Assessment of intraoral mucosal pain induced by the application of capsaicin

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Abstract

Objective: To develop an objective method for assessing nociceptive behavior in an animal model of capsaicin-induced intraoral pain. Changes in nociceptive responses were also examined after injury to the inferior alveolar nerve (IAN).

Design: Nociceptive responses evoked by the intraoral application of various doses of capsaicin were analyzed in lightly anaesthetized rats. The number of c-Fos protein-like immunoreactive (Fos-LI) neurons in the medullary dorsal horn (MDH) induced by the intraoral application of capsaicin was measured. Behavioral and c-Fos responses were also examined 14 days after injury to the IAN.

Results: Larger doses of intraoral capsaicin (1, 10 and 100 μg) induced vigorous licking behavior and c-Fos response in the MDH in a reproducible manner. The magnitudes of both behavioral activity and the c-Fos response from the 10 and 100 μg doses of capsaicin were significantly greater than that by the 1μg dose. Injury to the IAN exaggerated the behavioral and c-Fos responses evoked by intraoral capsaicin.

Conclusions: The intraoral application of capsaicin is a valid and reliable method for studying intraoral pain and hyperalgesia following nerve injury.

Keywords: capsaicin; behavior; c-Fos; medullary dorsal horn; immunohistochemistry; nerve injury

Abbreviations: DAB, diaminobenzidine; Fos-LI, c-Fos protein-like immunoreactive; IAN, inferior alveolar nerve; MDH, medullary dorsal horn; PAP, peroxidase anti-peroxidase; PB, phosphate buffer; PBS, phosphate-buffered saline; TRP, transient receptor potential; TRPV1, transient receptor potential vanilloid 1.

1. Introduction

Assessing pain levels arising from an orofacial region in animal studies is essential for elucidating the mechanisms for the pathophysiology of orofacial pain syndromes including trigeminal neuralgia, burning mouth syndrome, and temporomandibular disorders. A widely used behavioral model of facial pain employs the formalin test [1-4]. A subcutaneous injection of a formalin solution into the face of a rat has been shown to induce the early and late phases of intense grooming activity towards the injected area. A previous study also demonstrated that capsaicin treatment in the orofacial region produced similar face grooming behavior [5]. In spite of the clinical prevalence of pain originating from intraoral tissues, experimental models to assess nocifensive behavior following intraoral noxious stimulation have not yet been established. Therefore, an appropriate experimental model must be established to develop effective treatments for atypical orofacial pain. The experimental manipulation of intraoral tissues is particularly difficult in awake-behaving animals. A lightly anesthetized rat model was shown to permit the assessment of spinal nocifensive behaviors similar to those of unanesthetized rats [6-9].

The use of capsaicin as a noxious stimulus offers several advantages, and is now a widely used tool to study pain mechanisms in both humans and animals. Capsaicin is known to be a specific excitant of C- and Aδ-fibers that convey nociceptive signals [10, 11].

Previous studies demonstrated that capsaicin bound to the transient receptor potential (TRP) vanilloid 1 (TRPV1) channel and induced cation influx in peripheral nerve fiber terminals [12, 13]. Human models of capsaicin-induced pain and hyperalgesia are now available, allowing for correlations between animal and human studies [14-18].

Previous studies reported that thermal or mechanical noxious stimulation applied to peripheral tissue induced an intense c-Fos protein-like immunoreactivity in the spinal dorsal horn with somatotopic represented manner [19, 20]. It has been shown that increasing the intensity of thermal or mechanical stimulation of the facial skin correlated to an increase in the magnitude of c-Fos protein-like immunoreactivity in the medullary dorsal horn (MDH) [21-23]. The induction of c-Fos protein-like immunoreactivity may be associated with the activity of primary afferent nociceptors elicited by noxious stimulation.

The aim of this study was to develop a rat model of capsaicin-induced pain in the intraoral region in order to study trigeminal pain mechanisms. A behavioral test for the nociceptive response evoked by applying capsaicin to the dorsal surface of the tongue was conducted in lightly anesthetized rats. The induction of c-Fos protein-like immunoreactivity in the MDH was also evaluated. We further examined changes in these responses following injury to the inferior alveolar nerve (IAN) as a neuropathic pain model.

2. Materials and Methods

2.1. Animals

Male Sprague-Dawley rats weighing 200-250 g were used in this study. Rats were housed at 20 ˚C with a daily light period of 12 h, and were fed food and water ad libitum. All surgical and experimental procedures described herein were reviewed and approved by the Animal Care and Use Committee, Okayama University (protocol no. OKU-2012354), Government Animal Protection and Management Law (No. 105), Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6), and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), revised 1996. Every attempt was made to minimize the number of animals used and reduce their suffering at all stages of the study.

2.2 Behavioral testing

We examined the effect of various doses of capsaicin (0.001, 0.01, 0.1, 1, 10, and 100 μg) on behavioral responses (n = 6 for each dose). Rats were lightly anesthetized by an i.p. injection of pentobarbital sodium (20 mg/kg). These rats had corneal and flexion reflexes, but not voluntary movement. A video camera was used to record the

nociceptive response to the intraoral capsaicin treatment. Capsaicin (Wako Co., Japan) was dissolved at 1.5% in a vehicle consisting of 10% ethanol, 10% Tween 80, and 80% saline, and was then diluted with distilled water to yield the final concentrations. A 10 μl volume of diluted capsaicin was applied to the dorsal surface of the tongue using a micropipette. Care was taken to prevent the micropipette tip from contacting tissue other than the lingual surface. Following the intraoral application of capsaicin, animals were monitored for nociceptive behavior over a 10 min period. The rats exhibited vigorous licking behavior, i.e., quick movement of the tongue licking intra- and perioral tissues after the application of capsaicin, which continued with or without brief (< 5 sec) intervals. Since this behavior started shortly after the capsaicin application and lasted for several minutes, evaluation the nociceptive response was based on measurement of two parameters; i.e., 1) latency of the onset of licking behavior after the application of capsaicin and 2) the duration of licking (the time period between the onset and termination of licking). Licking behavior rarely reappeared after an interval longer than 5 sec. The termination of licking was determined when the rats showed no more licking behavior for over 5 sec. The behavioral test was performed by an investigator who was blinded to the group assignment of the rat.

2.3. Immunohistochemistry

We also examined the effect of intraoral capsaicin (0, 1, 10, and 100 μg) on the induction of c-Fos protein-like immunoreactivity in the MDH (n = 5 for each dose). Larger doses of capsaicin (10, and 100 μg) was lethal in lightly anesthetized rats and the most rats could not survive for an hour. Therefore, different groups of animals were examined for c-Fos protein-like immunoreactivity after the intraoral application of capsaicin with deep anesthesia. Rats were anesthetized by an i.p. injection of pentobarbital sodium (40-50 mg/kg) and a 10 μl volume of diluted capsaicin or a vehicle was applied to the dorsal surface of the tongue using a micropipette. Two hours after the application, rats were re-anesthetized and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brainstem with the upper cervical spinal cord attached was dissected out, postfixed in the same fixative for 24 h, and then immersed in 20% sucrose in 0.02 M phosphate-buffered saline (PBS, pH 7.4) for 48 h.

Fifty μm-thick transverse frozen sections from the level of the obex to the first cervical level were serially collected in phosphate-buffered saline (PBS, pH 7.4). Alternate series of free-floating sections were processed for immunohistochemistry for the c-Fos protein using the peroxidase anti-peroxidase (PAP) method, as described previously [24]. Briefly, sections were incubated for 1 h with 0.3% H_2O_2 in 80% methanol to quench endogenous peroxidase activity. After incubation with 3% normal goat serum for 1 h, sections were reacted with a rabbit anti-c-Fos antibody (1:8,000; Santa Cruz

Biotechnology, Santa Cruz, CA, USA) for 72 h at 4 ˚C. These sections were then sequentially incubated with goat anti-rabbit IgG (1:300; Cappel West Chester, PA, USA) and PAP complex (1:3,000; Cappel). The reaction products were visualized by nickel ammonium sulfate-intensified diaminobenzidine (DAB) histochemistry. Sections were mounted on glass slides, air-dried, dehydrated in graded alcohol solutions, cleared in xylene, and coverslipped with a mounting medium (Entellan, Merck, Darmstadt, Germany). Since c-Fos protein-like immunoreactivity was restricted to the neuronal nucleus, c-Fos protein-like immunoreactive profiles were hereafter referred to as c-Fos protein-like immunoreactive (Fos-LI) neurons. Using a camera lucida drawing tube, a dark field image of the brainstem section was traced and a bright field image of the Fos-LI neuron profile was plotted on white paper. The rostrocaudal distribution of Fos-LI neurons in the MDH was evaluated by plotting the number of Fos-LI neurons against the distance from the obex. To statistically analyze the magnitude of Fos-LI induction, 5 sections containing the largest number of Fos-LI neurons were selected and the average of 5 sections was recorded for each rat.

2.4. Effect of the IAN injury on capsaicin-induced nociceptive behavior and the c-Fos response

The IAN injury was performed under anesthesia by an i.p. injection of

pentobarbital sodium (40-50 mg/kg). The external aspect of the mandible was exposed by a skin incision and blunt dissection through the masseter muscle. The mandibular canal was exposed by drilling off the overlying bone, and the IAN was ligated firmly with 7-0 silk. The exposed nerve was transected just distal to the ligation, and soft tissues were then sutured. Exposure of the right IAN was also performed in sham-operated animals; however, nerve ligation and transection were omitted. IAN-injured rats and the corresponding sham-operated control animals were used for the histological examination (n = 5 for each surgery) and behavioral test (n = 6 for each surgery).

Rats were lightly anesthetized by an i.p. injection of pentobarbital sodium (20 mg/kg) 14 days after IAN injury or sham surgery for the behavioral test. A 10 µl volume of capsaicin in a dose of 1 µg was applied to the dorsal surface of the tongue using a micropipette as described above. This dose was confirmed to be the minimal dose with which all rats exhibited licking behavior. The latency and duration of licking were measured. The behavioral test was performed by an investigator who was blinded to the group assignment of the rat.

Rats were anesthetized with an i.p. injection of pentobarbital sodium (40-50 mg/kg) 14 days after the IAN injury or sham surgery, and 100 µg of capsaicin was applied to the dorsal surface of the tongue for histological examinations. This dose was confirmed to induce c-Fos protein-like immunoreactivity in the MDH in a reproducible manner; the lower dose of capsaicin the greater the variance in number of neurons

exhibiting the immunoreactivity was. Two hours after the application of capsaicin, rats were perfused transcardially with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brainstem with the upper cervical spinal cord attached was dissected out and processed for c-Fos immunohistochemistry as described above.

2.5. Statistical analysis

Five animals were included in each group for the histochemical experiments, and 6 animals were included in each group for the behavioral analysis. Results are presented as the mean ± SEM. Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by the post-hoc Tukey-Kramer test or Student's *t*-test. The criterion used to determine significance was $P < 0.05$.

3. Results

3.1. Nociceptive responses by the intraoral application of capsaicin

Animals maintained on light anesthesia displayed no spontaneous movements prior to being stimulated. Vigorous licking behavior was observed following the intraoral application of diluted capsaicin, which diminished within several minutes. Although rubbing of the mouth with the forepaw, head rotation, and hindpaw flinching were occasionally observed, licking was the only reliable and reproducible response in lightly anesthetized rats. The effects of various doses of capsaicin were assessed to investigate whether licking behavior was directly related to the intensity of noxious stimulation. Table 1 shows the incidence of licking behavior following the application of each dose of capsaicin. Application of the smallest dose of capsaicin (0.001 μg) did not induce licking behavior. Capsaicin doses of 0.01 and 0.1μg induced nociceptive behavior in 17% and 67% of rats, respectively. Larger doses of capsaicin (1, 10, and 100 μg) produced licking behavior in all rats tested. The licking latency and duration of licking were also measured following the application of these doses of capsaicin. The latency and duration of licking following the application of 1μg capsaicin were approximately 6-7 s and 80-90 s, respectively. The latencies of licking from the 10 and 100 μg doses of capsaicin were significantly shorter than that by the 1μg dose (Fig. 1) (n = 6 for each

group). Furthermore, the durations of licking by the 10 and 100 μg doses of capsaicin were significantly longer than that by the 1μg dose (Fig. 1). Taken together, these results demonstrate that licking behavior is directly related to the degree of noxious input.

3.2. Induction of c-Fos protein-like immunoreactivity by the intraoral application of capsaicin

A vehicle and smaller doses of capsaicin (below 0.1μg) induced few Fos-LI neurons per section of the MDH (Fig. 2). The application of larger doses of capsaicin (1, 10 and 100 μg) induced c-Fos protein-like immunoreactivity in the MDH on both sides (Fig. 2). These neurons were distributed densely in the dorsomedial 1/3 of the superficial laminae of the rostral MDH (Fig. 2A, B). A smaller number of Fos-LI neurons were also found in the deep laminae of the MDH. Along the rostro-caudal axis, they were concentrated at 0.0-3.0 mm caudal to the obex (Fig. 2B). No significant difference was observed in the distributions of Fos-LI neurons between each side. The number of Fos-LI neurons in the MDH for numerical analysis was counted separately for laminae I/II and laminae III/IV. The number of Fos-LI neurons after the application of 1μg capsaicin was approximately 10 per laminae I/II section of the MDH. The topographic distributions of Fos-LI neurons were similar on both sides, and no

significant difference was observed between each side. The number of Fos-LI neurons significantly increased in the superficial laminae after the application of 10 and 100 μg capsaicin (mean number ± SEM, 23.5 ± 3.30 on the left and 21.7 ± 3.81 on the right side for 10 μg; 35.9 ± 2.89 on the left and 36.3 ± 2.26 on the right side for 100 μg; Fig. 3). A slight increase was also observed in the deep laminae.

3.3 Effect of the IAN injury on the licking behavior and c-Fos inducibility evoked by intraoral capsaicin

All rats showed nociceptive responses following the application of 1μg capsaicin. The latency and duration of licking in sham-operated control rats were 5.53 ± 1.39 s and 86.4 ± 16.3 s, respectively. The licking latency was significantly shorter in rats with chronically injured IAN (2.15 ± 0.36 s) than in sham-operated controls (Fig. 4A) (n = 6 for each group). Furthermore, the duration of licking was significantly longer in the IAN-injured group (153 ± 10.3 s) than in the sham-injured group (Fig. 4B).

Intraoral capsaicin (100 μg) induced a large number of Fos-LI neurons in the MDH on both sides (Fig. 5). No significant difference was observed between the distributions of capsaicin-induced Fos-LI neurons following the sham surgery and IAN transection. The number of Fos-LI neurons was significantly higher in the superficial laminae on the ipsilateral side 14 days after IAN injury than after the sham surgery (mean number ±

SEM, 52.9 ± 5.65 for the IAN injured group; 32.7 ± 4.19 for the sham surgery group; Fig. 5). A slight increase was also found on the contralateral side.

4. Discussion

The present study showed that the intraoral application of capsaicin produced reproducible nociceptive behavior and induced Fos-LI neurons in the MDH. The magnitudes of both behavioral activity and the c-Fos response from the 10 and 100 μg doses of capsaicin were significantly greater than that by the 1μg dose. Moreover, the injury to the IAN exaggerated nociceptive behavior and the c-Fos response evoked by the intraoral application of capsaicin.

In this study, the nociceptive behavior evoked by intraoral capsaicin was examined in lightly anesthetized rats. Previous studies demonstrated that a lightly anesthetized rat model permitted the assessment of nocifensive behaviors similar to those of unanesthetized rats [6-9]. An advantage of this lightly anesthetized rat model is that experimental conditions could be reliably manipulated. This model allowed us to apply capsaicin solution intraorally, which cannot be easily achieved in awake animals.

Our results revealed reproducible nociceptive effects of intraoral capsaicin. Larger doses of capsaicin resulted in an increase in the incidence of licking behavior. The licking latency was significantly reduced within a range of 1 to 100 μg, and the duration of licking was significantly prolonged with an increase in the doses of capsaicin applied. These results were consistent with previous reports showing a similar positive correlation with doses up to 1.6 μg using the paw capsaicin test in mice [25]. Previous

studies also demonstrated that the magnitude of pain sensation in humans correlated with the concentration of capsaicin [16, 26, 27]. A recent study using Orofacial Pain Assessment Device (OPAD) showed that capsaicin applied to the cheeks induced nociceptive behavior in awake rats [28].

The distribution of Fos-LI neurons in the medullary and spinal dorsal horns following noxious mechanical and thermal stimulation was shown to be somatotopically organized, and the number of these neurons increased in a stimulus intensity-dependent manner [19, 21-23, 29, 30]. In support of the findings of previous studies, the distribution of Fos-LI neurons in this study was largely restricted to the dorsomedial part of the MDH, and resembled the central terminal field of primary neurons innervating the lingual nerve [31-33]. Therefore, these Fos-LI neurons appear to have been induced by the application of capsaicin to the receptive field of the lingual nerve. Consistent with the behavioral results obtained in this study, the number of Fos-LI neurons induced by larger doses of capsaicin (10 and 100 μg) was significantly greater than that by a smaller dose of capsaicin (1 μg). Similarities in the changes observed in the above morphological and behavioral parameters strongly suggest that the capsaicin-induced increase in the c-Fos response may reflect the sensation of pain.

A growing body of evidence has indicated that peripheral nerve injury induces abnormal pain sensation such as hyperalgesia and allodynia [34]. In addition to the pain as a direct result of the injury itself, aberrant chronic pain was shown to manifest

outside as well as within the peripheral territory of the injured nerve [21, 24, 35]. In this study, we examined changes in behavioral responses and c-Fos inducibility in the MDH evoked by intraoral capsaicin after injury to the IAN. Behavioral analysis in the present study showing enhanced nociceptive responses evoked by the intraoral application of capsaicin in the IAN-injured rats confirmed that peripheral nerve injury caused hyperalgesia in the area adjacent to the denervated area. The number of Fos-LI neurons induced by intraoral capsaicin was significantly increased in the superficial laminae (I/II) on the ipsilateral side of the MDH 14 days after the IAN injury. The MDH was previously shown to normally receive the central projection of trigeminal primary nociceptors [36-40]; therefore, the presently demonstrated enhanced c-Fos protein-like immunoreactivity after the IAN injury may reflect post-traumatic neuropathic pain manifesting in the intraoral and/or perioral regions. A similar injury-induced hyperinducibility of c-Fos was reported for the MDH, in which the number of Fos-LI neurons following noxious stimulation of the whisker pad was significantly increased in IAN injured rats [21]. Similarly in the spinal dorsal horn, transection of a peripheral nerve enhanced neuronal c-Fos induction in response to the stimulation of spared primary nociceptors [24]. We recently reported that the number of Fos-LI neurons induced by electrical stimulation of the lingual nerve at C-fiber intensity (10 mA) was significantly increased in the MDH of rats with chronically injured IAN [33]. These changes may be involved in the expansion of hyperalgesia to the area adjacent to the denervated area.

It is interesting that the injury-induced hyperinducebility of c-Fos was largely limited to the superficial laminae of the MDH ipsilateral to the injury. The effect of injury was barely discernible in either deeper laminae or the contralateral side. Therefore increase of c-Fos response appeared restricted in the vicinity of central terminal field of the injured IAN's primary neurons. Several theoretical explanations are possible for the presently observed c-Fos hyperinducebility. For example injured axons within the neuroma become sensitive to chemical adrenaline [41, 42]. Increase in circulating adrenaline due to noxious stimulation may evoke pathophysiological excitation of injured IAN primary neurons. Moreover, ephaptic connections established between injured and uninjured primary neurons might have exaggerated the capsaicin stimulation. In other instances second order nociceptive neurons in the MDH may receive convergent synaptic input from multiple primary nociceptors; loss of dominant primary input from the IAN might have potentiated excitability of second order nociceptors and, thereby, unmasked previously silent synapses from the lingual nerve's primary neurons. We have to await further investigation before we can pinpoint any one of these as the cause for increase in c-Fos response.

In summary, the present study showed that the application of intraoral capsaicin is a valid and reliable method for studying trigeminal pain and hyperalgesia after nerve injury. The magnitude of behavioral responses and number of Fos-LI neurons in the MDH induced by intraoral capsaicin strongly correlated with the stimulus intensity. We

also demonstrated that IAN injury exaggerated nociceptive behavior and c-Fos protein-like immunoreactivity in the MDH evoked by intraoral capsaicin. The intraoral application of capsaicin represents a useful tool for studying the mechanisms mediating intraoral nociception and identifying analgesic compounds to treat nerve injury-induced hyperalgesia and allodynia in the orofacial region.

Author contribution

Three authors (R. Terayama, S. Iida and T. Sugimoto) prepared the study design, examined and analyzed the data and wrote the manuscript. Three authors (K. Maruhama, H. Tsuchiya and M. Mizutani) contributed to the behavioral experiments.

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Competing interests

The authors declare that they have no conflicts of interest.

Ethical approval

All experiments was approved by the Animal Care and Use Committee, Okayama University (protocol no. OKU-2012354).

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References

1. Clavelou P, Dallel R, Orliaguet T, Woda A, Raboisson P. The orofacial formalin test in rats: effects of different formalin concentrations. Pain. 1995 Sep;**62**(3):295-301.

2. Clavelou P, Pajot J, Dallel R, Raboisson P. Application of the formalin test to the study of orofacial pain in the rat. Neurosci Lett. 1989 Sep 11;**103**(3):349-53.

3. Eisenberg E, Vos BP, Strassman AM. The NMDA antagonist Memantine blocks pain behavior in a rat model of formalin-induced facial pain. Pain. 1993 Sep;**54**(3):301-7.

4. Eisenberg E, Vos BP, Strassman AM. The peripheral antinociceptive effect of morphine in a rat model of facial pain. Neuroscience. 1996 May;**72**(2):519-25.

5. Pelissier T, Pajot J, Dallel R. The orofacial capsaicin test in rats: effects of different capsaicin concentrations and morphine. Pain. 2002 Mar;**96**(1-2):81-7.

6. Sandkuhler J, Gebhart GF. Characterization of inhibition of a spinal nociceptive reflex by stimulation medially and laterally in the midbrain and medulla in the pentobarbital-anesthetized rat. Brain Res. 1984 Jul 2;**305**(1):67-76.

7. Terayama R, Dubner R, Ren K. The roles of NMDA receptor activation and nucleus reticularis gigantocellularis in the time-dependent changes in descending inhibition after inflammation. Pain. 2002 May;**97**(1-2):171-81.

8. Terayama R, Guan Y, Dubner R, Ren K. Activity-induced plasticity in brain stem pain modulatory circuitry after inflammation. Neuroreport. 2000 Jun 26;**11**(9):1915-9.

9. Okada-Ogawa A, Suzuki I, Sessle BJ, Chiang CY, Salter MW, Dostrovsky JO, et al. Astroglia in medullary dorsal horn (trigeminal spinal subnucleus caudalis) are involved in trigeminal neuropathic pain mechanisms. J Neurosci. 2009 Sep 9;**29**(36):11161-71.

10. LaMotte RH, Lundberg LE, Torebjork HE. Pain, hyperalgesia and activity in nociceptive C units in humans after intradermal injection of capsaicin. J Physiol. 1992 Mar;**448**:749-64.

11. Ringkamp M, Peng YB, Wu G, Hartke TV, Campbell JN, Meyer RA. Capsaicin responses in heat-sensitive and heat-insensitive A-fiber nociceptors. J Neurosci. 2001 Jun 15;**21**(12):4460-8.

12. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature. 1997 Oct 23;**389**(6653):816-24.

13. Levine JD, Alessandri-Haber N. TRP channels: targets for the relief of pain. Biochim Biophys Acta. 2007 Aug;**1772**(8):989-1003.

14. Green BG, Shaffer GS. The sensory response to capsaicin during repeated topical exposures: differential effects on sensations of itching and pungency. Pain. 1993

Jun;**53**(3):323-34.

15. Pertens E, Urschel-Gysbers BA, Holmes M, Pal R, Foerster A, Kril Y, et al. Intraspinal and behavioral consequences of nerve growth factor-induced nociceptive sprouting and nerve growth factor-induced hyperalgesia compared in adult rats. J Comp Neurol. 1999 Jul 19;**410**(1):73-89.

16. Simone DA, Baumann TK, LaMotte RH. Dose-dependent pain and mechanical hyperalgesia in humans after intradermal injection of capsaicin. Pain. 1989 Jul;**38**(1):99-107.

17. Zhao NN, Whittle T, Murray GM, Peck CC. The effects of capsaicin-induced intraoral mucosal pain on jaw movements in humans. J Orofac Pain. 2012 Fall;**26**(4):277-87.

18. Ngom PI, Dubray C, Woda A, Dallel R. A human oral capsaicin pain model to assess topical anesthetic-analgesic drugs. Neurosci Lett. 2001 Dec 28;**316**(3):149-52.

19. Hunt SP, Pini A, Evan G. Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. Nature. 1987;**328**(6131):632-4.

20. Williams S, Evan GI, Hunt SP. Changing patterns of c-fos induction in spinal neurons following thermal cutaneous stimulation in the rat. Neuroscience. 1990;**36**(1):73-81.

21. Nomura H, Ogawa A, Tashiro A, Morimoto T, Hu JW, Iwata K. Induction of Fos protein-like immunoreactivity in the trigeminal spinal nucleus caudalis and upper

cervical cord following noxious and non-noxious mechanical stimulation of the whisker pad of the rat with an inferior alveolar nerve transection. Pain. 2002;**95**(3):225-38.

22. Strassman AM, Vos BP. Somatotopic and laminar organization of fos-like immunoreactivity in the medullary and upper cervical dorsal horn induced by noxious facial stimulation in the rat. J Comp Neurol. 1993;**331**(4):495-516.

23. Strassman AM, Vos BP, Mineta Y, Naderi S, Borsook D, Burstein R. Fos-like immunoreactivity in the superficial medullary dorsal horn induced by noxious and innocuous thermal stimulation of facial skin in the rat. J Neurophysiol. 1993;**70**(5):1811-21.

24. Sugimoto T, Ichikawa H, Hijiya H, Mitani S, Nakago T. c-Fos expression by dorsal horn neurons chronically deafferented by peripheral nerve section in response to spared, somatotopically inappropriate nociceptive primary input. Brain Res. 1993;**621**(1):161-6.

25. Sakurada T, Katsumata K, Tan-No K, Sakurada S, Kisara K. The capsaicin test in mice for evaluating tachykinin antagonists in the spinal cord. Neuropharmacology. 1992 Dec;**31**(12):1279-85.

26. Green BG, Rentmeister-Bryant H. Temporal characteristics of capsaicin desensitization and stimulus-induced recovery in the oral cavity. Physiol Behav. 1998 Aug;**65**(1):141-9.

27. LaMotte RH, Shain CN, Simone DA, Tsai EF. Neurogenic hyperalgesia:

psychophysical studies of underlying mechanisms. J Neurophysiol. 1991 Jul;**66**(1):190-211.

28. Anderson EM, Jenkins AC, Caudle RM, Neubert JK. The effects of a co-application of menthol and capsaicin on nociceptive behaviors of the rat on the operant orofacial pain assessment device. PLoS One. 2014;**9**(2):e89137.

29. Bullitt E. Expression of c-fos-like protein as a marker for neuronal activity following noxious stimulation in the rat. J Comp Neurol. 1990;**296**(4):517-30.

30. Terayama R, Nagamatsu N, Ikeda T, Nakamura T, Rahman OI, Sakoda S, et al. Differential expression of Fos protein after transection of the rat infraorbital nerve in the trigeminal nucleus caudalis. Brain Res. 1997;**768**(1-2):135-46.

31. Takemura M, Sugimoto T, Sakai A. Topographic organization of central terminal region of different sensory branches of the rat mandibular nerve. Exp Neurol. 1987 Jun;**96**(3):540-57.

32. Takemura M, Sugimoto T, Shigenaga Y. Difference in central projection of primary afferents innervating facial and intraoral structures in the rat. Exp Neurol. 1991 Mar;**111**(3):324-31.

33. Fujisawa N, Terayama R, Yamaguchi D, Omura S, Yamashiro T, Sugimoto T. Fos protein-like immunoreactive neurons induced by electrical stimulation in the trigeminal sensory nuclear complex of rats with chronically injured peripheral nerve. Exp Brain Res. 2012 Jun;**219**(2):191-201.

34. Zimmermann M. Pathobiology of neuropathic pain. Eur J Pharmacol. 2001 Oct 19;**429**(1-3):23-37.

35. Sugimoto T, Ichikawa H, Mitani S, Hitsu A, Nakago T, Nakago T. Changes in c-fos induction in dorsal horn neurons by hindpaw formalin stimulation following tibial neurotomy. Brain Res. 1994;**642**(1-2):348-54.

36. Dubner R, Bennett GJ. Spinal and trigeminal mechanisms of nociception. Annu Rev Neurosci. 1983;**6**:381-418.

37. Sessle BJ. Acute and chronic craniofacial pain: brainstem mechanisms of nociceptive transmission and neuroplasticity, and their clinical correlates. Crit Rev Oral Biol Med. 2000;**11**(1):57-91.

38. Sugimoto T, Fujiyoshi Y, He YF, Xiao C, Ichikawa H. Trigeminal primary projection to the rat brain stem sensory trigeminal nuclear complex and surrounding structures revealed by anterograde transport of cholera toxin B subunit-conjugated and Bandeiraea simplicifolia isolectin B4-conjugated horseradish peroxidase. Neurosci Res. 1997 Aug;**28**(4):361-71.

39. Sugimoto T, Fujiyoshi Y, Xiao C, He YF, Ichikawa H. Central projection of calcitonin gene-related peptide (CGRP)- and substance P (SP)-immunoreactive trigeminal primary neurons in the rat. J Comp Neurol. 1997 Feb 17;**378**(3):425-42.

40. Woda A. Pain in the trigeminal system: from orofacial nociception to neural network modeling. J Dent Res. 2003 Oct;**82**(10):764-8.

41. Korenman EM, Devor M. Ectopic adrenergic sensitivity in damaged peripheral nerve axons in the rat. Exp Neurol. 1981;**72**(1):63-81.

42. Wall PD, Gutnick M. Properties of afferent nerve impulses originating from a neuroma. Nature. 1974 Apr 26;**248**(5451):740-3.

Figure legends

Figure 1

The latency (A) and duration (B) of licking induced by the intraoral application of capsaicin. Licking latencies following the application of larger doses of capsaicin (10 and 100 μg) were significantly shorter than that of the smaller dose (1 μg). The durations of licking induced by larger doses of capsaicin were significantly longer than that by the 1μg dose. Each bar represents the mean value ± SEM of 6 rats. Asterisks indicate significant differences from the 1μg-dose group (*P < 0.05; **P < 0.01, ANOVA with the post-hoc Tukey-Kramer test).

Figure 2

(A) Immunohistochemistry for c-Fos in the MDH following the intraoral application of various doses of capsaicin. (B) Camera-lucida drawings of Fos-LI neurons in the MDH. The number at the bottom of each drawing indicates distances in mm rostral to the obex. Rectangles in B indicate the field of micrographs in A. (C) The rostro-caudal distribution of Fos-LI neurons in the superficial laminae (I/II) and deep laminae (III/IV) of the MDH. Representative data from one animal in each group are shown. The numbers per section of neurons on the right and left sides were plotted against the rostro-caudal axis. Scale bar = 100 μm. Cu, cuneate nucleus; LRt, lateral reticular

nucleus; Tr, spinal trigeminal tract.

Figure 3

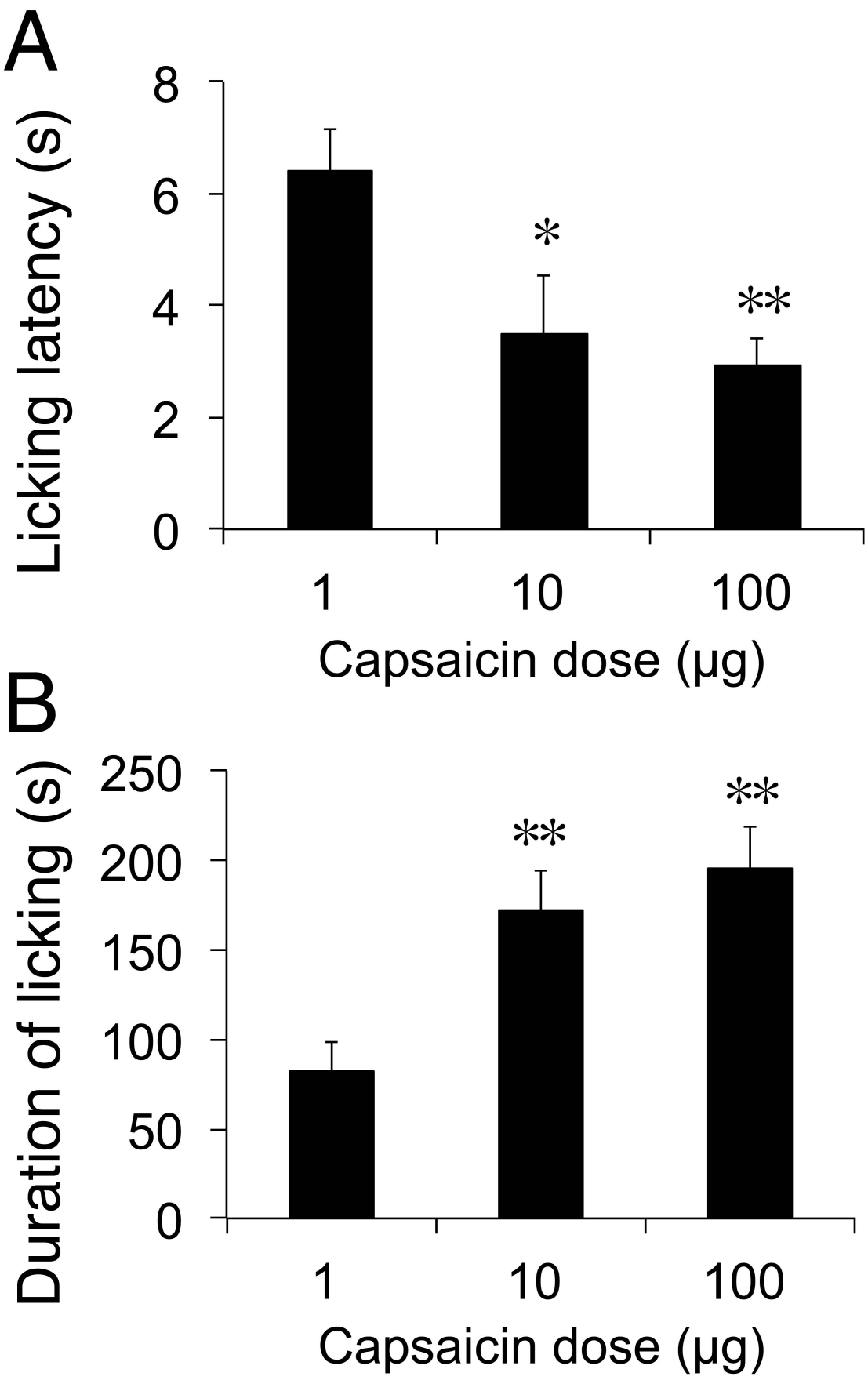
Effect of increasing doses of capsaicin on the induction of Fos-LI neurons in the MDH. The numbers per section of Fos-LI neurons in the superficial and deep laminae of the MDH induced by the intraoral application of capsaicin are shown. The number of Fos-LI neurons induced in the superficial laminae of the MDH following the application of larger doses of capsaicin (10 and 100 μg) was significantly higher than that of the smaller dose (1 μg). Each bar represents the mean value ± SEM of 5 individual experiments. Asterisks indicate significant differences from the 1μg-dose group at each side (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, ANOVA with the post-hoc Tukey-Kramer test). Sharps indicate significant differences from the 10μg-dose group at each side. (#$P < 0.05$, ANOVA with the post-hoc Tukey-Kramer test).

Figure 4

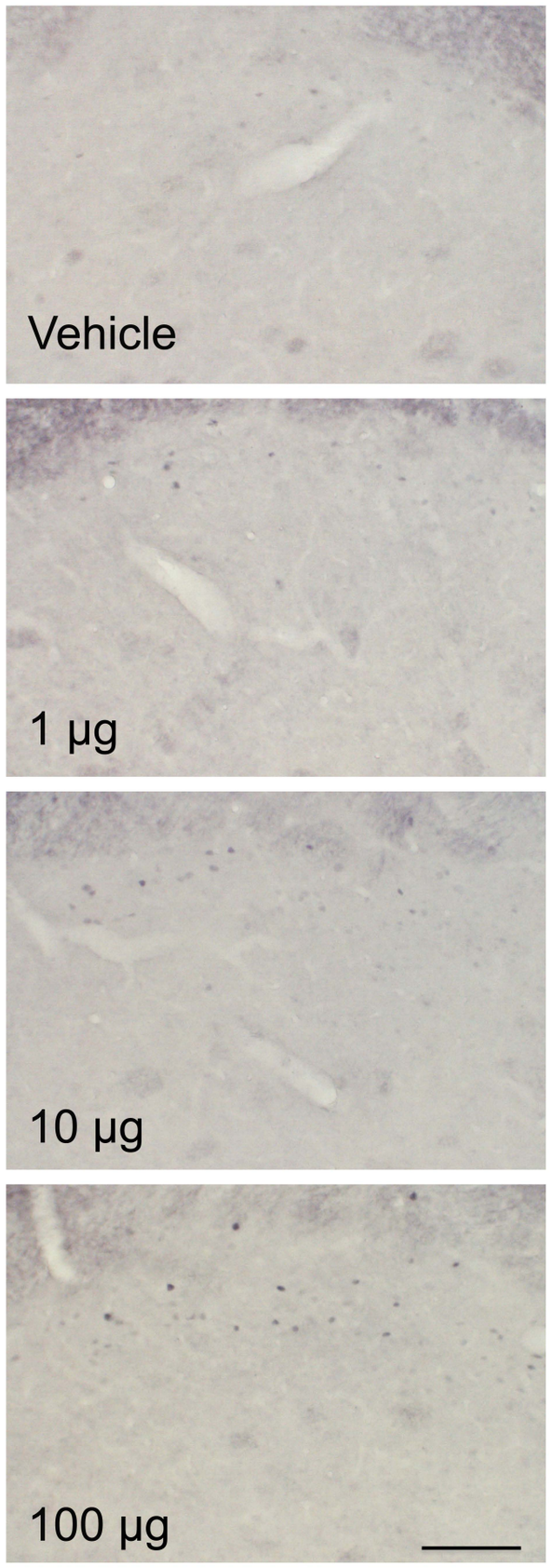
The latency (A) and duration (B) of licking induced by the intraoral application of 1μg capsaicin in lightly anesthetized rats. The latency and duration of licking in the IAN-injured group (Injury) were significantly different from those in the sham-operated group (Sham). Each bar represents the mean value ± SEM of 6 rats (*$P < 0.05$; **$P < 0.01$, the Student's *t*-test).

Figure 5

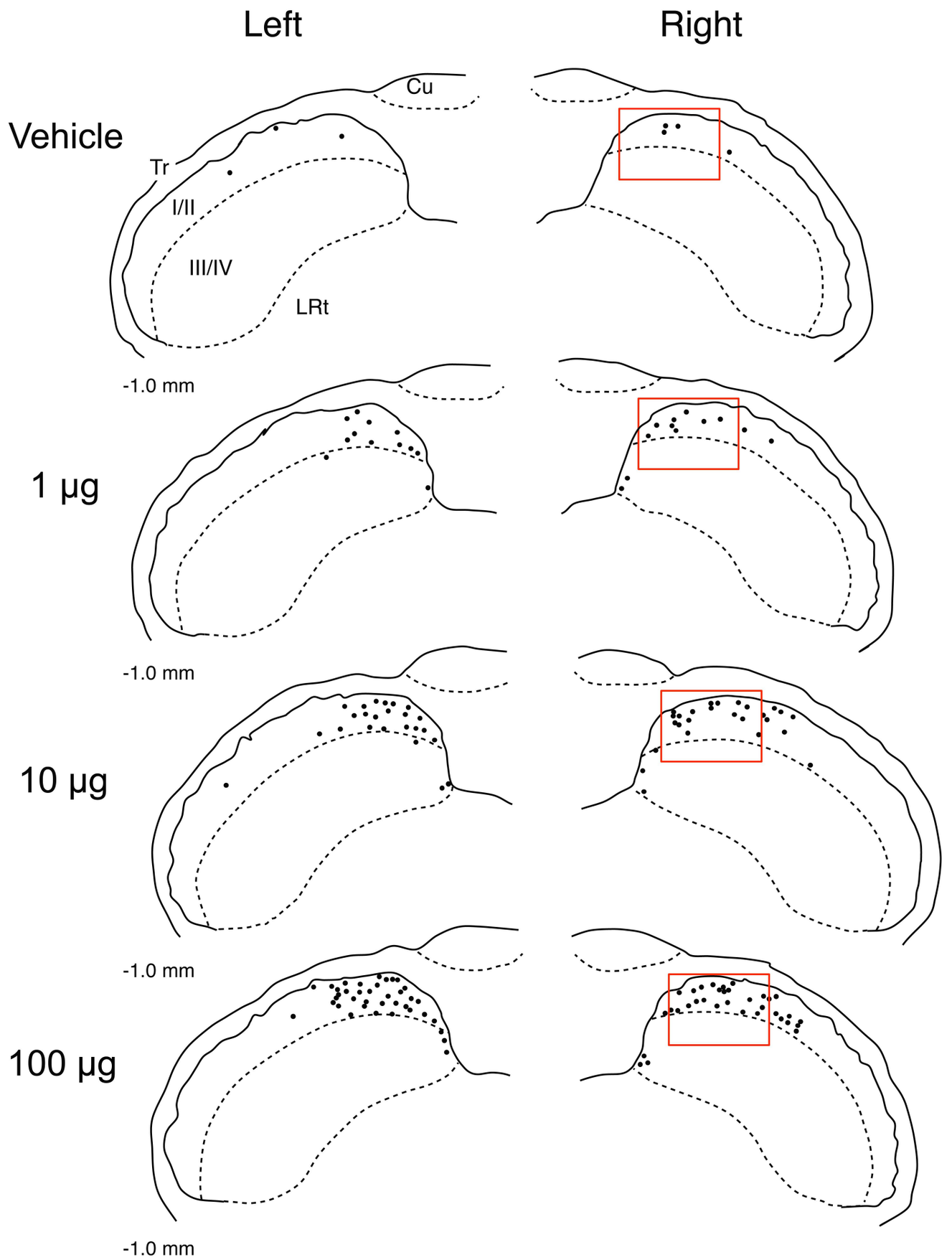
The number per section of Fos-LI neurons in the superficial (A) and deep laminae (B) of the MDH following the intraoral application of 100 μg capsaicin 14 days after the IAN injury (Injury) and sham surgery (Sham). The number of Fos-LI neurons found in both the superficial and deep laminae of the MDH ipsilateral to the injury was significantly higher in the IAN-injured group than in the sham-operated group. Each bar represents the mean value ± SEM of 5 individual experiments. Asterisks indicate significant differences from the sham-operated groups (*$P < 0.05$, the Student's *t*-test).


A
Licking latency (s)
8
6
4
2
0
*
**
1
10
100
Capsaicin dose (μg)
B
Duration of licking (s)
250
200
150
100
50
0
**
**
1
10
100
Capsaicin dose (μg)

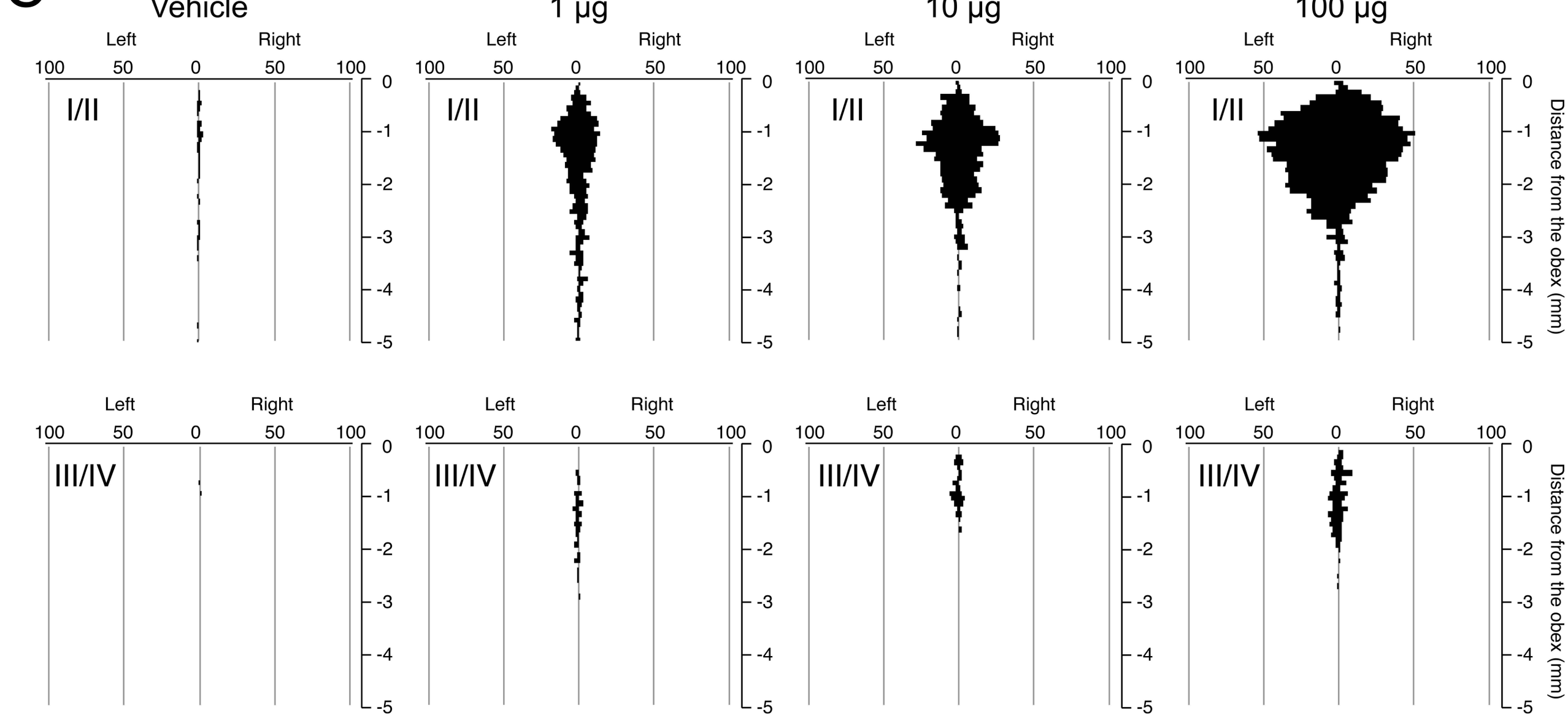
A

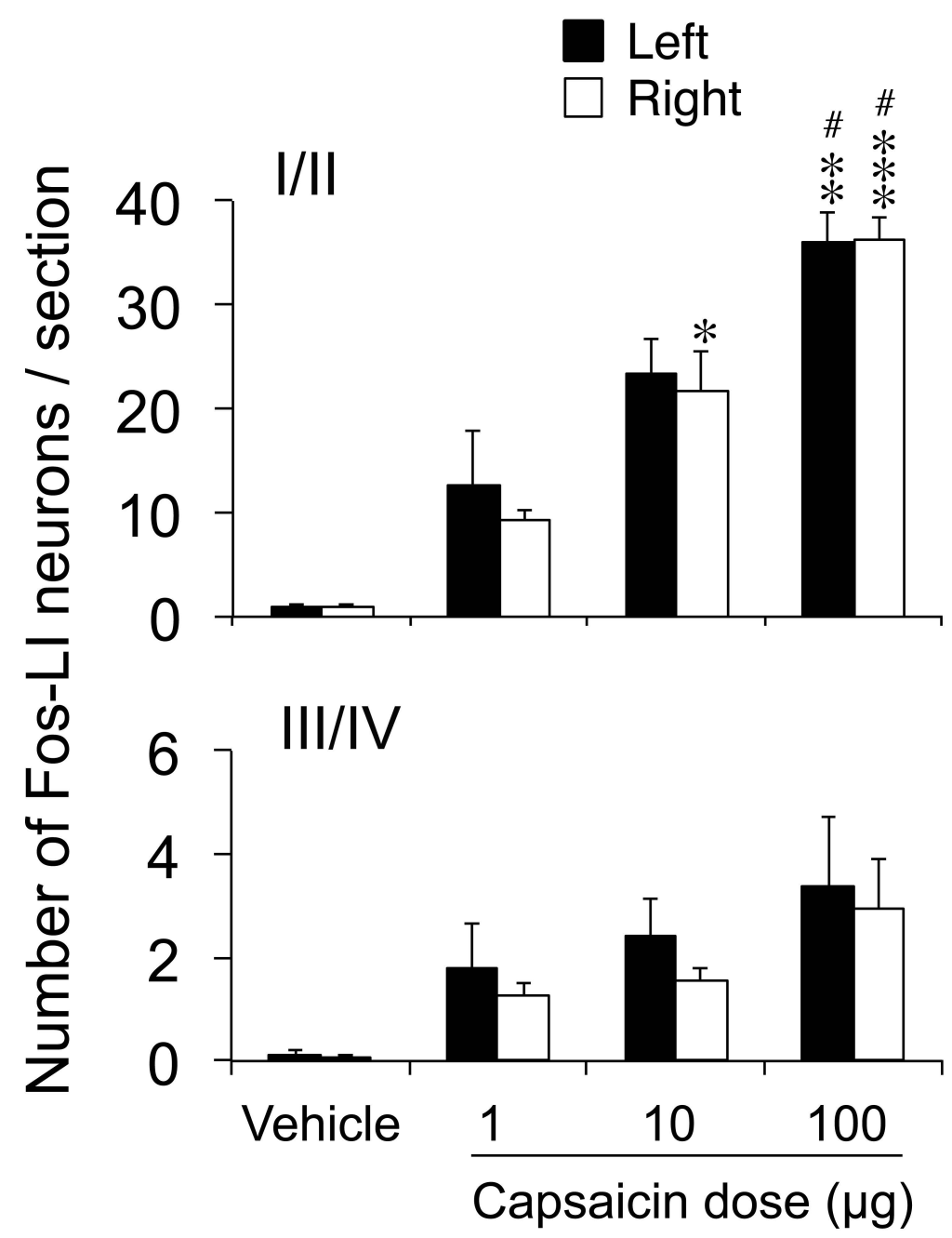

Vehicle
1 µg
10 µg
100 µg

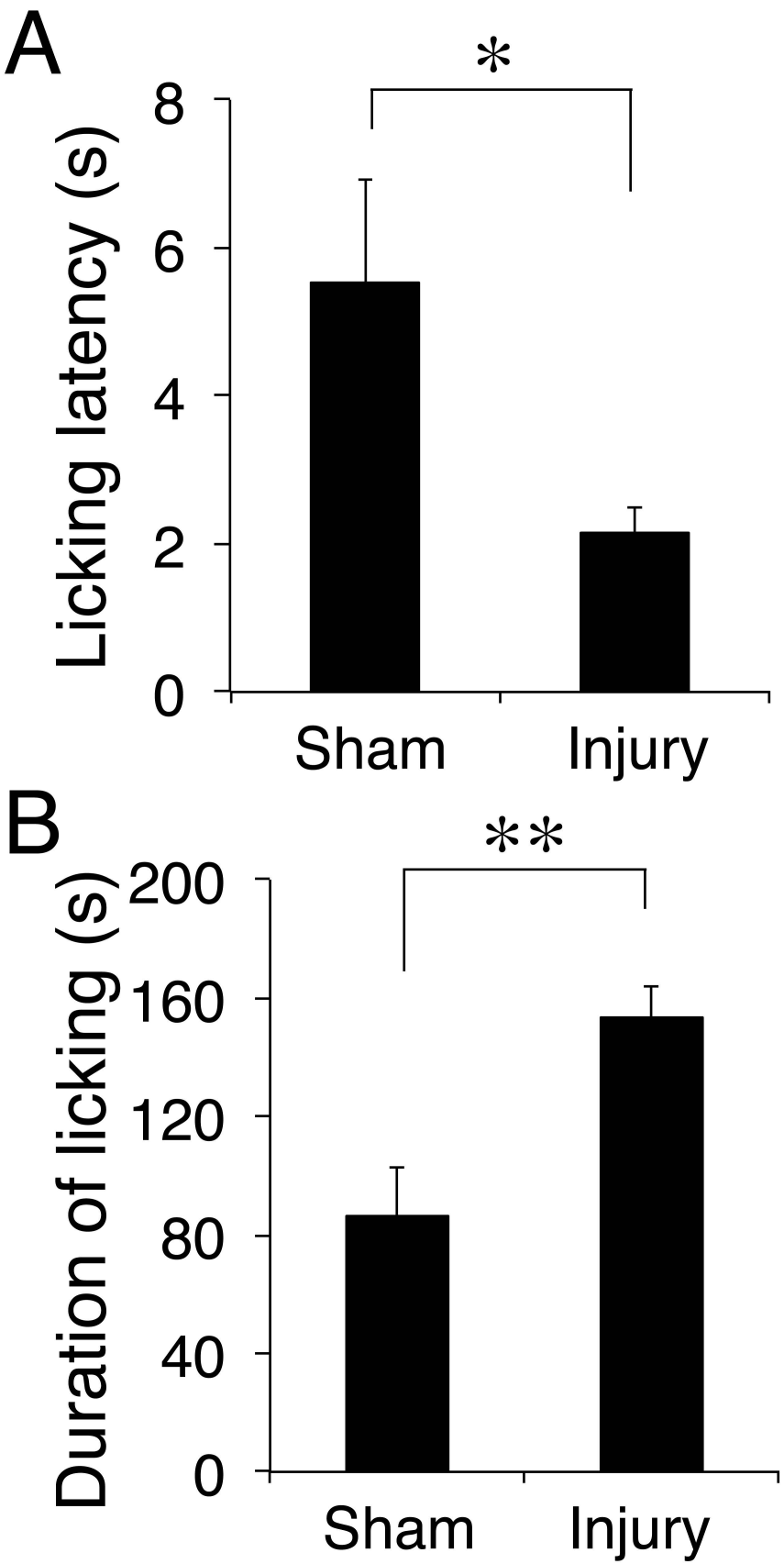
B


Left
Right
Vehicle
Cu
Tr
I/II
III/IV
LRt
-1.0 mm
1 µg
-1.0 mm
10 µg
-1.0 mm
100 µg
-1.0 mm

C


Vehicle
1 µg
10 µg
100 µg
Left
Right
100 50 0 50 100
I/II
III/IV
0 -1 -2 -3 -4 -5
Distance from the obex (mm)


Left
Right
I/II
III/IV
Number of Fos-LI neurons / section
40
30
20
10
0
6
4
2
0
#
****
#
****
*
Vehicle
1
10
100
Capsaicin dose (μg)


A
*
Licking latency (s)
8
6
4
2
0
Sham
Injury
B
**
Duration of licking (s)
200
160
120
80
40
0
Sham
Injury

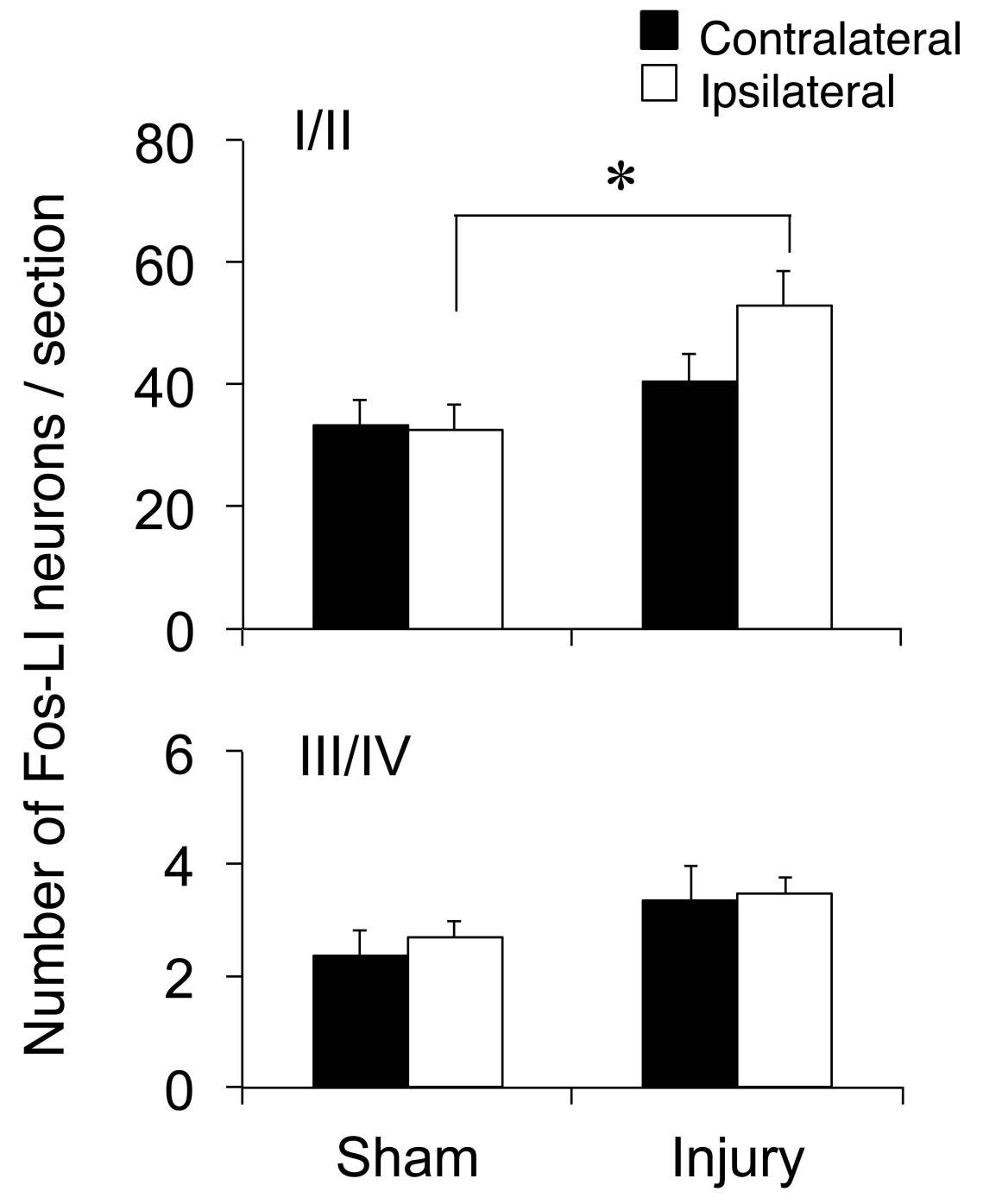

Contralateral
Ipsilateral
I/II
*
80
60
40
20
0
III/IV
6
4
2
0
Number of Fos-LI neurons / section
Sham
Injury

Table 1. Incidence of licking behavior following the intraoral application of capsaicin

Capsaicin Dose (μg)	Incidence
0.001	0% (0/6)
0.01	17% (1/6)
0.1	67% (4/6)
1	100% (6/6)
10	100% (6/6)
100	100% (6/6)