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치의학박사학위논문

The effects of pain-specific local anesthetics (QX-314 and capsaicin cocktail) on brainstem neuronal activity following tooth extraction

QX-314와 캡사이신 혼합물을 이용한 통증 특이적 국소마취법이 발치 후 뇌간 신경세포 활성에 미치는 효과에 관한 연구

2014 년 2 월

서울대학교 대학원

치의과학과 신경생물학전공

Batbayar BADRAL

ABSTRACT

The effects of pain-specific local anesthetics (QX-314 and capsaicin cocktail) on brainstem neuronal activity following tooth extraction

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This study was aimed to examine brainstem neuronal changes in trigeminal pathways (trigeminal spinal nucleus) after tooth extraction in rats by quantifying immunohistochemical markers for central sensitization (*c-Fos*) and to compare the efficacy of different local anaesthetics (lidocaine and QX-314/capsaicin cocktail) on the expression of *c-Fos* after tooth extraction. Adult male Sprague-Dawley rats received infiltration injection of anesthetics 10 min prior to lower molar tooth extraction. Neuronal activation was determined by immunohistochemistry for the proto-oncogene protein *c-Fos* in transverse sections of the trigeminal subnucleus caudalis (Sp5C). After tooth extraction, *c-*

Fos-like immunoreactivity (Fos-LI) detected in the dorsomedial region of bilateral Sp5C was highest at 2 hrs ($p < .01$ *vs*. naïve ipsilateral) and declined to pre-injury levels by 8 hrs. Pre-administration of the QX-314/capsaicin cocktail significantly reduced to sham levels Fos-LI examined 2 hrs after tooth extraction; reduced Fos-LI was also observed with the conventional local anesthetic lidocaine. Pulpal anesthesia by infiltration injection was confirmed by inhibition of the jaw-opening reflex in response to electrical tooth pulp stimulation. The results suggest that the QX-314/capsaicin cocktail anesthetics is effective in reducing neuronal activation following tooth extraction. Thus, a selective pain fiber 'nociceptive anesthetic' strategy may provide an effective local anesthetic option for dental patients in the clinic.

Keywords: capsaicin, central sensitization, lidocaine, orofacial pain, trigeminal caudal nucleus, QX-314.

Student Number: 2009-31330

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ABBREVIATIONS

TMD; temporomandibular joint disorder

CNS; central nervous system

TRPV1; Transient receptor potential vanilloid 1

Fos-LI; c-Fos-like immunoreactivity

Sp5C; trigeminal spinal nucleus caudalis

MDH; medullary dorsal horn

dEMG; digastric muscle electromyogram

IANT; inferior alveolar nerve transection

JOR; jaw-opening reflex

H&E; hematoxylin and eosin

Introduction

Early neuronal activation in the orofacial pain pathway following tooth extraction can lead to plastic changes in the central nervous system (CNS), such as central sensitization, resulting in hypersensitivity characteristic of trigeminal neuropathic pain. Thus, blockade of this initial CNS activation may be crucial for the prevention of neuropathic pain following tooth extraction. Conventional local anesthetic agents such as lidocaine are routinely used to block pain sensation during tooth extraction in the clinic. However, nonspecific blockade of all nerve fibers leads to undesirable sensory and motor loss in adjacent areas. A new approach to local anesthetic has utilized a cocktail containing the charged lidocaine derivative, QX-314, in combination with the TRPV1 receptor agonist capsaicin to block transmission in pain fibers. I have recently used this approach to anesthetize nociceptive neurons selectively while sparing non-nociceptive sensory and motor neurons in the trigeminal system. Neuronal activation in the CNS following peripheral noxious stimuli can be identified by the expression of c-Fos protein.

Tactile, thermal and nociceptive information from the oral cavity reaches the trigeminal nuclei (caudalis, interpolaris and oralis) via several branches of the mandibular and maxillary divisions of the trigeminal nerve. Nociceptive afferent fibers, in particular, are distributed primarily to nucleus caudalis, where they synaptically contact second-order neurons in superficial layers of caudalis and in the interstitial nucleus of the trigeminal tract (paratrigeminal nucleus). (Hamilton and Norgren, 1984;

Hunt et al., 1987; Norgren, 1984; Schults, 1992; Segade et al., 1990; Strassman and Vos, 1993; Torvik, 1956).

Several studies have also shown that the caudal part of the spinal trigeminal nucleus, subnucleus caudalis, is also an important site that relays nociceptive input from orofacial structures (for review, see Sessle, 1987a,b, 2000) including the dentition (Coimbra and Coimbra, 1994; Oakden and Boissonade, 1998; Sugimoto et al., 1998; Byers et al., 2000; Sessle, 2000). The expression of Fos protein has been used to identify the location of neurons that respond to experimentally applied nociceptive stimuli (Bullitt, 1989, 1990, 1991; Bullitt et al., 1992; Hunt et al., 1987). C-fos-like immunereactivity has been observed in the trigeminal nucleus after experimental tooth extraction and movement in rat (Satoshi Wakisaka et al., 1992). The C-fos gene product is synthesised within second-order neurons in response to noxious stimulation of peripheral tissues. Fos 'activation' is thought to correlate with gene expression changes in the post-synaptic neuron and thus may be a marker for synaptic plasticity. C-fos expression is rapidly upregulated after noxious stimulation (<2 hr). Therefore C-fos labelling provides a marker for the activation of nociceptive input from the tooth to the brain stem after tooth extraction.

Lidocaine is widely used to anaesthatise sensory nerves prior to tooth extraction in patients but leads to sensory and motor loss in adjacent areas. Experimental studies have also shown that lidocaine did not interfere c-Fos expression after pulp exposure. However bupivacaine did reduce c-Fos (Maria et al., 2005). The persistence of c-Fos and microglia suggests that lidocaine may have only temporary or incomplete blocking effect on nociceptive nerves, even though other sensations are

blocked for some time after injection.

A new approach is to selectively block nociceptive nerves by the combined application of the charged quaternary anaesthetic derivative QX-314 with the TRPV1 activator, capsaicin. This method has the benefit of prolonged nociceptive blockade compared to lidocaine alone (Kim et al., 2010). Key to the efficacy of the QX314-capsaicin cocktail is the expression of the TRPV1 receptor in the neuron to be aneasthatised.

Therefore identification of nociceptive neurons is a crucial prerequisite for this study. Tooth pulp neurons are thought to be mainly composed of nociceptive sensory neurons (Cook and McCleskey, 1997). Many classification methods have been developed to serve the purpose, based on differential conduction velocity, expression of several nociception-related receptor proteins, the size of soma, and degree of myelination.

Tooth extraction commonly leads to long-term pain in dental patients that may derive from plastic changes to peripheral and central neurons innervating the extracted tooth pulp, ligaments and surrounding tissues. Neuropathic pain caused by peripheral nerve injury is a common occurrence after tooth extraction [Berge, 2002; Tay and Zuniga, 2007], but the mechanism underlying neuropathic pain is unclear.

Purpose

I therefore will examine the effects of local anesthetics on nociceptive neuronal activation in second-order brainstem neurons after experimental tooth extraction by quantifying c-Fos-like immunoreactivity (Fos-LI) in the trigeminal spinal nucleus caudalis (Sp5C), a region critical in the processing of nociceptive signaling from the orofacial region. First I will confirm the anatomy of the spinal trigeminal nucleus in the rat using neuronal Nissl stain technique. I will also develop the DAB staining technique on a positive control experiment involving transection of the inferior alveolar nerve (IAN) to confirm detection of c-Fos in the brain stem.

The specific aims of this thesis are to: 1) To examine neuronal changes in trigeminal pathways (Trigeminal spinal nucleus) after tooth extraction pain in rats by quantifying immunohistochemical markers for central sensitization (*c-Fos*). 2) To compare the efficacy of different local anaesthetics (lidocaine and QX-314 cocktail) on the expression of *c-Fos* after tooth extraction. 3) To examine the efficiency of pulp anesthesia by recording tooth pulp stimulation-evoked jaw opening reflex.

Materials and Methods

1. Animals

All surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the School of Dentistry, Seoul National

University, and are reported according to the ARRIVE guidelines (Kilkenny *et al.*, 2010). Animal treatments were performed according to the Guidelines of the International Association for the Study of Pain. Eighty male Sprague–Dawley (SD) adult rats (Orient Bio Inc., Seongnam, Korea) weighing between 200-250g, were housed at a temperature of 23 ± 2 ^{0}C with a 12-hour light–dark cycle (light on 08:00 to 20:00), and provided with standard laboratory fed food and water *ad libitum*. The animals will be allowed to habituate to the housing facilities for 1 week before the experiments, and efforts will be made to limit distress to the animals.

2. Surgery and experimental groups

2.1. Inferior alveolar nerve transection (IANT)

A positive control experiment involving transection of the inferior alveolar nerve (IAN) to confirm detection of c-Fos in the brain stem. Four male Sprague-Dawley rats weighing 250–300 g (ipsilateral to the transection: n = 2, contralateral to transection: n = 2) were initially anesthetized with pentobarbital sodium (50 mg/kg ip). For the IAN transection, rats were placed on a warm mat, and a small incision was made on the surface of the facial skin over the masseteric muscle and to the alveolar bone through the masseteric muscle. The surface of the alveolar bone was exposed, and the bone surface covering the IAN was removed and the IAN was exposed. The IAN was tightly ligated at two points of the nerve trunk at just above the angle of the mandible and 1 mm proximal from the angle of the mandibular bone (Koichi Iwata *et al*., 2000). After surgery, capsaicin (0.5 μg/μL) was injected of 60 μL to IAN **(Figure.**

1). The brain stem removed 2h after inferior alveolar nerve transection and injection capsaicin.

2.2. Tooth extraction

Each rat (200-250 g) were anaesthetized with an intraperitoneal injection of urethane (i.p., 1.2g/kg., Sigma, St. Louis, Mo) and were then immobilized on the operation table in a supine position. Lower right 1st and 2nd molars were separated with a scalpel and extracted by the leverage technique; the left (contralateral) side remained intact in all rats **(Figure. 2).** Sham extraction was performed by opening of the mouth for 5 min., approximately the same time period as the tooth extraction procedures took. Naïve rats (n = 3) received no treatment. A total of 80 rats will undergo either tooth extraction (pain model), sham or Naïve group and others.

The animals will be divided into 8 groups:

Group: 1. Lower right first and second molars extraction (n=3 *per* time point: 2hours, 4hours, 8hours, 1day and 3days)

Group: 2. Lower right first and second molars extraction with local anaesthetic *(*2% lidocaine with 1:100,000 epinephrine). Fixation at 2h (n=3)

Group: 3. Lower right first and second molars extraction with local anaesthetic (Vehicle (20% Ethanol (96%), 5% Tween80, 75% Normal saline)). Fixation at 2h(c-Fos) n=3

Group: 4. Lower right first and second molars extraction with local anaesthetic (+Capsaicin(0.5 μg/μL)). Fixation at 2h(c-Fos) n=3

Group: 5 Lower right first and second molars extraction with local anaesthetic (+QX- 314(1%)). Fixation at 2h(c-Fos) n=3

Group: 6. Lower right first and second molars extraction with local anaesthetic (+QX-314 coctail(1% QX314 + 0.5μg/μl capsaicin), new strategy). Fixation at 2h(c-Fos) n=3

Group: 7. Sham group (n=3 rats *per* time point)

Group: 8. Normal control (Naïve) group (n=3 rats *per* time point)

For local anesthetic experiments, I used a 1cc anesthetic syringe to inject a total volume of 70 μL (**Figure. 3)** was administered as two bolus injections in a vehicle: a single lateral (buccal side) injection of 50 μL and a single medial (lingual side) injection of 20 μL 10 min prior to tooth extraction. For an average 200-g rat, a 70-μL administration of lidocaine- (20 mg/mL, 2%, with 1:100,000 epinephrine), QX-314- (10 mg/mL, 1%), or capsaicin- (0.5 μg/μL) containing solutions was equivalent to a dose of 7 mg/kg, 3.5 mg/kg, and 0.175 mg/kg, respectively. All compounds were purchased from Sigma–Aldrich, except for lidocaine, which was obtained from Gwangmyeong Pharmaceuticals Ltd. (Seoul, Korea).

Figure 1. Inferior alveolar nerve transection (IANT) – Positive control

A) Transverse cut under purpose of declaring activation of c-Fos-like immune-reactivity of subnucleus caudalis. Obex: (–1.6 mm). B) There are many detection c-fos neurons appear in ipsilateral. Two hours after IANT+capsaicin injection. C) There are no detection c-fos neurons appear in contralateral. Scale bar = 100μm.

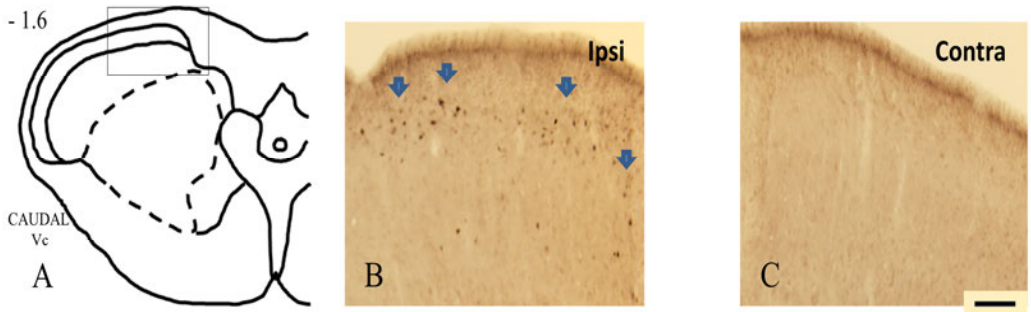

- 1.6
CAUDAL
Vc
A
Ipsi
B
Contra
C

Figure 2. Method of tooth extraction in the 7 week old rat

A) Intraoral image of rat. B) Molars were extracted by leverage technique after separation with a scalple. C) Intra oral image just after removed the molars. D) 1st and 2nd molars after extraction shown.

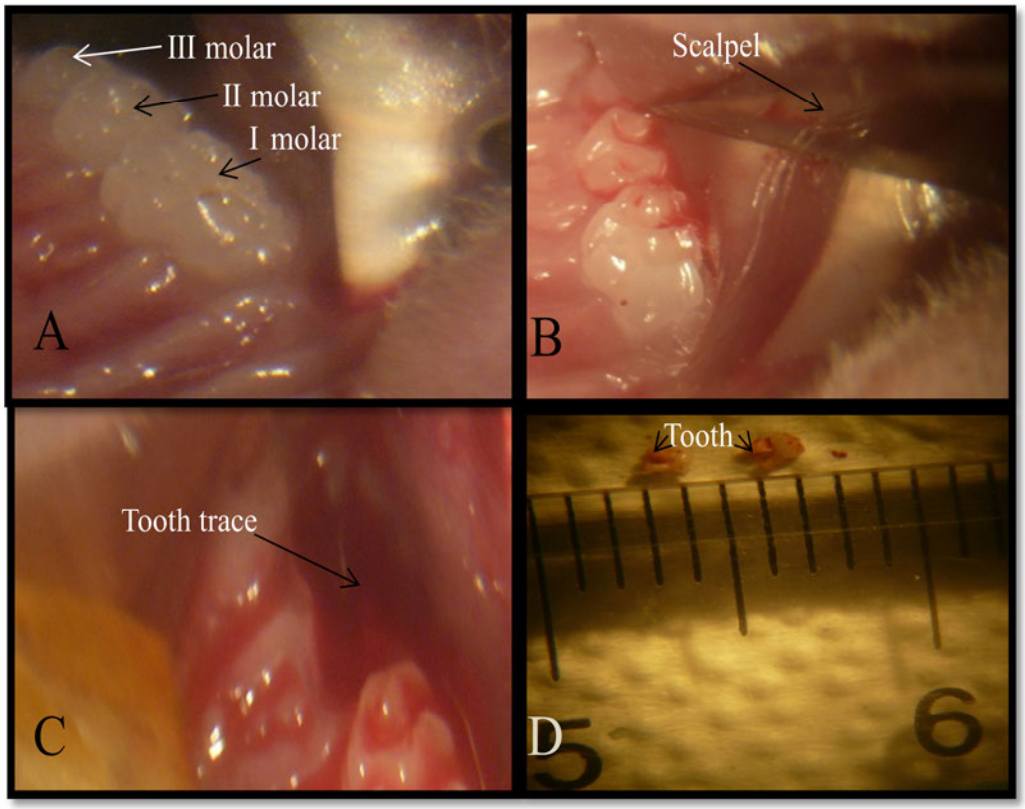

III molar
II molar
I molar
A
Scalpel
B
Tooth trace
C
Tooth
D
5
6

Figure 3. Local aneasthetic treatment calculation

Injected vestibule and lingual 10 min before tooth extraction. Fixation at 2h(c-Fos). A) Single bolus of lidocaine(mg). B) Volume anaesthetic injection(ml).

$$A)\quad \frac{7mg \times W(g)}{1000g} = X_1\,(mg) \qquad B)\quad \frac{X_1\,(mg) \times 1.8ml}{36mg} = X_2\,(ml)$$

3. Nissl stain, Immunohistochemistry and Histology

3.1. Nissl staining

For Nissl stain, sections (40 μm) were dehydrated through alcohol series to de-fat and then returned through the series back to distilled water before immersion in 0.1% cresyl violet solution (cresyl violet acetate (0.1g), distilled water (100ml), add 0.3 ml of glacial acetic acid just before use and filter) for 15 min. All sections were dehydrated in an alcohol series, cleared in xylene, and cover-slipped with synthetic mountant (Thermo Scientific, Waltham, MA, USA). Images were acquired by light microscopy at x10 and 40x magnification, with a CCD camera (Fluoview, SIS, Münster, Germany) attached to the microscope.

3.2. Immunohistochemistry in the medullary dorsal horn (MDH)

Rats were sacrificed 2 hrs, 4 hrs, 8 hrs, 1 day, and 3 days after tooth extraction (n = 3 *per* time-point). For local anesthetic experiments, rats were sacrificed at 2 hrs after tooth extraction for immunohistochemical analysis of c-Fos. Under pentobarbital anesthesia (50 mg/kg, i.p.), rats were transcardially perfused sequentially with physiological saline (including 500 U/L heparin) followed by 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS) (pH 7.0). The brainstem was then transferred to 30% sucrose in PBS for 48 hrs. Frozen transverse cryotome sections (50 μm) were collected in PBS (pH 7.0 with 0.08% sodium azide).

3.2.1. DAB staining(C-Fos-like immunoreactive)

The brainstem containing the trigeminal nucleus was removed and incubated, in 30% sucrose for 48h at 4°C. Cryostat sections (50 μm thickness) were cut serially between subnuclei oralis and caudalis; every 3rd section was reserved and stored in 0.1M PBS. Free-floating sections were rinsed in 0.1M TBS and preincubated for 2h at room temperature in 5% normal goat serum (NGS), 5% FBS and 0.3% Triton X-100 in TBS at pH 7.4. To block endogenous peroxidase activity, the sections were placed in 3% H_2O_2 for 20 min before preincubation (to quench peroxidase activity). Sections were incubated for 48 h at 4°C with c-Fos (Oncogene Sciences Ab5; 1:5.000) diluted in 1% normal goat serum with 0.75% Triton X-100 in TBS, with gentle agitation. After three 10 min washes in TBS, incubated 1:200 for 2h at room temperature with the secondary antibody (biotinylated goat anti-rabbit-IgG, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), washed again in TBS and then subjected to an ABC reaction (peroxidase-conjugated egg-white avidin, Vectastain, Vector Laboratories). Reaction products were visualized with diaminobenzidine DAB (0.05%)-H2O2 (0.01%) in DW for 1minut. Sections were then rinsed with DW and mounted on gelatin-coated glass slides, air dried, dehydrated, and cover-slipped.

The locations of Fos-like immunoreactive nuclei will be observed under a light microscope at x10 and x40 magnification and plotted onto A CCD camera(Fluoview, SIS, Münster, Germany) lucida drawings by confocal microscopy(FV-300, Olympus, Tokyo, Japan) of brainstem sections.

3.3. Hematoxylin and Eosin (H&E) staining

For local anesthetic experiments, rats were sacrificed at 24 hrs for injection site histological analysis. For hematoxylin and eosin (H&E) stain, the ipsilateral mandible was fixed with 4% PFA and dehydrated in a graded series of ethanol. Un-decalcified specimens were embedded in plasticresin (Technovit 7200, Heraeus Kulzer GmbH & Co., Frankfurt, Germany), and ground sections (15–20 μm thick) were prepared by a sawing and grinding technique (EXAKT® Apparatebau GmbH & Co., Norderstedt, Germany) as explained elsewhere (Donath and Breuner, 1982). The ground sections were stained with hematoxylin and eosin prior to histological evaluation *via* optical microscopy.

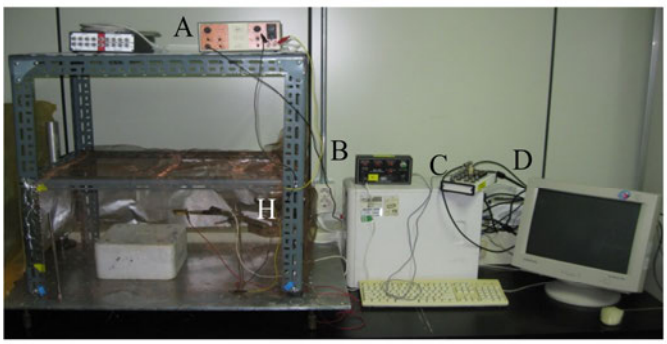
4. Tooth Pulp Stimulation and dEMG Recording

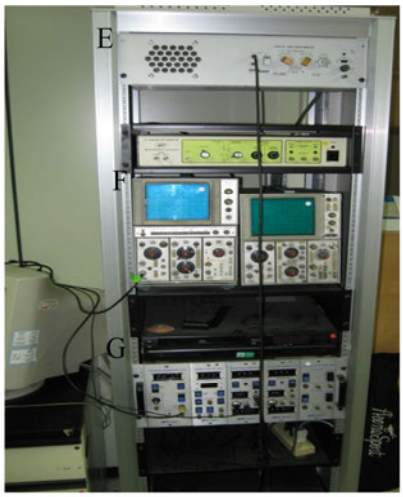
Electrical stimulation of pulpal afferents and recording of the jaw-opening reflex in the form of digastric muscle electromyogram (dEMG) were performed in adult SD rats (300-400 g) as previously described (Kim *et al.*, 2010). *Briefly*, under urethane anesthesia, a cavity was drilled in the first right mandibular molar. A stainless steel electrode was then inserted into the pulp and secured by filling the cavity with hydraulic temporary restorative (Caviton, GC Corporation, Tokyo, Japan); a reference electrode was hypodermically inserted into the lower lip. Stimulations were applied at 1.5 to 2.5 times threshold intensity with a stimulus isolator (A365, World Precision Instruments, Sarasota, FL, USA) at 30-second intervals. To record the dEMG, we inserted a pair of copper wires into the ipsilateral anterior belly of the digastric muscle,

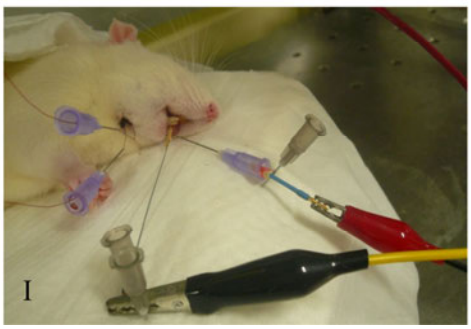
connected *via* an amplifier (Dam80, World Precision Instruments, USA) to an analog-digital converter (NI-DAQ, National Instruments, Austin, TX, USA) and recorded on a PC equipped with Igor Pro software (Ver. 4.0, Wavemetrics Inc., Portland, OR, USA). After establishment of a baseline dEMG response (20-30 min), QX cocktail or lidocaine (with epinephrine) was injected as a single bolus (70 µL/200 g) on the buccal side of the stimulated tooth. Data were normalized to the average dEMG peak-to-peak amplitude during 10 min prior to injection and displayed as mean ± SEM **(Figure. 4).**

Figure 4. Electrical stimulation of pulpal afferents and recording of the jaw-opening reflex in the form of digastric muscle electromyogram (dEMG) performed in adult SD rats

A) Stimulus isolator. B) Amplifier. C) A/D converter. D) Personal computer. E) Speaker. F) Oscilloscope. G) Interval generator. H) Faraday cage. I) A cavity was drilled in the first right mandibular molar. A stainless steel electrode was then inserted into the pulp and secured by filling the cavity, a reference electrode was hypodermically inserted into the lower lip. I inserted a pair of copper wires into the ipsilateral anterior belly of the digastric muscle. J) To record the digastric muscle EMG.


A
B
C
D
H


E
F
G


I

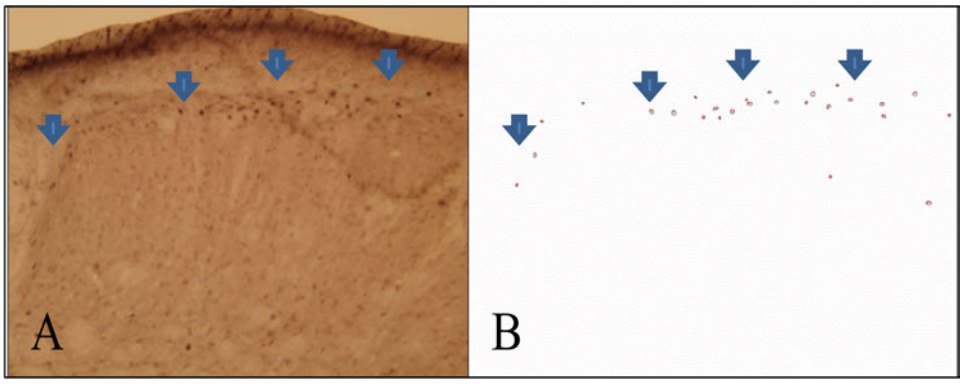

J

5. Analysis and Statistics

Transverse sections of the Sp5C (-1.6 mm to 0 mm from obex) (Paxinos and Watson, 2007) were collected and examined bilaterally for Fos-LI (Figure. 1A). The location of the obex and Sp5C were determined by comparison with equivalent Nissl-stained sections (Fig. 1B). The numbers of Fos-LI nuclei were determined in the superficial laminae (I-II) of bilateral Sp5C for each section, excluding tract and deeper lamina. Counting was performed with a custom-designed macro function with ImageJ software (NIH, Bethesda, MD, USA). *Briefly*, scaled images of the boxed region highlighted in Fig. 1B were converted to 8-bit monochrome and background-subtracted. Intensity threshold was adjusted to a pre-determined level to reveal darkened Fos-LI nuclei only **(Figure. 5A and B).** The number of Fos-LI nuclei within superficial laminae I and II only was automatically detected by the Analyze Particles function. Automatic analyses were compared with those obtained by a blind observer (manual counting) to ensure accuracy. Seven sections *per* animal were analyzed *per* experimental group by an experimenter blind to the experimental group. Data are presented as mean ± SEM. Statistical significances of the effects of time on Fos-LI within ipsi- and contralateral groups were analyzed by one-way analysis of variance (ANOVA) and Dunnett's multiple comparison test with Prism software (version 5.01, Graphpad Software Inc., La Jolla, CA, USA). The effect of nociceptive anesthetic pre-treatment on Fos-LI within ipsilateral Sp5C was analyzed with the unpaired Student's *t* test. *p* values less than .05 were considered to be significant.

Figure 5. Method for quantifying c-fos labelling in the brainstem

A) Light micrograph of dorsal-ventral brain stem showing c-fos labelling 2h after tooth extraction. B) Analysis summary image of count function in ImageJ software. Original light micrograph was converted to back/white image, and threshold adjusted to a pre-determined level to reveal darkened c-fos nuclei. Number of c-fos 'spots' was automatically counted with the count function.


A
B

Results

1. Brain stem anatomical location

For finding out anatomy of brainstem in detail. Perfuse fixation was made under analgesia of naïve rat with urethane, took out the brainstem, stained by method of Nissle stain. As a result of it, I defined Obex and detected there is nucleus caudalis at about - 1.6mm from obex **(Figure. 6A and B).** I used transverse cut under purpose of declaring activation of c-Fos-like immune-reactivity using a positive control injury: inferior alveolar nerve transection. There is more activation on ipsilateral part of subnucleus caudalis neuron after IANT. c-Fos was detected as dark staining mainly in ipsilateral region 2h after inferior alveolar nerve transection. For c-Fos location in horizontal brainstem slice, horizontal cut on brain stem after tooth extraction. c-Fos is more detected in the subnucleus caudalis region. All further results of C-fos detection are reported from the nucleus caudalis **(Figure. 7).**

Figure 6. Brain stem anatomy

Anatomical location in the Sp5C of naïve rat. A) Anatomical location of brainstem sections for c-Fos immunohistochemistry. Morphology of brainstem at level of Sp5C, -1.6 mm caudal from the obex. B) Nissl stain of transverse brainstem section -1.6 mm caudal from the obex. Square indicates region of analysis in dorsomedial Sp5C. Scale bar = 500 μm.

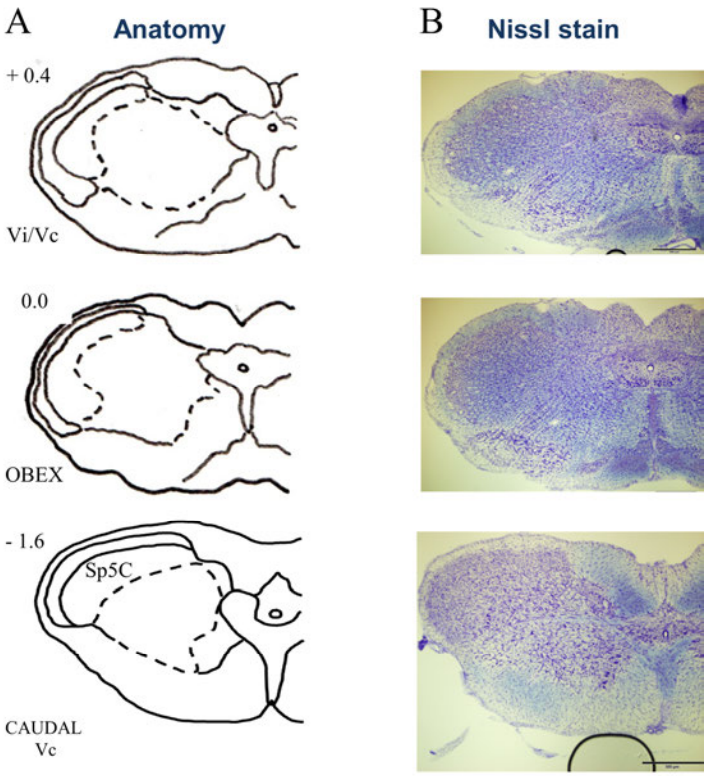
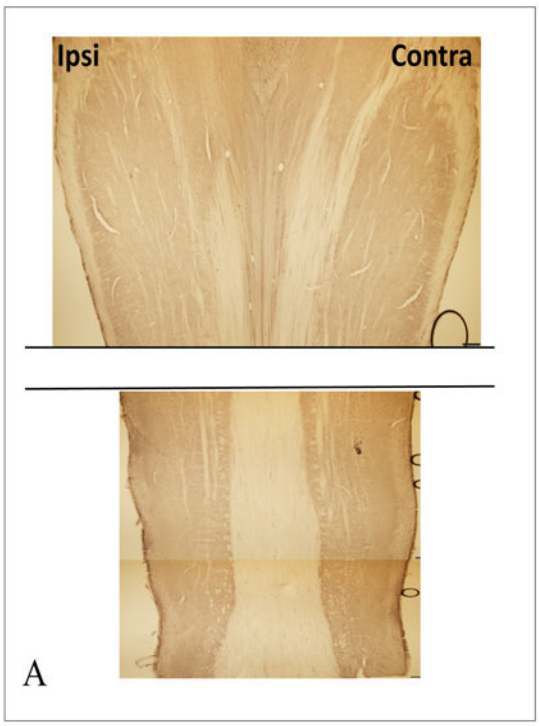

A
Anatomy
+ 0.4
Vi/Vc
0.0
OBEX
- 1.6
Sp5C
CAUDAL
Vc
B
Nissl stain

Figure 7. c-Fos location in horizontal brainstem slice

A) c-Fos location in horizontal brainstem tissue section two hours after tooth extraction. Scale bar = 400μm. B) High magnification of hotizontal section showing sub regions of trigeminal nucleus (Vo-Oralis, Vi-Intrapolaris, Vc-Caudalis). Note c-fos labelling at high magnification within Vc region (inset). C) Schematic digram of horizontal brain stem section.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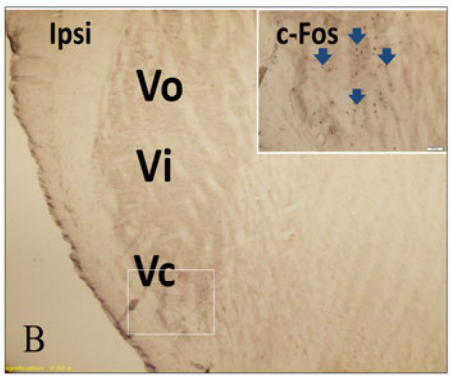
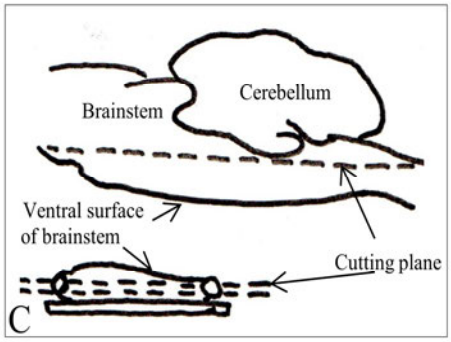

Ipsi
c-Fos
Vo
Vi
Vc
B


Brainstem
Cerebellum
Ventral surface
of brainstem
Cutting plane
C

2. Time course of lower molar tooth extraction-induced Fos-LI in the Sp5C

Fos-LI was minimally detected in the Sp5C of naïve animals **(Figure. 8).** Extraction of right lower (mandibular) 1st and 2nd molars enhanced Fos-LI in the superficial dorsomedial portion of the medullary dorsal horn, corresponding to the termination of the inferior alveolar nerve (Piao *et al.*, 2006). Fos-LI was significantly increased within the ispilateral dorsal horn and, to a lesser extent, within the contralateral Sp5C **(Figures. 8 and 9).** Temporal analysis revealed that Fos-LI peaked at 2 hrs after tooth extraction both ipsi- (46 ± 10 Fos-LI nuclei) and contralaterally (27 ± 6 Fos-LI nuclei) to tooth extraction injury compared with naïve animals (9 ± 4 and 7 ± 3 Fos-LI, respectively). Fos-LI was decreased at the 4-hour timepoint, and by 8 hrs after tooth extraction, Fos-LI returned to basal levels **(Figures. 9).** Injection of lidocaine (2%) 10 min prior to tooth extraction decreased detection of Fos-LI within the ipsilateral Sp5C by more than 50% (19 ± 1 Fos-LI nuclei, n = 3 rats) compared with untreated tooth extraction at 2 hrs (#$p < .05$, Student's *t* test) **(Figure. 9).**

Figure 8. Time course of lower molar tooth extraction-induced Fos-LI in the Sp5C

Photomicrographs of DAB-stained Fos-LI in bilateral dorsomedial Sp5C from naïve rats, 2 hrs after tooth extraction and pre-treatment with lidocaine (2h+Lidocaine). Scale bar = 100 μm.

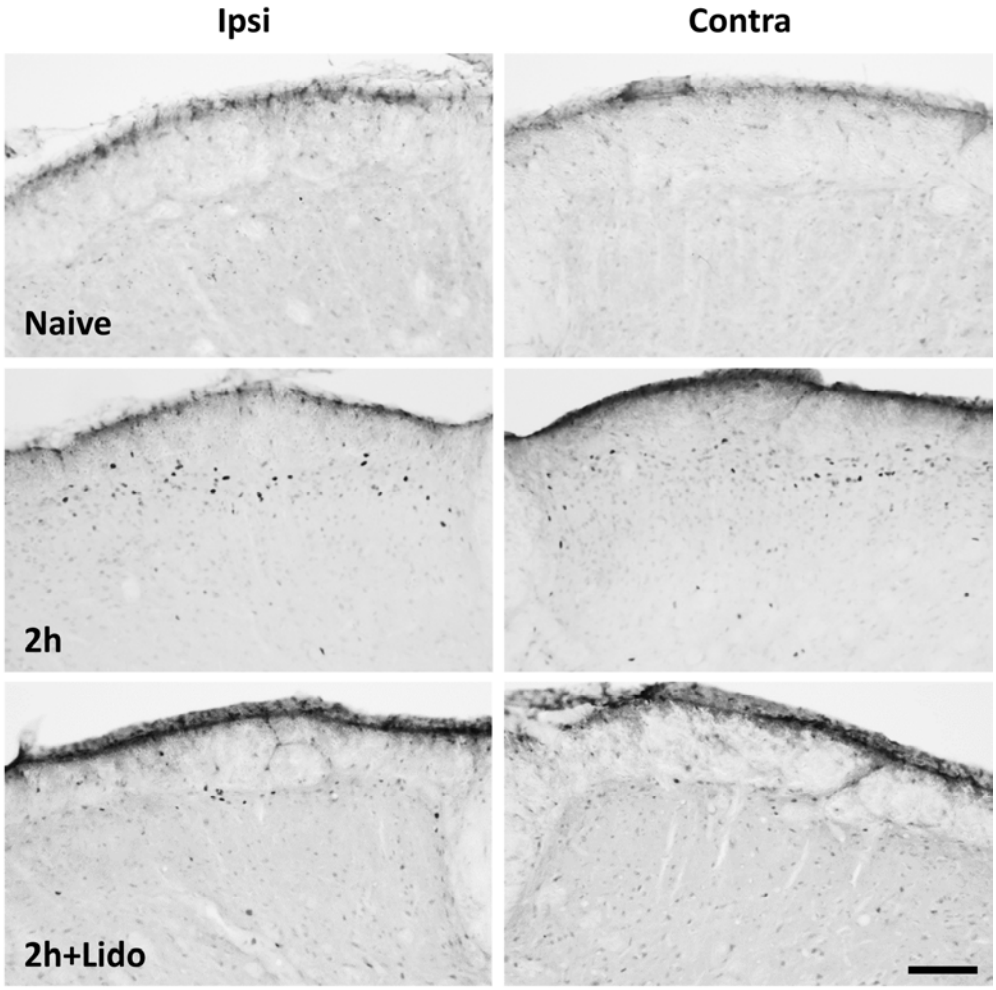
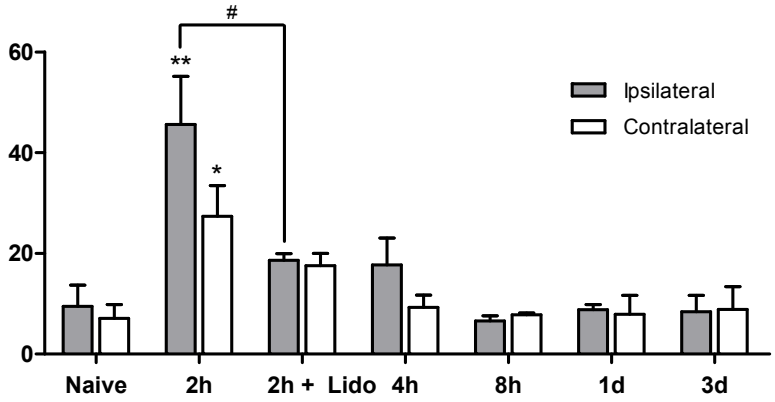

Ipsi
Contra
Naive
2h
2h+Lido

Figure 9. Time course of c-Fos expression in the rat brainstem after unilateral tooth extraction

Quantification of Fos-LI in ipsi- (gray bars) and contralateral (white bars) Sp5C. $^{**}p < .01$ *vs*. naïve ipsilateral, $^{*}p < .05$ *vs*. naïve contralateral. One-way ANOVA; $\#p < .05$ *vs*. untreated tooth extraction at 2 hrs, Student's *t* test.


#
60
40
20
0
**
*
Ipsilateral
Contralateral
Naive
2h
2h + Lido
4h
8h
1d
3d

3. Effect of local injection on c-Fos expression in the rat brainstem 2 hrs after unilateral tooth extraction

I next determined the effects of the QX cocktail and its constituent components on c-Fos expression in the Sp5C 2 hrs after tooth extraction **(Figure. 10).** Only pre-treatment of QX-314 in combination with the TRPV1 agonist capsaicin (QX cocktail) reduced ipsilateral Fos-LI (9 ± 2 Fos-LI nuclei, n = 5 rats) in comparison with vehicle injection (30 ± 8 Fos-LI nuclei, n = 5 rats) (#$p < .05$ *vs.* vehicle, Student's *t* test) and to a level comparable with sham surgery (11 ± 3 Fos-LI nuclei, n = 3 rats) **(Figure. 11).**

Figure 10. Effect of local injection on c-Fos expression in the rat brainstem 2 hrs after unilateral tooth extraction

Photomicrographs of DAB-stained Fos-LI in ipsilateral dorsomedial Sp5C from rats 2 hrs after tooth extraction receiving pre-treatment injection of vehicle (20% ethanol, 5% tween-80 in physiological saline), 1% QX-314 + 0.5 μg/μL capsaicin (+QX cocktail), 0.5 μg/μL capsaicin (+Capsaicin), or 1% QX-314 alone (+QX-314) 10 min prior to tooth extraction. Scale bar = 100 μm.

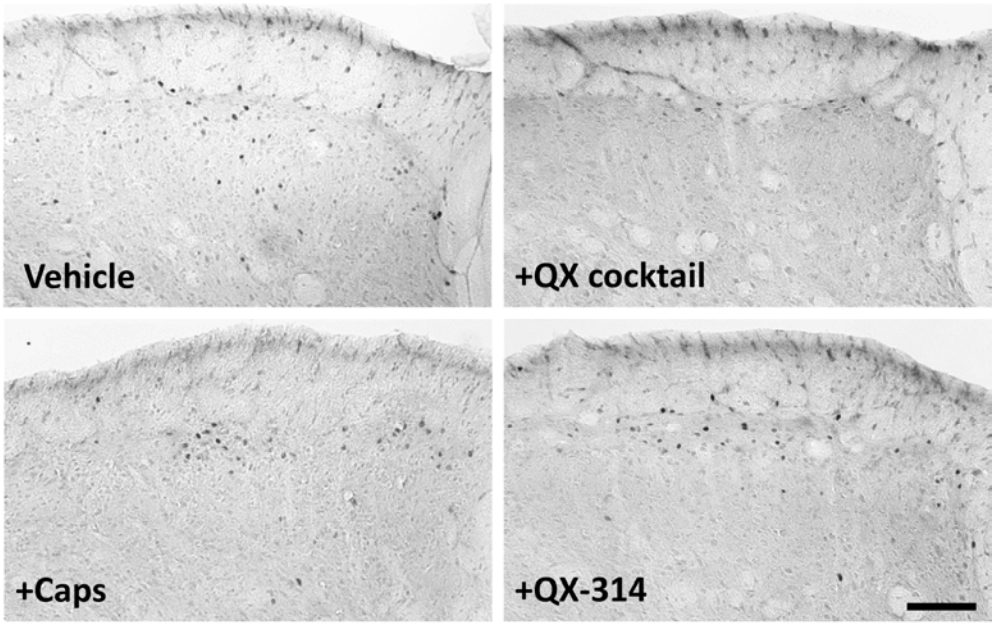
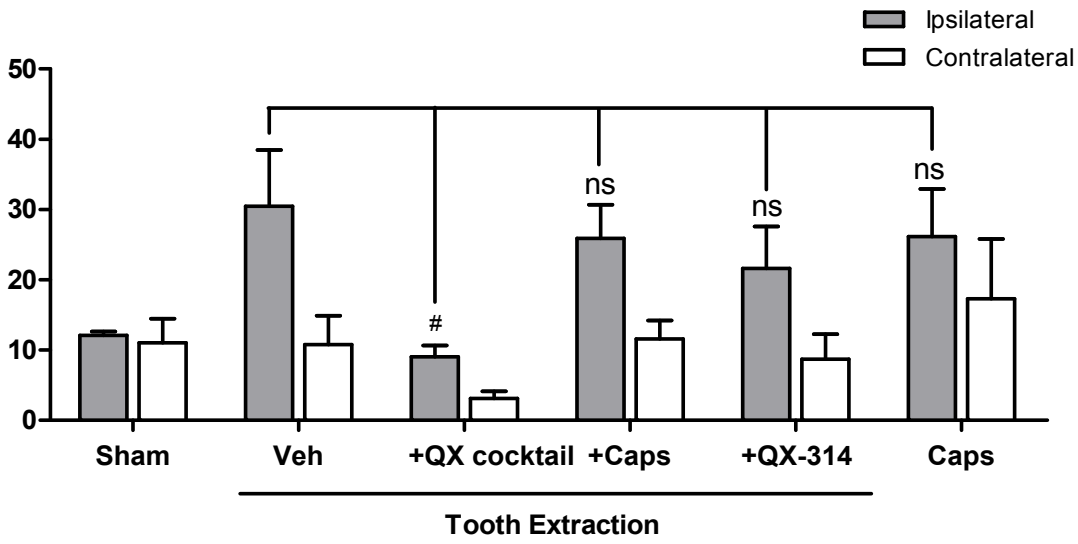

Vehicle
+QX cocktail
+Caps
+QX-314

Figure 11. Quantification of Fos-LI nuclei in ipsi- (grey bars) and contralateral (white bars) dorsomedial Sp5C in rats after sham surgery and in rats pre-treated with vehicle, QX cocktail, capsaicin or QX-314 alone, or capsaicin without tooth extraction

Injection of either QX-314 (22 ± 6 Fos-LI nuclei, n = 5 rats) (p = .2 *vs*. vehicle, Student's t test) or capsaicin (26 ± 5 Fos-LI nuclei, n = 8 rats) (p = .3 *vs*. vehicle, Student's t test) alone had no significant effect on tooth extraction-induced Fos-LI. Injection of capsaicin alone in otherwise naïve animals produced a measurable increase in ipsilateral Fos-LI (26 ± 7 Fos-LI nuclei, n = 4 rats) that was not significantly different from tooth extraction with vehicle injection (p = .4 *vs.* vehicle, Student's t test). #$p < .05$ *vs*. vehicle, unpaired Student's t test; ns, not significantly different from vehicle treatment. All capsaicin and QX-314-containing solutions (including QX cocktail) were constituted in vehicle solution.


Ipsilateral
Contralateral
50
40
30
20
10
0
#
ns
ns
ns
Sham
Veh
+QX cocktail
+Caps
+QX-314
Caps
Tooth Extraction

4. Effect of infiltration injection on tooth pulp stimulation-induced jaw-opening reflex (JOR)

To examine whether infiltration injection could deliver effective concentrations of anesthetic agents to pulpal afferents, we recorded the jaw-opening reflex (JOR) in response to electrical stimulation of the first mandibular molar. Injection of lidocaine (2%) into the mucobuccal fold adjacent to the stimulated tooth produced a rapid inhibition of the digastric muscle electromyogram (dEMG), which recovered 2 hrs after injection (n = 4 rats) **(Figure. 12).** Injection of QX cocktail in the same manner effectively reduced dEMG amplitude after 20 min with partial recovery 8 hrs after injection (n = 5 rats) **(Figure. 13).**

Figure 12. Effect of infiltration injection on tooth pulp stimulationinduced jaw-opening reflex (JOR) (Lidocaine 2%)

JOR was recorded in the form of a digastric muscle EMG (dEMG) in response to periodic electrical stimulation of the molar tooth pulp. After establishment of baseline dEMG, rats received a single buccal injection of lidocaine (2%) at time zero (arrow). Example voltage waveform dEMG traces (shown at right) were sampled from time points indicated from a representative experiment: (1) baseline, (2) 5 to 20 min postinjection, and (3) recovery.

Injection of lidocaine (2%) into the mucobuccal fold adjacent to the stimulated tooth produced a rapid inhibition of the digastric muscle electromyogram (dEMG) to 6% of control within 5 min (n = 4 rats) (traces 1 and 2), which recovered 2 hrs after injection (trace 3).

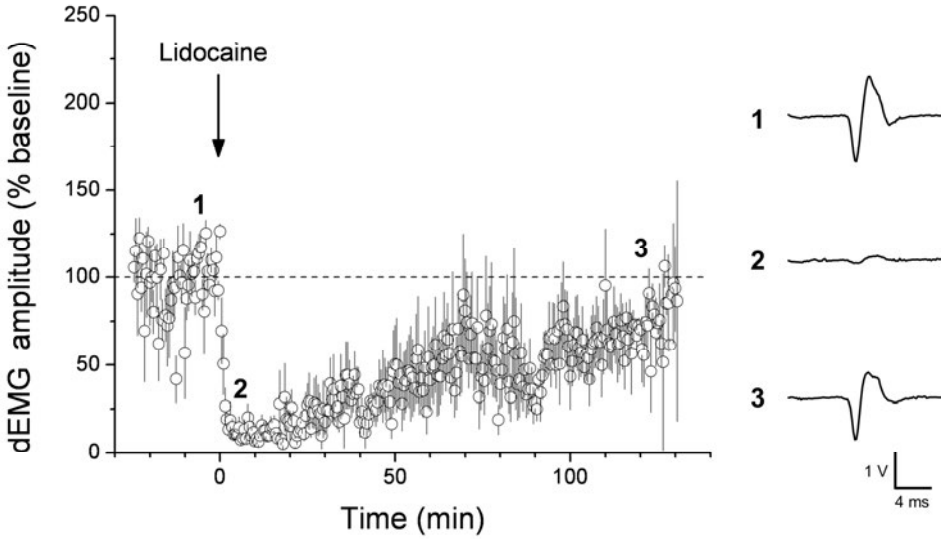
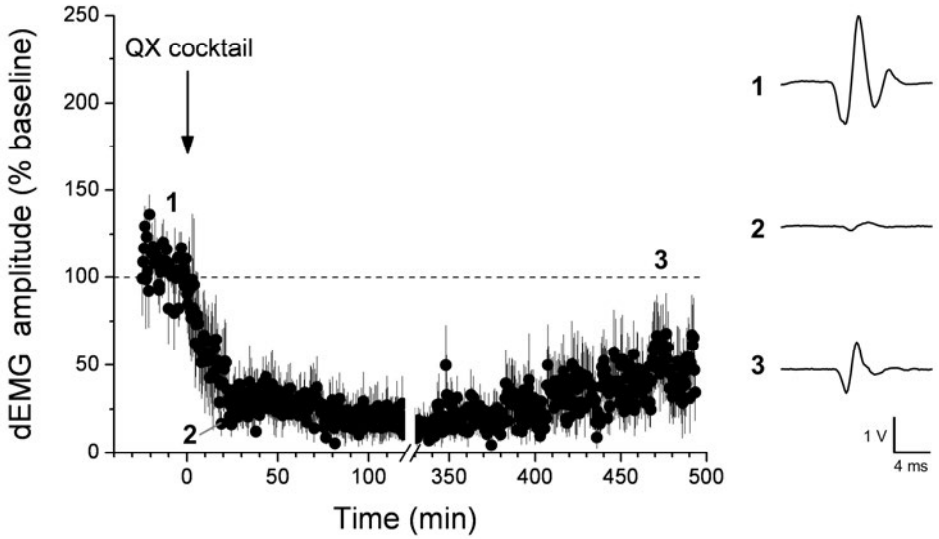

dEMG amplitude (% baseline)
250
200
150
100
50
0
Lidocaine
1
2
3
0
50
100
Time (min)
1
2
3
1 V
4 ms

Figure 13. Effect of infiltration injection on tooth pulp stimulationinduced jaw-opening reflex (JOR) (QX cocktail)

JOR was recorded in the form of a digastric muscle EMG (dEMG) in response to periodic electrical stimulation of the molar tooth pulp. After establishment of baseline dEMG, rats received a single buccal injection of QX cocktail at time zero (arrow).

Injection of QX cocktail reduced the dEMG amplitude to 50% of control after 10 min and 16% of control after 20 min (n = 5 rats) (traces 1 and 2). I observed partial recovery of the dEMG amplitude 8 hrs after injection (trace 3). Note the long recovery time of the QX cocktail compared with lidocaine. The abscissa of the QX cocktail-injected rat dEMG time-course is broken between 150 and 300 min for clear display of the onset and recovery of dEMG inhibition.

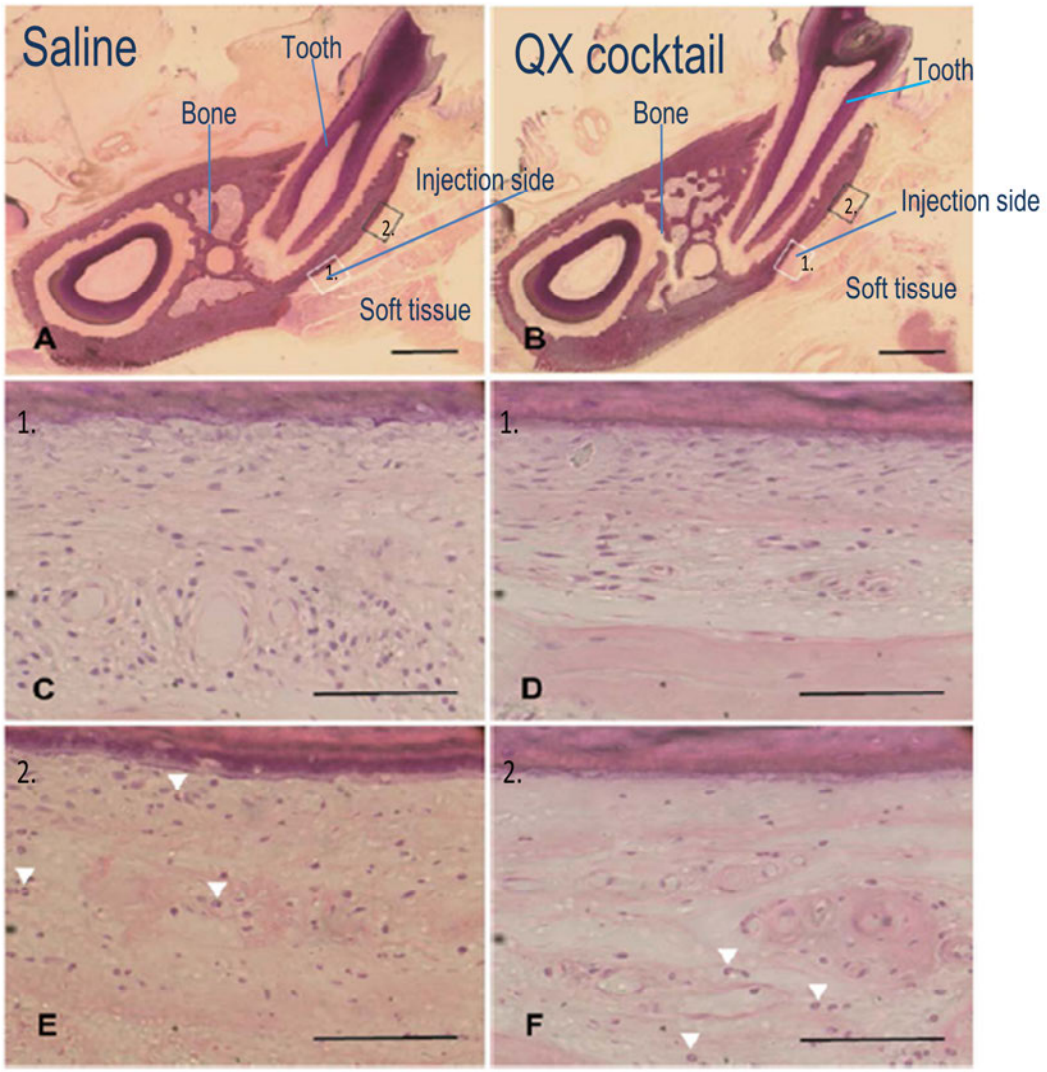

dEMG amplitude (% baseline)
250
200
150
100
50
0
QX cocktail
1
2
3
0
50
100
350
400
450
500
Time (min)
1
2
3
1 V
4 ms

5. Histological examination of hematoxylin and eosin (H&E)-stained peripheral tissues at the site of injection

Finally, I examined the gross morphology of mandibular tissue sections 24 hrs after injection of saline **(Figures. 14A, 14C, 14E)** or QX cocktail **(Figs. 14B, 14D, 14F).** Histological examination at high magnification showed no alterations in soft tissues near the site of injection in either group **(Figures. 14C, 14D)**, while edema and slight infiltration of polymorphonuclear leukocytes appeared in soft tissues superficial to the site of injection in both saline and QX cocktail-injected rats **(Figures. 14E, 14F)**, probably due to injection trauma.

Figure 14. Histological examination of hematoxylin and eosin (H&E)-stained peripheral tissues at the site of injection

Photomicrograph of mandibular cross-section from rat 24 hrs after injection of saline (A) or QX cocktail (B) at low magnification. (C, D) High magnification of injection site (white-boxed or 1 regions in A and B, respectively). (E, F) High magnification of region superficial to injection site (near cervical area) and overlying gingiva (black-boxed or 2 regions in A and B, respectively). Scale bars = 1 mm (A, B) and 100 μm (C-F); arrowheads indicate polymorphonuclear leukocytes.


Saline
Tooth
Bone
Injection side
2.
1.
Soft tissue
A
QX cocktail
Tooth
Bone
2.
Injection side
1.
Soft tissue
B
1.
C
1.
D
2.
E
2.
F

Discussion

In the present study, I report that lower molar extraction from adult rats resulted in a temporary enhancement of Fos-LI in the brain stem. c-Fos activation in the medullary dorsal horn has been demonstrated by diverse peripheral stimuli in the orofacial area (Strassman and Vos, 1993; Nomura *et al*., 2002). My results are consistent with those from an earlier study which identified Fos-LI in the trigeminal nucleus after experimental tooth extraction and pulp exposure in rats (Wakisaka *et al*., 1992). Fos-LI also followed a time-course similar to that of previous studies, peaking at 2 hrs after injury and returning to baseline 8 hrs after injury. The region of the medullary dorsal horn affected by these changes was located within the dorsomedial portion of the superficial layer of the Sp5C and is consistent with findings from previous studies on c-Fos expression following inferior alveolar nerve transection (Nomura *et al*., 2002; Piao *et al.*, 2006), suggesting that lower molar tooth extraction results in damage to tooth- and periodontium-innervating branches of the inferior alveolar nerve. In my study, I focused my analysis on the Sp5C region, although we cannot rule out the effects of tooth extraction in more rostral areas of the trigeminal nucleus. In addition, c-Fos expression may not account for all aspects of tooth-extraction-induced nociception (Sabino *et al.*, 2002). Interestingly, I observed enhanced Fos-LI in the contralateral Sp5C after tooth extraction, which was also attenuated by lidocaine pre-treatment. This observation may be accounted for either by indirect projections or by the possibility that nerves innervating the lower molars are among the small population of trigeminal afferent nerves that project bilaterally to the medullary dorsal horn (Jacquin *et al*., 1982). I further found that a cocktail of QX-314

and capsaicin given just 10 min prior to tooth extraction reduced Fos-LI, suggesting attenuation of neuronal activation in the brainstem. The anesthetic effect of the QX cocktail in the orofacial area is dependent on and selective for TRPV1 expressed in trigeminal nociceptive neurons (Kim *et al.*, 2010; Zakir *et al.*, 2012). Thus, c-Fos expression after tooth extraction likely results from activation of nociceptive fibers alone. Indeed, injection of capsaicin alone produced Fos-LI levels similar to those of tooth extraction with or without capsaicin included in the vehicle, suggesting that both capsaicin and tooth extraction stimuli excite an equivalent population of nociceptive afferent fibers. Infiltration anesthetic by injection into the mucobuccal fold allows injectate to pass through the alveolar bone (Tateno *et al.*, 2008). Here i used tooth pulp stimulation-evoked dEMG recordings to demonstrate that the infiltration injection of lidocaine in adult rats results in rapid (< 5 min) reduction of the JOR, thus validating the technique as an effective method for anesthesia of pulpal afferents. Inhibition of the JOR in response to injection of the QX cocktail displayed slower onset (10-20 min) but lasted more than 4 times longer than lidocaine. This supports my observed effect of the QX cocktail on c-Fos expression, which has been shown to require a slow and prolonged phase of nociceptive activity mediated by C-fibers (Herdegen *et al.*, 1991; Buritova *et al.*, 1997). The effectiveness of lidocaine to reduce c-fos expression in my study differs from that in a previous report (Locher-Claus *et al.*, 2005) but may be explained by differences in the nature of the tooth injury, site of procedure, and timing of injection prior to injury. Because of the presence of potential irritant compounds, I examined whether the QX cocktail had any effect on peripheral tissues local to the injection site. Histological examination of H&E stained mandibular tissue sections from QX cocktail-injected and saline-injected controls revealed comparable tissue

edema and leukocyte infiltration, as indicated by the presence of polymorphonuclear lymphocytes. While I cannot rule out more subtle inflammatory changes, this result suggests a minimal inflammatory effect of the components of the nociceptive anesthetic.

Summary

In summary this thesis reports on the effectiveness of the QX cocktail – a mixture of the TRPV1 agonist capsaicin and the membrane impermeant lidocaine derivative QX-314 – to prevent one of the sign of neuronal activation in the brain stem after molar tooth extraction. Specifically we were able to show that injection of the QX cocktail 10 mins prior to tooth extraction attenuated subsequent brainstem c-fos expression. The infiltration method of injection of the QX cocktail effectively anesthatized pulpal afferent fibres as measure by the jaw-opening reflex (JOR). Finally, that the QX cocktail did not cause any serious inflammation at injection site.

Conclusion

A selective pain fiber anesthetic strategy, such as the QX cocktail, is sufficient for preventing one of the early signs of central neuronal activation following tooth extraction and may provide a clinically effective local anesthetic option for dental patients that potentially avoids the sensory and motor complications of traditional local anesthetics.

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국문 초록

QX-314와 캡사이신 혼합물을 이용한 통증 특이적 국소마취법이 발치 후 뇌간 신경세포 활성에 미치는 효과에 관한 연구

서울대학교 대학원 치의과학과 신경생물학 전공

바드랄 바트바야르

본 연구는 발치에 의해서 뇌간 신경세포의 활성에 변화가 유도되는지를 삼차신경 척수감각핵 미측소핵 (Sp5C)에서 c-Fos 발현 양상의 변화를 분석하여 알아보았고, 또한 새로운 개념의 통증 특이적 국소마취제로서 QX-314와 capsaicin 혼합물의 효용성을, 일반적으로 가장 많이 사용되는 리도카인과 그 효과를 비교 평가하여 알아보았다. 성체 수컷 흰쥐(Sprague-Dawley)의 아래 어금니 발치 십 분 전 침윤마취(QX-314와 capsaicin 혼합물 혹은 리도카인)를 시행했다. 발치 이후 신경세포 활성화 여부는 면역조직화학법을 이용하여 원종양유전자 단백질인 c-Fos의 발현을 Sp5C에서 확인하였다. 발치 이후 신경활성의 지표인 c-Fos의 발현은 Sp5C의 양측 등내측에서 2시간이 지난 뒤 가장 높았고 ($p < .01$ vs. naïve ipsilateral) 8시간이 경과한 이후에는 발치를 시행하기 이전 수준으로 c-Fos의 발현이 감소하였다. 발치 시행 전 QX-314 와 capsaicin 혼합물의 투여는 발치 2시간 후 c-Fos의

발현 수준을 발치를 시행하지 않은 동물에서 나타난 c-Fos의 발현 수준까지 현저히 감소시켰다. 이런 마취에 의해 나타난 c-Fos 발현 감소 효과는 일반적인 국소 마취제인 리도카인에 의해서도 관찰되었다. 또한 치아 전기 자극에 의한 개구 반사 작용이 침윤 치수 마취에 의해 억제됨을 확인함으로써 QX-314와 capsaicin 혼합물 마취제가 발치 후 삼차신경 척수감각핵 미측소핵에서 통증정보를 전달하는 신경세포의 활성화를 효과적으로 감소시키는 것을 확인하였다.

상기 연구 결과는 치과에서 사용할 수 있는 통증 특이적 국소마취법의 기초를 제공함으로써, 향 후 임상에서 치과 환자들에게 효과적으로 사용될 수 있을 가능성을 제시하고 있다.

주요어: 캡사이신, 중추과민화, 리도카인, 구강안면통증, 삼차신경 척수감각핵 미측소핵, QX-314.

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