Expression of vasoactive intestinal peptide, calcitonin gene-related peptide, substance P, and intermediate neurofilaments in nasal mucosal nerve fibers of horses without nasal disease

Brendan M. Corcoran, MVB, PhD; Ian G. Mayhew BVSc, PhD; Caroline N. Hahn DVM, MSc; Dean R. Prince, DVM

Objective—To determine the distribution of nerve fibers containing calcitonin gene-related peptide (CGRP), substance P (SP), vasoactive intestinal peptide (VIP), and intermediate neurofilaments in nasal mucosa of horses.

Animals—6 horses without evidence of nasal disease.

Procedure—Full-thickness nasal tissue specimens were obtained from the rostral portion of the nasal septum at necropsy, and fluorescence immunohistochemistry was performed to assess mucosal distribution of nerve fibers.

Results—Nerve fibers with CGRP-like immunoreactivity (CGRP-Li) formed a dense subepithelial network, and a large number of fibers were found coursing between epithelial cells. Fibers with CGRP-Li were also associated with blood vessels and mucous glands. Fibers with SP-like immunoreactivity (SP-Li) had a similar distribution and density. In contrast, there were few fibers with VIP-like immunoreactivity. Fibers containing intermediate neurofilaments were prominent and appeared as large nerve fiber bundles mainly adjacent to the nasal septum but also close to mucous glands and within the lamina propria. Intermediate neurofilaments were also identified in single nerve fibers at all sites, but the density of fibers with intermediate neurofilaments did not match that of fibers with CGRP- or SP-Li.

Conclusions—The density and distribution of nerve fibers containing SP- or CGRP-Li in nasal mucosa of horses was similar to that reported for other species. However, expression of VIP in nerve fibers was low. Antibodies against intermediate neurofilaments identified many nerve fibers in nasal mucosa of horses but did not appear to identify small diameter fibers expressing SP or VIP. (Am J Vet Res 2000;61:1619–1624)

Nerve fibers immunoreactive for nonadrenergic, noncholinergic (NANC) neurotransmitters, such as vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), substance P (SP), and neuropeptide Y, are widely distributed in the nasal mucosa of many mammalian species. A variety of other NANC neurotransmitters (eg, peptide histidine isoleucine, peptide histidine methionine, helospectin, gastrin-releasing peptide, galanin, and nitric oxide) have also been identified in mammalian nasal mucosa. Many of these neurotransmitters co-localize with each other or with noradrenaline and acetylcholine, resulting in an extremely complex innervation pattern. Along with the classic cholinergic and adrenergic innervation, all of the NANC neurotransmitters are believed to be involved in control and maintenance of nasal mucosal function, and it is hypothesized that alterations in nasal innervation play a role in the etiopathogenesis of disease. However, despite the large body of information on nasal innervation in several species, to our knowledge there are no reports describing the NANC innervation of nasal mucosa in healthy horses.

Although many neurotransmitters have been found in mammalian nasal mucosa, most of the studies to date have primarily investigated SP, CGRP, and VIP. In part, this is because these 3 neurotransmitters are readily identifiable in nasal mucosa and play a role in disease. Vasoactive intestinal peptide is known to act as a vasodilator in the nasal passages and is believed to be involved in the control of mucus gland function, whereas SP and CGRP are sensory neuropeptides that are believed to have a complex local role in neurogenic inflammation and a central reflex role that affects the nasal response to noxious stimulation.

The purpose of the study reported here was to assess the expression of VIP, CGRP, and SP in nerve fibers within the nasal mucosa of horses without evidence of nasal disease. A further objective was to investigate whether identification of the structural neurofilaments L, M, and H in neuronal tissue would serve as a means to map nasal mucosal innervation.

Materials and Methods

Tissue specimens—Tissue specimens were collected from the rostral portion of the nasal cavity of 6 horses euthanatized for reasons other than respiratory disease. None of the horses had a history of nasal disease. Specimens included the nasal septum and consisted of mucosa from both nasal passages. Full-thickness specimens of the jejunum were also obtained from each horse; these specimens were used as positive controls. Tissue was collected from both sites within 10 minutes of euthanasia and immediately placed into Zamboni fixative (2% formaldehyde, 15% picric acid; pH 7.2) for 8
hours. Specimens were dehydrated in serial alcohol solutions (70, 80, 95, and 100%), cleared in xylene, and rehydrated (100, 80, and 50% alcohol; 3 changes of 0.1M PBS solution, pH 7.1). Each step in the process lasted 5 minutes. Tissue blocks were mounted on cork with an embedding matrix and frozen in isopentane cooled by liquid nitrogen. Ten-micron thick cryostat sections were cut and collected onto glass slides coated with gelatin and chrome alum. Sections were air dried for 60 minutes to improve tissue adherence to the slides prior to application of antibodies.

**Immunohistochemistry**—All primary antibodies used were polyclonal and raised in rabbits. Prior to use, antibodies were diluted with 0.1M PBS solution containing 0.03% Triton X-100 and refrigerated. Primary antibodies were against the neurotransmitters VIP<sup>3</sup>, SP<sup>4</sup> (sequence homologous for human, rat and porcine VIP), SP<sup>5</sup> (sequence homologous for all species), and CGRP<sup>6</sup> (human). We also used an antibody cocktail (Pan-N) containing polyclonal antibodies against bovine structural intermediate neurofilaments L, M, and H<sup>7</sup> to identify dendrites and axons in mature neurons. The extent of amino acid sequence homology between equine VIP and CGRP and human forms of these peptides is not known, and, therefore, the degree of crossreactivity between the antibodies used in this study and the native equine peptides cannot be stated. However, the degree of crossreactivity reported for peptides from several other species is high, and this is likely to be the case for horses. The same situation applies to equine neurofilaments.

Anti-VIP and -CGRP antibodies were applied at a dilution of 1:400, anti-SP at 1:800, and Pan-N at a dilution of 1:100. Slides incubated with anti-SP antibody were preincubated for 30 minutes at room temperature (approx 20 °C) with 10% normal horse serum in PBS solution to block non-specific staining. This procedure was not necessary for the other antibodies. Slides were incubated with the primary antibody at room temperature in moisture boxes for 18 hours, hand washed with 0.1 M PBS solution, and incubated with an affinity-purified fluorescein isothiocyanate-(FITC-)
d conjugated goat anti-rabbit IgG antibody at a dilution of 1:20 for 90 minutes at room temperature. After washing again with 0.1M PBS solution and careful blotting with filter paper, specimens were covered with glass coverslips, using a PBS-buffered glycerol (2:1) solution.

Negative control slides included nasal and jejunal tissue specimens incubated with PBS solution alone, with secondary antibody (ie, FITC-conjugated goat anti-rabbit IgG) alone, and with anti-VIP, anti-CGRP, and anti-SP antibodies that had been preabsorbed for 1 hour at room temperature with VIP, CGRP, or SP (1 mmol/ml), respectively. Adjacent 10-µm sections were also stained with H&E for assessment of tissue morphology. At least 3 serial sections from each tissue specimen and for each antibody were examined by use of fluorescence microscopy.<sup>1</sup> Distribution of immunoreactive structures was noted, and a subjective assessment of the extent of immunostaining was made by one of the authors (BMC).

**Results**

**Morphology**—Overall morphology of the 2 tissue types (nasal mucosa and jejunum) was assessed as good after examination of H&E stained sections. Nasal specimens had no histologic evidence of either active or chronic disease. The nasal mucosa consisted of a pseudostratified columnar epithelium (respiratory epithelium) overlying a lamina propria that contained seromucous glands and blood vessels. Interspersed between this layer and the nasal septal cartilage were bands of connective tissue, which we presumed were collagen fibers. Large nerve-fiber bundles could be readily recognized in all sections and were particularly apparent close to the nasal septal cartilage. At no time were neurons identified.

**Distribution of nerve fibers in control specimens**—Immunostaining of nerve fibers was not detected in any of the negative controls. The distribution and density of nerve fibers with VIP-like immunoreactivity (VIP-Li) in jejunal specimens (positive controls) was similar to that previously reported in horses.<sup>2</sup> A large number of such fibers were seen extending into villi, in the lamina propria, and in the junction between the circular and longitudinal muscle layers (myenteric plexus). Fibers and neurons with VIP-Li were readily visible in the myenteric plexus, but nerve cell bodies were not identifiable in the region of the submucous plexus. Structures with VIP-Li were also visible within the smooth muscle layers.

The distribution of nerve fibers with CGRP-like immunoreactivity (CGRP-Li) and SP-like immunoreactivity (SP-Li) in jejunal specimens was also similar to that previously reported and similar to that of fibers with VIP-Li. However, the intensity and quality of immunostaining for CGRP and SP was less than for VIP. Nerve fibers with VIP-Li, SP-Li, and CGRP-Li had distinct varicosities along their length.

The distribution of fibers that stained with Pan-N was similar to that for fibers with VIP-Li, SP-Li, and CGRP-Li, except that Pan-N-positive fibers were not seen in the intestinal villi. The degree of Pan-N staining was particularly intense in the myenteric plexus. Pan-N staining was identified from its linear appearance; varicosities were not detected along nerve fiber lengths.

**Distribution of nerve fibers in nasal mucosal specimens**—An extensive network of single and multiple nerve fiber strands were clearly delineated with Pan-N (Fig 1). Many of the nerve fibers coursed close to the nasal septal cartilage as distinct nerve fiber bundles. Other fibers were found in close association with mucous glands and blood vessels and in close proximity to the epithelium. However, only few Pan-N-positive fibers were detected coursing beneath the epithelium and between epithelial cells. This degree of staining contrasts with that seen for the other antibodies. Staining with Pan-N did not appear to allow identification of nerve fibers with CGRP- or SP-Li at these sites (ie, beneath the epithelium and between epithelial cells).

Nerve fibers with CGRP-Li were present in abundance throughout nasal tissue sections, particularly within the lamina propria and other submucosal sites (Fig 2). Numerous distinct single fibers were also detected coursing through the epithelial layer toward the luminal surface. The overall density of nerve fibers with CGRP-Li in the nasal mucosa was comparable to that of Pan-N-positive fibers. Nerve fiber bundles close to the nasal septum, as delineated by staining with Pan-N, had little CGRP-Li; only a small number of nerve fibers with CGRP-Li were visible in this area. We did not double-stain slides to assess the degree of co-expression of intermediate neurofilaments and CGRP.
Nerve fibers with SP-Li had a similar distribution to that of fibers with CGRP-Li; the most extensive staining was detected close to the epithelium (Fig 3). Despite the addition of a blocking step prior to incubating slides with anti-SP antibody, we still detected a high degree of background staining. This made identification of immunoreactive nerve fibers difficult, but a dense fiber network could be more readily seen using filters to modify the fluorescence excitation band width. However, use of such filters affected the intensity of

Figure 1—Photomicrographs of nasal mucosal tissue specimens from a horse without evidence of nasal disease after staining with antibodies against intermediate neurofilaments (L, M, and H), obtained by use of fluorescence microscopy. Nerve fibers coursing close to the nasal septal cartilage as distinct nerve fiber bundles (NFB, white arrows) are evident in the left panel, whereas single and multiple nerve fiber strands can be seen in proximity to the epithelium (arrows; right panel). Bar = 100 µm. EP = Epithelium. L = Nasal lumen. MG = Mucous glands.

Figure 2—Photomicrographs of nasal mucosal tissue specimens from a horse without evidence of nasal disease after staining with a polyclonal antibody against calcitonin gene-related peptide, obtained by use of fluorescence microscopy. Upper left panel—Nerve fibers are evident coursing beneath the epithelium (EP; white arrows), with many distinct single fibers passing through the epithelial layer toward the luminal surface (L; white and black arrowheads). Bar = 100 µm. Upper right panel—Nerve fiber bundles are evident coursing beneath the epithelium (EP; large asterisks), and fibers can be seen branching off toward the airway lumen (white arrow). Bar = 50 µm. Lower left panel—Nerve fibers are evident in close association with mucous glands (MG; white arrows). Bar = 50 µm. Lower right panel—Nerve fibers are evident in close association with blood vessels (BV; black arrows) and mucous glands (white arrows) beneath the epithelial layer. Bar = 50 µm. LP = Lamina propria.
tems may have an important role to play in nasal function, and it is postulated that these neuronal systems, more recently identified NANC innervation, is an example of nasal mucosa in many other mammalian species. The present study was limited to the investigation of the pattern of nasal mucosal innervation, using antibodies against intermediate neurofilaments and 3 NANC neurotransmitters. Results of this study provide baseline data that will enable initiation of further studies regarding the possible role of altered innervation in the etiopathogenesis of nasal disease in horses. It is believed that most SP- and CGRP-containing nerve fibers in mammalian nasal mucosa are unmyelinated sensory afferents. However, this is yet to be proven in horses. The density of nerve fibers with SP- or CGRP-Li coursing beneath the epithelium in nasal mucosal specimens was more pronounced than that seen subepithelially elsewhere in the respiratory tract of horses. Compared with the intensity of staining with anti-CGRP antibodies, the overall visibility of nerve fibers stained with anti-SP antibodies was disappointing because of the normal background tissue fluorescence.

Discussion
To our knowledge, there are no reports describing innervation of nasal mucosa in healthy horses or horses with nasal disease. There is, however, an extensive body of information describing the complexity of nasal mucosal innervation in many other mammalian species. The present study was limited to the investigation of the pattern of nasal mucosal innervation, using antibodies against intermediate neurofilaments and 3 NANC neurotransmitters. Results of this study provide baseline data that will enable initiation of further studies regarding the possible role of altered innervation in the etiopathogenesis of nasal disease in horses. It is well recognized that a combination of classic adrenergic and cholinergic innervation, together with the more recently identified NANC innervation, is an important factor in the control of normal nasal mucosal function, and it is postulated that these neuronal systems may have an important role to play in nasal disease.

Antibodies against the low, medium, and high molecular weight neurofilaments were used in the present study to attempt to map the total innervation pattern of nasal mucosa in horses. We hoped that this would allow quantitative assessment of the total innervation pattern, and, therefore, would be of value for subsequent assessment of innervation in disease states. Staining with Pan-N did allow us to successfully identify a large number of nerve fibers at all sites in the nasal mucosa. Many of these were single fibers, but large nerve fiber bundles were also seen running parallel to the nasal septum. The density of nerve fibers was unexpected, as the nasal mucosa is a highly innervated structure in all species studied to date. However, we had hoped that most neuronal structures would have stained with Pan-N, but Pan-N did not appear to stain all nerve fibers with CGRP- or SP-Li, particularly in the vicinity of the epithelium. Pan-N is an antibody cocktail designed to identify all neurofilament epitopes, both phosphorylated and nonphosphorylated, in all axons and dendrites. However, the degree of axonal expression of neurofilaments decreases with axon size and can be low in small diameter unmyelinated axons in the periphery of tissues; nerve fibers with SP- or CGRP-Li are believed to be in this category. Microtubule density in axons increases with a decrease in fiber width, and it is likely that antibodies raised against microtubule-associated protein would be better for identification of small, unmyelinated axons in the periphery of the nasal mucosa of horses.

An extensive network of fibers with CGRP-Li coursing beneath the respiratory epithelium in the nasal mucosa, with fibers extending intraepithelially toward the nasal lumen, has been described in humans, rats, guinea pigs, and rabbits. We detected a similar pattern in horses. It is believed that most SP- and CGRP-containing nerve fibers in mammalian nasal mucosa are unmyelinated sensory afferents. However, this is yet to be proven in horses. The density of nerve fibers with SP- or CGRP-Li coursing beneath the epithelium in nasal mucosal specimens was more pronounced than that seen subepithelially elsewhere in the respiratory tract of horses. Compared with the intensity of staining with anti-CGRP antibodies, the overall visibility of nerve fibers stained with anti-SP antibodies was disappointing because of the normal background staining. We previously used a variety of blocking techniques and several different anti-SP antibodies to overcome this problem, but have found that only preincubation with 10% normal horse serum lessens the degree of background staining. Nevertheless, we are confident that we identified nerve fibers with SP-Li in the nasal mucosa of these horses. Using retrograde labeling and denervation procedures, nerve fibers with CGRP- or SP-Li have been shown to originate from nerve cell bodies in the trigeminal ganglion, indicating the sensory function of these fibers. These sensory nerve fibers have a small diameter, are unmyelinated, and have free nerve endings, morphology that is consistent with a nociceptive function. Furthermore, as fibers with CGRP-Li have a region-specific distribution in rats (ie, greatest density is in areas directly exposed to inhaled air), it is reasonable to postulate these fibers have a role in the local and central responses to nasal irritation and inflammation. Fibers with CGRP- or SP-Li are also found in the nasal mucosa in association with blood vessels and mucous glands and are believed to have a role (possibly through local and central reflex mechanisms) in vasodilatation and mucus secretion. Because of these potential functions, it has been suggested that topical application of capsaicin, which depletes SP and CGRP from nerve endings, could be used therapeutically to...
control rhinorrhea and reflex nasal responses to noxious stimuli.36

Calcitonin gene-related peptide is usually found colocalized with SP and, although comparisons between species must be viewed with caution, the relative densities of nerve fibers with CGRP- or SP-Li in the epithelial zone of human and rat nasal mucosa appears to be similar.32,34,35 However, CGRP-Li is more readily demonstrable than SP-Li in the nasal mucosa of all species studied to date; Hauser-Kronberger et al30 found only CGRP-Li in nerve fibers associated with secretory ducts in nasal and laryngeal mucosa from humans. In the present study, nerve fibers with CGRP-Li were more readily identified in association with mucous glands than those with SP-Li. These results together with results of Hauser-Kronberger et al30 suggest that CGRP is of greater functional importance for control of mucus secretion.

The density of nerve fibers with SP- or CGRP-Li in nasal mucosa can change under certain conditions, and this has potential clinical implications. Experimentally induced chronic hypoxia has been shown to increase nerve fiber density in the nasal and tracheobronchial mucosa of rats, suggesting an as yet unidentified role for the primary sensory neurons in hypoxic adaptation.33 Of greater relevance to respiratory medicine are the results of Fang and Shen.3 They found that in humans with allergic or chronic hypertrophic rhinitis, innervation with, and distribution of, fibers with VIP-Li and SP-Li were altered. Tissue concentrations of VIP and SP in affected patients were also altered, compared with healthy humans. These results again suggest a role for neuropeptides in the etiopathogenesis of nasal disease.

Vasoactive intestinal peptide acts as a vasodilator in nasal mucosa and is contained within an atropine-resistant parasympathetic system.39 Nerve fibers that contain VIP are mainly found in association with blood vessels and mucous glands, and it is believed that these structures are regulated by VIP.4,9,20,21,32 In general, VIP-containing fibers are absent from the epithelium,22 although 2 studies have reported fibers coursing beneath and between respiratory epithelial cells in rat nasal mucosa.33 Most of these fibers appear to innervate goblet cells, but, unlike fibers with SP- or CGRP-Li, they do not extend to the mucosal surface.32 Nerve fibers with VIP-Li have been shown to originate from neurons in the sphenopalatine and pterygopalatine ganglia,3,10 and, in common with many NANC neurotransmitters, VIP often co-localizes with other neurotransmitters, including nitric oxide, helospectin, peptide histidine methionine, and acetylcholine.4,5,55

Nevertheless, the identification of fibers with VIP-Li in the nasal mucosa of all species studied to date is at variance with our findings in horses. The apparent absence or low expression of VIP in nasal mucosal nerve fibers of horses was an unexpected finding, because in other species, innervation with VIP-containing fibers has an important role in nasal mucosal function. A species variation in the density of such fibers was reported by Uddman et al,21 whereby these fibers were dense in nasal mucosa of cats, less dense in rabbits, and nearly absent in guinea pigs. However, in another comparative study evaluating VIP in nasal mucosa of guinea pigs,3 there was no indication of significant differences, compared with cats, pigs, rats, and humans.5 Similarly, Yokoyama et al52 demonstrated an intense innervation of glandular structures with VIP-containing fibers in guinea pigs. It may be that the findings of Uddman et al21 were attributable to technical problems or, alternatively, VIP-containing fibers may be localized to specific structures in guinea pigs. Indeed, the demonstration of region-specific distribution of CGRP-containing fibers in the nasal mucosa of rats5 suggests that similar distribution studies should be performed in all species. In the present study, tissue was only collected from the rostral portion of the nasal septum and possible regional variations in distribution of nerve fibers with VIP-Li cannot be discounted.

References

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