Received: 2 March 2021 | Revised: 29 April 2021 | Accepted: 18 May 2021

DOI: 10.1111/joor.13211

ORIGINAL ARTICLE

JOURNAL OF ORAL REHABILITATION WILEY

Astrocytes in the rostral ventromedial medulla contribute to the maintenance of oro-facial hyperalgesia induced by late removal of dental occlusal interference

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Funding information
Natural Science Foundation of China, Grant/Award Number: 81970955, 81771096, 81800998 and 81870788; Natural Science Foundation of Beijing, China, Grant/Award Number: 7192231

Abstract

Background: Astrocytes in the rostral ventromedial medulla (RVM) contribute to descending pain modulation, but their role in oro-facial pain induced by persistent experimental dental occlusal interference (PEOI) or following EOI removal (REOI) is unknown.

Objective: To explore the involvement of RVM astrocytes in PEOI-induced oro-facial hyperalgesia or its maintenance following REOI.

Methods: Male rats were randomly assigned into five groups: sham-EOI, postoperative day 6 and 14 of PEOI (PEOI 6 d and PEOI 14 d), postoperative day 6 following REOI on day 3 (REOI 3 d) and postoperative day 14 following REOI on day 8 (REOI 8 d). The nociceptive head withdrawal threshold (HWT) and activities of RVM ON- or OFF-cells were recorded before and after intra-RVM astrocyte gap junction blocker carbenoxolone (CBX) microinjection. RVM astrocytes were labelled immunohistochemically with glial fibrillary acidic protein (GFAP) and analysed semi-quantitatively.

Results: Persistent experimental dental occlusal interference-induced oro-facial hyperalgesia, as reflected in decreased HWTs, was partially inhibited by REOI at day 3 but not at day 8 after EOI placement. Increased GFAP-staining area occurred only in REOI 8 d group in which CBX could inhibit the maintained hyperalgesia; CBX was ineffective in inhibiting hyperalgesia in PEOI 14 d group. OFF-cell activities showed no change, but the spontaneous activity and responses of ON-cells were significantly enhanced that could be suppressed by CBX in REOI 8 d group.

Conclusion: Rostral ventromedial medulla astrocytes may not participate in PEOI-induced oro-facial hyperalgesia or hyperalgesia inhibition by early REOI but are involved in the maintenance of oro-facial hyperalgesia by late REOI.

KEYWORDS

astrocyte, carbenoxolone, dental occlusal interference, oro-facial pain, pain modulation, rostral ventromedial medulla

1 | INTRODUCTION

Oro-facial myofascial pain is common and has considerable socio-economic impact, especially if it is chronic.[1-3] Besides, some patients do not recover from oro-facial myofascial pain following rehabilitative therapies such as modifications to the dental occlusion to remove potential occlusal causes of the pain.[4,5] There is however variability between individuals in their responses to such rehabilitative therapies, and this may complicate clinical management. We previously established a rat model of sustained oro-facial hyperalgesia by the intraoral placement of a crown producing a persistent experimental dental occlusal interference (PEOI)[6] to test effects of removal of the EOI (REOI) at different time points and found that the oro-facial hyperalgesia could be inhibited by REOI when the crown was kept in place for <5 days, but not when it was kept in place for 6 days or longer.[7,8] Therefore, we divided the time course of oro-facial hyperalgesia into three stages: an initial stage (postoperative days 0–6) during which hyperalgesia was initiated and reversible, a chronification stage (postoperative days 7–8) during which hyperalgesia transitioned from being reversible to being irreversible and a maintenance stage (postoperative day 9 and beyond) when hyperalgesia became sustained and irreversible. The possible mechanisms underlying the PEOI-induced hyperalgesia were suggested to include neuronal and glial activation in the medullary dorsal horn (MDH)[9] and potentiation of synaptic transmission in the thalamus to the anterior cingulate cortex (ACC).[10]

Also noteworthy are our recent findings that adaptive neuroplasticity of ON- and OFF-cells in the rostral ventromedial medulla (RVM) participated in the chronification, maintenance and inhibition of the PEOI-induced oro-facial hyperalgesia.[11] The RVM is part of the descending pain modulation system which exerts 'top-down' control on ascending nociceptive transmission.[12-17] The RVM integrates sensory and descending inputs that it receives, and through its projections to the spinal dorsal horn (SDH) and MDH (also known as trigeminal subnucleus caudalis), the RVM conveys both descending facilitatory and inhibitory influences on nociceptive transmission in the SDH and MDH.[12,13,18,19] Increased activity of the RVM's ON-cells and decreased activity of its OFF-cells provide a net pronociceptive influence (descending facilitation prevailing over descending inhibition) that contributes to the persistence of hyperalgesia in peripheral neuropathic[15,20,21] and inflammatory hyperalgesia[18,22,23] models.

There is evidence that activation of astrocytes in the RVM contributes to the enhancement of descending facilitation that occurs in models of neuropathic pain and inflammatory pain, through the involvement of cytokines, as well as glial-neuronal interactions.[24,25] The reciprocal action between astrocytes and neurons involves many cytokines (e.g. TNF-α, IL-1β and related receptors) as well as the gap junctions that exist mainly on astrocytes rather than neurons.[26-29] For example, intrathecal (i.t.) injection of the astrocyte gap junction blocker carbenoxolone (CBX) attenuates nociceptive behaviour and trigeminal central sensitisation produced in functionally identified MDH neurons in models of trigeminal neuropathic pain[30] or inflammatory pain.[31] In view of the evidence that activation of astrocytes in the RVM contributes to pain modulation, it is possible that the modulatory influence of the RVM in PEOI-induced oro-facial hyperalgesia, or in its maintenance despite REOI during the chronification stage, involves not only neurons but also astrocytes. Therefore, we hypothesise that astrocytes may interact with neurons in the RVM and contribute to descending modulation of the PEOI-induced oro-facial hyperalgesia or the maintenance of hyperalgesia following EOI removal in the chronification stage. To address this hypothesis, we developed different oro-facial hyperalgesia models induced by PEOI as well as modified models induced by REOI at different time points to explore the involvement of RVM astrocytes in PEOI-induced oro-facial hyperalgesia or its maintenance following REOI.

2 | MATERIALS AND METHODS

2.1 | Animals

Ninety adult male Sprague Dawley rats initially weighing 180–200 g (Beijing Vital River Laboratory Animal Technology Co. Ltd.) were randomly assigned into different groups by using a simple randomised design. Among them, 35 rats were used for behavioural assessment (15 of these rats were also utilised for immunofluorescent experiments); 20 rats were used for cannula implantation and subsequent CBX microinjection (two of these rats were excluded from data analysis due to complications arising from cannula implantation surgery), and the remaining 35 rats were used for electrophysiological recordings. All rats were housed in individually ventilated cages in the animal facility of Peking University School and Hospital of Stomatology with steady temperature and humidity and under a 12-h light/dark cycle. The food and water were available ad libitum. The project was approved by the Institutional Animal Care and Use Committee of Peking University (IACUC number: LA2019353) and was also in accordance with the guidelines recommended by the International Association for the Study of Pain[32] and the Animal Research: Reporting of In Vivo Experiments.[33] For all the data reported in our study, the observer was blinded to the treatments of rats.

2.2 | Application of PEOI or REOI

The application of PEOI has been previously described in detail[6]; it involved the placement of a crown on the right maxillary first molar (Figure 1A). REOI was applied using a sharp probe to remove the crown (Figure 1B). For the sham-EOI rats, the same procedures were performed without the placement of an actual crown. Based on previous studies,[6-8] REOI 3 d and REOI 8 d (REOI on postoperative days 3 and 8, respectively, after placement of the crown) were chosen to establish hyperalgesia inhibition or maintenance models. Rats were randomly assigned into five groups: sham-EOI, postoperative day 6 of PEOI (PEOI 6 d), postoperative day 6 of REOI 3 d (REOI 3 d),

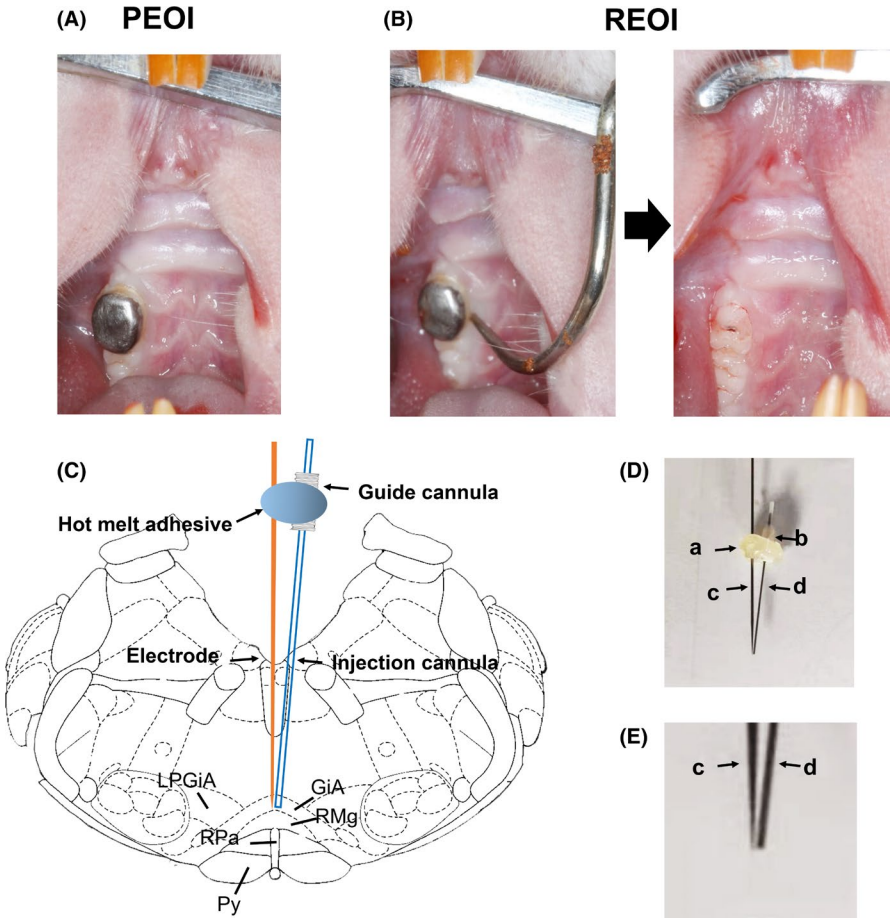

FIGURE 1 The schematic diagrams of PEOI and REOI, as well as the apparatus combining a recording microlectrode and injection cannula. A, Photograph of application of persistent EOI by cementing a crown on the right maxillary first molar (PEOI). B, The process of removing the EOI with a probe (REOI). C, Schematic diagram of the apparatus fabricated with an injection cannula attached to a stainless-steel microelectrode and reconstruction graph of injection sites. D, Image of the combined apparatus. E, Enlarged localised view of the tips of the injection cannula and the microelectrode. a, hot melt adhesive; b, injection cannula; c, microelectrode; d, guide cannula. EOI, experimental dental occlusal interference. PEOI, persistent experimental dental occlusal interference. REOI, removal of experimental dental occlusal interference. RMg, nucleus raphe magnus; GiA, nucleus gigantocellularis pars α; LPGiA, lateral paragigantocellularis nucleus pars α; Py, pyramidal tract.

postoperative day 14 of PEOI (PEOI 14 d) and postoperative day 14 of REOI 8 d (REOI 8 d) groups.

2.3 | Behavioural testing

Measurement of head withdrawal threshold (HWT) in response to stimulation to the belly of masseter on both sides through a modified electronic von-Frey anesthesiometer (BIO-EVF3, Bioseb)[6] was used as the evaluation of nociception in the sham-EOI, REOI 3 d, PEOI 6 d, REOI 8 d and PEOI 14 d rats. Detailed procedures have been described previously.[6] HWTs before and after the administration of CBX were measured in the sham-EOI, REOI 8 d and PEOI 14 d groups (see below) at the following time points: pre-surgery (baseline); pre-drug microinjection (pre), after artificial cerebrospinal fluid (aCSF) microinjection and at 15 and 60 min after CBX microinjection on postoperative day 14. The time points of 15 and 60 min after CBX microinjection were based on the literature[34] and confirmed by preliminary experiments.

2.4 | The immunofluorescent staining

After the HWT measurements, the sham-EOI, REOI 3 d, PEOI 6 d, REOI 8 d and PEOI 14 d rats were anesthetised with an overdose of sodium pentobarbital (100 mg/kg, intraperitoneally) and perfused transcardially with 250 ml of body-temperature normal saline (0.9%), followed by 300 ml precooled 4% paraformaldehyde (Sigma-Aldrich) in phosphate-buffered saline (PBS; pH 7.4). Brainstems (from 1 mm caudal to 4 mm rostral to the obex) were isolated and placed in the same fixative for post-fixation at 4℃ overnight and then dehydrated in 30% sucrose. Twenty-micron-thick brainstem sections were prepared for free-floating immunohistochemical staining. After blocking the sections with 10% normal donkey serum for 2 h, sections were incubated with the primary antibody against glial fibrillary acid protein (GFAP, an astrocyte marker; 1:400, Cell Signaling) for 2 days at 4℃. Brainstem sections were then incubated for 2 h at room temperature in Alexa Fluor 488-conjugated donkey anti-mouse secondary antibody (1:200, Proteintech). The primary antibody was replaced by PBS in selected sections as non-specific background staining. The stained sections were observed via a fluorescence microscope (BX51; Olympus), and the microscopic photographs were captured with a charge-coupled device camera (DP71; Olympus). Astrocytic scoring of activation was based on morphological changes according to well-accepted scoring categories.[35] Semi-quantitative analyses were performed on images digitised with constant exposure time and gain setting. Astrocytes were semi-quantified, in terms of the mean fluorescent intensity and the area of immunofluorescence, by using Image J software (National Institutes of Health). For the

semi-quantification of immunofluorescent staining data, the analyst was blinded to the treatments of rats. All measurements were made from five representative sections per animal with three animals used per group.

2.5 | Cannula implantation and drug microinjection

Cannula implantation surgeries were operated after adaptation and baseline HWT measurements in sham-EOI, REOI 8 d or PEOI 14 d groups. The rat was placed in a stereotaxic apparatus, and a guide cannula (Eicom, CXG-X) was lowered into RVM with the following stereotaxic coordinates: AP: −10.92 mm from bregma; DV: +10 mm from the cranial surface; L: 0 mm from the midline. And the guide cannula was immobilised with dental cement above the bone surface. Then, a dummy cannula (Eicom, CXD-X) was inserted into the guide cannula to preserve the dosing channel until the time of injection. After recovery for 7 days, relevant treatments were applied to these groups and the HWTs were measured before and after drug microinjection on postoperative day 14. Under isoflurane inhalation anaesthesia (4% induction, 1.5%–2% maintenance), either aCSF or CBX (0.5 μg) in a volume of 0.5 μl was delivered with an inserted injection tube (protruding for 0.5 mm after insertion into the guide cannula) that was attached to a Hamilton microsyringe (RWD Life Science Company) via PE-10 polyethylene tubing for 2 min by using a microinjection pump (Model 310 plus, KD Scientific Inc.). A delay period of 2 min before retracting the injection tube was ensured for complete diffusion of the drug. When the experiment was finished, direct blue dye in a volume of 0.5 μl was injected for verification of the injection sites by unaided viewing. CBX (1 μg/μl, Sigma-Aldrich) was diluted in aCSF containing 147 mM NaCl, 3.0 mM KCl, 0.8 mM $MgCl_2$, 1.2 mM $CaCl_2$, 2.0 mM NaH_2PO_4 and 2.0 mM Na_2HPO_4. The dosage and volume were the same as in earlier studies.[22,30,36]

2.6 | Neuron recording and stimulation procedures

The extracellular single-unit electrophysiological recordings of ON- and OFF-cells in the RVM were performed on postoperative day 14 in the sham-EOI and REOI 8 d groups. These rats were anesthetised with urethane (1 g/kg, i.p.) injection and were placed into a stereotaxic apparatus with a feedback-controlled heating blanket to maintain the body temperature. The stereotaxic coordinates used were AP: −9.9 to −11.5 mm from bregma; DV: +9.4 to +10.6 mm from the cranial surface; L: −1.1 to 1.1 mm from the midline. After a small craniotomy and removal of the dura, a stainless-steel recording microelectrode (tip impedance 10–18 MΩ at 1 kHz; A-M Systems) was lowered into the RVM by using an electronic micromanipulator (Model 2662, David Kopf Instrument). During the whole recording period, additional injections of urethane (200–300 mg/kg/h, i.p.) were periodically made to maintain anaesthesia. Neuronal activity was recorded, amplified and displayed on a storage oscilloscope; then, it would be isolated off-line and functionally classified as ON-, OFF- or NEUTRAL-cells.[17] ON-cells respond with a burst-like increase in their firing rates (>20% above baseline level); OFF-cells respond with a temporal decrease or pause in their firing rates (>20% below baseline level), respectively, to noxious peripheral stimulation; NEUTRAL-cells are unaffected (change of the firing rates < 10%) by noxious stimuli with an unclear role in nociception.[22,37-39] Only the ON-cells and OFF-cells whose signal-to-noise ratio of action potentials was greater than or equal to 3:1 were further studied.

The ongoing basal activity of RVM ON-cells or OFF-cell was recorded over a 1- to 2-min period before any stimulation. The mean firing rates during the consecutive 20 s before any stimulation were counted as the spontaneous discharge rates. The responses of RVM neurons were measured by recording the activity change of the neuron in response to a von-Frey filament force of 180 g and pinch with smooth forceps (with a force that can cause pain when applied to the experimenter's skin) applied within the skin overlying masticatory muscles; these stimuli lasted for 3 s (for ON-cells) and 7 s (for OFF-cells), respectively. The von-Frey filaments were applied so that they only exerted mechanical force on superficial tissues, whereas the pinch forceps could stimulate both the superficial and deep tissues.[40] An increase in activity evoked by a mechanical stimulus, defined as the response for ON-cells, was calculated by subtracting the spontaneous discharge rate from the stimulus-evoked discharge rate. A decrease in activity evoked by a mechanical stimulus, defined as the response for OFF-cells, was calculated by subtracting the spontaneous discharge rate from the stimulus-evoked discharge rate. Only the activity of one or two neurons was recorded and studied continuously for each rat.

At the end of the recording, the recording site was marked by an electrolytic lesion (50 μA for 30 s). Animals were perfused with 4% paraformaldehyde to acquire the fixed brainstems, and 50-μm-thick brainstem sections were sliced for subsequent microscopic examination and histological verification of recording sites by referring to a standard brain atlas of the rat.[41]

2.7 | Electrophysiological recordings before and after CBX microinjection in the RVM

An apparatus consisting of an injection cannula attached to a stainless steel recording microelectrode was fabricated (Figure 1C–E). The apparatus was lowered into the RVM without the injection cannula initially. When the tip of the microelectrode was approaching the RVM region, an injection cannula was inserted into the RVM through a modified guide cannula in the apparatus. The tip of the injection cannula and the microelectrode tip were 200–300 μm apart. The apparatus was further lowered by using an electronic micromanipulator (see above) until that an ON-cell was isolated. The neuron's baseline spontaneous activity and responses to a von-Frey filament force of 180 g and pinch stimulation were recorded before the CBX microinjection into the recording site through the injection cannula (see above). The spontaneous activity and responses to von-Frey

filament force of 180 g and to pinch stimulation of the ON-cell were recorded at 5, 15 and 30 min post-CBX microinjection. The choice of these time periods was guided by the literature.[9]

2.8 | Statistical analyses

SPSS v20.0 (SPSS) was employed for statistical analyses. All data were valued as mean ± SEM. Two-way analysis of variance (ANOVA) was executed to compare HWTs of ipsilateral and contralateral masseters among all groups. One-way ANOVA was used to compare immunofluorescent staining area and mean fluorescence intensity of GFAP in all groups. Changes of HWTs of ipsilateral and contralateral masseters in sham-EOI, REOI 8 d and PEOI 14 d groups before and after CBX microinjection were compared by three-way repeated measures ANOVA. All analyses were followed by the post hoc Bonferroni test. The spontaneous discharge rates and variation in responses between the sham-EOI and REOI 8 d groups were compared by independent sample *T* test. All the data were found to be normally distributed by using a single-sample Kolmogorov-Smirnov test (all $p > .05$). Differences were considered significant at $p < .05$.

3 | RESULTS

3.1 | Head withdrawal thresholds following EOI placement and removal

The HWTs of ipsilateral and contralateral masseter muscles showed no significant difference in all groups ($p > .05$; Figure 2). The HWTs evoked by stimulation of the masseter muscles on both sides decreased significantly in REOI 3 d, PEOI 6 d, REOI 8 d and PEOI 14 d rats compared with that in the sham-EOI rats ($p < .01$; Figure 2). The decreased HWTs in REOI 3 d rats were inhibited significantly compared with the HWTs in PEOI 6 d rats ($p < .05$; Figure 2). No significant difference was found in the HWTs between REOI 8 d rats and PEOI 14 d rats ($p > .05$; Figure 2). These results suggest that PEOI-induced oro-facial hyperalgesia reflected in decreased HWT can be partially inhibited by EOI removal on postoperative day 3 but cannot be inhibited by removing the EOI on postoperative day 8.

3.2 | Activation of astrocytes in the RVM following REOI on day 8

We examined whether PEOI or REOI induced astrocyte activation in the RVM area through immunofluorescent labelling with GFAP. Astrocytes exhibited extensive fine processes and were well-spaced in sham-EOI rats. Activation of astrocytes in the RVM was observed in REOI 8 d rats, but no astrocytic activation was apparent in REOI 3 d, PEOI 6 d and PEOI 14 d rats (Figure 3A,B). Semi-quantitative analyses of the GFAP-staining area in the RVM revealed a significant increase in labelled astrocytes in REOI 8 d rats ($p < .05$; Figure 3C,D).

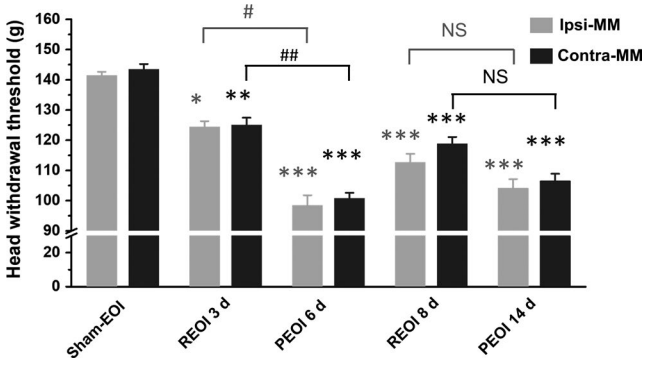

FIGURE 2 The changes in head withdrawal thresholds (HWTs) following EOI placement and EOI removal. The HWTs evoked by stimulation of masseter muscles on both sides decreased in REOI 3 d, PEOI 6 d, REOI 8 d and PEOI 14 d groups compared with sham-EOI group ($n = 7$ for each group). $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, vs. sham-EOI group. # $p < .05$. ##$p < .01$. REOI 3 d group vs. PEOI 6 d group. NS, no significant difference. Two-way repeated measures ANOVA, followed by the post hoc Bonferroni test. EOI, experimental dental occlusal interference. PEOI, persistent experimental dental occlusal interference. REOI, removal of experimental dental occlusal interference. PEOI 6 d or PEOI 14 d, HWT on postoperative day 6 or 14 of PEOI rats. REOI 3 d, HWT on postoperative day 6 of REOI 3 d group (REOI on postoperative day 3). REOI 8 d, on postoperative day 14 of REOI 8 d group (REOI on postoperative day 8). Ipsi-MM, ipsilateral masseter muscle. Contra-MM, contralateral masseter muscle

However, semi-quantitative analyses of the GFAP-staining area in REOI 3 d, PEOI 6 d and PEOI 14 d groups and the mean GFAP-fluorescence intensity in the RVM in all groups of rats showed no significant change ($p > .05$; Figure 3C,D).

3.3 | Maintenance of oro-facial hyperalgesia following REOI on day 8 is inhibited by an astrocyte gap junction blocker CBX

Since the immunofluorescent labelling findings indicated RVM astrocytic activations in REOI 8 d rats but not in other groups, the relationship between the maintenance of oro-facial hyperalgesia following EOI removal on postoperative day 8 and the involvement of RVM astrocytes was further studied. This was done by testing the effects of an astrocyte gap junction blocker CBX on the maintenance of oro-facial hyperalgesia on postoperative day 14 in REOI 8 d and PEOI 14 d rats. The experimental design and timeline are displayed in Figure 4A. The factor of the testing sites showed no significant main effect ($p > .05$). In the sham-EOI rats, no significant change of HWT compared with the baseline level of HWT was observed during the whole experimental process ($p > .05$; Figure 4B). On postoperative day 14 of the PEOI 14 d and REOI 8 d rats, there was no significant change in HWT after aCSF microinjection compared with pre-drug microinjection ($p > .05$; Figure 4B). In contrast, CBX reversed the HWT at 15 min post-microinjection (but not at 60 min post-microinjection) in REOI 8 d rats ($p < .001$, with significant main

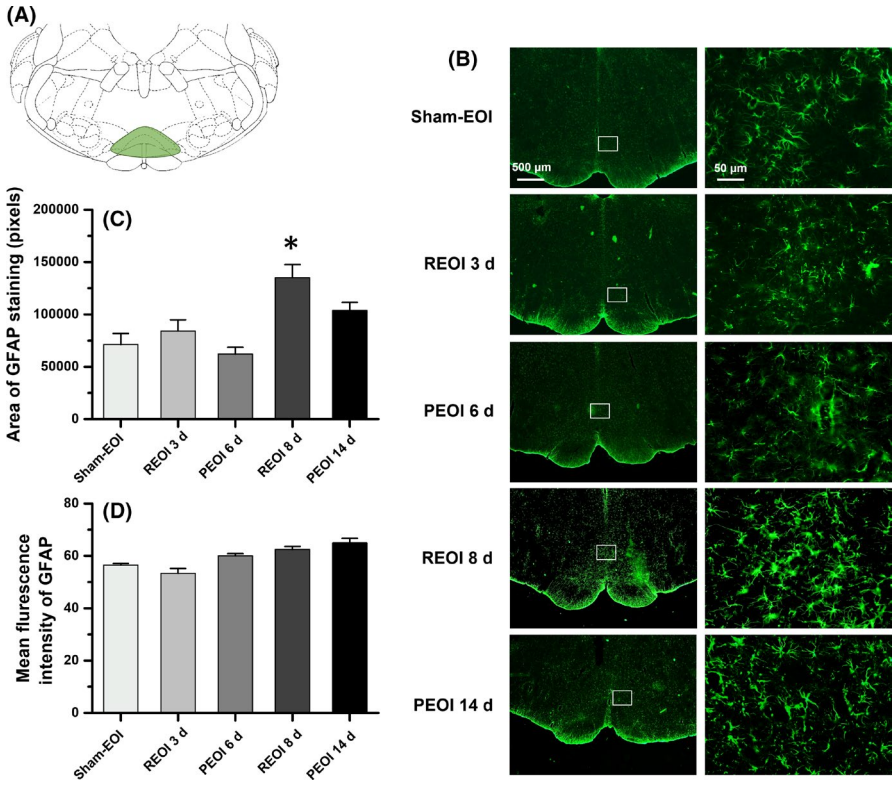

FIGURE 3 Upregulation of GFAP in reactive astrocytes in the RVM following REOI 8 d. A, Schematic diagram of the brainstem section. The region of RVM is illustrated as the green area. B, GFAP-positive cells in the RVM following the placement or removal of EOI. Low magnification photographs and an area of RVM are illustrated in the rectangular zone (left). Scale bar, 500 μm. High magnification photographs (right) of the rectangular areas in the left photographs. Scale bar, 50 μm. Astrocytes exhibited extensive fine processes and were well-spaced in sham-EOI rats. No activation of astrocytes was apparent in REOI 3 d, PEOI 6 d and PEOI 14 d rats. Activation of astrocytes was observed in REOI 8 d rats. C, Semi-quantitative analyses of GFAP-staining area show a significant increase in REOI 8 d rats ($n = 3$ for each group). D, Semi-quantitative analyses of GFAP-mean fluorescence intensity exhibit no significant change in different groups ($n = 3$ for each group). $^*p < .05$. vs. the sham-EOI group. One-way ANOVA, followed by the post hoc Bonferroni test. EOI, experimental dental occlusal interference. PEOI, persistent experimental dental occlusal interference. REOI, removal of experimental dental occlusal interference. PEOI 6 d or PEOI 14 d, GFAP staining or intensity on postoperative day 6 or 14 of PEOI rats. REOI 3 d, GFAP staining or intensity on postoperative day 6 of REOI 3 d group (REOI on postoperative day 3). REOI 8 d, GFAP staining or intensity on postoperative day 14 of REOI 8 d group (REOI on postoperative day 8)

effects as well as the interaction of treatment and time; Figure 4B). However, CBX microinjection had no obvious influence on HWT either at 15 min or at 60 min post-microinjection in PEOI 14 d rats ($p > .05$; Figure 4B).

3.4 | ON- and OFF-cell activity following REOI on day 8 and effect of CBX

Because the astrocytic activation and the inhibition of maintained hyperalgesia by CBX-induced suppression of astrocytes only occurred on postoperative day 14 of REOI 8 d rats, electrophysiological recordings were performed on postoperative day 14 of the REOI 8 d and sham-EOI rats to further explore the involvement of RVM neurons and astrocytes in the maintenance of oro-facial hyperalgesia induced by EOI removal. In total, the activity of 24 ON-cells and 20 OFF-cells was recorded and their recording sites were verified histologically (Figure 5A). ON-cell spontaneous activity and responses (reflected as an increase in activity) to a von-Frey filament force of 180 g and pinch applied to the oro-facial region showed significant increases on postoperative day 14 of REOI 8 d rats compared with those in sham-EOI rats ($p < .05$; Figure 5B–D). For OFF-cells in contrast, their spontaneous activity and responses to the von-Frey filament force of 180 g and pinch (reflected as a decrease in activity) exhibited no apparent change in REOI 8 d rats compared with those in sham-EOI rats (data not shown). In addition, we were able to maintain neuronal recording in a REOI 8 d rat for a sufficient length of time to be able to test also the effect of CBX administration on the activity of one of the ON-cells that had been altered following EOI removal on postoperative day 8. The spontaneous activity of the ON-cell declined, and the neuron also responded with considerably less of an increase in activity in response to a von-Frey filament force of 180 g and pinch stimulation at 15 and 30 min after CBX microinjection (Figure 6).

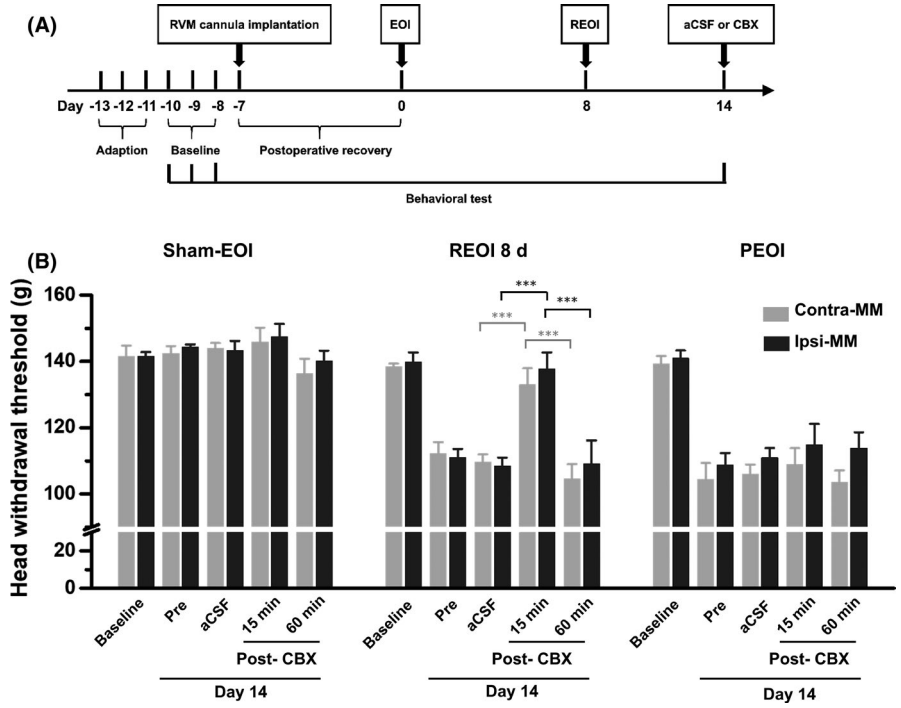

FIGURE 4 Effects of CBX on head withdrawal thresholds (HWTs) on postoperative day 14 following PEOI or REOI 8 d. A, Schematic diagram of the experimental design and timeline of HWT measurements. Adaptation: on days 4, 5 and 6 before the cannula implantation surgery, rats were placed in the testing environment 30 min/day and were comforted in the experimenter's palm. Baseline HWT was acquired by measuring the mean HWT for three consecutive days before any operation. After recovery for 7 days after cannula implantation, EOI was placed and then removed or not removed on day 8. The aCSF or CBX was microinjected into the RVM on postoperative day 14. B, HWTs of ipsilateral and contralateral MMs were measured before surgery (baseline), before microinjection (pre), after aCSF microinjection, and at 15 and 60 min post-CBX microinjection on postoperative day 14 in sham-EOI, REOI 8 d and PEOI groups ($n = 6$ for each group). *** $p < .001$. Three-way repeated measures ANOVA, followed by the post hoc Bonferroni test. RVM, rostral ventromedial medulla. EOI, experimental dental occlusal interference. PEOI, persistent experimental dental occlusal interference. REOI, removal of experimental dental occlusal interference. aCSF, artificial cerebrospinal fluid. CBX, carbenoxolone. REOI 8 d, HWT on postoperative day 14 of REOI 8 d group (REOI on postoperative day 8). Ipsi-MM, ipsilateral masseter muscle. Contra-MM, contralateral masseter muscle; HWT, head withdrawal threshold

4 | DISCUSSION

Oro-facial myofascial pain associated with dental occlusal interference is a common disorder that negatively impacts the lives of affected individuals, especially when the interference becomes persistent and the pain turns chronic.[1,19,42,43] The rat model of PEOI-induced oro-facial hyperalgesia used in this study simulated the chronic myofascial oro-facial pain induced in patients by a dental occlusal interference. Furthermore, the use of the modified models by REOI at different stages in the present study also provided an approach simulating the clinical rehabilitative procedure of removing the dental occlusal interference. This clinically relevant approach allowed for interruption of the EOI-evoked nociceptive afferent inputs into the central nervous system (CNS) by early removal (ie during the initial stage) or by late removal (ie during chronification stage) of the EOI, thereby allowing us to test whether early or late removal of the EOI inhibited the EOI-induced persistent pain state and provided insights into mechanisms that may contribute to the inhibition of the oro-facial hyperalgesia.

While the roles of neuronal circuits in the CNS have long been a focus of studies of mechanisms underlying the expression and modulation of pain, glial cells are increasingly being found to play an important part in persistent as well as acute pain states induced by nerve injury or inflammation.[24,25,28,44] Accumulating evidence indicates that activation of astrocytes in the MDH and SDH constitutes part of the process underlying central sensitisation of ascending nociceptive pathways in acute and chronic pain conditions.[9,19,24,25,28,30,31] Although activation of the descending modulatory system in the CNS that influences nociceptive transmission has been considered to be a result of neuronal plasticity,[13,16,45] we now show evidence that activated astrocytes in the RVM are crucially involved in descending facilitatory influences that contribute to the maintenance of oro-facial pain despite removal of the EOI several days after its placement (during the chronification stage, on postoperative day 8).

The behavioural observations (Figure 2) that removing the EOI at an early stage (ie the initial stage, on postoperative day 3) resulted in the inhibition of oro-facial hyperalgesia, while removing

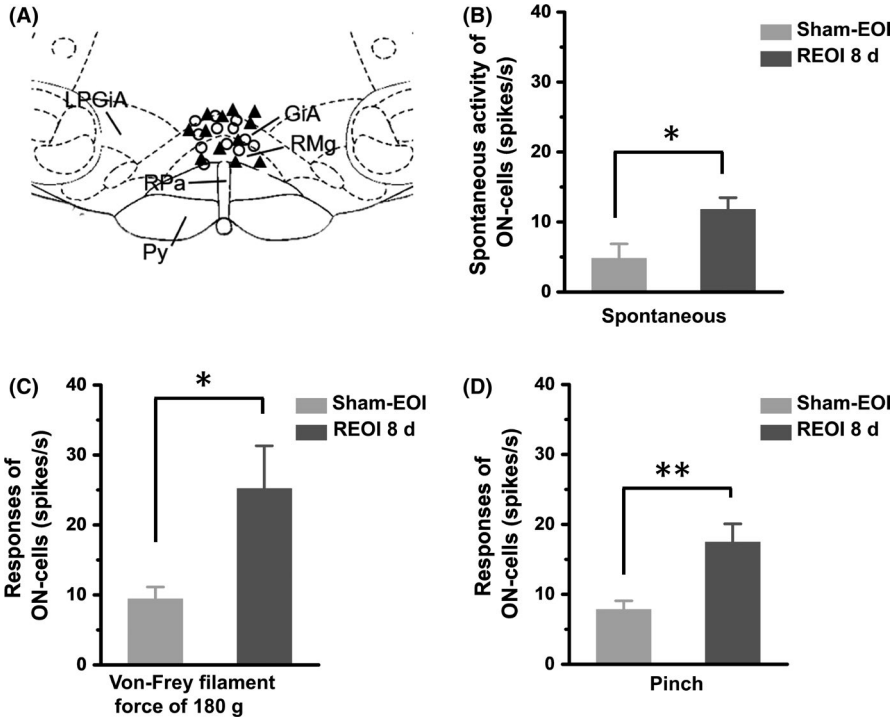

FIGURE 5 Enhanced properties of ON-cells following REOI 8 d. A, Reconstruction graph of histological verification of the sham-EOI (circles) and REOI 8 d (black triangles) groups. Spontaneous activity of the ON-cells (B), the responses (noxious-evoked discharges rates - spontaneous discharge rates) of ON-cells evoked by von-Frey filament force of 180 g (C) and pinch stimulation (D) increased significantly in REOI 8 d rats. $^{*}p < .05$. $^{**}p < .01$. Independent sample *T* test. EOI, experimental dental occlusal interference. REOI, removal of experimental dental occlusal interference. REOI 8 d, spontaneous activity or responses on postoperative day 14 of REOI 8 d group (REOI on postoperative day 8). GiA, nucleus gigantocellularis pars α; LPGiA, lateral paragigantocellularis nucleus pars α; Py, pyramidal tract; RMg, nucleus raphe magnus

the EOI at the later chronification stage (on postoperative day 8) did not inhibit the hyperalgesia revalidated previous findings.[7,8] But in addition, the present study has documented for the first time that astrocytes in the RVM are activated following late EOI removal and may be involved in the maintenance of the oro-facial hyperalgesia. Interestingly, we found no astrocytic activation associated with PEOI in both the early and late stages of the oro-facial hyperalgesia or associated with the early removal of the EOI (Figure 3). This lack of RVM astrocytic activation in the presence of PEOI in the current study contrasts with the reports of RVM astrocytic activation in models of hyperalgesia induced by chronic constriction of the infraorbital nerve[24] and with the PEOI-induced astrocytic activation in the MDH that is accompanied by oro-facial hyperalgesia,[9] both of which lasted from 3 d to at least 28 d. While this latter comparison emphasises the differences between RVM and MDH in the involvement of their resident astrocytes in the modulation of oro-facial hyperalgesia produced by the same type of peripheral manipulation (ie PEOI), the difference between the present study and the earlier RVM study[24] in the occurrence of RVM astrocytic activation is likely due to the different modalities of manipulation. Compared with chronic nerve constriction, the EOI would produce nociceptive afferent inputs that are relatively mild and so may not have been sufficient to induce prolonged astrocytic activation in the RVM with or without EOI removal. The PEOI-induced oro-facial hyperalgesia and its inhibition by early removal of the EOI may be due to changes in peripheral processes and neuronal and glial mechanisms in the MDH, as suggested by previous PEOI studies.[9,46]

Other CNS areas involved in the descending pain-modulatory systems such as the ACC may also have participated in the maintenance of pain on postoperative day 14 induced by PEOI although our earlier study suggests that the ACC may have not been involved in the maintenance of hyperalgesia following late EOI removal on postoperative day 8, which more likely involves mainly the descending facilitatory influence of RVM ON-cells.[10,11] Undoubtedly, there are complex interactions between EOI-related nociceptive inputs and the RVM as well as higher brain areas involved in descending modulation of nociceptive transmission,[12,13,15,18] and further studies are needed to unravel these interactions, including the role of glioplasticity as well as neuroplasticity in the RVM.

Since astrocytes are highly interconnected by gap junctions,[29,31] the present study's investigation of the role of RVM astrocytes in the EOI models also included the possibility that gap junction mechanisms were engaged in maintaining the oro-facial hyperalgesia following the late removal of the EOI. Gap junctions consist of a pair of hemichannels (connexons), and each connexon is composed of six protein subunits called connexins (Cx). Cx43 is the most abundant subunit expressed in astrocytes and indeed is only found in astrocytes.[47] It has been

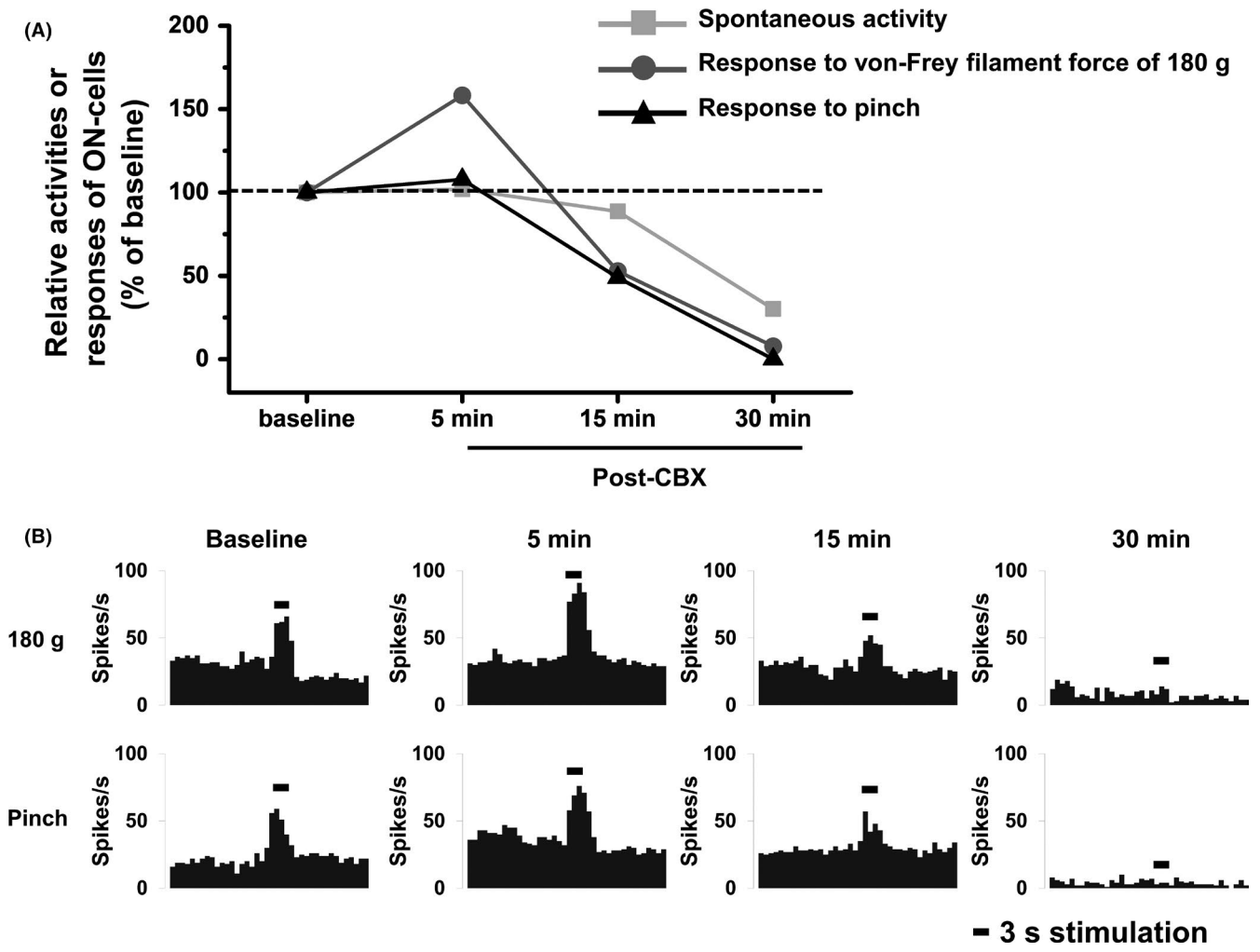

FIGURE 6 An example of the effects of CBX on activities of ON-cell following REOI 8 d. A, Relative changes of spontaneous activity and evoked responses (noxious-evoked discharge rates - spontaneous discharge rates) to von-Frey filament force of 180 g and pinch stimulation of an ON-cell before-microinjection (baseline) and at 5, 15 and 30 min post-CBX microinjection in the RVM. These features declined at 15 and 30 min post-CBX microinjection. B, Representative frequency histograms showing the changes of the ON-cell spontaneous activity and evoked responses (reflected as an increase in activity) by von-Frey filament force of 180 g or pinch stimulation (lasting for 3 s) before-microinjection (baseline) and at 5, 15 and 30 min post-CBX microinjection. Since no effect of the vehicle (aCSF) was observed in the HWT measurement on postoperative day 14 in REOI 8 d rats (see text), aCSF was not applied during the neuronal recording. Each histogram represents neuronal activity over 40 s period. EOI, experimental dental occlusal interference. REOI 8 d, spontaneous activity or responses on postoperative day 14 of REOI 8 d group (removal of EOI on postoperative day 8). RVM, rostral ventromedial medulla. CBX, carbenoxolone

well-documented that CBX can potently block Cx43 gap junctions and hemichannels, and thus, CBX is considered an astrocyte gap junction blocker.[31,48] The present study found that the intra-RVM microinjection of CBX did not affect oro-facial hyperalgesia associated with a persistent EOI but did inhibit the maintenance of oro-facial hyperalgesia following the late removal of the EOI (on postoperative day 8, see Figure 4). This novel finding provided further evidence that astrocytes may play an important role in the maintenance of oro-facial hyperalgesia after the late EOI removal but have no obvious impact on oro-facial hyperalgesia when a persistent EOI is present. The activity of astrocytes can be markedly increased through astrocyte-astrocyte gap junction connections in pathological pain states, and this process can be selectively blocked by CBX.[31,48] Astrocytic activation and upregulation of the Cx43 gap junction protein occur in the SDH following neuropathic injuries and these effects can be relieved by CBX.[34,48] CBX-induced blockade of Cx43 gap junctions in the MDH alleviates trigeminal nerve injury-induced oro-facial hypersensitivity and central sensitisation in the MDH.[30,31]

Although no studies have yet addressed whether upregulation of Cx43 gap junctions occurs in the RVM following the late EOI removal or whether deactivation of astrocytes in the RVM occurs after CBX microinjection, the present study has documented that intra-RVM CBX microinjection to block RVM gap junctions did inhibit the maintained oro-facial hyperalgesia following the late EOI removal on postoperative day 8 as well as suppress the spontaneous activity and responses of an ON-cell tested with intra-RVM CBX (Figures 4 and 6). Our electrophysiological recordings revealed that the spontaneous activity and response features of ON-cells in the

RVM were enhanced following the late EOI removal (on postoperative day 8, Figure 5). Interestingly, the activities of OFF-cells were not affected following REOI 8 d, possibly because the EOI-induced nociceptive inputs only persisted for 8 days and were insufficient to change OFF-cell activity. These findings for ON-cells and OFF-cells are consistent with earlier reports of a net pronociceptive influence exerted by sensitised pain-modulating neurons in the RVM following peripheral nerve injury[21] peripheral neuropathy[20] or inflammation.[18] Nonetheless, the findings also raise the following intriguing questions related to neuronal and astrocytic interactions in the RVM in response to removal of a nociceptive input producing hyperalgesia. Is it possible that the ON-cell enhanced excitability occurring after EOI removal involved the astrocytic activation that was occurring at this time? Astrocytic processes have been shown to contribute to neuronal hyperexcitability in other CNS areas, such as the MDH[30,31,49] or SDH[27,48] and hippocampus,[50] and so activated RVM astrocytes may have contributed to the ON-cell hyperexcitability. Another question is what processes in the RVM underlie our CBX-related finding indicating that an astrocytic-dependent enhanced descending facilitation from the RVM may be involved in the maintenance of oro-facial hyperalgesia after EOI removal? CBX may inhibit astrocytic-cytokine-neuronal interactions in the RVM[24] and thereby suppress the activation of ON-cells, and so the CBX finding may have involved such interactions resulting in inhibition of the oro-facial hyperalgesia. However, whether cytokines in the RVM are involved in the astrocytic-dependent RVM facilitatory influence on oro-facial hyperalgesia is not yet clear. Also unclear is what accounts for the activation of RVM astrocytes only after EOI removal in the later stage? Perhaps the persistent EOI-induced nociceptive afferent inputs suppress RVM astrocytic activation and that this activation can only be expressed once these inputs are eliminated by removal of the EOI. Further studies are needed to address these questions bearing on the mechanisms underlying the interactive role of neurons and astrocytes of the RVM in the descending control of oro-facial pain. It is also important for future studies to investigate the activity patterns of ON- and OFF-cells in the RVM and the role of RVM astrocytes in other oro-facial pain models, as well as the role in EOI-induced oro-facial hyperalgesia of other CNS areas that are components of the descending pain-modulatory system.[13,14,16]

Some limitations of the present study should also be noted. We also did not show directly that PEOI placement, or its removal, was accompanied respectively by increased or decreased nociceptive afferent inputs. Nevertheless, these changes are likely to have occurred since a previous study demonstrated that EOI can induce increased expression in the trigeminal ganglion of some cellular receptors associated with nociception.[46] In addition, since the present study only investigated male rats, future studies are needed to test whether there are sex differences in the RVM astrocytic influences in this model.

In summary, the present study has provided the first demonstration that astrocytes in the RVM in male rats may not be involved in either PEOI-induced oro-facial hyperalgesia or in the resolution of hyperalgesia by early EOI removal, but that activated astrocytes in the RVM do participate in the maintenance of oro-facial hyperalgesia following late EOI removal, likely by promoting descending facilitation from the RVM. The findings suggest that oro-facial discomfort due to a dental occlusal alteration should be treated as early as possible in order to minimise the possibility of the development of a chronic pain state. Furthermore, given the involvement of RVM astrocytes in the maintenance of the oro-facial hyperalgesia and neurons in the RVM, they might be future therapeutic targets for the development of novel clinical approaches to manage chronic oro-facial pain.

ACKNOWLEDGEMENTS

This research was supported by the Natural Science Foundation of China (No. 81970955, Y. Cao; No. 81771096, Q.-F. Xie; No. 81800998, X.-X. Xu; No. 81870788, K.-Y. Fu) and the Natural Science Foundation of Beijing, China (No. 7192231, Y. Cao).

CONFLICT OF INTEREST

All authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

S.-Y. Mo: Conceptualisation, Methodology, Investigation, Validation, Visualisation, Formal analysis, Data curation and Writing—original draft. S.-S. Bai: Methodology, Investigation, Data curation, Formal analysis and Writing—Reviewing and editing. X.-X. Xu and Y. Liu: Methodology, Investigation, Data curation and Formal analysis. K.-Y. Fu: Funding acquisition, Formal analysis and Supervision. Barry J. Sessle: Formal analysis, Supervision and Writing—Reviewing and editing. Y. Cao: Funding acquisition, Conceptualisation, Methodology, Supervision, Investigation, Resources, Data curation and Writing—Reviewing and editing. Q.-F. Xie: Funding acquisition, Conceptualisation, Methodology, Supervision, Resources, Data curation and Writing—Reviewing and editing. All the authors approved this work for publication.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/joor.13211.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Mo S-Y, Bai S-S, Xu X-X, et al. Astrocytes in the rostral ventromedial medulla contribute to the maintenance of oro-facial hyperalgesia induced by late removal of dental occlusal interference. *J Oral Rehabil*. 2022;49:207–218. https://doi.org/10.1111/joor.13211