Signaling Interaction between Facial and Meningeal Inputs of the Trigeminal System Mediates Peripheral Neurostimulation Analgesia in a Rat Model of Migraine

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Abstract—Peripheral neurostimulation within the trigeminal nerve territory has been used for pain alleviation during migraine attacks, but the mechanistic basis of this non-invasive intervention is still poorly understood. In this study, we investigated the therapeutic role of peripheral stimulation of the trigeminal nerve, which provides homosegmental innervation to intracranial structures, by assessing analgesic effects in a nitroglycerin (NTG)-induced rat model of migraine. As a result of neurogenic inflammatory responses in the trigeminal nervous system, plasma protein extravasation was induced in facial skin by applying noxious stimulation to the dura mater. Noxious chemical stimulation of the dura mater led to protein extravasation in facial cutaneous tissues and caused mechanical sensitivity. Trigeminal ganglion (TG) neurons were double-labeled via retrograde tracing to detect bifurcated axons. Extracellular recordings of wide dynamic range (WDR) neurons in the spinal trigeminal nucleus caudalis (Sp5C) demonstrated the convergence and interaction of inputs from facial tissues and the dura mater. Peripheral neurostimulation of homotopic facial tissues represented segmental pain inhibition on cephalic cutaneous allodynia in the migraine model. The results indicated that facial territories and intracranial structures were directly connected with each other through bifurcated double-labeled neurons in the TG and through second-order WDR neurons. Homotopic stimulation at the C-fiber intensity threshold resulted in much stronger inhibition of analgesia than the same intensity of heterotopic stimulation. These results provide novel evidence for the neurological bases through which peripheral neurostimulation may be effective in treating migraine in clinical practice. © 2020 The Authors. Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Key words: peripheral neurostimulation, migraine, trigeminal nerve, wide dynamic range neuron, spinal trigeminal nucleus caudalis, meninges.

INTRODUCTION

Migraine is a common neurological disorder that is estimated to affect 15–18% of the population in the United States (Lipton et al., 2001) and 9.3% of the population in China (Yu et al., 2011). Patients suffering from migraine often experience hypersensitivity with spontaneous pain, hyperalgesia, and cephalic cutaneous allodynia during disease onset (Mathew et al., 2004). Despite the high prevalence of migraine, drugs used as prophylactics are generally far from ideal due to undesirable side effects (Goadsby et al., 2002).

In recent decades, there has been a growing interest in the use of neurostimulation methods as migraine interventions (Magis and Schoenen, 2012). Progress has been driven by technological advances and the relative paucity of effective pharmacological interventions for migraine. Different from lesional procedures such as deep brain stimulation, non-invasive neural stimulation treatments, including transcatheter peripheral nerve stimulation, transcranial magnetic stimulation, supraorbital nerve stimulation, or electroacupuncture, have been widely used to treat different migraine subtypes in clinical practice (Linde et al., 2009; Vaisman et al., 2012; Schoenen et al., 2013; Ulloa et al., 2017; Zhao et al., 2017). Similar to surgical anesthetics that inhibit neuronal signals to decrease pain, these therapeutic methods similarly interfere with neuromodulatory neuronal efferent networks. However, the mechanisms underlying the efficacy of peripheral neurostimulation techniques for treating migraine are still being investigated (Yang et al., 2011; Magis and Schoenen, 2012).
Current evidence indicates that migraine pain arises from nociceptors in intracranial vessels and sinuses (Blau, 1978; Olesen and Edvinsson, 1997). Activation of these intracranial structures (including the dura mater, pia, arachnoid, and dural blood vessels) via mechanical, electrical, or chemical stimulation in different animal models gives rise to headache phenotypes that are remarkably similar to the referred pain of migraineurs (Ellrich et al., 1999; Messlinger and Ellrich, 2001). The trigeminal ganglion (TG) is proposed to be a migraine pain amplifier that detects nociceptive signals carried from peripheral terminals by C- and Aδ-fibers (Edvinsson et al., 2018). Strong, and long-lasting peripheral nociceptive inputs also lead to central sensitization of second-order trigeminovascular neurons in the spinal trigeminal nucleus caudalis (Sp5C) and upper cervical spinal cord, which together are known as the trigeminocervical complex. This central sensitization is considered to be the pathological mechanism underlying the transition of migraine onset to a chronic state (Bartsch and Goadsby, 2003; Noseda and Burstein, 2013). The convergence of nociceptive inputs in the TG and Sp5C from intracranial structures and extracranial tissue contributes to the craniofacial referred pain syndromes that occur during migraines.

As peripheral nerve stimulation of facial tissues and occipital muscles occurs via the sameafferent pathway as migraine, we attempted to use a rat model of migraine induced by nitroglycerin (NTG) to clarify the analgesic effects of neurostimulation. We investigated: (i) the interaction between inputs from facial tissues and intracranial afferents by assessing neurogenic inflammation and cutaneous mechanical thresholds, (ii) the double-labeled neurons in the TG with bifurcated afferents that are responsible for axon reflexes between extra- and intracranial structures, (iii) the ongoing activity of wide dynamic range (WDR) neurons in the Sp5C under both physiological and pathological conditions, and (iv) the analgesic effects of peripheral neurostimulation on behavioral cephalic mechanical allodynia in migraine model rats.

**EXPERIMENTAL PROCEDURES**

**Animals**

Male Sprague Dawley rats with body weight ranging from 280 to 310 g were obtained from the Laboratory Animal Center of China Academy of Military Medical Sciences [license number: SCXK-(Military)-2012-0004]. This study was carried out in accordance with the recommendations of the Guideline on the Humane Care and Use of Laboratory Animals issued by the Ministry of Science and Technology of the People’s Republic of China in 2006. The protocol was approved by the Institutional Animal Welfare and Use Committee of the Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences (no. 20170109). Rats were kept in an animal house maintained at 21 ± 2 °C under alternating 12-h cycles of dark and light. Food and water were available *ad libitum*. During testing, the core body temperature of the animals was maintained at 37.0 ± 0.5 °C by a feedback-controlled electric heating blanket (H-KWDY-III; Nanjing Xin Xiao Yuan Biotech, China). All efforts were made to minimize the number of animals used and mitigate their suffering.

**Meningeal plasma protein extravasation observation**

Rats were deeply anesthetized with pentobarbital (50 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO, USA) and then placed on a heating blanket. The hair on the face was carefully removed with a Pet Trimmer (CP-5000, Shenzhen Codos Electrical Appliances Co., Shenzhen, China) to facilitate further observation. Care was taken not to cause any injury to the skin before and during the procedure. Next, 5% Evans Blue (EB) dye (50 mg/kg; 50 mg/ml in saline, Sigma-Aldrich) was injected through the caudal vein. Two stainless electrodes spaced approximately 1.0 cm apart were inserted into stimulation sites, and 3.0 mA electrical stimuli comprising square waves of 0.6 ms duration were generated by a Constant Current Isolated Stimulator (DS3; Digitimer Ltd., Welwyn Garden City, UK) and delivered at a frequency of 15 Hz for 40 min through stimulating electrodes on the upper eyelid (innervated by the ophthalmic nerve) of rats in the experimental group (*n* = 10 rats), or the anterolateral side of the hindlimb (innervated by the peroneal nerve) of rats in the control group (*n* = 6 rats). After delivering the electrical stimuli, animals were transcendicularly perfused with a prewash of saline for 2 min to flush all of the blood out of the intravascular compartment without removing the extravagated dye (Markowitz et al., 1987). The hair on the head was then removed with depilatory cream (Veet, Reckitt Benckiser Group, Slough, UK) to allow visual inspection for EB dye spots. The brain and dura mater were dissected out, and the specimens were fixed in paraformaldehyde (PFA) for another 2 h to prepare them before photos were acquired with a digital camera (Canon, Tokyo, Japan).

**Plasma extravasation observation and mechanical sensitivity detection in facial tissues**

Rats were tested for their withdrawal response to mechanical stimulation on EB and control spots with an electronic von Frey aesthesiometer (model no. 2390; IITC Life Science, Woodland Hills, CA, USA). The apparatus was supplied with a plastic rigid tip coupled with a force transducer. Briefly, under pentobarbital anesthesia (50 mg/kg, i.p.; Sigma-Aldrich), the hair of the animal was carefully shaved with the Pet Trimmer. A 5 mm diameter rounded cranial window was drilled into the parietal bone to expose the superior sagittal sinus while leaving the dura mater intact (Paxinos and Watson, 2005). A small piece of Spongostane (Xiangen Medical Technology Development Co., China) with 2 μl 15% mustard oil (Sigma-Aldrich) was applied to the superior sagittal sinus of rats in the experimental group (*n* = 8 rats) for 30 min immediately after EB dye injection. For the control group (*n* = 3 rats), saline was applied in place of mustard oil using the same protocol. In both groups, the exposed cranial window of each animal was carefully sealed with Parafilm (Parafilm “M”; American National
Can, Chicago, IL, USA). The locations of observed EB extravagate spots on facial tissues were recorded and used to locate the corresponding control spots, contralateral to the EB spots. At 5 h after anesthesia application, the mechanical force required to elicit an avoidant withdrawal of the head was recorded for five stimulus presentations at approximately 1-min intervals. EB leakage sometimes was due to a lack of care in carrying out the procedure, and the relevant dye spots were excluded from further counting and from the mechanical sensitivity evaluation. The mean values of the five readings of von Frey withdrawal threshold (VFT) were used for analysis.

Retrograde tracer injections and animal treatment
Fluoro-Gold (FG; Biotium, Fremont, CA, USA) and cholera toxin subunit B (CTB; List Biological Labs, Campbell, CA, USA) were used to visualize the sensory innervation of the ipsilateral dura mater and facial tissues. A total of five Sprague Dawley rats were used for the retrograde tracing experiments involving application of FG to facial tissues and CTB to the dura mater. Under anesthesia with pentobarbital (30 mg/kg, i.p.; Sigma-Aldrich), 2% FG was injected subcutaneously into three points (2 μl per point) corresponding to the territories innervated by the ophthalmic (V1), maxillary (V2), and mandibular (V3) branches of the trigeminal nerve in the rat head (Fig. 2A), while 2 μl 1% CTB was administered to the dura mater in the same rat, close to the middle meningeal artery (MMA) (Fig. 2C). The experimental procedures for the application of tracer to the dura mater were carried out as described previously (Liu et al., 2008). Briefly, rats were mounted in a stereotaxic holder (Narishige, Tokyo, Japan), and a small craniotomy was performed at the temporal region to expose the MMA (white arrow in Fig. 2C). Next, 2 μl 1% CTB was applied to the central part of the bone window with a Hamilton syringe and covered with a piece of Spongostane (Xiangen Medical Technology Development Co.). A piece of Parafilm “M” (American National Can) was used to cover the bone window, which was sealed with bone wax to prevent contamination of surrounding tissues.

Tissue preparation and immunohistochemistry
Five days after application of FG and CTB application, rats were deeply anesthetized with pentobarbital (50 mg/kg, i.p.; Sigma-Aldrich) and transcardially perfused with 4% PFA. The TG ipsilateral to the side of tracer application was dissected out and post-fixed in 4% PFA for 2 h and then immersed in 25% sucrose overnight. Serial horizontal sections of TG were cut at a thickness of 30 μm on a cryostat (Thermo Fisher Scientific, Waltham, MA, USA) and mounted on gelatin-coated glass slides (Electron Microscopy Sciences, Hatfield, PA, USA). Based on the methods described in a previous study (Brumovsky et al., 2012), serial sections of the TG were cut and mounted on four gelatin-coated glass slides. Every one in four slides (one or two per animal) was chosen for immunohistochemistry to reveal FG- and CTB-labeling. Sections were pre-incubated 1 h in 3% normal donkey serum and then incubated in rabbit anti-FG (1:1000; Chemicon, Temecula, CA, USA) and goat anti-CTB (1:1000; List Biological Labs) in phosphate-buffered saline containing 1% Triton X-100 (PBS-T) overnight at 4 °C. After three rinses in PBS, the sections were incubated for 1 h in Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody (1:500; Molecular Probes, Eugene, OR, USA) and Alexa Fluor 594-conjugated donkey anti-goat secondary antibody (1:500; Molecular Probes) following further rinses in PBS. Finally, the sections were air-dried and coverslipped with 50% glycerin to improve visualization of labeling.

Labeled neuron observation and cell counting
Images of TG sections were captured using a laser-scanning confocal microscope (FV1200; Olympus, Tokyo, Japan). The numbers of FG- and CTB-labeled neurons in the TG were counted in the five rats that had FG applied to facial tissues and CTB to dura mater. Six images (630 × 630 μm²) were used from each rat for cell counting. Whole images for the TG sections were acquired and composed using a BX53 microscope equipped with DP73 camera (Olympus).

Electrophysiological recording in the Sp5C
The convergence of dural and facial afferent inputs onto neurons was investigated by extracellular recording of Sp5C neurons in male rats. After anesthesia with 10% urethane (1.2g/kg, i.p.; Sigma-Aldrich), rats were fixed onto a stereotaxic frame (Narishige) and secured with auricular bars. The left common carotid artery was cannulated for arterial pressure monitoring. Additional anesthetic (urethane 0.3g/kg, i.p.) was given if the animal showed large fluctuations in baseline arterial pressure, an increased heart rate, or a withdrawal response after pinching the paw. Heart rate was continuously monitored and maintained at 330–460 beats/min. After tracheal cannulation, animals breathed spontaneously, and their core temperature was maintained at 37.0 ± 0.5°C by a feedback-controlled electric heating blanket. An incision was made along the midline of the scalp, extending caudally to the cervical segments C2–3. The skin and periosteum were retracted and fixed with thread to expose the skull. The atlanto-occipital ligament including the underlying spinal dura was cut to expose the medulla oblongata. Then, to expose the dural receptive field (RF) and apply stimulation, a craniotomy of the parietal bone was made from bregma to lambda (Paxinos and Watson, 2005) on both sides using a drill that was continuously cooled with saline (Fig. 3A). Special care was taken to avoid lesioning the exposed dura mater and to prevent bleeding from dural blood vessels. During the procedure and throughout the experiment, the dura mater was protected from drying with warm liquid paraffin (Sigma-Aldrich). A total of 35 rats were ultimately used in this experiment.

Identification of neurons and RF mapping
To minimize vibration, the entire preparation (including the micromanipulators and stereotaxic frame holding the
animal) was placed onto an air pressure-supported table. Neurons within the Sp5C were characterized according to their facial cutaneous and deep RFs. A tungsten electrode (impedance 8 MΩ; FSH, Bowdoin, ME, USA) was first zeroed on the obex for subsequent calculations of antero-posterior and lateral recording coordinates. Recording tracks were positioned 0–2.5 mm posterior and 1.6–2.5 mm lateral to the obex using a micromanipulator (Narishg). The facial RFs were assessed in all three territories of the trigeminal nerve for both non-noxious and noxious stimuli in each rat. Non-noxious stimulation was applied to the RF by gently touching the facial tissue with von Frey filaments (model no. 2390, IITC Life Science). Units were also tested for corneal input by mechanical stimulation using a 1.0-g von Frey filament (IITC Life Science). Noxious mechanical stimulation consisted of pinching the facial tissue with blunt forceps, as previously described (Chiang et al., 1994; Shimizu et al., 2000).

The A- and C-fiber thresholds (Ta, Tc) were defined as the stimulation intensities required to evoke neuronal activity with a conductive velocity of >2 m/s or 0.5–2 m/s respectively (Burstein et al., 1998). They were calculated according to the distances between the stimulus and recording sites, divided by a fixed latency (Meng et al., 1997). Electrical stimulation of 2.5 times Tc was applied to the facial RF in this part of the experiment.

According to facial RF properties, neurons that responded to both non-noxious and noxious stimuli were classified as WDR neurons and were selected for further studies. Additionally, neuronal activity modulation was also tested by applying a noxious pinch to the hindpaw to exclude neurons belonging to the subnucleus reticularis dorsalis, located medial to the Sp5C (Villanueva et al., 1996).

When a WDR neuron sensitive to cutaneous stimulation of the trigeminal nerve was identified, it was tested for convergent input from the dura mater. The position and size of dural RFs were determined with 4.0-g custom-designed filaments. The mechanical stimulation applied to the dura mater for neuron identification was sufficient to activate C-fibers. The neuronal response to repeated non-noxious stimulation consisted of pinching the facial tissue with blunt forceps, as previously described (Chiang et al., 1994; Shimizu et al., 2000).

The protocols and status assessments used for cephalic cutaneous mechanical sensory testing are similar to previous studies (Oshinsky and Gomonchareonsiri, 2007; Oshinsky et al., 2012; Boyer et al., 2014, 2017; Dallel et al., 2018). Behavior testing was performed between 9:00 AM and 17:00 PM. Thirty experimental animals were acclimatized in a plastic holding restraint for 15 min per day over a 3-days training period before testing. This plastic tube restraint allowed the rats to turn around their head and escape the stimulus (Fig. 6B). Cephalic withdrawal thresholds were determined using an electronic von Frey anesthesiometer (model no. 2390; IITC Life Science, USA) fitted with a supplied rigid tip (0.8 mm diameter) applied to the midline of the forehead between the eyes (Dallel et al., 2018). The same volume of 0.9% saline was injected as control in the same animal 30 min before NTG administration. WDR neuron responses were continuously monitored during and after saline and NTG injection. A > 125% increase in discharge frequency of WDR neurons after saline or NTG injection was considered to indicate success of the experimental model, and those data were used for further study. After stabilization of background discharge, electrical stimulation at 1-Tc intensity was administered to facial territories or the hindlimb to inhibit nociceptive discharges evoked in the migraine model. The orders of stimulus sites were balanced across involved WDR neurons: the neuron was firstly assessed with stimulation of the hindlimb, followed by facial stimulation; the next involved neuron was tested in the opposite stimulation order. The analgesic effects of electrical stimulation at two different sites were compared in this series of experiments and are shown as percentage change in values before and after electrical stimulation.

At the end of each experiment, the recording site in the brainstem was labeled and verified by microscopic examination. The brain and spinal cord were removed, post-fixed in 10% formalin for 24 h, immersed in 25% sucrose overnight, and cryosectioned at 40 μm. Only recording data corresponding to histologically-confirmed Sp5C recording sites were included in statistical analyses.

NTG administration
To investigate the interactive relationship between facial territories and meninges under pathological conditions and to understand the therapeutic effect of peripheral neurostimulation, NTG (Yimin Pharmaceutical Co., Beijing, China) was administered i.p. at 10 mg/kg. This dose reliably induced migraine-like allodynia in preliminary studies (Costa et al., 2005; Pradhan et al., 2014; Sufka et al., 2016). The same volume of 0.9% saline was injected as control in the same animal 30 min before NTG administration. WDR neuron responses were continuously monitored during and after saline and NTG injection. A > 125% increase in discharge frequency of WDR neurons after saline or NTG injection was considered to indicate success of the experimental model, and those data were used for further study. After stabilization of background discharge, electrical stimulation at 1-Tc intensity was administered to facial territories or the hindlimb to inhibit nociceptive discharges evoked in the migraine model. The orders of stimulus sites were balanced across involved WDR neurons: the neuron was firstly assessed with stimulation of the hindlimb, followed by facial stimulation; the next involved neuron was tested in the opposite stimulation order. The analgesic effects of electrical stimulation at two different sites were compared in this series of experiments and are shown as percentage change in values before and after electrical stimulation.

Cephalic cutaneous mechanical sensory testing
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To test the effects of NTG and the analgesic effects of peripheral electrical stimulation on cephalic mechanical allodynia, experiment animals were randomly distributed into five different treatment groups: (A) Sham group (sensory test only), (B) Saline group (0.9% saline, 10 mg/kg, i.p.), (C) NTG group (nitroglycerin, 10 mg/kg, i.p., Yimin Pharmaceutical Co., Beijing, China), (D) NTG + Hindlimb Sti. group (NTG, 10 mg/kg, i.p.) and (E) NTG + Facial Sti. group (NTG, 10 mg/kg, i.p.). In groups (D) and (E), 10-min electrical stimulation (2.5 mA, 10 Hz) with 0.2-ms duration was applied on the anterolateral side of the hindlimb (innervated by the peroneal nerve) or facial tissues (innervated by mandibular nerve), respectively, after NTG administration. Responses to von Frey stimulus in all groups of animals were tested at baseline and 30, 60, 90, 120 and 150 min after NTG or saline injection (Fig. 6A). The evaluator was blinded to the groups. The experiment ultimately used 29 animals (5–6 per group). One rat with no response to the stimulus was excluded from the experiment.

**Statistical analysis**

Data were analyzed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). All data are expressed as mean ± standard error of the mean (SEM). Data sets of EB-labeled and control spots with normal distributions were analyzed using paired t-tests for differences within groups. For the electrophysiology experiment, paired t-tests were performed for pre- and post-stimulation comparisons if data were normally distributed. Wilcoxon signed-rank tests were conducted when the assumption of normality was not met. Differences between stimulation of facial tissues and hindlimbs after NTG-administration are expressed as a percentage of change and were compared by two-tailed Student’s t-test. Neural recording data were further estimated by comparing values pre- and post-stimuli using mixed model regression, with time and interaction between time and research subjects as fixed effects. The analysis was performed using the PROC GLM procedure in SAS 9.3 software (SAS Institute, Cary, NC, USA). Kruskal–Wallis H tests were conducted to determine the main effects of drug administration and electrical stimulation on the development of cephalic cutaneous allodynia as the assumption of normality was not met. If a significant main or interaction effect was apparent in the saline group (Fig. 1A), the pia mater is hardly separated from the brain’s surface, the blue-stained area on the cerebrum is contaminated by the pia mater. Similar staining was not apparent on the contralateral side or in the control group (n = 6 rats) receiving electrical stimulation to the hindlimb (innervation area of the peroneal nerve; Fig. 1A). These results indicate that peripheral electrical stimulation of facial territories of the trigeminal nerve causes neurogenic plasma protein extravasation in intracranial tissues.

**Chemical stimulation of the dura mater leads to protein extravasation in facial cutaneous tissues and causes mechanical sensitivity**

In the second series of experiments, we further investigated whether noxious chemical stimulation of the dura mater induces facial cutaneous neurogenic inflammation. EB spots appeared on the facial area approximately 15–20 min after dye injection and remained for 2 h during the observation period. Approximately 1–4 blue spots were observed on the facial areas of each of the 8 rats in the mustard oil stimulation group (Fig. 1D–F), whereas no spots were apparent in the saline group (n = 3 rats; data not shown). The majority of the EB spots were located on the territories of the ophthalmic nerve (8/10 spots), with a separate distribution in the territory innervated by the mandibular nerve.

After the animals regained consciousness, a rigid von Frey filament coupled to a force transducer was used to accurately measure and calculate VFTs for EB-labeled spots and control spots. The VFTs of EB spots were substantially less than those of control spots (12.830 ± 1.603 g for EB spots vs. 17.180 ± 2.168 g for control spots, t9 = −3.958, P = 0.003, paired t-test; Fig. 1G, H). These data corresponded well with the previous facial electrical stimulation experiments, suggesting that neurogenic inflammation can be induced bi-directionally between the two terminals of the trigeminal nerve innervating the facial tissues and dura mater.

**Neuronal labeling in the TG**

The numerous primary sensory neurons of the TG were labeled by applying neuronal tracers to the facial tissues and dura mater. Sensory neurons innervating facial tissues were observed in the V1, V2, and V3 branches of the TG (Fig. 2B), while sensory neurons associated with the dura mater were detected mainly in the V1 and V2 branches of the TG, and sparsely in the V3 branch (Fig. 2D). In contrast, double-labeled sensory neurons were scattered in the V1/V2 branches (Fig. 2E–G). The numbers of labeled sensory neurons were additionally counted in five rats in which FG and CTB were applied to...
the facial tissues and dura mater, respectively. In these rats, the numbers of FG- and CTB-labeled neurons in the TG were 187 and 68 respectively. Among these labeled neurons, there were 8 neurons labeled with both FG and CTB, accounting for 4.3% (8/187) and 11.8% (8/68) of the number of FG- and CTB-labeled neurons, respectively, and approximately 3.24% of all total labeled neurons observed in the TG (Fig. 2H).

Convergence and interaction of facial and dural afferents of WDR neurons in the Sp5C

In this phase of the experiment, 196 neurons in the Sp5C showing excitation in response to facial stimulation were characterized in 35 rats. Twenty WDR neurons in 11 rats receiving convergent inputs from both facial and dural primary afferents were ultimately selected for analysis. The recording sites in the Sp5C subnucleus were located 0–1.56 mm caudal and 1.8–2.5 mm lateral to the obex, with a recording depth ranging from 510 to 1610 μm from the dorsal surface of the medulla (Fig. 3C). The observed locations of WDR neurons were consistent with a region-specific arrangement into three trigeminal divisions reported in preliminary morphological studies (Paxinos, 2004; Wang et al., 2018).

According to the latencies of neuronal discharges and distances between the locations of the stimulation and recording sites, the mean stimulus threshold for A-fibers was 0.72 ± 0.34 mA (ranging from 0.57 to 0.82 mA), and the mean stimulus threshold for C-fibers was 4.33 ± 1.08 mA (ranging from 2.68 to 5.24 mA). Electrical stimulation of the facial RF at 2.5 Tc caused an initial short-latency response of 5–18 ms, which is within the A-fiber conduction velocity range, followed by additional long-latency responses between 30 and 100 ms, consistent with C-fiber activity (Bartsch and Goadsby, 2003).

Units responding to both non-noxious and noxious stimulation of the facial RF were considered WDR neurons. Most RFs (11/20) were located in the ophthalmic branch of the trigeminal nerve, including inputs to the cornea. In five other cases, the RFs were found within the territory of the maxillary branch, and in four other cases, the RFs were located within the mandibular branch. We used 4.0-g custom-designed filaments were used to stimulate the C-fibers of dural afferents to identify dural RFs. The results demonstrated that the intracranial excitatory RFs were generally small and were distributed within the sagittal sinus and the transverse sinus, as shown in Fig. 3A for a facial-dural convergent WDR neuron.

Stimulation of facial and dural RFs induced an immediate increase in the spontaneous activity of WDR neurons (Fig. 4). Non-noxious touch applied to the facial RF increased WDR neuron activity from 5.27 ± 0.47 Hz to 14.57 ± 0.73 Hz ($t_{19} = -3.958$, $**P = 0.003$, paired t-test; $n = 20$ dots in eight rats). Each color represents the data from one rat, as in (D). Data are presented as mean ± SEM. Scale bar = 5 mm in (A–C, E and F). Abbreviations: EB, Evans blue; VFT, von Frey threshold.
± 1.37 Hz ($t_{19} = -9.244$, $P < 0.001$, paired $t$-test). 2.5-Tc electrical stimulation to the facial RF increased WDR neuron activity from 7.06 ± 0.91 Hz to 22.85 ± 1.78 Hz ($t_{19} = -9.067$, $P < 0.001$, paired $t$-test). These convergent WDR neurons were also activated by noxious stimulation applied to the dura mater. After filament stimulation to the dura mater, the spontaneous activity of convergent WDR neurons increased from 3.52 ± 0.36 Hz to 6.49 ± 0.68 Hz ($t_{19} = -6.471$, $P < 0.001$, paired $t$-test). Similarly, noxious heat stimulation (46–52°C) applied to the dural RF for 20 s increased the neuronal response from 3.74 ± 0.65 Hz to 6.63 ± 0.80 Hz ($t_{19} = -6.129$, $P < 0.001$, paired $t$-test).

The PROC GLM analysis of neural activity before and after facial touch stimuli revealed a significant effect of Time ($R^2 = 0.748974$, $F = 7.59$, $P < 0.0001$; Time effect: $F = 72.20$, $P < 0.0001$) and no significant influence of Subject ($F = 1.13$, $P = 0.3730$). For neural activity pre- and post-electrical stimulation of facial tissues, the PROC GLM analysis showed no significant influence of Subject ($R^2 = 0.769998$, $F = 8.52$, $P < 0.0001$; Time effect: $F = 74.31$, $P < 0.0001$; Subject: $F = 1.94$, $P = 0.0811$). However, for neural activity pre- and post-mechanical stimulation on the dural RF, the PROC GLM analysis revealed a significant Time × Subject interaction ($R^2 = 0.766874$, $F = 8.37$, $P < 0.0001$), including a significant effect from Time ($F = 32.39$, $P < 0.0001$) and Subject ($F = 5.97$, $P < 0.0001$). Similarly, the PROC GLM analysis on neuronal discharges before and after dural heat stimuli revealed a significant Time × Subject interaction ($R^2 = 0.545677$, $F = 3.06$, $P = 0.0083$; Time effect: $F = 9.51$, $P = 0.0046$; Subject: $F = 2.41$, $P = 0.0325$).
Inhibition of homotopic or heterotopic electrical stimulation of WDR neurons in a migraine model

To investigate the analgesic effects of peripheral electrical neurostimulation based on convergence and interaction of WDR neurons in the Sp5C, 16 out of 20 units selected from 10 rats were studied in a model of acute migraine induced by 10 mg/kg NTG administration. This dose evokes significant and prolonged mechanical and thermal hypersensitivity in rodents (Bates et al., 2010; Sufka et al., 2016). In the present experiment, no significant differences in neuronal activity were observed after injection of 0.9% saline (5.98 ± 1.05 Hz vs. 5.78 ± 1.01 Hz, \( t_{11} = -1.242, P = 0.240 \), paired \( t \)-test). Most units (12/16) demonstrated an increase in discharge frequency after NTG injection and were considered to be successful models of migraine for use in further analysis.

Four WDR neurons with less than 125% increase of discharge frequency after NTG administration were excluded in further study. NTG enhanced neuronal activity approximately 3 min after administration, and this lasted for an additional 1 h during the subsequent observation period. As shown in Fig. 5A, C, NTG injection significantly increased the discharge frequency compared to background activity (12.02 ± 2.16 Hz vs. 5.98 ± 1.05 Hz, \( Z = -2.903, \ P = 0.004 \), Wilcoxon signed-rank test). In addition, the PROC GLM analysis revealed a significant Time × Subject interaction (\( R^2 = 0.07126, \ F = 3.2, \ P = 0.0258 \); Time effect: \( F = 10.60, \ P = 0.0063 \); Subject effect: \( F = 2.40, \ P = 0.0734 \) after NTG injection.

As shown in Fig. 5B, D, electrical stimulation at 1 Tc of either the facial tissues or hindlimb inhibited WDR neuron responses after NTG administration (facial: 6.58 ± 1.51 Hz vs. 13.45 ± 2.85 Hz, \( Z = -3.059, \ P = 0.002 \), Wilcoxon signed-rank test; hindlimb: 5.83 ± 1.17 Hz vs. 9.18 ± 1.43 Hz, \( t_{11} = -6.940, \ P < 0.001 \), paired \( t \)-test). Analysis of neural activity after facial electrical stimulation with the PROC GLM model revealed a significant Time × Subject interaction (\( R^2 = 0.8187, \ F = 5.87, \ P = 0.0020 \), including significant effects of Time (\( F = 13.14, \ P = 0.0031 \)) and Subject (\( F = 5.06, \ P = 0.0045 \)). Analysis of neural activity after electrical stimulation to the hindlimb showed a significant Time × Subject interaction (\( R^2 = 0.7244, \ F = 0.0207, \ P = 0.0207 \), including significant effects for Time (\( F = 6.18, \ P = 0.0273 \)) and Subject (\( F = 3.11, \ P = 0.0314 \)).

At the same stimulation intensity (1 Tc), facial electrical stimulation produced a significantly stronger inhibitory effect on WDR neuron activity compared to hindlimb stimulation (Fig. 5E; \( -51.92 ± 3.62 \% \) for facial stimulation vs. \( -38.48 ± 4.53 \% \) for hindlimb stimulation, \( t_{22} = -2.376, \ P = 0.027 \), Student’s \( t \)-test). There was no statistically significant in Stimulation Site × Subject interaction according to the PROC GLM analysis (\( R^2 = 0.4179, \ F = 0.93, \ P = 0.5347 \)). In summary, 1-Tc electrical stimulation of facial territories of the trigeminal nerve elicited more effective inhibition of WDR neuron firing than stimulation of the heterotopic hindlimb area after NTG administration, with no significant influences between-subject.

Effects of peripheral neurostimulation on NTG-evoked cephalic cutaneous mechanical sensitivity

To determine if NTG-treated rats developed enhanced cutaneous mechanical sensitivity to an acute migraine-like event, we tested changes in cephalic responses to mechanical stimulation following the administration of NTG or saline at different time points. Using electrical von Frey testing, we identified a time-dependent development of cephalic cutaneous allodynia in NTG-injected animals, but not in Sham and Saline-treated animals (Fig. 6).

Before injection, there were no differences in baseline withdrawal response to cephalic von Frey stimuli between rats treated with drugs or rats in the Sham group (\( H_4 = 4.384, \ P = 0.356 \), Wilcoxon test). In the Saline group, there was no significant difference compared with the Sham group (56.401 ± 1.160 g for Saline group and 56.786 ± 0.866 g for Sham group, \( H_4 = 215.509, \ P = 0.249 \)). Compared to the Saline group, the response threshold in the NTG group was significantly decreased after NTG injection (42.043 ± 0.798 g vs. 38.48 ± 4.53% for hindlimb stimulation, \( t_{22} = -2.376, \ P = 0.027 \), Student’s \( t \)-test).
56.401 ± 1.160 g, \( H_4 = 215.509, P < 0.001 \). Consistent with previous studies (Farkas et al., 2016; Ferrari et al., 2016; Dallel et al., 2018), cephalic VFTs were significantly lower than baseline VFTs 30 min after NTG injection in the NTG group and were prominent at 150 min during testing, indicating that NTG induced acute and persistent static cephalic mechanical allodynia (Fig. 6C). Detailed results are presented in Table 1.

To study the effects of peripheral neurostimulation on cephalic cutaneous allodynia development and the differences between homotopic and heterotopic stimuli, 2.5-mA electrical stimulation on facial tissues or the ipsilateral hindlimb was applied in NTG-induced migraine model rats. Compared to the NTG group, electrical stimulation of facial tissues after NTG administration slightly reduced the cephalic pain behavior in rats (47.936 ± 0.713 g vs. 42.043 ± 0.798 g, \( H_4 = 215.509, P < 0.001 \)) compared to the NTG group at the same time point. VFTs in the NTG + Hindlimb Sti. group was not significantly different compared with the NTG group during the testing period (43.771 ± 1.458 g vs. 42.043 ± 0.798 g, \( H_4 = 68.925, P = 0.607 \), Table 1). In addition, compared to 2.5-mA electrical stimulation on the hindlimb, electrical stimulation at the same intensity was also effective at attenuating the NTG-induced decrease in the cephalic mechanical threshold (47.936 ± 0.713 g in the NTG + Facial Sti. group vs. 43.083 ± 1.348 g in the NTG + Hindlimb Sti. group, \( H_4 = 77.024, P = 0.007 \), Table 1). The above results suggest that NTG-evoked allodynia was time dependent and could be attenuated with 2.5-mA electrical stimulation of facial tissues but not the hindlimb.

**DISCUSSION**

In this study, we explored the convergence of rat dura mater and referred facial tissue signals by trigeminal nerve afferents in the TG and in WDR neurons in the Sp5C, which are implicated to the pathogenesis of migraine. The effects of peripheral neurostimulation on cephalic cutaneous mechanical sensitivity were also evaluated in NTG-induced migraine model rats. Our results demonstrate that inputs from the meninges and signals from peripheral electrical stimulation are integrated within the same primary sensory neurons in the TG and the same second-order WDR neurons in the Sp5C. These processes might produce an efficient analgesic effect in our model of migraine.
Neurogenic inflammation is consistent with an interaction between sensory inputs from facial tissues and intracranial structures

Neurogenic inflammation has been widely studied in animal models of migraine and is assumed to suggest the bifurcate distribution of cutaneous polymodal nociceptor endings of the associated stimulated nerve afferents (Kenins, 1981). The phenomenon of plasma protein extravasation has been regarded as indicative of neurophysiological connections between two related territories, such as somato-somato (Meyer et al., 1984; Schmelz et al., 1996) or somato-visceral correlations (Ben et al., 2012; Kim et al., 2017). Activation of unmyelinated cutaneous afferents via intense thermal or mechanical stimulation, irritant chemical application, or antidromic nerve stimulation leads to increased vascular permeability and plasma extravasation. Subsequent release of vasoactive substances such as substance P, calcitonin gene-related peptide, histamine, and serotonin cause sensitization and hyperalgesia in associated cutaneous regions (He et al., 2017).

In the first phase of our study, both electrical stimulation of facial tissues and noxious mustard oil application onto the dura mater led to plasma extravasation in other areas innervated by trigeminal nerve endings, indicating a link between the two terminal territories. By examining the withdraw threshold of EB spots after mustard oil application onto the dura mater, we demonstrated that allogenic chemicals caused increased plasma protein extravasation and cutaneous sensitization. This corroborates existing evidence of bi-directional neural connectivity between the extracranial facial regions and intracranial structures. Interestingly, electrical stimulation of the hindlimb did not affect plasma extravasation in the dura mater, further confirming the close connection between facial tissues and intracranial structures. Neuroanatomy studies have shown that like visceral tissues, the dura mater is innervated by a large proportion of sympathetic fibers and unmyelinated fibers (Andres et al., 1987; Strassman et al., 2004). The participation of sympathetic nerve signals and increased expression of nociceptive neuropeptides after noxious stimulation are considered to be the neurophysiological mechanisms underlying the sensitization of the cutaneous area (Mayberg et al., 1984; Garcia-Poblete et al., 2003; He et al., 2017). Although the dorsal side of the dura mater cannot be clearly observed in a whole-mounted view, the possibility that plasma extravasation occurs in the dorsal dura mater cannot be fully excluded. To the best of our knowledge, although plasma extravasation in the dura mater and the relationship between the dura mater and the corresponding TG (Lundblad et al., 2015) have been investigated in prior studies of migraine pathogenesis (McDonald et al., 1996), there are no reports concerning the relationship between facial tissues and the dura mater, as investigated in the present study.

Bifurcated sensory innervation may be responsible for the axon reflex between facial tissues and the dura mater

Similar to previous studies (Andres et al., 1987; Schueler et al., 2013, 2014; Wang et al., 2018), we observed that...
numerous sensory neurons were labeled in the TG after the application of neuronal tracers to the facial tissues or to the dura mater. However, present study provided further evidences to demonstrate that there is a small number of trigeminal sensory neurons labeled simultaneously by two kinds of neuronal tracers when they were indepen-
dently applied to the facial tissues and the dura mater in the same rat. The existence of double-labeled trigeminal sensory neurons indicates that they send out bifur-
cated axons that innervate both the facial tissues and the dura mater. These direct neural connec-
tions might be the structural basis of the axon reflex between the extra- and intracranial tissues, sug-
gest that it might be possible to use extracranial electrical stimulation to modulate intracranial pathologies including migraine (Mayberg et al., 1981; Borges and Moskowitz, 1983). Similar neural connections also exist between the body and viscera, and this somato-visceral correlation is an important biological phenomenon under both physiological and pathological conditions (Sinclair et al., 1948; Alles and Dom, 1985). From the perspective of neural correlation, migraine can be regarded as a kind of referred headache that might be success-
fully alleviated by somatic stimulation of facial tissues by therapies such as transcutaneous electrical nerve stimulation (Magis and Schoenen, 2012).

Since the tracers were only applied to the limited regions of the facial tissues and the dura mater, the percentage of double-labeled trigeminal sensory neurons was low. As a result, most labeled trigeminal sensory neurons were associated either with facial tissues or with the dura mater, not both. Although these neurons were not found to innervate both regions, considering their adjacent locations within the same TG, they might still influence each other (Mayberg et al., 1981; O’Connor and van der Kooy,

Fig. 6. NTG-induced cephalic cutaneous allodynia was attenuated by electrical stimulation of facial tissues, but not the hindlimb. (A) Schematic representation of the experimental design. Rats in five groups were all tested for VFT at baseline and at 30-min intervals for 150 min (red arrow below) after NTG or saline i.p. injection (black arrow above). Rats in groups (D, E) received electrical stimulation (red line) immediately after NTG administration. (B) The picture shows a rat fitted with the plastic holding aperture used for cutaneous sensory threshold testing with a rigid von Frey filament coupled with a force transducer. Threshold was noted when rats quickly retracted their head away from the holding aperture used for cutaneous sensory threshold testing with a rigid von Frey filament coupled (red line) immediately after NTG administration. (C) Time course of the VFT of the face in five groups of rats after drug injections. The Saline group showed no obvious differences compared with the Sham group ($H_4 = 215.509, P = 0.249$, Kruskal Wallis $H$ test). Cutaneous allodynia was markedly attenuated in the NTG + Facial Sti. group ($H_4 = 215.509, ***P < 0.001$ vs. NTG group, Kruskal Wallis $H$ test), but not the NTG + Hindlimb Sti. group ($H_4 = 215.509, P = 0.607$ vs. NTG group, Kruskal Wallis $H$ test). Note that the VFT in the NTG + Facial Sti. group was significantly different compared with that in the NTG + Hindlimb Sti. group ($H_4 = 215.509, ***P < 0.001$, Kruskal Wallis $H$ test) during testing period. $N_{Sham} = 6$; $N_{Saline} = 5$; $N_{NTG} = 6$; $N_{NTG + Hindlimb Sti.} = 6$; $N_{NTG + Facial Sti.} = 6$. Data are presented as mean ± SEM. Abbreviations: inj., injection; i.p., intraperitoneal; B, Baseline; NTG, nitroglycerin; VFT, von Frey threshold.

Table 1. VFTs at different time points in five groups. All variables are presented as mean ± SEM.

| Group          | Baseline  | 30 min     | 60 min     | 90 min     | 120 min    | 150 min    |
|----------------|-----------|------------|------------|------------|------------|------------|
| Sham group     | 56.833    | 58.166 ± 1.928 | 53.047 ± 2.821 | 55.620 ± 2.006 | 59.703 ± 1.978 | 57.345 ± 1.978 |
| Saline group   | 53.334    | 55.613 ± 2.758 | 53.948 ± 2.575 | 57.968 ± 3.369 | 58.432 ± 3.308 | 59.113 ± 2.501 |
| NTG group      | 57.373    | 37.870 ± 1.518 | 32.560 ± 1.315*** | 41.827 ± 1.688*** | 39.270 ± 1.032*** | 43.359 ± 1.056*** |
| NTG + Hindlimb Sti. group | 57.461    | 40.734 ± 1.458** | 35.680 ± 1.348*** | 43.845 ± 1.519*** | 38.775 ± 1.087*** | 42.000 ± 1.519*** |
| NTG + Facial Sti. group | 56.483    | 43.797 ± 1.458** | 43.771 ± 1.458** | 48.433 ± 1.318** | 46.238 ± 1.318** | 48.893 ± 1.318** |

*P < 0.05, **P < 0.01, ***P < 0.001 vs. Saline group; ’P < 0.05, ’’P < 0.01 vs. NTG group; ’’’P < 0.05, ’’’’P < 0.01 vs. NTG + Hindlimb Sti. group. Abbreviations: VFT, von Frey threshold; Sti., Stimulation.
Recent studies have indicated that neuron-to-neuron or neuron-to-glial cells in the TG or dorsal root ganglion can be activated and interacted via each other by chemical substances under conditions of inflammation or mechanical hyperalgesia (Takeda et al., 2005, 2009; Kim et al., 2016). What’s more, as in vivo single-neuron recording in the TG (Zhao and Levy, 2014) or dorsal root ganglion (Ma et al., 2010) has only been achieved by few laboratories, interactions between primary afferent neurons still remains a concern for further research. In summary, trigeminal sensory innervation of the extracranial and intracranial tissues is a complex phenomenon that requires further investigation to be fully understood, especially under pathological conditions.

With respect to our double-labeling results, existing publications describe similar findings (Strassman et al., 2004; Schueler et al., 2013, 2014), while others (Mayberg et al., 1984; O’Connor and van der Kooy, 1986; Liu et al., 2008) found no mandibular division of the TG parallel to the MMA. The most likely reason for this discrepancy in routes between the application site of the tracer and the dura site is that it would depend on whether the spinous nerve travelled along the middle cranial fossa and followed MMA branches on the lateral side of the cerebrum (Andres et al., 1987; Strassman et al., 2004). It should be noted that although part of the dural and facial innervations originate from sensory neurons of the dorsal root ganglion in the upper cervical segments (Liu et al., 2008; Wang et al., 2018), in the present study we paid comparatively more attention to innervations originating in the TG.

Convergence of inputs from facial territories and the dura mater to WDR neurons is responsible for analgesic effects after peripheral electrical stimulation

As previously stated in the introduction, central sensitization of second-order neurons in the Sp5C and the upper cervical components contributes to increased cutaneous sensitivity, hyperalgesia, and allodynia in the trigeminal territories during migraine attack. Therefore, in this study we investigated the electrophysiological activity of WDR neurons in the Sp5C with convergent inputs from both dura mater and facial tissues. WDR neurons, also known as multireceptive or convergent neurons, include interneurons involved in polysynaptic reflexes, along with neurons projecting to ascending pathways, such as the spinothalamic and spinoreticular tracts (Morgan, 1998; Le Bars, 2002; Mørch et al., 2007). Impulses from the center of RFs mediated by A-fibers and C-fibers are both capable of exciting the same WDR neuron; therefore, these fibers have been studied in various animal models of somatic-visceral interaction and in referred pain studies (Cervero and Tattersall, 1986; Ness and Gebhart, 1991; Rong et al., 2005).

In the present study, we collected data regarding the location at which low-threshold mechanoreceptive (touch sensations) and nociceptive (pinching, electrical stimulation, mechanical stimulation, and heat) afferent inputs converge from facial territories and dura mater. The main projection of supraorbital nerve fibers onto WDR neurons in the Sp5C supplying the meninges is in accordance with the innervation of both structures by the ophthalmic division of the trigeminal nerve. It was previously reported that facial RFs of WDR neurons with meningeal inputs were preferentially restricted to the ophthalmic division (Davis and Dostrovsky, 1986; Strassman et al., 1986). Nonetheless, our electrophysiological results showed that these also extend to the maxillary and mandibular regions in some cases (9/20 neurons). Together with the neurogenic inflammation findings and the neural tracing results mentioned above, the evidence indicates that the first trigeminal division innervates most of the intracranial structures. The convergence of facial and meningeal afferent inputs onto both the TG and Sp5C is the most likely underlying neurophysiological mechanism of referred orofacial pain or hyperalgesia that frequently accompanying migraine attacks (Mathew et al., 2004; Goadsby et al., 2017).

There is strong evidence from animal experiments and clinical research that NTG can sensitize second-order neurons in the trigeminal nucleus caudalis and upper cervical spinal cord (Lambert et al., 2000; Jones et al., 2001; Di Clemente et al., 2009). According to previous studies, NTG-evoked migraine syndrome is considered to arise at least partly through action at perivascular afferent terminals of the dura mater; this can be alleviated by sumatriptan administration (Buzzi and Moskowitz, 1990; Goadsby and Hoskin, 1996). In view of the convergence of inputs from intracranial structures and facial tissues, and the hypothesis that NTG activates the ongoing discharge rate of the Sp5C neurons, we measured NTG-induced changes in WDR neurons. The results showed that NTG-derived sensitization of WDR neurons was significantly attenuated by peripheral electrical stimulation in both homotopic and heterotopic regions, whereas homotopic electrical stimulation produced a profound analgesic effect compared with heterotopic stimulation with same intensity (1 Tc). Homotopic stimulation at the C-fiber intensity may involve both local analgesia, according to the Gate Control Theory (Braz et al., 2014; Mendell, 2014), and supraspinal inhibition associated with trans-segmental mechanisms of descending pathway inhibition, such as diffuse noxious inhibitory control (DNIC, Le Bars, 2002). As DNIC was mainly involved in the analgesia effects of noxious stimuli on heterotopic regions of the body, the effects of hindlimb stimulation with 1-Tc intensity only involves this heterosegmental analgesia (Meng et al., 1997; Dallelo et al., 1999; Le Bars, 2002; Laprot et al., 2009, 2011). Besides, the involvement of DNIC was qualitatively and quantitatively dependent on peripheral C-fiber-evoked responses in the Sp5C (Villanueva and Le Bars, 1985, 1986), Sp5O (Dallelo et al., 1999) and spinal cord (Zhu et al., 2004; Zhi et al., 2017). The balance between A- and C-fiber inputs is supposed to be underlying mechanism for pain alleviation (Melzack and Wall, 1965). Due to much shorter distance between stimulation and recording sites at the trigeminal level (stimuli on facial tissues) than in the lambar spinal cord (stimuli on hindlimb), the inhibitory effects of facial stimulation were supposed to be induced with both A- and C-fiber activation. Therefore, it is a reason-
able explanation for the differential analgesic effect resulting from the peripheral stimulation of two heterosegmental regions. This suggests that homotopic facial territories are more closely related to intracranial structures than to the hindlimb, which suggests that electrical stimulation might be effective in clinical practice.

It needed to mention that we also considered the involvement of research subjects in in vivo single unit recording experiment. With the analysis of mixed model regression, pre- and post-stimulation on dural RF represented a significant Time × Subject interaction. This indicated that the effects of stimulation were statistically influenced according to inadequate numbers of research subject. However, the short distance from recording site to dural RF is highly considered to be an implicit factor but cannot be fully excluded during the experiment. In addition, the statistical analysis also represented a Time × Subject interaction before and after peripheral ES stimulation, but no statistically significant in Stimulation Site × Subject interaction with PROC GLM analysis. Consist with the Student's t-test, this indicated that ES of facial territories elicited more effective inhibition on WDR neuron firing than stimulation of hindlimb region after NTG injection. Further research on second-order neuron activity after peripheral stimulation would be more conceivable with the consideration of stimulation site and sufficient animals involved.

Effects of peripheral neurostimulation on cephalic cutaneous mechanical allodynia during migraine attack are highly related to stimulated site and intensity

Facial cutaneous allodynia is a neurologic condition induced by increased excitability of the spinal cord and trigeminal nucleus caudalis and has been long proposed as a clinical predictor of migraine attack and therapeutic treatment outcome (Sessle et al., 1986; Burstein et al., 2000a,b; Scher et al., 2003; Bigal et al., 2008; Lipton et al., 2008). Patients suffering from migraine often experience severe cephalic and extra-cephalic mechanical allodynia on the ipsilateral forearm (Burstein et al., 2000,b). Sensitization of meningeal perivascular nociceptors and sensitization of central trigeminal neurons that receive convergent inputs form the meninges and facial tissues are one possible explanation for cutaneous allodynia accompany migraine (Burstein et al., 2000,b, 2004). Coupled with traditional physiological measures such as electrophysiology and molecular studies, behavioral testing will allow researchers to study the associated symptoms of migraine and other trigeminal disorders (Oshinsky et al., 2012).

To fully clarify the effect of peripheral neurostimulation on cephalic cutaneous allodynia, we evaluated the spontaneous allodynia of facial skin induced by NTG administration and the different effects of the same intensity of homotopic and heterotopic electrical stimulation based on previously described methods (Oshinsky and Gomonchareonsiri, 2007; Oshinsky et al., 2012; Boyer et al., 2014, 2017; Dallel et al., 2018). The intensity (2.5 mA) used in the behavioral test was proposed to be equivalent to C-fiber activity according to our electrophysiology study. We demonstrated that facial cutaneous alodynia developed rapidly within 20 min and lasted for the following 150 min in trigeminal allodynia rats, which was consistent with previous animal and clinic-based studies (Burstein et al., 2000; Bigal et al., 2008; Ferrari et al., 2016; Dallel et al., 2018). Meanwhile, 2.5-mA electrical stimulation on facial tissues, but not the ipsilateral hindlimb, significantly attenuated mechanical sensitivity, indicating a close association with intracranial structures during migraine treatment.

In addition, it should be noted that much of the previous researches that concentrate on cephalic mechanical sensitivity test are carried out by classic manual von Frey filaments (Oshinsky and Gomonchareonsiri, 2007; Oshinsky et al., 2012; Boyer et al., 2017; Dallel et al., 2018). The maximum withdrawal thresholds in these studies range from 6 g to 10 g, which are much smaller than the threshold scores (50–60 g) we have assessed. This discrepancy could be attributed to the different von Frey apparatus we used. Compared to classic manual von Frey filament of varying forces, this electrical apparatus allows investigator accurately measure the precise pressure applied to test tissue with one rigid tip without changing series of weighted tips during experiment procedure (Martinov et al., 2013). As the testing surface and filament material can influence the results of von Frey, it is possible that the withdrawal values obtained by classic von Frey filament or electronic apparatus with a rigid filament are not directly comparable. Still, irrespective of the apparatus used, the endpoint is withdrawal to an abnormally aversive stimulus, and thus both technique can measure mechanical allodynia.

In this study, we investigated the convergence and interaction of sensory inputs from facial tissues and intracranial structures through the trigeminal spinal tract, as well as the differences in analgesic effects between homotopic and heterotopic peripheral electrical stimulation after NTG administration. These mechanistic studies are important for clinical applications and promoting the use of non-invasive neurostimulation techniques. Furthermore, they are helpful for identifying effective therapeutic approaches to combine peripheral nerve stimulation with conventional pharmacological treatments. In addition, the analgesic effects during peripheral stimulation might involve bifurcating trigeminal afferents and/or the convergence of signaling in WDR neurons. The different effects of these mechanisms were not compared in this present study. Further studies of the analgesic effects of homotopic and heterotopic electrical stimulation during migraine, as well as of other established preventive therapies, will help to better clarify the contributions of convergent sensory inputs from separate territories and ascertain the mechanisms involved.

ACKNOWLEDGEMENTS

This study was supported by the China Postdoctoral Science Foundation (No. 2017M620087) and National Natural Science Foundation of China (No. 81674075,
No. 81804210). We thank Professor Zhao Yufeng for her constructive suggestions for statistical analysis and Kristin Schoepfer for her assistance with editorial and grammatical corrections. We thank Ge Wen from Guangzhou Bioillus Co. Ltd for editing the graphical abstract.

AUTHOR CONTRIBUTIONS

SW conducted the experimental work and wrote the first draft of the manuscript. JW performed the immunohistochemistry experiments. KL organized the data base, WB helped in retrograde tracing experiment and gave valuable feedback in writing the manuscript. XC and SH performed the statistical analysis, and XG and BZ conceived the study and took responsibility for drafting the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

DECLARATIONS OF INTEREST

None.

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