Original Article

Tooth Movement Activates the Central Amygdala and the Lateral Hypothalamus by the Magnitude of the Force Applied

Ana Paula Ribeiro Novaes[a]; Maria José Alves da Rocha[b]; Christie Ramos Andrade Leite-Panissi[b]

ABSTRACT

Objective: To determine if the magnitude of the force used to induce incisor tooth movement promotes distinct activation in cells in the central amygdala (CEA) and lateral hypothalamus (LH) of rats. Also, the effect of morphine on Fos immunoreactivity (Fos-IR) was investigated in these nuclei.

Materials and Methods: Adult male rats were anesthetized and divided into six groups: only anesthetized (control), without orthodontic appliance (OA), OA but without force, OA activated with 30*g* or 70*g*, OA with 70*g* in animals pretreated with morphine (2 mg/kg, intraperitoneal). Three hours after the onset of the experiment the rats were reanesthetized and perfused with 4% paraformaldehyde. The brains were removed and fixed, and sections containing CEA and LH were processed for Fos protein immunohistochemistry.

Results: The results show that in the control group, the intramuscular injection of a ketamine/xylazine mixture did not induce Fos-IR cells in the CEA or in the LH. Again, the without force group showed a little Fos-IR. However, in the 70*g* group the Fos-IR was the biggest observed ($P < .05$, Tukey) in the CEA and LH compared with the other groups. In the 30*g* group, the Fos-IR did not differ from the control group, the without OA group, and the without force group. Furthermore, pretreatment with morphine in the 70*g* group reduced Fos-IR in these regions.

Conclusions: Tooth movement promotes Fos-IR in the CEA and LH according to the magnitude of the force applied. (*Angle Orthod.* 2010;80:111–115.)

KEY WORDS: Fos-protein; Orthodontic movement; Forebrain

INTRODUCTION

Orthodontic correction that involves the entire dental arch normally causes discomfort and unpleasant sensations, such as pain. These sensations are usually momentary and disappear gradually.[1] The acute force loaded onto teeth may be regarded as a noxious stimulus to the primary afferents in the tooth pulp and periodontal ligament and is transmitted to the central nervous system. Anatomic studies using transganglionic transport of neuronal tracers have shown that tooth afferents project to the principal sensory and spinal nuclei of the trigeminal system.[2]

Accordingly, tooth movement in rats was seen to induce c-Fos expression in neurons localized in the rat trigeminal nucleus complex as revealed by Fos-immunoreactivity (Fos-IR).[3–5] It was also seen to activate neurons in the parabrachial nucleus, which is one of the important relay nuclei for processing information from the orofacial regions.[6,7] Preadministration of morphine drastically reduced c-Fos expression in the trigeminal nucleus complex and all subnuclei of the parabrachial nucleus.[3,8] These results suggest that the appearance of Fos-IR cells in these nuclei may be at least partly due to noxious stimulation and/or stress related to pain.

The parabrachial nucleus is known as a relay site for nociceptive pathways, the spino(trigemino)parabrachiohypothalamic[9] and the spino(trigemino)pontoamygdaloid pathways.[10] Both pathways are involved in the affective-emotional (fear, aggression), behavioral

[a] Graduate PhD student, Department of Morphology, Stomatology and Physiology, School of Dentistry of Ribeirão Preto, University of São Paulo, São Paulo, Brazil.

[b] Associate Professor, Department of Morphology, Stomatology and Physiology, School of Dentistry of Ribeirão Preto, University of São Paulo, São Paulo, Brazil.

Corresponding author: Dr Christie Ramos Andrade Leite-Panissi, Faculdade de Odontologia de Ribeirão Preto – USP, Departamento de Morfologia, Estomatologia e Fisiologia, Ribeirão Preto, São Paulo, Brazil
(e-mail: christie@forp.usp.br)

Accepted: March 2009. Submitted: October 2008.

(vocalization, fight, freezing), and autonomic (cardiovascular and respiratory responses) reactions to noxious events.[9,10] Particularly, masseter muscle inflammation promotes activation of trigeminal neurons that project to rostral sites such as the submedius thalamic nucleus, parabrachial nucleus, and lateral hypothalamus.[11] Furthermore, a high level of stress and anxiety in rats has been found 24 hours after beginning experimental tooth movement.[12] Taken together, these results suggest that the orofacial noxious stimulation promotes the activation of pathways related to affective-emotional aspects of pain.

Despite the important psychological meaning attributed to the face and oral cavity, there is only one report on the activation of the amygdala and hypothalamus after experimental tooth movement. The investigators described c-Fos expression 24 hours (only period analyzed) after tooth movement induced by the Waldo and Rothblatt method, which consists of inserting an elastic between the rat molars.[13] The response was thought to be due to the inflammatory event that occurs within the periodontal tissues during this time and can release mediators, such as prostaglandins and substance P, which are related to the mechanism of pain sensation.[14,15] However, clinical observation indicates that discomfort or pain usually appears already a few hours after force application.[1] Therefore it is important to address the activation of these structures after an early period of tooth movement. Moreover, despite structural differences between sensory receptors in the periodontal ligament in molars and incisors,[16] only one report exists, so far, on neural activity after movement of incisor teeth.[4] This previous study used a spring-activated appliance, which allowed for quantification of the force applied and analyzed regions related to nociception and antinociception, but no information regarding the activation of amygdala and hypothalamus was provided.

The aim of this study was to investigate whether the magnitude of force applied to the incisor tooth influences c-Fos expression response in the central amygdala (CEA) and lateral hypothalamus (LH) of rats in an early period of tooth movement. The effect of morphine on the induction of Fos-IR cells was further examined to analyze the influence of noxious stimulus on the c-Fos expression in these nuclei.

MATERIALS AND METHODS

Forty male Wistar rats weighing 230–250 g were kept in Plexiglas wall cages in a room maintained at 24 ± 1°C, on a 12-hour light cycle (lights on at 7 AM), with free access to water and food. The experiments were carried out according to and with the approval of the ethical recommendations of the Committee for Animal Care and Use of the University of São Paulo, Campus of Ribeirão Preto (Process 04.1.681.53.4). All efforts were made to minimize animal suffering.

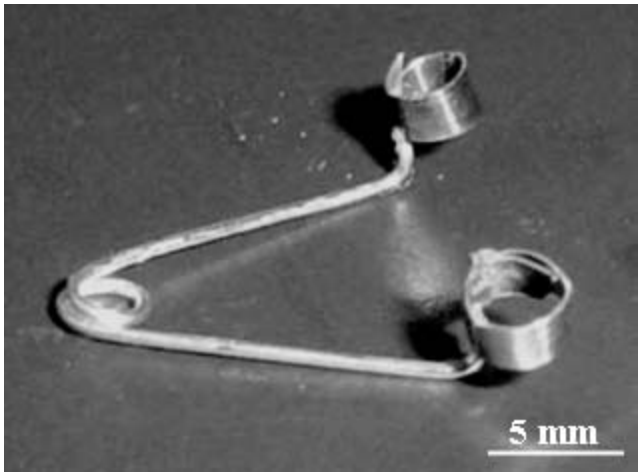

Figure 1. View of the orthodontic appliance used in this study.

Experimental Procedure

The experiments were performed between 8 AM and 12 AM. Before the experiments, the rats were anesthetized by an intramuscular injection of ketamine (100 mg/kg) plus xylazine (14 mg/kg) and divided into six experimental groups. In the control group (n = 3) the rats were only submitted to the anesthetized procedure. In the without orthodontic appliance (OA) group (n = 8), the mouths of the rats were opened for 1–2 minutes without the insertion of the OA. In the without force group, the rats received an inactivated OA that was fixed on the maxillary incisors (n = 9). In the activated groups, an OA activated with 30*g* (n = 8) or 70*g* (n = 8) was fixed on the maxillary incisors. The last group (n = 4) was pretreated with morphine sulphate (2 mg/kg, intraperioneally [IP]) (Sigma, Ronkonkoma, NY), 30 minutes before the induction of tooth movement by an OA activated with 70*g*.

Orthodontic Appliance

A fixed OA was constructed based on the model of Magdalena et al.[4] It consisted of a torsion spring made of 0.016 inches of stainless steel, with each edge welded to two stainless steel rings (orthodontic bands) of 0.004 × 0.06 inches that were cut open in the middle so that they could be fixed to the right and left incisors (Figure 1). By using a dynamometer, the appliance was activated (or not) with a force of 30*g* or 70*g* and briefly heated to memorize the force. After that, the appliance was fitted with the torsion spring and adapted to the rat's palate in such a way that the orthodontic band could be cemented to the incisor with zinc oxyphosphate.

Fos-Protein Immunohistochemistry

The rats were deeply anesthetized 3 hours after the onset of the experimental procedure with an intramuscular injection of ketamine (100 mg/kg) plus xylazine (14 mg/kg) and transcardially perfused with 200 mL phosphate buffered saline (PBS, 0.01M, pH 7.4) followed by 200 mL of paraformaldehyde at 4% in 0.1M phosphate buffer (pH 7.4) at 4°C. The encephalons were rapidly removed and soaked in the same fixative solution for 2 hours (at 4°C) and then cryoprotected by overnight soaking in 30% sucrose/phosphate buffer, after which the brains were frozen in isopentane (at −40°C). Subsequently, 40-μm coronal sections were cut inside a cryostat and processed for Fos immunocytochemistry. Briefly, tissue sections were successively washed and incubated for 20 hours with the primary Fos antibody (1: 2,000; SC 7202, Santa Cruz Biotechnology, Santa Cruz, Calif). The sections were then processed using the avidin biotin immunoperoxidase method (Vectastain ABC Kit; Vector Laboratories, Burlingame, Calif) and Fos-IR was revealed by the addition of the chromogen 3,3′-diaminobenzidine (DAB; Sigma) and 1% hydrogen peroxide. The polyclonal anti-c-Fos antibody was omitted in negative controls. The slices were rinsed in PBS, mounted on gelatin-pretreated slides, dehydrated through an ethanol series, xylene-cleared, and coverslipped for microscopic observations. For all experiments, tissues from the control and experimental rats were always processed in the same assay. The brain regions analyzed in this study were the central amygdala and lateral hypothalamus (HL).

Quantitative Analysis

The sections were analyzed using light microscopy and labeled neurons were recorded by the use of an image analysis system (Zeiss KS 300). The anatomic description of brain regions followed the Paxinos and Watson atlas.[17] For a cell to be considered to express Fos-IR, the nucleus of the neuron had to be of an appropriate size (cell neuron diameter ranging from approximately 8 to 15 μm) and shape (oval or round) and be distinct from the background at ×10 magnification. For quantitative analysis of Fos-labeled cells, three consecutive sections of the brain region of interest were taken from each rat. In each section, the number of Fos-positive neurons was counted unilaterally in the study area by one observer blinded to the treatment using a light microscope with ×10 objective and the absence or presence of labeled neurons was registered using an image analysis system (Image J). The data are presented as mean ± standard error of the mean and were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey test. The level of significance was set as $P < .05$.

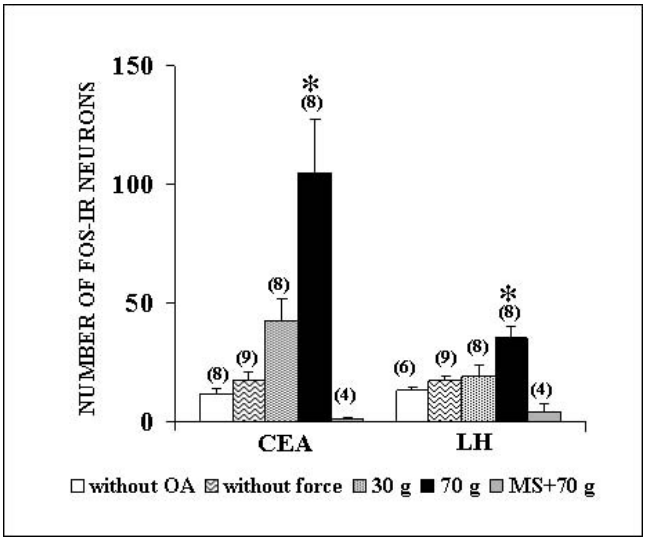

Figure 2. Number of Fos-IR cells (mean ± standard error of the mean) in the central amygdala (CEA) and lateral hypothalamus (LH) in the without orthodontic appliance (without OA) group, in the group that received the inactivated (without force) or activated (30*g*) or (70*g*) OA, and in the group that was pretreated with morphine sulphate (MS, 2 mg/kg) before the insertion of an OA activated with a 70*g*. *$P < .05$ compared with other groups by the Tukey test. Number of rats analyzed in parentheses.

RESULTS

To evaluate the effect of the anesthesia, the number of Fos-IR cells was analyzed in the area studied in control rats, that is, rats that were only submitted to the intramuscular injection of ketamine/xylazine mixture for 3 hours. In this group, no Fos-IR cells were detected in the CEA or in the LH. Furthermore, in the without OA, a small number of Fos-IR cells were scattered in the CEA (11 ± 2) and in the LH (13 ± 1). In the rats that received the inactivated OA (without force) a few Fos-IR cells were observed in both areas (17 ± 3 and 17 ± 2, CEA and LH, respectively). No significant differences in the numbers of Fos-IR cells were seen between without OA and without force rats (Figure 2).

In the groups in which the OA appliance was inserted, the number of Fos-IR cells increased according to the magnitude of the mechanical force applied to the appliance. In the rats that received the OA activated with 30*g*, the number of Fos-IR cells was 42 ± 10 in the CEA and 19 ± 4 in the LH (Figure 2). Tooth movement caused by the OA activated with 70*g* promoted even higher levels of c-Fos expression in both nuclei. Again, the largest number of Fos-IR cells was seen in the CEA, 104 ± 22, whereas in the LH the count was 35 ± 4. Statistical analysis demonstrated a significant difference in this group (70*g*) for both nuclei ($F4, 31 = 11.437$, $P < .001$; $F4, 27 = 7.880$, $P < .001$ ANOVA for CEA and LH, respectively) compared with

the other groups ($P < .01$, Tukey). In addition, there was no significant difference among the without OA group, the without force group, and the group that received an OA activated with 30*g* in both areas studied.

Morphine sulphate pretreatment (2 mg/kg, IP) before the insertion of an OA activated with 70*g* ($P < .001$, Tukey) reduced the number of Fos-IR cells in both nuclei (1 ± 1 and 4 ± 3, CEA and LH, respectively, Figure 2).

DISCUSSION

The present study demonstrates that force applied to the incisor promotes c-Fos expression in cells located in the CEA and LH possibly related to affective-emotional, behavioral, and autonomic reactions to pain. The response was clearly observable 3 hours after the insertion of an activated OA and demonstrating that the intensity of c-Fos expression in the CEA and the LH depends on the magnitude of the force applied.

Several studies have demonstrated that pretreatment with morphine decreases the number of Fos-IR cells in the spinal cord horn caused by chemical, mechanical, and visceral noxious stimuli.[18,19] The dose-dependent effects of morphine on the number of Fos-IR cells in the superficial layers of the ipsilateral trigeminal subnucleus caudalis was observed after experimental tooth movement.[3] A reduction of Fos-IR cells in the parabrachial nucleus by morphine treatment, which was reversible by pretreatment with naloxone, was seen after tooth movement.[8] We are aware that some dissociation between c-Fos and pain can occur,[20,21] but the fact that in the present study morphine treatment reduced c-Fos expression in the CEA and LH of rats that received the activated (70*g*) appliance reinforces the idea that these nuclei, like the trigeminal subnucleus caudalis[3] and parabrachial nucleus,[8] are at least partly stimulated by pain and/or stress related to pain.

The areas studied in this report, CEA and LH, have been proposed as important brain regions that trigger and mediate functions involving nociceptive and aversive stimulation.[9,10] In fact, the amygdala nuclear complex provides emotional significance of sensory stimuli, including pain. The CEA is the effector, which via its connection with the bed nucleus of the stria terminalis, hypothalamus, and brain stem, initiates autonomic, endocrine, and motor outputs that are critical for expression of emotional response,[22] including conditioned fear in response to pain. Furthermore, the hypothalamus has a central role of integrated autonomic and endocrine response for homeostasis and adaptation to internal or external stimuli.[23] Particularly, the lateral hypothalamus areas are involved in arousal, autonomic control, and pain modulation.[24]

The LH also appears to be a target for some direct nociceptive inputs from the spinal and medullary dorsal horn.[25–27] Furthermore, nociceptive signals originated from innervation territory of the trigeminal nerve reach and the hypothalamus through both the direct trigeminohypothalamic tract and the indirect reticulohypothalamic tract. This suggests that highly prioritized painful signals are transferred in parallel channels to ensure that this critical information reaches the hypothalamus.[26,27] Particularly, the unilateral inflammation of the masseter, a model of orofacial deep tissue injury, induces Fos protein expression in the neurons located into the caudal ventrolateral medulla, lateral reticular nucleus, and nucleus tractus solitarius that project to the parabrachial nucleus and lateral hypothalamus.[11]

The appearance of Fos-IR cells in the CEA and LH in this and other studies[11,13] may be due to the fact that these areas receive information through nociceptive pathways, such as the spino(trigemino)parabrachiohypothalamic,[9] the spinohypothalamic,[25] and the spino(trigemino)pontoamygdaloid tract.[10] The external portion of the lateral parabrachial area receives numerous afferents from the superficial laminae of the spinal cord dorsal horn and from the trigeminal subnucleus caudalis and sends a major projection to the CEA.[10] In addition, input to the LH from the parabrachial nucleus arises from a region around the internal lateral subnucleus of the parabrachial nucleus.[28] In addition, evidence suggests that the parabrachial-lateral hypothalamus pathway is involved in arousal mechanisms in response to somatosensory and/or noxious events generated in the orofacial region.[10]

Activation of the descending bulbospinal pathways could be another explanation for the Fos-IR neurons observed in these studied areas. The CEA and LH exhibited a substantial number of retrogradely labeled cells after the injection of cholera toxin b subunit (a sensitive retrograde tracer) into the dorsal raphe nucleus.[29] It is interesting to note in this context that experimental tooth movement increased serotonin and its metabolite (5-HIAA) levels in the medulla, which indicates that nociception induced by tooth movement activates the descending bulbospinal pathways[30]; in particular, the dorsal raphe nucleus and periaqueductal gray are also activated after experimental molar or incisor movement in rats.[4,31]

Few Fos-IR cells were observed in the CEA and LH nuclei of without OA rats and rats that received the OA without activation. The without force rats, however, did show more Fos-IR cells than the without OA rats, but these numbers were still considerably lower than those of the experimental rats, indicating that some of the Fos-IR cells in the CEA and LH were possibly induced by the insertion and fixation of the activated OA on the maxillary incisors. Thus, it is possible to suggest that the

number of Fos-IR cells increased in intensity according to the magnitude of force applied to the OA.

CONCLUSIONS

- The CEA and LH are activated 3 hours after OA insertion on the incisors of rats.
- Experimental tooth movement promotes c-Fos expression in the CEA and LH according to the magnitude of the force applied to the OA.
- The reduction in Fos-IR cells observed by morphine pretreatment suggests that pain stimulation plays a critical role in the activation of these brain nuclei.

REFERENCES

1. Ngan P, Kess B, Wilson S. Perception of discomfort by patients undergoing orthodontic treatment. *Am J Orthod Dentofacial Orthop.* 1989;96:47–53.
2. Marfurt CF, Turner DF. The central projections of tooth pulp afferent neurons in the rat as determined by the transganglionic transport of horseradish peroxidase. *J Comp Neurol.* 1984;223:535–547.
3. Aihara Y, Maeda T, Hanada K, Wakisaka S. Effects of morphine on the distribution of Fos protein in the trigeminal subnucleus caudalis neurons during experimental tooth movement of the rat molar. *Brain Res.* 1999;819:48–57.
4. Magdalena CM, Navarro VP, Park DM, Stuani MB, Rocha MJ. C-fos expression in rat brain nuclei following incisor tooth movement. *J Dent Res.* 2004;83:50–54.
5. Watanabe M, Tanaka E, Suemune S, et al. Expression of c-Fos protein in the trigeminal nuclear complex resulting from quantified force application to the rat molar. *J Oral Rehabil.* 2003;30:1128–1137.
6. Fujiyoshi Y, Yamashiro T, Deguchi T, Sugimoto T, Takano-Yamamoto T. The difference in temporal distribution of c-Fos immunoreactive neurons between the medullary dorsal horn and the trigeminal subnucleus oralis in the rat following experimental tooth movement. *Neurosci Lett.* 2000; 283:205–208.
7. Yamashiro T, Nakagawa K, Satoh K, Moriyama H, Takada K. *c-fos* expression in the trigeminal sensory complex and pontine parabrachial areas following experimental tooth movement. *Neuroreport.* 1997;8:2351–2353.
8. Hiroshima K, Maeda T, Hanada K, Wakisaka S. Temporal and spatial distribution of Fos protein in the parabrachial nucleus neurons during experimental tooth movement of the rat molar. *Brain Res.* 2001;908:161–173.
9. Bester H, Menendez L, Besson JM, Bernard JF. Spino(trigemimo)parabrachiohypothalamic pathway: electrophysiological evidence for an involvement in pain processes. *J Neurophysiol.* 1995;73:568–585.
10. Bernard JF, Peschanski M, Besson JM. A possible spino (trigemino)-ponto-amygdaloid pathway for pain. *Neurosci Lett.* 1989;100:83–88.
11. Ikeda T, Terayama R, Jue SS, Sugiyo S, Dubner R, Ren K. Differential rostral projections of caudal brainstem neurons receiving trigeminal input after masseter inflammation. *J Comp Neurol.* 2003;465:220–233.
12. Yozgatian JH, Zeredo JL, Hotokezaka H, Koga Y, Toda K, Yoshida N. Emotional stress-and pain-related behaviors evoked by experimental tooth movement. *Angle Orthod.* 2008;78:487–494.
13. Yamashiro T, Satoh K, Nakagawa K, Moriyama H, Yagi T, Takada K. Expression of Fos in the rat forebrain following experimental tooth movement. *J Dent Res.* 1998;77:1920–1925.
14. Grieve WG III, Johnson GK, Moore RN, Reinhardt RA, Dubois LM. Prostaglandin E (PGE) and interleukin-1 beta (IL-1 beta) levels in gingival crevicular fluid during human orthodontic tooth movement. *Am J Orthod Dentofacial Orthop.* 1994;105:369–374.
15. Saito M, Saito S, Ngan PW, Shanfeld J, Davidovitch Z. Interleukin 1 beta and prostaglandin E are involved in the response of periodontal cells to mechanical stress in vivo and in vitro. *Am J Orthod Dentofacial Orthop.* 1991;99:226–240.
16. Jayawardena CK, Takahashi N, Takano Y. A unique localization of mechanoreceptors in the periodontal tissue of guinea pig teeth. *Arch Histol Cytol.* 2002;65:233–244.
17. Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates.* 3rd ed. San Diego, Calif: Academic Press; 1997.
18. Abbadie C, Besson JM. Effects of morphine and naloxone on basal and evoked Fos-like immunoreactivity in lumbar spinal cord neurons of arthritic rats. *Pain.* 1993;52:29–39.
19. Hammond DL, Presley RW, Gogas KR, Basbaum AI. Morphine or U-50,488 expresses Fos protein-like immunoreactivity in the spinal cord and nucleus tractus solitarii evoked by a noxious visceral stimulus in the rat. *J Comp Neurol.* 1992;315:244–253.
20. Harris JA, Westbrook RF, Bentivoglio M. Fos expression in the spinal cord is suppressed in rats displaying conditioned hypoalgesia. *Behav Neurosci.* 1995;109:320–328.
21. Presley RW, Menétrey D, Levine JD, Basbaum AI. Systemic morphine suppresses noxious stimulus-evoked Fos protein-like immunoreactivity in the rat spinal cord. *J Neurosci.* 1990;10:323–335.
22. Davis M, Whalen PJ. The amygdala: vigilance and emotion. *Mol Psychiatry.* 2001;6:13–34.
23. Benarroch EE. Pain-autonomic interactions. *Neurol Sci.* 2006;27:130–133.
24. Abrahanson EE, Moore RY. The posterior hypothalamic area: chemoarchitecture and afferent connections. *Brain Res.* 2001;889:1–22.
25. Giesler GJ. The spino-hypothalamic tract. In: Besson JM, Guildbaud G, and Ollat H, eds. *Forebrain Areas Involved in Pain Processing.* Paris: Eurotext; 1995:13–25.
26. Malick A, Burstein R. Cells of origin of the trigeminohypothalamic tract in the rat. *J Comp Neurol.* 1998;400:125–144.
27. Malick A, Strassman RM, Burstein R. Trigeminohypothalamic and reticulohypothalamic tract neurons in the upper cervical spinal cord and caudal medulla of the rat. *Neurophysiol.* 2000;84:2078–2112.
28. Feil K, Herbert H. Topographic organization of spinal and trigeminal somatosensory pathways to the rat parabrachial and Kölliker-Fuse nuclei. *J Comp Neurol.* 1995;353:506–528.
29. Peyron C, Petit JM, Rampon C, Jouvet M, Luppi PH. Forebrain afferents to the rat dorsal raphe nucleus demonstrated by retrograde and anterograde tracing methods. *Neuroscience.* 1998;82:443–468.
30. Yamashiro T, Fukunaga T, Kabuto H, Ogawa N, Takano-Yamamoto T. Activation of the bulbospinal serotonergic system during experimental tooth movement in the rat. *J Dent Res.* 2001;80:1854–1857.
31. Hattori Y, Watanabe M, Iwabe T, et al. Administration of MK-801 decreases *c-Fos* expression in the trigeminal sensory nuclear complex but increases it in the midbrain during experimental movement of rat molars. *Brain Res.* 2004; 1021:183–191.