〔ORIGINAL〕

Lingual nerve stimulation-induced c-Fos expression in the trigeminal spinal nucleus

Yasumasa SAKURAI[1], Seishi ECHIGO[1], Satoshi KUCHIIWA[2], Hiroshi IZUMI[3]

[1] Clinics of Dentistry for Oral Surgery, Tohoku University Dental Hospital, Sendai 980–8575, Japan,
[2] Department of Neuroanatomy, Field of Neurology, Kagoshima University Graduate School of Medical and Dental Sciences, Sakuragaoka, Kagoshima, 890–8520, Japan,
[3] Department of Oral Physiology, School of Dentistry, Health Sciences University of Hokkaido, Ishikari–Tobetsu, Hokkaido, 061–0293, Japan

Abstract

The aim of the present study was to investigate whether electrical stimulation of the unilateral central cut ends of the lingual nerve produces trigeminal–parasympathetic reflex vasodilatation in both sides of the lower lip and stimulates neurons in the trigeminal nuclear complex in rats subjected to cervical vagosympathectomy, deeply anesthetized with urethane and artificially ventilated. Immunohistochemical detection of c–Fos expression was used to assess the impact of prolonged lingual nerve stimulation. We found that unilateral lingual nerve stimulation at 10 min intervals for 200 min produced the following: (1)consistent blood flow increases predominantly in the ipsilateral side of the lower lip, (2) more profound expression of c–Fos protein ipsilaterally in all subnuclei of the trigeminal spinal nucleus (Vsp) except the trigeminal subnucleus oralis, (3) a greater number of c–Fos–positive neurons in the ipsilateral trigeminal subnucleus interpolaris/caudalis transition zone (Vi/Vc) compared with the four other areas (trigeminal subnucleus caudalis/upper cervical spinal cord transition zone, subnucleus caudalis, subnucleus interpolaris, and subnucleus oralis) of the Vsp, and (4) no statistically significant increase in c–Fos expression in all subnuclei of the Vsp of the contralateral side in comparison with the control rats. The present studies and our previous data suggest that impulses elicited by electrical stimulation of the lingual nerve converge on the ipsilateral Vi/Vc in the Vsp and that the parasympathetic vasodilator neurons and salivatory nucleus, after receiving projections from the Vi/Vc in the Vsp, project to the lower lip via the otic ganglion.

Key word : c–Fos, trigeminal spimal nucleus, parasympathetic reflex, lingual nerve

1. Introduction

The trigeminal spinal nucleus (Vsp) has been suggested as responsible for parasympathetic reflex vasodilatation in the cat lower lip (Izumi and Nakamura, 2000 ; Koeda et al., 2003). However, it is still unknown which subnuclei of the Vsp, namely oralis (Vo), interpolaris (Vi), or caudalis (Vc), is more deeply involved in this trigeminal–parasympathetic reflex. The study of synaptically linked multineural networks in the brain is crucial to understanding reflex pathways.

Electrophysiologic and neuroanatomic techniques (e.g., nerve degeneration or axonal transport of enzymes by anterograde tracings) have long been used to identify the second– or higher–order cells and subnuclei involved in specific reflex pathways. However, technical constraints limit the mapping of large regions of the trigeminal system (Chattipakorn et al., 2002) by electrophysiologic methods, and artifacts may cause false mapping of synaptically linked neural pathways elicited by cell necrosis and virus spreading to glia cells or nonrelevant neurons via ventricular diffusion by neuroanatomic methods. On the other hand, the c–Fos proto–oncogene provides a novel avenue for research and a useful marker to study polysynaptic functional pathways in the central nervous system (Suwanprathes et al., 2003).

To date, few studies have examined the functional relationship between parasympathetic reflex vasodilatation and c–Fos ex-

受付：平成18年10月31日
Corresponding author (Hiroshi IZUMI), E-mail : izumih@hoku-iryo-u.ac.jp

pression in the subnucleus of the Vsp produced by stimulation of the central cut end of the lingual nerve in rats. The aim of the present study was two-fold : (1) to identify sites within the subnucleus of the Vsp that may be active after lingual nerve-mediated sensitization by measuring c-Fos expression within the trigeminal complex, and (2) to examine the functional significance of parasympathetic reflex vasodilatation and c-Fos expression in the ipsilateral and contralateral Vsp of rats when the central cut end of one side of the lingual nerve was electrically stimulated.

2. Materials and methods

2.1.Preparation of animals

The experimental protocols were reviewed by the Committee on the Ethics of Animal Experiments at Tohoku University School of Medicine and were performed in accordance with both the Guidelines for Animal Experiments issued by the Tohoku University School of Medicine and The Law (No.105) and Notification (No.6) issued by the Japanese Government.

Twenty-one adult Wistar rats, unselected as to sex and weighing 260-450g, were initially sedated with inhalation anesthetic (3% isoflurane) and then anesthetized with urethane (1.0g/kg intravenously [IV]). The anesthetic was supplemented if and when necessary throughout the experiment. A femoral artery was cannulated for the measurement of systemic arterial blood pressure (SABP). One femoral vein was cannulated to allow drug injection. The anesthetized animals were intubated and then paralyzed by IV injection of pancuronium bromide (Mioblock ; Organ, Teknika, The Netherlands.) at 0.4 mg/kg initially and supplemented with 0.6 mg/kg every hour or so after testing the level of anesthesia (see below). The animals were artificially ventilated through the tracheal cannula with a mixture of 50% air and 50% O_2 (ventilator Model SN-480-6 ; Shinano, Tokyo, Japan). End-tidal CO_2 was kept at 35-40 mm Hg with the aid of an infrared analyzer (Capnomac Ultima ; Datex Co., Helsinki, Finland)(Izumi, 1999 ; Izumi and Ito, 1998 ; Izumi and Nakamura, 2000), and rectal temperature was maintained at 37-38 ℃ using a heating pad.

In all experiments, the cervical vagi and superior cervical sympathetic trunks were cut bilaterally in the neck before any stimulation to eliminate reflexes mediated by the vagus nerve and sympathetic effects on the orofacial area, respectively.

The criterion for an adequate depth of anesthesia was the absence of any precipitate changes in SABP as reflex responses to a fairly minor noxious stimulus (such as pinching the upper lip for approximately 2 s). If the depth of anesthesia was considered inadequate, additional urethane was administered (i.e., intermittent doses of 100 mg/kg IV). Once an adequate depth of anesthesia had been attained, supplementary doses of pancuronium were given approximately every 60 min to maintain immobilization during periods of stimulation.

2.2.Electrical stimulation of the lingual nerve

The lingual nerve has proven to be the most suitable of the afferent nerves investigated so far for eliciting reflex activation of parasympathetic nerve fibers mediating blood flow and salivation responses in the lower lip and submandibular gland [18-21, 31, 40].

To elicit parasympathetic reflex vasodilatation in the lower lip, we electrically stimulated the central cut end of the right (ipsilateral) lingual nerve 20 times every 10 min, using a 20 s train of 2 ms rectangular pulses at a frequency of 10 Hz and an intensity of 20 V (Fig.1). A bipolar silver electrode attached to a Nihon Kohden Model SEN-7103 Stimulator (Tokyo, Japan) was used for this procedure.

The rats were assigned to one of two experimental groups. The first group of animals (n=12) received lingual nerve stimulation. Electrical stimulation of the right lingual nerve was done as above in a single rat. The left (contralateral) lingual nerve was set with a bipolar silver electrode but was not stimulated. The second group of animals (n=9) underwent preparation and application of a bipolar silver electrode bilaterally, but were not stimulated (sham-operation group).

2.3.Measurement of lower lip blood flow

Changes in lower lip blood flow (LBF) were monitored (Fig.2) using a laser Doppler flowmeter (model ALF21D ; Advance,

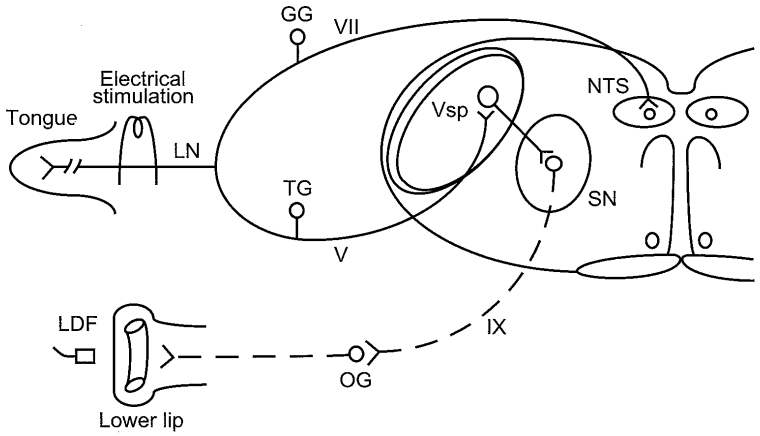

Fig.1. Schematic representation of the sites used for electrical stimulation and blood flow measurement. Stimulation sites : central cut end of the lingual nerve (LN). Blood flow measurement sites : lower lip (by laser–Doppler flowmeter, LDF). TG, trigeminal ganglion ; GG, geniculate ganglion ; Vsp, trigeminal spinal nucleus ; NTS, nucleus tractus solitarius ; SN, salivatory nucleus ; IX, glossopharyngeal nerve ; OG, otic ganglion.

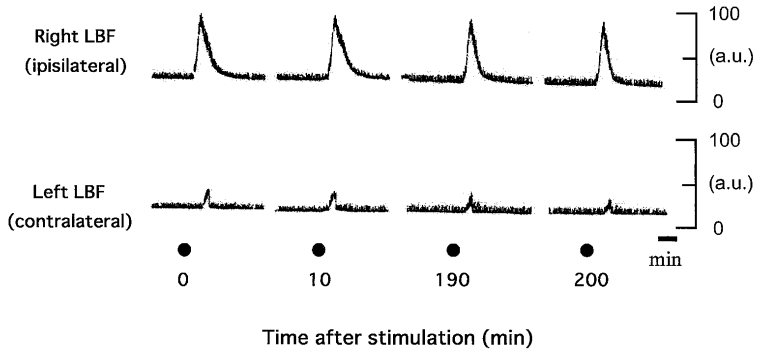

Fig.2. Typical example of changes in right (ipsilateral) and left (contralateral) lower lip blood flow [LBF ; in arbitrary units (a.u.)] elicited by electrical stimulation of the central cut end of right lingual nerve. Right lingual nerve was stimulated where indicated (filled circle) every 10 min for 20 s with pulses of 2 ms duration at 20 V intensity and 10 Hz frequency. Stimulus times (0, 10, 190, 200 min after first stimulation) are shown below the stimulus markers.

Tokyo, Japan), as descried previously [Izumi, 1999 ; Izumi and Ito, 1998 ; Izumi and Nakamura, 2000]. The probe was placed against the lower lip without exerting any pressure on the tissue. The LBF changes were assessed by measuring the height of the responses on the chart. Flow levels are expressed in arbitrary units (a.u. ; Fig. 2).

2.4.Tissue preparation and immunohistochemistry

All experimental rats were killed 2 h after the last stimulation. All animals received a lethal dose of sodium pentobarbital, which was perfused through the heart with 500 ml cold saline, followed by 500 ml cold fixative (4% paraformaldehyde, 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4). The caudal brainstem and upper cervical spinal cord were removed from the skull. The tissues were placed in 30% sucrose in 0.1 M phosphate buffer for 3–7 days. The brainstem was cut on a microtome into 50 μm–thick, transverse frozen sections. Every third section was collected in 0.02 M phosphate–buffered saline (PBS) and then processed for c–Fos immunocytochemistry by the peroxidase–antiperoxidase method, as described previously [Cheng et al., 2002]. After blocking endoperoxidase with 0.15% H_2O_2, we preincubated the floating sections in a blocking solution of 0.3% Triton X–100, 1% normal goat serum, 0.05% sodium azide and 0.3% bovine serum albumin (BSA) in 0.02 M PBS, pH 7.4, and then incubated them for 3 days at 4℃ in a solution containing rabbit anti–c–Fos antibody (1 : 5000 ; Chemicon International, Temecula, CA) diluted in the blocking solution. After several rinses with 0.02 M PBS, the sections were immersed in a solution of goat anti–rabbit IgG (1 : 2000 ; EY Laboratories) overnight at 4℃, and following several more rinses, the sections were incubated in rabbit peroxidase anti–peroxidase IgG (1 : 2000 ; DAKO, Denmark) overnight at 4℃. The second and third antibodies were diluted in 0.02 M PBS containing 0.3% BSA and 1% normal goat serum. Following several rinses with 0.1 M Tris–HCl buffer, pH 7.6, the sections were treated with 0.02% diaminobenzidine and 0.6% nickel ammonium in

0.001% hydrogen peroxidase. After final washes, the sections were mounted on gelatin-chrome alum-coated glass slides, air dried, dehydrated with ethanol, cleared in xylene, and placed under cover slipps. Some adjacent sections were counterstained with thionin, and nuclei were localized according to the atlas of Paxinos and Watson (1998).

2.5.Data analysis

All sections of the animals were examined under an Olympus light microscope (Provis AX 70) with a FUJIX digital camera (HC-2500 ; FUJIFILM). For each nucleus, the number of Fos-like immunoreactive (Fos-LI) neurons in each section was counted and averaged. The tissue sections were within 3.3 mm rostral and 1.5 mm caudal to the obex. We detected only oval, densely stained nuclei.

The general view is that the Vsp is cytoarchitecturally divided into three subnuclei (pars caudalis, pars interpolaris, and pars oralis). However, the borders between the nuclei are not always clear, so we separated the Vsp into five parts based on the position of the obex, as follows : (1) the pars caudalis and the upper spinal cord transition zone (Vc/C_1 ; 1.2-1.5 mm posterior to the obex), (2) the main part of the pars caudalis (Vc ; 1.2 mm posterior to 0.2 mm anterior to the obex), (3) the pars interpolaris and the caudalis transition zone (Vi/Vc ; 0.2-0.8 mm anterior to the obex), (4) the main part of the pars interpolaris (Vi ; 0.8-2.0 mm anterior to the obex), and (5) the pars oralis (Vo ; 2.0-3.3 mm anterior to the obex).

All counts were made by one investigator to maintain consistency in application of the criteria used to select profiles as c-Fos-positive cells and to reduce the likelihood of subject variability. All numerical data of c-Fos-positive neurons are given as mean±SE. Analysis of variance (ANOVA) was used for group comparisons, followed by Scheff's test to further delineate the differences between specific parts. The significance level was set at $P<0.05$ for all elements.

3. Results

The resting mean SABP value obtained in our rats was 116.20±20.20 mmHg (n=21).

3.1.Effects of electrical stimulation of the right lingual nerve on lower lip blood flow

Fig.2 shows typical recordings of the evoked changes in LBF (on both sides) following right lingual nerve stimulation at 20 times every 10 min, using a 20 s train of 2 ms rectangular pulses at a frequency of 10 Hz and an intensity of 20 V in a single rat. The right LBF increased markedly, and the left LBF increased slightly. LBF consistently increased on both sides predominantly when the right lingual nerve was stimulated at 10 min intervals for 200 min. The parasympathetic reflex vasodilatation in the lower lip was evoked at all stimulations consistently on both sides.

3.2.Fos-like immunoreactivity in trigeminal neurons

Fig.3 shows the typical photomicrographs of the c-Fos expression in the Vi/Vc after electrical stimulation of the central cut end of the right lingual nerve. Fos-LI neurons were concentrated in the dorsomedial part of the Vi/Vc. The number of Fos-LI

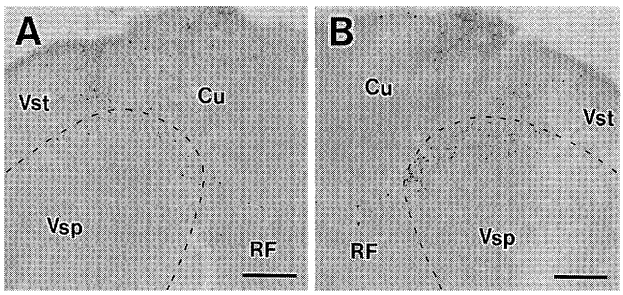

Fig.3. Photomicrograph illustrating an example of c-Fos positive neurons in 50 μm sections of the trigeminal subnucleus interpolaris/caudalis (Vi/Vc) transition zone after electrical stimulation of the central cut end of the right ingual nerve. (A) represents the left (contralateral) side of the Vi/Vc transition zone and (B) represents the right (ipsilateral) side of the Vi/Vc transition zone, respectively. Vst, spinal tract of the trigeminal nerve ; Cu, cuneate nucleus ; RF, reticular formation. Scale bar, 500 μm

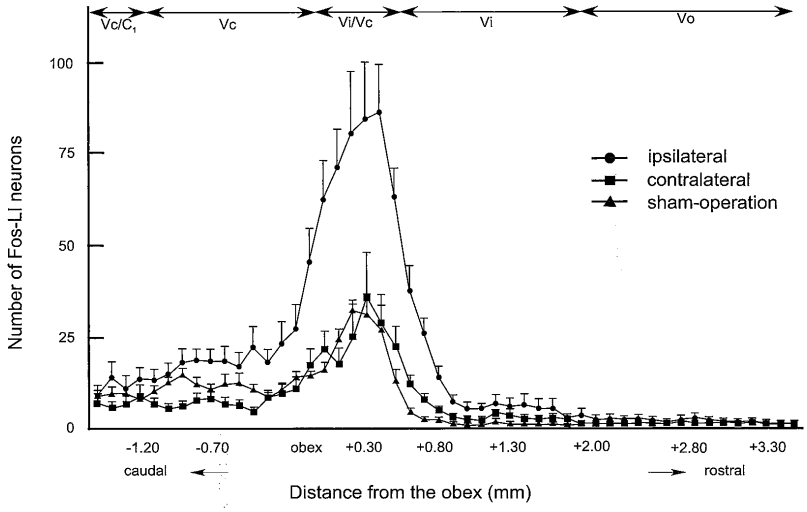

Fig.4. The distribution of Fos–LI neurons within the right (ipsilateral) side (circles), the left side (squeres) and sham–operation (triangles) of Vsp in five sites [trigeiminal subnucleus caudalis/upper cervical spinal cord (Vc/C_1) transition zone, subnucleus caudalis (Vc), subnucleus interpolaris/caudalis (Vi/Vc) transition zone, subnucleus interpolaris (Vi), subnucleus oralis (Vo) in relation to the distance from the obex following the right lingual nerve stimulation for 20 times every 10 min for 20 s with pulses of 2 ms duration at 20 V intensity and 10 Hz frequency. The Fos–LI in the Vsp exhibited the distribution with one peak centered at the periobex level, and there was the great difference of the number of Fos–LI neurons between the right side and the left side or sham–operation at that area. Values = Mean ± S.E.

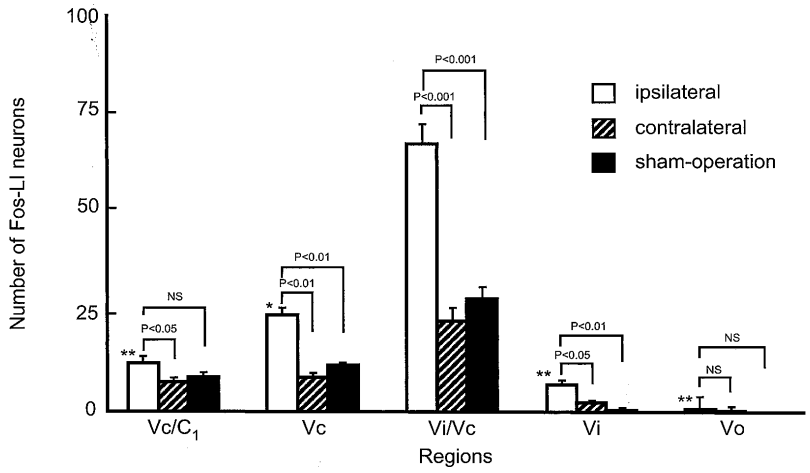

Fig.5. The average number of Fos–LI neurons per section in the right side (ipsilateral ; open columns), left side (contralateral ; hatched columns) and sham–operation (filled columns) of trigeiminal subnucleus caudalis/upper cervical spinal cord (Vc/C_1) transition zone, subnucleus caudalis (Vc), subnucleus interpolaris/caudalis (Vi/Vc) transition zone, subnucleus interpolaris (Vi), subnucleus oralis (Vo) evoked by the right lingual nerve stimulation. The condition of electrical stimulation of the lingual nerve was at 20 times every 10 min, using a 20 s train of 2 ms rectangular pulses at a frequency of 10 Hz and at intensity of 20 V in rats (n=21). Values = Mean±S.E. Statistical significance of difference among the right, left sides and the sham–operation were assessed by means of ANOVA followed by the Sheffe's test ($P<0.05$, 0.01, 0.001). Brackets indicate significant difference between 2 columns. The asteriskes * and ** denote statistically difference at $P<0.01$ and $P<0.001$, compared the ipsilateral Vi/Vc with the ipsilateral Vc/C_1, Vc, Vi and Vo, respectively. NS represents not significant.

neurons at the Vi/Vc was significantly greater on the right side (B) than the left side (A) and when compared with sham–operated rats ($P<0.001$). No significant difference was observed between the left side and sham–operated rats.

Fig.4 shows the mean±SE of the c–Fos–positive neurons per section in the right (ipsilateral) side, left (contralateral) side and sham–operated rats for Vc/C_1, Vc, Vi/Vc, Vi, and Vo evoked by right lingual nerve stimulation. The Fos–LI neurons in the Vsp exhibited a distribution with one peak centered at the periobex level and showed a marked difference near the obex in the number of Fos–LI neurons in the Vsp between the right and left sides or sham–operated rats. This area with the peak distribution was the Vi/Vc.

Fig.5 shows the statistical numbers of Fos–LI neurons in the Vc/C_1, Vc, Vi/Vc, Vi and Vo of 21 adult Wistar rats at the

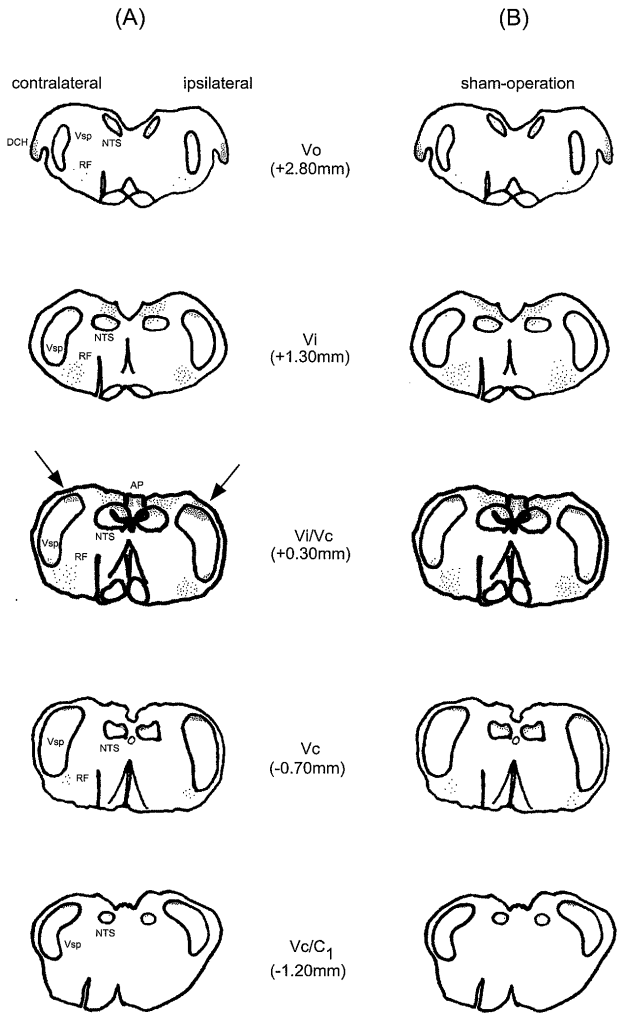

Fig.6. Schematic illustrations of the Fos-LI at the Vsp (Vo, Vi, Vi/Vc, Vc and Vc/C_1), nucleus tractus solitarius (NTS), area postrema, lateral reticular nucleus, pyramidal tract and dorsal cochlear nucleus following electrical stimulation. The right lingual nerve was stimulated 20 times every 10 min for 20 s with pulses of 2 ms duration at 20 V intensity and 10 Hz frequency. Each dot represents one Fos-LI labeled nucleus. The numbers in the parenthesis indicate the rostrocaudal distance (mm) from the obex (0). Data for each group (the Vc/C_1 transition zone, Vc, Vi/Vc transition zone, Vi and Vo) are from one individual rat respectively. At the Vi/Vc transition zone there was the robust Fos-LI expression in the right dorsal medial part of the Vsp, and the Fos-LI neurons were visually different between the right side and the left side. At the other nuclei there was the Fos-LI expression, but the number of Fos-LI neurons in the right side, left side and sham-operation was not statistically different, respectively ($P > 0.05$). AP, Area postrema ; RF, reticular formation ; DCH dorsal cochlear

right (ipsilateral) side, left (contralateral) side, and after sham-operation. Data are shown as the average number per section.

The number of Fos-LI neurons was significantly greater on the right side than the left side in the Vc/C_1, Vc, Vi/Vc, and Vi, but not in the Vo (Vc/C_1 : $P<0.05$; Vc : $P<0.01$; Vi/Vc : $P<0.001$; Vi : $P<0.05$; Vo : $P>0.05$). The number of Fos-LI neurons was also significantly greater on the right side than in the sham-operated rats in the Vc, Vi/Vc, and Vi, but not in the Vc/C_1 or Vo (Vc : $P<0.01$; Vi/Vc : $P<0.001$; Vi : $P<0.01$; Vc/C_1 : $P>0.05$; Vo : $P>0.05$). The number of Fos-LI neurons was not significantly different between the left side and the sham-operated rats in any region (Vc/C_1, Vc, Vi/Vc, Vi, Vo : $P>0.05$).

In the right side of the Vsp, the number of Fos-LI neurons at the Vi/Vc was significantly greater than in the Vc/C_1, Vc, Vi, and Vo (Vc/C_1 : $P<0.001$; Vc : $P<0.01$; Vi : $P<0.001$; Vo : $P<0.001$).

Fig.6 shows the typical distribution of c-Fos expression in the Vsp (Vc/C_1, Vc, Vi/Vc, Vi, and Vo), nucleus of the solitary tract (NTS), area postrema, lateral reticular nucleus and dorsal cochlear nucleus following electrical stimulation of the right lingual nerve under the conditions described earlier. Fos-LI neurons are shown in schematic drawings of coronal sections in those regions. At the Vi/Vc, robust c-Fos expression was found in the right dorsomedial part of the Vsp, and the Fos-LI neurons were visually different between the right and left sides.

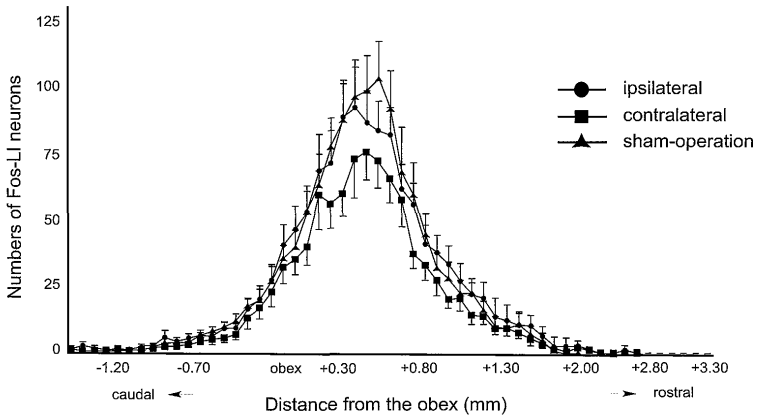

Fig.7. The distribution of Fos–LI neurons in the nucleus tractus solitarius (NTS) in relation to the distance from the obex following the right lingual nerve stimulation 20 times every 10 min for 20 s with pulses of 2 ms duration at 20 V intensity and 10 Hz frequency. The Fos–LI in the NTS exhibited the distribution with one peak centered the point of 0.5 mm rostral from the obex. No difference of the number of Fos–LI neurons was observed between the right and left sides or sham–operation at all area. Values = Mean±S.E.

After electrical stimulation of the right lingual nerve, the Fos–LI neurons were expressed in the NTS, area postrema, lateral reticular nucleus, and dorsal cochlear nucleus. In these nuclei, the Fos–LI neurons were expressed about equally on the right side, left side, and after sham–operation.

Fig.7 shows the distribution of Fos–LI neurons in the NTS on the right side, left side, and sham–operated rats. The Fos–LI neurons exhibited a distribution with one peak centered at 0.4 mm rostral from the obex, and the numbers of Fos–LI neurons on the right side, left side, and after sham–operation were not significantly different ($P>0.05$).

4. Discussion

We have previously proposed the presence of a parasympathetic reflex vasodilator mechanism serving the orofacial areas in the cat and rat (Izumi et al., 2002 ; Mizuta et al., 2000, 2002 ; Mizuta and Izumi, 2004). Although the afferent and efferent pathways involved in this reflex response to somatic sensory stimulation have now been well studied (Izumi et al., 2002, 2003), the central mechanism in the brainstem remains uncertain. The study of synaptically linked multineuronal networks in the brain is crucial to understanding reflex pathways.

Recently we have suggested that the Vsp is an important bulbar relay for lingual nerve evoked parasympathetic vasodilatation in the cat lower lip (Mizuta et al., 2000, 2002 ; Mizuta and Izumi, 2004). This proposal is based on the fact that unilateral microinjection (1 μl/ml) of either lidocaine (2%) or kainic acid (10 mM) into the Vsp ipsilateral to the stimulated lingual nerve led to a reversible or irreversible reduction in reflex vasodilatation, but had no effect on vasodilatation elicited by stimulation of the contralateral lingual nerve. However, microinjection of these agents at a volume of 1 μl into the brainstem seems to spread approximately 1-1.5 mm in radius, judging from our previous experiment using horseradish peroxidase (HRP) (1 μl, 10%) as a histologic marker microinjected into the salivatory nucleus (Izumi et al., 2002). The question thus arises as to which subnucleus of the Vsp is more deeply involved in the above trigeminal–parasympathetic reflex (Izumi et al., 2002 ; Mizuta et al., 2000, 2002 ; Mizuta and Izumi, 2004), given that the Vsp consists of three subnuclei : caudalis (Vc), interpolaris (Vi), and oralis (Vo).

Electrophysiologic techniques (i.e., extracellularly evoked single–unit response to trigeminal nerve stimulation) or conventional neuroanatomic techniques (e.g., nerve degeneration or axonal transport of enzymes by anterograde route) have not allowed precise identification of the second– or higher–order cells and subnuclei involved in specific trigeminal reflex pathways. Numerous studies have established that expression of immediate–early genes can be induced within the central nervous system under various conditions (Oakden and Boissonade, 1998 ; Ro et al., 2003 ; Strassman and Vos, 1993 ; Suwanprathes et al., 2003 ; Yoshida et al., 1991). Among them, c–Fos is the best characterized. The c–Fos proto–oncogene provides a novel avenue for research and a useful marker to study polysynaptic functional pathways in the central nervous system (Suwan-

prathes et al., 2003). The expression of c-Fos is specific to the nuclei of the second- and higher-order neurons in specific pathways ; it does not occur in closely associated glial, ependymal, or endothelial cells (Bullitt, 1990 ; Hunt et al., 1987 ; Morgan and Curran, 1991 ; Munglani and Hunt, 1995). The induction of Fos-LI neurons in the medullary and rostral cervical spinal dorsal horns after noxious orofacial stimulation has been studied systematically (Bereiter, 1997 ; Bullitt, 1990 ; Hunt et al., 1987 ; Lu et al., 1993, Lu and Bereiter, 1995 ; Meng and Bereiter, 1996 ; Strassman and Vos, 1993). Noxious orofacial stimulation induces Fos-LI neurons in widespread brainstem structures, including the Vsp, the NTS, the paratrigeminal nucleus, the lateral reticular nucleus, and the inferior olivary nucleus. Our previous finding that the Vsp, but not the NTS, is involved in the parasympathetic reflex vasodilatation in the lower lip induced by lingual nerve stimulation (Mizuta et al., 2002) has led us to focus on neural activation in the Vsp following lingual nerve stimulation.

The aim of the present study was to investigate whether electrical stimulation of the unilateral lingual nerve produces trigeminal-parasympathetic reflex vasodilatation in both sides of the lower lip and stimulates neurons in the trigeminal nuclear complex. Immunohistochemical detection of c-Fos expression was used to assess the impact of prolonged lingual nerve stimulation. In previous studies, Fos-LI neurons have been used as a marker of neural activity in the trigeminal system (Bullitt, 1990 ; Chattipakorn et al., 2002 ; Hunt et al., 1987). The primary findings of the present study are that unilateral lingual nerve stimulation dose the following : (1) elicits consistent blood flow increases predominantly in the ipsilateral side of the lower lip when stimulated at 10 min intervals for 200 min ; (2) evokes more profound c-Fos expression ipsilaterally in all subnuclei of the Vsp except Vo ; and (3) results in marked increases of c-Fos expression in the ipsilateral Vi/Vc compared with the other subnuclei both in the ipsilateral and contralateral sides.

No consensus has been reached about the best anesthetic to use when studying the relationship between trigeminal-parasympathetic reflex vasodilatation and c-Fos expression in the Vsp. Urethane was chosen in the present study because we recently observed that urethane anesthesia preserves trigeminal-parasympathetic reflex vasodilatation compared with other anesthetics, such as pentobarbital and isoflurane (Ito et al., 1998). However, it has been reported that urethane anesthesia by itself induced bilateral c-Fos expression in autonomic brain nuclei related to cardiovascular regulation such as the NTS, area postrema, and paraventricular hypothalamus (Rocha and Herbert,1997). Furthermore, it was suggested that the influence of urethane on c-Fos expression in the brainstem nuclei could be explained, at least in part, by its cardiovascular effects because urethane induces a decrease in arterial blood pressure and heart rate (Natarajan and Morrison, 1999 : Rocha and Herbert, 1997). These findings suggest that urethane might not be suitable for c-Fos protein analysis in studies investigating trigeminal mediated autonomic reflex responses in the brainstem. However, whatever mechanism is involved in c-Fos expression by urethane, a marked difference in c-Fos expression was observed not only between the ipsilateral and contralateral sides of each subnucleus of the Vsp, but also among subnuclei of the Vsp by itself (Vi/Vc sites were more sensitive) following lingual nerve stimulation. These results suggest that urethane can be useful for simultaneous measurements of trigeminal-parasympathetic reflex vasodilatation and c-Fos expression following lingual nerve stimulation.

As shown in Fig.2, lingual nerve stimulation elicited a marked increase in ipsilateral LBF and a slight increase in the contralateral LBF. These increases occurred consistently over 0-200 min on both the ipsilateral and contralateral sides. After electrical stimulation of the right lingual nerve for 20 times, c-Fos-positive neurons were measured on both sides of the subnucleus of the Vsp (Fig.4) ipsilateral and contralateral to lingual nerve stimulation as well as after sham-operation (Figs.4 and 5). All levels of the Vsp seem to play an important role in full expression of the response to lingual nerve stimulation. In particular, c-Fos expression within the Vsp was concentrated in the Vi/Vc. The number of c-Fos-positive neurons in the ipsilateral Vi/Vc was much higher than in the four other subnuclei (Vc/C_1,Vc, Vi, Vo) of the Vsp. It has been reported recently that inflammation of either the pulp (Byers et al, 2000 ; Chattipakorn et al., 1999, 2002 ; Coimbra and Coimbra, 1994 ; Oakden and Boissonade, 1998) or the masseter muscle (Imbe et al., 1999 ; Ro et al., 2003) or thermal and chemical stimulation of the cornea (Bereiter, 1997 ; Bereiter et al., 2000 ; Lu and Bereiter, 1995 ; Meng and Bereiter, 1996) induced a similar amount of c-Fos expression in a group of neurons located in the Vi/Vc and in the Vc. However, as shown in Figs.4 and 5, lingual nerve stimulation induced a larger amount of c-Fos expression in the Vi/Vc than in the other subnuclei of the Vsp. Meng and Bereiter (1996) compared the magnitudes of the bimodal peaks of c-Fos in the Vsp produced by thermal (50℃) and chemical

(mustard oil) stimuli to rat cornea and suggested that select features of corneal stimuli, such as modality, are encoded differently by neurons in the Vi/Vc as compared with those located in the Vc/C_1. This proposal may be supported by the selective action of morphine, which caused a significant dose-related reduction in the number of c-Fos-positive neurons at the Vc/C_1, but not at the Vi/Vc, after corneal stimulation with mustard oil (Bereiter, 1997 ; Bereiter and Bereiter, 2000 ; Bereiter et al., 1994, 2000 ; Meng and Bereiter, 1996). This latter result is in good agreement with our previous experimental finding that IV administration of morphine did not reduce the parasympathetic mediated reflex vasodilatation following lingual nerve stimulation [Izumi et al., 1997]. Several lines of evidence suggest that the c-Fos expression observed in the Vi/Vc and Vc/C_1 after peripheral trigeminal nociceptive stimulation may be involved in autonomic responses and nociceptive transmission to the central nervous system, respectively. This possibility may be supported by experimental data showing that neurons in the ventral portion of the Vi/Vc and Vc/C_1 project into autonomic control areas such as the NTS (Menetrey and Basbaum, 1987 ; Meng and Bereiter, 1996) and the nucleus submedius of the thalamus (Meng and Bereiter, 1996 ; Yoshida et al., 1991). On the basis of the present data and previous studies, it seems likely that impulses elicited by electrical stimulation of the lingual nerve converge on the ipsilateral Vi/Vc in the Vsp and that the parasympathetic vasodilator neurons, and salivatory nucleus, after receiving projections from the Vsp (Mizuta et al., 2002, Mizuta and Izumi, 2004), project to the lower lip via the otic ganglion (Kuchiiwa et al., 1992).

Comparison of the results obtained by thermal or chemical stimulation of the corneal surface and those by electrical stimulation of the lingual nerve shown in the present experiment suggests some variation. The c-Fos-positive neurons appeared bimodally distributed after corneal stimulation but uniformly distributed after lingual nerve stimulation (Fig.4). This discrepancy may be due to the difference in of choice of anesthetic (urethane vs chloralose) or the difference in the site used to elicit trigeminal nerve stimulation. No statistically significant difference in the amount of c-Fos expression could be observed in all subnuclei of the Vsp between the contralateral side and the sham-operated rats (Fig.4), suggesting that c-Fos expression in the contralateral side after lingual nerve stimulation is due primarily to the effect of urethane.

Although the present level of understanding suggests that the Vi/Vc mediates the parasympathetic reflex vasodilatory response in the lower lip following lingual nerve stimulation, more work is needed to reach a definite conclusion on this point. In the meantime, we propose that the afferent information induced by lingual nerve stimulation electrically enters the Vi/Vc via the trigeminal ganglion, and after this input, the secondary information enters in the inferior salivatory nucleus, and the parasympathetic vasodilatory reflex occurs in the lower lip by the glossopharyngeal nerve via the otic ganglion.

References

Bereiter DA. Morphine and somatostatin analogue reduce c-fos expression in trigeminal subnucleus caudalis produced by corneal stimulation in the rat. Neuroscience 77 : 863-874, 1997.

Bereiter DA and Bereiter DF. Morphine and NMDA receptor antagonism reduce c-fos expression in spinal trigeminal nucleus produced by acute injury to the TMJ region. Pain 85 : 65-77, 2000.

Bereiter DA, Bereiter DF, Hirata H and Hu JW. c-fos expression in trigeminal spinal nucleus after electrical stimulation of the hypoglossal nerve in the rat. Somatosens Mot Res 17 : 229-237, 2000.

Bereiter DA, Hathaway CB and Benetti AP. Caudal portions of the spinal trigeminal complex are necessary for autonomic responses and display Fos-like immunoreactivity after corneal stimulation in the cat. Brain Res 657 : 73-82, 1994.

Bullitt E. Expression of c-fos-like protein as a marker for neuronal activity following noxious stimulation in the rat. J Comp Neurol 296 : 517-530, 1990.

Byers MR, Chudler EH and Iadarola MJ. Chronic tooth pulp inflammation causes transient and persistent expression of Fos in dynorphin-rich regions of rat brainstem. Brain Res 861 : 191-207, 2000.

Chattipakorn SC, Light AR, Willcockson HH, Narhi M and Maixner W. The effect of fentanyl on c-fos expression in the trigeminal brainstem complex produced by pulpal heat stimulation in the ferret. Pain 82 : 207-215, 1999.

Chattipakorn, SC, Sigurdsson A, Light AR, Narhi M and Maixner W. Trigeminal c-Fos expression and behavioral responses to pulpal inflammation in ferrets. Pain 99 : 61-69, 2002.

Cheng SB, Kuchiiwa S, Nagamoto I, Akasaki Y, Uchida M, Tominaga M, Hashiguchi W, Kuchiiwa T and Nakagawa S.2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin treatment induces c-Fos expression in the forebrain of the Long-Evans rat. Brain Res 931 : 176-180, 2002.

Coimbra F and Coimbra A. Dental noxious input reaches the subnucleus caudalis of the trigeminal complex in the rat, as shown by c-fos expression upon thermal or mechanical stimulation. Neurosci Lett 173 ; 201-204, 1994.

Hunt SP, Pini A and Evan G. Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. Nature 318 : 631-634, 1987.

Imbe H, Dubner R and Ren K. Masseteric inflammation-induced Fos protein expression in the trigeminal interpolaris/caudalis transition zone : contribution of somatosensory-vagal-adrenal integration. Brain Res 84 : 165-175, 1999.

Ito Y, Izumi H, Sato M, Karita K and Iwatsuki N. Suppression of parasympathetic reflex vasodilatation in the lower lip of the cat by isoflurane, propofol, ketamine and pentobarbital : implications for mechanisms underlying the production of anaesthesia. Br J Anaesth 81 : 563-568, 1998.

Izumi H. Functional roles played by the sympathetic supply to lip blood vessels in the cat. Am J Physiol 277 : R682-R689, 1999.

Izumi H, Date H, Mizuta K, Nakamura I and Kuchiiwa S. Reduction in parasympathetic reflex vasodilatation following stereotaxic earbar insertion : importance of reduced afferent input. Brain Res 961 : 53-62, 2003.

Izumi H and Ito Y. Sympathetic attenuation of parasympathetic vasodilatation in oro-facial areas in the cat. J Physiol 510 : 915-921, 1998.

Izumi H, Ito Y, Sato M, Karita K and Iwatsuki N. Effects of inhalation anesthetics on parasympathetic reflex vasodilation in the lower lip and palate of the cat. Am J Physiol 273 : R168-R174, 1997.

Izumi H and Karita K. Parasympathetic-mediated reflex salivation and vasodilatation in the cat submandibular gland. Am J Physiol 267 : R 747-R753, 1994.

Izumi H and Karita K. Low-frequency subthreshold sympathetic stimulation augments maximal reflex parasympathetic salivary secretion in cats. Am J Physiol 268 : R1188-R1195, 1995.

Izumi H and Karita K. Salivary secretion in cat submandibular gland mediated by chorda tympani afferents. Am J Physiol 268 : R438-R 444, 1995.

Izumi H, Mizuta K and Kuchiiwa S. Simultaneous measurement of parasympathetic reflex vasodilator and arterial blood pressure responses in the cat. Brain Res 952 ; 61-70, 2002.

Izumi H and Nakamura I. Nifedipine-induced inhibition of parasympathetic-mediated vasodilation in the orofacial areas of the cat. Am J Physiol 279 : R331-R339, 2000.

Koeda S, Yasuda M and Izumi H. Species differences in the reflex effects of lingual afferent nerve stimulation on lip blood flow and arterial pressure. J Comp Physiol [B] 173 : 629-636, 2003.

Kuchiiwa S, Izumi H, Karita K and Nakagawa S. Origins of parasympathetic postganglionic vasodilator fibers supplying the lips and gingivae ; an WGA-HRP study in the cat. Neurosci Lett 142 : 237-240, 1992.

Lu J and Bereiter DA. Acute injection of adrenal steroids reduces cornea-evoked expression of c-fos within the spinal trigeminal nucleus of adrenalectomized rats. Neuroscience 66 : 933-941, 1995.

Lu J, Hathaway CB and Bereiter DA. Adrenalectomy enhances Fos-like immunoreactivity within the spinal trigeminal nucleus induced by noxious thermal stimulation of the cornea. Neuroscience 54 : 809-818, 1993.

Menetrey D and Basbaum AI. Spinal and trigeminal projections to the nucleus of the solitary tract : a possible substrate for somatovisceral and viscerovisceral reflex activation. J Comp Neurol 255 : 439-450, 1987.

Meng ID and Bereiter DA. Differential distribution of Fos-like immunoreactivity in the spinal trigeminal nucleus after noxious and innocuous thermal and chemical stimulation of rat cornea. Neuroscience 72 : 243-154, 1996.

Mizuta K and Izumi H. Bulbar pathway for contralateral lingual nerve-evoked reflex vasodilatation in cat palate. Brain Res 1020 : 86-94, 2004.

Mizuta K, Karita K and Izumi H. Parasympathetic reflex vasodilatation in rat submandibular gland. Am J Physiol 279 : R677-R683, 2000.

Mizuta K, Kuchiiwa S, Saito T, Mayanagi H, Karita K and Izumi H. Involvement of trigeminal spinal nucleus in parasympathetic reflex vasodilatation in cat lower lip. Am J Physiol 282 : R492-R500, 2002.

Morgan JI and Curran T. Stimulus-transcription coupling in the nervous system : involvement of the inducible proto-oncogenes fos and jun. Annu Rev Neurosci 14 : 421-451, 1991.

Munglani R and Hunt SP. Molecular biology of pain. Br J Anaesth 75 : 186-192, 1995.

Natarajan M and Morrison SF. Adrenal epinephrine secretion is not regulated by sympathoinhibitory neurons in the caudal ventrolateral

medulla. Brain Res 827 : 169–175, 1999.

Oakden EL and Boissonade FM. Fos expression in the ferret trigeminal nuclear complex following tooth pulp stimulation. Neuroscience 84 : 1197–1208, 1998.

Paxinos G and Watson C. The Rat Brain. Academic Press, California, 1998.

Ro JY, Harriott A, Crouse U and Capra NF. Innocuous jaw movements increase c–fos expression in trigeminal sensory nuclei produced by masseter muscle inflammation. Pain 104 : 539–548, 2003.

Rocha MJ and Herbert H. Effects of anesthetics on Fos protein expression in autonomic brain nuclei related to cardiovascular regulation. Neuropharmacology 36 : 1779–1781, 1997.

Sato A, Izumi H, Nakamura I and Karita K. Differences in parasympathetic vasodilator and salivary responses in the cat submandibular gland between lingual and chorda–lingual nerve stimulation. J Dent Res 80 : 484–489, 2001.

Strassman AM and 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 331 : 495–516, 1993.

Suwanprathes P, Ngu M, Ing A, Hunt G and Seow F. c–Fos immunoreactivity in the brain after esophageal acid stimulation. Am J Med 115 Suppl 3A : 31S–38S, 2003.

Yoshida A, Dostrovsky JO, Sessle BJ and Chiang CY. Trigeminal projections to the nucleus submedius of the thalamus in the rat. J Comp Neurol 307 : 609–625, 1991.