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Expression of Fos protein in brainstem after application of L-menthol to the rat nasal mucosa

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Abstract

There are two functional pathways for the nasotrigeminal reflex: the spinal nucleus of trigeminal nerve (SPV) to the Kölliker-Fuse (KF) nucleus and the nucleus of solitary tract (NTS) to the lateral parabrachial nucleus (PBl). Although stimulation of the nasal mucosa by cool temperature induces respiratory depression, it is still unknown whether these nuclei are activated. In the present study, we examined the expression of Fos protein in rat brainstem neurons after nasal application of L-menthol, which is known to activate cold-sensitive nasal receptors. Application of L-menthol, but not paraffin oil, decreased the respiratory rate from 99.7 ± 15.6 to 78.5 ± 7.3 min^{-1}. Furthermore, a significantly higher density of Fos-immunoreactive cells was observed in the SPV and KF in the L-menthol rats than in the controls. In the SPV, the density of Fos-immunoreactive cells was highest at approximately 0.5 mm rostral to the obex in both the L-menthol (48.5 ± 11.5 cells/section) and paraffin oil (26.0 ± 9.6 cells/section) groups. In the KF, the mean density of Fos-immunoreactive cells was highest at approximately 5.0 mm rostral to the obex in both groups (L-menthol: 67.8 ± 14.0 cells/section, control: 41.0 ± 12.7 cells/section). The present study suggests that the SPV-KF pathway is important for the cold-induced respiratory depression.

Keywords: Nasotrigeminal reflex; Sensory nerve; Fos; Respiratory modulation

The nasal mucosa contains various sensory receptors for cardiorespiratory reflexes, such as drive receptors for nasal muscle activation, baroreceptors for pressure changes within the nasal cavity, thermoreceptors for temperature within the nasal cavity and chemoreceptors for chemical irritants [23]. Of these receptors, the nasal thermoreceptors are mainly activated by cool temperature (15 °C), and also by L-menthol [18,22,29,33]. It has been reported L-menthol or cold stimulation of the nasal mucosa induces respiratory depression similar to reflexes induced by other nasal stimuli, e.g., ammonia [32], nicotine and capsaicin [24], carbon dioxide [1] and air [30]. The neuronal circuitry of respiratory depression evoked by mustard oil [3], carbon dioxide [3], water [6,13] and air [32] to the nasal mucosa has been studied by Fos protein immunohistochemistry. Fos is an immediate early gene encoding transcription factor and can participate in long-term alteration of cellular function [4,13]. The spinal nucleus of the trigeminal nerve (SPV), the Kölliker-Fuse (KF) nucleus, the nucleus of solitary tract (NTS) and the lateral parabrachial nucleus (PBl) are the important nuclei activated by nasal stimulation [3,7,11,13,31]. The pathway activated by nasal sensory receptors runs through the SPV via the ethmoidal nerve (EN5), and the pathway activated by baroreceptor and chemoreceptor afferents run through the NTS to PBl [7]. Tracer studies have demonstrated that the KF receives input from the caudal portion of SPV (SPVc) afferents, and the PBl is projected from the medial region of the NTS afferents [9,10,19,20,26]. The nasotrigeminal afferents terminate in the SPV and NTS [2,12,14,18]. Thus, two functional pathways are suggested for the nasotrigeminal afferents; the SPV to KF and the NTS to PBl. The KF is a portion of parabrachial (PB) complex, which forms the pneumotaxic center [6,8,27]. The KF represents the sensory–autonomic interface relaying trigeminal input from the

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nasal mucosa to respiratory cell groups in the medulla or spinal cord [5]. The PBl is also a portion of the PB complex, and is an important cardiovascular and visceral relay station [7]. However, the afferent pathway of L-menthol stimulation of nasal mucosa has not yet been established.

In the present study, we examined the expression of Fos protein in the rat brainstem neurons after nasal application of L-menthol that activated cold-sensitive receptors to determine the neural circuitry for nasal cold stimulation.

Fourteen male Wistar rats (8-week-old, 160–170 g) were used in the present study. The animals were anesthetized with urethane (1.5 g/kg; intraperitoneal injection) and divided into two experimental groups according to the applied stimulation procedure: (1) L-menthol group ($n = 6$), 10 μl of 10 mM L-menthol in paraffin oil was injected into the right nasal cavity via thin polyethylene cannula (0.2 mm in diameter); (2) paraffin oil group ($n = 6$). To examine the non-specific effects of the injection procedure, 10 μl of pure paraffin oil was injected into the right nasal cavity. To examine the background Fos immunoreactivity and the effects of anesthesia, the animals those did not undergo any treatment was also used ($n = 2$). The microinjector (IM-9B Microinjector, Narishige Co., Tokyo) was used for the application of liquid chemicals.

Changes in respiration rate and electrocardiogram (E.C.G.) were monitored in the L-menthol and paraffin oil groups. Respiration rate was detected by dynamic air-pressure sensor (MI Lab Co., Japan) [16,28]. The dynamic air-pressure sensor was used to convert body weight shifts from respiratory movements to pressure fluctuations. It includes a pad containing air. The pad is placed under the rats' bodies. Acoustic signals received by the pad are converted to electric signals. Detected signals were recorded and analyzed with BIMUTUS II (Kissei Com Co., Japan). E.C.G. was recorded by use of a bioelectric amplifier (1253A in Polygraph System 360, NEC-Sanei Co., Japan).

At 1 h after the stimulation procedures, the rats were perfused transcardially with Ringer's solution (200 ml) followed by Zamboni's fixative (4% paraformaldehyde, 0.5% picric acid in 0.1 M phosphate buffer; pH 7.4). The brains were removed and immersed in the same fixative overnight at 4 °C. Then the brains were rinsed (3× 10 min) in phosphate-buffered saline (PBS; pH 7.4) and soaked in 30% sucrose in PBS and frozen. Serial transverse sections (50 μm) of the brainstem were cut on the cryostat and collected in PBS. The sections were divided into two series. One series of sections was processed for Nissl stain while the other was used for Fos immunohistochemistry. For immunohistochemistry, free-floating sections were first rinsed in 0.5% Triton X-100 in PBS overnight at 4 °C. In order to block non-specific peroxidase activity, the sections were incubated in PBS containing 0.3% H_2O_2 for 30 min at room temperature, and then rinsed in PBS (3× 10 min). To prevent non-specific binding sites, sections were incubated for 1 h in non-immune donkey serum (1:50) and rinsed in PBS (3× 10 min). The sections were then incubated for 2 days at 4 °C with rabbit polyclonal antiserum against Fos (Ab-5, 1:50000, Oncogene Research Products, Cambridge, MA). After incubation, the sections were rinsed in PBS (3× 10 min) and incubated for 1 h in biotinylated anti-rabbit IgG donkey serum (Jackson ImmunoResearch Products, West Grove, PA) and washed in PBS (3× 10 min). The sections were then incubated in an avidin–biotin–peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) for 1 h. After washing with PBS (3× 10 min), sections were incubated in 0.02% 3,3′-diaminobenzidine tetrahydrochloride in Tris–HCl buffer solution (pH 7.4) in the presence of 0.006% H_2O_2 for 15–30 min at room temperature. Finally, after two washes in PBS, sections were mounted on gelatin-coated slides, air-dried, dehydrated in alcohols, cleared in xylene, and coverslipped. The brainstem sections were observed with a light microscope. We focused on respiration-related nuclei; the SPV (from 0.75 mm rostral to the obex to 0.25 mm caudal to the obex), KF (from 5.0 mm rostral to the obex to 5.5 mm rostral to the obex), NTS (from 0.75 mm rostral to the obex to 0.25 mm caudal to the obex) and PBl (from 5.0 mm rostral to the obex to 5.5 mm rostral to the obex). The numbers of positive neurons in the SPV, KF, NTS and PBl were counted in each section. Statistical analyses were performed by t-test with $P < 0.05$ considered statistically significant.

Distinct respiratory depression was observed in the L-menthol group (Fig. 1). After nasal application of L-menthol, respiratory rate decreased from 99.7 ± 15.6 to 78.5 ± 7.3 min^{-1} with increased heart rate from 270 to 284 BPM (Fig. 1). On the other hand, application of paraffin oil resulted in only a slight decrease of respiratory rate from 97.0 ± 8.0 to 91.8 ± 6.9 min^{-1} with increased heart rate from 268 to 276 BPM (Fig. 1). The E.C.G. was not different between L-menthol rats and paraffin oil rats.

Immunohistochemically, Fos-immunoreactive cells were observed in the brainstem including SPV, NTS, KF and PBl. In the SPV and KF, significant differences were observed in the

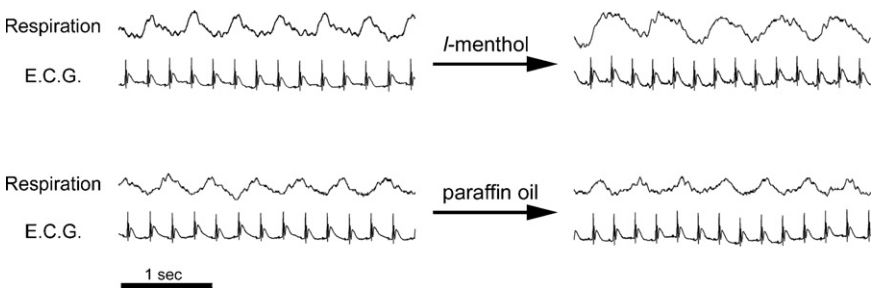

Fig. 1. Changes in respiratory rate and electrocardiogram (E.C.G.) after application of L-menthol or paraffin oil to the right nasal cavity. Application of L-menthol decreased the respiratory rate from 99.7 ± 15.6 to 78.5 ± 7.3 min^{-1} and increased heart rate from 270 to 284 BPM.

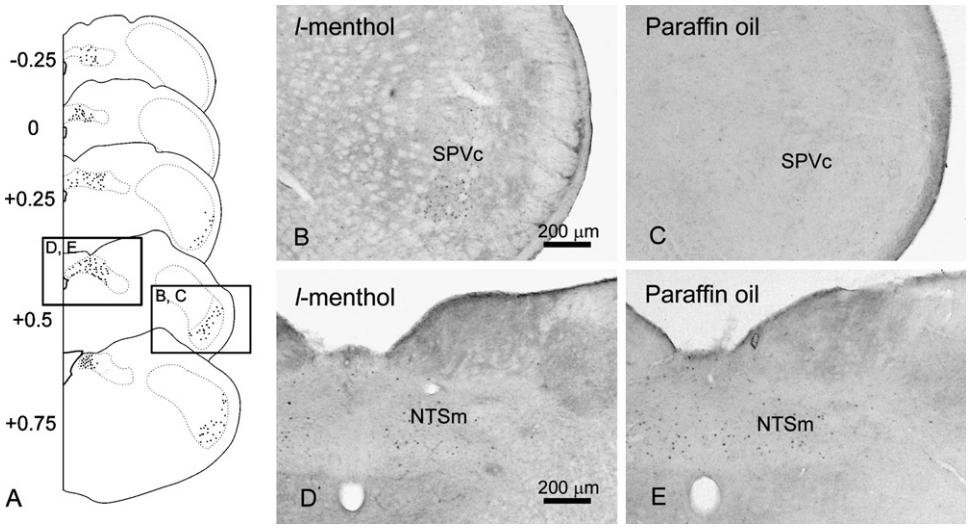

Fig. 2. (A) Schematic illustration showing the distribution of Fos-immunoreactive cells in the medulla of the L-menthol group. Numbers indicate distance in millimeters rostral (−) and caudal (+) to the obex. Photomicrographs showing Fos-immunoreactive cells in the SPVc (B and C) and NTSm (D and E) at approximately 0.5 mm rostral to the obex. Fos-immunoreactive cells were mainly observed at approximately 0.25–0.75 mm rostral to the obex in the SPV, and at approximately 0.75 mm rostral to 0.25 mm caudal to the obex in the NTS. In the SPV, a higher density of Fos-immunoreactive cells was observed in the L-menthol group than in the paraffin oil group. SPV, spinal nucleus of trigeminal nerve; SPVc, caudal part of SPV; NTS, nucleus of solitary tract; NTSm, medial part of NTS.

density of Fos-immunoreactive cells between L-menthol group and paraffin oil group (Figs. 2–4). In the non-treatment rats, Fos-immunoreactive cells were hardly observed in the SPV, NTS, KF and PBl.

In the SPV, Fos-immunoreactive cells were mainly observed in the caudal portion. A significantly higher density of Fos-immunoreactive cells was noted in sections at approximately 0.75–0.25 mm rostral from obex in the L-menthol group than in the paraffin oil group (Figs. 2B, C and 4). In the L-menthol group, the density of Fos-immunoreactive cells was highest (48.5 ± 11.5 cells/section) at approximately 0.5 mm rostral to the obex. In the paraffin oil group, the density of Fos-immunoreactive cells was also highest (26.0 ± 9.6 cells/section) at approximately 0.5 mm rostral to the obex.

In the NTS, Fos-immunoreactive cells were mainly observed in the medial part (NTSm). There was no significant difference in the density of Fos-immunoreactive cells between L-menthol group and paraffin oil group (Figs. 2D, E and 4B). In the L-menthol group, the density of Fos-immunoreactive cells was highest (48.8 ± 8.8 cells/section) at approximately 0.5 mm rostral to the obex. In the paraffin oil group, the density of Fos-immunoreactive cells was also highest (51.8 ± 14.2 cells/section) at approximately 0.5 mm rostral to the obex.

In the KF, Fos-immunoreactive cells were mainly observed in the caudal part. They were observed at approximately 5.0 mm rostral to the obex, and their density was significantly larger in the L-menthol group than in the paraffin oil group (Figs. 3B, C and 4C). The mean density of Fos-immunoreactive cells was 67.8 ± 14.0 cells/section in the L-menthol group and 41.0 ± 12.7 cells/section in the paraffin oil group.

In the PBl, Fos-immunoreactive cells were mainly observed at approximately 5.0 mm rostral to the obex. There was no significant difference in the density of Fos-immunoreactive cells between L-menthol group and paraffin oil group (Figs. 3B, C and 4D). The mean density of Fos-immunoreactive cells was 67.2 ± 24.0 cells/section in the L-menthol group and 61.3 ± 21.1 cells/section in the paraffin oil group.

In the present study, nasal application of L-menthol, but not paraffin oil, resulted in respiratory depression. The sensory

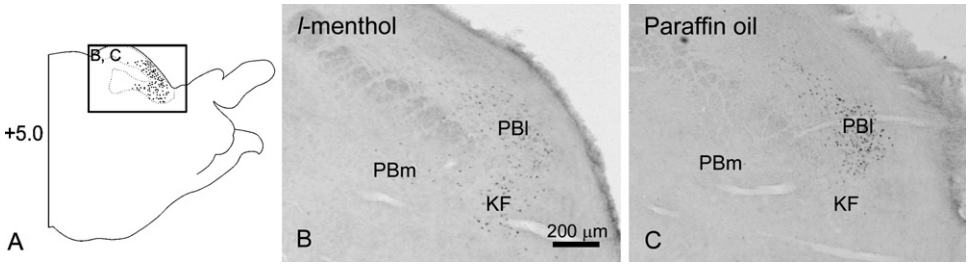

Fig. 3. (A) Schematic illustration showing the distribution of Fos-immunoreactive cells in the pons of the L-menthol group. Photomicrographs showing Fos-immunoreactive cells in the KF and PBl at approximately 5.0 mm rostral to the obex in the L-menthol group (B) and paraffin oil group (C). Fos-immunoreactive cells were mainly observed at approximately 5.0 mm rostral to the obex in the KF and PBl. In the KF, a higher density of Fos-immunoreactive cells was observed in the L-menthol group than in the paraffin oil group. KF, Kölliker-Fuse nucleus; PBl, lateral parabrachial nucleus; PBm, medial part of parabrachial nucleus.

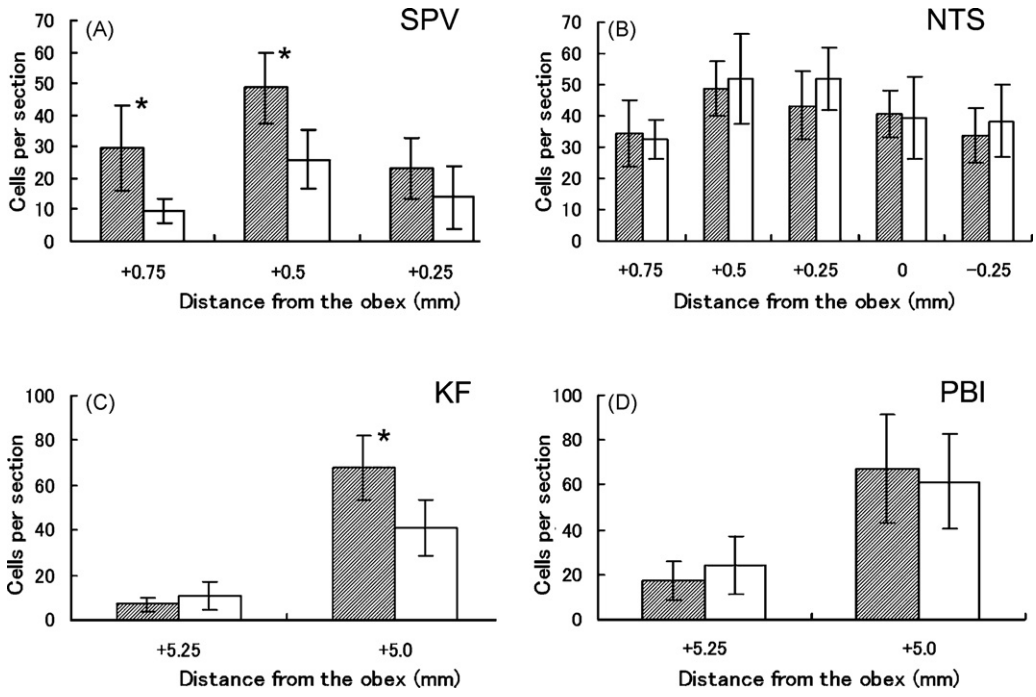

Fig. 4. Mean density of Fos-immunoreactive cells/section in the SPV (A), NTS (B), KF (C) and PBl (D). Numbers below each bar indicate the distance in millimeters rostral (−) and caudal (+) to the obex. Data are mean ± S.D. *$P < 0.05$, between L-menthol group (closed bars) and paraffin oil group (open bars) in the SPV and the KF. SPV, spinal nucleus of trigeminal nerve. SPVc, caudal part of SPV; NTS, nucleus of solitary tract; NTSm, medial part of NTS; KF, Kölliker-Fuse nucleus; PBl, lateral parabrachial nucleus.

molecule for L-menthol receptor is thought to be TRPM8, a member of the TRP family, which is sensitive to low temperature (8–28 °C) and L-menthol [15,21,30]. Nasal cold receptors that are stimulated by cool air or water can be stimulated by L-menthol [17,22,29,33]. Many physiological studies have demonstrated that nasal cold receptors evoke respiratory depression [17,22,29,33]. Thus, it seems that L-menthol stimulated nasal cold receptors in the present study.

Few Fos-immunoreactive cells were observed in non-treatment rats. This result indicates that there is no influence on respiratory depression by anesthesia. Therefore, respiratory-related nuclei (SPV, NTS, KF and PBl) were activated by L-menthol and paraffin oil in the present study.

A previous study demonstrated that nasotrigeminal afferents run through the ethmoidal nerve, a branch of the ophthalmic division of the trigeminal nerve [12]. In the nasotrigeminal reflex, electrophysiological study has demonstrated that nasotrigeminal afferent pathways are conveyed to the central nervous system via EN5 [24]. In the SPV, the intense Fos-immunoreactivity was mainly observed in its caudal part because EN5 afferents terminate in this part [3,18]. These tracer studies and the present results suggest that the SPV receives primary afferent input from L-menthol stimulation of the nasal mucosa. The KF is a component of the parabrachial complex, which forms the pneumotaxic center [6,8,27]. Although Fos expression in the KF is reported to be mainly in its medial and caudal parts as determined by Fos immunohistochemistry in other nasal stimulation studies [7,13,31], Fos-immunoreactive cells were observed in the caudal part of the KF in the present L-menthol study. Therefore, the caudal part of the KF may play an important role in the respiratory depression evoked by nasal cold receptors. Since the KF mediates respiratory regulation circuit from the SPVc [6], the present results suggest that the KF receives secondary afferent inputs from the nasal cold receptors. In addition, tracer studies demonstrated a prominent projection from the SPVc to the KF [5,9,19,26]. In conclusion, the SPV-KF pathway may be the afferent pathway for respiratory depression by low temperature within the nasal cavity.

Dutschmann and Herbert (1997) suggested that nasal cavity pressure stimulation runs through the NTS to the PBl. In the present study, intense Fos expression was observed in the medial part of the NTS and the PBl in both L-menthol group and paraffin oil group. Since a similar respiratory reflex is evoked by stimulation of nasal mechanoreceptors [25], Fos-immunoreactive cells observed in these nuclei in the present study reflect from the slight respiratory depression induced by stimulation of the nasal mechanoreceptors by nasal application of paraffin oil. Thus, the NTS-PBl nasotrigeminal afferent pathway may play an important role in paraffin oil stimulation.

In conclusion, the present study demonstrated that the SPV and KF were activated by L-menthol injection to the nasal cavity. Because L-menthol and cold air would stimulate same receptor molecules, it suggested that the SPV-KF pathway is important for cold-induced respiratory depression.

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