Involvement of medullary microglial activation in facial skin incision-induced mechanical allodynia following neonatal facial injury

Tomoyuki Matsui
Nihon University Graduate School of Dentistry
Major in Pediatric Dentistry
(Directors: Profs. Tetsuo Shirakawa and Masamichi Shinoda
and Assist. Prof. Suzuro Hitomi)

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This thesis is based on the following article and additional result in terms of IL-1β expression in the trigeminal spinal subnucleus interpolaris/caudalis (Fig. 2c): Matsui T, Hitomi S, Hayashi Y, Shibuta I, Otsuji J, Ando M, Inada T, Soma K, Iwata K, Shirakawa T, Shinoda M (2021) Microglial activation in the trigeminal spinal subnucleus interpolaris/caudalis modulates orofacial incisional mechanical pain hypersensitivity associated with orofacial injury in infancy. J Oral Sci, in press.

Abstract

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

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

Results: The mechanical pain hypersensitivity induced by AFI was significantly exacerbated and prolonged by NFI. The number of Iba1-immunoreactive cells in the trigeminal spinal nucleus following AFI was increased by NFI, suggesting that NFI 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 NFI.

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

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 to change the neuronal circuits in adulthood, leading mechanical hypersensitivity [3]. Recently, facial skin incision in neonates (NFI) 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 neonates can result in dysfunction of neural circuitries in the peripheral nervous system in adulthood, leading sensory defects in orofacial regions. However, the central neurological mechanism underlying the increase of the mechanical hypersensitivity by AFI following NFI was 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 which is released from primary afferents is facilitated in the spinal dorsal horn. ATP has been reported to activate microglia via $P2X_4$ receptors [8,9]. The activated microglia releases brain-derived neurotrophic factor (BDNF), which acts on TrkB receptors expressed in spinal dorsal horn neurons, leading 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 NFI, 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 the rats with AFI following NFI.

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 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℃ 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

NFI was performed under the 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 the 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). The 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 the 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. The 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 the increased facial incision-induced mechanical hypersensitivity in adulthood following NFI, the intraperitoneal administration of minocycline hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) at 30 mg/kg diluted in saline

were performed under the 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 were received an equivalent volume of saline (Vehicle). MHWTs after AFI following NFI 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 the 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℃. 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 donkey serum for 1.5 h in room temperature at 23°C, and goat polyclonal anti-Ibal antibody (1:500, ab5076; Abcam) and rabbit anti-interleukin-1 beta (IL-1β) antibody (1:200, BS-0812R; BIOSS) for 72 h at 4°C. After washing in PBS, the sections were incubated with Alexa Fluor 568-conjugated donkey anti-goat IgG and Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:1000; A11057 and A21206, respectively, 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 (twenty 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. N represents the number of tested rats. 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 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 ($P < 0.05$), so 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 the mechanical hypersensitivity by AFI following NFI

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, NFI+Sham 60 g, Sham+AFI 60 g, NFI+AFI 50 g; Day 2: Sham+Sham 50 g, NFI+Sham 50 g, Sham+AFI 4 g, NFI+AFI 8 g) (Fig. 1). The decrease was exacerbated in the rats with NFI from day 2 to day 12, indicating prolongation of facial skin incision-induced mechanical hypersensitivity by NFI. The NFI did not change MHWT in the facial skin in adulthood.

Microglial activation and IL-1β expression in the Vi/Vc following AFI

Ibal immunoreactivity in the Vi/Vc to which is projected by primary nociceptive neurons innervating the facial skin was analyzed on day 10 after AFI (Fig. 2). The number of Ibal-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 NFI (P = 0.07) at 720 μm from the obex. The results suggest that NFI enhances microglial activation in the Vi/Vc associated with AFI. Since IL-1β released from activated microglia is known to be involved in modulation of Vc nociceptive neuronal activity [8], the change in expression of IL-1β in the Vi/Vc following AFI was examined. The IL-1β immunoreactivity in the Vi/Vc by AFI was enhanced by NFI (Fig. 2c).

Effects of minocycline on microglial hyperactivation associated with the NFI

To assess the effects of minocycline on microglial hyperactivation associated with NFI, 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 NFI. Intraperitoneal successive administration of minocycline significantly inhibited the increase in the number of Iba1-IR cells in the Vi/Vc after AFI with NFI (Fig. 3).

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

To determine whether the microglial hyperactivation accompanied with NFI is involved in the enhancement of the mechanical hypersensitivity induce 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 with NFI 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 NFI induces the enhancement of the mechanical hypersensitivity by AFI.

There was no significant difference between MHWT in the rats received minocycline and that of rats received AFI only (Fig. 4).

Discussion

The present study demonstrated that experience of NFI causes prolongation of mechanical hypersensitivity induced by re-incision in the same site in adulthood, in agreement with the results of the 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 NFI. Following AFI with NFI, 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 NFI 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 contributes to the activation of microglia and astrocytes in the spinal dorsal horn [8,11-13]. Activated microglia releases BDNF and IL-1β [14,15], which bind to TrkB and IL-1R1, respectively, and lead incisional pain in adult rats. In the present study, the AFI-induced mechanical hypersensitivity was 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-1β, 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. A further research is needed for elucidating the mechanism in detail.

Neonatal tissue injury can result persistent changes in the peripheral and spinal nociceptive processing [17]. For instances, peripheral inflammation in the neonatal period has evoked hyperexcitability in spinal dorsal horn neurons and the 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 the previous studies, the present study showed that NFI induced the long-lasting enhancement of the 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

hyperactivation 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, NFI-induced microglial alteration likely involved in the increase in microglial activation following AFI. In addition, expression of IL-1β in the Vi/Vc after AFI was increased by NFI. Taken together, the enhancement of microglial hyperactivation by AFI with NFI increased production and release of some cytokines including IL-1β and neurotrophic factors and those 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 glycinergic input onto the spinal dorsal horn neurons by skin incision in the hind paw during the neonatal period [20], the decrease of inhibitory transmission also likely involved in the pathogenesis of incision-induced mechanical pain hypersensitivity following NFI.

In the present study, the successive intraperitoneal administrations of minocycline inhibited microglial activation and long-lasting prolongation of the mechanical hypersensitivity by AFI with NFI. 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-1β, IL-6 and IL-18, 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 NFI.

In conclusion, the present study indicates that the NFI 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.

Acknowledgments

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Figures

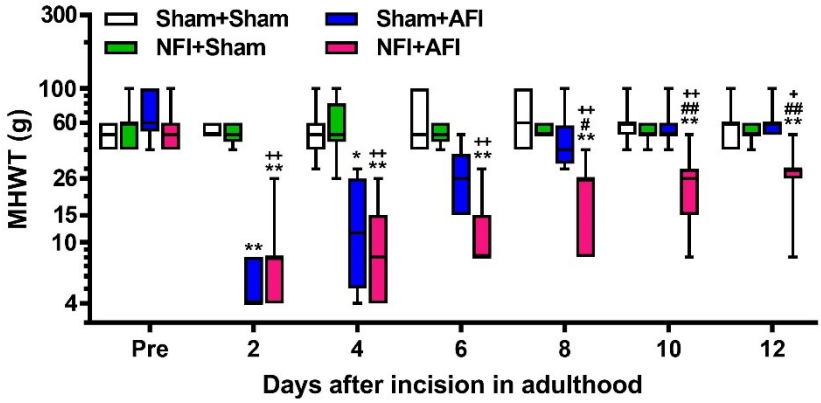

Figure 1

Fig. 1. Changes in mechanical head withdrawal thresholds in facial skin following facial skin incisions in NFI and AFI. Sham+Sham, the sham treatment in both neonate and adulthood, n = 7; NFI+Sham, NFI and the sham treatment in adulthood, n = 9; Sham+AFI, the sham treatment in neonate and AFI, n = 8; NFI+AFI, NFI and AFI, n = 10. Data are presented as median and interquartile range (25-75%). The upper and lower whiskers represent the maximum and minimum values, respectively. The Kruskal-Wallis test followed by Dunn's multiple comparisons test. *: $P < 0.05$, **: $P < 0.01$ compared with the value on Sham+Sham. #: $P < 0.05$, ##: $P < 0.01$ compared with the value on Sham+AFI. +: $P < 0.05$, ++: $P < 0.01$ compared with the value on NFI+Sham.

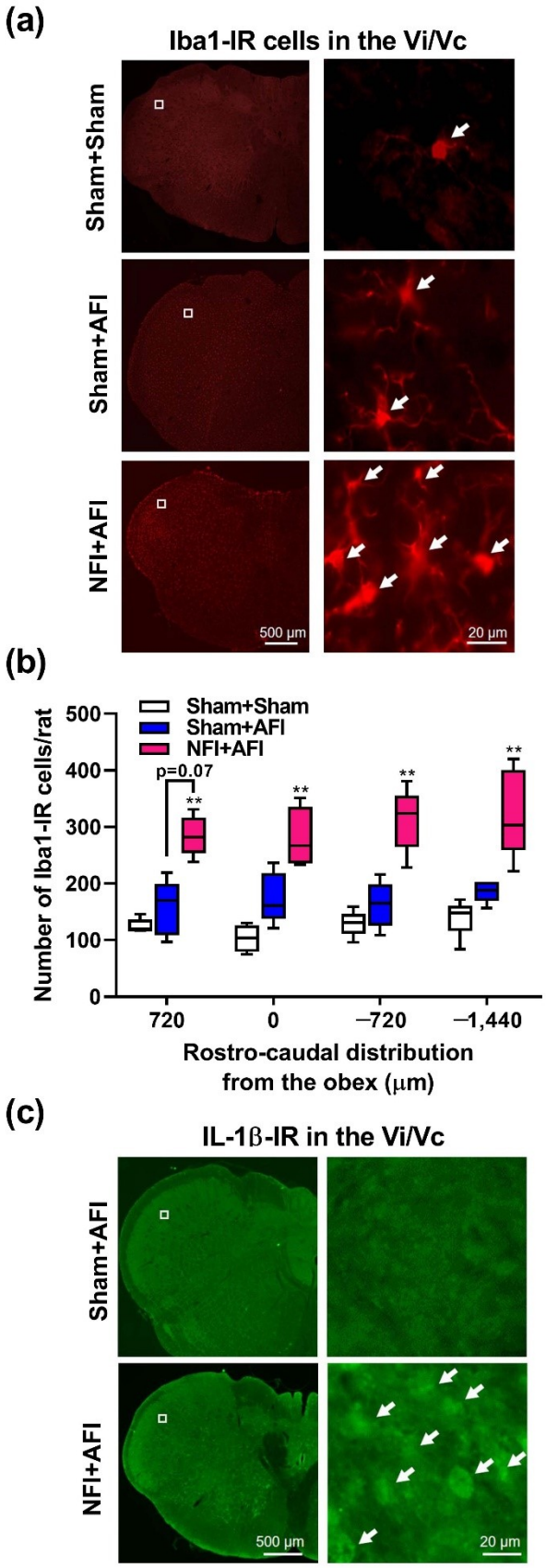
(a)
Iba1-IR cells in the Vi/Vc
Sham+Sham
Sham+AFI
NFI+AFI
500 μm
20 μm
(b)
Sham+Sham
Sham+AFI
NFI+AFI
p=0.07
**
Number of Iba1-IR cells/rat
500
400
300
200
100
0
720
0
−720
−1,440
Rostro-caudal distribution
from the obex (μm)
(c)
IL-1β-IR in the Vi/Vc
Sham+AFI
NFI+AFI
500 μm
20 μm

Figure 2

Fig. 2. Microglial activation and IL-1β expression in the trigeminal spinal nucleus interpolaris/caudalis (Vi/Vc) on day 10 after AFI. Sham+Sham, the sham treatment in both infant and adulthood; Sham+AFI, the sham treatment in AFI; NFI+AFI, facial incision in neonate (NFI) and AFI. (a) Photomicrographs of Ibal-IR cells in the Vi/Vc of in Sham+Sham, Sham+AFI and NFI+AFI. The arrows indicate Ibal-IR cells. The right microphotographs are enlarged images in each open square shown in the left panels. (b) The number of Ibal-IR cells in the Vi/Vc in NFI+AFI, Sham+AFI, and Sham+Sham. Data are presented as median and interquartile range (25-75%). The upper and lower whiskers represent the maximum and minimum values, respectively. n = 5 in each group. The Kruskal-Wallis test followed by Dunn's multiple comparisons test. **: $P < 0.01$ compared with the value in Sham+Sham. (c) Photomicrographs of IL-1β-immunoreactivity in the Vi/Vc of Sham+AFI and NFI+AFI. The arrows indicate IL-1β immunoreactivie cells. The right microphotographs are enlarged images in each open square shown in the left panels.

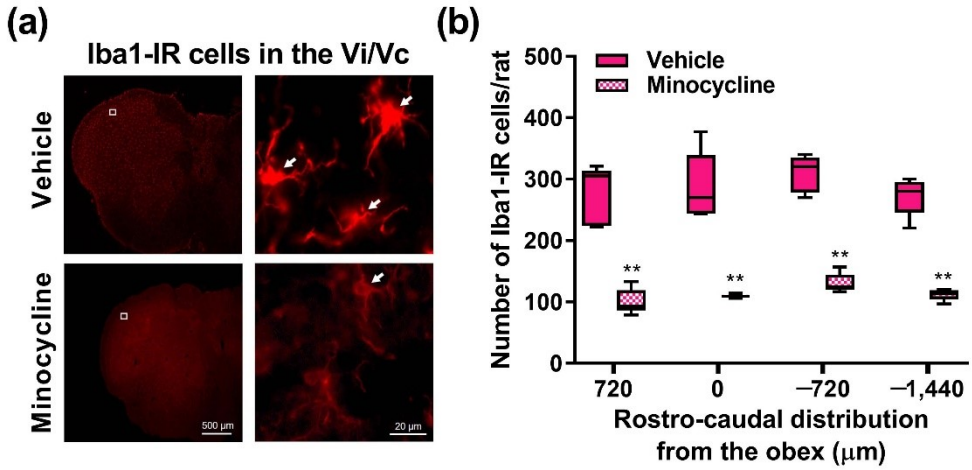

Figure 3

Fig. 3. Effect of minocycline on microglial activation on day 10 after facial skin incision in adulthood (AFI) with the neonatal facial skin incision (NFI). (a) Photomicrographs of Ibal-immunoreactivity (IR) cells in the trigeminal spinal nucleus interpolaris/caudalis (Vi/Vc) following minocycline and vehicle administration. The arrows indicate Ibal-IR cells. The right microphotographs are enlarged images in each open square shown in the left panels. (b) The number of Ibal-IR cells in the Vi/Vc after minocycline and vehicle administration. Data are presented as median and interquartile range (25-75%). The upper and lower whiskers represent the maximum and minimum values, respectively. n = 5 in each group. Mann-Whitney test. **: $P < 0.01$ compared with the value in each Vehicle.

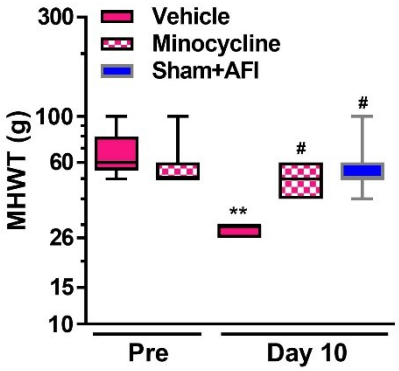

Figure 4

Fig. 4. Effects of minocycline on mechanical head withdrawal threshold (MHWT) in the whisker pad skin pre and on day 10 after facial skin incision in adulthood (AFI) with the neonatal facial skin incision (NFI). Sham+AFI, Sham treatment in neonate and AFI. Sham+AFI data are already shown in Figure 1. Data are presented as median and interquartile range (25-75%). The upper and lower whiskers represent the maximum and minimum values, respectively. Vehicle, n = 5; Minocycline, n = 7. The Kruskal-Wallis test followed by Dunn's multiple comparisons test. **: $P < 0.01$ compared with the value on pre in vehicle. #: $P < 0.05$ compared with the value on day 10 in Vehicle.