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Distribution of calcitonin gene-related peptide nerve fibers in the canine larynx

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Summary. Immunohistochemistry was used to investigate the distribution pattern of calcitonin gene-related peptide (CGRP) nerve fibers in the laryngeal mucosa, glands and intrinsic muscles of the dog. CGRP immunoreactive nerve fibers were found more frequently than substance P immunoreative nerve fibers in every region of the larynx. In the epithelia, CGRP nerve fibers were mainly found in the epiglottis, arytenoid region and subglottis. Many taste buds were observed in the arytenoid region and were densely innervated by the CGRP nerve fibers. In the lamina propria, the plexus of CGRP nerve fibers was present, with some of these fibers associated with blood vessels. Laryngeal glands were also innervated by a few CGRP nerve fibers. In the intrinsic laryngeal muscles, abundant immunoreactivity was observed and many motor end-plate-like structures were found with CGRP immunoreactivity. These findings strongly suggest that CGRP plays an important role in all of the sensory, motor and autonomic nervous systems of the larynx.

Key words: Calcitonin gene-related peptide – Canine larynx – Immunohistochemistry

Introduction

The innervation of the larynx by several kinds of neuropeptides in addition to the classical neurotransmitters has been shown in previous reports such as ours which demonstrated the distribution of substance P (SP) nerve fibers in the canine larynx [6]. It is well known that calcitonin gene-related peptide (CGRP), one of these neuropeptides, is distributed throughout the central and peripheral nervous systems as a neurotransmitter or a neuromodulator [9, 18]. Although the existence of CGRP nerve fibers in the larynx has also been documented [9], the details concerning its laryngeal innervation are still lacking.

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In the larynx, sensorimotor innervation has an important function that coordinates the complex and delicate vocal cord movements and reflexes that influence the activity of laryngeal motoneurons [20]. Autonomic innervation is also important in regulating laryngeal blood flow and secretion [4, 5]. It is therefore important to investigate in detail and systematically the existence of CGRP in the larynx to help understand its fine sensory, motor and autonomic innervations, since CGRP is considered to exist in these three nervous systems in other organs [3, 17]. In the present study, we used immunohistochemistry to attempt to demonstrate the detailed distribution pattern of CGRP nerve fibers in the mucosa, glands and intrinsic muscles of the canine larynx.

Materials and methods

Five young dogs of both sexes, weighing 1–2.5 kg, were used in this experiment. Under deep anesthesia with i.p. pentobarbital (30 mg/kg), each animal was perfused through the left cardiac ventricle with 0.1 *M* phosphate-buffered saline (PBS) followed by ice-cold fixative containing 4% paraformaldehyde, 0.2% picric acid and 0.35% glutaraldehyde in 0.1 *M* phosphate buffer (PB). After perfusion fixation, the larynx was removed and postfixed with 4% paraformaldehyde and 0.2% picric acid in 0.1 *M* PB for 1 day. The extirpated larynx was next immersed in 0.1 *M* PBS containing 20% sucrose for 1 day. It was then sectioned at 20 μm on a freezing microtome. These sections were processed for immunohistochemistry. Free-floating sections were treated with 1% bovine serum for 1 h and incubated with anti-CGRP serum (Amersham, × 5000) for 4 days at 4°C. Sections were then incubated with biotinylated anti-rabbit IgG (× 1000) for 1 day and with avidin-biotin complex (× 1000) for 1 day. Sections were washed in 50 m*M* TRIS buffer. This was followed by incubation with 0.01% 3,3′ diamidinobenzidine and 0.3% nickel ammonium sulfate. The sections were next mounted on chrome-coated glass slides, air-dried and washed with tap water. Finally, tissues were dehydrated in a graded series of ethanol, cleaned in xylene and covered with Entellan (Merck, Darmstadt, FRG).

Results

CGRP immunoreactive nerve fibers were found in every region of the larynx. In the epithelia of the laryngeal mu-

cosa. CGRP nerve fibers were especially found in the epiglottis, posterior glottis and subglottis (Table 1). In the glottis, abundant CGRP nerve fibers were observed in the posterior glottic epithelium, although there were very few in the anterior glottis. Most of these fibers with varicosities reached the surface of the epithelium and appeared to be free-ended (Fig. 1A). In the epithelium of the laryngeal surface of the epiglottis and the posterior glottis, taste buds were observed and were densely innervated by CGRP nerve fibers (Fig. 1B). In the lamina propria, the plexus of CGRP nerve fibers was commonly found and some of the CGRP nerve fibers were clearly associated with blood vessels (Fig. 1C). No CGRP immunoreactive epithelial cells were found.

Many CGRP nerve fibers were found in the region of laryngeal glands (Fig. 1D). Some of these fibers appeared to terminate in glandular cells, while most fibers were observed to lie parallel to blood vessels.

Table 1. Distribution of calcitonin gene-related peptide (CGRP) terminals in the canine laryngeal epithelium

Region	Epiglottis	False cord	Glottis		Subglottis
			Anterior	Posterior	
CGRP	++	+	±	+++	++

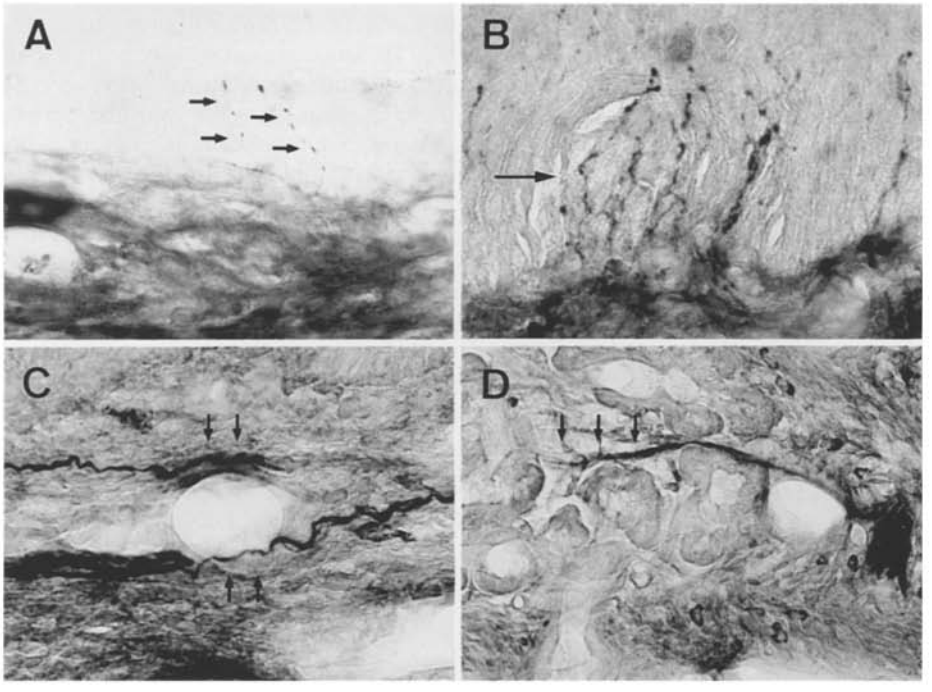

Fig. 1. A Subglottic epithelium: free-ended nerve fibers with varicosities reach the surface of the epithelium (→), × 440. **B** In the epithelium of the arytenoid region, many taste buds are observed and densely innervated by the calcitonin gene-related peptide (CGRP) nerve fibers (→), × 440. **C** In the lamina propria of the laryngeal epithelium, the plexus of CGRP nerve fibers is commonly found and some of these fibers (→) are clearly associated with blood vessels, × 440. **D** Many CGRP nerve fibers are observed in the glandular region and some of these fibers (→) appear to terminate in glandular cells, × 220

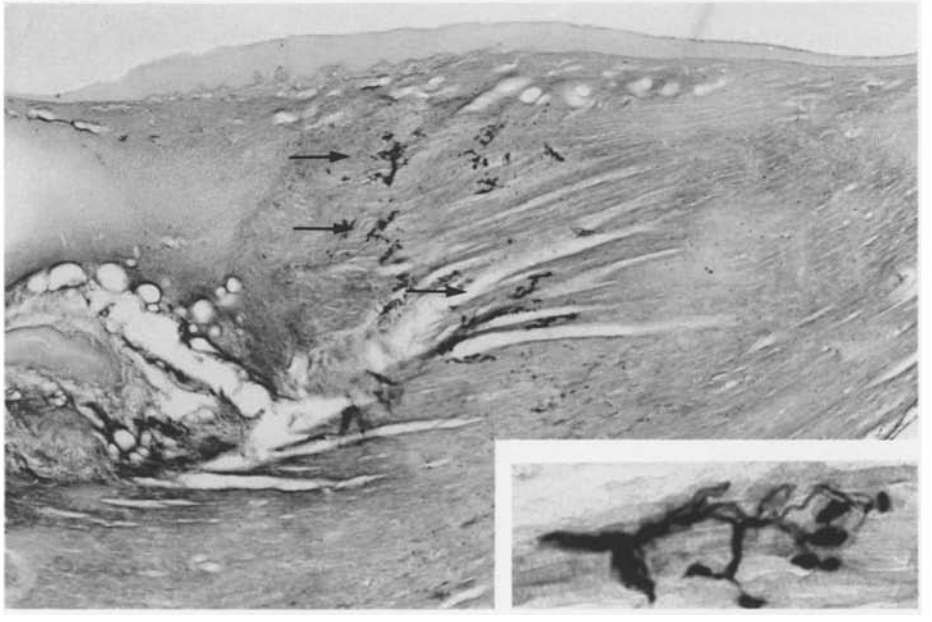

Fig. 2. Thyroarytenoid muscle having abundant CGRP immunoreactivity in the middle of the muscle (→), × 44. *Inset:* Under high magnification, motor end-plate-like structures with CGRP immunoreactivity can be observed, × 440

In the intrinsic laryngeal muscles abundant immunoreactivity was also observed, as well as motor end-plate-like structures with CGRP immunoreactivity (Fig. 2). Some CGRP fibers were also associated with blood vessels. These findings were consistent in all intrinsic laryngeal muscles.

Discussion

In the present study, we have meticulously clarified the distribution pattern of the CGRP nerve fibers in the canine larynx using immunohistochemistry.

The larynx has three principle sets of intrinsic afferent neural systems, operated respectively from receptors located in the laryngeal mucosa, the capsule of the intercartilaginous joints of the larynx, and the laryngeal muscles themselves [20]. The present study showed that many CGRP nerve fibers with varicosities reached the surface of the epithelium and appeared to be free-ended, although we could not detect CGRP nerve fibers in the capsules of the intercartilaginous joints. This finding agrees with Koizumi's [8] neurohistological report that free sensory nerve endings extend into the epithelial layers of the laryngeal mucosa of the dog. CGRP is also now well known to act as a neurotransmitter in the sensory nervous systems [15]. Our present findings and these reports strongly suggest that CGRP must have some relationship to the sensory innervation of the larynx.

It is noteworthy that the quantity of CGRP nerve fibers in the epithelia is different in the various regions of the larynx. In the laryngeal mucosa, the existence of pain receptors, mechanoreceptors and chemoreceptors has been indicated physiologically [7, 16, 19]. From the anatomical point of view, CGRP nerve fibers distributed densely in the epiglottis and subglottis could participate in mechanoreception and chemoreception. It should be noted that CGRP nerve fibers were distributed abundantly in the cartilaginous part of the vocal cord, although very few CGRP nerve fibers were observed in the membranous part of the vocal cord. This membranous part is considered to be the vibratory structure and no delicate sensory innervation is necessary. On the other hand, the cartilaginous part of the vocal cord could play an important role in mechanoreception for vocal cord movement. It is now known that the arytenoid region and epiglottis are richly supplied with taste buds [11, 13]. Nakano and Muto [11] reported that the taste buds in the arytenoid region are considered to mediate reflex responses of apnea and swallowing during chemical stimulation of the larynx. Terenghi et al. [18] reported that CGRP nerve fibers innervate the taste buds in the posterior surface of the epiglottis of the rat, although the taste buds in the arytenoid region were not mentioned at all. Our present study showed that the arytenoid region of the dog is richly supplied with taste buds and these structures are also densely innervated by CGRP nerve fibers. This finding and the role of the taste buds in the arytenoid region stated above also indicate that CGRP plays an important role in the sensory nervous system of the larynx.

Regarding the neurotransmitter of the laryngeal sensory system, we have already reported on the participation of SP [6]. The overlapping distribution of SP and CGRP in the central and peripheral nervous systems is currently known [2, 10]. In the present study, CGRP nerve fibers were more frequently observed in the laryngeal epithelia, as compared with the lesser distribution pattern of SP nerve fibers in the same tissues [6]. This finding suggests that CGRP plays a more important role than SP in laryngeal sensory innervation.

CGRP is now also known to be a neurotransmitter or a neuromodulator in the autonomic nervous system [3]. Our present findings that the laryngeal glands and vessels are innervated by CGRP nerve fibers reconfirm that CGRP could be a neurotransmitter or a neuromodulator of the laryngeal autonomic nervous system.

CGRP has been found in the motor neurons of a variety of mammals [1, 14]. Takami et al. [17] reported that CGRP pharmacologically enhances the muscle contraction of the diaphragm in stimulating the phrenic nerve. New and Mudge [12] have suggested that CGRP may be a motor-neuron-derived trophic factor that increases acetylcholine receptor synthesis at vertebrate neuromuscular junctions. That the intrinsic laryngeal muscles are innervated by CGRP nerve fibers suggests that CGRP also participates in the laryngeal motor system, although the exact function of CGRP is still unknown.

Our findings indicate that CGRP plays an important role in all of the sensory, motor and autonomic nervous systems of the larynx. What this role actually is remains to be defined.

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References

1. Gibson SJ, Polak JM, Bloom SR et al. (1984) Calcitonin gene-related peptide immunoreactivity in the spinal cord of man and eight of other species. J Neurosci 4:3101–3111
2. Gulbenkian S, Merighi A, Wharton J, Varndell IM, Polak JM (1986) Ultrastructural evidence for the coexistence of calcitonin gene-related peptide and substance P in secretory vesicles of peripheral nerves in the guinea pig. J Neurocytol 15:535–542
3. Hanko J, Hardebo JE, Kahrstrom J, Owman C, Sundler F (1985) Calcitonin gene-related peptide is present in mammalian cerebrovascular nerve fibers and dilates pial and peripheral arteries. Neurosci Lett 57:91–95
4. Hisa Y (1982) Fluorescence histochemical studies on the noradrenergic innervation of the canine larynx. Acta Anat (Basel) 113:15–25
5. Hisa Y, Matsui T, Fukui K, Ibata Y, Mizukoshi O (1982) Ultrastructural and fluorescence histochemical studies on the sympathetic innervation of the canine laryngeal glands. Acta Otolaryngol (Stockh) 93:119–122
6. Hisa Y, Sato F, Fukui K, Ibata Y, Mizukoshi O (1985) Substance P nerve fibers in the canine larynx by PAP immunohistochemistry. Acta Otolaryngol (Stockh) 100:128–133
7. Kirchner JA, Suzuki M (1968) Laryngeal reflexes and voice production. Ann NY Acad Sci 155:98–109
8. Koizumi H (1953) On the sensory innervation of the larynx in the dog. Tohoku J Exp Med 58:199–210

9. McWilliam PN, Maqbool A, Batten TFC (1989) Distribution of calcitonin gene-related peptide-like immunoreactivity in the nucleus ambiguus of the cat. J Comp Neurol 282:206–214
10. Morishima Y, Takagi H, Kawai Y, Emson PC, Hillyard CJ, Girgis SI, MacIntire I (1986) Ultrastructural observation of nerve fibers containing both substance P and calcitonin gene-related peptide in the nucleus tractus solitarii of the rat: a combination of immunofluorescence and PAP methods. Brain Res 379:157–161
11. Nakano T, 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
12. New HV, Mudge AW (1986) Calcitonin gene-related peptide regulates muscle acetylcholine receptor synthesis. Nature 323: 809–811
13. Palmieri GA, Asole A, Panu R, Sanna L, Farina V (1983) On the presence. structure and probable function role of taste buds located on the laryngeal surface of the epiglottis in some domestic animals. Arch Anat Histol Embryol 66:55–66
14. Rosenfeld MG, Mermord J-J, Amara SG et al. (1983) Production of a novel neuropeptide enclosed by the calcitonin gene via tissue-specific RNA processing. Nature 304:129–135
15. Silverman JD, Kruger L (1989) Calcitonin gene-related peptide immunoreactive innervation of the rat head with emphasis on specialized sensory structures. J Comp Neurol 280:303–330
16. Suzuki M, Kirchner JA (1967) Larnygeal reflex pathways related to rate and rhythm of the heart. Ann Otol Rhinol Laryngol 76:774–780
17. Takami K, Kawai Y, Uchida S, Tohyama M, Shiotani Y, Yoshida H, Emson PC, Girgis S, Hillyard CJ, MacIntire I (1985) Effect of calcitonin gene-related peptide on contraction of striated muscles in the mouse. Neurosci Lett 60:227–230
18. Terenghi G, Polak JM, Rodrigo J, Mulderry PK, Bloom SR (1986) Calcitonin gene-related immunoreactive nerves in the tongue, epiglottis and pharynx of the rat: occurence, distribution and origin. Brain Res 365:1–14
19. Widdicombe J (1973) Supplementary statement II. In: Wyke BD (ed) Ventilatory and phonatory control systems. Oxford University Press, London, pp 465–468
20. Wyke BD, Kirchner JA (1976) Neurology of the larynx. In: Hinchcliffe R, Harrison D (eds) Scientific foundation of otolaryngology. Heinemann, London, pp 546–574