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ORIGINAL PAPER

Effects of Restraint Water-Immersion Stress-Induced Gastric Mucosal Damage on Astrocytes and Neurons in the Nucleus Raphe Magnus of Rats via the ERK1/2 Signaling Pathway

Fangcheng Fan[1] · Mengzhu Yang[2] · Xiwen Geng[3] · Xiaoli Ma[4] · Haiji Sun[1]

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Abstract
Restraint water-immersion stress (RWIS) consists of psychological and physical stimulation, and it has been utilized in the research of gastric mucosal damage. It has been shown by previous studies that the nucleus raphe magnus (NRM) is closely involved in the gastrointestinal function, but its functions on the stress-induced gastric mucosal injury (SGMI) have not been thoroughly elucidated to date. Consequently, in this research, we aim to measure the expression of astrocytic glial fibrillary acidic protein (GFAP), neuronal c-Fos, and phosphorylation extracellular signal regulated kinase 1/2 (p-ERK1/2) in the process of RWIS with immunohistochemistry and western blot methods. What is more, we detect the relation between astrocytes and neurons throughout the stress procedure and explore the regulation of the ERK1/2 signaling pathway on the activity of astrocytes and neurons after RWIS. The results indicated that all three proteins expression multiplied following peaked 3 h substantially. The SMGI, astrocyte and neuron activity were affected after the astrocytotoxin L-A-aminohexanedioic acid (L-AA) and c-fos antisense oligonucleotide (ASO) injections. After the injection of PD98059, the gastric mucosal injury, astrocyte and neuron activity significantly fell off. These results suggested that RWIS-induced activity of astrocytes and neurons in the NRM may play a significant part in gastric mucosa damage via the ERK1/2 signaling pathway.

Keywords Nucleus Raphe Magnus · Restraint water-immersion stress · ERK1/2 signaling pathway · Astrocytes · Neurons

Introduction

The traditional definition of stress is as a stimulus, often referred to as a certain psychological and physiological stressor, such as gastric ulcers, inflammation, and diabetes. The brain activates a series of neuropeptide-secreting systems under stress, which gives rise to the release of hormones from varieties of spread synapses [1]. There are many factors behind the pathogenesis of gastric ulcers, such as inflammation, thermal injury, or the endocrine system [2]. A stress model designated Restraint water-immersion stress (RWIS), which is including psychological and physical stimulations. Several symptoms are caused by the model, such as hypothermia, gastrointestinal disorders, and rises in gastric acidity and gastric acid secretion. RWIS is a normally applied animal model in the research of stress-induced gastric mucosal damage injury (SGMI) [3].

The nucleus raphe magnus (NRM) is a crucial section of the central nervous system circuit complicated in the modulation of nociceptive responses. The NRM is clustered with serotonergic neurons and participates in descending pain and other wide-spectrum behaviors [4]. Serotonergic neurons in the NRM are mediated according to numerous neurotransmitters, like noradrenaline (NA), glutamate and γ-aminobutyric acid (GABA) [5]. In the NRM, the serotoninergic and GABAergic neurons could inhibit spinal nociceptive transmission, and glutamatergic projections from synapses in other regions onto GABAergic neurons

✉ Xiaoli Ma
mxl7125@126.com

✉ Haiji Sun
sunhj5018@126.com

[1] Key Laboratory of Animal Resistance Biology of Shandong Province, College of Life Science, Shandong Normal University, Jinan 250014, China

[2] Qingdao No. 31 Middle School, Qingdao 266041, China

[3] Shandong Traditional Chinese Medicine University, Jinan 250014, China

[4] Central Laboratory, Jinan Central Hospital Affiliated to Shandong University, Jinan 250013, China

can in turn inhibit serotonergic neurons [6]. Some experiments indicate the involvement of the NRM in stress and tension [7]. Former examinations have proven that the NRM is complicated in the regulation of tonic immobility stress via a relation between GABAergic and opioidergic systems, and the opioidergic action may be caused by the inhibition of GABAergic neurons [8]. A raised ventilatory reply to hypoxia was detected by virtue of ibotenic acid lesions in the NMR, which implied that this nucleus exerts an inhibitory regulator of respiratory throughout hypoxic stress [9].

At the spinal level, nociceptive transmission's descending restriction is regulated by the NRM serotoninergic pathways and the descending locus coeruleus (LC) noradrenergic pathways [10]. Recent studies that use electrical stimulation and lesion techniques to manipulate NRM activity have demonstrated that NRM stimulation reduced the amplitude of gastric constriction, while LC lesioning could abolish the effects of NRM stimulation [11]. Serotonergic neurotransmission is related to acute stressful life events, and it has been known that the dysfunction of the serotonergic system is complicated in the evolution of affective disorders [12]. Serotonin is believed to allow for the structural plasticity of the brain and be involved in regulating motivational subjects for rewarding brain stimulations [13]. It is linked to several diverse brain functions and is activated by a variety of signaling pathways in many neurons [14]. It has been confirmed that analgesia can be created through electrical stimulation of the NRM, which is rich in serotonergic neurons [15].

Besides, a large quantity of testimony points out that the NRM is in a similar way involved in the regulation of gastric functions. Electrical damage to the NRM increased gastric acid output and serum gastrin levels, which could be prevented by vagotomy [16]. Based on this evidence, it is sensible to assume that the brain nuclei play a function in gastric mucosal damage. This study aimed at investigating the participation of the NRM in RWIS. C-fos, as an inducible transcriptional factor, is involved in important cellular events, including cell proliferation, differentiation and survival [17, 18].The expression of c-fos has been used widely as a marker of neuronal activity. The expression of glial fibrillary acidic protein (GFAP), as a marker of astrocytic activation, significantly increased in respond to various physiological or noxious stimuli [19]. Our previous research had shown that Fos upregulation correlates with RWIS-induced gastric ulcer. However, it is not clear that whether astrocytes participate in RWIS-induced gastric mucosal damage. Much of the previous research implies p-ERK activation is involved in Fos expression [20].Therefore, We examined the expression of neuronal c-Fos, the glial fibrillary acidic protein (GFAP) expression in astrocytes and the phosphorylation of extracellular signal-regulated kinase 1/2 (p-ERK1/2) through immunohistochemistry and western blotting. We microinjected antisense oligonucleotide (ASO) to inhibit c-Fos [13] and astrocytotoxin L-A-aminohexanedioic acid (L-AA) [21] and tested the neuron-astrocyte network. The ERK1/2 signaling pathway inhibitor PD98059 [22] was utilized during RWIS to identify its role in the regulation of neurons and astrocytes.

Materials and Methods

Animals

Wistar male rats, 220–250 g (from Shandong University) were used as experimental animals. All rats were housed in an environmentally controlled (22 ± 2 °C) room, free food and water, a normal 12:12 h light/dark cycle (light on at 6:00 and off at 18:00) instead of natural circadian rhythm of light. The rats need to fast for 24 h and drink freely ahead of the stress protocols.

Stress Protocols

The rats were separated into 6 groups randomly. Except for the control group, the test groups were treated with RWIS (0.5 h, 1 h, 3 h, and 5 h respectively). After inhalation of ether anesthesia, the test rats were secured onto a board with their limbs. The animals were then vertically submerged into cool water as they were awake with the water temperature 21 ± 1 °C. To exclude the effect of circadian rhythm, the experiments were carried out between 8:00 and 13:00. In this research, all experiments were conducted according to the guidelines of National Institutions of Health for the Use and Care of Lab Animals and took following to the Shandong Normal University's Ethics Review Board.

Drugs and Injections

In the lateral ventricle of the rats, a stainless steel guideline cannula was implanted unilaterally according to the coordinate: lateral: 1.8 mm, antero-posterior: 0.8 mm and dorsoventral: 3.8 mm. After surgical treatment, the rats were fed in clean environment, free food and water. A week later, the surgically operated animals were separated into three groups randomly: the ASO group, the L-AA group and the control group. Phosphorothioate-modified ASO (50 μg dissolved in 10 μL physiological saline, Sangon Biotechnology Co.); astroglial toxin L-AA (dissolved 100 nmol in 10 μL physiological saline, Sigma St. Louis, MO, USA); or the physiological saline (control, 10 μL physiological saline) were then injected into the lateral ventricle. Each microinjection lasted ten minutes, and then the rats underwent RWIS for 1 h. Immunohistochemistry was conducted to observe the GFAP and c-Fos expression.

PD98059 is an inhibitor of ERK1/2 signaling pathway. To further study the signaling pathway participation in the damage of gastric mucosal induced by RWIS, PD98059 was injected into the lateral ventricle. One week after surgical operation, the rats were separated into 2 groups at random: the control group (10 μL physiological saline); and the PD98059 group (0.2 mg dissolved in 10 μL DMSO). The microinjection was processed in same method, and then the rats were treated with RWIS for 3 h. The expression of GFAP, c-Fos and p-ERK1/2 were gauged subsequently with immunohistochemistry.

Gastric Mucosal Injury Evaluation

The animals were euthanized according to narcotic overdose (100 mg/kg dead body weight) after the RWIS. Their stomachs were taken out and secured within half an hour in 4% paraformaldehyde which was dissolved in 0.1 mol/L PB. The stomachs were incised along the greater curvature and adequately rinsed with saline solution. The scale of gastric ulcer was determined by dissection microscope. The gastric erosion index (EI) was ascertained in accord with the Guth method [23]. Scores were calculated by the length of the damage. Lengths ≤ 1 mm were given a score of 1; lengths between 1 mm and 2 mm were given a score of 2; and the others were derived in turn. When the length was larger than 1 mm, the score was doubled. In a rat, the accumulative scores of all lesions were defined as the EI of the rat.

Immunohistochemistry

The brains of the rats were cut off after they were sacrificed, and fixed in 4% paraformaldehyde. The whole brains were fixed for 12 h and then infiltrated with 20% sucrose solution (dissolved in 0.1 mol/L PB) at 4 °C for 24 h. Brain tissues were sliced up at 30 micrometers using frozen section method. The sections were incubated overnight in three primary antibody separately: rabbit anti-c-Fos (1:500; Santa Cruz Biotechnology, USA), mouse anti-GFAP (1:500; Chemicon, USA), and rabbit anti-p-ERK1/2 (1:1000; Cell Signaling Technology, USA). Then the sections were incubated in the secondary antibody incubation solution for 2 h (room temperature). The labeled tissue sections were revealed using 10 min incubation with diaminobenzidine.

Evaluation of Immunohistochemistry

Microscopic analyze of the brain sections was used under identical conditions. Paxinos and Watson defined the nuclear boundaries and nomenclature of the rat brain stereotaxic atlas [24]. All types of labeled cells were quantified with the software of Image-Pro Plus 7.0 (Media Cybernetics Inc, USA). Three inconsecutive sections of both astrocytes and neurons were calculated in each animal, the mean numbers of each section per 0.01 mm^2 were shown as immunohistochemical measure.

Western Blot

After RWIS, we collected the NRM and extracted the proteins. After the SDS-PAGE, the gel was cut according to the location of the corresponding protein band. Proteins were then transferred to PVDF blotting membranes and incubated with four kinds of primary antibodies separately overnight at 4 °C: rabbit anti-GAPDH (1:500; Goodhere Biotechnology, China), anti-c-Fos (1:500; Santa Cruz Biotechnology, USA) and anti-GFAP (1:500; Chemicon, USA), and rabbit anti-p-ERK1/2 (1:1000; Cell Signaling Technology). Subsequently, the membranes were incubated 1 h for the secondary antibodies at room temperature. Chemiluminescence detection methods were used to examine the blot, the optical density (OD) of the bands was calculated with Quantity One.

Statistical Analysis

As mean values ± SEM, all of the results were reported. The SPSS 16.0 software (SPSS, Chicago, IL, USA) was utilized for analyzing the values. Statistical analysis was executed using applying a *t* test or a repeated measure one-way analysis of variance (ANOVA) acted in accordance with a Dunnett's post hoc analysis. $*P < 0.05$ was considered to be statistically significant.

Results

Model of Gastric Mucosal Injury During RWIS

As illustrated in Fig. 1a–e, the extent of ulcerations gradually increased with the extension of stress time, and the comparison between the test group and the normal control was significant different. Through the photograph, the gastric wall and its adjacent structures could be seen clearly in the control group (Fig. 1f). The lesions that formed visibly increased macroscopically on the surface as deep red areas or linear streaks, with time increasing the presence of lesions for the rats under RWIS. It may be concluded that the experimental rat model induced by RWIS was successful.

Increased c-Fos Expression of the Nucleus Raphe Magnus

To detect neuronal activity in the NRM after RWIS, a detection marker c-Fos was assessed. In control animals, the cells were slenderly immunoreactive and were few in figure. Numerous c-Fos-immunoreactive cells were noticed

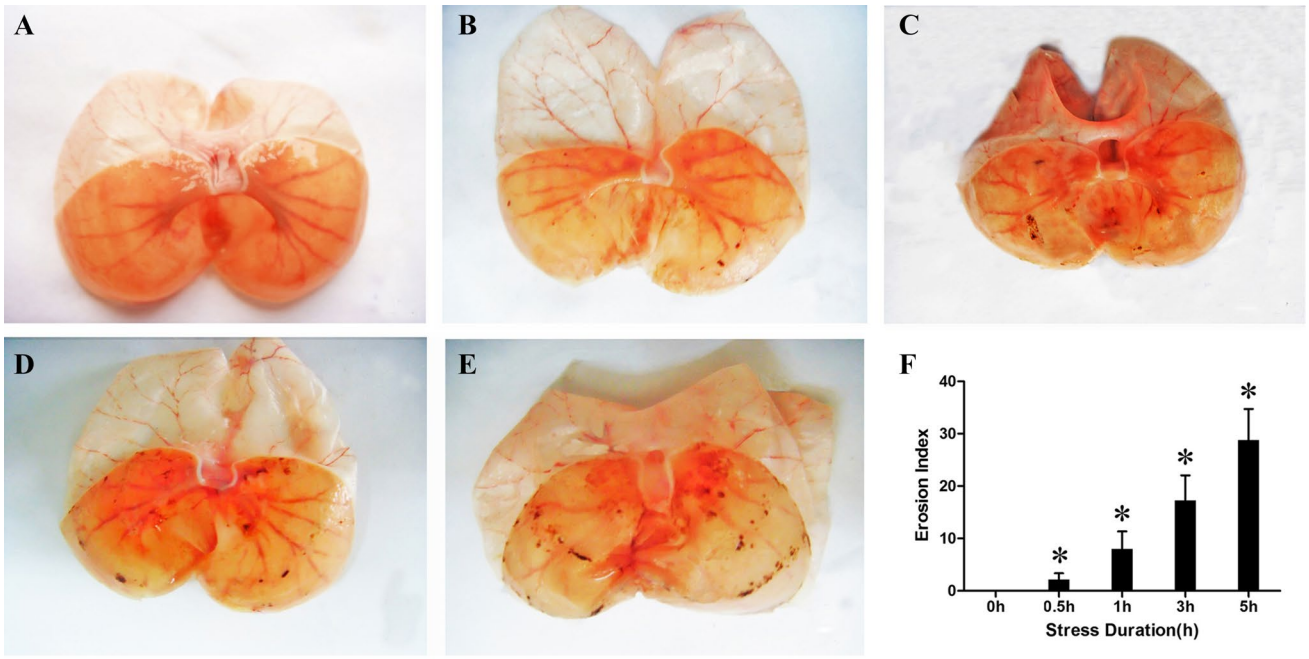

Fig. 1 Effects of RWIS on gastric mucosal damage: control (**a**), RWIS 0.5 h (**b**) 1 h (**c**), 3 h (**d**), and 5 h (**e**), **f** Quantification of erosion index (EI). All data are expressed as the mean ± SEM (n = 6). *$P < 0.05$ vs. control group

gradually increasing after RWIS, peaking at 3 h (Fig. 2a–f). As illustrated in Fig. 2g, h, those of immunoreactivity were matched by the western blot's results. The experimental results show that the NRM neurons are involved in RWIS.

Increased GFAP Expression of the Nucleus Raphe Magnus

The NRM of rats was studied to observe whether the expression of the astrocyte marker, GFAP, reacts to RWIS. In sections immunostained with antibodies against GFAP in different time periods of stress, low levels of GFAP were detected in the normal control. The positive cells were obviously enhanced after RWIS, the highest GFAP-positive cells were detected at 3 h (Fig. 3a–e). There was a significant rise in the numbers of GFAP in the test groups compared to the normal control group ($P < 0.05$) (Fig. 3f). The experimental data of the western blot analysis matched those of immunoreactivity (Fig. 3g, h). These experimental data indeed suggest that astrocytes in the NRM participate in RWIS.

Increased p-ERK1/2 Expression of the Nucleus Raphe Magnus

To examine the actions of the ERK1/2 signaling pathway after RWIS, p-ERK1/2 were subjected to western blot and immunoreactivity. The radical level of p-ERK1/2 was relatively low in control animals as illustrated in Fig. 4. When rats were assessed in the different experimental conditions, there was a gradual increase after each treatment, which peaked at 3 h (Fig. 4a–e). Significant difference was manifested by the test group compared to the normal control (Fig. 4f). Those of immunoreactivity were matched by the experimental data of western blot, as illustrated in Fig. 4g, h. This finding indicated that in the NRM, the ERK1/2 signaling pathway contributes to stress reaction.

GFAP and c-Fos Expression and Gastric Mucosal Damage Were Lessened by L-AA or ASO Treatment

The actions of neuron-astrocyte network during RWIS were determined by lateral ventricle injection of L-AA or ASO. Ulcers in experimental rats were prevented by microinjection of L-AA or ASO through RWIS, when compared to the control (Fig. 5a–d). The experimental data showed that the active neurons and astrocytes in the NRM were affected by the L-AA or ASO injection (Fig. 5e–g, i–k). Significant difference was observed by the injection groups compared to the control ($P < 0.05$) (Fig. 5h, l). The present examination proved that the interaction of astrocytes and neurons in the NRM are convoluted in SGMI.

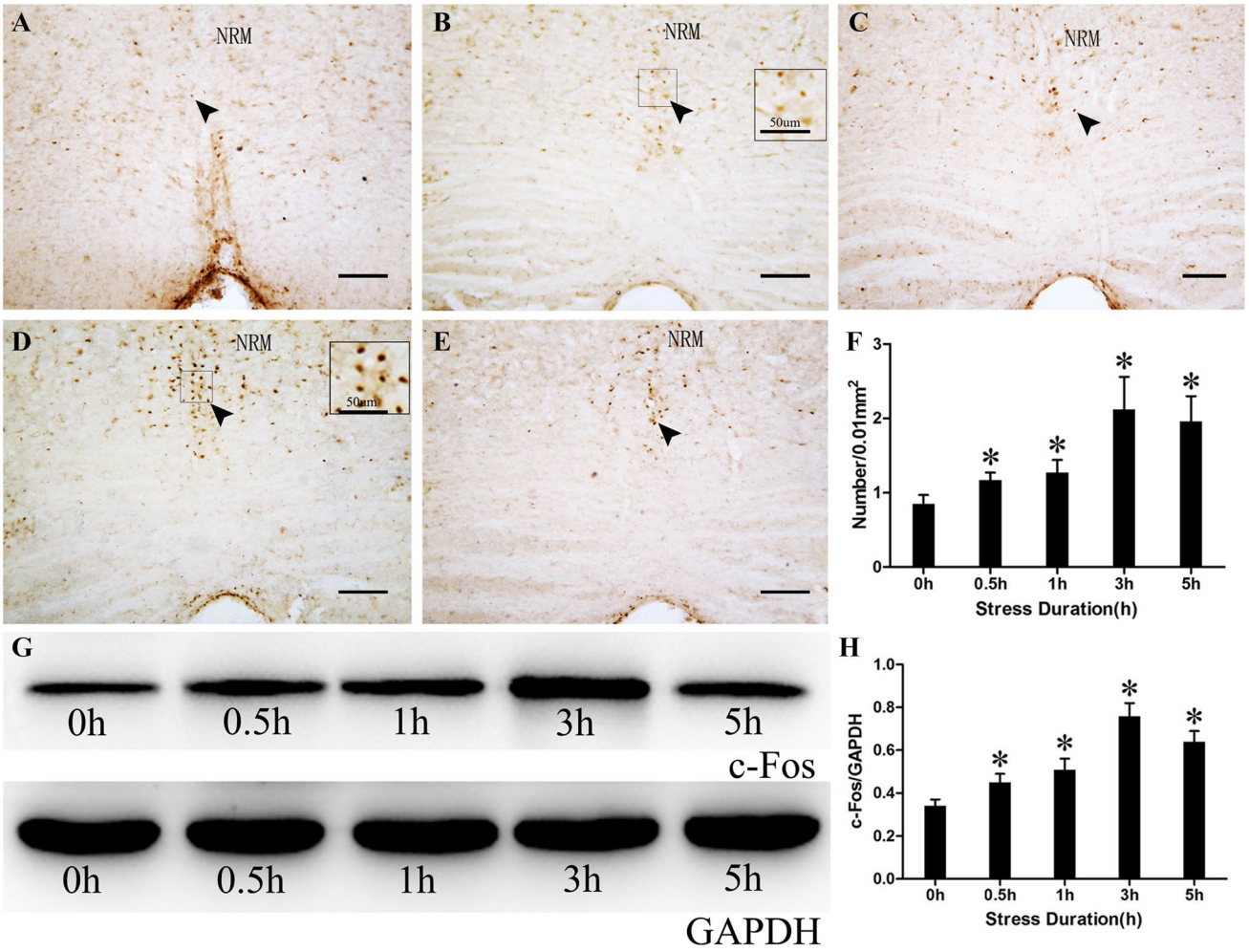

Fig. 2 Micrographs of c-Fos immunoreactivity and western blots in the NRM: control (**a**), RWIS 0.5 h (**b**) 1 h (**c**), 3 h (**d**), and 5 h (**e**) (100×); Scale bars, 100 μm. **f** Quantification of c-Fos. The square in the panel is 400×. **g** Western blot of c-Fos protein. **h** Quantification of c-Fos/GAPDH. All data are expressed as the mean ± SEM (n = 6). *$P < 0.05$ vs. control group

GFAP and c-Fos Expression and Gastric Mucosal Damage Were Lessened by PD98059 Treatment

PD98059 was microinjected into the lateral ventricle to explore the function of the ERK1/2 signaling pathway in the process of RWIS, and the comparison between the drug group and the normal control was significant different (Fig. 6a, e, and i). A clear inhibitory result was been shown on the expression of p-ERK cells (Fig. 6b, f, and j). Moreover, the microinjection of PD98059 had an inhibitory result on both the expression of neurons and astrocytes (Fig. 6c, d, g, h, and j). These data suggested that the ERK1/2 signaling pathway may regulate the activation of neuron-astrocyte network and participated in SMGI.

Discussion

The NRM is the prime source in the brain stem of the axons projecting to the spinal cord and consists of major 5-HT fibers [25]. Serotonergic neurons in the NRM connect to other synaptic nuclei in the CNS, with the position and site of 5-HT influencing gastric motility as excitation or inhibition [26]. The present study demonstrated whether neurons, astrocytes, and the ERK1/2 signaling pathway participate in RWIS, assessed the neuron-astrocyte network, and assessed the effects of injecting inhibitor PD98059 on gastric mucosal damage. Experimental results illustrated that: in the NRM, GFAP, c-Fos, and p-ERK1/2 expression gradually raised after RWIS; the EI and the expression of neurons

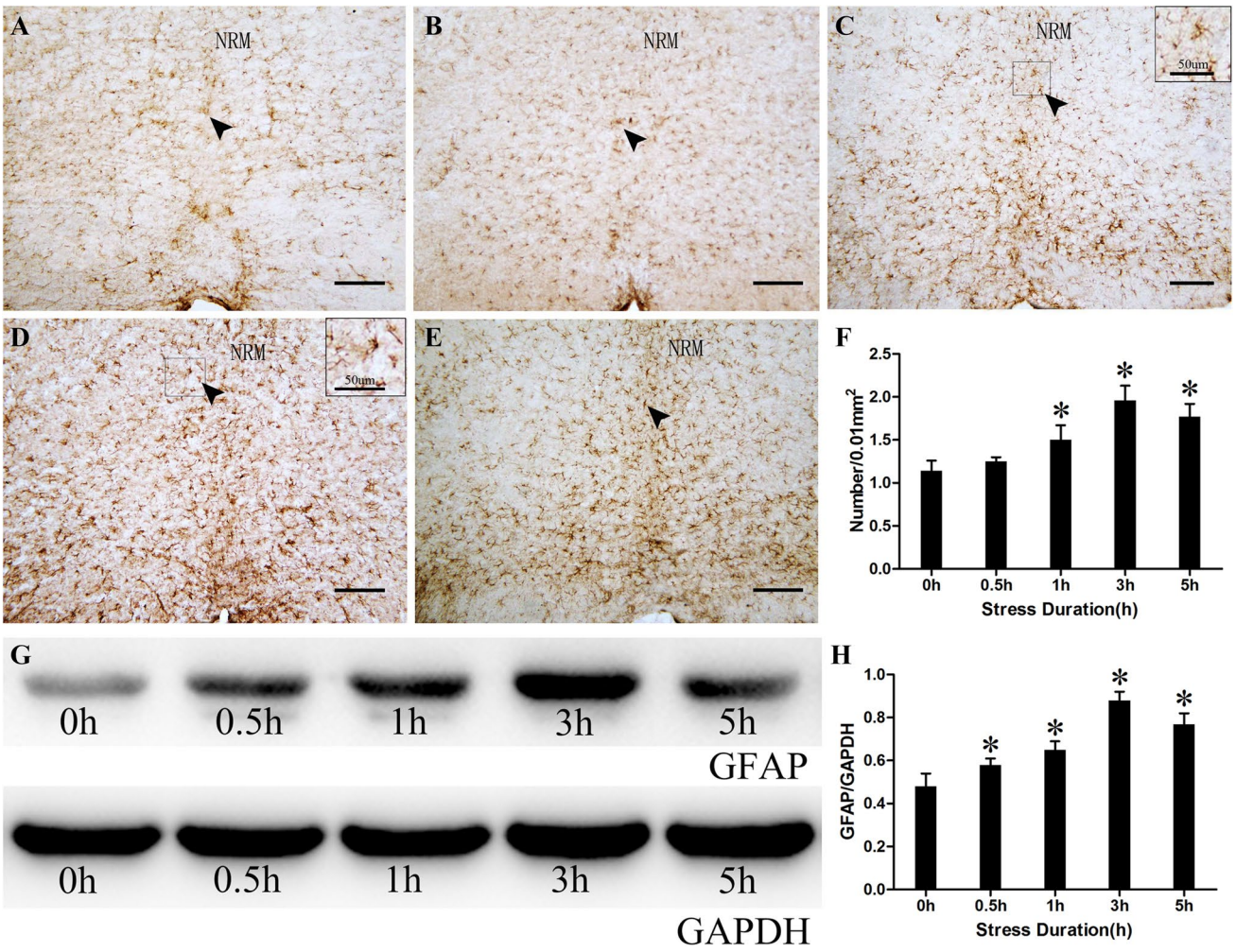

Fig. 3 Micrographs of GFAP immunoreactivity and western blots in the NRM: control (**a**), RWIS 0.5 h (**b**) 1 h (**c**), 3 h (**d**), and 5 h (**e**) (100×); Scale bars, 100 μm. **f** Quantification of GFAP. The square in the panel is 400×. (**g**) Western blot analysis of GFAP protein. **h** Quantification of GFAP/GAPDH. All data are expressed as the mean ± SEM (n = 6). *$P < 0.05$ vs. control group

and astrocytes fell off after the misconnection of L-AA or ASO; and the activity of neurons and astrocytes and gastric mucosal injury were restrained after lateral ventricle injection of PD98059.

Neuronal and Astrocytic Activations Were Enhanced by RWIS

It is confirmed that NRM plays an extremely significant part in going down pain and other wide-spectrum behaviors [27]. It has been shown that after stimulating the NRM can suppress the sensation of nuclear energy and activate the serotonergic mechanism [28]. In our study, we examined neuronal c-Fos expression, and the increase of the protein expression after stress demonstrated that neurons in the NRM take part in RWIS. RWIS includes anxiety, hypothermia, and many other stimuli that can cause stress gastric mucosal damage. It has been confirmed that the neurons of the locus coeruleus (LC), the ventromedial hypothalamic nucleus (VMH) and other brain nuclei are involved in RWIS [29, 30]. Serotonergic neurons and noradrenergic neurons respond to pain stimulation together, and 5-HT is mediated by NA [5, 31]. This indicates that the LC influences the NRM, and commonly participates in some stress reaction processes. These discoveries consequently suggest that the RWIS-induced malfunction of neurons in the NRM joins in the RWIS, and contributes to the SGMI.

Astrocytes are a component of supportive glial cells in the CNS and reactive astrocytes are used as a reliable and sensitive marker for detecting CNS injuries [32]. In previous experiments, astrocyte [Ca^{2+}] release is mediated by many neurotransmitters, including glutamate, GABA, 5-HT and several peptides. The release of [Ca^{2+}] leads to a release of glutamate from astrocytes, which is a very important

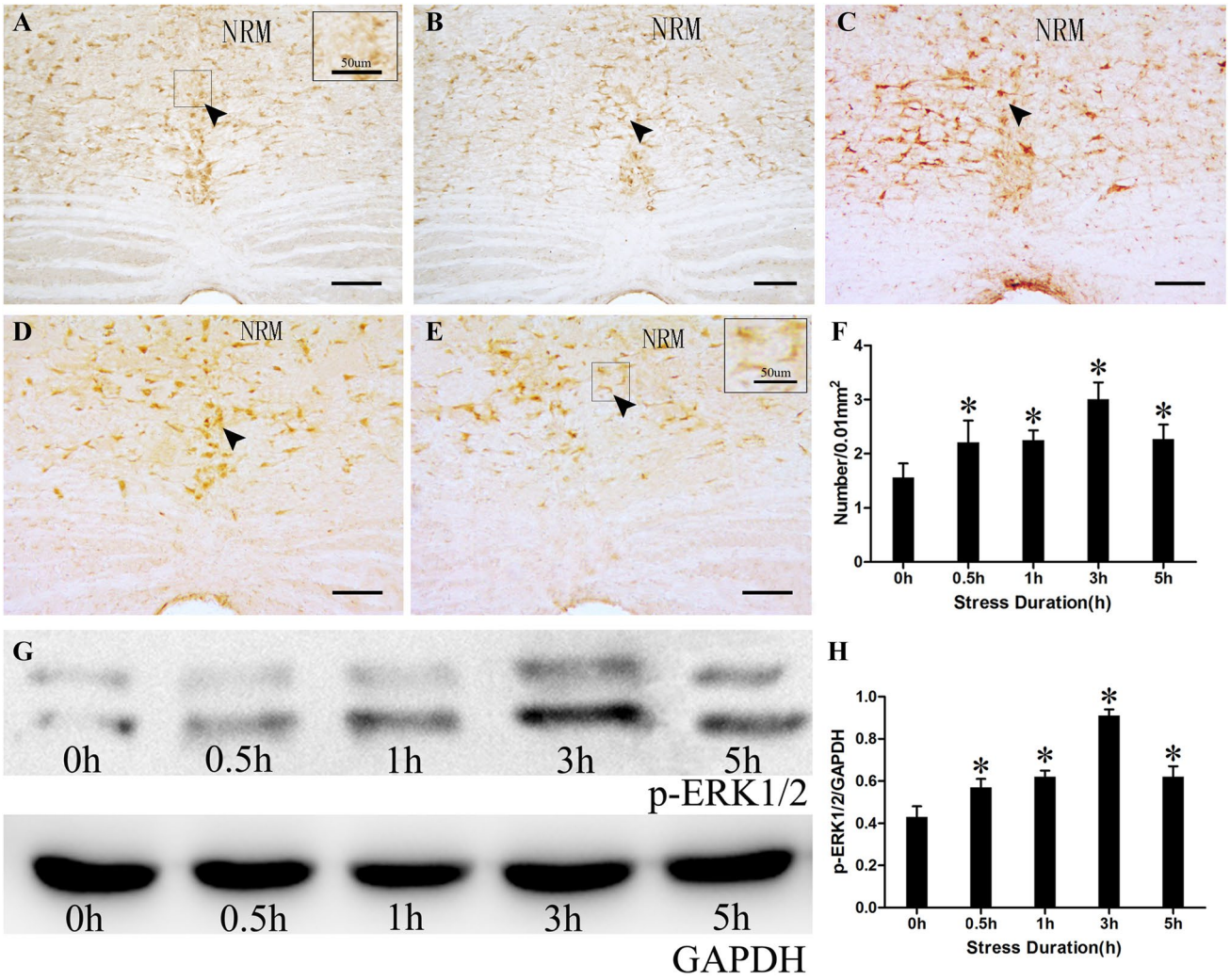

Fig. 4 Micrographs of p-ERK1/2 immunoreactivity and western blots in the NRM: control (**a**), RWIS 0.5 h (**b**) 1 h (**c**), 3 h (**d**), and 5 h (**e**) (100×); Scale bars, 100 μm. **f** Quantification of p-ERK1/2. The square in the panel is 400×. **g** Western blot analysis of p-ERK1/2 protein. **h** Quantification of p-ERK1/2/GAPDH. All data are expressed as the mean ± SEM (n = 6). $^{*}P < 0.05$ vs. control group

regulator of neurotransmission [33]. It has been reported that GFAP expression in the astrocytes is regulated through several inter and intracellular signaling molecules exhibiting both regional and local variability [34]. In the present research, over RWIS, GFAP expression considerably raised compared to the normal contral. Astrocytes in the NRM may be consequently activated and involved in gastric malfunction, which participates in SGMI.

Neuron-Astrocyte Network in the NRM Contributes to Gastric Mucosal Damage

Neurons are a signaling cell type in the CNS, and glial cells are the most important component of cerebral volume [35]. The basis for information processing in the CNS is considered to be neurons; one type of glial cell known as an astrocyte is now regarded as another cell type that is important for information processing. It has been shown that the neurotransmitters transmitted by neurons are also detected in astrocytes [36]. Astrocyte [Ca^{2+}] signals arouse the release of neurotransmitters, which join in the excitability and plasticity of neurons. Based on these calcium signals, the neurotransmitters can also return to neurons [37]. The astrocyte-neuron network has been previously described in the CNS [38]. Our hypothesis of the astrocyte-neuron network exists in the NRM, and we used the lateral ventricle to inject L-AA or ASO to inhibit astrocytes or activated neurons in the NRM that was induced by RWIS. After the microinjection of L-AA or ASO, the SGMI was substantially lessened and the expression of neurons and astrocytes was substantially reduced. While ASO was injected, the expression points of astrocytes and neurons were all decreased,

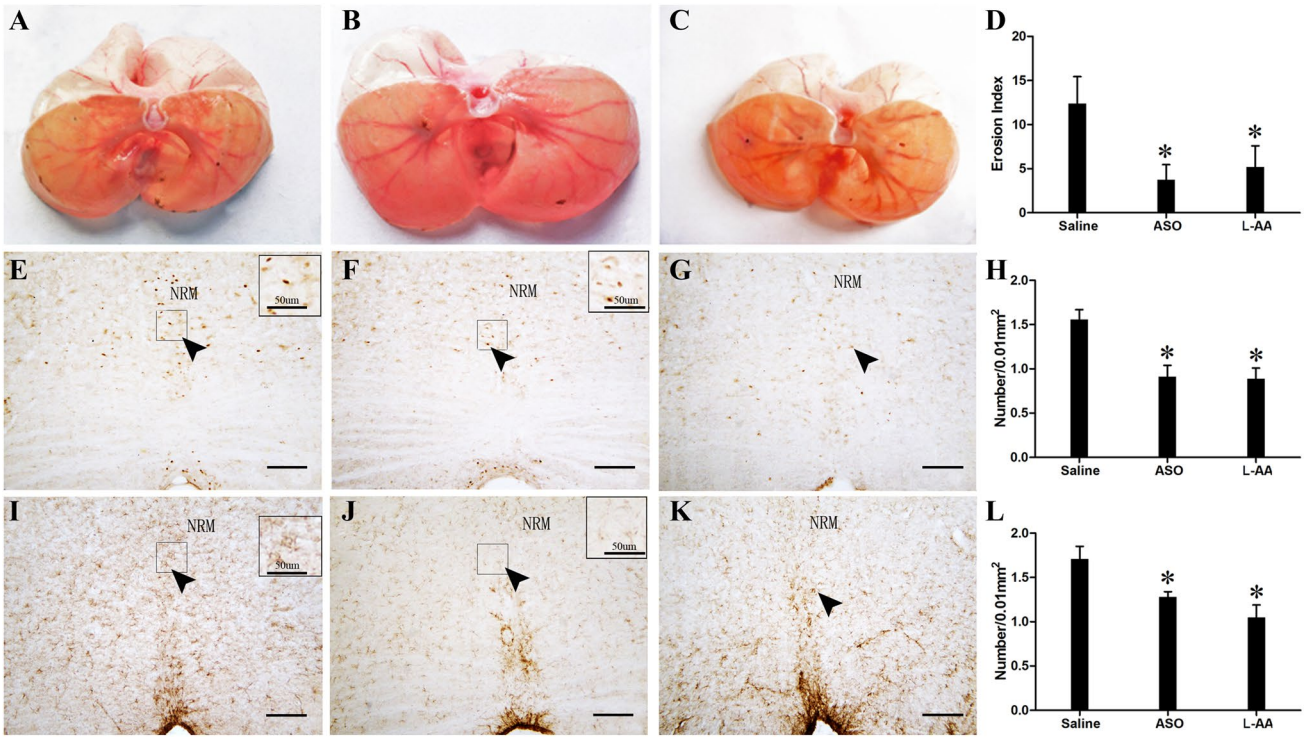

Fig. 5 Microinjection of ASO or L-AA, induced by RWIS. **a** Saline group. **b** ASO group. **c** L-AA group. **d** Quantification of erosion index (EI). **e–g** c-Fos expression induced by injecting saline, ASO or L-AA (100×); Scale bars, 100 μm. **h** Quantification of c-Fos. **i–k** GFAP expression induced by injecting saline, ASO or L-AA (100×); Scale bars, 100 μm. **l** Quantification of GFAP. The square in the panel is 400×. All data are expressed as the mean ± SEM (n = 6). *$P < 0.05$ vs. control group

and the same finding was detected for L-AA. These results might recommend that astrocytes and neurons were activated through RWIS and regulate each other in the NRM. The neuron-astrocyte network in the NRM may play a significant function in information that processes under RWIS statuses and may take part in SGMI.

ERK1/2 Signaling Pathway in the NRM Contributes to Gastric Mucosal Damage

Extracellular signal-regulated kinase (ERK) is activated through extensive stimulus and stress response and involved in stress reactions [39]. In neurons, ERK can play a function in promoting cell death or supporting cell survival [40]. Researches have reported that the ERK is mediated by 5-HT which regulates the neuronal plasticity and neuronal gene expression [41]. In present study, an assessment of p-ERK1/2 immunoreactive protein expression was completed by us in the process of RWIS. These experimental results suggested that the proteins expression were increased obviously, indicating that ERK was involved in RWIS. Phosphorylation of active ERK1/2 regulates neuronal activity significantly [42]. We found that the expression of the two proteins raised with the increase of stress time, the maximum was observed at 3 h, and dropped at 5 h. The expression of both proteins demonstrated similar curves. We pretreated the lateral ventricle with the inhibitor PD98059. As anticipated, this signaling pathway was completely blocked by PD98059. Because of this, the indexes of rat ulcers were notably reduced and the neuronal activity in the NRM was limited. These results suggested that the ERK1/2 signaling pathway was activated in NRM, regulated neuronal activity in the process of RWIS and was involved in the SGMI.

A recent study show that ERK1/2 signaling pathway joins in regulating the activation of astrocyte through releasing [Ca^{2+}] from intracellular stores [43]. It has been shown that stimulation by thrombin-activated astrocytes occurs through the ERK1/2 signaling pathway [44]. PD98059 was utilized to explain the interaction between ERK and astrocytes. Our results showed that after the microinjection of PD98059, GFAP expression substantially fell off compared to the control group. These experimental data illustrated that astrocytic activation induced by RWIS is mediated through the signaling pathway. It is in consequence possible that the ERK1/2 signaling pathway possesses a meaning function in neuron-astrocyte network's regulation under RWIS statuses.

In conclusion, the present results indicate that astrocytic and neuronal activity in the NRM significantly increased after RWIS. In the NRM, there is a neuron-astrocyte network

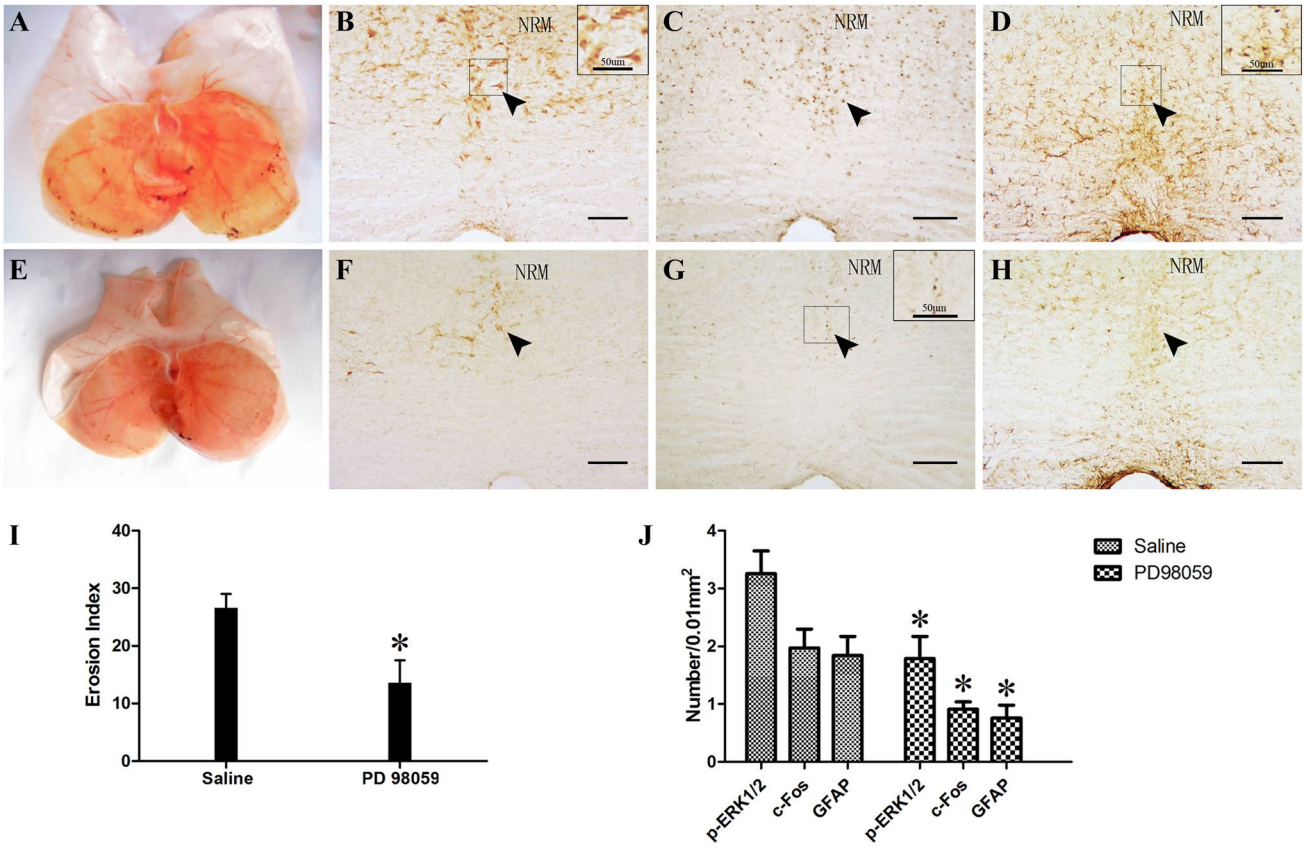

Fig. 6 Microinjection of PD98059, induced by RWIS. **a** Saline group. **e** PD98059 group. **i** Quantification of erosion index (EI). **b–d** p-ERK1/2, c-Fos, and GFAP expression induced by injecting saline (100×). **f–h** p-ERK1/2, c-Fos, and GFAP expression induced by injecting PD98059 (100×); Scale bars, 100 μm. The square in the panel is 400×. **j** Quantification of p-ERK1/2, c-Fos, and GFAP. All data are expressed as the mean ± SEM (n = 6). *$P < 0.05$ vs. control group

regulates the mechanism of the SGMI via the ERK1/2 signaling pathway.

Authors Contribution XM and HS formulated the study's design. FC, MY, and XG carried the learning out and analyzed the experimental data. The manuscript was drafted by FC. XM and HS corrected the paper for significant noetic content.

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