Preganglionic and Sensory Origins of Calcitonin Gene-Related Peptide-like and Substance P-like Immunoreactivities in Bullfrog Sympathetic Ganglia

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These experiments further define the organization of peptidergic pathways in the paravertebral sympathetic system of the bullfrog. Populations of axons and synaptic boutons in sympathetic ganglia 9 and 10 were found to express calcitonin gene-related peptide-like immunoreactivity (CGRP-IR) and substance P-like immunoreactivity (SP-IR). CGRP-IR is present in fibers that run through the ganglia and in boutons that make contact with almost half of the principal neurons. SP-IR is also present in fibers within the ganglia and in a rare class of synaptic boutons that are found on <1% of the principal neurons. Both forms of immunoreactivity are coexpressed in some nerve fibers and in the rare synaptic boutons that contain SP-IR. Neuropeptide Y-like immunoreactivity (NPY-IR), a marker for C-type postganglionic neurons, was used to identify the postsynaptic targets of boutons containing CGRP-IR and SP-IR. Ninety-five percent of the ganglion cells contacted by CGRP-IR boutons were negative for NPY-IR and are therefore likely to be B-type postganglionic neurons. Similarly, 100% of the ganglion cells contacted by boutons containing SP-IR were negative for NPY-IR.

Lesions of the sympathetic chain demonstrated that synaptic boutons containing CGRP-IR arise from neurons whose axons enter the chain rostral to ganglion 7. Cutting the chain between ganglia 8 and 9 eliminates all preganglionic B and C inputs to ganglia 9 and 10. The destruction of the preganglionic C pathway by this lesion was verified by staining ganglia 9 and 10 for luteinizing hormone releasing hormone (LHRH). This lesion also eliminated boutons containing CGRP-IR and drastically reduced the number of ganglionic fibers that stained for CGRP-IR and SP-IR. By contrast, cutting the sympathetic chain between ganglia 6 and 7 spared LHRH-IR in the preganglionic C pathway but still eliminated the boutons that normally express CGRP-IR and reduced the amount of staining for SP-IR.

CGRP-IR in the sympathetic ganglia arises from pregan-

glionic and sensory neurons whereas ganglionic SP-IR is purely sensory in origin. In the spinal cord, the preganglionic B and C neurons that innervate ganglia 9 and 10 are located in different segments. In segments that contain preganglionic B cells, but not those that contain C cells, there were 243 ± 37 (mean $\pm$ SD) neurons in the intermediolateral cell column that express CGRP-IR. However, no cell bodies containing SP-IR were found in this region of the spinal cord. Retrograde tracing with the fluorescent dye fast blue was used to label preganglionic neurons and sensory neurons that project to ganglia 9 and 10. Paradoxically, we were unable to detect CGRP-IR in axotomized preganglionic neurons labeled with fast blue. In addition to labeling spinal neurons, retrograde transport of fast blue from the sympathetic chain labeled 374 ± 176 sensory neurons located in ipsilateral dorsal root ganglia 4-8. When dye-filled sensory neurons were double labeled for peptides, we found that 70% contain both CGRP-IR and SP-IR, 8% contain only SP-IR, 6% contain only CGRP-IR, and 16% contain neither form of immunoreactivity.

Together the results suggest that CGRP-IR in sympathetic ganglia 9 and 10 originates from 2 sources: preganglionic B cells that selectively form synapses with 92% of postganglionic B cells, and sensory neurons that selectively form synapses with 1.4% of postganglionic B cells. The rare synapses between sensory neurons and B-type ganglion cells always coexpress SP-IR and CGRP-IR. The presence in postganglionic rami communicantes of fibers that are positive for CGRP-IR and SP-IR indicates that some sensory fibers project by way of the sympathetic chain ganglia to targets in the periphery.

Calcitonin gene-related peptide (CGRP) and substance P (SP) are expressed, either individually or together, by several subpopulations of neurons that interact with non-neuronal peripheral tissues or with neurons in autonomic ganglia. CGRP is present in many motoneurons that innervate striated muscles, in a large subset of sensory neurons (Rosenfeld et al., 1983), and also in small subsets of preganglionic autonomic neurons (Senba and Tohyama, 1988) and postganglionic sympathetic neurons (Landis and Fredieu, 1986; Lindh et al., 1987). SP is present in a subpopulation of sensory neurons in dorsal root ganglia (Hökfelt et al., 1975) and also in subsets of preganglionic autonomic neurons (Erichsen et al., 1982a, b; Krukoff et al., 1985; Bowers et al., 1986) and enteric neurons (Pearse and Polak, 1975; Schultzberg et al., 1978; Furness and Costa, 1987). Coexpression of both neuropeptides occurs throughout the pri-

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mary sensory system where most neurons containing SP are also positive for CGRP (Gibson et al., 1984), a finding that has been replicated in many laboratories (see Ju et al., 1987). This complex pattern of cellular expression suggests that CGRP and SP may influence many aspects of peripheral nervous function including the activity of the sympathetic nervous system.

Sympathetic ganglia are potentially an important site of physiological interaction between visceral motor systems and sensory systems that utilize peptide transmitters. The existence of such peripheral reflex pathways has been elegantly demonstrated in isolated preparations where, in the absence of the spinal cord, distension of the colon elicits noncholinergic slow synaptic potentials in sympathetic neurons of the inferior mesenteric ganglion (Kreulen and Peters, 1986). Although the transmitter(s) mediating these synaptic events have not been positively identified, SP and CGRP are 2 candidates under consideration. In support of this possibility many para- and prevertebral sympathetic ganglia contain axons that are immunoreactive for CGRP and/or SP (Hökfelt et al., 1977; Gamse et al., 1981; Matthews and Cuello, 1982; Jan and Jan, 1982; Lee et al., 1987). These axons are generally thought to be of sensory origin, and this has been demonstrated directly in mammalian prevertebral sympathetic ganglia through the use of retrograde tracing in combination with immunocytochemistry (Dalsgaard et al., 1982). To date, several electrophysiological actions of CGRP and SP have been elucidated in autonomic ganglia. Exogenously applied CGRP enhances the calcium-dependent shoulder and afterhyperpolarization of action potentials in parasympathetic neurons of the mammalian bladder ganglion (Nohmi et al., 1986) and produces a slow excitatory depolarization of mammalian AH/type 2 myenteric neurons (Palmer et al., 1986). Substance P appears to mediate slow excitatory postsynaptic potentials (EPSPs) in sympathetic neurons of the mammalian inferior mesenteric ganglion (Dun and Jiang, 1982; Tsunoo et al., 1982; Dun and Kiraly, 1983; Konishi et al., 1983) and possibly in mammalian myenteric neurons (Katayama and North, 1978) and in parasympathetic choroid neurons of the avian ciliary ganglion (Dryer and Chiappinelli, 1985). In tissue culture, SP modulates the currents induced by the nicotinic action of acetylcholine on avian sympathetic and parasympathetic neurons (Role, 1984). However, the functional significance of these actions of CGRP and SP remain largely unknown, and this is especially true for paravertebral sympathetic ganglia.

The present experiments were undertaken to elucidate the cellular origins of SP and CGRP in the paravertebral sympathetic system of the bullfrog. We focused attention on ganglia 9 and 10 because their cellular anatomy is simpler than that of analogous mammalian ganglia and that of prevertebral ganglia. Nonetheless, independent anatomical, physiological, and functional criteria can be used to distinguish subclasses of pre- and postganglionic neurons in the frog system. Identifying the origins of the fibers that contain CGRP and SP may therefore provide a basis for investigating the ganglionic functions of these 2 neuropeptides. Within bullfrog ganglia 9 and 10, Jan and Jan (1982) first demonstrated the presence of axons that contain SP-like immunoreactivity, and they suggested that the SP-positive axons are probably sensory through fibers. Jan and Jan (1982) also found that SP produces a slow excitatory depolarization of B cells but concluded that the slow peptidergic EPSP seen in response to stimulation of the preganglionic C pathway is mediated by luteinizing hormone releasing hormone (LHRH) and not SP. Subsequent biochemical analyses of extracts from the

cardiac ganglion indicate that bullfrog SP is similar, but not identical, to mammalian SP (Bowers et al., 1986). Comparable studies have not been done to characterize the frog antigens recognized by antibodies raised against mammalian CGRP. Consequently, the immunoreactivities demonstrated in bullfrog tissues with antibodies raised against SP and CGRP are properly referred to as SP-like immunoreactivity and CGRP-like immunoreactivity. For ease of description, we use the abbreviations SP-IR and CGRP-IR in this paper.

Recently, Kuramoto and Fujita (1986) discovered axons and synaptic boutons in bullfrog ganglia 9 and 10 that contain CGRP-IR and found that a small number of the immunoreactive boutons also contain SP-IR. They observed that many of the immunoreactive boutons were in contact with the larger ganglion cells and proposed that the CGRP-IR and/or SP-IR was localized in neurons that selectively innervate ganglionic B cells. Their novel hypothesis was further supported by double-labeling experiments which showed that CGRP-IR does not coexist with LHRH in preganglionic C axons. However, it was not possible to identify the cellular sources of the CGRP-IR and SP-IR based on these observations.

In this study we have replicated some of the earlier experiments described above and have performed 4 additional sets of experiments to establish the origins of fibers containing CGRP-IR and SP-IR in ganglia 9 and 10. First, we have taken advantage of the fact that neuropeptide Y-like immunoreactivity (NPY-IR) is a marker for postganglionic C cells (Horn et al., 1987) and used double-labeling procedures to identify the cells contacted by immunoreactive synaptic boutons. Second, nerve lesions have been used to characterize the segmental origins of the immunoreactive axons and boutons in sympathetic ganglia 9 and 10. Third, we have exploited the fact that the cell bodies of preganglionic B and C neurons projecting to ganglia 9 and 10 are located in different spinal segments (Horn and Stofer, 1988a) by examining those segments of the cord for immunoreactive cell bodies. Fourth and finally, retrograde tracing with fast blue, a fluorescent dye, has been combined with indirect immunofluorescence to characterize the immunoreactivity of sensory neurons that project to ganglia 9 and 10. Some experiments described in this paper have appeared in abstract form (Horn and Stofer, 1987, 1988c; Stofer and Horn, 1988).

Materials and Methods

Preparation of animals

Fixation. All experiments were performed on 4–6 inch bullfrogs of both sexes (Charles D. Sullivan Co., Nashville, TN). Prior to histological processing, each animal was anesthetized by immersion for 45 min in 0.4% tricaine and perfused through the heart with 50–75 ml of Ringer's solution followed by 150–200 ml of freshly made cold 4% paraformaldehyde in 50 mM NaCl, 50 mM Na phosphate buffer (pH = 7.3). Then sympathetic and sensory ganglia and the spinal cord were dissected and postfixed for 1 hr at 4°C. Tissues were washed overnight at 4°C in 25% sucrose in 100 mM Na phosphate buffer (pH = 7.3), embedded in Tissueteck (Miles), and sectioned on a cryostat at 10–48 μ m. Sections were collected directly onto gelatin-coated glass slides and stored at –20°C prior to further processing.

Lesions. In 4 experiments, the paravertebral sympathetic chain was cut between ganglia 6 and 7 or between ganglia 8 and 9. Surgery was performed under tricaine anesthesia using a lateral abdominal approach (Horn and Stofer, 1988a). Two to 4 mm of the sympathetic chain were removed in order to prevent regeneration. After making the lesion, the abdominal muscles and skin were closed with separate sets of closely spaced 6-0 silk sutures. Care was taken to keep the frogs covered with moist tissues during recovery from anesthesia, and postoperative frogs were housed in isolation. After allowing 16–21 d for nerve degeneration, animals were prepared for histological procedures as described above.

Table 1. Descriptions of primary and secondary antisera

Antigen	Host species	Label	Dilution	Code or catalog no.	Source or reference
CGRP	Rabbit	—	1:3200	CA-08-220	Cambridge Research Biochemicals
SP	Rabbit	—	1:1600	CA-08-335	Cambridge Research Biochemicals
SP	Rat	—	1:400	MCA 75S	Serotec Ltd. ^a
NPY	Rabbit	—	1:800	RPN 1702	Amersham Corporation
NPY	Sheep	—	1:1000	—	Drs. J. R. Oliver and W. W. Blessing ^b
LHRH	Rabbit	—	1:1000	4468	Dr. A. Silverman ^c
Rabbit	Goat	Rhodamine	1:1000	6330	TAGO Inc.
Rabbit	Goat	Fluorescein	1:1000	6230	TAGO Inc.
Rabbit	Goat	HRP	1:800	6430	TAGO Inc.
Rat	Sheep	Rhodamine	1:200	AAR04R	Serotec Ltd.
Goat	Swine	Fluorescein	1:600	605-270	Boehringer Mannheim Biochemicals

^a Monoclonal antibody from hybrid clone NC1/34 HL described by Cuello et al. (1979).

^b Centre for Neuroscience, Flinders University of South Australia, Bedford Park, Australia (see Blessing et al., 1986).

^c Department of Anatomy and Cell Biology, College of Physicians & Surgeons, Columbia University, New York, NY.

Retrograde labeling with fast blue. In 6 bullfrogs, fast blue (Sigma) was used to retrogradely label spinal and sensory neurons that project into sympathetic ganglia 9 and 10. Our method for applying fast blue was similar to that used previously (Horn and Stofer, 1988a) for labeling preganglionic neurons with horseradish peroxidase (HRP). Under tricaine anesthesia, the sympathetic chain was exposed and cut at the level of ganglion 9. A polyethylene cuff (PE190) was placed around the rostral end of the cut chain and filled with 2–4 μ l of 2% fast blue in water. The stock solution of fast blue was vortexed just prior to use in order to suspend undissolved particles. The cuff was then sealed with a mixture of Vaseline and mineral oil, and the animal was closed and cared for in the same manner used in lesion experiments. After survival times of 12–28 d, animals were perfused and tissues prepared for sectioning. Two of 3 animals that were used only for counting the numbers of retrogradely labeled sensory neurons were perfused with fixative containing 8% paraformaldehyde. All other dye-labeled animals were perfused with fixative containing 4% paraformaldehyde.

Immunocytochemistry

The dilutions and sources of the primary and secondary antibodies used in this work are listed in Table 1. The method for immunocytochemistry was identical, with 2 exceptions, to that previously described (Horn et al., 1987). The exceptions were a variation in the amount of triton X-100 added to primary antibody solutions and a nickel intensification method used to react HRP-coupled secondary antibodies (J. P. Hornung, personal communication). The concentration of triton was varied with section thickness: 10 μ m, 0.1–0.2%; 20 μ m, 0.2%; 48 μ m, 0.4%. In some experiments, sections of the spinal cord were reacted with a secondary goat anti-rabbit antibody coupled to HRP. After a 2-hr incubation in this antibody, the sections were washed 3 times in 100 mM Tris buffer (pH = 7.4). The washed sections were preincubated for 10 min in 0.2% NiNH_4SO_4 in 100 mM Tris buffer and then placed for 10 min in a chromagen solution containing 0.05% 3,3'-diaminobenzidine, 0.2% NiNH_4SO_4 , and 100 mM Tris buffer. The reaction was started by applying fresh chromagen solution to which 0.01% H_2O_2 had been added. After reacting for 5–10 min, the tissue was washed in Tris buffer, dehydrated, cleared, and coverslipped.

Double labeling. Four combinations of primary antibodies were used: (1) Rabbit anti-CGRP and sheep anti-NPY, (2) rabbit anti-LHRH and sheep anti-NPY, (3) rabbit anti-NPY and rat anti-SP, and (4) rabbit anti-CGRP and rat anti-SP. For all combinations, sections were incubated overnight at 4°C in the presence of both antibodies, triton X-100 (see above for concentrations), and 1% preimmune serum. Calf serum was used with the 2 combinations that included sheep anti-NPY serum, and goat serum was used with the other 2 antibody combinations. After incubation in primary antibodies, the sections were washed 3 times for 5 min in PBS. The secondary antibodies used with primary antibody combinations 1 and 2 were always a goat anti-rabbit serum and a swine anti-goat serum. To preclude the possibility of spurious binding between these secondary antisera, they were applied sequentially to the tissue

with an intervening blocking step (Horn and Stofer, 1988b). In these cases, the sections were initially incubated in diluted swine anti-goat antibody coupled to fluorescein and 1% calf serum in PBS. After 30 min at room temperature, the sections were washed 3 times with PBS. Unbound sites on the anti-goat serum were then blocked by incubating the sections for 30 min in 1% goat serum. The sections were then washed 3 times in PBS followed by incubation for 30 min at room temperature in diluted goat anti-rabbit antisera and 1% goat serum in PBS. Finally, the sections were washed with PBS and coverslipped with 1:1 PBS: glycerin. Control experiments (below) demonstrated that there was no binding between secondary antibodies in sections that were processed in this manner. With combinations 3 and 4 of primary antibodies, the blocking step was omitted because the secondary antibodies used under these conditions were not directed against each other. In these cases, the sections were washed after incubation in primary antibodies, and both secondary antibodies were applied simultaneously in the presence of 1% goat serum and allowed to react for 30 min at room temperature prior to final washing and coverslipping.

Controls. Several types of controls were run to test the specificity of the observed staining. Previous preabsorption controls with authentic NPY and LHRH have shown that 10^{-7} M concentrations of these peptides selectively block the staining produced in bullfrog sympathetic ganglia by the anti-LHRH serum and by both anti-NPY sera and that 10^{-5} M concentrations of authentic CGRP, SP, neurotensin, and bombesin do not block the staining produced by these antibodies (Horn et al., 1987; Horn and Stofer, 1988b). For the present study, additional preabsorption controls were run to test the rabbit anti-CGRP, rabbit anti-SP, and rat anti-SP antibodies. Each of the 3 antibodies was preincubated for 24 hr at 4°C in the presence of 10^{-5} , 10^{-6} , and 10^{-7} M concentrations of CGRP, SP, LHRH, bombesin, and neurotensin. Authentic peptides were obtained from Bachem, except for LHRH, which came from Peninsula. Sections of sympathetic ganglia, dorsal root ganglia, and spinal cord were stained with each of the preabsorbed antibodies. Staining by all 3 antibodies in each tissue was selectively blocked by 10^{-7} M concentrations of appropriate peptides. For the rabbit anti-CGRP and rat anti-SP, 10^{-5} M concentrations of inappropriate peptides had no effect on staining. However, staining with the rabbit anti-SP was partially inhibited by 10^{-5} M CGRP and neurotensin but unaffected by 10^{-6} M concentrations of these peptides. None of the secondary antibodies bound to the 3 types of tissue in the absence of primary antibodies.

Additional control experiments were run to test for inappropriate binding in double label experiments between secondary and primary antibodies and between secondary antibodies. Controls of this type for double-labeling with rabbit anti-LHRH and sheep anti-NPY have already been described (Horn and Stofer, 1988b). For the other 3 double-label combinations described above, the following controls were run with sections of sympathetic ganglia, dorsal root ganglia, and spinal cord. To test for inappropriate binding between secondary and primary antibodies, each primary antibody was reacted with the inappropriate secondary antibody used in each of the 3 double-label combinations.

All tissue treated in this way was negative. To test for possible binding between the secondary antibodies, each primary was then run with both secondaries from each of the 3 double-label combinations. In all cases, only the appropriate immunofluorescent staining was seen.

Analysis

Processed sections were viewed under a Zeiss Standard microscope equipped for brightfield and epifluorescence (HBO 100) with filter sets for fast blue (48-77-02), fluorescein (48-77-10), and rhodamine (48-77-15). Photographs were taken with an Olympus OM-4 camera back and Ilford HP5 black and white film.

In 2 sets of experiments, numbers of labeled spinal neurons and sensory neurons were estimated from counts of labeled cell profiles in serially sectioned tissue. In the first case, we counted those neuronal profiles in the intermediolateral cell column that contained CGRP-IR. These cells were labeled with an HRP-coupled secondary antibody and counted under brightfield illumination at a magnification of 400 $\times$. In the second case, sensory neuron profiles that had been labeled retrogradely with fast blue were counted under epifluorescent illumination at 250 $\times$. In both cases, measurements of cell size were then made. For the spinal cells a camera lucida was used to draw a series of 318 cells at a magnification of 1000 $\times$, and for the sensory cells, photographs were made of 71 fields containing 321 fluorescent profiles. Areas of the labeled cell profiles were measured from the drawings and photographic prints using an Apple Macintosh computer, a digitizing tablet (MacTablet, Summagraphics, Fairfield, CT), and a program called MacMeasure (version 1.9) that was given to us by W. Rasband of the Research Services Branch at NIMH. The raw counts of labeled cell profiles were then adjusted for double counting of split cells using correction factors calculated from section thickness and average diameters of labeled profiles (Abercrombie, 1946).

Results

Localization of CGRP-IR and SP-IR in sympathetic ganglia 9 and 10

Several populations of axons and synaptic boutons within sympathetic ganglia 9 and 10 are positive for CGRP-IR and SP-IR (Fig. 1). Immunoreactive principal neurons were never observed. Axons showing both forms of immunoreactivity are also present in the sympathetic chain at the rostral end of ganglion 9 and in the postganglionic rami communicantes of ganglia 9 and 10. The monopolar shape of amphibian autonomic neurons and the size of presynaptic nerve terminals enable one to identify with light microscopy synaptic boutons that make contact with the somata and initial axon segments of principal neurons (McMahan and Kuffler, 1971). We found that many of the larger cell bodies in ganglia 9 and 10 are covered with synaptic boutons containing CGRP-IR (Fig. 1*A*). The immunoreactive boutons are several microns in diameter and are connected to one another by smaller processes on the surfaces of the postganglionic neurons. When viewed in section (10 μ m), immunoreactive boutons often appear to form halos around postganglionic cell bodies. Immunoreactive processes are also seen to form spirals around the initial axon segments of some postganglionic neurons. Although synaptic boutons containing SP-IR are also present in ganglia 9 and 10, they are much rarer than those containing CGRP-IR. It was generally necessary to examine several sections before finding SP-IR boutons in contact with a ganglion cell. Figure 1*B* illustrates the largest cluster of such cells that was found during this study. It contains 5 relatively large ganglion cells that are heavily invested with SP-IR boutons. Typically, no more than 2 or 3 such cells were observed in a single section. Another distinctive feature of synaptic boutons containing SP-IR was that they tended to be more densely clustered on the somata and axon hillocks of principal neurons than did most boutons containing CGRP-IR (compare Fig. 1*B* with Fig. 1*A*).

Double-labeling the ganglia with a rabbit antiserum to CGRP and a rat monoclonal antibody to SP revealed that some axons express both forms of immunoreactivity (Fig. 2). In addition, there are fibers that contain only CGRP-IR or SP-IR. No attempt was made to quantify the relative numbers of axons containing different combinations of CGRP-IR and SP-IR as it would require resolution beyond that of light microscopy; however, all 3 possible combinations of immunoreactivity are present in fibers located among cell bodies in the ganglia (Fig. 2, *A, B*), in large fiber bundles within the sympathetic chain (Fig. 2, *C, D*), and in the rami communicantes of ganglia 9 and 10 (Fig. 2, *E, F*). Since the rami of ganglia 9 and 10 do not contain preganglionic axons, it seems likely that at least some of the immunoreactive axons arise from a different source, possibly sensory neurons.

One would predict the coexpression of CGRP-IR and SP-IR in synaptic boutons to be infrequent based on the finding that CGRP-IR boutons contact large numbers of ganglion cells whereas SP-IR boutons are rare. Double labeling confirmed this prediction (Fig. 2, *A, B*). However, occasional neurons were found to be in contact with synaptic boutons that were positive for both CGRP-IR and SP-IR (Fig. 2, *C, D*). Careful examination of these cells revealed that both forms of immunoreactivity were coexpressed in the same boutons and not in separate boutons converging on a common postsynaptic target (Figs. 2, *C, D*; 6, *A, B*). To estimate the frequency of these rare cells, ganglia 9 and 10 were removed unilaterally from 3 frogs and serially sectioned an 10 μ m. Every second section was then double-labeled for SP and CGRP. A total of 89 cell profiles contacted by boutons with SP-IR were found, and 100% were also positive for CGRP-IR. Based on previous cell counts (Horn et al., 1987), the 89 profiles covered with double-labeled boutons represent <1% of all principal neurons.

In addition to postganglionic neurons, bullfrog sympathetic ganglia contain small islands of chromaffin cells. These cells are also known as small intensely fluorescent (SIF) cells. We never observed SIF cells containing either CGRP-IR or SP-IR. Some SIF cells are innervated by preganglionic C fibers (Dunn and Marshall, 1985), and some SIF cell clusters contain axons that are immunoreactive for LHRH (Horn and Stofer, 1988b). By contrast, the occurrence in SIF cell clusters of fibers that contain CGRP-IR and/or SP-IR is exceedingly rare. During the present experiments, we observed only one example of a SIF cell cluster containing a few fibers that were positive for both CGRP-IR and SP-IR.

Synaptic contacts with postganglionic B cells

The next series of experiments was designed to identify the principal neurons that receive inputs from boutons expressing CGRP-IR and SP-IR. Sympathetic neurons in ganglia 9 and 10 are classified as fast B, slow B, and C-type cells on the basis of electrophysiological criteria: a) Action potential conduction velocities of postganglionic axons and b) selective innervation by preganglionic B and C axons (Dodd and Horn, 1983). More recent studies have shown that NPY-IR is a marker for electrophysiologically identified C cells (Horn et al., 1987) and that double labeling for LHRH, a marker for preganglionic C cells (Jan et al., 1980), and for NPY-IR can be used to demonstrate the specificity of synaptic connections between pre- and postganglionic elements of the sympathetic C pathway (Horn and Stofer, 1988b). At present, there are no anatomical markers that distinguish fast B from slow B neurons.

Figure 1. Immunofluorescence in sympathetic ganglion 9 after processing with primary antibodies to CGRP (*A*) and to SP (*B*) and a secondary antisera coupled to rhodamine. *A*, CGRP-IR is prominent in synaptic boutons on the surface of postganglionic neurons. Five examples of neurons covered by immunoreactive boutons are numbered (1–5). In addition, there are immunoreactive fibers running through the field (arrows). Note that many of the labeled fibers have a beaded appearance. *B*, An unusual example of 5 principal neurons (numbered 6–10) that are covered with SP-IR boutons. This field is unusual because neurons are contacted by SP-IR boutons that are generally rare and scattered throughout the ganglia (see text). Note that SP-IR boutons on each neuron tend to be more densely packed than those containing CGRP-IR. Fibers containing SP-IR (arrows) are commonly seen in sympathetic ganglia 9 and 10.

Figure 2. Three examples of double labeling in sympathetic ganglion 9 for CGRP-IR (*A, C, E*) and SP-IR (*B, D, F*). Fluorescein and rhodamine coupled secondary antisera, respectively, were used to visualize the 2 forms of immunofluorescence. Panels *A* and *B* depict a field in the center of the ganglion that is composed primarily of cell bodies, many of which are surrounded by boutons containing CGRP-IR (*A*). Five examples are numbered 1–5. In panel *B* it can be seen that none of the boutons contacting these 5 cells are positive for SP-IR. *A* and *B* also contain stained axons that are either double labeled (*double arrows*) or single labeled (*single arrows*). The field depicted in *C* and *D* was located at the rostral end of ganglion 9 and contains a large fiber tract (the sympathetic chain) and a scattering of postganglionic neurons. In addition to single- and double-labeled fibers running along the chain, note the presence of one neuron at the top of the field (*curved arrow*). Its axon hillock region is surrounded by synaptic boutons that are double labeled for CGRP-IR and SP-IR. Panels *E* and *F* depict a postganglionic ramus at its juncture with the body of ganglion 9. The ramus contains immunofluorescent axons that are single labeled (*single arrows*) and double labeled (*double arrows*).

Figure 3. Double immunofluorescence in sympathetic ganglion 9 for CGRP-IR with rhodamine (*A*) and NPY-IR with fluorescein (*B*). Note that most of the neurons at the left of the field are devoid of synaptic boutons with CGRP-IR and are positive for NPY-IR. Running up through the middle of the field are a group of neurons that are surrounded by CGRP-IR boutons. Ten of these neurons are numbered (1–10), and it can be seen that they are all negative for NPY-IR. Exceptions to this pattern are present above neuron 9 at the top of the field. These cells are positive for NPY-IR and are in contact with CGRP-IR boutons.

After double labeling ganglion sections with sheep anti-NPY serum and rabbit anti-CGRP serum, we found that the great majority of principal neurons contacted by synaptic boutons containing CGRP-IR were negative for NPY-IR and that most NPY-negative profiles were contacted by immunoreactive boutons (Fig. 3). In other words, CGRP-IR boutons selectively form contacts with the majority of postganglionic B cells. To quantify our observations, microscope fields from 28 double-labeled non-adjacent sections (3 animals) were photographed at a magnification of $250\times$ and analyzed. The sample contained a total of 1,413 postganglionic cell profiles, 50.3% of which were positive for NPY-IR. This level of NPY-IR expression is consistent with estimates based on more extensive cell counts in serially sectioned ganglia (Horn et al., 1987). In the present sample of sections, 91.9% (686 of 746) of NPY-negative cell profiles and 3.8% (25 of 667) of NPY-IR cell profiles were contacted by CGRP-IR boutons. Conversely, when the photographs were scored first for CGRP-IR boutons, 95.0% (400 of 421) of the bouton clusters were found to be in contact with NPY-negative cells.

A similar approach was used to identify the relatively rare neurons that are contacted by boutons containing SP-IR. Remaining alternate serial sections from the 3 animals that had been used to count neurons contacted by boutons that coexpress SP-IR and CGRP-IR were double-labeled with rabbit antiserum to NPY and monoclonal rat antibody to SP. In this set of sections there were 86 neuron profiles contacted by boutons with SP-

IR, all of which were negative for NPY-IR. Examples of the staining are illustrated in Figure 4.

Thus it appears that 2 groups of CGRP-IR boutons selectively contact B-type postganglionic neurons. The major group of boutons does not contain SP-IR and contacts 92% of the ganglion cells that are negative for NPY-IR. The minor group of boutons coexpresses SP-IR and forms contacts only with neurons that are negative for NPY-IR. In the 3 sets of serial ganglion sections that were stained for SP-IR, there were a total of 175 cell profiles in contact with SP-IR boutons (89 from SP/CGRP double label and 86 from SP/NPY double label). We previously estimated that ganglia 9 and 10 contain 3167 principal neurons that are negative for NPY-IR and that the correction factor for double-counting these cells in $10\text{-}\mu\text{m}$ sections is 0.71 (Horn et al., 1987). This implies that 1.4% of B cells are contacted by boutons containing both CGRP-IR and SP-IR.

Lesions of the sympathetic chain

To determine the segmental origins of the fibers and synaptic boutons in ganglia 9 and 10 that contain CGRP-IR and SP-IR, we cut the sympathetic chain in several animals. Immunoreactivity in sympathetic ganglia from lesioned and control sides was compared after allowing 16–21 d for degeneration.

In 2 animals, the paravertebral chain was cut between ganglia 6 and 7. Based on electrophysiological evidence (e.g., Jan and Jan, 1982; Dodd and Horn, 1983), this procedure should selectively destroy the preganglionic B input to ganglia 9 and 10.

Figure 4. Two examples of double immunofluorescence in sympathetic ganglia 9 and 10 for SP-IR with rhodamine (*A, C*) and NPY-IR with fluorescein (*B, D*). Panel *A* illustrates the typical appearance of a ganglion cell (1) covered with SP-IR boutons. It has a relatively large diameter and is the only cell of its kind in the field. The axon hillock is to the left of the soma. At the bottom of the field there is a large fiber bundle with numerous axons containing SP-IR. Panel *B* demonstrates that cell 1 does not contain NPY-IR and is therefore a B cell. Panel *C* illustrates a second example of a neuron (2) covered by SP-IR boutons and negative for NPY-IR (*D*). Once again, cell 2 is the only cell of its kind in the field.

To verify that the C pathway remained intact after the lesion, some sections were double labeled using rabbit antiserum to LHRH and sheep antiserum to NPY. LHRH-IR is localized in preganglionic C cells (Jan et al., 1980; Jan and Jan, 1982) that selectively form synapses on postganglionic C cells containing NPY-IR (Horn and Stofer, 1988b). As expected, the pattern of double labeling for LHRH-IR and NPY-IR that was seen after cutting the preganglionic B pathway (Fig. 5, *A, B*) is indistinguishable from that seen in control ganglia. However, this lesion almost entirely eliminated the synaptic boutons that contain only CGRP-IR (Fig. 6). Although a few axons containing both CGRP-IR and SP-IR were still present after this lesion, it was our impression that their prevalence was reduced (compare Fig. 6 with Fig. 2). In addition, there always remained in the lesioned ganglia a few cells which were contacted by boutons coexpressing CGRP-IR and SP-IR (Fig. 6, *A, B*).

In 2 other animals, the paravertebral chain was cut between ganglia 8 and 9. This procedure destroys both the preganglionic B and C inputs to ganglia 9 and 10. After this lesion, boutons containing LHRH-IR were absent and the pattern of cellular staining for NPY-IR appeared normal (Fig. 5, *C, D*). Cutting the chain at this level also eliminated the presence of boutons

containing CGRP-IR and of most axons containing CGRP-IR and/or SP-IR (Fig. 7). Although a few boutons containing SP-IR remained in these ganglia, we did not attempt to count them owing to their scarcity. Together the effects of both types of lesions show that, in ganglia 9 and 10, virtually all CGRP-IR and SP-IR arises from axons in the sympathetic chain and that the synaptic boutons containing only CGRP-IR arise from axons that enter the chain rostral to ganglion 7.

Localization of CGRP-IR and SP-IR in the spinal cord

To test the possibility that immunoreactive synaptic boutons in sympathetic ganglia 9 and 10 belong to preganglionic neurons, we labeled the spinal cord for CGRP-IR and SP-IR. CGRP-IR is present in 3 areas: Motorneurons in the ventral horn, fibers in the dorsal horn, and neurons in the intermediolateral cell column (Fig. 8). In a previous tracing study, Horn and Stofer (1988a) showed that preganglionic neurons supplying the ninth and tenth ganglia are located in the intermediolateral cell column. In transverse sections of the spinal cord, the column occupies the lateral half of the white matter and extends from the level of the central canal for about 150 μ m in the dorsal direction. In the rostrocaudal axis, the column of preganglionic neu-

Figure 5. Double immunofluorescence in sympathetic ganglia 9 and 10 for LHRH-IR with rhodamine (*A, C*) and NPY-IR with fluorescein (*B, D*) after lesions of the sympathetic chain. Panels *A* and *B* depict a field in ganglion 9 from an animal in which the sympathetic chain had been cut between ganglia 6 and 7. Its appearance is indistinguishable from unlesioned controls. Many of the principal neurons, especially the smaller ones, are in contact with LHRH-IR synaptic boutons (*A*). Virtually every such cell is positive for NPY-IR (*B*). The arrows labeled 1–3 point to 3 examples of immunoreactive boutons (*A*) and the neurons that they contact (*B*). Panels *C* and *D* depict a field in ganglion 10 from an animal in which the sympathetic chain had been cut between ganglia 8 and 9. In this case, LHRH-IR is entirely eliminated (*C*), but the distribution of ganglion cells containing NPY-IR appears normal. The arrows labeled 4–6 point to 3 examples of neurons that are devoid of immunoreactive synaptic boutons (*C*) yet express NPY-IR (*D*). These results demonstrate that the first lesion spares the preganglionic C pathway and that the second lesion destroys this pathway.

rons that projects to ganglia 9 and 10 extends from a point slightly caudal ($\approx 300 \mu\text{m}$) to the entry zones of dorsal root 4 to a point approximately midway between the entry zones of roots 7 and 8. All the preganglionic B neurons in this pool are located rostral to dorsal root 6, and all the preganglionic C neurons are located caudal to dorsal root 6. In the present study, we found CGRP-IR in neurons that lie in the intermediolateral cell column, but only in sections cut rostral to dorsal root 6 (Fig.

8). These immunoreactive neurons are similar in size to preganglionic neurons. Occasional axons containing CGRP-IR were found to extend from the dorsal horn into the intermediolateral cell column and possibly make contact with the cell bodies and proximal dendrites of neurons containing CGRP-IR (Fig. 8C).

The pool of neurons that are located in the intermediolateral cell column and contain CGRP-IR was reconstructed in 3 animals. Each spinal cord was cut into serial $48\text{-}\mu\text{m}$ sections be-

Figure 6. Double immunofluorescence in sympathetic ganglia 9 and 10 for CGRP-IR with fluorescein (*A*) and SP-IR with rhodamine (*B*) and for CGRP-IR with rhodamine (*C*) and NPY-IR with fluorescein (*D*) after cutting the sympathetic chain between ganglia 6 and 7. This lesion eliminates virtually all of the synaptic boutons that contain CGRP-IR (*A* and *C*) and reduces the number of CGRP-IR fibers. Arrows in panels *A* and *C* point to some of the CGRP-IR fibers that remain after the lesion. The curved arrow in panel *A* points to CGRP-IR synaptic boutons covering the surface of one ganglion cell. These boutons also contain SP-IR (curved arrow in *B*). Comparison of the straight arrows in panels *A* and *B* shows that many of the stained fibers that remain after the lesion contain both CGRP-IR and SP-IR. Panel *D* shows that the pattern of cellular staining for NPY-IR is normal. None of the 4 NPY-negative neurons, numbered 1–4, in panels *C* and *D* are in contact with CGRP-IR boutons.

Figure 7. Double immunofluorescence in sympathetic ganglia 9 and 10 for CGRP-IR with fluorescein (A) and SP-IR with rhodamine (B) and CGRP-IR with rhodamine (C) and NPY-IR with fluorescein (D) after cutting the sympathetic chain between ganglia 8 and 9. This lesion eliminates virtually all immunoreactive synaptic boutons (A, B, and C) but does not affect cellular staining for NPY-IR (D). Arrows point to a few remaining fibers that contain CGRP-IR (A and C) and SP-IR (B). Four examples (1–4) of neurons that are negative for NPY-IR and devoid of CGRP-IR boutons are numbered in panels C and D.

Figure 8. Staining for CGRP-IR in the spinal cord using secondary antiserum coupled to HRP. *A*, Section cut slightly caudal to dorsal root 4 contains immunoreactive fibers in the dorsal horn, motoneurons (*mn*) in the ventral horn, and relatively small neurons in the intermediolateral cell column (*iml*). *B*, Higher-magnification view of labeled neurons in the section shown in panel *A*. *C*, Another example of an immunoreactive neuron in the intermediolateral cell column from a section cut between the entry zones of dorsal roots 4 and 5. Note that immunoreactive fibers from the dorsal horn appear to make contact (arrows) with the labeled cell body and one of its dendrites. *D*, Low-power view of CGRP-IR in a section cut at the level of spinal nerve 3. Fibers in the dorsal horn and brachial motoneurons in the ventral horn are labeled. There are no labeled cells in the intermediolateral cell column. *E*, CGRP-IR in a section cut slightly caudal to the entry zone of dorsal root 7. Again, fibers in the dorsal horn and motoneurons are immunoreactive, but there are no labeled cells in the intermediolateral cell column.

tween the levels of spinal roots 3 and 7 and processed with an HRP-coupled second antibody. We then counted the number of immunoreactive neurons in the intermediolateral cell column on both sides of the cord. The areas of labeled profiles were also measured and were used to calculate the average cell diameter.

In a sample of 318 profiles drawn in equal numbers from each of the 6 intermediolateral cell columns the average diameter was $16.5 \pm 3.0 \mu\text{m}$ ($\pm\text{SD}$). After a correction factor of 0.744 was applied to the raw counts (Abercrombie, 1946), there were 279 ± 53 (mean $\pm$ SD) neurons containing CGRP-IR in each

Figure 9. Segmental distribution of neurons in the intermediolateral cell column that contain CGRP-IR. The spinal cord was serially sectioned (48 μ m) and immunoreactive cell profiles were counted in each section and are plotted. Panels A, B, and C are data from the left sides of 3 spinal cords. Segmental boundaries are marked and labeled on the upper axis of each plot.

of the 6 cell columns. The rostrocaudal extent of the column is illustrated in Figure 9 where the number of labeled cell profiles in each section is plotted for the left sides of the 3 cords. Data from the right sides were similar in appearance. The most striking feature of these plots is the high density of labeled cells between the levels of spinal nerves 4 and 5. Moving caudally, the number of labeled cells tapers off and then disappears somewhere between spinal roots 5 and 6. Both the density profile and the caudal boundary of the pool of immunoreactive neurons thus coincide with that of preganglionic B neurons projecting to ganglia 9 and 10 (compare Fig. 9 with figure 9 in Horn and Stofer, 1988a). The rostral boundary of the pool of immunoreactive neurons was observed to be slightly rostral to nerve 4 (Fig. 9A) or to coincide with nerve 4 (Fig. 9, B, C). Based on retrograde tracing (Horn and Stofer, 1988a), none of the immunoreactive neurons lying rostral to the level of nerve 4 are likely to project to sympathetic ganglia 9 and 10. Discounting

these cells, there were a total of 243 ± 37 (mean $\pm$ SD, $n = 6$) immunoreactive neurons in the intermediolateral cell column between the levels of spinal nerves 4 and 6.

In 4 other frogs, preganglionic neurons were retrogradely labeled by applying fast blue to the sympathetic chain at the level of ganglion 9 and then processed for CGRP-IR using secondary antibodies coupled to HRP and to rhodamine. Using either label, it was possible to visualize CGRP-IR in some neurons within the intermediolateral cell column. However, despite numerous attempts in which the conditions of staining were varied, we were unable to detect clear-cut immunoreactivity in the fast blue-labeled neurons. This indicates either that cells in the intermediolateral column that contain CGRP-IR are not preganglionic B neurons or that axotomy inhibits the expression of CGRP-IR in these cells. Although neither possibility can be eliminated, we favor the latter based on the other results of this study (see Discussion).

Figure 10. SP-IR in the spinal cord between the levels of spinal nerves 4 and 5. This 20- μ m section was incubated with rabbit antiserum to SP and a secondary antibody coupled to HRP. Immunoreactive axons are present in Lissauer's tract, the dorsal horn (*dh*) and in gray matter dorsal to central canal (marked with an *X*). Small arrows point to the medially located immunoreactive fibers and to a faint band of fibers that extends laterally to the intermediolateral cell column (*iml*). SP-IR is not present in cell bodies in this section.

In contrast to the preceding results, SP-IR in the spinal cord between the levels of spinal roots 3 and 8 was localized primarily in fibers (Fig. 10). The densest staining was seen in the dorsal horn and in fibers located dorsal to the central canal. In some sections, there was also faint SP-IR extending laterally from the central canal to the region of the intermediolateral cell column. Only a few spinal neurons were found to contain SP-IR, and they were located around the central canal at the level of nerve 8, a position that is medial and caudal to the pool of preganglionic neurons projecting to ganglia 9 and 10. The same overall pattern of staining for SP-IR in the cord was seen in normal animals and in animals with lesions of the sympathetic chain.

Spinal neurons therefore are unlikely to be the source of SP-IR in sympathetic ganglia.

Sensory neurons that project to sympathetic ganglia

The final series of experiments was designed to characterize CGRP-IR and SP-IR in sensory neurons and to determine which of these cells project to sympathetic ganglia 9 and 10. Large populations of sensory neurons in dorsal root ganglia 2 through 10 contain one or both forms of immunoreactivity (Fig. 11). The size distribution of sensory neurons in these ganglia is quite broad; some cell bodies are distinctly large (40–70 μ m) whereas others are very small (10–30 μ m). Although we did not systematically assess the distribution of immunoreactive sensory neurons, it was our impression that CGRP-IR is present in the majority of sensory neurons including cells of all sizes. By contrast, SP-IR was present in a smaller subset of sensory neurons, most of which appeared to be relatively small. In double-labeled material (Fig. 11), some neurons were found to contain both SP-IR and CGRP-IR, and others were found to express only one form of immunoreactivity or neither form of immunoreactivity.

To identify the sensory neurons that project to sympathetic ganglia 9 and 10, the sympathetic chain was cut at the level of ganglion 9 in 3 animals and placed in a cuff containing fast blue. Dorsal root ganglia 2–8 were then removed, serially sectioned and examined for cell profiles that were retrogradely labeled with fast blue. All of the labeled sensory neurons were found in ipsilateral dorsal root ganglia 4–8. The labeled profiles were counted and their segmental distribution is expressed in Figure 12 as the percent of total labeled profiles. Note that the number of labeled neurons per ganglion increases monotonically as one moves caudally from dorsal root ganglion 4 to ganglion 8 and that two thirds of the labeled cells are in ganglia 7 and 8.

In order to estimate the actual number of sensory neurons labeled with fast blue, we used the method of Abercrombie (1946) to correct the counts of cell profiles. Because many cell nuclei were obscured by the dye, correction factors were calculated using the average diameter of labeled cell profiles. The diameters were obtained by measuring the areas of labeled pro-

Figure 11. Double immunofluorescence in dorsal root ganglion 6 for CGRP-IR with fluorescein (*A*) and SP-IR with rhodamine (*B*). Ten cells in this field have been numbered. Cells 1–5 coexpress both forms of immunoreactivity, cells 6–8 express only CGRP-IR, and cells 9 and 10 are negative for CGRP-IR and SP-IR.

Figure 12. The segmental distribution of sensory neurons that project to sympathetic ganglia 9 and 10. Data from 3 animals in which the sympathetic chain was cut between ganglia 8 and 9 and exposed to fast blue. After retrograde transport of the dye, there were labeled sensory neurons in ipsilateral dorsal root ganglia 4–8. For each animal, the labeled cell profiles were counted in each ganglion and then expressed as a percentage of the total labeled profiles in that animal. The means $\pm$ SD of these percentages are plotted in the histogram. Note that the relative number of sensory neurons projecting to sympathetic ganglia 9 and 10 increases from dorsal root ganglion 4 to ganglion 8.

files from photographs on non-adjacent sections of ganglia 4–8. In 10- μ m sections the average diameter of 137 labeled profiles was $21.6 \pm 4.3 \mu\text{m}$ (mean $\pm$ SD), and in 20- μ m sections the average diameter of 184 labeled profiles was $22.0 \pm 4.5 \mu\text{m}$. Both sets of diameters had unimodal distributions that were not significantly different ($p = 0.204$ for 10 μm data and $p = 0.298$ for 20 μm data) from the normal distribution (Kolmogorov–Smirnov one sample test). After correction factors of 0.316 (10- μ m sections) and 0.455 (20- μ m sections) were applied, there were a total of 374 ± 176 (mean $\pm$ SD) labeled sensory neurons ($n = 3$ animals) in ganglia 4–8.

To assess the peptide content of sensory neurons that project to sympathetic ganglia 9 and 10, 3 additional animals were labeled with fast blue, and sections of dorsal root ganglia 4–8 were double-labeled for CGRP-IR and SP-IR. Figure 13 illustrates an example of the triple labeling. A sample of 1531 fast blue-labeled cell profiles from ganglia 4–8 in 3 animals was then scored for immunoreactivity. Overall, 70% of the cell profiles were positive for both CGRP-IR and SP-IR, 6% contained only CGRP-IR, 8% contained only SP-IR, and 16% were not immunoreactive for either peptide. Although the distribution of peptide expression in fast blue-labeled neurons varied between segmental ganglia (Fig. 14), the differences were relatively small and followed no obvious trend.

Discussion

The results of this study, in conjunction with earlier work by others, support 4 primary conclusions that are listed below and schematically presented in Figure 15. The evidence is summarized in the succeeding discussion.

1. Preganglionic B, but not C, neurons express CGRP-IR. In sympathetic ganglia 9 and 10, the CGRP-IR containing axons of preganglionic B cells selectively form synapses on postganglionic B neurons that lack NPY-IR.

2. There is a large sensory projection from dorsal root ganglia 4–8 into sympathetic ganglia 9 and 10. Many of these neurons project through the ganglia to the periphery.

3. All SP-IR in sympathetic ganglia 9 and 10 is localized in

Figure 13. Double immunofluorescence in sensory neurons of dorsal root ganglion 8 after retrograde labeling with fast blue. *A* depicts fast blue fluorescence, *B* depicts CGRP-IR with fluorescein, and *C* shows SP-IR with rhodamine. This field contains 3 dye-labeled neurons, 2 of which coexpress CGRP-IR and SP-IR. The third dye-labeled cell is negative for both forms of immunoreactivity.

Figure 14. The segmental distribution of immunoreactive sensory neurons that project to sympathetic ganglia 9 and 10. Data from 3 animals in which the sympathetic chain had first been cut between ganglia 8 and 9 and exposed to fast blue. A sample of sections from dorsal root ganglia 4–8 were then double-labeled for CGRP-IR and SP-IR, and the cell profiles that contained fast blue were scored for immunoreactivity. In each ganglion, the numbers of cells containing the 4 possible combinations of immunoreactivity are expressed as percentages of the total fast-blue-labeled profiles at that segmental level. The number of cell profiles contributing to each bar is listed above in parentheses. The bar at the right presents the combined data from the 5 sensory ganglia. Overall, 84% of the neurons contained at least one form of immunoreactivity, and 70% coexpressed CGRP-IR and SP-IR.

sensory fibers. The majority of sensory neurons that project to these ganglia coexpress CGRP-IR and SP-IR, and the remainder express only CGRP-IR or SP-IR or neither peptide.

4. Some sensory neurons that coexpress CGRP-IR and SP-IR selectively form synapses with a small population of postganglionic B neurons in ganglia 9 and 10.

Confirmation of earlier observations

Jan and Jan (1982) first established the presence of axons in sympathetic ganglia 9 and 10 that contain SP-IR. They also demonstrated that postganglionic C neurons generally are smaller than B cells and that LHRH-IR is localized in preganglionic C neurons that selectively innervate the smaller postganglionic neurons (Jan et al., 1980). More recently, Kuramoto and Fujita (1986) confirmed the presence of fibers containing SP-IR and discovered the rare population of synaptic boutons that coexpress SP-IR and CGRP-IR. In addition, they found that many of the larger ganglion cells are contacted by boutons containing only CGRP-IR and that these cells are distinct from those innervated by fibers containing LHRH. They pointed out that the fibers containing CGRP-IR and SP-IR could originate from preganglionic and sensory neurons and proposed that these fibers selectively innervate B-type postganglionic neurons. In the present work we confirmed that subpopulations of axons and synaptic boutons within ganglia 9 and 10 express CGRP-IR and SP-IR, and we did additional experiments to identify these axons and boutons.

CGRP-IR in preganglionic B neurons

The most direct proof that preganglionic B cells contain CGRP-IR would be to demonstrate that retrogradely labeled neurons in the spinal cord are immunoreactive. Although this experiment yielded a negative result, we believe that other indirect evidence strongly supports our first conclusion. Double labeling in ganglia 9 and 10 demonstrated that the vast majority of synaptic boutons containing CGRP-IR are negative for SP-IR (Fig. 2). Consequently, the source of the boutons must be preganglionic and/or sensory neurons that express only CGRP-IR. The lesion experiments demonstrate that such fibers enter the sympathetic chain rostral to ganglion 7 (Figs. 6, 7). Preganglionic

B, but not C, cells are segmentally distributed in this manner (Dodd and Horn, 1983; Horn and Stofer, 1988a). In addition, a population of sensory neurons in dorsal root ganglia 4 through 6 have axons that enter the sympathetic chain rostral to ganglion 7 and project to the ninth and tenth ganglia (Fig. 12). One could therefore argue that sensory neurons can account for the presence of CGRP-IR boutons in ganglia 9 and 10 and for the effects of chain lesions on these boutons. Pursuing this hypothesis, dorsal root ganglia 4, 5, and 6 contain respectively 4.6%, 9.1%, and 19.4% of the 374 sensory neurons that project to ganglia 9 and 10 (Fig. 12). Of these neurons, 9.6%, 6.1%, and 7.6% in ganglia 4, 5, and 6 contain only CGRP-IR (Fig. 14). Based on these numbers, one can estimate that a total of 9 sensory neurons containing only CGRP-IR project from dorsal root ganglia 4, 5, and 6 to sympathetic ganglia 9 and 10. The targets in ganglia 9 and 10 consist primarily of 3167 neurons that are negative for NPY-IR (Horn et al., 1987). Based on the present finding that 91.9% of such neurons are contacted by boutons containing CGRP-IR, this implies that 9 sensory neurons form synaptic boutons on 2914 postganglionic B neurons. Although divergence of this magnitude is possible, the corollary that half of the neurons in a paravertebral ganglion receive sensory input is without precedent. Instead, we favor a more parsimonious interpretation of the data. The postganglionic B neurons in the ninth and tenth ganglia are already known to be innervated by 137 preganglionic B neurons (Horn and Stofer, 1988a). Moreover, the present study demonstrates that 243 neurons in the appropriate region of the spinal cord contain CGRP-IR (Fig. 9). The rostrocaudal distribution and density of the immunoreactive spinal neurons closely parallels that of retrogradely labeled preganglionic B cells. The finding that more spinal neurons contain CGRP-IR than were labeled by transport of HRP is probably due to the fact that some preganglionic neurons in this region of the spinal cord project to ganglia other than 9 and 10 (Robertson, 1987). We therefore favor the conclusion that preganglionic B neurons contain CGRP-IR and that axotomy suppresses its expression. In this vein, it is interesting that Senba and Tohyama (1988) recently demonstrated CGRP-IR in preganglionic neurons of the rat. However, in their experiment, they injected fast blue into the pelvic ganglion rather than applying it to a cut nerve. In any

A. Origins Of Caudally Projecting Axons In The Sympathetic Chain

B. Pathways Within Sympathetic Ganglia 9 & 10

Figure 15. Summary of peptidergic pathways through sympathetic ganglia 9 and 10. *A*, Spinal segments 4 through 8 each contribute both preganglionic and sensory fibers that project via the paravertebral chain to sympathetic ganglia 9 and 10. *B*, Preganglionic B and C cells are located in contiguous segments of the spinal cord (Horn and Stofer, 1988a). Preganglionic B cells utilize acetylcholine (*Ach*) as their primary neurotransmitter and express CGRP-IR. They selectively innervate the B-type postganglionic neurons. Frog sympathetic neurons use epinephrine (*Epi*) as their primary transmitter (von Euler, 1971). B cells project selectively into cutaneous branches of peripheral nerves (Horn et al., 1988) where they innervate nonvascular targets (Yoshimura, 1979; Horn et al., 1986). Preganglionic C cells use *Ach* and LHRH as cotransmitters (Jan and Jan, 1982). They selectively innervate C-type postganglionic neurons which use *Epi* as a transmitter and express NPY-IR (Horn et al., 1987). C cells selectively innervate blood vessels (Yoshimura, 1979; Horn et al., 1986). Sensory neurons that project to ganglia 9 and 10 can be divided into 4 groups based on the expression of CGRP-IR and SP-IR. Some of the sensory neurons that coexpress CGRP-IR and SP-IR selectively innervate a small population of B-type postganglionic neurons. The peripheral targets of sensory fibers projecting through sympathetic ganglia are unknown.

event, the available data do not rule out the possibility that a few sensory neurons containing only CGRP-IR form synapses on B neurons in ganglia 9 and 10. Another experiment to test our interpretation of the data would be to double-label the sympathetic ganglia for CGRP and choline acetyltransferase, the cholinergic marker enzyme known to be present in preganglionic, but not sensory, neurons. Unfortunately, the antibody to this enzyme which is readily available (Boehringer Mannheim Biochemicals) does not react with frog tissues (J. P. Horn and W. D. Stofer, unpublished observation).

Sensory axons in the sympathetic chain

The existence of a large sensory projection into ganglia 9 and 10 was demonstrated by the retrograde labeling with fast blue

of 374 sensory neurons in dorsal root ganglia 4–8 (Fig. 11). This finding is remarkable when one considers that only 338 preganglionic neurons project to these same ganglia (Horn and Stofer, 1988a). By comparison, in the cat, the cervical sympathetic trunk contains axons from 104 sensory neurons in spinal ganglia and axons from 4656 preganglionic neurons (Wong and Leong, 1984), and the lumbar sympathetic trunk contains axons from 650 sensory neurons and axons from 4500 preganglionic neurons (Janig and McLachlan, 1986). Although the ratio of sensory to preganglionic fibers in the paravertebral system is higher in the bullfrog than in the cat, it is interesting that the segmental overlap between sensory and preganglionic neurons that project into the lumbar sympathetic trunk in the cat is analogous to that in the bullfrog (compare figure 1 in Janig and

McLachlan, 1986, with Fig. 11 and with figure 8 in Horn and Stofer, 1988a). In each species, the sensory and preganglionic fibers projecting to a particular chain ganglion arise from roughly the same segments.

It seems likely that most of the sensory axons in the frog paravertebral system are through fibers that continue on to innervate targets in the periphery. This conclusion is supported by the finding that the postganglionic rami communicantes of ganglia 9 and 10 contain many fibers with CGRP-IR and/or SP-IR (Fig. 2) and by electrophysiological evidence that these rami do not contain preganglionic fibers (Dodd and Horn, 1983). It should also be noted that our retrograde labeling procedure precluded the detection of sensory neurons in dorsal root ganglia 9 and 10 that project to sympathetic ganglia 9 and 10. The observation that cutting the sympathetic chain between ganglia 8 and 9 eliminates most, but not all, SP-IR and CGRP-IR in these ganglia suggests that a few such cells may exist.

The conclusion that SP-IR in sympathetic ganglia 9 and 10 is purely sensory in origin is supported by 2 findings. First, it is present in sensory neurons projecting to the ganglia (Figs. 13, 14); second, the intermediolateral cell column in the spinal cord does not contain cell bodies that are positive for SP-IR (Fig. 10). Inagaki et al. (1981) reported a similar distribution of SP-IR in the spinal cord of the bullfrog. These data also support the conclusion that the synaptic boutons in sympathetic ganglia 9 and 10 which coexpress CGRP-IR and SP-IR are sensory in nature. The selective innervation by these sensory neurons of postganglionic B neurons is supported by the finding that such cells are always negative for NPY-IR (Fig. 4).

An interesting question is raised by the finding that 3.8% of postganglionic neurons containing NPY-IR are contacted by boutons containing CGRP-IR. Does this finding imply that our use of NPY-IR as a marker for C cells is at fault, or does it represent a projection from preganglionic B cells to postganglionic C cells? Horn et al. (1987) reported that 88.3% of electrophysiologically identified C cells contained NPY-IR and that 98.9% of identified B cells were negative for NPY-IR. It is uncertain whether some C cells do not express NPY-IR or whether the damage produced by double impalements and Lucifer yellow injection simply eliminates the immunoreactivity of some C cells. This necessitates that we qualify our present use of NPY-IR as a marker for C cells. In the present experiments we found that 91.9% of the cells that were negative for NPY-IR and 3.8% of the cells that were positive for NPY-IR were contacted by CGRP-IR boutons. If a few of the cells that were negative for NPY-IR were not B cells, then our data would underestimate by a few percent the proportions of B and C cells receiving such inputs. For similar reasons, we may have failed to detect a small number of contacts between C cells and sensory boutons that contain CGRP-IR and SP-IR. By contrast, those contacts between NPY-IR cells and boutons containing only CGRP-IR are extremely likely to represent connections between preganglionic B and postganglionic C cells. In support of this possibility, Smith and Weight (1986) reported the existence of occasional synapses between preganglionic B cells and postganglionic C cells. Additional experiments will be required to fully resolve this issue.

As mentioned in the introduction, CGRP and SP alter the electrophysiological properties of autonomic neurons, but the physiological significance of these actions is poorly understood. Figure 15 summarizes our present understanding of peptidergic and nonpeptidergic pathways in the bullfrog sympathetic system

and its relation to the innervation of peripheral targets. At this time, only LHRH fulfills all the requirements for identification as a neurotransmitter (Jan et al., 1980; Jan and Jan, 1982). It remains for future work to determine whether CGRP, SP, and NPY function as transmitters in the bullfrog sympathetic system and to identify the peripheral targets of fast B and slow B neurons, of sympathetic neurons that receive sensory inputs, and of sensory neurons that simply project through the ganglion. It may also be important to consider the trophic effects of these neuropeptides. In this regard, it is interesting to note that the kinetic properties of nicotinic acetylcholine receptors on bullfrog sympathetic neurons are influenced by the source (B or C) of their preganglionic innervation (Marshall, 1985, 1986) and that CGRP stimulates the synthesis of nicotinic receptors in skeletal muscle (Fontaine et al., 1986; New and Mudge, 1986).

References

- Abercrombie, M. (1946) Estimation of nuclear population from microtome sections. *Anat. Rec.* 94: 239–247.
- Blessing, W. W., P. R. C. Howe, T. J. Joh, J. R. Oliver, and J. O. Willoughby (1986) Distribution of tyrosine hydroxylase and neuropeptide Y-like immunoreactive neurons in rabbit medulla oblongata, with attention to colocalization studies, presumptive adrenaline-synthesizing perikarya, and vagal preganglionic cells. *J. Comp. Neurol.* 248: 285–300.
- Bowers, C. W., L. J. Jan, and Y. N. Jan (1986) A substance P-like peptide in bullfrog autonomic nerve terminals: Anatomy, biochemistry and physiology. *Neuroscience* 19: 343–356.
- Cuello, A. C., G. Galfre, and C. Milstein (1979) Detection of substance P in the central nervous system by a monoclonal antibody. *Proc. Natl. Acad. Sci. USA* 76: 3532–3536.
- Dalsgaard, C.-J., T. Hökfelt, L.-G. Elfvin, L. Skirboll, and P. Emson (1982) Substance P-containing primary sensory neurons projecting to the inferior mesenteric ganglion: Evidence from combined retrograde tracing and immunohistochemistry. *Neuroscience* 7: 647–654.
- Dodd, J., and J. P. Horn (1983) A reclassification of B and C neurones in the ninth and tenth paravertebral sympathetic ganglia of the bullfrog. *J. Physiol. (Lond.)* 334: 255–269.
- Dryer, S. E., and V. A. Chiapinelli (1985) Properties of choroid and ciliary neurons in the avian ciliary ganglion and evidence for substance P as a neurotransmitter. *J. Neurosci.* 5: 2654–2661.
- Dun, N. J., and Z. G. Jiang (1982) Non-cholinergic excitatory transmission in inferior mesenteric ganglia of the guinea-pig: Possible mediation by substance P. *J. Physiol. (Lond.)* 325: 145–159.
- Dun, N. J., and M. Kiraly (1983) Capsaicin causes release of a substance P-like peptide in guinea-pig inferior mesenteric ganglia. *J. Physiol. (Lond.)* 340: 107–120.
- Dunn, P. M., and L. M. Marshall (1985) Innervation of small intensely fluorescent cells in frog sympathetic ganglia. *Brain Res.* 339: 371–374.
- Erichsen, J. T., H. J. Karten, W. D. Eldred, and N. C. Brecha (1982a) Localization of substance P-like and enkephalin-like immunoreactivity within preganglionic terminals of the avian ciliary ganglion: Light and electron microscopy. *J. Neurosci.* 2: 994–1003.
- Erichsen, J. T., A. Reiner, and H. J. Karten (1982b) Co-occurrence of substance P-like and leu-enkephalin-like immunoreactivities in neurones and fibres of the avian nervous system. *Nature* 295: 407–410.
- Fontaine, B., A. Klarsfeld, T. Hökfelt, and J. P. Changeux (1986) Calcitonin gene-related peptide, a peptide present in spinal cord motoneurons, increases the number of acetylcholine receptors in primary cultures of chick myotubes. *Neurosci. Lett.* 71: 59–65.
- Furness, J. B., and M. Costa (1987) *The Enteric Nervous System*, Churchill Livingstone, New York.
- Gamse, R., A. Wax, R. E. Zigmond, and S. E. Leeman (1981) Immunoreactive substance P in sympathetic ganglia: Distribution and sensitivity towards capsaicin. *Neuroscience* 6: 437–441.
- Gibson, S. J., J. M. Polak, S. R. Bloom, I. M. Sabate, P. M. Mulderry, M. A. Ghatei, G. P. McGregor, J. F. B. Morrison, J. S. Kelly, R. M. Evans, and M. G. Rosenfeld (1984) Calcitonin gene-related peptide immunoreactivity in the spinal cord of man and of eight other species. *J. Neurosci.* 4: 3101–3111.

- Hökfelt, T., J. O. Kellerth, G. Nilsson, and B. Pernow (1975) Substance P: Localization in the central nervous system and in some primary sensory neurons. *Science* 190: 889-890.
- Hökfelt, T., L.-G. Elfvin, M. Schultzberg, M. Goldstein, and G. Nilsson (1977) On the occurrence of substance P-containing fibers in sympathetic ganglia: Immunohistochemical evidence. *Brain Res.* 132: 29-41.
- Horn, J. P., and W. D. Stofer (1987) Calcitonin gene-related peptide-like immunoreactivity appears to be present in preganglionic sympathetic B-type, but not C-type, neurons in the bullfrog. *Soc. Neurosci. Abstr.* 13: 297.
- Horn, J. P., and W. D. Stofer (1988a) Spinal origins of preganglionic B and C neurons that innervate paravertebral ganglia nine and ten of the bullfrog. *J. Comp. Neurol.* 268: 71-83.
- Horn, J. P., and W. D. Stofer (1988b) Double labeling of the paravertebral sympathetic C system in the bullfrog with antisera to LHRH and NPY. *J. Auton. Nerv. Syst.* 23: 17-24.
- Horn, J. P., and W. D. Stofer (1988c) Segmental origins and possible vascular destination of peptide containing sensory fibers in bullfrog sympathetic ganglia. *Soc. Neurosci. Abstr.* 14: 355.
- Horn, J. P., S. Fatherazi, and W. D. Stofer (1986) Selective innervation of blood vessels in the bullfrog by sympathetic C neurons that contain neuropeptide Y-like immunoreactivity. *Soc. Neurosci. Abstr.* 12: 542.
- Horn, J. P., W. D. Stofer, and S. Fatherazi (1987) Neuropeptide Y-like immunoreactivity in bullfrog sympathetic ganglia is restricted to C cells. *J. Neurosci.* 7: 1717-1727.
- Horn, J. P., S. Fatherazi, and W. D. Stofer (1988) Differential projections of B and C sympathetic axons in peripheral nerves of the bullfrog. *J. Comp. Neurol.* 278: 570-580.
- Inagaki, S., E. Senba, S. Shiosaka, H. Takagi, Y. Kawai, K. Takatsuki, M. Sakanaka, T. Matsuzaki, and M. Tohyama (1981) Regional distribution of substance P-like immunoreactivity in the frog brain and spinal cord: Immunohistochemical analysis. *J. Comp. Neurol.* 201: 243-254.
- Jan, L. Y., and Y. N. Jan (1982) Peptidergic transmission in sympathetic ganglia of the frog. *J. Physiol. (Lond.)* 327: 219-246.
- Jan, L. Y., Y. N. Jan, and M. S. Brownfield (1980) Peptidergic transmitters in synaptic boutons of sympathetic ganglia. *Nature* 288: 380-382.
- Janig, W., and E. McLachlan (1986) The sympathetic and sensory components of the caudal lumbar sympathetic trunk in the cat. *J. Comp. Neurol.* 245: 62-73.
- Ju, G., T. Hökfelt, E. Brodin, J. Fahrenkrug, J. A. Fischer, P. Frey, R. P. Elde, and J. C. Brown (1987) Primary sensory neurons of the rat showing calcitonin gene-related peptide immunoreactivity and their relation to substance P-, somatostatin-, galanin-, vasoactive intestinal polypeptide- and cholecystokinin-immunoreactive ganglion cells. *Cell Tissue Res.* 247: 417-431.
- Katayama, Y., and R. A. North (1978) Does substance P mediate slow synaptic excitation within the myenteric plexus? *Nature* 274: 387-388.
- Konishi, S., M. Otsuka, K. Folkers, and S. Rosell (1983) A substance P antagonist blocks non-cholinergic slow excitatory postsynaptic potential in guinea-pig sympathetic ganglia. *Acta Physiol. Scand.* 117: 157-160.
- Kreulen, D. L., and S. Peters (1986) Non-cholinergic transmission in a sympathetic ganglion of the guinea-pig elicited by colon distension. *J. Physiol. (Lond.)* 374: 315-334.
- Krukoff, T. L., J. Ciriello, and F. R. Caleresu (1985) Segmental distribution of peptide-like immunoreactivity in cell bodies of the thoracolumbar sympathetic nuclei of the cat. *J. Comp. Neurol.* 240: 90-102.
- Kuramoto, H., and T. Fujita (1986) An immunohistochemical study of calcitonin gene-related peptide (CGRP)-containing nerve fibers in the sympathetic ganglia of bullfrogs. *Biomed. Res.* 7: 349-357.
- Landis, S. C., and J. R. Fredieu (1986) Coexistence of calcitonin gene-related peptide and vasoactive intestinal peptide in cholinergic sympathetic innervation of rat sweat glands. *Brain Res.* 377: 177-181.
- Lee, Y., N. Hayashi, C. J. Hillyard, S. I. Girgis, I. MacIntyre, P. C. Emson, and M. Tohyama (1987) Calcitonin gene-related peptide-like immunoreactive sensory fibers form synaptic contact with sympathetic neurons in the rat celiac ganglion. *Brain Res.* 407: 149-151.
- Lindh, B., J. M. Lundberg, T. Hökfelt, L. G. Elfvin, J. Fahrenkrug, and J. Fischer (1987) Coexistence of CGRP- and VIP-like immunoreactivities in a population of neurons in the cat stellate ganglion. *Acta Physiol. Scand.* 131: 475-476.
- Marshall, L. M. (1985) Presynaptic control of synaptic channel kinetics in sympathetic neurones. *Nature* 317: 621-623.
- Marshall, L. M. (1986) Different synaptic channel kinetics in sympathetic B and C neurons of the bullfrog. *J. Neurosci.* 6: 590-593.
- Mathews, M. R., and A. C. Cuello (1982) Substance P-immunoreactive peripheral branches of sensory neurons innervate guinea pig sympathetic neurons. *Proc. Natl. Acad. Sci. USA* 79: 1668-1672.
- McMahan, U. J., and S. W. Kuffler (1971) Visual identification of synaptic boutons on living ganglion cells and varicosities in postganglionic axons in the heart of the frog. *Proc. R. Soc. Lond. B.* 177: 485-508.
- New, H. V., and A. W. Mudge (1986) Calcitonin gene-related peptide regulates muscle acetylcholine receptor synthesis. *Nature* 323: 809-811.
- Nohmi, M., P. Shinnick-Gallagher, P. W. Gean, J. P. Gallagher, and C. W. Cooper (1986) Calcitonin and calcitonin gene related peptide enhance calcium-dependent potentials. *Brain Res.* 367: 346-350.
- Palmer, J. M., M. Schemann, K. Tamura, and J. D. Wood (1986) Calcitonin gene-related peptide excites myenteric neurons. *Eur. J. Pharmacol.* 132: 163-170.
- Pearse, A. G. E., and J. M. Polak (1975) Immunocytochemical localization of substance P in mammalian intestine. *Histochemistry* 41: 373-375.
- Robertson, D. R. (1987) Sympathetic preganglionic neurons in frog spinal cord. *J. Auton. Nerv. Syst.* 18: 1-11.
- Role, L. W. (1984) Substance P modulation of acetylcholine-induced currents in embryonic chicken sympathetic and ciliary ganglion neurons. *Proc. Natl. Acad. Sci. USA* 81: 2924-2928.
- Rosenfeld, M. G., J. Mermod, S. G. Amara, L. W. Swanson, P. E. Sawchenko, J. River, W. W. Vale, and R. M. Evans (1983) Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. *Nature* 304: 129-135.
- Schultzberg, M., C. F. Dreyfus, M. D. Gershon, T. Hökfelt, R. P. Elde, G. Nilsson, S. Said, and M. Goldstein (1978) VIP-, enkephalin-, substance P- and somatostatin-like immunoreactivity in neurons intrinsic to the intestine: Immunohistochemical evidence from organotypic tissue cultures. *Brain Res.* 155: 239-248.
- Senba, E., and M. Tohyama (1988) Calcitonin gene-related peptide containing autonomic efferent pathways to the pelvic ganglion of the rat. *Brain Res.* 449: 386-390.
- Smith, P. A., and F. F. Weight (1986) The pathway for the slow inhibitory postsynaptic potential in bullfrog sympathetic ganglia. *J. Neurophysiol.* 56: 823-834.
- Stofer, W. D., and J. P. Horn (1988) A rare class of bullfrog sympathetic neurons that are encrusted with substance P containing synaptic boutons. *Soc. Neurosci. Abstr.* 14: 355.
- Tsunoo, A., S. Konishi, and M. Otsuka (1982) Substance P as an excitatory transmitter of primary afferent neurons in guinea-pig sympathetic ganglia. *Neuroscience* 7: 2025-2037.
- von Euler, U. S. (1971) Adrenergic neurotransmitter functions. *Science* 173: 202-206.
- Wong, W. C., and S. K. Leong (1984) The origin of postganglionic and sensory axons in the cervical sympathetic trunk of the cat: A horseradish peroxidase study. *J. Comp. Neurol.* 222: 161-165.
- Yoshimura, M. (1979) Tonic and reflex discharges of sympathetic ganglion cells. *J. Kurume Med. Assoc.* 42: 1221-1238.