Locus coeruleus activation contributes to masseter muscle overactivity induced by chronic restraint stress in mice

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It is commonly accepted that exposure to stress may cause overactivity in the orofacial muscles, leading to consistent muscle pain, which is the main symptom of temporomandibular disorders. The central neural mechanism underlying this process, however, remains unclear. The locus coeruleus is considered to play an important role in stress-related behavioral changes. Therefore, the present study was designed to examine the role of locus coeruleus neurons in masseter overactivity induced by stress. C57BL/6 mice were subjected to chronic restraint stress for 14 days to establish an animal model. The behavioral changes and the electromyography of the masseter muscle in mice were measured. The expression of Fos in locus coeruleus was observed by immunofluorescence staining to assess neuronal activation. A chemogenetic test was used to inhibit locus coeruleus neuronal activity, and the behavioral changes and electromyography of the masseter muscle were observed again. The results exhibited that chronic restraint stress could induce anxiety-like behavior, overactivity of the masseter muscle, and significant activation of locus coeruleus neurons in mice. Furthermore, inhibition of noradrenergic neuron activity within the locus coeruleus could alleviate stress-induced anxiety behavior and masseter muscle overactivity. Activation of noradrenergic neurons in locus coeruleus induced by stress may be one of the central regulatory mechanisms for stress-induced anxiety-like behaviors and overactivity of masseter muscles. *NeuroReport* **35: 763–770 Copyright © 2024 The Author(s). Published by Wolters Kluwer Health, Inc.**

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Introduction

Orofacial muscle pain, which is the most common clinical symptom of temporomandibular disorders (TMD), has been complained by approximately 80% of patients with TMD and severely affects the normal life of patients [1]. It has been reported that TMD patients with orofacial muscle pain tend to have higher levels of anxiety or depression than the normal population [2,3]. Furthermore, people subjected to chronic psychological stress are more prone to hypertension and fatigue in the orofacial muscles, which result in consistent pain in the jaw muscles and temporomandibular joints [4,5]. Therefore, stress-induced hypertension and fatigue of the orofacial muscles are important precipitating factors for the orofacial muscle pain in TMD.

Myoelectricity level is an important indicator for evaluating the functional status of the orofacial muscles and assessing the development and therapeutic effects of TMD [6]. Clinical studies have shown that the myoelectric activity of the masseter muscles of TMD patients is significantly higher than that of the normal population [7,8]; and animal studies have also confirmed that experimental animals under exposure to stress condition showed anxiety-like behaviors, elevated activity, and abnormal energy metabolism of masseter muscles [9,10]. However, it is still not clear whether there are potential neural pathways in the central neural systems through which stress could induce masticatory muscle overactivity.

The locus coeruleus, located in the brainstem, is considered to be closely related to the stress response [11]. The neurons in the locus coeruleus are mainly noradrenergic neurons, which synthesize and secrete the neurotransmitter noradrenaline, playing an important role in the regulation of stress-related behavioral changes, emotions, and

cognitions [12]. Anatomically, the locus coeruleus is in close proximity to the mesencephalic trigeminal nucleus (Vme), which is an important nucleus that regulates the movement of the masticatory muscles [13]. Moreover, locus coeruleus also sends out neural projections to the Vme to modulate the excitability of Vme neurons [14,15]. Our previous study has confirmed that chronic stress can lead to elevated excitability of Vme neurons, contributing to overactivity of masseter muscle [16]. Consequently, whether the overactivity of masseter muscle caused by chronic stress is relevant to the locus coeruleus neuron activation, still needs to be further explored.

Therefore, in view of previous studies and observations, we hypothesized that chronic stress could induce enhanced locus coeruleus neuron excitability, which then contributes to masticatory muscle hyperfunction. In the present study, the animal model was established by chronic restraint. The masseter muscle was typically chosen for myoelectricity testing because of its superficial location and the strongest masticatory force among orofacial muscles [6,17]. Besides, the designer receptors exclusively activated by designer drugs (DREADDs) method was used to investigate the involvement of locus coeruleus neurons in masseter muscle overactivity induced by chronic stress.

Methods

Animals

A total of 40 SPF-grade male C57BL/6 mice (6–8 weeks old) were used in this study, which was provided by the Laboratory Animal Center of Fourth Military Medical University. The mice were raised in a room with controlled temperature (25 ± 1 °C), humidity (50 ± 5%), 12-hour light/dark cycle, and ad libitum food and water intake. All animal experiments were approved by the Ethics Committee of the School of Stomatology, Fourth Military Medical University (No. K9-2022-020) in accordance with the National Institutes of Health guidelines for the care and use of experimental animals.

This study consists of two parts of experiments. In the first part, 16 mice were randomly divided into the control (CON) group and chronic restraint stress (CRS) group ($n = 8$) to investigate whether CRS could induce behavioral changes, masseter overactivity, and activation of locus coeruleus neurons. In the second part, 24 mice were randomly divided into CON + saline group, CRS + saline group, and CRS + CNO (clozapine-n-oxide) group ($n = 8$). Chemical and genetic techniques were adopted to inhibit the activation of noradrenergic neurons in locus coeruleus, aiming to observe the involvement of locus coeruleus neurons in masseter overactivity elicited by CRS.

Animal model of restraint stress

The animal model of restraint stress used in this study was established by referring to previous studies [16]. The mice in the CRS group were placed in 50 ml centrifuge tubes with several ventilation holes in the wall; they were continuously restrained for 4 h starting at 8 : 00 a.m. every day for a total of 14 days. During the restraint stress, the mice were not fed or watered. The CON group mice were raised normally in cages.

Behavioral tests

An elevated plus maze (EPM) experiment was used to detect the anxiety level of the animals [18]. The EPM system (RD1208-EP; Shanghai Mobile Datum Corporation, Shanghai, China) was located 50 cm from the ground and consisted of two opposite open arms, two opposite closed arms, and a central activity area. As the experiment began, the mice were placed in the central area and allowed to move freely for 5 min, and their activities were recorded through a camera and processed with an automatic analysis system (Shanghai Mobile Datum Information Technology). The percentage of retention time in open arms and the percentage of entries into open arms of each group were used as parameters for assessing the anxiety level.

Electromyography recordings of masseter muscle

The customized electrodes (KedouBC Technology Corporation, Suzhou, China) were used for electromyography (EMG) level recording of masseter muscles in mice. An insulated flexible wire (length: 2.5 cm, diameter: 0.5 mm) was used, with one end soldered to a stainless steel microneedle (length: 2 mm, diameter: 0.15 mm) and the other end soldered to a female connector, and a silver wire to serve as a ground wire. Before the beginning of the experiment, the electrodes were placed in the left masseter muscles of mice according to a previous study with a little modification [19]. After anesthesia with intraperitoneal injection of sodium pentobarbital (35 mg/kg), the skin on the left cheek and the top of the skull was prepared, sterilized, and then incised. The skin of the left cheek was incised to expose the masseter muscle, and the electrode microneedle was inserted into the muscle belly and fixed with a suture. The insulated wires walked subcutaneously through the posterior ear and threaded out at the cranial incision, and the female connector was fixed to the cranial vault bone with dental cements. Antibiotics (cefotiam hydrochloride, 66 mg/kg) and analgesics (flurbiprofen axetil, 3.3 mg/kg) were administered intraperitoneally before allowing the mice to recover.

Experiments were started 1 week after electrode implant surgery. On the next day of the last restraint stress, the EMG level of masseter muscles of mice in the awake state was recorded by MP46 physiological recorder (BIOPAC, Grand Rapids, Michigan, USA). After acclimatization to the connecting cable for 30 min, and then the EMG signals of the masseter muscles of the mice in the waking state (30 min for each mouse) were collected

and processed with Biopac Student Lab software (Grand Rapids, Michigan, USA). To minimize the influence of the electrical baseline of each EMG data, the EMG was recalibrated based on the median value of each EMG data. Then the integral electromyography (iEMG) was obtained by calculating the sum of the areas under the curve, and the root mean square (RMS) was obtained by RMS processing of the EMG signals and getting the mean value. The muscle activity level is generally quantified with iEMG and RMS values [20].

Immunofluorescence staining

After deep anesthesia with sodium pentobarbital intraperitoneally (60 mg/kg), mice were perfused transcardially with 100 mL of 0.01 M PBS (pH 7.4), followed by 200 ml 0.1 M phosphate buffer (pH 7.4) that contained 4% paraformaldehyde. and brain tissues were removed for postfixation and transferred to 30% sucrose in 0.1 M phosphate buffer for dehydration. Frozen sections (30 μm thick) were cut with a cryostat (Leica CM1800; Leica, Heidelberg, Germany) and collected in 0.01 M PBS. The sections were rinsed, and blocked with 10% donkey serum for 30 min at room temperature, then incubated overnight with primary antibody: mouse anti-Fos (1 : 500, ab11959; Abcam, Cambridge, UK), rabbit anti-tyrosine hydroxylase (1 : 500, AB152; Millipore, Boston, Massachusetts, USA) at room temperature. After being rinsed, sections were incubated with secondary antibody: A594 donkey anti-mouse IgG (1 : 500, A-21203; Thermo Fisher Scientific, Waltham, Massachusetts, USA), A647 donkey anti-rabbit IgG (1 : 500, A-31573; Thermo Fisher Scientific) for 4 h at room temperature. The staining slices were observed under a laser confocal microscope (FV1000; Olympus, Tokyo, Japan) and photographed, and the number of Fos-positive neurons in each group of locus coeruleus nuclei was calculated using the Image-Pro Plus image analysis system (Media Cybernetics, Bethesda, Maryland, USA). The numbers of Fos-labeled neurons in locus coeruleus were quantified in three sections per animal per group.

Injection of designer receptors exclusively activated by designer drugs

Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (35 mg/kg) and were fixed on a stereotaxic apparatus (RWD Life Science, Shenzhen, China). The skin on the head was prepared, sterilized, and incised to fully expose the skull. According to the mouse brain stereotaxic atlas [21], the position of locus coeruleus was obtained and a glass micropipette filled with rAAV-TH-hM4D(Gi)-mCherry-WPRE-hGH pA (PT-3960, 2.0E + 12vg/ml, 0.05 μl; BrainVTA, Wuhan, China) connected to a microsyringe (1 μl; Hamilton, Reno, Nevada, USA) was advanced into the left locus coeruleus (anteroposterior: –5.50 mm; mediolateral: +0.90 mm; dorsoventral: –3.70 mm). The DREADDs injections were made by pressure with a microinjection pump (RWD Life Science) the pipette was held in place for 15 min after the injection to ensure the tracer absorption into the tissue and reduction of possible spread. Then the electrodes for EMG were implanted as mentioned before, and the experiments were performed after 1 week of recovery. On the next day of the last restraint stress, mice in each group were injected intraperitoneally with CNO (1 mg/kg; Sigma, St. Louis, Missouri, USA) or saline, and the behavioral tests and EMG recordings of masseter muscle were carried out after 30–40 min of CNO administration. Afterward, the mice were perfused and sliced for visualization of the injection sites in locus coeruleus.

Statistical analysis

All experimental data were expressed in mean ± SD and analyzed using SPSS 19.0 statistical software (SPSS Inc., Chicago, Illinois, USA). The first part of the experimental data was analyzed by independent-samples Student's *t*-test; the second part of the experimental data was analyzed by one-way analysis of variance with Tukey's post-hoc test. *P* value less than 0.05 was considered statistically significant.

Results

Chronic restraint stress leads to anxiety-like behavior and enhanced levels of electromyographic activity of the masseter muscle in mice

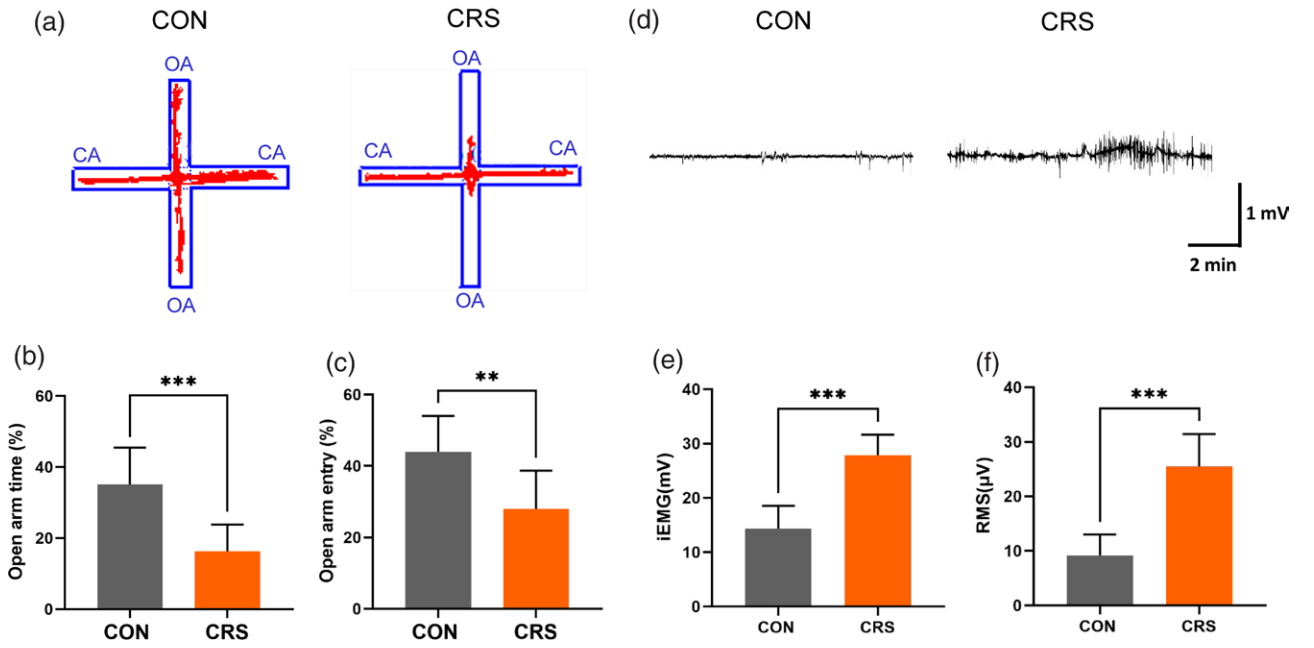
The EPM test was conducted to assess the effects of CRS on the behavioral changes of mice. Figure 1a shows the representative movement trails of mice from CON and CRS groups in the EPM tests. The percentage of entries into open arms and the percentage of retention time in open arms were significantly lower in the stress group than in the control group of mice ($P < 0.001$, $P < 0.01$, Fig. 1b and c), suggesting that CRS could lead to anxiety-like behaviors in mice.

The EMG recording was used to evaluate the activities of the masseter muscle of mice after CRS. Figure 1d shows the representative EMG wave figure of the masseter muscle in mice from CON and CRS groups. After being subjected to 14 days of restraint stress, the levels of iEMG and RMS of masseter muscle of mice in the stress group were significantly higher than those of the control group ($P < 0.001$ and $P < 0.001$, Fig. 1e and f), suggesting that CRS could induce overactivity of masseter muscle in mice.

Chronic restraint stress causes significant activation of locus coeruleus neurons

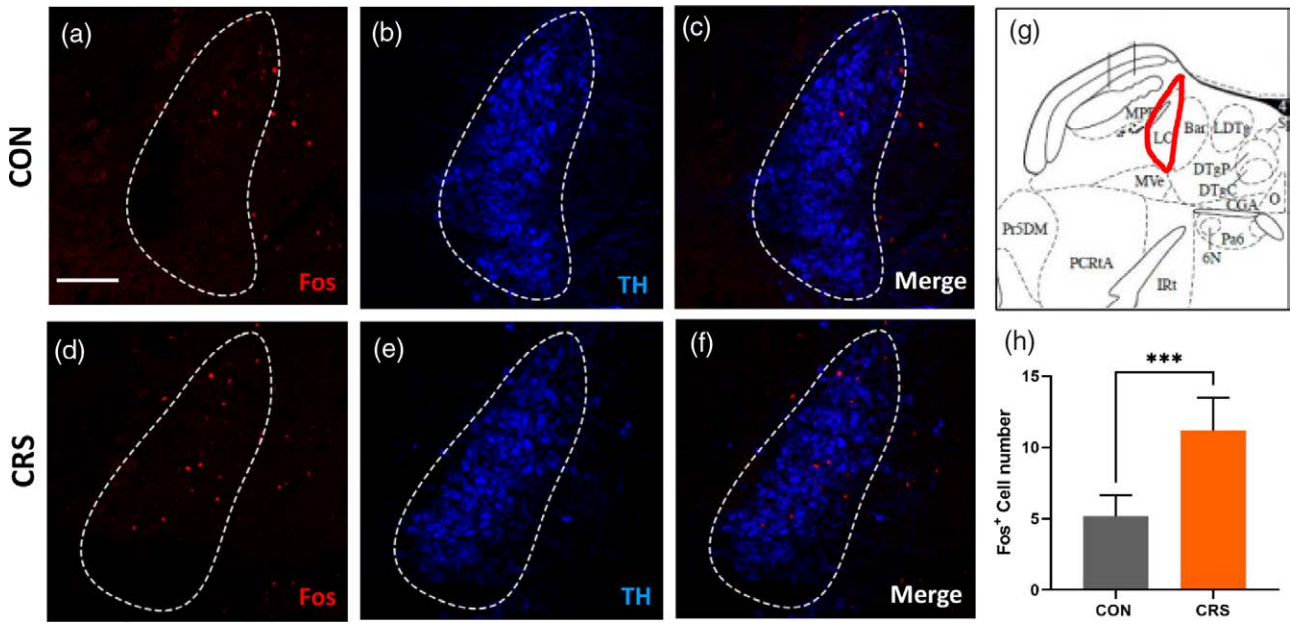
To assess neuronal activation in locus coeruleus, the expression of Fos was observed by immunofluorescence staining. Figure 2a–f shows the immunofluorescence staining results of Fos in locus coeruleus from CON and CRS groups after CRS. Blue fluorescence is shown as tyrosine hydroxylase–positive neurons, which serve as a location marker for locus coeruleus. Red fluorescence shows the nuclei of Fos-positive neurons. Figure 2g refers to the region of staining

Fig. 1



Chronic restraint stress caused anxiety-like behavioral changes and elevated masseter EMG levels. (a) Representative movement trials of mice from the CON and CRS groups in the elevated plus maze test ($n = 8$/group). The percentage of the OAs retention time (b) and OAs entries (c) were significantly different between the CON and CRS groups. (d) Representative EMG wave figures of masseter muscle in mice from CON and CRS groups ($n = 6$/group). The levels of iEMG (e) and root mean square (f) of masseter muscle in CRS mice were significantly higher than those in CON mice. CA, closed arm; CON, control; CRS, chronic restraint stress; iEMG, integral electromyography; OA, open arm. $^{**}P < 0.01$; $^{***}P < 0.001$, independent-samples Student's *t*-test.

Fig. 2



Chronic restraint stress-induced neuron activation in LC. (a–f) Double immunofluorescence of Fos and TH in LC from CON and CRS mice. (g) The region of staining, with the red outline referring to LC. (h) Data analysis showing that the number of Fos-positive neurons in LC from CRS group was significantly higher than that in the CON group ($n = 6$/group). Bar=100 μm. CON, control; CRS, chronic restraint stress; LC, locus coeruleus; TH, tyrosine hydroxylase. $^{***}P < 0.001$, independent-samples Student's *t*-test.

in locus coeruleus (Bregma –5.52 mm) [20]. The number of Fos-positive neurons at the locus coeruleus in the CRS group was significantly higher than that in the CON group ($P < 0.001$, Fig. 2h), suggesting that CRS could lead to significant activation of locus coeruleus neurons.

Inhibition of noradrenergic neuron activity within the locus coeruleus alleviates restraint of stress-induced anxiety behavior

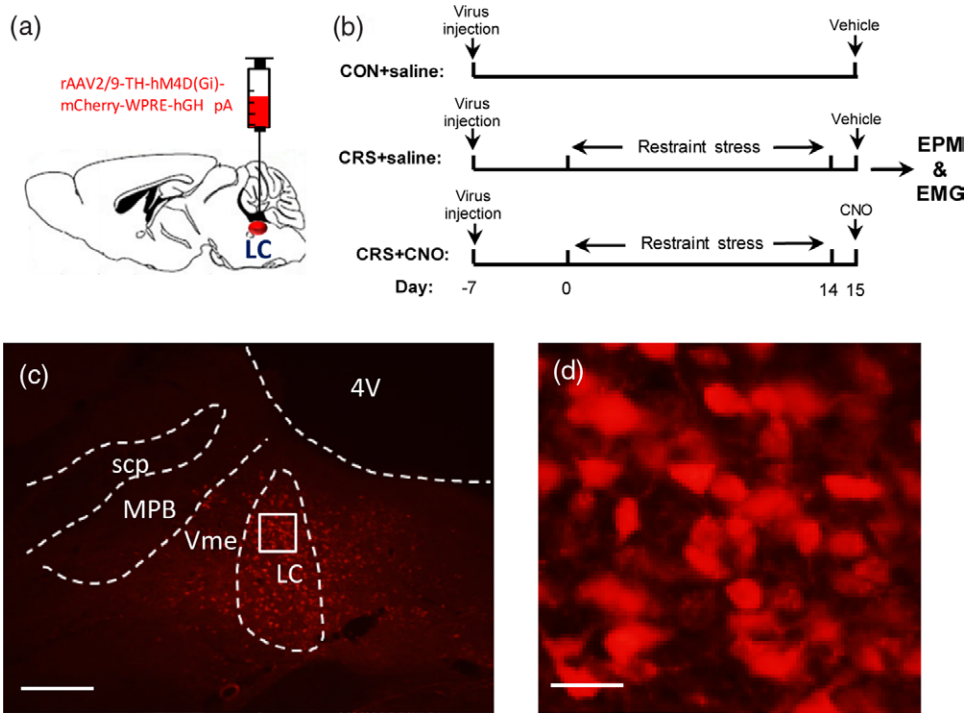
To further verify the role of locus coeruleus in the process of restraint stress-induced overactivity of the masseter muscle, we used the DREADDs method to specifically inhibit the activation of noradrenergic neurons in locus coeruleus (Fig. 3a). Figure 3b shows the procedure of the second part experiment. The validity of the viral vector infection was evaluated by mCherry expression in the injection site (Fig. 3c). Higher-magnification images showed that the DREADDs red fluorescence had been successfully transfected into locus coeruleus neurons (Fig. 3d), which ensured that administration of CNO could effectively inhibit the activation of noradrenergic neurons in the locus coeruleus.

In Figure 4, representative movement trails of mice from behavioral results are shown (Fig. 4a). The mice in the CRS + saline group had a significantly lower percentage of retention time in open arms and percentage of retention time in open arms in the EPM than those in the CON + saline group ($P < 0.001$, $P < 0.001$, Fig. 4b and c), whereas the mice in the CRS + CNO group had a significantly higher percentage of retention time in open arms and the percentage of entries into open arms in the EPM than the mice in the CRS+ saline group ($P < 0.05$, $P < 0.05$, Fig. 4b and c), suggesting that inhibiting the activation of noradrenergic neurons in the locus coeruleus could alleviate the anxiety-like behavior caused by chronic stress.

Inhibition of noradrenergic neuron activity within the locus coeruleus relieves restraint stress-induced masseter muscle overactivity

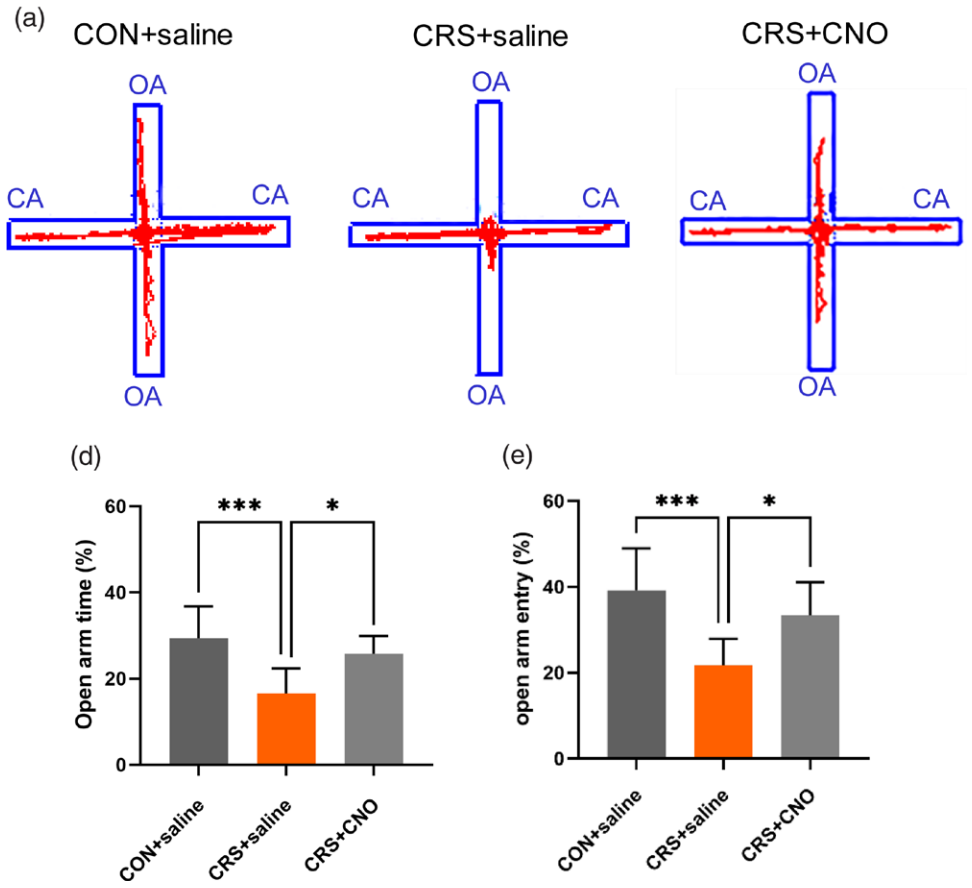
Figure 5 shows the EMG recording results from CON + saline, CRS+ saline, and CRS + CNO groups. The representative EMG wave figure of masseter muscle was shown in Fig. 5a. Statistical analysis revealed that the iEMG and RMS levels of the masseter muscle of the mice in CRS + saline group were significantly higher than those of the CON + saline group ($P < 0.001$, $P < 0.001$, Fig. 5b and c), whereas the iEMG and RMS levels of the masseter muscle of the mice in CRS + CNO group were significantly lower than those of the CRS + saline group ($P < 0.01$, $P < 0.05$, Fig. 5b

Fig. 3



Designer receptors exclusively activated by designer drugs injected into the LC. (a) Virus injection strategy. (b) Timeline for virus injection, restraint stress, and behavioral tests or EMG recording. (c) Injection of viral vectors into the LC. The framed area in (c) is magnified in (d). Successful transfection was morphologically verified by mCherry knocked into the neurons. Bar = 200 μm in (c); bar = 30 μm in (d). CNO, clozapine-n-oxide; CON, control; CRS, chronic restraint stress; EMG, electromyography; EPM, elevated plus maze test; LC, locus coeruleus.

Fig. 4



Inhibition of noradrenergic neuron activity in locus coeruleus alleviates anxiety-like behavior induced by restraint stress. (a) Representative movement trials of mice from the CON + saline, CRS + saline and CRS + CNO groups in the EPM tests ($n = 8$/group). (b and c) The mice in the CRS + saline group had a significantly lower percentage of retention time in OAs and percentage of retention time in OAs than those in the CON + saline group. Mice in the CRS + CNO group had a significantly higher percentage of retention time in OAs and the percentage of entries into OAs than the mice in the CRS + saline group. CA, closed arm; CNO, clozapine-n-oxide; CON, control; CRS, chronic restraint stress; OA, OA, open arm. *$P < 0.05$; ***$P < 0.001$, one-way analysis of variance with Tukey's post-hoc test.

and c). The results suggested that inhibiting the activation of noradrenergic neurons in the locus coeruleus could attenuate the overactivity of masseter muscle caused by restraint stress.

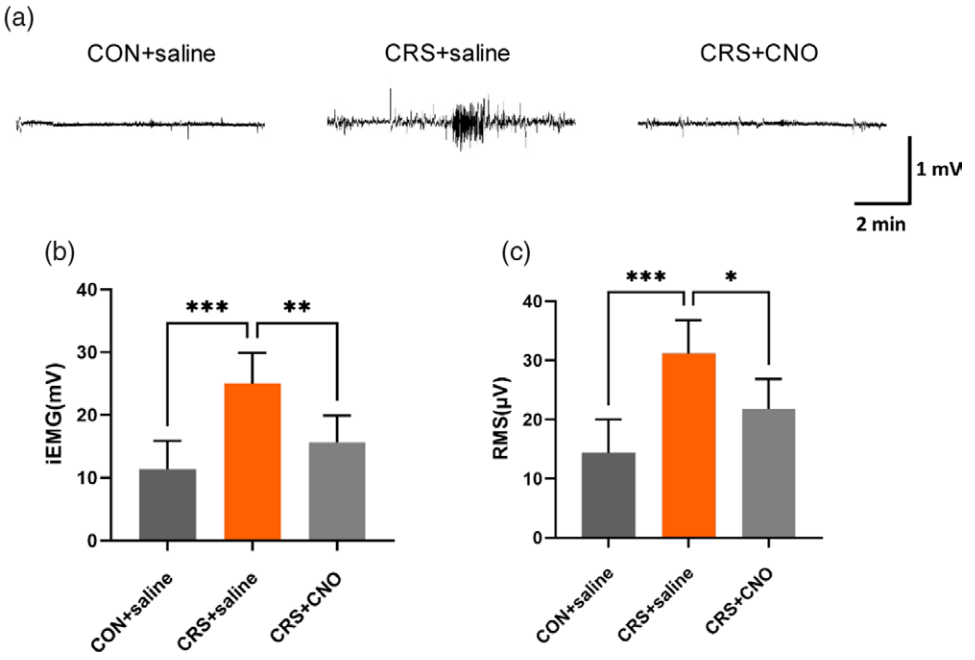
Discussion

Numerous studies have concluded that psychological stress is an important risk factor for the occurrence and development of orofacial muscle pain, which is the most common clinical symptom of TMD [2–5]. Exploring through which central neural mechanisms that psychological stress affects the occurrence and development of TMD is of great significance for dentists to better understand the etiology of TMD and seek more targeted treatment. The present study exhibited that chronic stress could cause significant anxiety-like behavior and overactivity of the masseter muscle, as well as obvious activation of neurons in locus coeruleus. Furthermore, inhibiting the noradrenergic neuron activation in locus coeruleus could reduce the overactivity of masseter induced by chronic stress, indicating that locus coeruleus may play an important role in the overwork of masticatory muscle elicited by chronic stress.

The animal stress model employed in the present study is restraint stress, which can produce physiological and psychological discomfort by restricting animal-free movement to a moderate extent, has been widely used in studies related to psychological stress because of its ease of operation, good reproducibility, and less tolerability [16]. The mice in this study were subjected to CRS for 14 consecutive days, and displayed a significantly reduced retention time and entry number in the open arms in the EPM test, suggesting that CRS could successfully induce anxiety-like emotions in mice, which is also consistent with the results of previous studies [18].

Relevant clinical studies have shown that there is a correlation between anxiety and the tension of the orofacial muscles, which is likely to be an important reason for aggravating

Fig. 5



Inhibition of noradrenergic neuron activity in LC decreased the elevated masseter electromyography level caused by restraint stress. (a) Representative electromyography wave figures of masseter muscle in mice from CON and CRS groups ($n = 6$/group). (b and c) The iEMG and RMS levels of the masseter muscle of the mice in CRS + saline group were significantly higher than those of the CON + saline group. Masseter muscle iEMG and RMS levels of the mice in CRS + CNO group were significantly lower than those of the CRS + saline group. CNO, clozapine-n-oxide; CON, control; CRS, chronic restraint stress; iEMG, integral electromyography; RMS, root mean square. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, one-way analysis of variance with Tukey's post-hoc test.

the symptoms of TMD [4,5]. Therefore, the present study further examined the level of EMG activity of the masseter muscles in mice subjected to stress. According to the anatomical characteristics of the orofacial region of mice and relevant literatures [19], we customized implantable needle electrodes, which could be implanted into the masseter muscles of mice and fixed on the cranial site, and be used for long-time muscle EMG signal acquisition under the awake state. The experimental results revealed that CRS could cause a significant elevation of masseter muscle EMG levels. The masseter muscles are the most important pair of maxillofacial muscles, and their movements are innervated by the trigeminal motor nucleus (Vmo), while the Vme modulates the proprioceptive afferents of the masticatory muscles and sends glutamatergic projections to the Vmo [22]. Previous studies by our research group have demonstrated that CRS leads to elevated excitability of Vme neurons and enhanced Vme–Vmo glutamatergic projections, which is associated with elevated masseter muscle tension due to stress [16]. However, whether the increased excitability of Vme neurons caused by stress is because of the influence of projections from other stress-related nuclei remains to be further explored.

The locus coeruleus, which is immediately adjacent to the medial Vme, has long been considered one of the stress-related centers in the brain, and is thought to be closely related to physiological functions such as attention, arousal, and memory, and mediates the stressful stress response [11,12]. Moreover, noradrenergic neurons in the locus coeruleus are the main site of noradrenaline synthesis within the brain, which is closely related to the stress response, and many psychiatric disorders such as anxiety disorders and depression have dysregulation of the noradrenergic system within the locus coeruleus [23]. Therefore, to confirm whether restraint stress can cause direct activation of locus coeruleus neurons, we examined the expression level of Fos protein, the expression product of the immediate early gene *c-fos*, within the locus coeruleus [24]. Tyrosine hydroxylase is a key enzyme in the process of cellular synthesis of noradrenaline, and therefore we used tyrosine hydroxylase to label locus coeruleus neurons in this study [25]. The results showed that after being subjected to 14 days of stress, the number of Fos-positive cells within locus coeruleus in the CRS group mice was significantly more than that in the control group mice, indicating that restraint stress could lead to significant activation of locus coeruleus neurons.

To verify the contribution of locus coeruleus neuronal activation in the overactivity of masseter muscle induced by stress, we further employed a DREADD approach in this study, which is the most commonly used chemogenetic receptor [26]. In the present study, we chose the inhibitory DREADDs hM4D(Gi) because of the activation of locus coeruleus neurons

after stress. Furthermore, the tyrosine hydroxylase promoter was added into the DREADDs to specifically inhibit noradrenergic neurons in locus coeruleus. After intraperitoneal injection of CNO or saline, the results showed that the mice in the CRS + saline group showed significant anxiety-like changes compared with the CON + saline group, and the anxiety level was significantly attenuated after the administration of CNO; on the other hand, the masseter muscle EMG level of the mice in CRS + saline group was significantly higher than that of the CON + saline group, whereas was significantly decreased after the administration of CNO. These findings further demonstrate that the activation of noradrenergic neurons in locus coeruleus may play an important role in the anxiety-like behavior as well as the elevated masseter muscle tension caused by restraint stress. Anatomically, the locus coeruleus is in close proximity to the Vme and has been demonstrated to send neural projections directly to the Vme [14,15]; and it has been reported that the cell membrane of Vme neurons contains α_{2A}-adrenergic receptors and can be activated by noradrenergic neural projections from the locus coeruleus, which subsequently leads to alterations in the electrophysiological properties of neurons in Vme [15]. Therefore, through the action of the locus coeruleus-Vme neural pathway, the locus coeruleus transmits neuronal activation information to the Vme, which in turn affects the motor status of the masticatory muscles and is likely to be one of the central mechanisms by which noradrenergic neurons in locus coeruleus are involved in the regulation of stress-induced overactivity of the masseter muscle.

In summary, the present study revealed that the activation of noradrenergic neurons in locus coeruleus induced by stress may be one of the central regulatory mechanisms for stress-induced anxiety-like behaviors and overactivity of masseter muscles. These findings provide a novel central mechanism underlying the correlation between psychological factors and TMD.

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Conflicts of interest

There are no conflicts of interest.

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