Modulatory effects of repeated psychophysical stress on masseter muscle nociception in the nucleus raphe magnus of rats

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Abstract

Psychophysical stress could increase nociception in the orofacial region including the masseter muscle, and neural changes in the brain contribute to increases in masseter muscle nociception under psychophysical stress conditions. The nucleus raphe magnus (NRM) located in the brain stem serves crucial roles to regulate nociception as descending pain controls. However, it remains unclear if neural activities in the NRM could be affected under psychophysical stress conditions. This study conducted experiments to assess (1) whether neural activity, indicated by Fos expression in the NRM evoked by masseter muscle injury, is modulated by the repeated forced swim stress (FST); (2) whether the selective serotonin reuptake inhibitor, fluoxetine, administered daily after FST could affect the number of Fos-positive neurons in the NRM. Results revealed that repeated forced swim stress significantly increased the number of Fos-positive neurons in the NRM evoked by masseter muscle injury. Fluoxetine inhibited increases in the number of Fos-positive neurons in the NRM in FST, but not in sham rats. These findings indicated that FST could increase in nociceptive neural activities in the NRM evoked by masseter muscle injury, which could be due to changes in serotonergic mechanisms at least, in part.

Key Words; pain, nucleus raphe magnus, repeated forced swim stress, masseter muscle, serotonin, rostral ventromedial medulla

Introduction

Exposure to repeated psychophysical stress has modulatory effects on the orofacial nociception (1). Multiple brain structures mediate the pain experience in a complex manner; nociceptive transmission can be affected by psychophysical stress conditions, which could influence neural functions in the brain components of pain circuits [2,3]. Previously repeated forced swim stress (FST), which can induce psychophysical stress conditions, enhanced nociceptive neural excitabilities in the trigeminal subnucleus caudalis (Vc) region [4-6], while Vc region is well documented to be a critical region to mediate nociception in deep orofacial tissues such as masseter muscle (MM) [7,8] and temporomandibular joint regions [4].

The nucleus raphe magnus (NRM), the area of the rostral ventromedial medulla (RVM) in the caudal brainstem, is a key relay for the descending pain controls to the trigeminal subnucleus caudalis (Vc) [9,10]. It is, therefore, possible that neural changes in the NRM under pathological conditions could cause the increases in nociception in the areas of trigeminal nerve territory [7,8]. Ample studies revealed the dysfunction of the NRM could affect nociceptive responses in spinal pain models, but it remains unclear if psychological stress conditions could modulate nociceptive neural activities in the NRM related to the trigeminal noxious inputs to the Vc region. Notably, neural functions in the NRM in trigeminal pain models appeared to be different from those in spinal pain models [11,12].

Serotonin (5HT) is a key factor in descending pain controls and in mediating psychological stress and nociceptive responses [2,3,6,13,14]. Serotonergic neurons could display nociceptive responses indicated by Fos protein expression in the NRM evoked by noxious stimuli to the peripheral regions [15,16]. Chronic restraint stress modulates tryptophan hydroxylase biosynthesis of serotonin production in the NRM [17], and antidepressant agents, such as the selective serotonin reuptake inhibitors (SSRIs), reduce tryptophan hydroxylase levels in the NRM [18]. Our recent results revealed that the daily administration of the SSRI, fluoxetine reduced nociceptive neural activities at the Vc region after FST [8]. These findings suggested that dysfunction of serotonergic mechanism in the brain could affect neural activity in the Vc region by adversely influencing the descending pain control pathways including NRM functions [13]. However, it remains unknown how SSRIs affect masseter muscle nociception in the NRM especially under FST conditions.

In this study, Fos immunohistochemical procedures were conducted to quantify the MM nociception in the NRM under repeated FST. Despite several limitations, Fos protein is often employed as a marker of neuronal excitability to assess neural function in the central nervous systems for psychophysical stress conditions and pain processing [19,20]. The aims of this study were to clarify if repeated psychophysical stress had modulatory effects on Fos expression in the area of NRM, and if repeated SSRI could affect Fos expression in the NRM after noxious stimulation to the masseter muscle.

Materials and methods

Animals

Experiments were conducted in accordance with International Association for the Study of Pain (21) and were reviewed by the Institutional Animal Care and Use Committee and approved by the President of Niigata University (#SA00351). All experiments performed in this study using animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. We made efforts to reduce and minimize the number of animals used for the experiments and their suffering. This report includes no studies with human participants. Sprague–Dawley rats (Male, 250-280 g, Charles River, Japan) were employed. Rats were housed in plastic cages (two rats per cage), and they can access to food and water for at least 5 days freely before stress conditioning being conducted. Cages were maintained at a temperature of 25 ± 2°C and were light-controlled protected units (12:12 h light : dark cycles with light beginning at 8:00 A.M.).

Repeated forced swim stress treatment (FST)

For repeated FST each rat was placed in a plastic cylinder (diameter 30 cm, height 50 cm) containing 20 cm water (25-27 °C) for 10 minutes/day between 09:00 A.M. and 11:00 A.M. for 3 days (Days -3, -2, and -1) [22,23] (**Fig. 1**). Fresh water was employed for each session. Sham rats served as the controls and were placed in an empty swim chamber under the same schedule. Rats were dried in a warm environment after each FST session. Noxious stimulation to Masseter muscle (MM) region was conducted with formalin on Day 0 at 24 hours after the last FST.

Masseter muscle (MM) injury

MM stimulation was induced with 0% formalin (i.e., saline, 0.05 mL), 1% formalin, and 5% formalin (0.05 mL) injected into the central portion of the left MM under general anesthesia with three types of mixed anesthetic agents. A combination anesthetic drug was prepared with 0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol. MM injection of formalin was conducted on Day 0 in the FST-conditioned group (0% formalin [n = 5]; 1% formalin [n = 5]; and 5% formalin [n = 5]) and in the repeated sham-conditioned group (0% formalin [n = 5]; 1% formalin [n = 5]; 5% formalin [n = 5]). Additional rats were included as the controls. These rats received no stimulus to the MM region with FST (FST-N.S. group; n = 5) or with sham FST (Sham-N.S. group, n = 5). Until rats were euthanized, the plane of anesthesia of rats was kept at which the rats showed no withdrawal reflex evoked by noxious pinch stimulation to the hindpaw. All rats were allowed to survive for 2 hours after MM stimulation.

Fluoxetine effects on Fos responses in the RVM after FST

Rats were divided into six groups, which included the vehicle group (i.e., saline, 1 mL/kg) and fluoxetine diluted in the saline-treated groups (0.1 mg/kg, 1 mg/kg) under FST or sham

conditions. Five rats were employed for each treatment. Drugs were given intraperitoneally 30 minutes after each FST and sham treatment on Day -3 to Day -1. Formalin (5% in saline, 0.05 mL) was injected to induce Fos expression in the RVM.

Tissue preparation and Fos immunohistochemistry.

Two hours after MM injury, rats were anesthetized deeply with three types of mixed anesthetic agents. They were perfused through the heart with 150 mL saline (4°C), followed by 400 mL of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (PFA, pH, 7.4; temperature, 4°C). The brainstem was removed and postfixed with PFA overnight. Next day, brainstems were placed in sucrose (30% in 0.1M PBS) for 2-3 days at 4°C. Brainstem transverse frozen sections (50 μm thick) were serially cut using a freezing microtome. Sections were collected in five wells containing 0.01 M PBS. The sections were processed for Fos immunohistochemistry from the brainstem (-10 mm to -11.5 mm caudal to the bregma). After washing several times, sections were incubated in 5% normal goat serum (NGS) for 120 minutes in affinity-purified mouse c-Fos monoclonal antibody (1:2,000, Abcam, Cambridge, MA, 4°C, 40 hours) or in rabbit serotonin polyclonal antibody (1:10,000; Immunostar, Hudson WI, USA, 4°C, 16 hours) in 0.01 M PBS containing Triton-X (0.3%) and 5% normal goat serum, biotinylated goat anti-mouse immunoglobulin G (IgG) antibody (1:300; Vector, Burlingame, CA, USA) at room temperature (RT), and avidin-biotin-peroxidase complex for 60 min at RT. Fos-positive nuclei were visualized by the incubation of sections in diaminobenzidine (DAB)-nickel solution, which was activated by 0.01% peroxidase. After sections were washed in tris-buffered saline (TBS) for twice (10 min each), those were mounted on untreated glass slides (Matsunami, Osaka, Japan). Sections on the slide glasses were dehydrated in ethanol series (70, 80, 90, 95, 100%), and then, cleared in xylene. Finally, sections mounted on the slide glass were cover-slipped. Specific Fos and 5HT staining was abolished by omitting the primary antibody. The experiment for double labeling of Fos- and serotonin- (5HT) immunoreactivity was conducted in the NRM to determine the neurochemical properties in Fos expressing cells. MM stimulation with 5% formalin was conducted. The procedures for Fos-immunostaining in detail are described above, while for 5HT immunostaining, after the completion of Fos-immunostaining, sections were incubated with rabbit serotonin polyclonal antibody (1:5,000; Immunostar, Hudson WI, USA), followed by biotinylated anti-rabbit IgG (1:200; Vector, Burlingame, CA, USA) for 2 hours at room temperature. 5HT immunoreactivity was visualized by DAB alone activated by 0.01% peroxidase.

Data analysis

In our experimental conditions, and consistent with the other report [24], Fos expressing cells were predominantly induced in the caudal area of RVM region. Using a light microscope, the number of Fos-positive cells was quantified in the NRM, nucleus reticularis gigantocellularis

pars alpha (GiA), ventral nucleus reticularis gigantcellularis (Gi), and nucleus lateralis paragigantocellularis (LPGi) at the level between -10 mm and -11.5 mm to the bregma (**Fig. 2A, B**). Under bright-field illumination, Fos-positive cells were counted if they contained a black regularly shaped nucleus that was surrounded by a brown-stained perinuclear cytoplasmic region with dendritic processes. On the other hand, 5HT positive cells were counted if they contained a well-defined nucleus that was surrounded by a brown-stained perinuclear cytoplasmic region which had dendritic processes. The number of serotonin-positive cells were counted in the NRM. Nuclear and laminar boundaries in the RVM were defined by other studies [17,25]. Fos- and 5HT-positive cells were quantified with 4-5 sections and average number of Fos-positive cells/section in each area was statistically analyzed by using analysis of variance, followed by Bonferroni tests. The percentage of double labeling cells are estimated by the number of both Fos- and 5HT-expressing cells in the total number of Fos positive cells with or without 5HT expression in the NRM. A probability level less than 0.05 was significant. Sections from each animal were observed at a magnification of 100× to quantify the number of Fos-positive cells. The examiner was blinded to the treatment groups.

Results

FST effects on Fos responses in the RVM.

FST with no stimulation (i.e., FST-N.S. group) or with saline (i.e., 0% formalin) administered into the MM showed a small change in the number of Fos expressing cells in each area of RVM, compared with the change in the sham rats (Fig 3, $P > 0.1$). These findings indicated that injection procedures alone had no effect on neural activity in the RVM region. FST effect on the number of Fos-positive cells in each area, after formalin injection was then tested. In general, Fos-positive cells were distributed in the caudal portion of the RVM in sham rats and FST rats (**Fig. 2A and 2B**). Compared with the saline (0% formalin) injection, an injection of 5% formalin to the MM region significantly increased the number of Fos-positive cells in the NRM ($P < 0.001$, **Fig. 3A**) and GiA ($P < 0.001$, **Fig. 3B**) in the sham rats and FST rats. Furthermore, compared with the sham rats, FST significantly caused increases in the number of Fos-positive cells in the NRM region evoked by 5% formalin ($P < 0.01$, **Fig. 3A**), indicating that FST could facilitate MM nociceptive responses in the NRM. In the Gi and LPGi, 1% and 5% formalin did not significantly enhanced Fos responses in comparison to saline injection under FST and the sham conditions (**Fig. 3C and 3D**). The percentages of Fos and 5HT double-labeled cells (**Fig 4**) in total number of Fos positive cells regardless of double labeling/section were significantly greater in sham rats (27.5 ± 2.1%, $P < 0.01$) than that in FST rats (17.6 ± 1.8%). Further, there is a small, but decreases in the number of 5HT positive cells in the NRM after FST (28.3 ± 2.1 cells/section in sham rats; 20.5 ± 0.6 cells/section in FST rats, $P < 0.05$). These results indicated that the reduction of the percentages of double labeling cells/total number of Fos-positive cells after FST was due to increases in the number of Fos expressing cells with decreases in that of 5HT expressing cells.

Effects of fluoxetine on Fos expression in the NRM after FST

The effects of fluoxetine on Fos expression in the NRM after injecting 5% formalin to the MM region was determined (**Fig 5A**), because our current results revealed that MM stimulation with formalin had greater influences on the number of Fos-positive cells, mainly in the NRM, compared with other areas in the RVM (**Fig. 3**). In FST rats, but not sham rats, fluozetine significantly decreased the number of Fos-positive cells in the NRM compared with the vehicle-treated rats (0.1 mg/kg, $P < 0.05$; 1 mg/kg, $P < 0.01$, **Fig. 5B**). The number of Fos positive cells was significantly increased in GiA in Sham and FST rats (Fig 3A), but fluoxetine did not reduce on Fos responses, significantly ($P > 0.1$).

Discussion

This study had several novel findings. First, repeated FST increased nociceptive neural activity, as indicated by Fos expression in the NRM, after formalin injection into the MM. These findings indicated that psychophysical stress conditions could affect the NRM function which might lead to the dysfunction of descending pain controls. Second, repeated administration of antidepressant drug just after each FST, SSRI, could prevent the FST-induced enhancement of Fos responses in the NRM by MM stimulation with formalin. These findings indicated that FST increased nociceptive responses in the NRM, which might be mediated by changes in serotonergic mechanisms at least, in part. Several areas in the RVM, such as the NRM, GiA and LPGi regions, regulate nociception in trigeminal and spinal pain models [12,24]. Imaging studies that employed glucose utilization showed increases in neural excitability in the NRM and GiA in rats with peripheral nerve injury and inflammation [26,27]. Furthermore, a recent report indicated the distinct roles of the NRM versus the LPGi in the regulation of neural activities in the spinal dorsal horn [29]. Our present results revealed that formalin injection into the MM increased Fos expression, especially in the NRM, but not in the Gi and LPGi regions, in the sham and FST rats. Furthermore, mapping studies using Fos immunoreactivity have indicated that the contribution of each area in the RVM to nociceptive responses could be dependent on various pain conditions. For example, Fos expression evoked by noxious stimulation to the hindpaw was greater in the NRM than in other areas under persistent monoarthritis of the temporomandibular joint (TMJ) [24], whereas those by forepaw or visceral stimulation could cause greater Fos expression in the GiA than in other areas [16]. Our present results demonstrated that nociceptive neural activity was much greater in the NRM than in other areas of the RVM, which indicated that the NRM was apparently more sensitive to MM injury, compared with other areas in the RVM under repeated FST condition. Therefore, this study focused on determining the effects of repeated and acute FST on neural activity, primarily in the NRM.

The NRM has descending pathways, which could regulate nociceptive neural activities in the trigeminal subnucleus caudalis (Vc) regions [10]. Changes intrinsic to the NRM are documented in various chronic pain conditions such as TMJ inflammation [24], headache [29], visceral pain [30] and peripheral nerve injury [31,32]. Mounting evidence revealed that repeated psychological stress could enhance nociceptive responses related to the spinal and trigeminal nerve inputs [33,34]. As nociceptive neural properties in the NRM based on trigeminal nerve inputs were not similar to that seen after spinal nerve inputs [11,12], it is important to assess the effects of the FST on functional changes in the NRM using trigeminal pain models rather than in spinal pain models. Our previous report showed that the facilitatory effects of FST on nociception in the MM regions occurred because of increases in Fos expression in the Vc region [8]. Our results showed significant increases in Fos

expression, primarily in the NRM evoked by MM injury after repeated FST, whereas the facilitatory effects of FST alone on Fos expression seemed to be less in other areas in the RVM. These findings suggested that enhanced neural activity in the NRM could have roles in increasing MM nociception with changes in neural activity in the Vc region. Serotonergic mechanisms are critically involved in the mediation of stress responses such as psychological distress [35,36] and stress-induced hyperalgesia [2,3]. Previous reports have indicated that stress conditionings influence serotonergic function such as the 5HT concentration in the central nervous systems [37,38]. In contrast, modulating serotonergic mechanisms by antidepressants, such as SSRIs, could relieve stress-induced depression-like behaviors and enhance nociception [8,23,39,40]. These findings suggest that serotonergic mechanisms have critical roles in mediating psychological distress and nociceptive processing. Many reports have implicated 5HT in regulating pain responses and have demonstrated that bulb-spinal/trigeminal serotonergic pathways exert modulatory influences on orofacial nociceptive signaling within the Vc and spinal cord [2,3]. However, evidence has also indicated that serotonergic inputs to the NRM can affect neural activity in the NRM [41]. Several 5HT receptor subtypes can regulate nociception in the NRM [42] and the level of a certain subtype of 5HT receptor in the NRM is altered under pathological conditions [43]. These findings suggest that the involvement of serotonergic mechanisms in regulating neural activities in the NRM could be sensitive to several pathological conditions and could modulate descending outputs to the Vc region, which could affect orofacial nociception. In our study, repeated SSRI administration after each FST could decrease enhanced Fos expression evoked by MM injury in the NRM in FST rats, but not in sham rats. These findings suggest that serotonergic function in the brain could be affected by repeated FST. At this point, it is unclear how SSRIs affect neural activity in the NRM: however, it could be direct actions of SSRIs in the neural activities in the NRM or indirect effects of SSRIs on neural activity in remote areas of the brain in which descending mechanisms can regulate neural activity in the NRM. Further investigations would be required to determine the action sites of SSRI which can inhibit Fos induction in the NRM after stress conditionings.

Previously neural recording experiments indicated that 5HT neurons in the NRM could be functionally classified as neutral cells, but not as on-cells and off-cells [44,45]. In our present data, FST increases the number of Fos positive cells in the NRM. However, the percentage of double labeling cells in the total number of Fos positive cells with or without 5HT expression were decreased under FST conditions. These findings indicated that most Fos-positive cells, which were increased after FST, were unlikely serotonergic neurons in the NRM, and most Fos positive cells, which were sensitive to FST, seemed to be On- or Off-cells. Further, it is possible that FST could induce phenotype changes in serotonergic neurons from neutral cells to On- or Off cells as

was seen in a persistent inflammatory pain model [46]. However, further functional experiments are definitely required.

In conclusion, FST increased the number of Fos positive cells in the NRM evoked by MM injury possibly via changes in serotonergic function. These findings suggested that psychophysical stress conditions could influence neural activities in the NRM which had modulatory roles on deep craniofacial nociception.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1

Experimental design. From Day -3 to Day -1 (10 min/day), the rats underwent daily sessions of repeated forced swim stress treatment (FST) or sham treatments. On Day 0, the rats were euthanized 2 hours after undergoing unilateral formalin injection into the masseter muscle (MM) in order to examine the number of Fos expressing cells in the rostral ventromedial medulla (RVM).

Figure 2

(A) The photomicrograph shows Fos expression in the rostral ventromedial medulla (RVM; bregma, -11.5 mm). Fos immunoreactivity in the RVM was evoked by the ipsilateral injection of 5% formalin into the masseter muscle, after repeated forced swim stress treatments (FST). **(B)** The schematic figures show the four areas in the rostral ventromedial medulla (RVM) areas employed in measuring the number of Fos-positive cells. Each dot represents five Fos-positive cells. The number below the coronal section indicates rostro-caudal distance from the bregma (mm).

Abbreviations: Gi, nucleus reticularis gigantocellularis; GiA, nucleus reticularis gigantocellularis pars alpha; LPGi, lateral nucleus reticularis paragigantocellularis; NRM, nucleus raphe magnus. The number "7" indicates the facial nucleus.

Figure 3

The effects of repeated forced swim stress treatments (FST) on the number of Fos-positive cells in each area of the rostral ventromedial medulla (RVM) such as the **(A)** NRM, **(B)** GiA, **(C)** Gi, and **(D)** LPGi.

Abbreviations: Gi, nucleus reticularis gigantocellularis; GiA, nucleus reticularis gigantocellularis pars alpha; LPGi, lateral nucleus reticularis paragigantocellularis; NRM, nucleus raphe magnus; N.S., no stimulus.

*** $P < 0.001$, versus the 0% group within the each stress treatment group.

§§ $P < 0.01$, versus the repeated sham rat with 5% formalin group.

Figure 4

The photomicrograph shows cells that are double-labeled with Fos and serotonin (5HT) (arrows), cells with Fos expression alone (arrow heads), and 5HT-positive cells (*) in the NRM (bregma, -11.5 mm). These cells are from a brain section from a rat that had undergone FST.

Figure 5

A. The photomicrographs show Fos expression in the Nucleus Raphe magnus (NRM; bregma, -11.5 mm) from the vehicle, 0.1 mg/kg and 1 mg/kg fluoxetine-treated rats in sham and FST groups. The number of Fos –positive cells were counted within the area shown by the triangle.

B. The effects of intraperitoneally administered fluoxetine at the doses of vehicle, 0.1 mg/kg, and 1 mg/kg on Fos expression in the NRM in the sham and FST groups**.**

*** $P < 0.001$, versus the sham rats.

§§ $P < 0.01$, versus the vehicle-treated FST rats.

§§§ $P < 0.001$, versus the vehicle-treated FST rats.

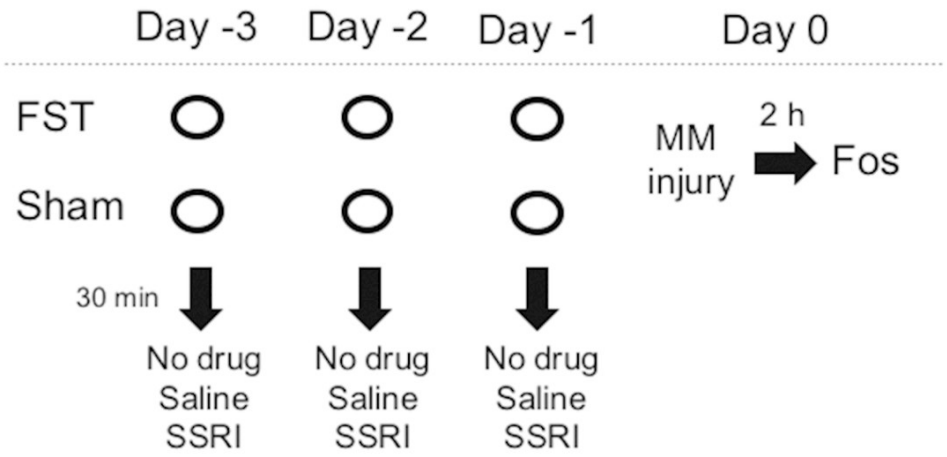
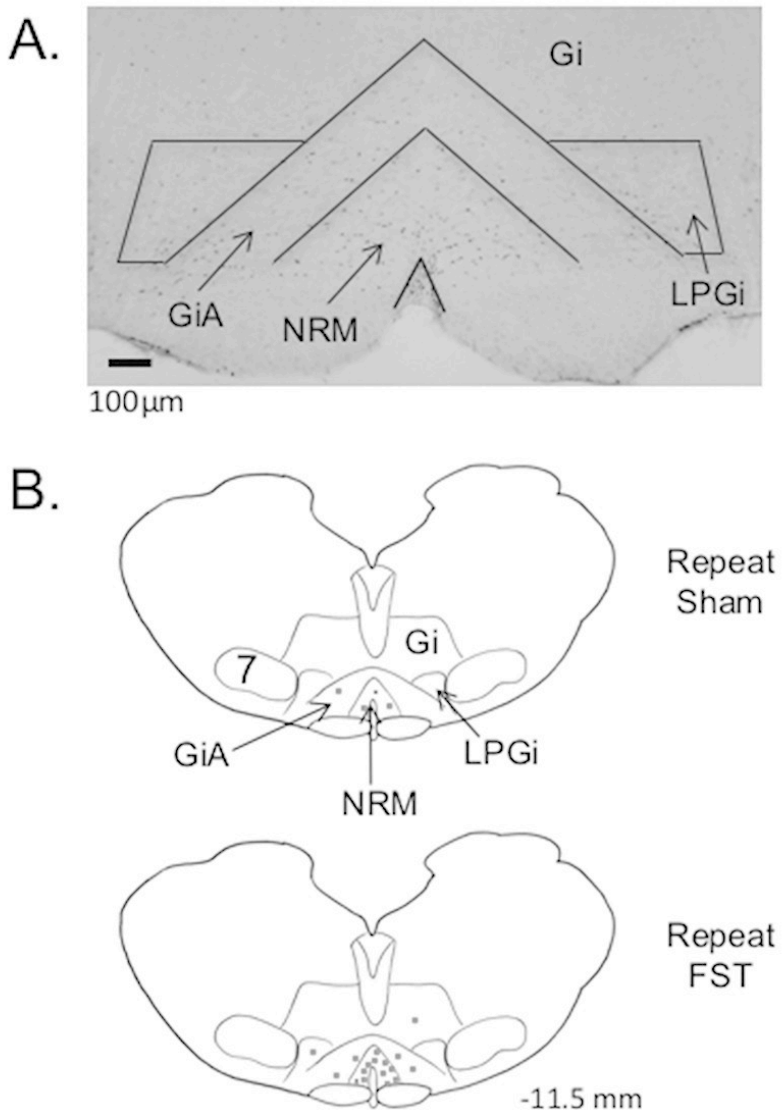


Figure 1Experimental design. From Day -3 to Day -1 (10 min/day), the rats underwent daily sessions of repeated forced swim stress treatment (FST) or sham treatments. On Day 0, the rats were euthanized 2 hours after undergoing unilateral formalin injection into the masseter muscle (MM) in order to examine the number of Fos expressing cells in the nucleus raphe magnus (NRM).

153x85mm (200 x 200 DPI)



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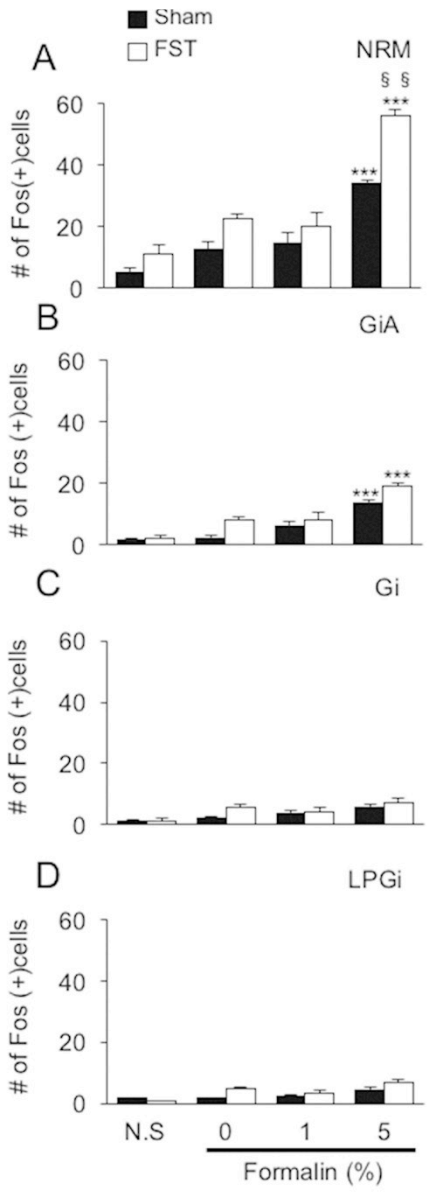

Figure 3
The effects of repeated forced swim stress treatments (FST) on the number of Fos-positive cells in each area of the rostral ventromedial medulla (RVM) such as the (A) NRM, (B) GiA, (C) Gi, and (D) LPGi.

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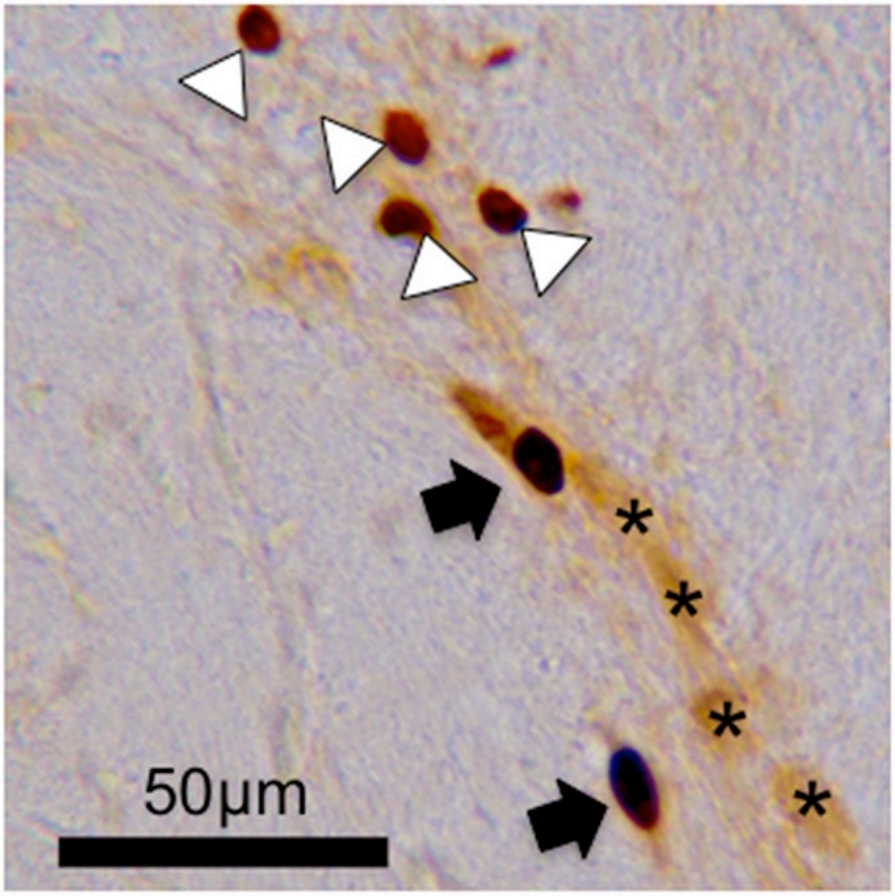

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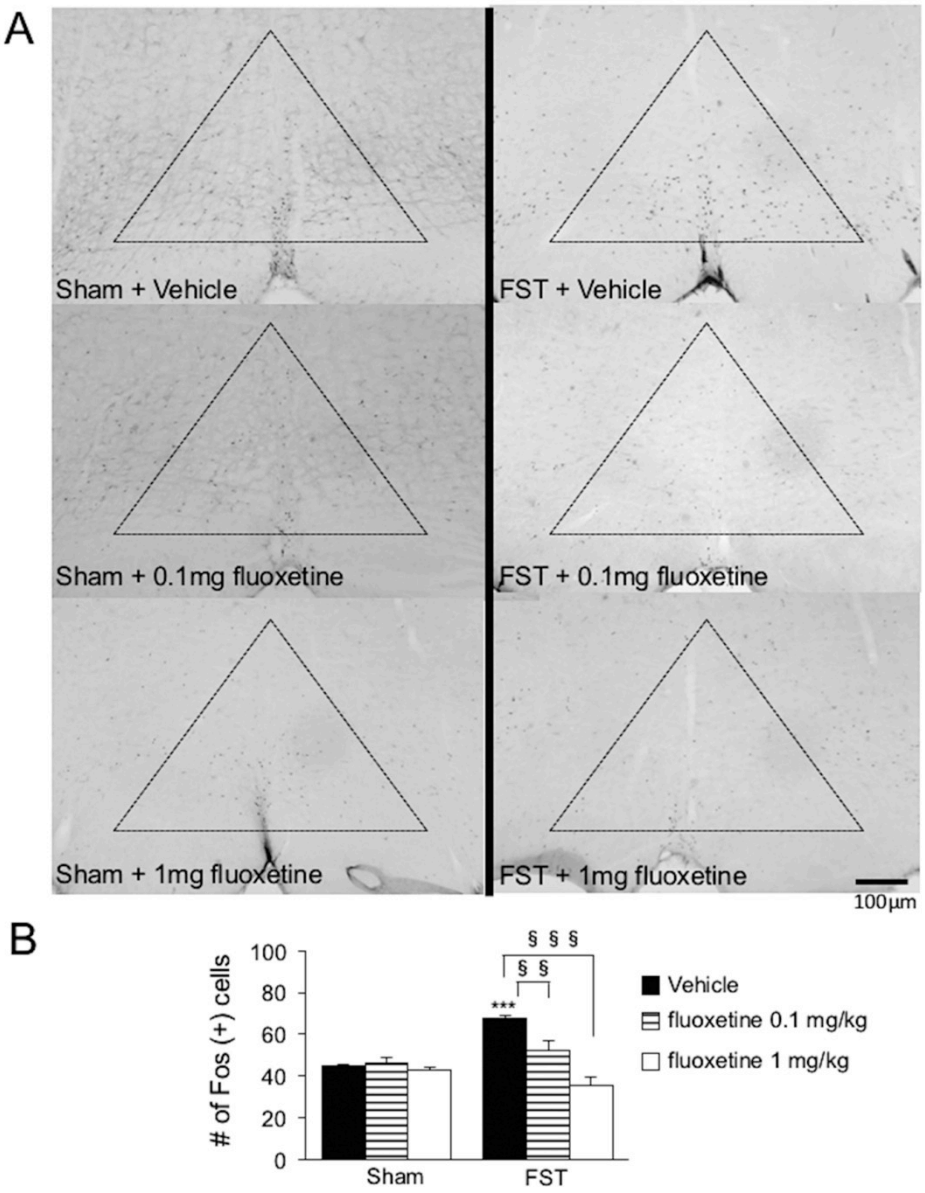

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