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An Improved Rodent Model of Trigeminal Neuropathic Pain by Unilateral Chronic Constriction Injury of Distal Infraorbital Nerve (dIoN-CCI)

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Abstract

The number of studies on trigeminal nerve injury using animal models remains limited. A rodent model of trigeminal neuropathic pain was first developed in the 1994, in which chronic constriction injury is induced by ligation of the infraorbital nerve (IoN-CCI). This animal model has served as a major tool to study trigeminal neuropathic pain. Unfortunately, the surgical procedure in this model is complicated and far more difficult than ligation of peripheral nerves (e.g. sciatic nerve). The aim of this study was to improve on the current surgical procedure of IoN ligation to induce trigeminal neuropathic pain in rats. We demonstrate that the IoN can be readily accessed through a small facial incision. Chronic constriction injury can be induced by ligation of a segment at the distal infraorbital nerve (dIoN-CCI). This dIoN-CCI procedure is simple, minimally invasive and time-saving. Our data show that the dIoN-CCI procedure consistently induced both acute and chronic nociceptive behaviors in rats. Daily gabapentin treatment attenuated mechanical allodynia and reduced face-grooming episodes in dIoN-CCI rats.

Perspective—The orofacial pain caused by trigeminal nerve damage is severe and perhaps more debilitating than other types of neuropathic pain. However, studies on trigeminal neuropathic pain remain limited. This is largely due to the lack of proper animal models given the complexity of the existing surgical procedure required to induce trigeminal nerve injury. Our improved dIoN-CCI

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model is likely to make it more accessible to study the cellular and molecular mechanisms of neuropathic pain caused by trigeminal nerve damage.

Keywords

Rat; distal; infraorbital nerve; orbital cavity; chronic constriction; mechanical allodynia; face-grooming

Background

Orofacial neuropathic pain caused by trigeminal nerve injury is a debilitating condition with limited therapeutic options^{11, 27}. Chronic constriction injury of the infraorbital nerve (IoN-CCI) was developed in 1994²⁵ and has been used as a major animal model to study trigeminal neuropathic pain disorders. IoN-CCI induces injury to a pure sensory nerve resulting in painful sensory disturbances in the IoN territory such as the mystacial vibrissae and its surrounding hairy skin areas^{7, 23}. Thus, IoN-CCI appears to be a suitable animal model to study neuropathic pain caused by trigeminal nerve injury.

However, trigeminal neuropathic pain disorders are not as widely studied as peripheral neuropathic pain conditions probably because of the complexity of the IoN-CCI procedure²⁵. Here we demonstrate a simplified, minimally invasive and time-saving surgical procedure to produce CCI by the ligation of a distal infraorbital nerve (dIoN-CCI). We compared and contrasted our procedure with the procedure originally developed by Vos et al²⁵ in which an intraorbital segment of the IoN was ligated. We also made comparison with a procedure in which the ligation site on the IoN is distal to the infraorbital foramen¹⁰. Our data show that the dIoN-CCI procedure consistently induced both acute and chronic nociceptive behaviors in rats that were comparable with those induced by more complex and more traumatic surgical procedures.

Methods

Animals

Adult male Sprague–Dawley rats weighing 250–270 g were purchased from Charles River Laboratories (Wilmington, MA). The experimental protocols were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee.

Surgical procedures

All surgical procedures were performed aseptically on rats anesthetized with Ketamine/xylazine (75 mg/Kg; 5 mg/Kg). The shaved skin surface was scrubbed with iodine, and 70% of isopropyl alcohol was used to remove excess iodine. We used different surgical procedures in this study, as listed below, in order to compare the behavioral outcome reflective of trigeminal neuropathic pain.

dIoN-CCI, ligation of a distal segment of the IoN outside the orbital cavity through a facial incision—The facial surface between the eye and whisker pad of the rat was gently shaved without damaging the whiskers. A 0.5 cm incision parallel to the mid-line

was made starting at the caudal end of the third row of whisker lines towards the ipsilateral orbit (Fig 1A). The superficial fascia was bluntly separated to expose the IoN trunk at its distal segment outside the orbital cavity (Fig 1B). Two chromic catgut ligatures (4-0) were loosely tied around the distal part of the IoN (2 mm apart) (Fig 1C, D). To ensure proper constriction of the IoN, a criterion proposed by Bennett and Xie² was followed. The ligatures were applied in such a way that the diameter of the IoN was reduced by a just noticeable amount and the circulation through the superficial vasculature was retarded but not cut off. The skin incision was closed with a polyester suture (4-0) (Fig 1E). Rats in sham groups underwent the same surgical procedure including skin incision and the IoN nerve dissection except for the actual nerve ligation.

IoN-CCI, ligation of an IoN segment inside the orbital cavity through a midline incision²⁵—The unilateral ligation of the IoN was performed as described by Vos et al²⁵. We shaved the head of the rat and fixed the head in a stereotaxic frame. We made a midline incision on the scalp to expose skull and nasal bone (Fig 1F). The boundaries of the orbit were exposed by tissue dissection, including the maxillary, frontal, lacrimal and zygomatic bones. We exposed the IoN by gently deflecting the orbital contents with a cotton swab. The IoN was then dissected from its surrounding tissues at its most rostral extent in the orbital cavity, which was just caudal to the infraorbital foramen (Fig 1G). Two chromic catgut ligatures (4-0) were loosely tied around the IoN (2 mm apart) (Fig 1H, I). The skin incision was closed with three polyester sutures (4-0) (Fig 1J).

IoN-CCI, ligation of an IoN segment outside of the orbital cavity through a midline incision¹⁰—A midline incision was made over the nose of the rat as described¹⁰ (Fig 1K). The left infraorbital nerve (IoN) was exposed just distal to the IoN foramen¹⁰ (Fig 1L). Two 4-0 chromic gut sutures were placed around the IoN just distal to the foramen¹⁰ (Fig 1M, N). Each suture was loosely placed around the nerve. The skin incision was closed with three polyester sutures (4-0) (Fig 1O).

Neuronal tracing—Anterograde and retrograde labeling tracer 3000 Da tetramethylrhodamine-conjugated dextran (Invitrogen, Carlsbad, CA, USA) was used to label the neurons in the trigeminal ganglion¹⁵. The IoN trunk at its distal segment outside the orbital cavity was exposed (Fig 1B). The IoN trunk was transected at the same site as for ligation (Fig 3B). To prevent tracer from contaminating the surrounding tissue, a small piece of parafilm was placed under the transected IoN. DMSO was applied to the transected surface of the IoN to increase the penetration of dextran. Dextran granules were applied directly onto the transected surface of the IoN with a micro spatula and held in place for one minute. A small dab of petroleum jelly was applied to the nerve end before sealing it with parafilm and superglue. At 2, 5, and 7 days after dextran application, rats were perfused with 4% of paraformaldehyde as described previously²⁹. Brains and the trigeminal ganglion were harvested, sectioned, mounted on the glass slides and examined on an Olympus fluorescence microscope²⁹. The site of facial nucleus in the brain section was determined based on stereotaxic coordinates of The Rat Brain⁸

Drug treatment

Gabapentin was administered daily by oral gavage using an autoclavable curved rat feeding tube with a ball tip (Kent Scientific, CT). The feeding volume was determined based on body weight at 0.1–0.2 mL/10 g⁴. The dose of gabapentin was 150 mg/Kg⁵.

Behavioral tests

All behavioral experiments were carried out with the investigators blinded to treatment conditions. Animals were habituated to the test environment for two consecutive days (30 minutes per day) before baseline testing. A clear Plexiglas enclosure (10 × 10 × 20 cm) (IITC Life Science, CA), in which a rat can move freely, was used for behavioral tests.

Mechanical allodynia—Orofacial sensitivity to mechanical stimulation was tested using von Frey filaments²⁵. The stimuli were applied within the IoN territory, near the center of the vibrissal pad. This area was stimulated on both sides of the face after surgery, i.e., ipsilateral and contralateral to the side where surgery had been performed. Stimuli were applied in an ascending order of intensity. The ipsilateral and contralateral sides were stimulated in a randomized order for each animal. The following criteria were used to determine a positive response to a filament: an immediate withdrawal reaction, attacking the filament by biting and grabbing, escaping by moving away from the filament, or asymmetric face stroke to the stimulated facial area. A threshold force of response (in grams) was defined as the first filament that evoked at least two reactions out of five applications²².

Face-grooming—The rat was placed into the Plexiglas enclosure and video recorded. Face-grooming episodes were counted over a period of 10 min after the rat was placed into the enclosure. A face-grooming episode was defined as an uninterrupted sequence of face-grooming actions²⁵.

Statistical analysis of behavioral data

Behavioral data were analyzed using the Mann-Whitney test. GraphPad5 software was used for the statistical analyses. All data were expressed as mean ± SEM and the statistically significant level was set at $P < 0.05$.

Results

A distal segment of the IoN was ligated through a facial incision

Fig. 1 illustrates our improved surgical procedure to produce chronic constriction injury to a distal part of the IoN in rats (dIoN-CCI) (Fig 1A–E). We show that a segment of the IoN trunk outside the orbital cavity could be readily exposed with a small incision in the facial area (Fig 1A). This incision site overlies the IoN trajectory and the incision reveals the IoN without the need of extensive facial tissue dissection (Fig 1B). The IoN trunk is readily accessible for ligation after being exposed (Fig 1C). The two ligatures are placed in the IoN trunk before the IoN branches into the whisker pad (Fig 2A). There is no visible bleeding during our surgical procedure. The choice of this incision site and size makes it possible to access the IoN without causing damage to either the infraorbital artery or the angular artery.

The incision site is parallel to the infraorbital artery and lies laterally off it¹⁹. The starting point of the incision site is inferior to the angular artery¹⁹, and a small incision of 0.5 cm makes it possible to avoid this artery in the procedure. Furthermore, minimal tissue dissection is necessary for exposing the IoN trunk through facial incision, causing less cutaneous nerve damage. In our hands, it took approximately 6–8 min to perform dIoN-CCI on a rat (n=10).

For comparison, we also performed other IoN-CCI surgeries based on the description by Vos et al²⁵ (Fig 1F–J) and Henry et al¹⁰ (Fig 1K–O). In both procedures, a midline incision was made in order to access the IoN (Fig 1F, K). However, the midline incision is far medial to the IoN trunk and does not overlie the IoN trajectory at the site of IoN ligation. In order to expose and access the IoN for ligation, the soft tissue has to be dissected out laterally towards the IoN from the skin incision site (Fig 1G, L). This dissection process requires meticulous care to reduce tissue trauma and to avoid damage to the facial artery, which makes it rather time consuming. In the Vos' procedure²⁵, the orbital contents were deflected to gain access to a segment of the IoN located inside the orbital cavity for ligation (Fig 1H, I; Fig 2A), which increased the risk of bleeding and damaging the orbital contents such as the lacrimal gland. It also took about 20–30 min to perform the Vos' procedure²⁵ (n=10) on a rat. The disturbance and potential damage to the orbital contents are avoided by ligating the IoN segment outside the orbital cavity (Fig 1M, N) in the procedure described by Henry et al¹⁰. However, the midline incision makes this procedure prone to bleeding and substantial tissue damage.

Furthermore, anatomical assessment shows that through our small facial incision (Fig 1A, B), we accessed the IoN trunk (Fig 2C and Fig 3A) but not any of the facial nerve branches. The marginal mandibular nerve (MMN) and buccal nerve (BN) branches of the facial nerve run laterally to supply the masseter muscle (Fig 3A). The ligation site on the IoN (Fig 3B) is located superior and medial to the convergence site of MMN and BN⁹.

Facial nerve afferents were not detected at the constriction site of dIoN-CCI

We used neuronal tracing to confirm that through our facial incision site we accessed the IoN trunk, but not any of facial nerve branches. Trigeminal afferents at the constriction site (CCI site) were applied with 3000 Da tetramethylrhodamine-conjugated dextran. Strong fluorescent labeling was observed in the neuron cell bodies in the trigeminal ganglion (or gasserian ganglion) at 2 days following the application of dextran (Fig 4A). This result indicates that dIoN-CCI ligation site is the trigeminal afferents. We did not observe any fluorescent signal in the neurons of the facial nucleus at 2, 5 and 7 days after application of dextran (Fig 4B), suggesting that the facial nerve afferents were not involved in dIoN-CCI site. Taken together, retrograde tracing using dextran confirmed that we accessed the trigeminal nerve trunk, but not any facial nerve branches, in dIoN-CCI procedure through our facial incision site.

dIoN-CCI induced prolonged pain behavior in rats

Next, we examined both evoked and non-evoked pain behavior in rats subjected to both dIoN-CCI and sham operation. Compared with sham rats, dIoN-CCI rats exhibited a

significant decrease in threshold toward mechanical stimuli in von Frey test on the ipsilateral side (Fig 5A), which started at 1 day after injury and lasted for at least one month. The response toward mechanical stimulation of the contralateral IoN territory remained unchanged in these same rats. dIoN-CCI rats also displayed an increase in asymmetric face-grooming episodes (Fig 5B) compared with sham rats. The count of face-grooming episodes was also increased in dIoN-CCI rats as compared with sham rats. Furthermore, daily gabapentin treatment attenuated mechanical allodynia (Fig 6A) and reduced face-grooming episodes (Fig 6B) in dIoN-CCI rats.

Discussion

Orofacial pain caused by damage to the trigeminal system is debilitating. Patients have difficulty with daily functions such as eating and talking. Compared with other types of peripheral neuropathic pain, there is a paucity of studies on the cellular and molecular mechanisms underlying trigeminal neuropathic pain. This is probably due to the complexity in performing surgical procedure on trigeminal nerve on animals. IoN-CCI²⁵, the application of complete Freund's adjuvant (CFA) to the orbital portion of the IoN³, and unilateral partial ligation of the IoN (pIONL)^{1, 28} are three commonly used animal models to study trigeminal neuropathic pain. All IoN-CCI procedures described and used by others are also complex and difficult to perform^{10, 12, 13, 25}. In this report, we showed an improved model of IoN-CCI by 1) using a 0.5 cm facial incision to access the IoN trunk outside the orbital cavity and 2) placing two ligatures at the distal IoN just before the IoN branches into the whisker pad.

Our anatomical assessment shows that the IoN exits the orbital cavity through infraorbital fissure located posterior lateral to the infraorbital foramen in Sprague-Dawley rats (Fig 2B). In contrast, the IoN exits the orbital cavity through the infraorbital foramen in human. A segment of the IoN trunk lies outside the orbital cavity before the IoN branches into the whisker pad. Current literature indicates that chronic constriction injury of the IoN has been performed by placing two chromic gut ligatures on the IoN trunk either inside^{13, 25} or outside^{10, 12} the orbital cavity. Both midline²⁵ and eye lid¹³ incisions have been used to gain access to the IoN segment inside the orbital cavity, and in either case the orbital contents are affected. The placement of ligatures on the intraorbital segment of the IoN presses the orbital contents and may cause eye discomfort, leading to increased grooming activities. In contrast, our dIoN-CCI procedure avoids the pressure that induces eye discomfort and resultant behavior presentations. Others have also used midline¹⁰ or intraoral incision¹² to access the IoN trunk outside the orbital cavity for ligation. Both types of incision are more invasive than our facial incision approach. The ligation of the infraorbital nerve (IoN) through an intraoral incision has been applied in both mice¹⁴ and rats²⁶ to the studies of the cellular mechanisms of neuropathic pain. However, intraoral incision directly affects feeding activities and causes unwanted pain around the incision site. Therefore, dIoN-CCI produces a more relevant nerve injury instead of a mixed nerve and tissue injury as previous models do.

We used both anatomical assessment and retrograde neuronal tracing method¹⁸ to confirm that we accessed and ligated the IoN trunk through our facial incision, but not any facial

nerve branches. These data support that the nociceptive phenotypes displayed in dIoN-CCI rats were mediated by trigeminal nerve injury. We noticed that the fluorescence signal from neuronal tracer dextran was detected in the neuron cell bodies in the trigeminal ganglion as early as two days after dextran was applied to the constriction site. It has been reported that when dextran was applied to the buccal nerve branch¹⁵, the fluorescence signal from dextran tracer was observed in the facial nucleus at 5 days after tracer application. Because the trigeminal nerve is much larger in size compared to the buccal nerve branch, it may take less time for the tracer to reach the neuron cell bodies in the trigeminal ganglion.

Overall, our dIoN-CCI procedure is less invasive and the territory of infraorbital nerve remains intact, so is the relevant cutaneous nerve. Thus, the behavior outcome of dIoN-CCI rats is more closely reflective of the “pure” IoN injury. As with other nerve ligation-induced chronic pain animal models, great care is needed to apply proper constriction to the infraorbital nerve to obtain consistent behavioral outcomes in animals^{2, 22}. Altogether, our procedure is simple, minimally invasive, and time-saving, which can be performed without the use of a stereotaxic instrument.

Animals subjected to IoN-CCI display behavioral phenotypes consistent with clinical symptoms caused by trigeminal nerve injury²¹. The improved dIoN-CCI surgical procedure is more suitable for studying trigeminal neuropathic pain. The behavioral outcome of dIoN-CCI rats exhibited the expected pain phenotype of IoN injury. These dIoN-CCI rats exhibited both acute and chronic hypersensitivity towards mechanical stimuli in the ipsilateral whisker pad, whereas the contralateral area was not affected. This is very similar to mononeuropathy induced by sciatic nerve ligation²². Painful sensory disturbance of the IoN territory leads to intense and persistent face-grooming activities in free moving rats²⁴. In our model, enhanced asymmetric face-grooming was observed in dIoN-CCI rats over a prolonged period of time. In contrast, the nociceptive behavior of rats subjected to previous IoN-CCI procedures varies depending on the specific type of procedure used in different studies^{6, 13, 16, 17}. Some studies showed that IoN-CCI rats exhibited an early phase hyposensitivity and a late phase hypersensitivity to mechanical stimulation in the IoN territory^{13, 25}. The early phase hyposensitivity lasted from 3 days¹³ to 14 days²⁵. One study showed that the rats did not exhibit any behavioral change in response to mechanical stimulation until 3 weeks after IoN-CCI¹⁶. In CFA-induced trigeminal pain model, mechanical hyperalgesia (by pin prick) increased gradually, peaked between 7–9 days and returned to the baseline at 11 days after CFA injection³. CFA rats showed a brief increase in face-grooming only on day 1 after CFA injection³. Thus, our dIoN-CCI surgical procedure has the advantage of producing consistent trigeminal pain behaviors at both acute and chronic phases. Consistent with a previous study⁵, gabapentin treatment attenuated mechanical allodynia and reduced face-grooming episodes in the dIoN-CCI rats.

Several new animal models have been developed to aid the study of orofacial pain. One study showed that direct injection of cobra venom factor (CVF) into the trunk of IoN induced nerve demyelination as well as behavioral changes in exploratory activity, face-grooming, etc.³⁰. A spared nerve injury (SNI) through segmental resection of three out of five main IoN branches (SNI-TN) was also developed recently as an approach to evaluating trigeminal pain²⁰. However, the sensitivity to mechanical stimuli, a hallmark feature of

neuropathic pain, has not been tested in either CVF injection or SNI-TN models^{20, 30}. Further studies are needed to evaluate the CVF and SNI-TN models and their role in the study of trigeminal neuropathic pain^{20, 30}. Recently, a new animal model was developed to induce facial pain in rats by chronic constriction of the buccal branch of the facial nerve¹⁵. The rats exhibited both acute and chronic mechanical allodynia after CCI injury¹⁵.

In summary, our improved dIoN-CCI surgical procedure makes this animal model more accessible, which would help facilitate studying the cellular and molecular mechanisms of trigeminal neuropathic pain. The dIoN-CCI procedure also makes this animal model useful to evaluate therapeutic strategies for both acute and chronic trigeminal neuropathic pain.

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Highlights

- We report an improved surgical procedure to induce trigeminal neuropathic pain in rats by unilateral chronic constriction injury of distal infraorbital nerve (dIoN-CCI).
- The dIoN-CCI model is easy to perform, minimally invasive, and time saving.
- The dIoN-CCI rats exhibited both evoked and spontaneous pain behaviors over a prolonged period.
- This is a more clinically relevant rodent model for orofacial pain resulting from trigeminal neuropathic pain.

Fig. 1. Surgical procedure showing chronic constriction of the IoN on rats. **A–E:** Simplified and improved procedure by ligation of a distal segment of the IoN trunk through a facial incision (dIoN-CCI). **F–J:** Ligation of intraorbital segment of the IoN through a midline incision as described by Vos et al²⁵. **K–O:** Ligation of a segment of the IoN outside the orbital cavity through a midline incision as described by Henry et al¹⁰.

Fig. 2.

Anatomy of the infraorbital nerve (IoN) of a Sprague-Dawley (SD) rat. **A:** The IoN trunk exits the orbital cavity through a fissure inferior to the infraorbital foramen. **B:** A segment of the IoN trunk lies at the bottom of the orbital cavity. **C:** The facial structural details of a SD rat. 1: infraorbital nerve, 2: infraorbital fissure (arrow), 3: infraorbital foramen, 4: zygomatic arch, 5: nasal bone. **D:** The infraorbital nerve and underlying anatomical structures. 1a: infraorbital nerve outside the orbital cavity, 1b: infraorbital nerve inside the orbital cavity, 2: zygomatic arch, 3: infraorbital foramen, 4: orbital cavity, 5: nasal bone.

Fig. 3.
Anatomy of the distal infraorbital nerve (IoN) and the facial nerve of a Sprague–Dawley (SD) rat. **A:** Facial nerve and the distal portion of the IoN trunk entering whisker pad. **B:** Our ligation site (arrow) on the IoN. 1: The IoN, 2: the marginal mandibular nerve (MMN) branch, 3: the buccal nerve (BN).

Fig. 4. Representative micrograph images of neuronal tracer dextran staining of the trigeminal ganglion and facial nucleus in a Sprague–Dawley (SD) rat. **A, B:** Trigeminal ganglion was harvested at 2 days after dextran staining. The neuron cell bodies were stained by dextran applied on the dIoN at the same site as for CCI. (A: 4x, B: 20x) **C, D:** At 7 days after applying dextran on the dIoN at the same site as for CCI, dextran staining was not observed in the neuron cell bodies in the facial nucleus. Highlighted area in C: facial nucleus. (C: 4x, D: 10x)

Fig. 5.

Time course of behavioral changes in rats subjected to our improved procedure dIoN-CCI.

A: Mechanical allodynia was increased in the IoN territory after dIoN-CCI. **B:** The dIoN-CCI rats showed increased face-grooming activities. (n = 6/group. Sham vs. dIoN-CCI: * p<0.05)

Fig. 6.

Improved behavioral outcome in the dIoN-CCI rats after gabapentin treatment. **A:** Mechanical allodynia was reduced in the IoN territory after gabapentin treatment. **B:** The dIoN-CCI rats showed decreased face-grooming activities after gabapentin treatment. The treatment started at 3 days after dIoN-CCI. (n = 6/group, before vs. after gabapentin treatment: * p<0.05)