Microglial activation in the trigeminal spinal subnucleus interpolaris/caudalis modulates orofacial incisional mechanical pain hypersensitivity associated with orofacial injury in infancy

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Abstract

Purpose: Infantile tissue injury induces sensory deficits in adulthood. Infantile facial incision (IFI) was reported to cause an enhancement of incision-induced mechanical hypersensitivity in adulthood due to acceleration of the trigeminal ganglion neuronal excitability. However, the effects of IFI on activation of microglia in the spinal trigeminal nucleus and its involvement in facial pain sensitivity is not well known.

Methods: A facial skin incision was made in the left whisker pad in infant (IFI) and/or adult rats (AFI). Mechanical head withdrawal threshold and microglial activation in the trigeminal spinal nucleus were analyzed.

Results: Mechanical pain hypersensitivity induced by IFI was significantly exacerbated and prolonged by IFI. The number of Iba1-immunoreactive cells in the trigeminal spinal nucleus following AFI was increased by IFI, suggesting that IFI facilitates microglial hyperactivation following AFI. Intraperitoneal administration of minocycline, a microglial activation inhibitor, suppressed the facial incision-induced microglial hyperactivation in the trigeminal spinal nucleus and the exacerbation of the facial mechanical pain hypersensitivity induced by IFI.

Conclusion: These results suggest that facial trauma in infants causes hyperactivation of microglia in the trigeminal spinal nucleus following IFI, leading to the prolongation of the facial mechanical pain hypersensitivity.

Keywords: facial incisional pain, infant, microglia, minocycline, trigeminal spinal nucleus

Introduction

Early tissue injury leads to change in somatosensory processing and pain signaling [1,2]. In animal studies, peripheral inflammation in the neonatal period has caused changes in the neuronal circuits in adulthood, leading to mechanical hypersensitivity [3]. Recently, facial skin incision in infants (IFI) causes an enhancement of the mechanical hypersensitivity induced by re-incision in the same facial region in adulthood (AFI), which is thought to be due to the enhancement of trigeminal ganglion (TG) neuronal excitability via activation of primary neuron-satellite glial cell communication in TG [4]. In this manner, peripheral tissue injury in infants can result in dysfunction of neural circuits in the peripheral nervous system in adulthood, leading to sensory defects in orofacial regions. However, the central neurological mechanism underlying the increase of the mechanical hypersensitivity by IFI following IFI is not well known.

Orofacial noxious information is conveyed to the trigeminal spinal nucleus interpolaris (Vi), caudalis (Vc) and upper cervical spinal cord by trigeminal nerve fibers [5-7]. Following peripheral nerve injury, the accumulation of ATP, released from primary afferents, is facilitated in the spinal dorsal horn. ATP has been reported to activate microglia via P2X receptors [8,9]. The activated microglia releases brain-derived neurotrophic factor (BDNF), which acts on TrkB receptors expressed in spinal dorsal horn neurons, leading to an enhancement of neuronal excitability responsible for orofacial pain [6].

To determine whether microglial activation in the Vi/Vc is related to the enhancement of the mechanical hypersensitivity by AFI following IFI, the present study analyzed the changes in the number of activated microglia in the Vi/Vc and mechanical head withdrawal threshold (MHWT) in the whisker pad skin in rats with AFI following IFI.

Materials and Methods

Animals
Male Sprague-Dawley rats (infant rats: 6-8 g, adulthood rats: 200-310 g, Japan SLC, Hamamatsu, Japan) were used. Infant rats were weaned on postnatal day 21. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the guidelines of the International Association for the Study of Pain [10]. All experiments were approved by the Animal Experimentation Committee at Nihon University (animal protocol number: AP17D029). All animals were monitored and maintained under environmental conditions (temperature: 21-23°C and humidity: 40-60%) with food pellets and water provided ad libitum. All procedures were performed during the light phase (12 h light/dark cycle, lights on 7:00-19:00). The number of animals and animal suffering were reduced maximally in all experiments.

Facial skin incision
IFI was performed under deep inhalation anesthesia using isoflurane (1-3% in 1 L/min, Mylan, Canonsburg, PA, USA) on postnatal day 4 (PD4) rats and AFI was performed under deep anesthesia with intraperitoneal administration of butorphanol at 2.5 mg/kg (Meiji Seika Pharmaceutical, Tokyo, Japan), medetomidine at 0.375 mg/kg (Xenoac, Fukushima, Japan), and midazolam at 2.0 mg/kg (Sand, Tokyo, Japan) on postnatal week 7 (PW7). A facial skin incision was made in parallel to the upper lip in the left whisker pad using a blade scalpel (#11, depth: 0.5 mm and length 2.5 mm for infancy; depth: 1 mm and length 10 mm for adulthood) and the incision was closed with silk thread (6-0). The incision site in adult rats was identical with that of infant rats. Sham treatment was conducted by facial skin suture without skin incision on PD4 or PW7.

Measurement of MHWT
To evaluate mechanical sensitivity in facial skin, MHWT was measured in the whisker pad skin. From a week before the measurement, PW7 rats were trained to be stable in the chamber. Mechanical stimulation was applied to the whisker pad skin (2 mm above the incision site) five times at 1 s intervals using von Frey filaments (Touch-Test Sensory Evaluator; North Coast Medical, Morgan Hill, CA, USA) in ascending order of the mechanical intensity (4, 8, 15, 26, 30, 40, 50, 60, and 100 g) at pre-incision and 2, 4, 6, 8, and 12 days after AFI. The lowest mechanical stimulus intensity at...
which the rats escaped 3 out of 5 times was defined as MHWT. The cutoff value was set as 100 g.

**Minocycline administration**

To assess whether microglial activation is involved in increased facial incision-induced mechanical hypersensitivity in adulthood following IFI, intraperitoneal administration of minocycline hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) at 30 mg/kg diluted in saline was performed under inhalation isoflurane anesthesia (2-4% in 1 L/min). The concentration of minocycline hydrochloride was determined according to a previous study [11]. Minocycline hydrochloride was administered daily from the day before the incision until day 5 after AFI. Control rats received an equivalent volume of saline (vehicle). MHWTs after AFI following IFI were measured before (pre) and on day 10 following minocycline hydrochloride or vehicle administration under the same conditions as described above.

**Immunohistochemistry**

Ten days after AFI and sham treatment, rats were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) under deep anesthesia with an intraperitoneal administration of butorphanol at 2.5 mg/kg, medetomidine at 0.375 mg/kg, and midazolam at 2.0 mg/kg. The brain including medulla and cervical cord was dissected and post-fixed with the same fixative for several days at 4°C. For cryoprotection, the tissues were transferred to 20% sucrose in phosphate buffered saline (PBS) overnight. The tissue was sectioned at a thickness of 30 μm using a freezing microtome (Leica, Tokyo, Japan). After rinsing in PBS, free-floating tissue sections were incubated in 3% normal goat serum for 1.5 h in room temperature. After rinsing in PBS, the sections were incubated with Alexa Fluor 568-conjugated anti-rabbit goat IgG (1:100; A11011, Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for 2 h. The sections were rinsed in PBS and then, mounted on MAS-coated glass (Matsunami, Tokyo, Japan) and cover-slipped using a mounting medium (PermaFluor, Thermo Fisher Scientific). Iba1-immunoreactive (IR) cells in the Vi/Vc were analyzed using a BZ-X810 (Keyence, Osaka, Japan). The number of Iba1-IR cells in the Vi/Vc region were counted and the mean number of the Iba1-IR cells (20 sections/rat) was obtained from each animal.

**Statistical analysis**

Data are presented as median and interquartile range (25-75%). In box-and-whisker plots, upper and lower whiskers represent the maximum and minimum values, respectively. The number of rats is presented in parentheses. Statistical analyses were conducted with GraphPad Prism ver. 8 (GraphPad Prism Software Inc., San Diego, CA, USA). A P-value of less than 0.05 was regarded as statistically significant. The Shapiro-Wilk normality test was conducted for the check of the normality for each group. The Brown-Forsythe test was conducted for the check of equality of variance. In the case that the results of the Shapiro-Wilk normality test and the Brown-Forsythe test did not show normality or homogeneity of variances (P < 0.05), non-parametric procedures were selected. The Kruskal-Wallis test followed by Dunn’s multiple comparisons test and Mann-Whitney test were used as the non-parametric procedures.

### Results

#### Changes in mechanical hypersensitivity by AFI following IFI

MHWT in the whisker pad skin was significantly decreased following AFI compared with that of sham operation on day 2 and 4 (row median values; Pre: Sham+Sham 50 g, IFI+Sham 60 g, Sham+AFI 60 g, IFI+AFI 50 g; Day 2: Sham+Sham 50 g, IFI+Sham 50 g, Sham+AFI 40 g, IFI+AFI 4 g) (Fig. 1). The decrease was exacerbated in rats with IFI from day 2 to day 12, indicating prolongation of facial skin incision-induced mechanical hypersensitivity by IFI. The IFI did not change MHWT in the facial skin in adulthood.

#### Microglial activation in the Vi/Vc following AFI

Iba1 immunoreactivity in the Vi/Vc, which is projected by primary nociceptive neurons innervating the facial skin, was analyzed on day 10 after AFI (Fig. 2). The number of Iba1-IR cells in the Vi/Vc by AFI were significantly further increased between 720 μm to −1,440 μm from the obex compared with that of sham treatments in both infant and adulthood (Sham+Sham). In addition, the number of Iba1-IR cells tended to be increased by IFI (P = 0.07) at 720 μm from the obex. The results suggest that IFI enhances microglial activation in the Vi/Vc associated with AFI.

#### Effects of minocycline on microglial hyperactivation associated with the IFI

To assess the effects of minocycline on microglial hyperactivation associated with IFI, minocycline or vehicle was administrated intraperitoneally before and daily for 7 consecutive days (days −1, 0, 1, 2, 3, 4, and 5) after AFI with IFI. Intraperitoneal successive administration of minocycline significantly inhibited the increase in the number of Iba1-IR cells in the Vi/Vc after IFI with AFI (Fig. 3).

#### Effects of minocycline on the enhancement of the mechanical hypersensitivity induced by AFI

To determine whether the microglial hyperactivation accompanied with IFI is involved in the enhancement of the mechanical hypersensitivity induced by AFI, MHWT was measured pre and on day 10 after re-incision with intraperitoneal successive administration of minocycline or vehicle. No significant difference was observed in MHWT pre-AFI between the rats administered minocycline and vehicle. The decrease in MHWT at 10 days after AFI compared with that of sham operation was significantly reduced in the rats administered minocycline (IFI+AFI compared with Sham+Sham) (Fig. 1).
days after AFI with IFI was significantly suppressed to the level of MHWT following AFI only (Sham+AFI) by the administration of minocycline ($P < 0.05$), indicating that microglial hyperactivation accompanied with IFI induces the enhancement of the mechanical hypersensitivity by AFI. There was no significant difference between MHWT in rats that received minocycline and those that received AFI only (Fig. 4).

**Discussion**

The present study demonstrated that experience of IFI causes prolongation of mechanical hypersensitivity induced by re-incision in the same site in adulthood, which is in agreement with the results of a previous study [4]. After AFI, microglial activation evaluated by Iba1 immunoreactivity was observed in the Vi/Vc projected by the second branch of the trigeminal nerve which innervates the whisker pad skin. The AFI-induced microglial activation was significantly enhanced by IFI. Following AFI with IFI, successive intraperitoneal administration of minocycline inhibited the microglial hyperactivation in the Vi/Vc and alleviated the prolongation of mechanical hypersensitivity. These results suggest that the IFT enhances microglial activation in the Vi/Vc after AFI, causing persistent facial injury-induced mechanical hypersensitivity. Skin incision injures distal nerve endings, leading to the release of various molecules such as ATP, neuropeptides, and the chemokine fractalkine from the central terminals of the primary afferent. These contribute to the activation of microglia and astrocytes in the spinal dorsal horn [8,11-13].

Activated microglia releases BDNF and interleukin (IL)-1β [14,15], which bind to TrkB and IL-1R1, respectively, lead to incisional pain in adult rats. In the present study, the AFI-induced mechanical hypersensitivity persisted until day 4 after the incision and then recovered around day 10. In contrast, the number of Iba1-IR cells tended to be higher, but not significantly, on day 10 after AFI only compared with sham rats. It has been reported that activated microglia associated with the orofacial pain hypersensitivity have two polarization states, affective M1 and protective M2. M1 produces pro-inflammatory mediators including IL-1, tumor necrosis factor-α, and IL-6 and contributes to neuropathic pain. The protective M2 produces anti-inflammatory mediators such as IL-4 and IL-10 [16]. Therefore, the protective M2 type of microglia may still remain activated even after the termination of the facial pain hypersensitivity. Currently, the mechanism of microglia M2 activation is still poorly understood. Further research is needed to elucidate the mechanism in detail.

Neonatal tissue injury can result in persistent changes in the peripheral and spinal nociceptive processing [17]. For instance, peripheral inflammation in the neonatal period has evoked hyperexcitability in spinal dorsal horn neurons and hyperexcitability has persisted into adulthood [18]. Additionally, hind paw incision at the first post-natal week has enhanced behavioral response following re-injury in adulthood [19]. In agreement with previous studies, this study showed that IFI induced long-lasting enhancement of mechanical hypersensitivity by AFI and that this prolonged hypersensitivity was suppressed by the inhibition of microglial hyperactivation in the Vi/Vc. It follows that microglial hyper-
activation in the Vi/Vc likely contributes to the long-lasting enhancement of mechanical hypersensitivity. Neonatal surgical injury has been reported to alter spinal neuroimmune reaction including microglial responses, causing the enhancement of injury-induced hyperalgesia in adulthood [2]. The microglial alteration occurred probably because microglia have an instructive role in development of synaptic connectivity and refinement of neuronal circuitry [17]. Therefore, in the present study, IFI-induced microglial alteration was likely involved in the increase in microglial activation following AFI. Taken together, the enhancement of microglial hyperactivation by AFI with IFI increased production and release of some neurotrophic factors and cytokines and this signaling enhanced neuronal excitability in the Vi/Vc, resulting in long-lasting prolongation of the mechanical hypersensitivity by AFI. Since inhibitory transmission in the adult has been decreased through a reduction in the inhibitory glycnergic input onto the spinal dorsal horn neurons by skin incision in the hind paw during the neonatal period [20], the decrease of inhibitory transmission was also likely involved in the pathogenesis of incision-induced mechanical pain hypersensitivity following IFI.

In the present study, the successive intraperitoneal administrations of minocycline inhibited microglial activation and long-lasting prolongation of the mechanical hypersensitivity by AFI with IFI. Minocycline prevented lipopolysaccharide-induced microglial and/or macrophage’s activation and pronociceptive but not antinociceptive cytokine production and release in the dorsal root ganglion and the spinal cord in adult rats [21]. Especially, minocycline decreases the pronociceptive cytokines including IL-6 and IL-1β, but not antinociceptive cytokines such as IL-1α, IL-4, and IL-10 in the spinal cord [22]. Therefore, minocycline administration in the present study was likely to inhibit M1 activation and caused inhibition of the mechanical hypersensitivity induced by IFI.

In conclusion, the present study indicates that the IFI enhances microglial activation in the Vi/Vc after AFI, causing the long-lasting mechanical hypersensitivity. Systemic minocycline administration will be a useful analgesic method for the pathological enhancement of orofacial traumatic pain hypersensitivity due to traumatic stress in infancy.

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Conflict of interest
The authors declared no potential conflicts of interest.

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