Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 6888–6892, August 1991
Neurobiology

Axon collaterals indicate broad intraspinal role for sacral preganglionic neurons

(intracellular injection/autonomic nervous system/parasympathetic nervous system/bladder/neurobiotin)

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Communicated by George B. Koelle, May 10, 1991

ABSTRACT **The classic view of preganglionic neurons in spinal autonomic nuclei is that they convey information exclusively from the central nervous system to autonomic neurons in peripheral ganglia. The present morphological study in the cat sacral spinal cord demonstrates that these neurons may also make abundant synaptic connections within the spinal cord. Neurons labeled intracellularly with neurobiotin or horseradish peroxidase exhibited an expansive distribution of axon collaterals in spinal cord laminae I, V, VII, VIII, IX, X, and the ventrolateral funiculi. This broad-ranging axon-collateral system, which has the potential for multiple neuronal contacts, indicates widespread integrative functions for sacral preganglionic neurons within the spinal cord, in addition to functions currently known in the periphery.**

The preganglionic neuron of the autonomic nervous system represents the first component of a two-neuron efferent pathway that carries information from the central nervous system to the visceral organs. These neurons, which are located in the spinal cord—many of them in the intermediolateral cell column—and in visceral motor nuclei in the brainstem, integrate afferent inputs from various central and peripheral sources and then convey efferent signals to ganglion cells in the peripheral nervous system.

According to traditional concepts, preganglionic neurons have purely peripheral functions and do not make efferent connections with other neurons in the central nervous system. However, in the sacral parasympathetic preganglionic pathways to the urinary bladder of the cat, a bilateral and intersegmental recurrent inhibitory mechanism has been identified using electrophysiological techniques (1–3). These findings raise the possibility that sacral preganglionic neurons have an axon-collateral system that makes synaptic contacts with inhibitory interneurons within the spinal cord similar to axon-collateral pathways that have been identified for somatic motoneurons (4). Numerous morphological studies have failed to confirm the existence of sacral preganglionic axon collaterals (5–9). However, in the present experiments extensive axon-collateral systems have been revealed by using intracellular labeling techniques.

MATERIALS AND METHODS

In these experiments, 20 male cats were anesthetized with diallylbarbituric acid (0.3 mg/kg) and urethane (2.4 mg/kg i.p.) supplemented with sodium pentobarbital (30 mg/kg i.v.), as needed. After a laminectomy, neurons in the sacral spinal cord were activated antidromically by electrical stimulation of the S2 ventral root and identified as preganglionic neurons by having stimulus thresholds two to three times higher than those of motoneurons and by their slow conduction velocities of 4–12 m/sec. These neurons were located in lateral lamina VII in an area of the sacral parasympathetic nucleus known to contain bladder preganglionic neurons (6, 8) and had conduction velocities typical of these cells (1, 10). Electrodes were judged to be intracellular when resting membrane potentials dropped 35–55 mv and evoked potentials increased suddenly from ≈10 mv to reach 50 mv or higher. Preganglionic neurons were injected with 4% horseradish peroxidase (HRP; Sigma VI) in 0.5 M KCl or with 2% (wt/vol) neurobiotin (Vector Laboratories) in 1.0 M KCl through beveled glass electrodes with resistances of 70–120 MΩ. The tracers were ejected for 3–10 min with current pulses (3–9 nA, 200-msec duration) delivered at a frequency of 2.5 Hz. After transport times of 1–9 hr, the cats were perfused with 0.1 M phosphate-buffered saline followed by 2% paraformaldehyde/2% glutaraldehyde/phosphate-buffered saline (pH 7.4) for the HRP tissue or 4% paraformaldehyde/0.2% picric acid/0.15 M phosphate buffer (pH 7.4) for the neurobiotin tissue. After postfixation for 4 hr, the tissue was cut with a vibratome into 50-μm sections and stored in phosphate-buffered saline. HRP tissue was processed in diaminobenzidine, according to Adams (11). Neurobiotin sections were processed using the avidin–biotin reaction with the ABC Elite kit, and instructions were supplied by Vector Laboratories; the chromagen was diaminobenzidine.

Sections were stained with toluidine blue, cleared in alcohol and xylene, mounted on glass slides with Depex, and stored at room temperature. Labeled cells were examined and reconstructed with a Leitz Orthoplan microscope and drawing tube.

RESULTS

The most striking morphological characteristic of the injected sacral preganglionic neurons was the broad distribution of axon collaterals, which heretofore have not been detected within the cat spinal cord (6, 7, 9, 12, 13). Of 26 neurons recovered from 11 cats, 16 had identifiable axon collaterals; 11 of these 16 were filled with HRP, and 5 were filled with neurobiotin; no collaterals could be detected for 10 of the HRP-filled cells. The axons of the labeled neurons were positively identified by tracing them into the ventral horn and often into the ventral rootlets to their exit from the cord. They were of fairly uniform diameter throughout, and some showed evidence of myelination. All processes identified as axon collaterals were verified by tracing them back to their parent axon. Fig. 1 is a composite map of seven complex axon-collateral systems originating from a neurobiotin-labeled preganglionic neuron. Collaterals at a and b had similar patterns but were separated by a few hundred microns in the longitudinal plane. They both extended medially

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Abbreviation: HRP, horseradish peroxidase.

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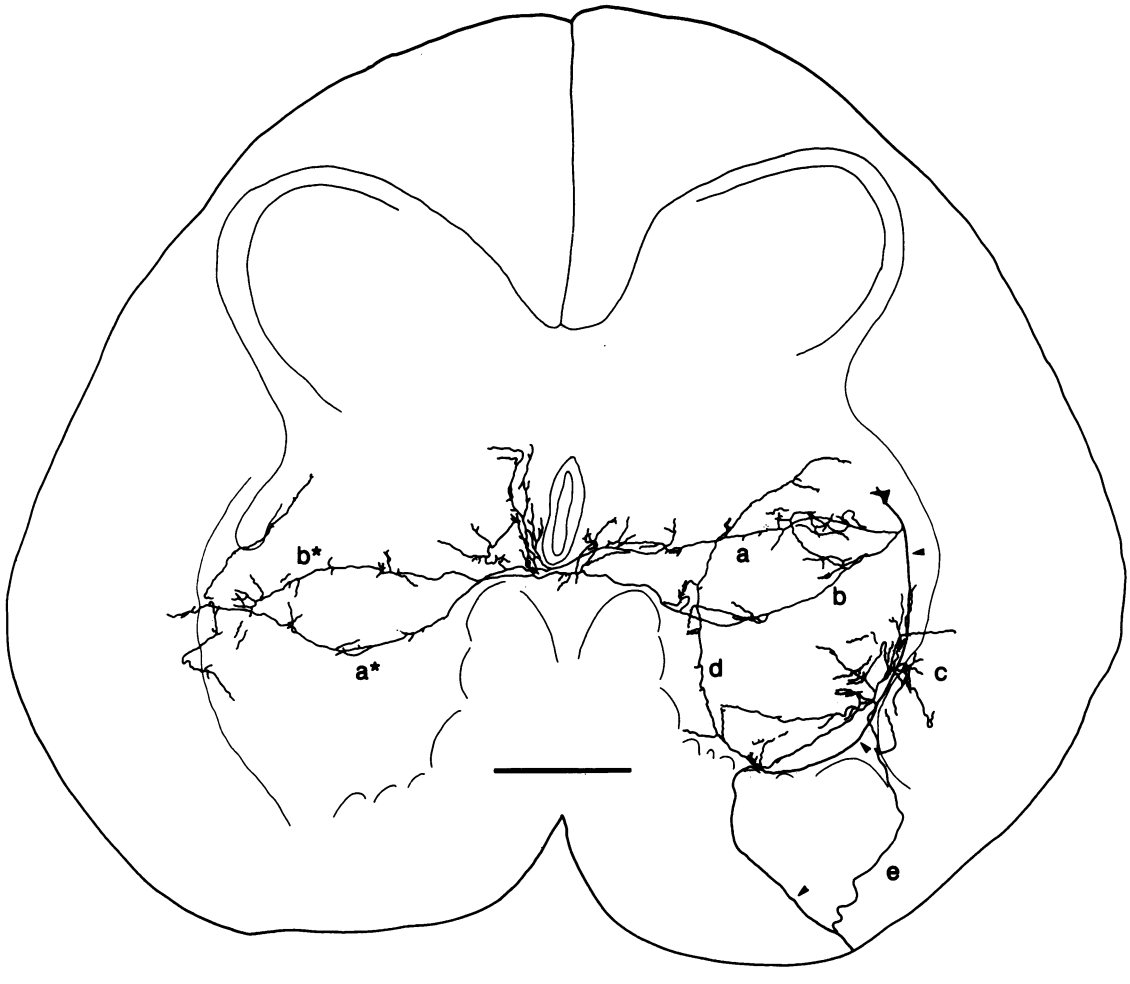

FIG. 1. Composite camera lucida drawing of seven axon collaterals originating from a neurobiotin-filled preganglionic neuron in the S2 spinal cord. The cell body shown without its dendrites is located in lateral lamina VII, the center of the lateral band of the sacral parasympathetic nucleus. The collaterals and their boutons are located in laminae VII, VIII, X, and the ventrolateral funiculi on both sides and in ipsilateral lamina IX. a and b are two collaterals that extend into the contralateral spinal cord (see a* and b*) to reach the region of the opposite parasympathetic nucleus. c identifies a region in the lateral ventral horn containing three collaterals; d denotes a single collateral that curves through the ventral horn and back into the nucleus; and e identifies an axon collateral that originated from the parent axon (arrowheads) in the ventral funiculus. (Bar = 500 μm.)

through lamina VII, beneath the central canal, through contralateral lamina VII, and into the region of the contralateral parasympathetic nucleus. All along this bilateral route the collaterals gave off multiple branches and numerous varicosities, which we presume to be boutons. We, therefore, refer to these as boutons throughout the remainder of this paper. The area around the central canal (lamina X) received an especially heavy innervation. Three smaller collaterals (c in Fig. 1) occupied the lateral ventral horn, whereas the sixth axon collateral (d) curved through the ventral horn and back into the ipsilateral parasympathetic nucleus. A seventh collateral (e) originated from the parent axon near its exit from the spinal cord and traveled rostrad in the ventral funiculus for >1000 μm, giving off five small terminal fields to the ventral horn along its route.

While the first cell's axon collaterals occupied the ventral horn and intermediate zone, collaterals of another labeled preganglionic neuron (Fig. 2) were distributed primarily in the dorsal aspect of the intermediate zone and dorsal horn. The dorsal-most primary collateral immediately divided into six complex secondary systems, which coursed through the ipsilateral autonomic nucleus in lamina VII and V. Branches continued into lamina I, the medial dorsal horn, and the contralateral dorsal gray commissure. The ventral primary collateral issued from the parent axon in the ventral funiculus and crossed beneath the central canal to reach the contralateral dorsal horn, a linear distance of ≈4500 μm from its origin. The majority of axon collaterals from each of these two neurons were located within 900 μm rostral and 500 μm caudal to their parent cell. Two other neurobiotin-labeled preganglionic neurons had similarly complex systems, while a fifth neurobiotin cell, though well-labeled, had only a few axon collaterals that were limited to the ipsilateral parasympathetic nucleus. These data indicate there are both a variety of preganglionic axon-collateral patterns as well as differences in the degree of complexity of these patterns.

Neurobiotin appears to be a superior tracer to HRP in these experiments, as it produced the most extensive labeling of these fine collateral systems, including all those traced contralaterally. The HRP-filled collaterals did not label as intensely and were observed only on the ipsilateral side of the spinal cord; most of these collaterals remained within the region of the parasympathetic nucleus and probably represent only a partial distribution of these processes.

In the gray matter, neurobiotin- and HRP-labeled boutons from all neurons were identified in laminae I, V, VII, VIII, IX, and X, although no single neuron was observed to send collaterals to all of these locations. For the two neurobiotin-injected preganglionic neurons described above (Figs. 1 and 2), a combined total of 1604 boutons was counted using a ×60

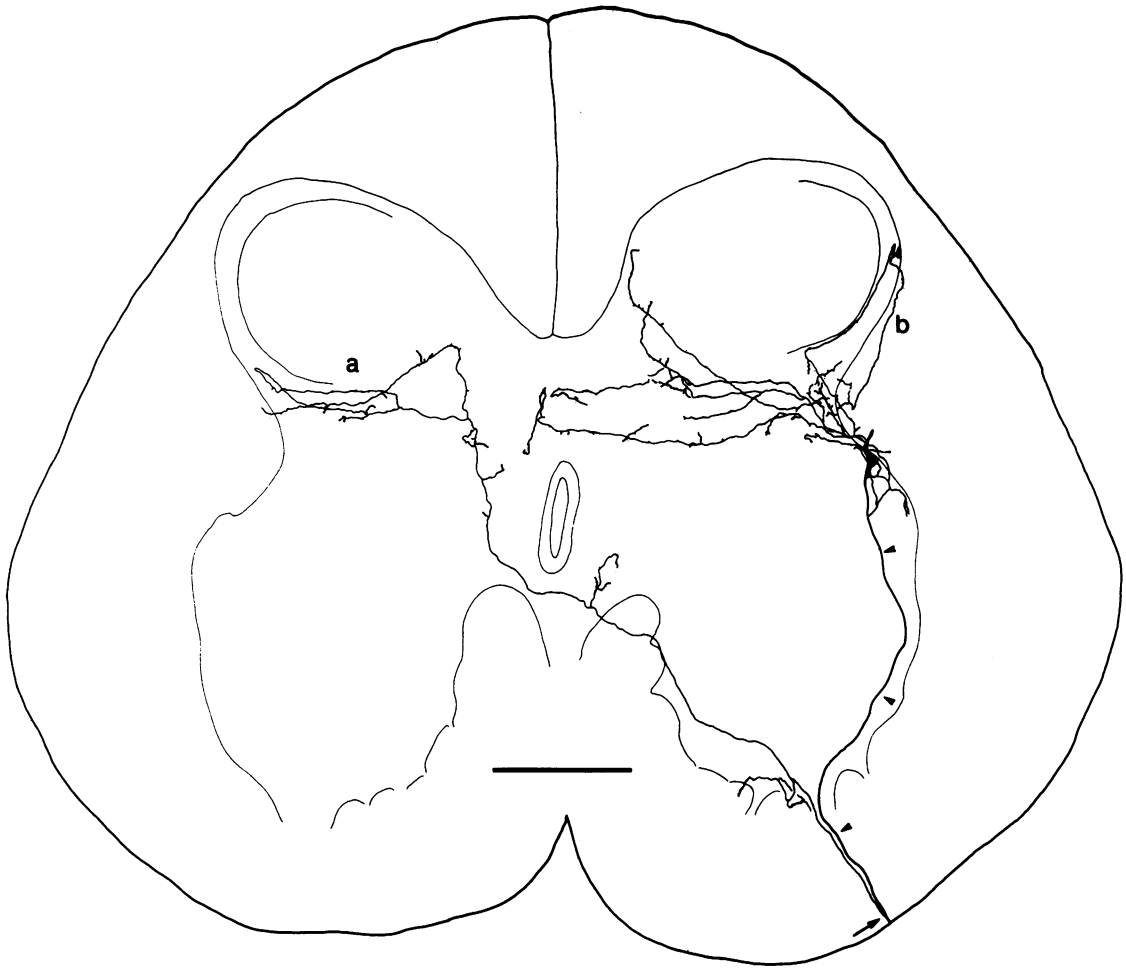

FIG. 2. Composite camera lucida drawing of a preganglionic neuron injected with neurobiotin, shown without its dendrites, located in a similar part of the parasympathetic nucleus as the previous cell (Fig. 1) but at a more caudal level of the S2 segment. a indicates an axon collateral that originated from its parent axon in the right ventral funiculus (single arrow) and then extended beneath the central canal, through left lamina VII to reach contralateral laminae I and V for a total linear distance of >4500 μm. b indicates axon collaterals forming branches and boutons in right lateral lamina V and extending well into lateral lamina I. Other branches extend through medial lamina V and into the contralateral dorsal commissure. Arrowheads mark the path of the parent axon. (Bar = 500 μm.)

oil-immersion lens. Although the majority of these boutons were not visibly apposed to neurons (Fig. 3*A*), a small number (<5%) were seen adjacent to and outlining the dendrites of Nissl-stained cell bodies (Fig. 3*B*). It seems likely that other boutons near cell bodies may be contacting unstained dendrites.

Most labeled boutons were located in the gray matter but some were also traced into the white matter. Fig. 4 is a drawing illustrating a portion of the branching pattern of axon collaterals and boutons in the ventrolateral funiculus from an experiment in which five preganglionic neurons in the same location were injected with HRP. The majority of these collaterals were located in narrow extensions of gray neuropile that penetrated between the longitudinal axons of the white matter.

DISCUSSION

Previous evidence of axon collaterals from sacral preganglionic neurons came solely from neurophysiological studies, demonstrating both ipsilateral and contralateral recurrent inhibition of bladder preganglionic neurons during electrical stimulation of the sacral ventral roots (1, 2). It was initially assumed that this recurrent inhibition was mediated by an inhibitory synaptic input to the preganglionic neurons, similar to the recurrent inhibition of somatic motor neurons (4). However, subsequent experiments indicated that this recurrent inhibition occurred at a site earlier on the micturition reflex pathway and possibly on the sensory limb at sites of bladder afferent termination in the dorsal horn (3). The bilateral axon collateral projection to lateral laminae I, V, and VII (see Fig. 2) in proximity to visceral sensory axons (9) and second-order interneurons (14) may be the morphological substrate for this inhibition.

It is clear that the complexity of the preganglionic axon collaterals varies considerably between neurons. This variation could be related, in part, to the efficiency of the intracellular labeling. For example, all cells filled with neurobiotin exhibited collaterals, whereas only 61% of the HRP cells had collaterals. HRP cells also exhibited more limited collateral systems, which were distributed only ipsilaterally and were less intensely labeled than those of neurobiotin cells. Thus, it would appear that neurobiotin may be a superior tracer. Among the neurobiotin-filled cells, label was more uniformly intense than with HRP, but there was also significant variation in the distribution patterns of axon collaterals (see Figs. 1 and 2). This variation may reflect morphological differences between discrete functional groups of preganglionic neurons because at least two major cell types—i.e., those innervating the sex organs and others that supply the lower urinary tract—are both present in the lateral sacral parasympathetic nucleus (6, 8). In future experiments, it will be important to label functionally identified preganglionic neurons to test this idea.

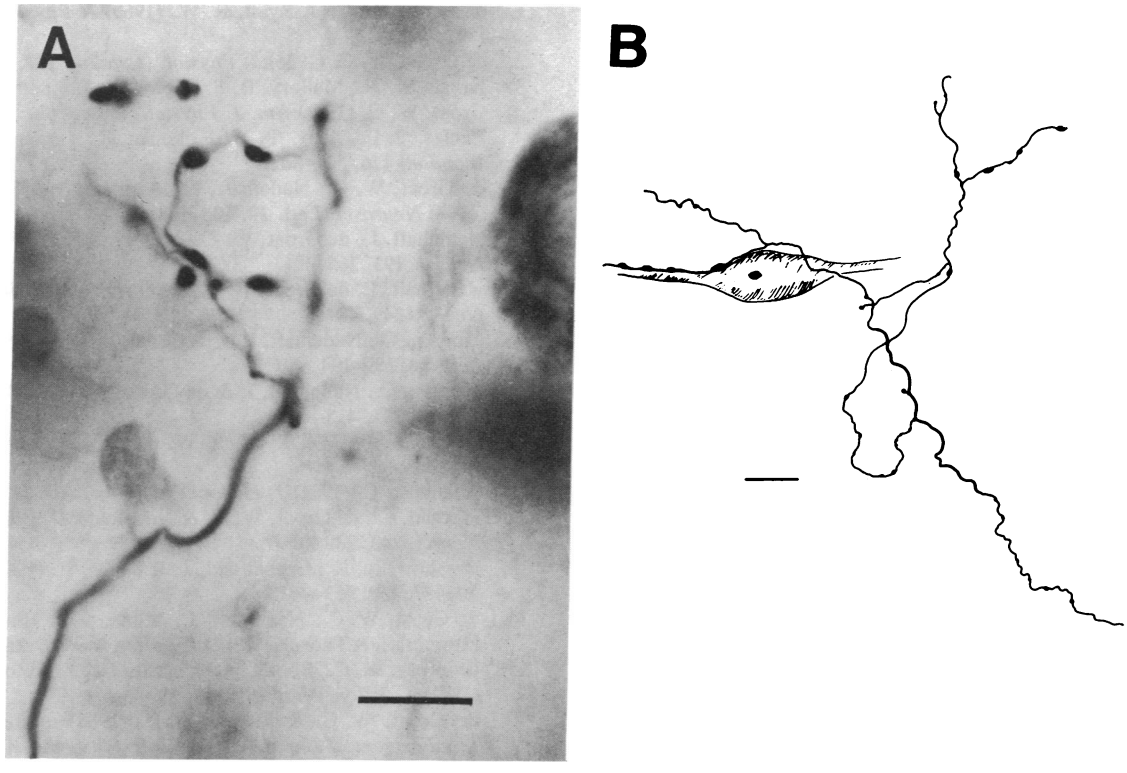

FIG. 3. (*A*) Neurobiotin-filled boutons in lamina VII medial to the sacral parasympathetic nucleus. Like the majority of boutons in these experiments they were not visibly in close proximity to other spinal neurons. (*B*) Composite camera lucida drawing of neurobiotin-filled boutons in close apposition to the soma and proximal dendrite of a small neuron in contralateral lamina VII. Less than 5% of boutons in these experiments were observed in close proximity to neurons. (Bars = 10 μm.)

Based on the large numbers of axon-collateral varicosities (presumably boutons) of sacral preganglionic neurons, it seems likely that these cells contact large numbers of spinal neurons and have a broad synaptic influence in the spinal cord. It follows, therefore, that the more complex of these sacral preganglionic neurons may have widespread integrative functions within the spinal cord. Various reciprocal functions, including those between the bladder and colon and between the viscera and striated sphincter muscles (15–17), might be mediated by collaterals terminating directly upon either preganglionic neurons, motoneurons, upon interneurons, or upon incoming axons in the lateral funiculus (see Figs. 1–4). For example, the inhibition of the colon that occurs during bladder contractions might be due, in part, to direct contact upon colon preganglionic neurons located in lamina V by axon collaterals from bladder preganglionic

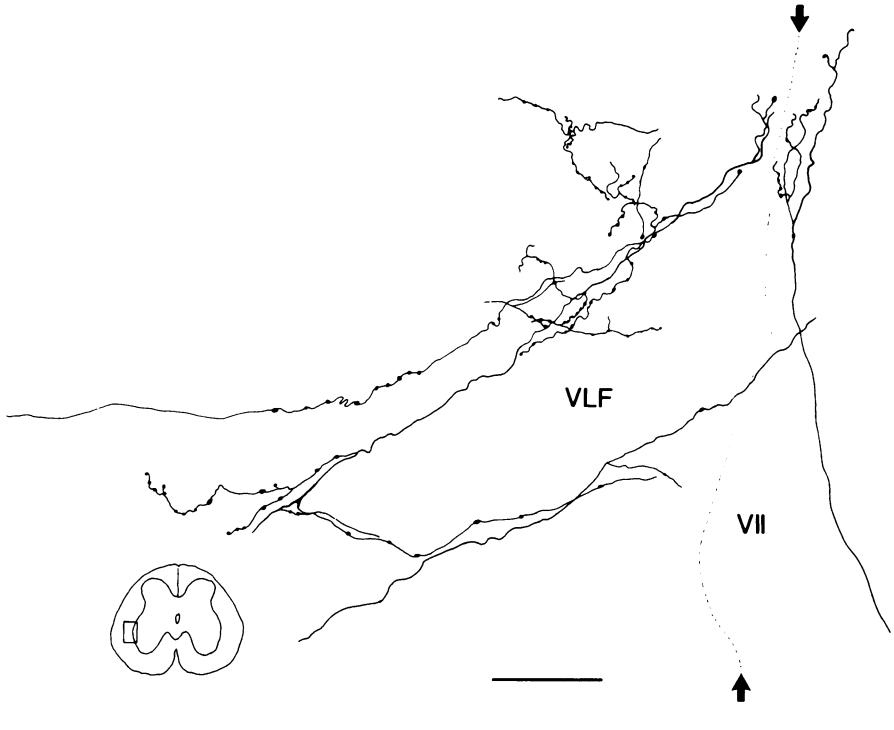

FIG. 4. Composite camera lucida drawing of HRP-filled axon collaterals in the ventrolateral funiculus (VLF) lateral to lamina VII (VII). Dotted line and arrows indicate the border between the edge of the gray and the VLF. These collaterals from parent axons some 50 μm caudal in the previous section were traced up to 350 μm into the white matter. (Bar = 50 μm.)

neurons located in lateral lamina VII (8, 15, 18). Likewise, the reciprocal relaxation of the urethral sphincter and contraction of the bladder detrusor, which occurs during voiding, may involve enkephalin-containing neurons that have been identified near the central canal and that reportedly project to and could inhibit the motoneurons in the ventral horn supplying the sphincter muscles (19, 20). Because neurons around the central canal receive axon collaterals from preganglionic neurons that would be excited during bladder contraction, the possibility of an axon collateral–interneuronal pathway for coordinating bladder–sphincter function is raised.

The occurrence of extensive sacral preganglionic axon collaterals also raises the question of whether preganglionic neurons at other levels of the neuraxis may likewise have extensive axon-collateral systems. Other investigators using Golgi staining or intracellular labeling with HRP to study sympathetic preganglionic neurons in the cat have found no evidence of axon collaterals (13, 21). Similarly, previous experiments on the sympathetic system in other species, including the neonatal rat (22, 23) and the pigeon (24), would indicate collaterals on sympathetic preganglionic neurons are rare and are limited in structure and extent. But, in view of the current results, it might be worthwhile to explore these regions again by using intracellular labeling with neurobiotin.

In conclusion, these data suggest that it may be necessary to change our view of the sacral preganglionic neuron. The presence of the large numbers of presumptive axon-collateral boutons suggests that sacral preganglionic neurons terminate upon spinal neurons and have the potential for broad synaptic influence within the spinal cord. Moreover, these cells that are located in the intermediate laminae along with interneurons do not serve as the final common pathway to target organs but instead make synaptic connections with autonomic ganglion cells in the periphery. Hence, it may be reasonable to view the sacral preganglionic neuron as representing a particular variety of interneuron having both central and peripheral functions.

The authors would like to thank D. H. Kita University of Tennessee, for his advice in the application of neurobiotin. This research was supported by Grant R01 NS 26585 from the National Institute of Neurological Disorders and Stroke.

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