Neurons in the dorsal column white matter of the spinal cord: Complex neuropil in an unexpected location

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ABSTRACT It is common to think of gray matter as the site of integration in neural circuits and white matter as the wires that connect different groups of neurons. The dorsal column (DC) white matter, for example, is the spinal cord axonal pathway through which a topographic map of the body is conveyed to the somatosensory cortex. We now describe a network of neurons located along the midline of the DCs. The neurons are present in several mammals, including primates and birds, and have a profuse dendritic arbor that expresses both the neuron-specific marker, microtubule-associated protein-2, and the neurokinin-1 receptor, a target of the neuropeptide, substance P. Electron microscopy and double immunostaining for synaptophysin and a marker of γ-aminobutyric acid-ergic terminals documented a rich synaptic input to these neurons. Finally, injection of a γ-aminobutyric acid type A receptor antagonist or of substance P into the cerebrospinal fluid of the rat spinal cord induced Fos expression and internalization of the neurokinin-1 receptor in these neurons, respectively, indicating that the DC neurons are under tonic inhibitory control and can respond to neurotransmitters that circulate in the cerebrospinal fluid.

Although neuronal cell bodies are concentrated in the gray matter of the spinal cord and brain, there are several exceptions. Most notable are two neuronal groups in the white matter of the lateral columns of the spinal cord, the lateral cervical nucleus (1) and the lateral spinal nucleus (2). The former is located at the first and second cervical segments and is found in all species. The latter is prominent in the rat and is present at all levels of the spinal cord. Importantly, electrophysiological and anatomical studies have established that neurons within these two groups can be readily distinguished from neurons in the neighboring dorsal horn gray matter.

To date, distinct neuronal cell groups have not been identified in the dorsal column (DC) white matter, which contains large-diameter primary afferent and second-order axons en route to the medulla (3). Here we provide evidence that the DCs, in fact, contain a population of neuronal cell bodies and a distinct neuropil. This finding was uncovered in the course of our studies of the organization of spinal cord neurons that express the neurokinin-1 (NK-1) receptor, which is targeted by the peptide substance P (SP). The cell bodies are concentrated near the midline of the DC white matter. In the present paper we establish that these cells are, in fact, neurons, that they receive a dense synaptic input and that they are under a profound tonic γ-aminobutyric acid (GABA)-ergic inhibitory control.

This paper was submitted directly (Track II) to the Proceedings office. Abbreviations: NK-1, neurokinin-1; MAP-2, microtubule-associated protein-2; SP, substance P; DC, dorsal column; GABA, γ-aminobutyric acid; P, phosphate buffer; EM, electron microscopy; CSF, cerebrospinal fluid.

Methods

Immunocytochemistry. All experiments were reviewed and approved by the Institutional Care and Animal Use Committee at the University of California, San Francisco. Most experiments were performed on male Sprague-Dawley rats (Bantin & Kingman, Fremont, CA), weighing 230–270 g. We also used spinal cord tissue from mice, cats, rhesus monkeys, and birds (Zebra finch). Under pentobarbital anesthesia the animals were perfused intracardially with PBS followed by 4% formaldehyde in 0.1 M phosphate buffer (PB). Immunocytochemistry was performed on 30-μm transverse or sagittal frozen sections of the spinal cord as described (4). For immunofluorescence, we followed the primary antisera with indocarbocyanine Cy-3-conjugated secondary antisera; for double immunofluorescence, we simultaneously used indocarbocyanine Cy-5-conjugated secondary antisera (both from Jackson ImmunoResearch, 1:600). Confocal images were obtained with an MRC 600 confocal microscope (Bio-Rad).

The following antisera were used: NK-1 receptor (1:5,000, S. Vigna, Duke University); the neuronal marker microtubule-associated protein (MAP-2, 1:1,000, Sigma); glial fibrillary acidic protein (1:500, Chemicon); synaptophysin (1:400, Boehringer Mannheim), which identifies synaptic terminals; serotonin (1:1,000, Eugene Tech, Ridgefield, NJ); neuronal nitric oxide synthase (1:5,000, D. Bredt, University of California, San Francisco); N-methyl-d-aspartate R1 (1:1,000, L. Jan, University of California, San Francisco); mu-opioid receptor (1:5,000, R. Elde, University of Minnesota, Minneapolis); somatostatin 2A receptor (1:1,000, A. Schonbrunn, University of Texas, Houston); SP (1:30,000, Peninsula Laboratories); GABA vesicle transporter (5) (1:1,000, R. Edwards, University of California, San Francisco); and Fos (1:30,000, D. Slamon, University of California, Los Angeles). For some tissue, we used the avidin-biotin protocol as described (6), with a nickel-intensified diaminobenzidine protocol and glucose oxidase to localize the reaction product. For double labeling, we omitted the nickel from one of the reactions, producing contrasting black and brown reaction products. Color photomicrographs were made from digitized microscope images, captured with Adobe Photoshop and printed on a Fujix printer.

Electron Microscopy (EM). For EM, rats were deeply anesthetized and perfused with 0.1 M PBS, pH 7.4 and then with 3.7% formaldehyde and 1% glutaraldehyde in PB. After postfixation for 1.5 hr, 30-μm mid sagittal sections of spinal cord were cut on a Vibratome, washed in PB, and treated with 50% ethanol for 45 min to improve antibody penetration (7).

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Sections were washed in Tris PBS and then immunostained for MAP-2, as above, with the nickel-intensified dianaminobenzidine reaction and processed for EM (8). Labeled neurons were chosen for EM analysis if they were from the middle of the DCs in sections that contained the central canal as well as longitudinally cut midsagittal blood vessels.

**Stimulation and Retrograde Tracing Protocols.** To determine whether the neurons in the DC white matter respond to peripheral stimuli, we used expression of the Fos protein as a marker of activity (8). Noxious stimuli were applied under anesthesia, 10–15 min after administration of sodium pentobarbital (40 mg/kg, i.p.). The mechanical stimulus consisted of hindpaw pinch with a hemostat for 2 min (n = 5). For thermal stimulation, we immersed the hindpaw in a 52°C water bath (n = 4). The visceral stimulus was an i.p. injection of 1.0 ml of 4% acetic acid.

To identify possible axonal targets of the cells in the DC white matter, we used retrograde tracing with Fluorogold. The tracer (0.2–0.6 μl) was injected into several brainstem, thalamic, or cerebellar sites by using coordinates taken from the atlas of Paxinos and Watson (9). The rats (n = 15; 2–4 per site) survived 7 days and then were perfused. We used fluorescence microscopy to colocalize retrogradely labeled cells and MAP-2 immunoreactive neurons.

To address the responsiveness of the DC cells to other forms of stimulation, we made direct cerebrospinal fluid (CSF) injections of SP (100 μg in 20 μl of saline; n = 5), the GABAA receptor antagonist bicuculline (10 μg in 20 μl of saline; n = 6), the GABAB receptor antagonist CG55848 (30 μg in 20 μl of saline; n = 4; kindly provided by Novartis, Basel, Switzerland), or the glycine receptor antagonist strychnine (100 μg in 20 μl of saline; n = 3). Under Halothane anesthesia, we made intrathecal injections via lumbar puncture by using a 30-gauge needle attached to a Hamilton syringe inserted between the S1 and S2 vertebrae. To monitor NK-1 receptor internalization, the rats were perfused 5 min after the injection, as described (10). To monitor Fos expression, the rats were perfused 2 hr after the injection of bicuculline or 90 min after the noxious stimulus. Finally, we evaluated the consequence of destroying unmyelinated primary afferent C fibers with the neurotoxin capsaicin. To this end, 1-day-old rats received an injection of capsaicin (100 mg/kg; s.c.), and the animals were studied at 3 months.

**RESULTS**

**Anatomy of the DC Neurons.** Our analysis of midsagittal sections of the spinal cord, which cut through the central canal and lamina X of the gray matter, revealed large numbers of NK-1 receptor-immunoreactive cells in the midline of the overlying DC white matter (Fig. 1). These cells also express the neuronal marker, MAP-2, but none immunostain for glial fibrillary acidic protein. Although not all spinal levels were examined in all species, we found comparable neurons in cervical, thoracic, lumbar, and sacral segments of the rat, in cervical and lumbar segments of the mouse, cat, and rhesus

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**Fig. 1.** (A) NK-1 receptor immunoreactive neurons (white arrowheads) are scattered through this 30-μm midsagittal section of the lumbar spinal cord of the adult rat. The dense NK-1 receptor immunoreactivity in lamina X (lam X) distinguishes the gray matter from the white matter of the DCs. (B and C) The neurons are also MAP-2 positive and have highly varicose dendrites that arborize extensively, often contacting the dorsal surface of the cord (red arrowheads). (Bars: 100 μm, A; 50 μm, B; and 25 μm, C.)
monkey, and in cervical segments of the bird (Zebra finch). The neurons are present in adult animals, and we have observed them in 3- and 13-day-old rats (Fig. 2E). The neurons average 15 μm in diameter, and we observed up to 25 cell bodies per mm² in a 30-μm thick sagittal section of the lumbar cord of the rat. A remarkable feature of these neurons is their planar dendritic arbor, reminiscent of the arbor of cerebellar Purkinje cell dendrites. Thus, dendritic branches of the neurons extend rostrocaudally and dorsoventrally, often to the cord surface and to the gray matter around the central canal, but only in the midsagittal plane. Individual dendrites could be followed up to 800 μm. When viewed in the transverse plane, we detected only a thin band of dendritic labeling (DC, Fig. 2A). In this particular case, the dendritic arbor is found along the border of a dorsal, V-shaped wedge of tissue near the midline of the DCs.

Because the neurons express the NK-1 receptor and because some SP-containing axons course in the DCs (11) we performed a double-label immunocytochemical study to examine the relationship between SP and the NK-1 receptor. Although we confirmed the observation that there are SP-containing axons in the DCs, we never found evidence for a SP-containing synaptic input to the DC neurons.

On the other hand, when we immunostained with a general marker of synaptic terminals, namely the synaptic vesicle antigen, synaptophysin, we found evidence that there is an extensive input to these neurons. Synapses were located on cell bodies and dendrites of every DC neuron (Fig. 3A). To confirm that these were true synaptic connections we performed EM immunocytochemistry on MAP-2 immunoreactive cell bodies and dendrites taken from the heart of the lumbar DC white matter (Fig. 4). We found that MAP-2 labeled cell bodies and dendrites received both symmetric and asymmetric synapses (Fig. 4C) and participated in complex synaptic arrangements, including glomeruli that contained a central terminal surrounded by dendrites and various vesicle-containing profiles.

To establish the connections made by these neurons, we performed a series of tract tracing studies to identify the target of their axons. The DC neurons proved too sparse to permit anterograde tracing studies, and we could not be certain that any terminal labeling did not derive from DC axons of passage. We therefore turned to retrograde tracing approaches.

Despite making Fluorogold injections into a variety of targets, including the DC nuclei, parabrachial nucleus, lateral thalamus, and cerebellum, we never retrogradely labeled the DC neurons. We also studied lumbar DC neurons after retrograde tracer injections into the cervical spinal cord, but never found labeling. Although we found labeled DC neurons in the cervical spinal cord after Fluorogold injections into the caudal medulla, this labeling was accompanied by considerable glial labeling and there was no labeling of DC neurons in the lumbar cord. For these reasons, we believe that the cervical labeling resulted from spillover of the tracer into the spinal CSF and uptake by dendrites of the DC neurons that contact the spinal cord surface. Finally, the neurons did not label after injection of Fluorogold into the sciatic nerve. This finding

![Image](https://example.com/image.png)
indicates that the DC neurons are not a spinal cord homologue of the mesencephalic nucleus of V, which contains cell bodies of primary afferent stretch receptors that innervate jaw muscles.

**Functional Properties of the DC Neurons.** The presence of a rich synaptic input to the DC neurons prompted the next series of studies. We monitored expression of the Fos protein to provide a measure of activity in response to different forms of peripheral stimulation. Because of the potential relationship between small-diameter primary afferents and these neurons, and because these neurons express the NK-1 receptor, we first studied the effect of noxious mechanical, thermal, or visceral stimulation, which evokes the release of SP (12, 13). Although we used stimuli that induced extensive Fos expression in neurons in the adjacent gray matter, notably in neurons of lamina I of the dorsal horn, which also express the NK-1 receptor, none of these stimulation protocols induced Fos expression in the DC neurons.

We previously have demonstrated that although many dorsal horn neurons express the NK-1 receptor over much of their somatic and dendritic surface, less than 15% of the receptors appose synaptic terminals (14). Exogenous SP or noxious stimulation, however, induces massive internalization of the NK-1 receptors, indicating that these nonsynaptic receptors are functional (4, 10). To test whether comparable responses exist in the DC neurons, we evaluated the effect of noxious thermal, mechanical, or visceral stimuli on the distribution of the NK-1 receptor. As for Fos induction, we found no evidence of a response in the DC neurons.

The absence of a response to peripheral stimulation directed our attention to another source of excitatory input. Specifically, because the DC neurons have dendrites that arborize extensively along the surface of the cord (Fig. 1 B and C), we hypothesized that they are ideally positioned to respond to compounds that circulate in the CSF. To test this possibility, we made CSF injections of SP and monitored the DC neurons for internalization of the NK-1 receptor. Fig. 3E illustrates that this stimulus produced a profound internalization of the receptor, in approximately one-third of the DC neurons. The greatest response was observed in the cell bodies, although labeling of the receptor within dendrites also was detected after intrathecal SP injection.

Because we could not activate these neurons by noxious stimulation we next tested the possibility that there is a significant inhibitory control of these neurons. Consistent with this hypothesis, we found that at least 80% of the synaptophysin profiles stain positively with a marker of GABAergic terminals, namely an antiserum directed against the GABA vesicular transporter (Fig. 3B). This marker of GABAergic terminals was found in synaptophysin profiles over the cell body and dendrites of the DC neurons. Occasionally we detected GABA vesicle transporter immunoreactive axons that extended dorsally from the gray matter surrounding the

![Fig. 3. Sagittal sections of the rat lumbar spinal cord provide information on the functional nature of the neurons of the DC white matter. (A) Labeling for synaptophysin (green) reveals a dense somatic and dendritic synaptic input to the DC neurons (stained for NK-1 receptor; red). (B) A large proportion of the synaptic input to the neurons (stained for MAP-2; red) are GABAergic as they express the GABA vesicle transporter (green). (C and D) Direct CSF injection of the GABA_A receptor antagonist, bicuculline, induces Fos expression (black nucleus; arrowheads in C) in approximately 50% of the DC neurons (identified by the brown MAP-2 immunoreaction product). (E) Direct CSF injection of SP induces internalization of the NK-1 receptor in approximately one-third of the DC neurons. The receptor is concentrated in cytoplasmic endosomes. (F) Approximately 20% of the neurons (stained for MAP-2; red) express neuronal nitric oxide synthase (green). In A, B, and F, superimposition of the confocal images produces a yellow image where the immunostaining of two antigens overlaps. (Bars: 25 µm, A; and 50 µm, B–F.)

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central canal, which suggests that the input to the DC neurons derives from local GABAergic neurons.

To test the hypothesis that there is a tonic GABAergic control of these neurons, we next evaluated, also in the rat, the effect of direct CSF injection of the GABA\(_A\) receptor antagonist bicuculline. To monitor activity of the neurons we again immunostained for the Fos protein. Bicuculline not only produced a seizure of the hindlimbs and tail but also induced expression of the Fos protein, in approximately 50% of the DC neurons (Fig. 3C and D). Importantly, neither the GABA\(_B\) receptor antagonist, CG558486, nor the glycine receptor antagonist, strychnine, induced Fos expression in these neurons, even though strychnine evoked seizures comparable to those produced by bicuculline.

To identify additional cytochemical features of the DC neurons we screened for several other neurotransmitters and receptors. We found no labeling with \(N\)-methyl-D-aspartate R1, somatostatin 2A, or mu-opioid receptor antisera, and none of the neurons stained positively for serotonin, which we examined because it is concentrated in the midline neurons of the brainstem raphe nuclei (15). On the other hand, approximately 20% of the neurons stained intensely for immunoreactive neuronal nitric oxide synthase (ref. 16; Fig. 3F).

**DISCUSSION**

Standard neuroanatomy textbooks describe the DCs as large white matter bundles through which large-diameter primary afferent fibers ascend to the DC nuclei of the medulla. In the rodent, the ventralmost portion of the DCs also contains axons of the corticospinal tract (17). Although neurons have been found in several white matter regions of the brain and spinal cord, we believe that the DC population that we have identified has not previously been described. Importantly, these DC neurons are clearly different from the isolated spinocerebellar tract neurons that Grant et al. (18) observed in the ventral part of DC white matter. First, the latter group is located ventrally, adjacent to Clarke’s column, and it is not on the midline. Second, the DC neurons that we identified do not retrogradely label from the cerebellum. Third, only the DC neurons along the midline have dendrites that arborize in the sagittal plane and immunostain for the NK-1 receptor. This population of neurons also differs from the isolated neurons described by Waldeyer (19) and later by the Scheibels (20). Those neurons are found in the white matter that is immediately dorsal to lamina I of the dorsal horn; we presume that they are displaced neurons of the marginal layer. Those neurons are also morphologically very distinct from the neurons in the DC white matter.

What is particularly puzzling is our inability to identify the axonal target of these neurons. We found no retrograde labeling after injections into the DC nuclei, which are targeted by primary afferent and postsynaptic (23, 24) DC axons, or into the parabrachial nucleus or lateral thalamus, both of which are...
targets of NK-1 receptor-positive neurons in the dorsal horn (25, 26). These results provide further evidence that the DC neurons are not merely displaced dorsal horn neurons. Lumbar DC neurons also did not label after tracer injections into the cervical cord, which indicates that the DC neurons are not part of a propriospinal network.

Noxious stimulation, which should evoke the release of SP from SP-containing afferents in the DCs, did not induce Fos expression in the DC neurons, as it readily does in neurons of the superficial dorsal horn (8). Importantly, because noxious visceral stimulation did not induce Fos, it is unlikely that these neurons are at the origin of the midline DC pathway recently implicated in visceral nociception (27). On the other hand, intrathecal injection of SP did induce NK-1 receptor internalization in these neurons, which indicates that they are responsive to SP. This result raises the possibility that the DC neurons can monitor the chemistry of the spinal cord CSF. On the other hand, although this population has some anatomical features reminiscent of the neuronal circumventricular organs, notably their proximity to the vascular system and CSF (28), we did not find specialized capillary loops in association with these neurons, as there are in circumventricular organs.

In part the difficulty in activating these neurons may result from their being under a profound tonic inhibitory control. We found a dense GABAergic synaptic input upon these neurons and EM analysis revealed that there is a complex neuropil through which these controls are exerted. Furthermore, removal of inhibitory control by intrathecal injection of bicuculline, but not of strychnine, induced Fos expression in the DC neurons. Conceivably, the strength of these controls (in the setting of injury, or during development) contributes to the activity that can be induced in the DC neurons.

Finally, the possibility that these neurons function during development should be considered. Their location relative to the remnants of the roof plate is of particular interest (29). It is possible that the midline planar dendiric arborization of the DC neurons contributes to the ipsilateral segregation of large-diameter afferents. In this regard, the neurons may serve a function comparable to that of the subplate neurons in the neocortex (30), which serve as guideposts for axons that eventually innervate overlying cortex. Finally, because GABA can depolarize spinal cord neurons during development (31), it also will be of interest to evaluate the effect of eliminating GABAergic controls on the response properties of developing DC neurons.

In summary, we have discovered an interesting population of neurons in the DC white matter of the spinal cord. In some respects the neurons are homogeneous, (e.g., all express the NK-1 receptor); however, heterogeneity is evident from the differential immunostaining for neuronal nitric oxide synthase. The neurons are under a tonic GABAergic control, but can be activated by SP that circulates in the CSF. Yet to be determined is the physiological stimulus to which the neurons respond in the normal animal.

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