 4 and 5. In detail, nitrite is reduced to nitrogen oxide, which reacts with Griess reagent that detected by absorbance at A540nm.

Plasmids, transfection and lentivirus preparation

RXFP1 sgRNA and control sgRNA plasmids were constructed by annealing the oligonucleotides of the target sequence and were cloned into lenticrispr V2 vector (Addgene).

HEK293T cells were obtained from the American Tissue Collection Center(ACTT, Manassas, VA, USA) and

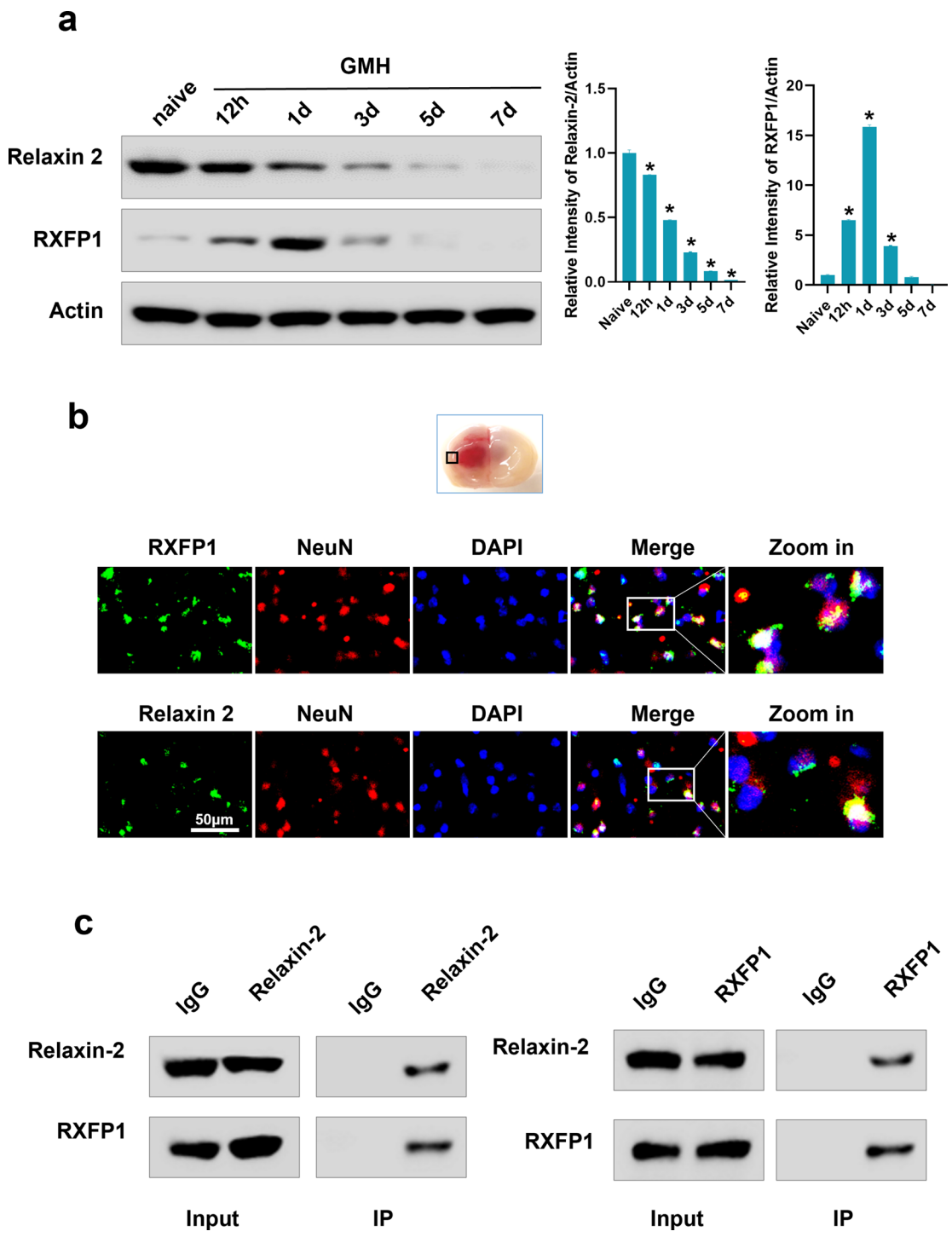

Fig. 1 Endogenous relaxin-2 interacts with RXFP1 and co-localizes in neurons on the first day after GMH. Western blot data illustrated that the expression of endogenous relaxin-2 was decreased at 12 h after GMH. The expression of RXFP1 increased at 12 h, peaked on the first day, and declined significantly on the third, fifth, and seventh day after GMH (**a**, $n = 6$, $^*p < 0.05$ vs. naïve). Double immunofluorescence staining showed that relaxin-2 and RXFP1 was expressed abundantly on neurons marked with NeuN on the first day after GMH (**b**, $n = 6$, bar = 50 μm). Co-IP confirmed that relaxin-2 binds with RXFP1 in the brain tissue on the first day after GMH (**c**, $n = 3$)

maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO_2. Target DNA (15 μg), psPAX2 (7.5 μg), pmD2.G (7.5 μg) and polyethylenimine (PEI, 60 μg) were mixed fully and added into HEK293T cells which is about 80% confluent in a 20 cm dish. The medium was changed 8–12 h after transfection. The viral supernatant was collected 72 h after the medium change, passed through a 0.45 μm filter, centrifuged at 3000 g for 10 min at 4°C, and further ultracentrifuged the supernatant at 28,000 rpm for 2 h at 4°C. Finally, discarded the supernatant, re-suspended the viral particles for quantification, aliquoted into 1×10^{10}TU/5μl/rat and stored at -80°C [18].

Western blot

Brain tissues were collected and stored in a -80°C freezer after perfused with cold PBS. After extraction of protein samples, protein quantification was performed using BCA protein assay kit (Thermo Fisher, #23225, U.S.A.). Equal amounts of protein (30 μg) were loaded into 7.5-12.5% SDS-page gel, then electrophoresed and transferred to nitrocellulose membranes (0.2 μm), which was blocked with 5% non-fat blocking grade milk (sigma-aldrich, U.S.A.) and incubated the following primary antibodies overnight at 4 °C: anti-RXFP1(1:1000, Biorbyt, USA, orb157275), anti-relaxin-2 (1:1000, Invitrogen, USA, PA5-76483), anti-Bcl2 (1:2000, CST, USA, #2876), anti-Bax (1:1500, CST, USA, #2772), anti-Romo-1 (1:1000, Proteintech, USA, 24200-1-AP), anti-Actin (1:10000, Proteintech, USA, 81115-1-RR), anti-phospho-p44/42 ERK (Thr202/Tyr204) (1:2000, CST, USA, #9101), p44/42 ERK (1:2000, CST, USA, #9102), anti-phospho-nNOS (Ser1417)(1:1000, Thermo Fisher, USA, PA1-032), and anti-nNOS (1:1000, Proteintech, USA, 29231-1-AP). On the second day, the membranes were incubated with the appropriate secondary antibodies (goat anti-mouse, Thermo Fisher, USA, #31430; goat anti-Rabbit, Thermo Fisher, USA, #65-6120) for 1 h at room temperature. Immunoblots were then visualized with ECL Substrates for High-Sensitivity Western Blot Detection (bio-rad, USA, # 1705061) and quantified with Image J software (ImageJ 1.5, NIH, USA). The results were normalized using β-Actin as an internal control.

Co-IP assay

The whole brains were lysed with RIPA lysis buffer and prepared for Co-IP assay. After the protein A/G beads (Thermo Fisher, USA, #88802) incubated with the primary antibodies [anti-RXFP1(1:500, Biorbyt, USA, orb157275) or anti-relaxin-2 (1:500, Invitrogen, USA, PA5-76483)] or IgG (1:500, Proteintech, USA, 10285-AP) for 6–8 h, the prepared brain lysate was added to the mixture of beads and antibodies overnight at 4 °C. Then, the mixture was washed with PBS containing 0.1% NP40 for three times and eluted using the elution buffer. Finally, the elution samples were detected by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), subsequently transferred membrane, expoused after incubating with ECL Substrates (bio-rad, USA, # 1705061).

Immunofluorescence

Double fluorescence staining was performed as described previously. Sections were blocked with 5% donkey serum for 1 h and incubated at 4 °C overnight with primary antibodies: rabbit anti-RXFP1 (1:200, Biorbyt, orb157275), Rabbit anti-relaxin-2 (1:200, Invitrogen, USA, PA5-76483), and mouse anti-NeuN antibody (1:200, Thermo Fisher, USA, MA5-33103) followed by incubation with appropriate fluorescence-conjugated secondary antibodies for 2 h at room temperature. Fluorescence microscopy and software were used to image the sections (Leica DMi8; Leica Microsystems, Wetzlar, Germany).

DHE staining

To illustrate the brain oxidative stress level in vivo, fresh frozen brain sections from Sham, GMH + vehicle, and GMH + rh-relaxin-2 were incubated with 2 μM fluorescent dye DHE, Thermo Fisher, USA, D23107) at 37 °C for 30 min in a humidified chamber and protected from light. The images were captured and the red fluorescent intensity was quantified by ImageJ software. The fluorescent intensity was expressed relative to that of Sham group.

TUNEL staining

To quantify the neuronal apoptosis, double staining of neuron marker NeuN and TUNEL staining was conducted by in situ Apoptosis Detection Kit (Roche, USA, #7791-13-1) according to the manufacturer's instructions at 1 day after GMH. The numbers of TUNEL-positive neurons was counted in the ipsilateral cortex. Data were presented as the ratio of TUNEL-positive neurons.

Fluoro-Jade C staining

Fluoro-Jade C (FJC) staining was performed according to the manufacturer's instruction (Fluoro-Jade C Ready-to-Dilute Staining Kit, Biosensis, USA, # TR-100). FJC positive neurons were counted in six sections per brain with ImageJ software to be analyzed. The data were presented with average number of FJC-positive neurons in the fields as cells/mm^2.

Open field test

A black rectangular box (100 cm×100 cm×45 cm) was used for the Open Field Test (OFT) as described previously [2]. The box was illuminated with three 30 W

fluorescence bulbs placed 2 m above the box. The pups were placed in the corner of the box and allowed 10 min of exploration. The total distance travelled and the percentage of time spent in the center were analyzed by tracking system.

T-maze

Each rat was placed at the start area of the T-maze and allowed to choose an arm to enter. Once the pup selected an arm, it was confined to that arm for 30 S and then returned to the start arm and confined there for 10 s. Afterwards the rat was allowed to choose an arm again. Ten trials were performed per rat. The total alternate numbers were recorded and repeated for 10 trials as corrected percentage for each animal.

Statistical analysis

All data were presented as a mean ± SD. All analysis were performed using GraphPad Prism 9 (GraphPad software). Differences between individual groups were first compared using analysis of variance (one-way ANOVA) and then *post hoc* testing were analyzed with Tukey or Student-Newman-Keuls multiple comparisons. Differences between two groups were compared using Student's *t test*. All reported p values were two-side, and a value of $p < 0.05$ was considered statistically significance.

Results

Endogenous relaxin-2 interacts with RXFP1 and both protein colocalized in neurons on the first day after GMH

Western blot data illustrated that the expression of endogenous relaxin-2 was decreased at 12 h after GMH. However, the expression of RXFP1 increased at 12 h, peaked on the first day, and declined significantly on the third, fifth, and seventh day after GMH (Fig. 1a). This is consistent with our previously results [16]. Additionally, double immunofluorescence staining showed that relaxin-2 and RXFP1 was expressed abundantly on neurons marked with NeuN on the first day after GMH (Fig. 1b). Furthermore, Co-IP confirmed that relaxin-2 binds with RXFP1 in the brain tissue on the first day after GMH (Fig. 1c).

Rh-relaxin-2 treatment inhibits neurons apoptosis and oxidative stress on the first day after GMH

Our previous data demonstrated that administration of rh-relaxin-2 (i.p. 60 μg/kg) improved neurological functions, attenuated degranulation of mast cells and neuroinflammation, and ameliorated post-hemorrhagic hydrocephalus (PHH) after GMH [16]. In order to explore whether rh-relaxin-2 inhibits neuronal oxidative stress and apoptosis after GMH, western blot analysis was performed to quantify the level of apoptosis makers Bcl-2 and Bax, and oxidative stress marker Romo1 on the first day after GMH in vivo. The results confirmed that relaxin-2 and RXFP1 were upregulated after treatment with rh-relaxin-2 (Fig. 2a, b and c). Moreover, Bcl-2 reduced remarkably post-GMH but raised significantly after treatment with rh-relanxin-2 (Fig. 2a and d). However, the level of Bax increased obviously after GMH and declined after treatment with rh-relaxin-2 (Fig. 2a and e). The alteration of Rmo1 is same as that of Bax (Fig. 2a and f). Furthermore, rh-relaxin-2 treatment improved the concentration of NO, even though it was declined after GMH (Fig. 2g).

Rh-relaxin-2 reduced neuronal apoptosis on the first day after GMH

TUNEL staining was used to see whether rh-relaxin-2 can reduce neuronal apoptosis on the first day after GMH. The TUNEL-positive neurons of the ipsilateral cortex in GMH + Vehicle group increased significantly compared with Sham group on the first day after GMH, but the treatment with rh-relaxin-2 could reduce the number of TUNEL-positive neurons (Fig. 3a and b). This result was consistent with the apoptosis markers Bcl-2 and Bax by western blot analysis.

Rh-relaxin-2 reduced the oxidative stress injury of brains on the first day after GMH

Oxidative stress level of ipsilateral hemisphere after GMH was measured by DHE staining as well as the expression of Romo1 by western blot (Fig. 2a). The fluorescence intensity of DHE in GMH + vehicle group was much higher than that of in Sham group. Rh-relaxin-2 treatment could significantly reduce the intensity of DHE in vivo (Fig. 4a and b).

Rh-relxin-2 reduced neuronal degeneration at 28 d after GMH

Fluoro-Jade C was performed to explore whether rh-relaxin-2 can attenuate neuronal degeneration in brain cortex tissue. Robust Fluoro-Jade C staining was observed in GMH + Vehicle group at 28 d after GMH. However, rh-relaxin-2 treatment significantly reduced the number of Fluoro-Jade C-positive neurons compared to GMH + Vehicle group (Fig. 5a and b).

Downregulation of RXFP1 attenuated the neuroprotective, anti-oxidative and anti-apoptosis effects induced by rh-relaxin-2 on the first day post-GMH

Lenti-virus with sgCtrl and sgRXFP1 was administered i.c.v. at 2 d prior to GMH to inhibit RXFP1 in brain (Fig. 6). The results showed that the expression of relaxin-2 was reduced significantly after GMH compared to Sham pups, knockdown of RXFP1 by lenti-virus does not affected the expression of relaxin-2(Fig. 6a and b). Western blot results showed that the expression of

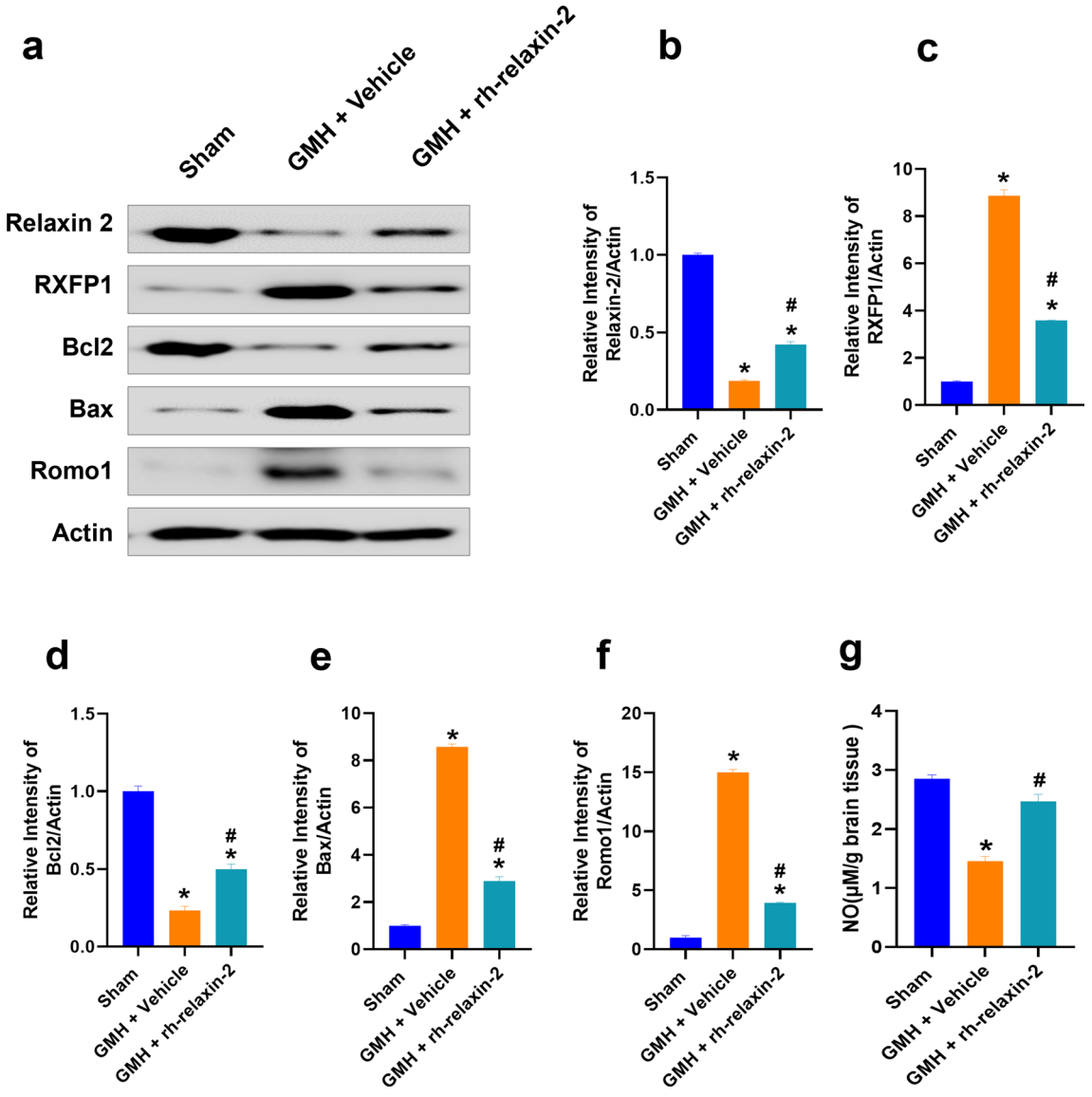

Fig. 2 Rh-relaxin-2 treatment inhibit neuronal apoptosis and oxidative stress on the first day after GMH. The rh-relaxin-2 treatment upregulated relaxin-2 (**a**, **b**) and downregulated RXFP1 (**a**, **c**). Moreover, The rh-relaxin-2 treatment recovered Bcl-2 (**a**, **d**) and downdregulated. the level of Bax (**a**, **e**) remarkably after GMH. The tendency of Rmo1 (**a**, **f**, $n=6$, the relative density of each protein has been normalized against the sham group) is same as that of Bax. Furthermore, rh-relaxin-2 treatment improved the concentration of NO (i, $n=3$, $^{*}p<0.05$ vs. Sham, $\#p<0.05$ vs. Vehicle group), even though it was declined after GMH

RXFP1 was increased significantly after GMH compared to Sham group (Fig. 6a and c). Knockdown of RXFP1 by lenti-virus dramatically reduced the expression of RXFP1, Phosphorylated ERK (Fig. 6a and d), phosphorylated nNOS (Fig. 6a and e), and Bcl2 (Fig. 6a and f), increased the expression of Bax (Fig. 6a and g) and Romo1 (Fig. 6a and h) in sgRXFP1 pups. Meanwhile, the concentration of NO in sgRXFP1 (Fig. 6i) pups brains was inhibited obviously after RXFP1 knockdown.

Inhibition of ERK reversed the neuroprotective effect of rh-relaxin-2 on the first day post-GMH

LY3214996 was used to abolish ERK (Fig. 7). The results showed that the expression of relaxin-2 was reduced significantly after GMH compared to Sham pups, After adding rh-relaxin-2, relaxin-2 was partially improved, but LY32149 did not affect the expression of relaxin-2 (Fig. 7a and b). Western blot results showed that the expression of RXFP1 was increased significantly after GMH compared to Sham pups, The expression of RXFP1 decreased after adding rh-relaxin-2, but LY321499 did not affect the expression of RXFP1 (Fig. 7a and c). The results demonstrated that phosphorylated ERK was

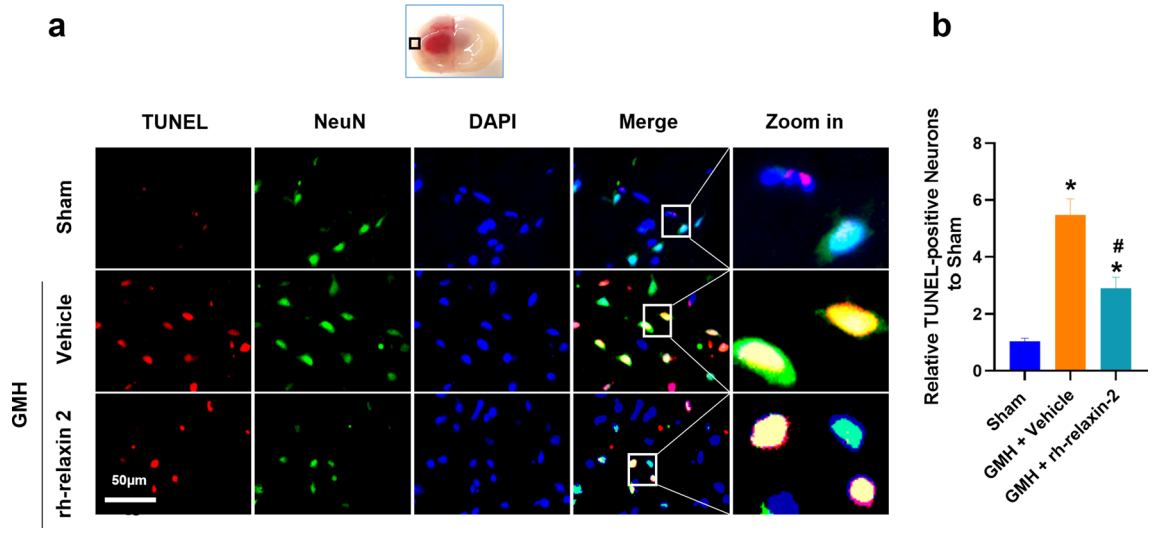

Fig. 3 Rh-relaxin-2 reduced neuronal apoptosis on the first day after GMH. Rh-relaxin-2 treatment reduced the TUNEL-positive neurons after GMH, while the number of TUNEL-positive neurons is significantly high in Sham and GMH + Vehicle group as well (**a**, **b**, $n = 3$, $^{*}p < 0.05$ vs. Sham, $\#p < 0.05$ vs. Vehicle group)

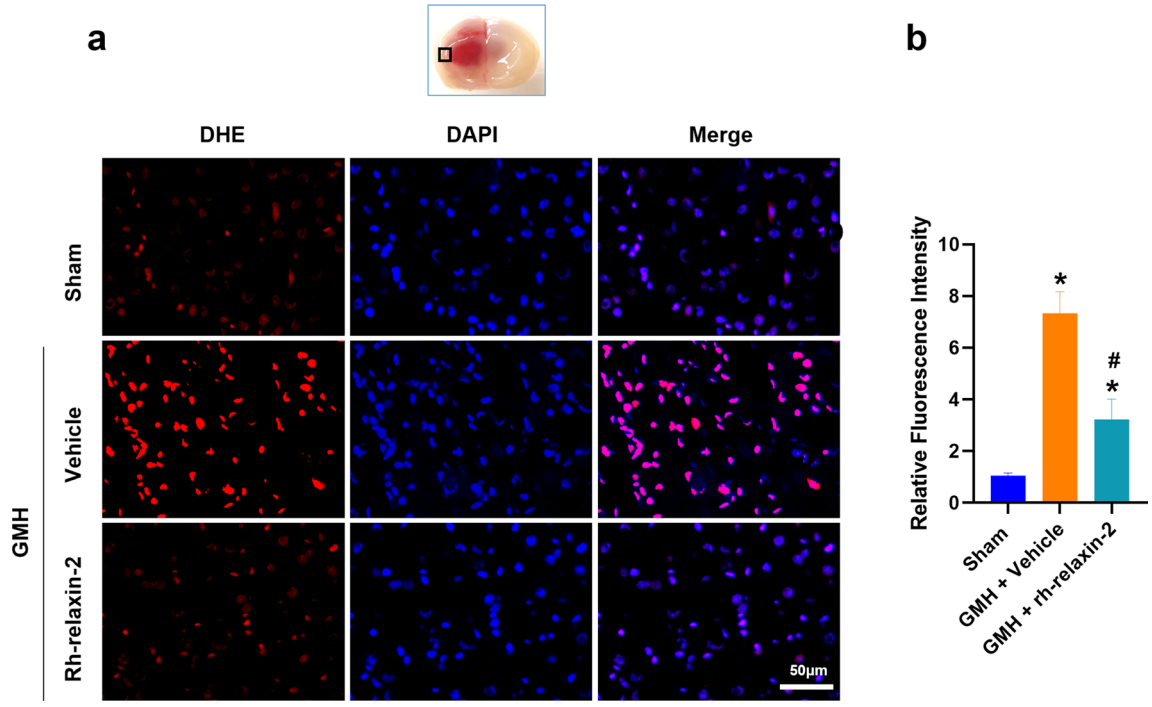

Fig. 4 Rh-relaxin-2 reduced the oxidative stress injury of brains on the first day after GMH. The intensity of DHE is significantly reduce in Rh-relaxin-2 treatment group as compare to the fluorescence intensity of DHE in GMH + vehicle group was much higher than that of Sham group (**a**, **b**, $n = 3$, $^{*}p < 0.05$ vs. Sham, $\#p < 0.05$ vs. Vehicle group

reduced significantly by LY3214996 on the first day after i.c.v. (Figure 7a and d). The expression of phosphorylated nNOS (Fig. 7a and e) and Bcl2 (Fig. 7a and f) also decreased subsequently. However, Bax (Fig. 7a and g) and Romo1 (Fig. 7a and h) expression elevated significantly on the first day after GMH. Additionally, the amount of NO measurement showed it was decreased evidently in the brains of LY3214996 treated pups (Fig. 7i).

Rh-relaxin-2 did not affect neurological behavior and the function of liver and kidney

The side effect of rh-relaxin-2 in treatment to pups with GMH was not unclear so far, therefore, we

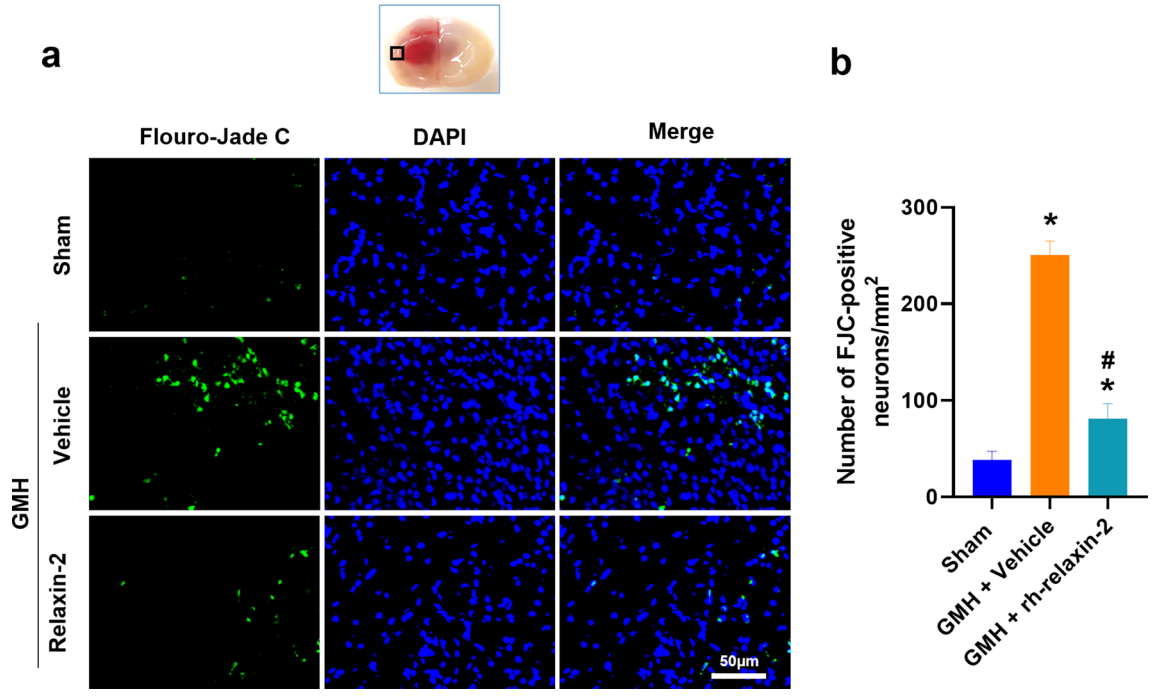

Fig. 5 Rh-relxin-2 reduced neuronal degeneration at 28 d after GMH. The Robust Fluoro-Jade C staining was observed in GMH + Vehicle group and rh-relaxin-2 treated group as well, on 28th day of GMH. The rh-relaxin-2 treatment significantly reduced the number of Fluoro-Jade C-positive neurons as compared to GMH + Vehicle group (**a**, **b**, $n = 3$, $^{*}p < 0.05$ vs. Sham, $\#p < 0.05$ vs. Vehicle group)

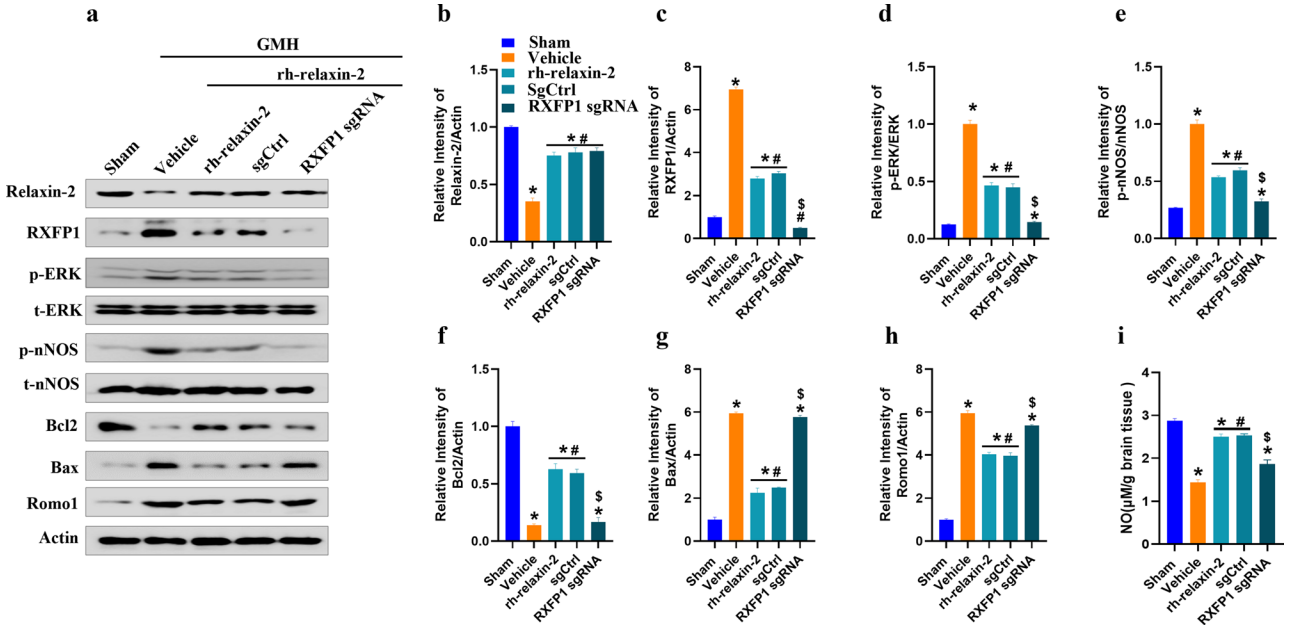

Fig. 6 Downregulation of RXFP1 attenuated the neuroprotective, anti-oxidative and anti-apoptosis effects induced by rh-relaxin-2 on the first day post-GMH. The expression of relaxin-2 was reduced significantly after GMH compared to Sham pups (**a**, **b**). Downregulation of RXFP1 (**a**, **c**) by lentivirus with sgRXFP1 significantly inhibited the expression of p-ERK (**a**, **d**), p-nNOS (**a**, **e**), Bcl2 (**a**, **f**), and the level of NO (i), however, increased the expression of Bax (**a**, **g**) and Romo1 (**a**, **h**) on the first day after GMH. $^{*}p < 0.05$ vs. Sham, $\#p < 0.05$ vs. Vehicle, $\$p < 0.05$ vs. sgCtrl. one-way ANOVA, Tukey's test, $n = 6$

performed mood-related neurobehavioral tests on the 28 d after GMH. There was no significance in total distance (Fig. 8a) and percentage of time in the center (Fig. 8b) between naïve and rh-relaxin-2 treatment in open field test. T-maze test revealed that the percentage of correct entries in rh-relaxin-2 treatment remained the same as naïve group (Fig. 8c). The body weight at 28 d was also no bias between rh-relaxin-2 treatment and naïve group

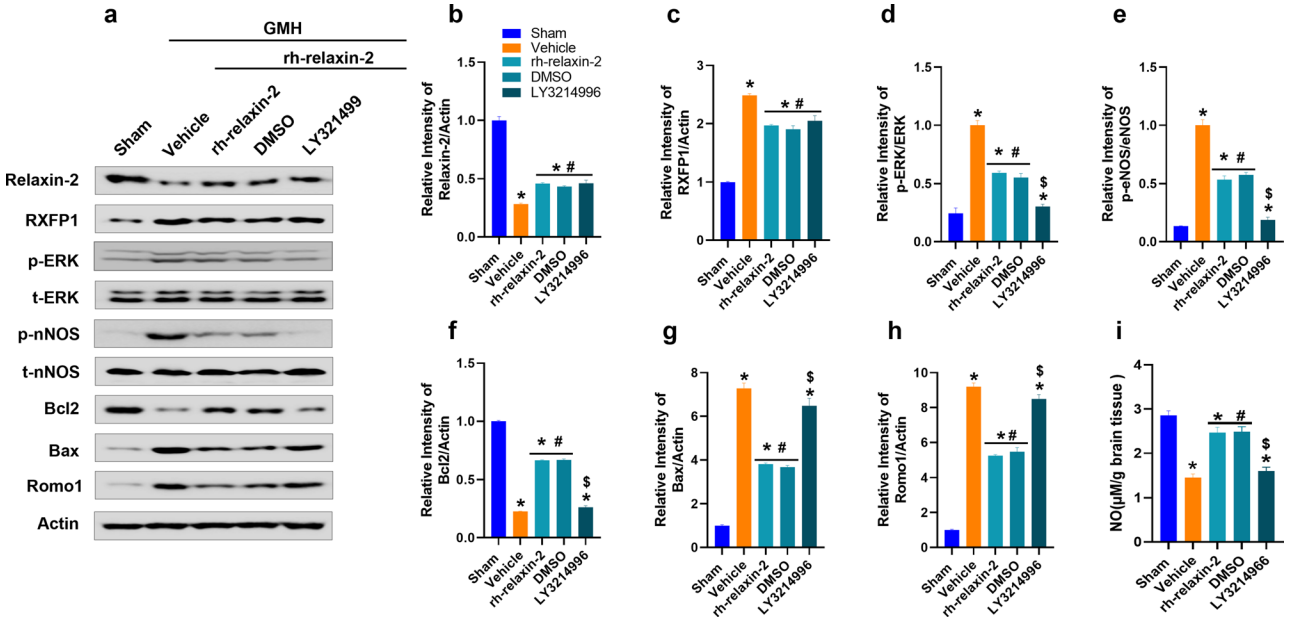

Fig. 7 Inhibition of ERK reversed the neuroprotective effect of rh-relaxin-2 on the first day post-GMH. The expression of relaxin-2 was reduced significantly after GMH compared to Sham pups, After adding rh-relaxin-2, relaxin-2 was partially improved, but LY32149 did not affect the expression of relaxin-2 (**a**, **b**). Western blot results showed that the expression of RXFP1 was increased significantly after GMH compared to Sham pups. The expression of RXFP1 decreased after adding rh-relaxin-2, but LY321499 did not affect the expression of RXFP1(**a**, **c**). LY3214996 significantly inhibited the expression of p-ERK (a, d), p-nNOS (a, e), Bcl2 (**a**, **f**), and the level of NO (i), however, increased the expression of Bax (**a**, **g**) and Romo1 (**a**, **g**) on the first day after GMH. *$p < 0.05$ vs. Sham, #$p < 0.05$ vs. Vehicle, \$$p < 0.05$ vs. corn oil group. One-way ANOVA, Tukey's test, $n = 6$

(Fig. 8d). Moreover, the level of AST (Fig. 8e), ALT (Fig. 8f), and Creatinine (Fig. 8g) in the serum was not differentiated in naïve and rh-relaxin-2 treatment.

Discussion

In this present study, we demonstrated the neuroprotective effects of rh-relaxin-2 and explored the potential mechanism of rh-relaxin-2 in oxidative stress and neuronal apoptosis which was at least in part mediated via RXFP1-ERK-nNOS-NO pathway signaling after GMH. Summary of our findings included that: Firstly, the level of endogenous relaxin 2 decreased, and its receptor RXFP1 increased at 12 h, peaked on the first day, and declined significantly from the third day in a time-dependent manner after GMH, and both of them expressed and bind in neurons. Secondly, administration of 60 μg/kg rh-relaxin-2 remarkably reduced oxidative stress and neuronal apoptosis after GMH, which was accompanied by upregulation of relaxin 2, RXFP1, Bcl-2 and NO, while downregulation of Bax and Romo1, in the brain on the first day after GMH in rats. Thirdly, inhibition of RXFP1 partially abolished the anti-oxidative and anti-apoptotic effects of rh-relaxin-2, and reversed the upregulation of relaxin 2, RXFP1, p-ERK, p-nNOS, and NO. The blockage of ERK also offset the rh-relaxin-2 beneficial effects and reversed the p-ERK, p-nNOS, and NO. Finally, the side effect experiment showed that there was not found the significance in neurological behavior, body weight, liver and kidney function between naïve and rh-relaxin-2 treatment after GMH on the 28th day.

GMH causes rapid and sustained inflammation throughout the brain suggests that anti-neuroinflammation may be a therapeutic target for GMH. However, increasingly evidence showed oxidative stress and neuronal apoptosis follow the acute neuroinflammatory response, which mainly concentrated in white matter [6]. Therefore, targeting oxidative stress and neuronal apoptotic injury is another very important therapeutic method for GMH except anti-neuroinflammation. Relaxin 2 is a natural peptide hormone that was first discovered in 1926 by Frederick Hisaw after his observation in pregnant pigs [10]. Currently, it is now widely known that relaxin 2 is synthesized in different tissues of non-pregnant women and in men. Furthermore, serelaxin (rh-relaxin-2) has permitted the study of its effects in cardiovascular, renal, hepatic, and brain tissue, as well as its evaluation in several randomized placebo controlled clinical trials, such as acute and chronic heart failure, systemic sclerosis, renal and hepatic impairment, and cirrhosis [10–14, 19]. RXFP1, the receptor of relaxin 2, is widely expressed in heart, kidney, lung, liver, blood vessels, and various areas of the brain. In the present study, we for the first time, demonstrated that intraperitoneal administration of rh-relaxin-2 can reduce oxidative stress injury and neuronal apoptosis in relaxin 2 and RXFP1 dependent manner in GMH. In detail, we found that there were significant

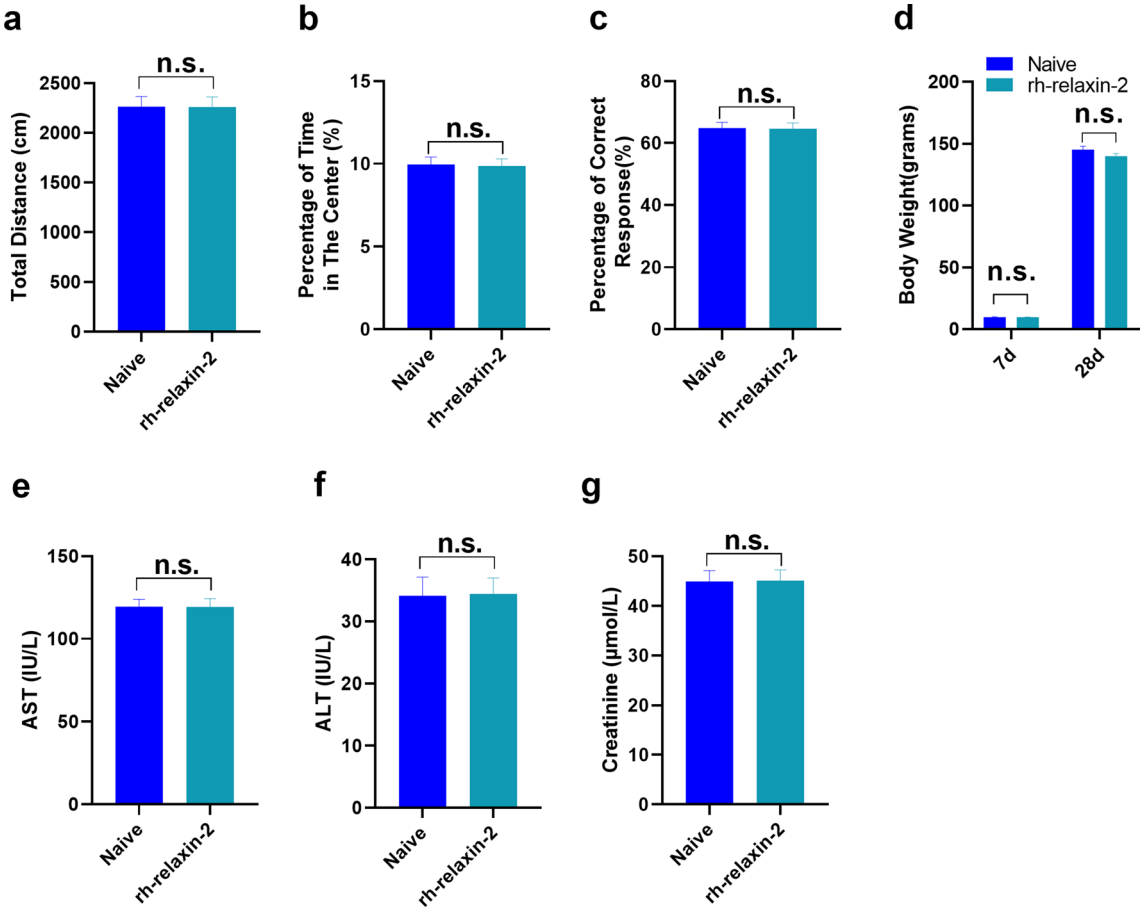

Fig. 8 Rh-relaxin-2 did not affect neurological behavior and the function of liver and kidney. The total distance (**a**) and percentage of time in the center (**b**) in the open field test, and the percentage of correct entries (**c**) in T-maze test show no significance between rh-relaxin-2 treatment and naïve group. The body weight (**d**), the level of AST (**e**), ALT (**f**), and CRE (**g**) were no bias at 28 d between rh-relaxin-2 treatment and naïve group. t-test, $n = 6$. n.s.=no significant

increases in the intensity of TUNEL-positive and DHE staining neurons in brain tissue at 24 h after GMH. Our data showed rh-relaxin-2 treatment can remarkably reduce oxidative stress and neuronal apoptosis as measured by DHE and TUNEL staining. In the western blot results, oxidative stress marker Romo1 and pro-apoptotic marker Bax were significantly reduced while anti-apoptotic marker Bcl-2 was increased in rh-relaxin-2 treated group. After lenti-virus with sgRXFP1 was administered, it reversed the protective effect of rh-relaxin-2 against oxidative stress and neuronal apoptosis, confirming the protective of relaxin 2 and RXFP1 after GMH. The same effect of rh-relaxin-2 happened in ischemia-reperfusion injury (IRI) after kidney transplantation, the team of Philipp Stiegler confirmed that relaxin 2 was beneficial to preserve the kidney graft, as an antioxidant, anti-apoptotic genes were upregulated, pro-apoptotic/necroptotic genes were downregulated [20].

The white matter injury in GMH model caused long-term cognitive impairments. It's interesting that our study showed that neurodegeneration were apparent even at 28 day after GMH as measured in Fluoro-Jade C staining. We found rh-relaxin-2 treatment with GMH can significantly reduce the neuronal degeneration as tested by Fluoro-Jade C staining, which was the first reported in GMH. Therefore, rh-relaxin-2 treatment significantly reduced the neuronal degeneration.

Growing studies have demonstrated that human relaxin-2 signals through RXFP1 to activate p-ERK and neuronal nitric oxide synthase (nNOS) pathway in human and rat renal myofibroblasts to inhibit TGF-β1 activity [15]. Consistent with these findings, our study showed that p-ERK was activated by the increased RXFP1 on the first day after GMH, and administration of rh-relaxin-2 inhibited the activation of p-ERK. Our added finding that RXFP1 or ERK inhibition attenuated the p-nNOS and NO confirms that RXFP1's ability to activate p-ERK is linked to signal through nNOS. A rapidly expanding body of data have indicated that the importance of nitric oxide in the physiology of the central nervous system and nNOS constitutes the predominant source of NO in neurons. Our experimental data showed

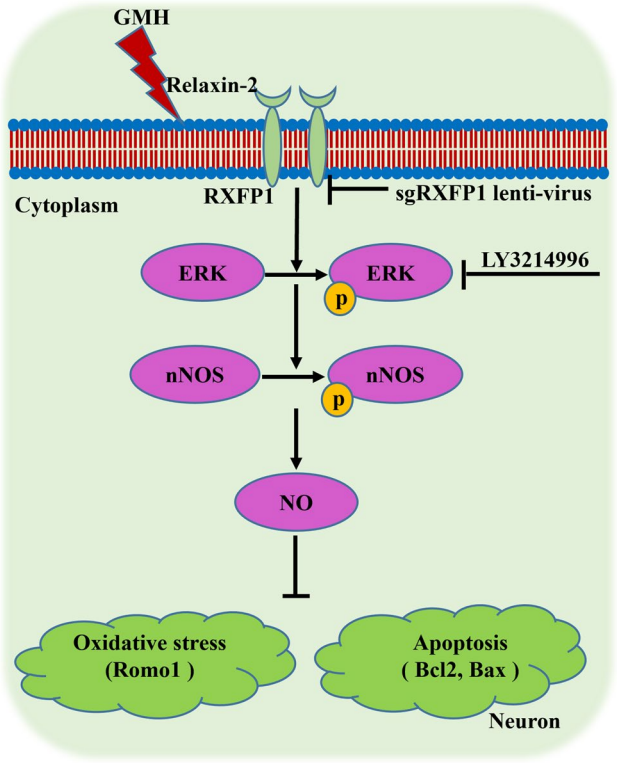

Fig. 9 Proposed mechanism

p-nNOS was markedly increased after GMH, which was in agreement with other central nervous disease, such as ischemic stroke and epilepsy. M.A. Moskowitz reported that mice lacking nNOS gene has a reduced infarct size after they are subjected to models of focal ischemia [21]. Also, blocking nNOS protected N2a cells against PILO-induced apoptosis and pyroptosis, indicating that nNOS may be involved in epileptogenesis by regulating cell death [22]. It's very interesting that administration of rh-relaxin-2 inhibited the expression of p-nNOS and relieve the oxidative stress and neuronal apoptotic injury in GMH. The underlying mechanism is likely that phosphorylation at the sites of Ser847/1412 increased nNOS activity, which catalyzes the oxidation of L-arginine to generate NO as product in neurons after GMH [23]. Additionally, in our current study, the long-term behavior test, namely open field test and T-maze, did not demonstrate a difference between naive and rh-relaxin-2 treated animals on the 28th day. Furthermore, there was not found the significance in neurological behavior, body weight, liver and kindey function between naïve and rh-relaxin-2 treatment after GMH on the 28th day. This data suggested that short-phase treatment with rh-relaxin-2 did not elicit significant mood related behavioral changes in animals nor did it alter the function of liver and kidney. Therefore, according to what has been discussed above, our findings, coupled with the previously research, suggested that re-relaxin-2 attenuates oxidative stress and neuronal apoptosis via ERK-nNOS-NO pathway after germinal matrix hemorrhage in rats.

There are several limitations in this current study. In vitro study would exclude the interplay between neurons and other cell types and further verify this signaling pathway that holds true in neurons reaction after GMH. And in the test for side effect, we need do more tests to reflect the effect of rh-relaxin-2 on other organs in the rats.

Conclusion

We demonstrated that intraperitoneal administration of rh-relaxin-2 attenuated oxidative stress and neuronal apoptosis through RXFP1-ERK-nNOS-NO pathway signaling after GMH. Rh-relaxin-2 may be an useful new therapeutic strategy against oxidative stress and neuronal apoptosis in GMH patients.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12987-024-00616-7.

Supplementary Material 1

Acknowledgements

Not applicable.

Author contributions

G.Z., P.L. and Y.S. designed the study; P.L. and J.L. draft the manuscript. X.Z., G.L., H.K., S.Z., and M.L. performed the IHC staining and analysis; K.R. performed the animal model and behavior test; Q.Z. carried out the Western Blot analysis; G.W., W.F., and G.Z. performed the statistical analysis on experimental data; G.L. performed the molecular biology experiment. All authors read and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

This study was conducted in full accordance with ethical principles and approved by the Medical Ethics Committee of the second Hospital Affiliated with Nanchang University (the 2nd affiliated hospital, Jiangxi Medical College, Nanchang University) and Nanfang Hospital of Southern Medical University. Our study did not involve human participants.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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