Histol Histopathol (2000) 15: 683-688

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Cellular and Molecular Biology

Changes in the immunoreactivity of substance P and calcitonin gene-related peptide in the laryngeal taste buds of chronically hypoxic rats

T. Kusakabe[1], T. Yoshida[2], H. Matsuda[2], Y. Yamamoto[3], Y. Hayashida[4], T. Kawakami[5] and T. Takenaka[1]

[1]Laboratory for Anatomy and Physiology, Department of Sport and Medical Science, Kokushikan University, Tokyo,

[2]Department of Otorhinolaryngology, Yokohama City University School of Medicine, Kanazawa-ku, Yokohama,

[3]Laboratory of Veterinary Anatomy, Department of Veterinary Science. Faculty of Agriculture, Gifu University, Gifu,

[4]Department of Systems Physiology, University of Occupational and Environmental Health, Kitakyushu,

[5]Department of Physiology, Kitasato University School of Medicine, Sagamihara, Japan

Summary. The distribution of substance P (SP)- and calcitonin gene-related peptide (CGRP)-immunoreactive nerve fibers in the taste buds of the epiglottis and aryepiglottic folds was compared between normoxic control and chronically isocapnic hypoxic rats (10% O_2 and 3-4% CO_2 for 3 months). In the normoxic laryngeal taste buds, SP- and CGRP-imrnunoreactive fibers were detected within the taste buds, where they appeared as thin processes with many varicosities. Most CGRP fibers showed coexistence with SP, but a few fibers showed the immunoreactivity of CGRP only. The density of intra- and subgemmal SP and CGRP fibers penetrating into the laryngeal taste buds was significantly higher in chronically hypoxic rats than in normoxic control rats. Water intake in the hypoxic rats was significantly lower than in the normoxic rats. These results indicate that the increased density of SP- and CGRP-containing nerve fibers within the laryngeal taste buds is a predominant feature of hypoxic adaptation. The altered peptidergic innervation and reduced water intake support the hypothesis that the laryngeal taste buds are involved in water reception, and that the water reception may be under the control of peptidergic innervation.

Key words: Chronic hypoxia, Laryngeal taste bud, Substance P, Calcitonin gene-related peptide, Immunohistochemistry

Introduction

Since the first description of extralingual taste buds by Verson (1868), these structures have been found in the hard and soft palate, buccal wall, pharynx, and larynx of a variety of mammals including humans (Lalonde and Eglitis, 1961; Kadanoff, 1969; Miller, 1977; Bradley et al., 1980; Palmieri et al., 1983; Stedman et al., 1983; Miller and Smith, 1984; Nakano and Muto, 1987; Yamamoto et al., 1997), and they account for a significant proportion, approximately 11% and 27% of the total rodent and human taste bud population, respectively (Travers and Nicklas, 1990). The lingual taste buds are generally considered to be specialized sensory receptors for detecting chemical substances (Miller, 1977), but the physiological role of the extralingual taste buds has remained controversial.

Recently, the distribution of two regulatory neuropeptides, substance P (SP) and calcitonin gene-related peptide (CGRP) immunoreactive fibers have been reported in the cat laryngeal taste buds (Shin et al., 1995). The possible origin of SP and CGRP has been suggested to be as primary sensory neurons. In view of the fact that approximately one-third of extraoral taste buds are located in the distal areas of the upper airway, it is useful for elucidation of the possible function of the extraoral taste buds to examine the distribution and abundance of SP and CGRP fibers in laryngeal taste buds under varying atmospheric conditions.

In the present study, the density of SP and CGRP immunoreactive nerve fibers in the taste buds located in and around the epiglottis were compared between normoxic and chronically hypoxic rats, and a possible role for them is discussed.

Materials and methods

Chronically hypoxic exposure

Wistar rats were placed in an air-tight acrylic chamber, and were exposed to chronic hypoxia (10% O_2 in N_2 and 3-4% CO_2 for 3 months). The CO_2 was added to the hypoxic gas mixture at the concentration of 3-4% because this much was necessary to maintain an

Offprint requests to: Dr. Tatsumi Kusakabe, Laboratory for Anatomy and Physiology, Department of Sport and Medical Science, Kokushikan University, 7-3-1 Nagayama, Tama-shi, Tokyo 206-8515, Japan. Fax: 81-42-339-7238. e-mail: kusakabe@kokushikan.ac.jp

arterial partial pressure of CO_2 close to that measured during the control period. This hypoxic exposure has been detailed in previous papers (Kusakabe et al., 1998; Matsuda et al., 1998), and the hypoxic condition has been confirmed to be isocapnic to rats in a previous study (Hayashida et al., 1996). Six rats were kept chronically in this chamber for 3 months with food and water available *ad libitum*. Six control rats were housed for 3 months in the same chamber ventilated by air at the same flow rate. The chamber was opened for 10 min every 3 days for animal husbandry.

The weight of the animals, and water and food intake were monitored for 3 weeks, from 7 to 10 weeks after the beginning of the hypoxic exposure, and the numerical values were expressed as the means for one week.

Tissue preparation

The animals were intraperitoneally anesthetized with sodium pentobarbital (0.05 mg/g), and perfused through a thin nylon tube inserted into the ventricle with 0.1M heparinized phosphate buffer saline (PBS), followed by freshly prepared Zamboni's fixative solution (0.2% picric acid and 4% paraformaldehyde in 0.1M PBS). The larynx was then removed, and immersed in the same fixative for an additional 8 h at 4 °C. After washing in PBS, the specimens were transferred to 30% sucrose in PBS at 4 °C for 24 h. The specimens including the epiglottis were cut serially at 15 μm on a cryostat, and mounted in two series on poly-L-lysine-coated slides.

All experiments with animals were performed in accord with "Principles of laboratory animal care" (NIH publ. no. 86-23, revised 1985).

Immunohistochemistry

The sections were processed for immunohistochemistry according to the peroxidase-antiperoxidase (PAP) method. They were incubated at 4 °C overnight with the primary antisera against SP and CGRP (1:1500; Cambridge Research Biochemicals, Northwich, UK). After rinsing in several changes of PBS, the sections were transferred for 2 h to anti-rabbit IgG (1:200; Organo Technica, Durham, USA). Next the sections were rinsed with several changes of PBS, and transferred for 2 h to rabbit PAP (1:200; Jackson Immuno Research, West Grove, USA). This immunostaining procedure has been detailed in a previous report (Kusakabe et al., 1991).

The sections of the chronically hypoxic epiglottis were also processed by an indirect double-labelling immunofluorescence method to examine whether SP and CGRP coexist in the nerve fibers within the taste buds, or not. The sections were first incubated overnight at 4 °C in guinea-pig antiserum against SP (1:500; Euro-Diagnostica, Sweden). Then they were incubated under the same conditions in rabbit antiserum against CGRP (1:1500; Cambridge Research Biochemicals, UK). After washing in PBS, the sections were incubated in a mixture of secondary antisera, rhodamine-conjugated goat anti-guinea-pig IgG (1:200; Organo Technica, USA), and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:200; Organo Technica, USA) for 2h at room temperature. The double immunostained sections were examined with a Zeiss Axiomat microscope equipped with a dual band filter set (Chroma Technol., USA) for simultaneous viewing of both red and green fluorescent dyes. This double immunostaining procedure has been detailed in previous reports (Kusakabe et al. 1996a,b). From the same area where immunofluorescence was observed, a differential interference-contrast (Nomarski) image was also taken with the same microscope.

Control

The reaction for neuropeptides was verified by treating sections with primary antibody which had been inactivated by overnight incubation with 50-100 μM of its peptide. To test the specificity of the antisera, the sections treated with SP antiserum were incubated with FITC-conjugated anti-rabbit IgG, and those treated with CGRP antisera were reacted with rhodamine-conjugated anti-guinea-pig IgG.

Data analysis

The density of immunoreactive fibers within the epiglottal taste buds of normoxic and chronically hypoxic rats was represented as the number of varicosities. In sections through the center of the taste bud, the number of varicosities was counted with an ARGUS 100 computer and image processor (Hamamatsu-Photonics, Japan) on 50 taste buds taken from six normoxic and six chronically hypoxic epiglottis. The value was expressed as mean±SD (n=50). Statistical comparisons between the control and experimental values were determined using Student's *t*-test.

Results

Figure 1 shows the changes in weight, and in water and food intake from the 7th to 10th week after the beginning of hypoxic exposure. Throughout the monitoring period, there were no significant changes in the water and food intake in either normoxic or hypoxic rats, although the animals' weight gradually increased. Water intake in the hypoxic rats was significantly lower than in the normoxic rats, but there was no significant change in food intake.

In the sections stained with hematoxylin and eosin, the taste buds of the control normoxic and chronically hypoxic rats were seen as pale corpuscles in the dark-stained stratified epithelium of the laryngeal surface of the epiglottis (Fig. 2), and the aryepiglottic folds. These taste buds, which were pierced by a luminal taste pore,

were not associated with papillae, although those in the dorsal lingua were generally associated. In the hematoxylin and eosin stained sections, there were no obvious morphological differences between normoxic and chronically hypoxic laryngeal taste buds.

Immunoreactivity for SP and CGRP was recognized in intra- and subgemmal nerve fibers of both normoxic and chronically hypoxic rats, and the immunoreactivity was also found within and just beneath the laryngeal epithelium around the taste buds (Figs. 3-6). SP- and CGRP-immunoreactive nerve fibers appeared as thin processes with many varicosities showing punctate structures. Some intragemmal fibers, which penetrated into the taste buds, reached to a point near the taste pore (Figs. 3-6). The mean number of varicosities per section of taste bud in intragemmal SP and CGRP fibers was 8.77±1.77 (n=50) and 10.32±2.66 (n=50), respectively, and that in subgemmal SP and CGRP fibers was 12.20±2.65 (n=50) and 14.13±2.83 (n=50), respectively (Fig. 7).

In the chronically hypoxic laryngeal taste buds, especially in the basal area, the relative abundance of SP and CGRP fibers was greater than in the normoxic ones (Figs. 5, 6). The mean number of varicosities in the intragemmal SP and CGRP fibers was significantly increased from 8.77±1.77 to 12.67±4.10 ($p<0.01$, n=50), and from 10.32±2.66 to 15.19±4.18 ($p<0.05$, n=50), respectively. That of subgemmal SP and CGRP fibers was also significantly increased from 12.20±2.65 to 14.13±4.82 ($p<0.01$, n=50), and from 13.13±2.83 to 18.43±4.87 ($p<0.01$, n=50), respectively (Fig. 7).

When the double-labelled sections for SP and CGRP were examined with the multiple dye filter system, most SP immunoreactive fibers in the chronically hypoxic laryngeal taste buds (Fig. 8) showed yellow fluorescence, which indicates the coexistence of SP and CGRP. A small number of fibers in the taste buds showed only immunoreactivity of CGRP.

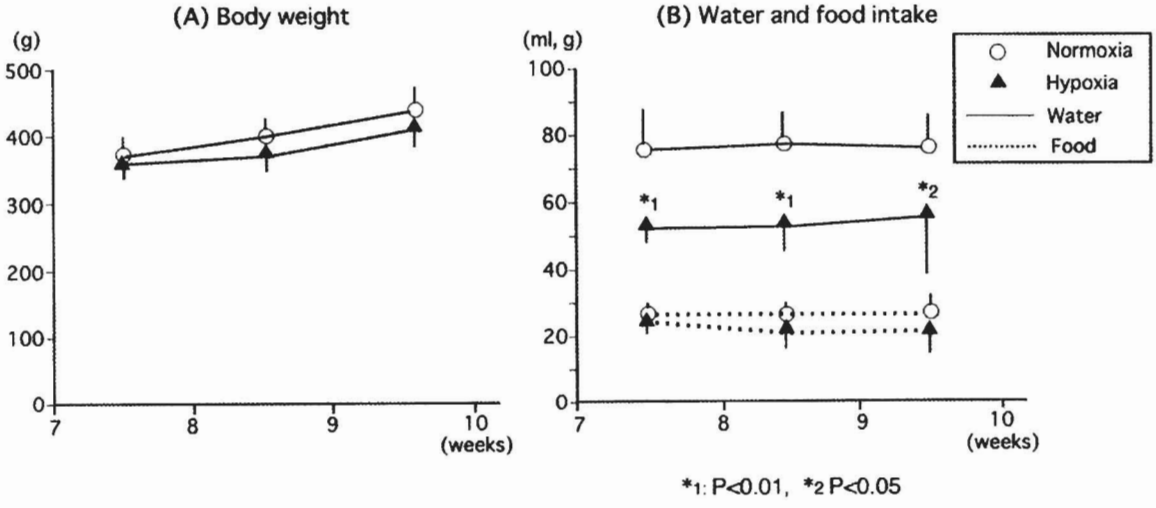
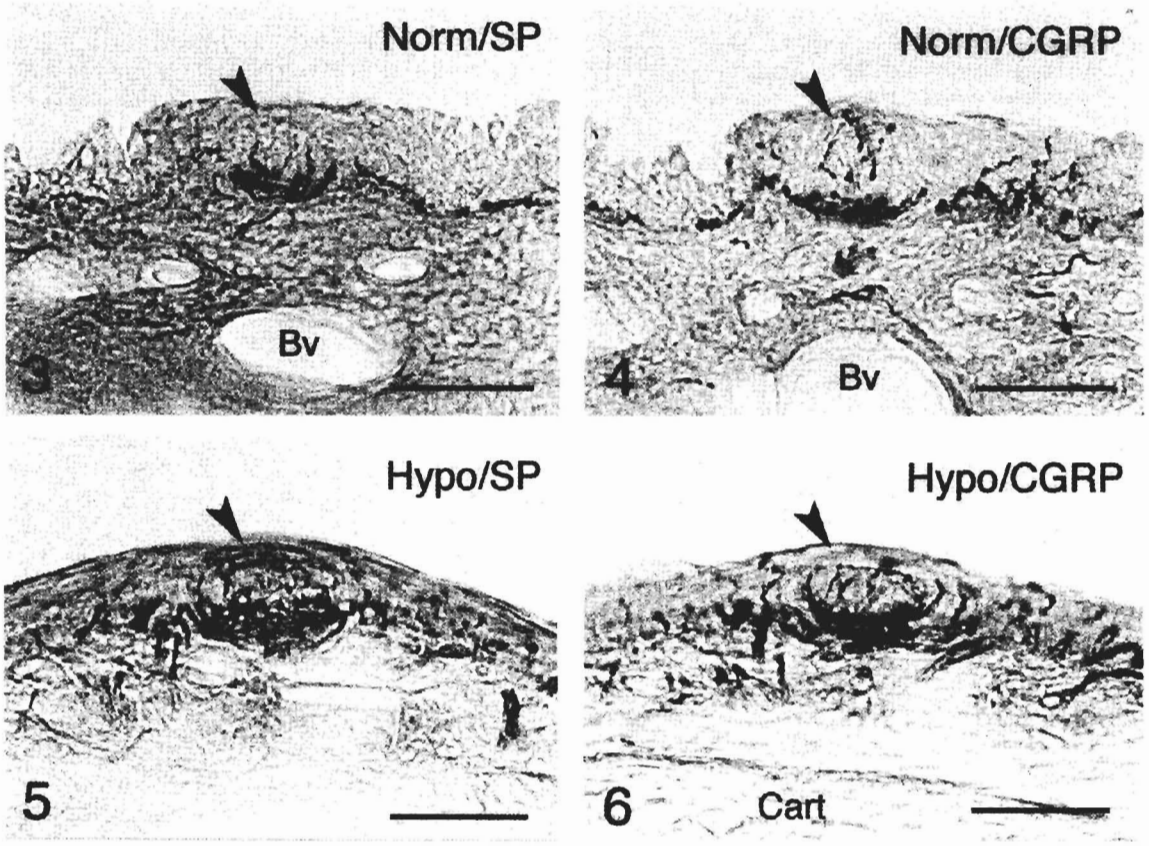


Fig. 1. Body weight **(A)**, and water and food intake **(B)** of the rats from the 7th to 10th week after the beginning of hypoxic exposure (triangle) and of the control rats (circle). The numerical values are weekly means±SD.
*: $p<0.01$;
**: $p<0.05$.

Fig. 2. Hematoxylin-eosin-stained section showing a taste bud (arrowhead) located in the laryngeal surface of the epiglottis. Bv: blood vessel; Cart: cartilage of the epiglottis. Bar: 50 μm.



Figs. 3-6. SP- and CGRP-immunoreactive nerve fibers in the taste buds (arrowhead) and in the laryngeal surface of the epithelium of the normoxic (Norm) **(Figs. 3 and 4)** and chronically hypoxic (Hypo) **(Figs. 5 and 6)** epiglottis. Note the remarkable increase in the density of SP and CGRP fibers in the chronically hypoxic laryngeal taste buds. Bv: blood vessel; Cart: cartilage of the epiglottis. Bar: 50 μm.

No nerve fibers with immunoreactivity for SP and CGRP were detected in the sections incubated in the preabsorbed antisera, and these two primary antisera did not show cross reactivity with the second antisera.

Discussion

The present study showed, for the first time, that the density of intra- and subgemmal SP and CGRP immunoreactive fibers within the laryngeal taste buds in chronically hypoxic rats was significantly higher (1.2-1.5 times) than in normoxic control rats. Thus, altered peptidergic innervation in the laryngeal taste buds could represent an important feature in acclimatization to the chronic hypoxia. Water intake during hypoxic exposure was significantly lower than in normoxic conditions. A similar significant decrease in water intake has been reported in rats receiving chemical stimulation of the laryngeal mucosa (Miyaoka et al., 1987). This suggests that the laryngeal taste buds may be involved in the water reception. Actually, it has been speculated that most information for water reception is generated in the laryngeal and pharyngeal regions (Harada and Smith, 1992; Hanamori and Ishiko, 1993). Storey and Johnson (1975) have suggested that water stimulation of laryngeal receptors can produce a significant central apnea or disruption of respiration. In the present study, however, it is not clear whether the reduced water intake

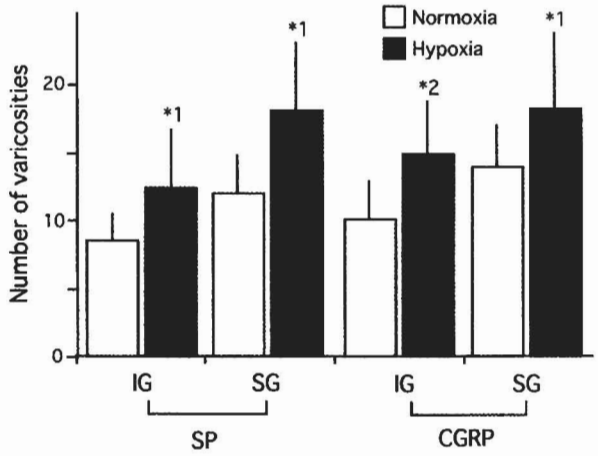


Fig. 7. Histograms comparing the absolute number of varicosities of intra (IG)- and subgemmal (SG) fibers per section in normoxic and chronically hypoxic laryngeal taste buds. IG: intragemmal fibers; SG: subgemmal fibers. *1: $p<0.01$; *2: $p<0.05$.

of hypoxic rats is due to an altered taste reception or is just an adaptation to the hypoxic condition. To clarify this, it is necessary to measure the urinary output of the animals and the vasopressin levels.

In addition, this study demonstrated the coexistence of CGRP in most SP-containing fibers within the

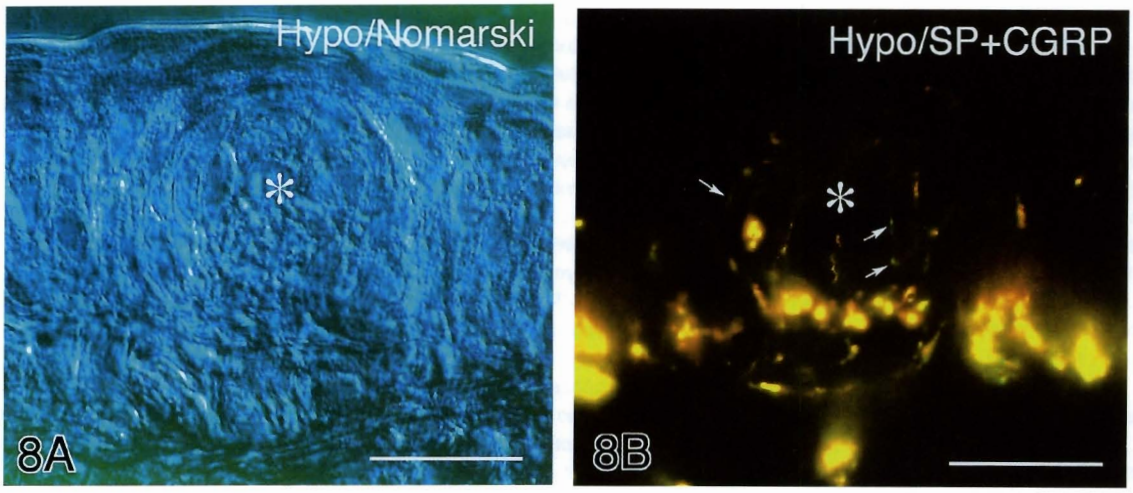


Fig. 8. A and B show the same area in the frozen section showing the hypoxic laryngeal taste bud (asterisk). **A.** Nomarski image. **B.** Yellow fluorescence showing coexistence of SP and CGRP in and around the laryngeal taste bud. Arrows indicate a small number of CGRP fibers without SP. Bar: 20 μm.

hypoxic laryngeal taste buds. We believe that co-release would synergistically effect water reception as a possible functions of the laryngeal taste buds. Considered together with these findings, the water reception may be one of the possible function of the laryngeal taste buds as previously suggested (Storey and Johnson, 1975; Miyaoka et al., 1987; Harada and Smith, 1992; Hanamori and Ishiko, 1993), and may be under the control of peptidergic innervation.

Several authors have also speculated that the laryngeal taste buds may take part in airway protection (Khaisman, 1976; Stedman et al., 1983; Travers and Nicklas, 1990; Shin et al., 1995). More recently, Yoshida et al. (1999) showed an increase in the relative abundance of SP and CGRP fibers within the chronically hypoxic rat laryngeal epithelium, and suggested that the increased intraepithelial fibers may be involved in airway defense mechanisms in the chronically hypoxic environment. According to these authors, there are two possible receptor sites for the stimuli, one being the laryngeal taste buds, and the other free endings within the laryngeal epithelium. The laryngeal mucosa is richly innervated by the superior laryngeal nerve which contains a number of sensory elements (Andrew, 1956; Andrew and Oliver, 1951), and the superior laryngeal nerve responds to water stimulation (Shingai, 1980). In the present study, SP and CGRP fibers were detected within the laryngeal epithelium as well as in the taste buds. Therefore, we have to consider whether chemical and mechanical stimuli are sensed by the laryngeal taste buds, or by free endings within the laryngeal epithelium around the taste buds. Because the density of SP and CGRP fibers in the hypoxic condition is increased both within the laryngeal taste buds and within the epithelium around the taste buds (Yoshida et al. 1999) is increased in the hypoxic condition, we cannot tell which is true. It is possible that both laryngeal taste buds and free endings are involved in the functions of the laryngeal taste buds. To clarify this, it is necessary to perform precise stimulation of the taste buds alone or of the laryngeal surfaces alone.

In conclusion, the increased density of SP- and CGRP-containing nerve fibers within the chronically hypoxic laryngeal taste buds is a predominant feature of hypoxic adaptation, and the altered peptidergic innervation and water intake support the hypothesis that the laryngeal taste buds are involved in water reception. This suggests that the regulation of water reception may be under the control of peptidergic innervation.

Acknowledgements. We wish to thank Ms. K. Haraguchi of the Department of Systems Physiology, University of Occupational and Environmental Health for hypoxic animal husbandry, and monitoring of water and food intake. We are also grateful to Dr. R.C. Goris of the Department of Anatomy, Yokohama City University School of Medicine, for his help in editing the manuscript.

References

Andrew B.L. (1956). A functional analysis of the myelinated fibers of the superior laryngeal nerve of the rat. J. Physiol. (Lond) 133, 420-432.

Andrew B.L. and Oliver J. (1951). The epiglottal taste buds of the rat. J. Physiol. (Lond) 114, 48P-49P.

Bradley R.M. and Cheal N.L. and Kim Y.H. (1980). Quantitative analysis of developing epiglottal taste buds in sheep. J. Anat. 130, 25-32.

Hanamori T. and Ishiko N. (1993) Cardiovascular responses to gustatory and mechanical stimulation of the nasopharynx in rats. Brain Res. 619, 214-222.

Harada S. and Smith D.V. (1992). Gustatory sensitivities of the hamster's soft palate. Chem. Senses 17, 37-51.

Hayashida Y., Hirakawa H., Nakamura T. and Maeda M. (1996). Chemoreceptors in autonomic responses to hypoxia in conscious rats. Adv. Exp. Med. Biol. 410, 439-442.

Kadanoff D. (1969). Uber die Endigungen der Nervenfasern in den Geschemacksknospen bei affen im Vergleich zum Menschen. Z. Mikrosk. Anat. Forsch. 81, 295-303.

Khaisman E.B. (1976). Particular features of the innervation of taste buds of the epiglottis in monkeys. Acta. Anat. 95, 101-115.

Kusakabe T., Anglade P. and Tsuji S. (1991). Localization of substance

P, CGRP, VIP, neuropeptide Y, and somatostatin immunoreactive nerve fibers in the carotid labyrinths of some amphibian species. Histochemistry 96, 255-260.

Kusakabe T., Kawakami T., Ono M. and Takenaka T. (1996a). Distribution of peptidergic nerve fibers in the bullfrog lingual papillae demonstrated by a combination of double immunofluorescence labelling and a multiple dye filter. Histochem. J. 28, 289-297.

Kusakabe T., Kawakami T., Ono M., Syoui N., Kurihara K. and Takenaka T. (1996b). Precise coexistence of regulatory peptides in the nerve fibers of the amphibian carotid labyrinth demonstrated by a combination of double immunofluorescence labelling and a multiple dye filter. Brain Res. 735, 307-310.

Kusakabe T., Hayashida Y., Matsuda H., Gono Y., Powell F.L., Ellisman M.H., Kawakami T. and Takenaka T. (1998). Hypoxic adaptation of the peptidergic innervation in the rat carotid body. Brain Res. 806, 165-174.

Lalonde E.R. and Eglitis J.A. (1961). Number and distribution of taste buds on the epiglottis, pharynx, larynx, soft palate and uvula in a human newborn. Anat. Rec. 140, 91-95.

Matsuda H., Kusakabe T., Hayashida Y., Furukawa M., Kawakami T., Takenaka T. and Tsukuda M. (1998). Substance P- and calcitonin gene-related peptide-containing nerve fibers in the nasal mucosa of chronically hypoxic rats. Brain Res. Bull. 45, 563-569.

Miller I.J. Jr. (1977). Gustatory receptors of the palate. In: Food Intake and Chemical Senses. Katsuki Y., Takagi S. and Oomura Y. (eds) University of Tokyo Press. Tokyo. pp 173-184.

Miller I.J. Jr. and Smith D.V. (1984). Quantitative taste bud distribution in the hamster. Physiol. Behav. 32, 275-285.

Miyaoka Y., Sakaguchi T., Yamazaki M. and Shingai T. (1987). Changes in water intake following pharyngolaryngeal deafferentation in the rat. Physiol. Behav. 40, 369-371.

Nakano T. and Muto H. (1987). Anatomical observations in the arytenoid region of the mouse with special reference to the function of the taste buds. Z. Mikrosk. Anat. Forsch. 101, 205-211.

Palmieri G., Asole A., Panu R., Sanna L. and Farina V. (1983). On the presence, structure and probable functional role of taste buds located on the laryngeal surface of the epiglottis in some domestic animals. Arch. Anat. Histol. Embryol. 66, 55-68.

Shin T., Nahm I., Maeyama T., Miyazaki J., Matsuo H. and Yu Y. (1995). Morphologic study of the laryngeal taste buds in the cat. Laryngoscope 195, 1315-1321.

Shingai T. (1980). Water fibers in the superior laryngeal nerve of the rat. Jpn. J. Physiol. 30, 305-307.

Storey A.T. and Johnson P. (1975). Laryngeal water receptors initiating apnea in the lamb. Exp. Neurol. 47, 42-55.

Stedman H.M., Bradley C.M., Mistretta C.M. and Bradley R.M. (1983). A quantitative study of cat epiglottal taste buds during development. J. Anat. 136, 821-827.

Travers S.T. and Nicklas K. (1990). Taste bud distribution in the rat pharynx and larynx. Anat. Rec. 227, 373-379.

Yamamoto Y., Atoji Y. and Suzuki Y. (1997). Innervation of taste buds in the canine larynx as revealed by immunohistochemistry for the various neurochemical markers. Tissue Cell 29, 339-346.

Yoshida T., Matsuda H., Hayashida Y., Gono Y., Nagahara T., Kawakami T., Takenaka T., Tsukuda M. and Kusakabe T. (1999). Changes in the distribution of substance P and calcitonin gene-related peptide immunoreactive nerve fibers in the laryngeal mucosa of chronically hypoxic rats. Histol. Histopathol. 14, 735-741.

Verson R. (1868). Beiträge zur Kenntnis des Kehlkopfes und der Trachea. Sbe. Akad. Wiss. Wien. 57, Maiheft.

Accepted January 19, 2000