exported into the outer membrane, thereby crossing the inner membrane. This requires a cleaved signal sequence and additional sorting signals in the mature part of the protein [7]. In contrast, mitochondrial porins are directly imported into the outer mitochondrial membrane; their insertion does not require cleavage of the NH_2-terminal signal sequence, but recent experiments suggest the involvement of carboxy-terminal sequences [5]. Interestingly, bacterial porins are thought to reach the outer membrane via periplasmic intermediates [7], whereas mitochondrial proteins destined to cross both membranes have been shown to go through 'contact-sites' of the outer and inner membrane [5,9].

How could one explain all the differences between bacterial and mitochondrial porins on the basis of the endosymbiotic theory? If the theory were right, there should be a common ancestor for eukaryotic and prokaryotic porins. The existence of such an ancestor would become more likely with the finding of a 'missing link', a protein sharing some of the features of both types of porins. The putative new pore discovered in porinless yeast mitochondria could be such a protein; with its cation selectivity and small exclusion limit it resembles bacterial porins, but it is localized in mitochondria [5].

Toxic pore proteins represent a completely different type of channel; both eukaryotic killer-toxin [4] and bacterial colicins [8] are secreted proteins which are made as larger precursors. Their unrelated cleavage and assembly pattern makes a common origin extremely unlikely. It is therefore interesting how both prokaryotes and lower eukaryotes invented the same trick to kill unpleasant neighbors by destroying their membrane potential. Unfortunately our knowledge about the structure and function of the yeast killer toxin is not large enough to allow a mechanistic comparison with bacterial colicins. The beautiful model presented by Pattus et al. [8] for the insertion of colicin A into its target membrane allows for the first time insight into the conformational change involved in channel opening after contact of the protein with the membrane. Similar work is needed also for eukaryotic and prokaryotic porins as well as yeast killer toxin before we will be able to fully understand and compare their mechanism of action.

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Research Articles

A new dopaminergic terminal plexus in the ventral horn of the rat spinal cord. Immunohistochemical studies at the light and electron microscopic levels

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Summary. It has been thought that the ventral motor column in the rat spinal cord is virtually free of dopaminergic fibers. However, a new dopaminergic terminal plexus was visualized at all spinal levels in the ventral horn using electron as well as light microscopic immunohistochemistry.
Key words. Anti-dopamine serum; rat; spinal cord; ventral horn; electron microscopy.

The existence of dopaminergic neurons in the vertebrate spinal cord was suggested first by biochemical studies [1,2]. These neurons had been demonstrated morphologically, using histofluorescence and tyrosine hydroxylase-immunohistochemical techniques. In these studies, DAergic innervation was found in a part of the spinal cord, but it was difficult to distinguish the dopamine from the approximately 10-fold more dense noradrenaline (NA) neu-

rons by the techniques used[3]. Recently, using the modified histofluorescence method on animals treated with neurotoxin to deplete noradrenaline, presumed spinal DA nerve terminals were detected mainly within the dorsal horn, the intermediolateral cell column and associated parts of the intermediate and central gray matter[4]. However, the ventral horn was virtually free of fluorescent fibers. It was also reported from biochemical studies that the DA-levels in the ventral horn were negligible in the thoracic and lumbar segments[5].

In the present study, we investigated DA innervation of the rat spinal cord, with an immunohistochemical technique utilizing highly sensitive antibody raised against DA. Antiserum to DA was prepared according to the method of Geffard et al.[6], and its specificity was described in detail in our previous paper[7]. Briefly, the antiserum was checked by several control tests and the enzyme-linked immunosorbent assay (ELISA) as described previously[8-10]. Our DA antiserum was found to cross-react slightly with NA ($< 0.1\%$) but not with adrenaline (AD), 5-hydroxytryptamine (5-HT), histamine (HA), octopamine (OA), L-3,4-dihydroxyphenylalanine (L-DOPA) or 5-hydroxytryptophan (5-HTP) in our ELISA system. Absorption of the antiserum with DA-glutaraldehyde-bovine serum albumin (DA-GA-BSA) completely abolished the immunostaining, but preabsorption with NA-GA-BSA did not affect the staining. The anti-dopamine serum treated by preabsorption with NA-GA-BSA conjugate was used for the studies described here.

Eight male Wistar rats (200–300 g) were used in the present immunohistochemical study. At first, the rats were anesthetized with an i.p. injection of sodium pentobarbital (50 mg/kg), and subsequently perfused via the ascending aorta with 400–800 ml of 5% GA in 0.01 M cacodylate buffer. After perfusion, the spinal cords were dissected out and sliced at 20 µm thickness. They were further processed for the PAP method. The procedures used for tissue preparation and immunohistochemical staining have been described elsewhere[11].

Light microscopy. DA-immunoreactive fibers were recognized throughout the whole of the gray matter at any level of spinal cord examined. In the dorsal horn and the intermediate zone surrounding the central canal, relatively dense DA-labeled fibers were found. The densest DA-labeled nerve terminals were found in the intermediolateral nucleus. These DA fibers formed a dense plexus of varicose terminals which terminated around unlabeled neurons. These findings were similar to those previously reported[12], however, DA-labeled fibers were also distinctly observed within the anterior horn, especially in the laminae, where fine varicose terminals were seen in the vicinity of the perikarya of motoneurons (fig. 1). These DA terminal plexi were constantly seen throughout the anterior column at all spinal levels, but their density was relatively higher in the cervical and lumbar cords than in the remaining part. The present immunohistochemical findings, observed in eight rats, show that a distinct DA-containing type of nerve plexus is located in the anterior motor column. (fig. 2), where this kind of

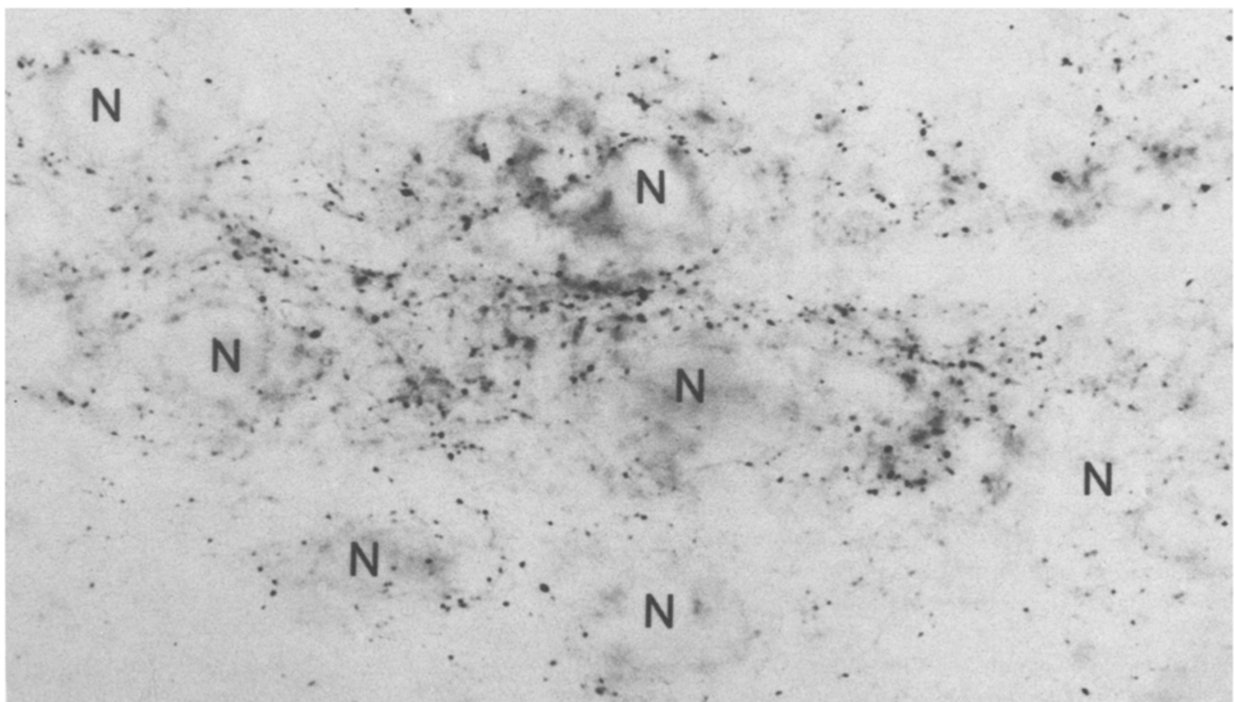


Figure 1. High magnification of DA-labeled fibers sparsely distributed in the ventral horn of the rat spinal cord. Varicosities on DA-labeled fibers can be seen in close contact with perikarya of motoneurons. N: nucleus of motoneurons.

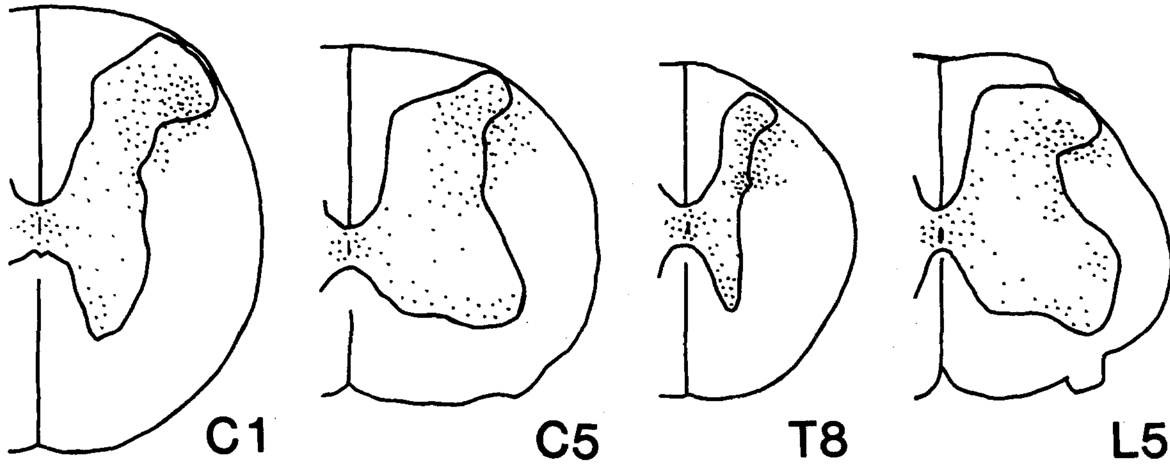
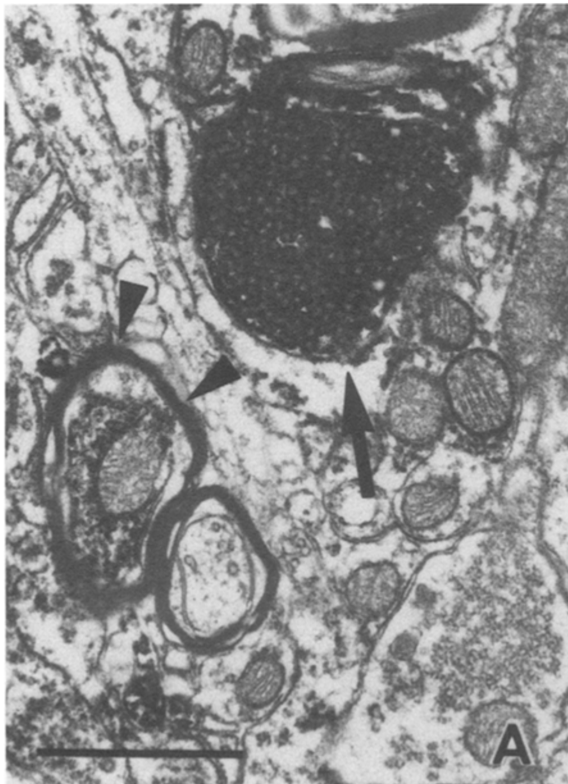


Figure 2. Schematic representation of the distribution of DA-labeled fibers and terminals in the spinal cord of the rat. Dots represent labeled fibers and terminals. The relatively dense DA-labeled innervation is also seen in the ventral horn at the cervical (C1, C5), thoracic (T8) and lumbar (L5).



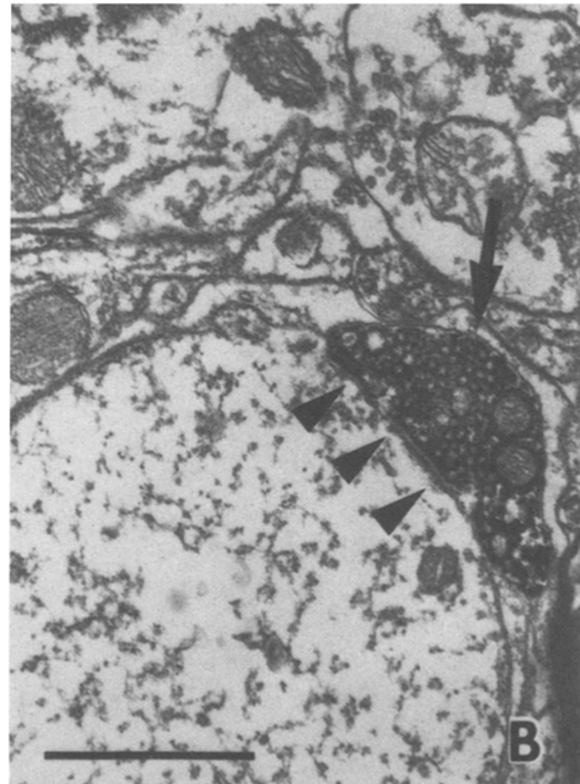


Figure 3. Electron micrographs of the DA-immunoreactive nerve terminals in the ventral horn of the rat spinal cord. These terminals contain heavily stained small vesicles (arrows). The DA-immunoreactive myelinated axon. Reaction products are seen in the axoplasmic matrix as indicated by the arrowhead in (A). The DA-immunoreactive terminal is observed to make synaptic contact (arrowhead, (B)) with a dendrite-like process. Bars = 0.5 μm.

innervation has so far not been defined histochemically.

Electron microscopy. After completion of the immunohistochemical reactions, sections were treated with 1% osmium tetroxide, dehydrated and embedded in Epok 812 on microscope slides. The sections were examined by light microscopy and the anterior horn was cut from the specimens and then re-embedded. Ultra-thin sections were examined by electron microscopy.

Electron microscopy revealed the presence of immunoreactive products in the anterior horn of all eight rats. These products were heavily stained and clearly distinguishable from non-reactive ones. The DA-immunoreac-

tive varicosities and terminals were packed with small round vesicles with a diameter of 30–45 nm, like ordinary synaptic vesicles. Some large granulated vesicles were also seen. The vesicle membranes gave these an overall darkened appearance (fig. 3). In the terminals the mitochondria were the main cell organelles. Some of the terminals were observed to make synaptic contact with presumably dendritic processes (fig. 3, A). Synaptic contacts with the perikarya of motoneuron were not observed. DA-immunoreactive fibers were mostly nonmyelinated, but occasionally had a myelin sheath (fig. 3, B).

In order to investigate the major source of DA-labeled fibers in the ventral horn of the spinal cord, we combined injection of the retrograde tracer WGA-HRP with DA-immunohistochemistry. Six male rats were used for this study in addition to the original eight. WGA-HRP was injected in a 10% suspension unilaterally into the lumbar spinal cord including the ventral horn. The injection volume was 0.1 µl. After 4–5 days survival, the rats were sacrificed. Rats were anesthetized and perfused intracardially with fixative (5% GA in 0.01 M cacodylate buffer). The brains were stored in 15% sucrose and then sliced at 22 µm thickness. They were processed for WGA-HRP staining with diaminobenzidine (DAB) + nickel ammonium sulfate, and for DA-immunohistochemistry using DAB alone. The retrogradely labeled DA-containing neurons (fig. 4) were seen only within the caudal hypothalamus, the so-called A 11 cell group[12]. These double-labeled neurons were found ipsilaterally to the injection site. The diencephalic A 11 cell group was the principal single source of this innervation, which is in agreement with previous reports[13–15]. We concluded that the diencephalic A 11 cell group was the origin of DA labeled fibers in the ventral horn as well as the dorsal horn, the intermediolateral cell column and associated parts of the intermediate and central gray matter.

These studies provide evidence for the existence of a new DAergic terminal plexus in the ventral horn, as well as those in the intermediolateral cell column, the dorsal horn and the area surrounding the central canal which had been previously noted. It was previously thought that the ventral horn was virtually free of DAergic innervation. The discovery of such innervation in the ventral horn was made possible by the use of an immunohistochemical technique utilizing a highly sensitive antibody raised against dopamine. Our findings were confirmed by immunohistochemical observation with both light and electron microscopes. Electron microscopy revealed DA-immunoreactive terminals packed with small vesicles and making synaptic contact. By means of double-label, retrograde staining for WGA-HRP and DA immunohistochemistry, the origin of DAergic projection to the ventral horn in the spinal cord was found to exist within the caudal hypothalamus (A 11). This descending hypothalamospinal DA system may be involved in somatic motor regulation.

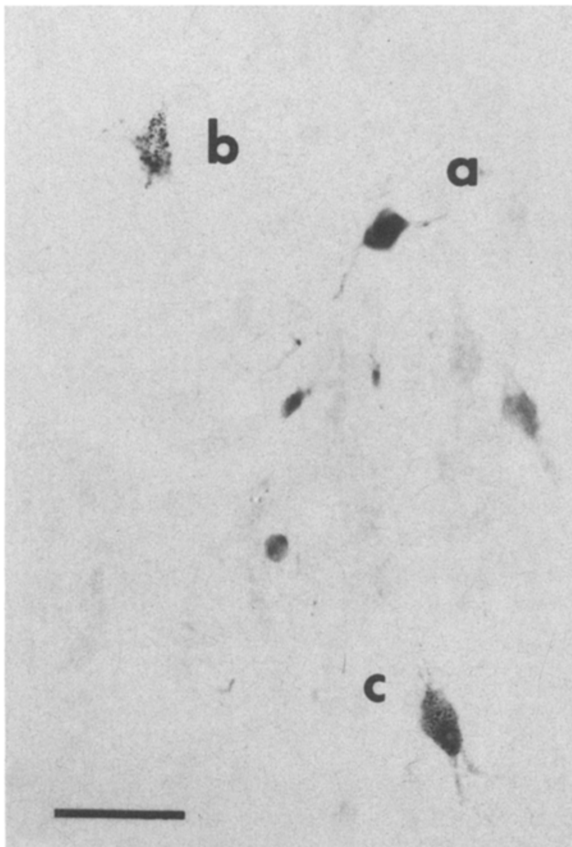


Figure 4. WGA-HRP tracing combined with DA immunohistochemistry within the caudal hypothalamus (A 11).
a is a non-labeled DA-immunoreactive neuron.
b is a cell labeled with WGA-HRP alone.
c is a labeled DA-containing neuron. Bar = 50 µm.

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