Abstract: Psychophysical stress can cause neural changes that increase nociception in the orofacial region, particularly the masseter muscle (MM). The nucleus raphe magnus (NRM), which is located in the brain stem, serves the crucial role of regulating nociception through descending modulatory pain control. However, it remains unclear if neural activities in the NRM are affected under psychophysical stress conditions. This study conducted experiments to assess (1) whether neural activity, indicated by Fos expression in an NRM that has experienced MM injury, is affected by the stress of repeated forced swim tests (FST); and (2) whether the selective serotonin reuptake inhibitor fluoxetine administered daily after an FST could affect the number of Fos-positive neurons in the NRM. Results revealed that the stress from repeated FSTs significantly increased the number of Fos-positive neurons in an NRM that had been affected by MM injury. Fluoxetine inhibited increases in the number of Fos-positive neurons in the NRM that occurred as a result of FSTs, but this was not observed in sham rats. These findings indicate that the stress from FSTs could increase nociceptive neural activity in an NRM that has experienced MM injury. This could be due, in part, to changes in serotonergic mechanisms.

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

Introduction

Exposure to repeated psychophysical stress has modulatory effects on orofacial nociception [1]. Multiple brain structures mediate the experience of pain in a complex manner. Nociceptive transmission can be affected by psychophysical stress conditions, which can influence neural functioning with regard to pain circuits in the brain [2,3]. Prior studies have found that repeated forced swim tests (FSTs), which can induce psychophysical stress conditions, enhanced nociceptive neural excitabilities in the trigeminal subnucleus caudalis (Vc) region [4-6], with the Vc region being well documented as a critical area for mediating nociception in deep orofacial tissues, such as masseter muscle (MM) [7,8] and temporomandibular joints [4].

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

Serotonin (5HT) is a key factor in descending pain control pathways as well as mediating psychological stress and nociceptive responses [2,3,6,13,14]. Serotonergic neurons display nociceptive responses indicated by Fos protein expression in an NRM subjected to noxious stimuli in the peripheral regions [15,16]. Chronic restraint stress modulates tryptophan hydroxylase biosynthesis of serotonin production in the NRM [17], and antidepressant agents, such as selective serotonin reuptake inhibitors (SSRIs), reduce tryptophan hydroxylase levels in the NRM [18]. Recent findings suggested that dysfunction of the serotonergic mechanism in the brain could affect neural activity in the Vc region by adversely influencing descending pain control pathways including NRM functions [13]. However, it remains unknown how SSRIs affect MM 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 FSTs. Despite several limitations, Fos protein is often employed as a marker of excitability to assess neural functioning in the central nervous system 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 NRM, and if administering daily doses of an SSRI could affect Fos expression in the NRM after noxious stimulation of the MM.

Materials and Methods

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

Repeated forced swim stress tests (FST)
For repeated FSTs, each rat was placed in a plastic cylinder (diameter 30 cm, height 50 cm) containing 20 cm water (25-27°C) for 10 min/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 used for each session. Sham rats served as the controls and were placed in an empty swim chamber on the same schedule. Rats were dried in a warm environment after each FST session. Noxious stimulation to the MM region was conducted with formalin on Day-0 at 24 h after the last FST.

Correspondence to Dr. Keiichiro Okamoto, Division of Oral Physiology, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan
E-mail: okamoto12@dent.niigata-u.ac.jp
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rats received no stimulus to the MM region after FSTs (FST-N.S. group; n = 5). Additional rats were included as the controls. These n% formalin = 5; 1 n and the sham-conditioned group (0 n = 5; and 5 n formalin group (0 n = 5; 5% formalin n = 5)). Additional rats were included as the controls. These rats received no stimulus to the MM region after FSTs (FST-N.S. group; n = 5), nor did the sham rat group who experienced FSTs (Sham-N.S. group, n = 5). Until the rats were euthanized, the plane of anesthesia of rats was kept at the point that the rats showed no withdrawal reflex evoked by noxious pinch stimulation to the hindpaw. All rats remained alive for 2 h 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 the group that received fluoxetine diluted in saline (0.1 mg/kg, 1 mg/kg) under FST or sham conditions. Five rats were employed for each treatment. Drugs were given intraperitoneally 30 min 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 h after MM injury, rats were deeply anesthetized 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. The next day, the brainstems were placed in sucrose (30% in 0.1 M 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 being washed several times, sections were incubated in 5% normal goat serum (NGS) for 120 min in affinity-purified mouse c-Fos monoclonal antibody (1:2,000, Abcam, Cambridge, MA, 4°C, 40 h) or in rabbit serotonin polyclonal antibody (1:10,000; Immunostar, Hudson WI, USA, 4°C, 16 h) 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 an avidin-biotin-peroxidase complex for 60 min at RT. Fos-positive nuclei were visualized by the incubation of sections in a diaminobenzidine (DAB)-nickel solution, which was activated by 0.01% peroxidase. After sections were washed in tris-buffered saline (TBS) twice (10 min each), they were mounted on untreated glass slides (Matsunami, Osaka, Japan). Sections on the slide glasses were dehydrated in an ethanol series (70, 80, 90, 95, and 100%), and then cleared in xylene. Finally, sections mounted on the slide glass were cover-slipped. Specific Fos and serotonin (5HT) staining was abolished by omitting the primary antibody. The experiment for double labeling of Fos- and 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 are described above. 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 h at RT. 5HT immunoreactivity was visualized by DAB alone activated by 0.01% peroxidase.

Data analysis
The experimental conditions, which were consistent with the initial report [24], Fos expressing cells were predominantly induced in the caudal area of the 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 gigantocellularis (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 which had 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 have been defined by other studies [17,25]. Fos- and 5HT-positive cells were quantified with 4-5 sections and an average number of Fos-positive cells/section in each area was
Results

The effects of FSTs on Fos responses in the RVM

The FST group with no stimulation (i.e. FST-N.S. group) or saline (i.e. 0% formalin) administered into the MM showed a small change in the number of Fos expressing cells in each area of the 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. The effect of FSTs 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, B). 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 rats experienced increases in the number of Fos-positive cells in the NRM region evoked by 5% formalin (\( P < 0.01 \), Fig. 3A), indicating that FST stress could facilitate MM nociceptive responses in the NRM. In the Gi and LPGi, 1% and 5% formalin did not significantly enhance Fos responses in comparison with saline injection under FST and sham conditions (Fig. 3C, D). The percentages of Fos and 5HT double-labeled cells (Fig. 4) in the 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 in FST rats (17.6 ± 1.8%). Further, there is a small decrease 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 FSTs was due to increases in the number of Fos expressing cells with decreases in 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 were determined (Fig. 5A). This location was chosen due to the findings that MM stimulation with formalin had a greater influence on the number of Fos-positive cells in the NRM compared with other areas in the RVM (Fig. 3). In FST rats, but not sham rats, fluoxetine 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. 5A), but fluoxetine did not reduce Fos responses significantly (\( P > 0.1 \)).

Fig. 3 The effects of repeated FSTs on the number of Fos-positive cells in each area of the RVM, such as the (A) NRM, (B) GiA, (C) Gi, and (D) LPGi. 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 each stress treatment group. §§\( P < 0.01 \), versus the repeated sham rat with 5% formalin group.

Fig. 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 an FST.
Discussion

This study had several novel findings. Firstly, repeated FSTs increased nociceptive neural activity, as was indicated by Fos expression in the NRM, after formalin injection into the MM. These findings indicate that psychophysical stress conditions can affect the functioning of the NRM, which might lead to the dysfunction of descending pain controls. Secondly, repeated administration of an SSRI just after each FST may have prevented the FST-induced enhancement of Fos responses in the NRM by MM injection with formalin. These findings indicate that FSTs increased nociceptive responses in the NRM, which might be mediated, in part, by changes in serotonergic mechanisms. Several areas in the RVM, such as the NRM, Gi and LPGi regions, regulate nociceptive input in the spinal and peripheral pain models [12,24]. Imaging studies that employ glucose utilization show increases in neural excitability in the NRM and GiA in rats with peripheral nerve injury and inflammation [26,27]. Furthermore, a recent report indicates the distinct roles of the NRM versus the LPGi in the regulation of neural activity in the spinal dorsal horn [29]. The results reveal that formalin injection into the MM increases Fos expression, especially in the NRM, but not in the Gi and LPGi regions for either group of rats. The NRM could be functionally classified as neutral cells, but not as ON-cells or OFF-cells [44,45]. In the present data, FSTs increase 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 indicate that most Fos-positive cells, which were increased after FST sessions, were likely serotonergic neurons in the NRM, and that most Fos-positive cells in the NRM were sensitive to several pathological conditions and could modulate descending outputs to the Vc region, which could affect orofacial nociception. Repeated SSRI administration after each FST was demonstrated to potentially enhance Fos expression evoked by MM injection in the NRM in FST rats but not in sham rats. These findings suggest that serotonergic functioning in the brain could be affected by repeated FSTs. At this point, it is unclear how SSRIs affect neural activity in the NRM. However, this effect could be related to the 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 SSRIs which can inhibit Fos induction in the NRM after stress conditioning.

Previous neural recording experiments indicate that 5HT neurons in the NRM could be functionally classified as neutral cells, but not as ON-cells and OFF-cells [44,45]. In the present data, FSTs increase 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 indicate that most Fos-positive cells, which were increased after FST sessions, were unlikely serotonergic neurons in the NRM, and that most Fos-positive cells, which were sensitive to FSTs, seemed to be ON- or OFF-cells. Furthermore, it is possible that FSTs 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 required to determine this relationship.

In conclusion, FSTs increased the number of Fos-positive cells in an NRM affected by MM injury, possibly via changes in serotonergic functioning. These findings suggest that psychophysical stress conditions could influence neural activities in the NRM, which plays modulatory roles in deep craniofacial nociception.

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References