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Neurochemical diversity of afferent neurons that transduce sensory signals from dog ventricular myocardium

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Abstract

While much is known about the influence of ventricular afferent neurons on cardiovascular function in the dog, identification of the neurochemicals transmitting cardiac afferent signals to central neurons is lacking. Accordingly, we identified ventricular afferent neurons in canine dorsal root ganglia (DRG) and nodose ganglia by retrograde labeling after injecting horseradish peroxidase (HRP) into the anterior right and left ventricles. Primary antibodies from three host species were used in immunohistochemical experiments to simultaneously evaluate afferent somata for the presence of HRP and markers for two neurotransmitters. Only a small percentage (2%) of afferent somata were labeled with HRP. About half of the HRP-identified ventricular afferent neurons in T₃ DRG also stained for substance P (SP), calcitonin gene-related peptide (CGRP), or neuronal nitric oxide synthase (nNOS), either alone or with two markers colocalized. Ventricular afferent neurons and the general population of T₃ DRG neurons showed the same labeling profiles; CGRP (alone or colocalized with SP) being the most common (30–40% of ventricular afferent somata in T₃ DRG). About 30% of the ventricular afferent neurons in T₂ DRG displayed CGRP immunoreactivity and binding of the putative nociceptive marker IB₄. Ventricular afferent neurons of the nodose ganglia were distinct from those in the DRG by having smaller size and lacking immunoreactivity for SP, CGRP, and nNOS. These findings suggest that ventricular sensory information is transferred to the central nervous system by relatively small populations of vagal and spinal afferent neurons and that spinal afferents use a variety of neurotransmitters.

Keywords

calcitonin gene-related peptide; neuronal nitric oxide synthase; substance P; ventricular afferent neuron; dorsal root ganglion; nodose ganglion

1. Introduction

Cardiac afferent neurons of the nodose and dorsal root ganglia are crucial elements in the hierarchy of neurons that comprise the cardiac nervous system (Brown 1967; Malliani et al.,

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1973; Uchida 1975; Malliani A., 1982; Armour and Kember 2004). The distal sensory neurites of these neurons, distributed primarily in the dorsal atria and the outflow tracts of the ventricles, continually transduce the chemical and mechanical milieu of the heart. Such information is transferred to second order neurons in the medulla and spinal cord by means of chemical mediators that are released from the central neurites of these afferent neurons. Sensory input from these neurons has a major role in the control of efferent neuronal output to the heart. Functional studies have identified a population of canine dorsal root ganglion (DRG) afferent neuronal somata that are activated by application of endogenous peptides (e.g., substance P) or other chemicals to their ventricular epicardial sensory neurites (Huang et al., 1995; Huang et al., 1996). Many of these sensory neurites, which lie concentrated in the anterior portions of the left and right ventricles, are polymodal in nature, responding to both chemical and mechanical stimuli (Armour and Kember, 2004). In contrast, ventricular neurites from vagal afferent neurons respond primarily to chemical stimuli (Armour and Kember, 2004). The neurotransmitters utilized by ventricular afferent neurons of the canine DRG and nodose ganglia remain unknown.

The goals of this project were to identify DRG and nodose ganglion neurons with neurites localized in the canine ventricular myocardium (i.e., ventricular afferents) and to determine the neurotransmitter phenotype of these neurons compared to the general population of afferent neurons in the same ganglia. The somata of ventricular afferent neurons were first identified via retrograde labeling with horseradish peroxidase (HRP) in the present study. Thereafter, immunostaining to identify HRP and neurochemical markers was employed to determine the presence and colocalization of substance P (SP) with calcitonin gene-related peptide (CGRP) or neuronal nitric oxide synthase (nNOS) in HRP-positive and HRP-negative somata. To further characterize canine DRG neurons, we also evaluated the presence of isolectin B₄ (IB₄) binding, which is a putative marker for a subpopulation of nociceptive neurons (Stucky and Lewin, 1999; Price and Flores, 2007). Finally, we determined the size of ventricular afferent neurons. Using these methods, we identified major physical and neurochemical differences between ventricular afferent neurons of the canine nodose ganglia and DRG.

2. Materials and methods

2.1. Surgical procedures and tracer injections

The experimental protocol was approved by the East Tennessee State University Committee on Animal Care and conforms to the Guide for Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication No. 85-23, revised 1996). Five adult, conditioned mongrel dogs of either sex (24–26 kg) were used in this study. Four received neuronal tracer injections and one served as a sham control. Anesthesia was induced by sodium thiopental (15–30 mg/kg, i.v.). After endotracheal intubation, mechanical ventilation was initiated (Hallowell EMC Model 2000, Hallowell EMC, Pittsfield, MA) and anesthesia was maintained with isoflurane (1–3%). The depth of anesthesia was determined by continuous monitoring of blood pressure and heart rate as well as corneal and pedal reflexes. Body temperature was maintained at 37°C by means of a circulating water heating pad (Gaymar T/Pump, Gaymar Industries Inc., Orchard Park, NY). A left thoracotomy at the fourth intercostal space was performed and an incision was made into the ventral pericardium to expose the ventricles. Sixteen 20µl boluses of tracer solution comprising 20% horseradish peroxidase (HRP, type VI, Sigma) and 0.2% cholera toxin B subunit-conjugated HRP (List Biological Laboratories) in sterile 5% dimethylsulfoxide/water were injected via a Hamilton syringe into the midwall regions of the conus (outflow tract) of the right ventricle (4 injections/animal), and then the ventral and lateral walls of the anterior left ventricle (12 injections/animal). For the sham control, vehicle was injected into these same sites. Slight pressure was applied to each injection site for about a minute after removing the needle to minimize leakage of tracer.

After the final tracer injection, the pericardium and thoracic cavity were closed and residual air within the thoracic cavity was withdrawn via a chest tube. Buprenorphine (0.005–0.01 mg/kg, i.m. or i.v.) was administered pre- and post-operatively for pain management, as needed. Antibiotic therapy (intramuscular cefazolin or oral cephalexin, 22–30 mg/kg) was administered twice daily prior to surgery and continued for seven days post-operatively. After allowing 7 days for retrograde transport of the tracers, the dogs were anesthetized in the manner depicted above and a dorsal laminectomy was performed to expose and remove the DRG at levels T₂ and T₃ of the spinal cord. Animals were then rotated, and a midline incision was made in the neck region so the nodose ganglia could be exposed and removed. A thoracotomy was performed to collect cardiac fat pads, which contain the intrinsic cardiac ganglia, for another study. After completing tissue collection, animals were euthanized by increasing the isoflurane to 5% and then removing the heart.

2.2 Tissue preparation

After being removed, the DRG and nodose ganglia were washed briefly in saline and then transferred to ice-cold fixative solution containing 4% paraformaldehyde and 0.2% picric acid in phosphate buffered saline (PBS, pH 7.3). Tissues were fixed for 24 h at 4° C. They were then transferred to 20% sucrose in PBS (4° C, 3 days) for cryoprotection, frozen in powdered dry ice, and stored in sealed tubes at –80° C until processing. Sections of ganglia were cut to a thickness of 16 µm using a Leica CM 3050S cryomicrotome. Adjacent sections were collected on separate chromealum gelatin coated slides such that six representative sets of tissue sections were obtained from each tissue sample. Slide-mounted sections were stored in sealed slideboxes at –20° C prior to staining.

2.3 Immunohistochemistry

Triple labeling of sections was performed at room temperature using standard methods. Briefly, slides were rinsed with 0.1 M PBS (pH 7.3), permeabilized in PBS containing 0.4% Triton X-100 and 0.5% bovine serum albumin (BSA), and blocked for 2 hours in PBS containing 10% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA), 1% BSA and 0.4% Triton X-100. Sections were then incubated overnight in buffer containing goat anti-HRP, rabbit anti-SP, and either mouse anti-CGRP or mouse anti-nNOS (Table 1). After washing with PBS, tissues were incubated for 2 hours with species-specific donkey secondary antibodies that were conjugated to AlexaFluor 488, 555 or 647 (Molecular Probes, Eugene, OR) and washed with PBS. Coverglasses were attached with Citifluor mounting medium (Ted Pella, Redding, CA) and sealed with clear nail polish. Negative control slides were processed similarly without using any primary antibodies. For one experiment, sections of T₂ DRG were incubated with biotinylated isolectin B₄ (IB₄; 6 µg/ml, Cat. No. B1105, Vector Laboratories, Burlingame, CA), goat anti-HRP and mouse anti-CGRP to determine if IB₄ binding revealed a unique population of spinal ventricular afferent neurons. AlexaFluor-conjugated streptavidin (1:200; Molecular Probes) was added to the secondary antibody mixture for identification of IB₄-positive neurons.

2.4. Data acquisition and analysis

Immunolabeled neurons were viewed and photographed using an Olympus BX41 fluorescence microscope equipped with an Optronics MagnaFire SP charge-coupled device camera. Separate images were acquired for all fields when viewed using filters for each of the three fluorophores. Stereo Investigator software (MicroBrightField, Williston, VT) was used to overlay digital images of each view and count the total number of neurons present and the number of neurons that were single, double, or triple labeled. The total number of neurons per section was determined from images obtained with the exposure time increased to show all somata from their background fluorescence. Long and short axes of HRP-labeled neurons

showing a clear nuclear region were measured using Stereo Investigator software. The cross-sectional areas of the same somata were determined by using the nucleator probe function of the software. The selectivity of fluorescence filters and the presence of colocalized markers were confirmed in several sections obtained from each animal by sequential scanning with a Leica TCS SP2 confocal microscope. Confocal analysis was used to evaluate all sections with HRP-labeled neurons in the experiment with IB₄.

Cell counts, axis lengths, and cross-sectional areas are reported as mean $\pm$ S.D. (n=4 for DRG and n=3 for nodose ganglion). In view of the small sample size for HRP-positive spinal afferents neurons showing SP and/or nNOS immunoreactivity, the incidence of these neurons is presented as a percentage for pooled data from all dogs followed by actual cell counts in parentheses. For the larger groups of HRP-labeled DRG neurons, a Student's *t* test was used to compare their staining pattern to that of the general population of afferent somata in DRG at the T₃ spinal level. Dimensions of different groups of HRP-labeled DRG neurons or different groups of nodose ganglion neurons were compared by separate one-way ANOVA. Dimensions of HRP-labeled neurons in DRG and nodose ganglia were compared by Student's *t* tests. A value of *P* < 0.05 was considered significant.

3. Results

3.1. Presence of HRP-labeled ventricular afferent neurons in T₃ DRG

A total of 13,988 afferent neuronal somata were identified by histological means in tissue sections collected from four canine right- and left-sided T₃ DRG (Table 2). Thus, approximately 1750 neurons were observed in each DRG studied. These sections represent one third of those collected from each ganglion. A total of 155 ventricular afferent neurons were identified by retrograde labeling with HRP. These somata represent about 1% of the right and left T₃ ganglia afferent neuronal population. More HRP-labeled neurons were identified in left-sided ganglia than right (56 versus 99). HRP immunoreactivity had a granular appearance and generally filled the cytoplasm of labeled neurons (Fig. 1 and Fig. 2). No HRP-labeled neurons were observed in 3,086 neurons identified in triple-stained sections obtained from T₃ ganglia of the sham control dog that was not injected with HRP.

3.2. Chemical profile of the general T₃ DRG neuronal population

In sections of T₃ DRG incubated with antibodies against CGRP and SP, about 21% of all neurons displayed immunoreactivity to CGRP alone, 13% to SP alone, and about 6% to both CGRP and SP (Fig. 1 and Table 2). Thus, approximately 60% of all neurons in this series did not stain for either of these neuropeptides. In a second series of sections incubated with antibodies against SP and nNOS, about 16% of all neurons displayed immunoreactivity to nNOS alone, 17% to SP alone, and about 5% to both nNOS and SP (Fig. 2 and Table 2). Similar staining profiles for these markers were observed in right- and left-sided T₃ DRG (Table 2). Approximately 62% of all T₃ neurons in this series did not stain for either of these neuronal markers. Additional immunohistochemistry was performed using the vesicular glutamate transporter (VGLUT) antibodies listed in Table 1, but no staining was observed.

3.3. Neurochemical profiles and dimensions of HRP-identified DRG ventricular afferent neurons

Ventricular afferent neurons identified by retrograde labeling with HRP displayed a diverse neurochemical profile similar to that of the general DRG neuronal population (Table 2). A total of 97 HRP-immunoreactive (HRP-IR) somata were identified in the first series of sections that were also incubated with antibodies against CGRP and SP. Right- and left-sided ganglia contained 34 and 63 HRP-IR neurons, respectively. Overall, these somata showed about the same frequency of CGRP immunoreactivity and colocalization of CGRP with SP as seen in

the general afferent neuronal population of the T₃ ganglia. However, fewer HRP-positive neurons stained for only SP, and HRP-IR neurons in right-sided ganglia showed a higher percentage of CGRP-IR neurons compared to the whole population (Table 2). Such differences might be attributed to the small sample size for HRP-IR neurons since no difference in SP staining (HRP-IR vs total) was observed in the next series of sections so studied. A total of 58 HRP-IR somata were identified in the second series of sections that were incubated with antibodies against nNOS and SP. Right- and left-sided ganglia contained 22 and 36 HRP-IR neurons, respectively. For nNOS and SP, no staining differences were noted right to left DRG or between HRP-IR neurons and the total T₃ neuronal population.

We measured the size of all HRP-IR DRG neurons with a visible nucleus, and summarized data are presented in Table 3. No differences in neuronal size were detected for ventricular afferent neurons having different neurochemical profiles.

3.4. IB₄ binding to T₂ DRG neurons

Further characterization of DRG neurons was obtained by evaluating IB₄ binding in sections from one animal. All sections were incubated with IB₄ and antibodies against HRP and CGRP. Sequential confocal scans were obtained for eleven fields that contained HRP-IR ventricular afferent neurons, and resulting images were evaluated. Neuronal and non-neuronal components of the ganglia showed IB₄ binding to varying degrees, but only 33% of the 493 neuronal somata in these fields were judged to be IB₄-positive. For these neurons, IB₄ staining occurred at the plasma membrane and within the cells where it had a wavy appearance (Fig. 3). Intraneuronal IB₄ labeling ranged from bright staining in most regions of the cytosol down to lower intensity staining that occupied less than half of the cross-sectional area. HRP-labeled ventricular afferents comprised about 6% (31 neurons) of the total neurons counted, and about 30% of these neurons were positive for IB₄ and CGRP (Fig. 3).

3.5. Neurochemical profile and dimensions of ventricular afferent neurons of the nodose ganglia

Two series of sections through the left nodose ganglion from three of the HRP-injected dogs were triple stained using antibodies against HRP and either CGRP and SP (Fig. 4) or SP and nNOS (Fig. 5). These sections contained a total of 1788 neurons, and 31 (~2%) of these were retrogradely labeled with HRP. None of these ventricular afferent neurons of the nodose ganglia showed immunoreactivity for CGRP, SP, or NOS but other neurons in the same fields stained positive for each of these markers (Fig. 4 and Fig. 5). In the first series, 4.8% of the neurons stained for SP, 18.6% for CGRP and only 1.6% for CGRP and SP. In the second series 4.7% of the neurons stained for SP, 11% for NOS and 3.8% for SP and NOS.

Neuronal size was evaluated for HRP-labeled ventricular afferents and for other neurons in the same images that showed immunoreactivity for specific neurochemical markers (Table 3). Ventricular afferent neurons showed a trend toward smaller size compared to other HRP-negative nodose ganglion neurons that were immunolabeled for CGRP, SP, or nNOS, but these differences did not reach statistical significance (e.g., $P=0.2362$ for ANOVA on cross-sectional area). However, ventricular afferent neurons of the nodose ganglia were significantly smaller than DRG ventricular afferent neurons (Table 3).

4. Discussion

Primary antibodies from three host species were used in this study to identify HRP-labeled ventricular afferent neurons in canine DRG and nodose ganglia and to evaluate the presence of markers for different putative neurotransmitters within these populations. Using this approach, we found that only small percentages (1~2%) of the sensory neurons in these ganglia

were associated with ventricular sensory neurites. The neurochemical profiles of ventricular afferent neurons in the DRG were similar to those observed in the general population of T₃ afferent neurons and occurred at about the same frequency. Staining for SP, CGRP or nNOS alone was associated with about 10–30% of the neurons identified. Only 5–10% of afferent cell bodies displayed double staining for SP and CGRP or SP and nNOS. Conversely, none of these three putative neurotransmitter markers were detected in 50–60% of the HRP-positive and HRP-negative neurons in T₃ DRG. A distinct population of ventricular afferent neurons was identified in nodose ganglia. These neurons were significantly smaller than DRG ventricular afferent neurons and lacked immunoreactivity for SP, CGRP and nNOS.

To maximize the labeling of ventricular afferent neurons in this study, we used cholera toxin B subunit-conjugated HRP in addition to unconjugated HRP. Multiple injections (16 per dog) of these combined tracers were made into the right and left ventricular outflow tracts as these regions contain the highest density of ventricular afferent neurites, based on results obtained from *in vivo* functional studies (Huang et al., 1995; Huang et al., 1996; Armour and Kember, 2004). We also selected T₃ ganglia for evaluation because previous anatomical and functional studies demonstrated that T₂–T₄ ganglia contain the largest population of DRG cardiac afferent neuronal somata (Vance and Bowker, 1983; Hopkins and Armour, 1989; Huang et al., 1995; Huang et al., 1996). In spite of these steps, only about 1~2% of the T₃ DRG neurons were associated with such sensory neurites (i.e., showed retrograde labeling with HRP). While it is likely that some ventricular afferent neurons were not labeled in our experiments, it is reasonable to assume that ventricular afferent neurons comprise only a small portion of the total afferent population since neurons in upper thoracic DRG have sensory fields in many other tissues, including the lungs and skin of the thorax and forelimbs (Hopkins & Armour, 1989). Likewise, retrograde labeling with HRP occurred in only a small percentage of nodose ganglion neurons. This finding was expected since the nodose ganglia provide sensory innervation to many other autonomic targets including blood vessels, airways, and viscera.

The ventricular afferent neurons identified in the DRG exhibited a variety of neurochemical phenotypes, based on the presence or absence of immunostaining for CGRP, SP, and nNOS. Five unique patterns of neurotransmitter/neuromodulator content are suggested by the single and double labeling patterns that we observed for these markers. Although CGRP might be co-localized with nNOS in some ventricular afferent neurons, we could not test this option as suitable antibodies from appropriate hosts were not available. Judging from the results obtained in this study, one or more additional neurotransmitters must be expressed by the majority of DRG neurons that were unlabeled after staining for the selected markers. Glutamate is the most likely neurotransmitter for many of these neurons (Li et al., 2001; Oliveira et al., 2003; Corbett et al., 2005), but we could not test this hypothesis since several antibodies against vesicular glutamate transporters failed to label neurons in sections of canine DRG or nodose ganglion. Negative results with these VGLUT antibodies is most likely due to a lack of reactivity with the corresponding canine VGLUT proteins since there is strong evidence that glutamate is a neurotransmitter for baroreceptor and cardiac mechanoreceptor afferents in the dog (Seagard et al., 1999; Seagard et al., 2000). The functional significance of such neurochemical diversity among DRG ventricular afferent neurons remains unknown. However, since neurites of most ventricular afferent neurons of the DRG are polymodal in nature, responding to chemical and mechanical stimuli (Huang et al., 1996), it is possible that cotransmitters associated with individual neurons might have a role to play in the differential transduction of these signals to central neurons.

The canine ventricular afferent neurons that colocalized SP and CGRP in this study probably function as nociceptors. Coexistence of these neuropeptides typifies a major group of nociceptive neurons that are further defined by the presence of transient receptor potential vanilloid subfamily type 1 channels, which confer sensitivity to capsaicin (Zahner et al.,

2003; Pan and Chen, 2004; Price and Flores, 2007). Both of these neuropeptides are released from isolated hearts during myocardial ischemia, after treatment with chemicals that are released from ischemic myocardium, and when hearts are challenged with capsaicin (Franco-Cereceda and Lisk, 2000; Hoover et al., 2000). While colocalization of SP and CGRP is nearly complete in cardiovascular afferent fibers of the guinea pig (Wharton et al., 1986; Franco-Cereceda et al., 1987; Gibbins et al., 1987), we found that CGRP-IR neurons outnumbered SP-IR neurons in canine DRG. This greater abundance of CGRP-positive neurons applied to the entire population, including HRP-labeled ventricular afferents. Some of these CGRP-positive ventricular afferents might be nociceptive neurons as well since we found that 30% of ventricular afferents in T₂ DRG showed both IB₄ binding and CGRP immunoreactivity. Binding of IB₄ has been used to identify a population of nociceptive neurons that lack SP/CGRP in the mouse (Stucky and Lewin, 1999), but studies of rat sensory ganglia show that IB₄ also labels a significant number of neuropeptide-positive sensory neurons in this species (Price and Flores, 2007).

No differences in long axis dimension or cross-sectional area were found between groups of ventricular afferent neurons in canine DRG but these neurons were significantly larger than HRP-labeled ventricular afferent neurons in the nodose ganglion. Another distinguishing feature of the HRP-labeled nodose ganglion neurons was the absence of immunoreactivity for SP, CGRP, and nNOS. However, these neural markers were detected in several nodose ganglion neurons that were unlabeled for HRP. It remains possible that SP, CGRP, and nNOS might be associated with vagal afferent neurons in the dogs that provide sensory innervation to the atria.

Previous work has shown that application of SP to the ventricular epicardium increases the activity of 65–85% of functionally identified ventricular afferents neurons in DRG (Huang et al., 1995; Huang et al., 1996). This finding suggests that ventricular sensory neurites possess neurokinin (i.e., SP) receptors. It has also been demonstrated that the spontaneous activity generated by these neurons is reduced significantly following application of a SP receptor antagonist to their receptor fields (Huang et al., 1995). Collectively, these observations support the contention that endogenous SP provides tonic activation to some ventricular neurites associated with these somata in the anesthetized dog. The SP-immunoreactive ventricular afferent neurons identified in the present study presumably represent one source of endogenous SP that could influence the activity of cardiac sensory neurites. Local release of SP from cardiac sensory neurites has also been suggested from evidence that SP-containing sensory fibers have both afferent and efferent functions (Maggi and Meli, 1988; Hoover et al., 2000). Such local release of SP and the subsequent activation of other ventricular sensory neurites could provide a mechanism for increasing the gain of cardiac sensory transduction. Reflex activation of the ischemia-sensitive spinal afferents augments SP release into lamina I–VII of the spinal cord (Hau et al., 2004) and may initiate central neural signaling of angina.

Nitrgergic vagal afferent neurons are known to innervate the heart (Calupca et al., 2000) and, as such, may also play a role in modulating autonomic efferent neuronal control of heart rate (Sears et al., 1998; Conlon and Kidd, 1999). To the best of our knowledge, this report provides the first definitive evidence for a population of DRG ventricular afferent neurons that express nitrgergic properties. Such neurons might have dual afferent and efferent functions as occurs with SP/CGRP containing afferent neurons. Interestingly, previous work has demonstrated that a selective inhibitor of nNOS attenuates the pressor reflex evoked by application of bradykinin to the left ventricular epicardium of anesthetized cats (Tjen et al., 2001). It is possible that the inhibition of local nitric oxide production by nitrgergic spinal afferent neurons contributes to this effect.

In conclusion, data obtained by retrograde tracing with HRP suggests that very limited populations of canine DRG and nodose ganglion neurons are associated with ventricular sensory neurites. About half of the ventricular afferent neurons identified in the DRG stained for CGRP, SP, or nNOS, either alone or with two markers colocalized. Separate staining showed that about 30% of the ventricular afferent neurons in the DRG exhibit CGRP immunoreactivity and binding of the putative nociceptive marker IB₄. Ventricular afferent neurons of the nodose ganglia are distinct from those in the DRG by having a smaller size and lacking immunoreactivity for SP, CGRP, and nNOS. Additional investigation is required to identify neurotransmitters that are utilized by ventricular afferent neurons of the nodose ganglion and the sizable population of DRG ventricular afferents, which lacked immunoreactivity for CGRP, SP, or nNOS.

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Fig. 1. Confocal microscopic images of single optical sections obtained by sequential scanning of a T₃ dorsal root ganglion tissue section that was triple-labeled for HRP (A, green), CGRP (B, red), and SP (C, blue). Arrowheads indicate an HRP-labeled cardiac afferent neuron (A) that also exhibits CGRP immunoreactivity (B) and appears yellow in the overlay (OVL) panel (D). CGRP and SP are colocalized in another neuron identified by arrows in panels B–D. Asterisks indicate another neuron that shows only SP immunoreactivity (C and D).

Fig. 2. Confocal microscopic images of single optical sections obtained by sequential scanning of a T₃ dorsal root ganglion tissue section that was triple-labeled for HRP (A, green), nNOS (B, red), and SP (C, blue). Arrowheads indicate an HRP-labeled cardiac afferent neuron (A) that also exhibits nNOS immunoreactivity (B) and appears yellow in the overlay (OVL) panel (D). Arrows in B and D indicate another neuron that shows only nNOS immunoreactivity. Asterisks in C and D indicate a neuron that only displayed SP immunoreactivity.

Fig. 3.

Confocal microscopic images obtained by sequential scanning of a T₂ DRG section that was triple-labeled for HRP (A, green), IB₄ (B, red), and CGRP (C, blue). Arrowheads indicate an IB₄-positive ventricular afferent neuron (B) that also shows CGRP immunoreactivity (C), and appears purple in the overlay (OVL) panel (D). The insert in panel B shows IB₄ labeling of the ventricular afferent neuron at a higher magnification. Arrows indicate another IB₄-positive neuron (B) that shows only CGRP immunoreactivity (C and D). Another image from the same section shows an HRP-positive neuron (E, asterisk) that lacks IB₄ binding (F, asterisk). Panels A–D are maximum projection images compiled from 20 optical sections spanning 7.8 μm. Panels E and F were compiled from 21 optical sections spanning 8 μm.

Fig. 4. Confocal microscopic images obtained by sequential scanning of a nodose ganglion tissue section that was triple labeled for HRP (A, green), CGRP (B, red), and SP (C, blue). Arrows indicate HRP-labeled cardiac afferent neurons (A) that exhibit no immunoreactivity for CGRP (B) or SP (C) and remain green in the overlay (OVL) panel (D). Asterisks indicate HRP-negative neurons that show immunoreactivity for CGRP (B) and SP (C). Panels A–D are maximum projection images compiled from 12 optical sections spanning 4.4 μm .

Fig. 5.

Confocal microscopic images obtained by sequential scanning of a nodose ganglion tissue section that was triple labeled for HRP (A, green), NOS (B, red), and SP (C, blue). Arrowhead indicates HRP-labeled cardiac afferent neuron (A) that exhibits no immunoreactivity for nNOS (B) or SP (C) and remains green in the overlay (OVL) panel (D). Asterisks indicate HRP-negative neurons that show immunoreactivity for nNOS (B) and SP(C). Panels A–D are maximum projection images compiled from 18 optical sections spanning 6.8 μm.

Table 1

Primary Antibodies

Antibody (abbreviation)	Host	Dilution	Supplier	Cat. No.
Substance P (SP)	Rabbit	1:1000	ImmunoStar	20064
Neuronal nitric oxide synthase (nNOS)	Mouse	1:1000	Sigma-Aldrich	N2280
Horseradish peroxidase (HRP)	Goat	1:15000	Sigma-Aldrich	P5774
Calcitonin gene-related peptide (CGRP)	Mouse	1:2500	Abcam	AB10987
Vesicular glutamate transporter 1	Guinea pig	1:100	Chemicon	AB5905
	Rabbit	1:100	Novus	NB1001837
Vesicular glutamate transporter 2	Rabbit	1:100	Synaptic Systems	135411

Table 2
Neurochemical profile of canine T₃ dorsal root ganglion neurons and the subpopulation of HRP-labeled ventricular afferents.

Sections stained for HRP, SP and CGRP			
Total neurons counted per dog ganglion (n=4)			
Right	1009 ± 301	%SP+	12.9 ± 5.7
Left	917 ± 244		12.7 ± 3.3
Combined	1925 ± 295		13.1 ± 4.7
HRP-labeled neurons counted per dog			
Right	9 ± 5 (0.89 ± 0.59 % of total)	%CGRP+	21.2 ± 8.9
Left	16 ± 9 (1.83 ± 1.30 % of total)		21.0 ± 7.4
Combined	24 ± 8 (1.26 ± 0.36 % of total)		21.1 ± 7.9
		%CGRP+ *	21.1 ± 7.9
			48.2 ± 6.0
			23.4 ± 17.2
			30.8 ± 17.3
Sections stained for HRP, SP and nNOS			
Total neurons counted per dog ganglion (n=4)			
Right	714 ± 231	%SP+	14.3 ± 6.8
Left	858 ± 424		17.7 ± 7.7
Combined	1572 ± 378		16.8 ± 6.6
HRP-labeled neurons counted per dog			
Right	6 ± 1 (0.83 ± 0.32 % of total)	%SP+	13.6 (3/22)
Left	9 ± 1 (1.39 ± 0.98 % of total)		13.9 (5/36)
Combined	15 ± 2 (0.98 ± 0.31 % of total)		13.8 (8/58)
		%nNOS+	16.2 ± 2.8
			16.6 ± 2.9
			16.3 ± 2.8
		%nNOS+	26.1 ± 11.7
			28.7 ± 21.3
			27.9 ± 16.7
		% (SP+ & nNOS+)	3.8 ± 2.5
			6.2 ± 2.0
			5.3 ± 1.6
		% (SP+ & CGRP+)	6.3 ± 5.1
			6.5 ± 3.6
			6.5 ± 4.3
		% (SP+ & CGRP+)	11.8 (4/34)
			9.5 (6/63)
			10.3 (10/97)
		%SP+ & nNOS+	3.8 ± 2.5
			6.2 ± 2.0
			5.3 ± 1.6
		% (SP+ & nNOS+)	9.1 (2/22)
			8.3 (3/36)
			8.6 (5/58)
		%SP+ only	51.0 ± 26.2
			48.6 ± 13.2
			49.3 ± 13.3

Values are mean % ± S.D. (n=4) or the % for pooled data from all 4 dogs (total marker-positive HRP-labeled neurons/total HRP-labeled neurons). *Significantly different from value for total population of neurons on same side.

* Significantly different from value for total population of neurons on same side.

Table 3Size of ventricular afferents neurons in canine T₃ DRG and left nodose ganglia.

	Long axis (μm)	Short Axis (μm)	Area (μm ²)
<u>DRG (n=4)</u>			
HRP only (28 neurons)	52 ± 4 *	30 ± 8	1371 ± 449 *
HRP + nNOS (11 neurons)	48 ± 8 *	29 ± 8	1161 ± 609
HRP + peptide (38 neurons)	51 ± 4 *	30 ± 5	1256 ± 310 *
<u>Nodose ganglia (n=3)</u>			
HRP only (31 neurons)	26 ± 11	19 ± 9	450 ± 282
CGRP (124 neurons)	41 ± 12	30 ± 7	1015 ± 482
SP (36 neurons)	51 ± 8	30 ± 4	1081 ± 352
nNOS (58 neurons)	39 ± 6	27 ± 6	912 ± 353

Values are means ± S.D.

* Significantly different from corresponding value for HRP-positive nodose ganglion neurons.